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THE IMMUNMODULATORY MECHANISMS OF EXTRACORPOREAL PHOTOPHERESIS

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A thesis submitted for partial fulfilment of the requirements of The Nottingham Trent University for the degree of Doctor of Philosophy

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Abstract

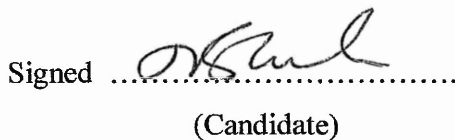
Extracorporeal photopheresis (ECP) was originally pioneered as a treatment for Cutaneous T cell Lymphoma (CTCL) (Edelson *et al.*, 1987). More recently, ECP therapy has been found to be effective in Graft versus Host Disease (GvHD) (Owsianowski *et al.*, 1994). ECP involves the separation and exposure of leucocytes to 8-Methoxypsoralen (8-MOP) and UVA, treated cells are subsequently re-infused (Edelson *et al.*, 1987). Lymphocytes demonstrating apoptotic markers are observed very early in the ECP process, immediately prior to re-infusion. The early apoptotic lymphocytes display phosphatidylserine externalisation, depolarisation of the mitochondrial membrane potential and a fall in Bcl-2/Bax ratio. Subsequent changes included the activation of the caspase cascade and expression of Fas-Ligand, observed 24 hours post ECP. The apoptotic lymphocytes are phagocytosed by monocytes following ECP. ECP-treated monocytes, unlike other UV therapies, retain the co-stimulatory markers necessary for the effective presentation of processed antigens. Immediately following ECP, the number of T-cells expressing TNFα and IFNγ falls, whilst levels of monocytes expressing TNFα are also reduced.

The anti-clonotypic immunomodulatory response, thought to be induced by ECP (Edelson *et al.,* 1994), may therefore be induced by the extensive immediate and progressive immunological challenge of infused apoptotic lymphocytes. This process is thought capable of removing the expanded malignant and autoreactive T cell clones present in patients treated using ECP (Edelson *et al.,* 1994). In addition, down-regulation of TNFα and IFNγ may benefit the T-cell mediated conditions where these cytokines play a pathological role.

Declaration

This work has not been accepted in substance for any other degree and is not being submitted in candidature for any other degree.

This is to certify that the work here was carried out by the candidate himself. Due acknowledgement is made of all the assistance received.



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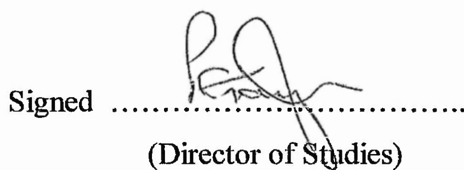

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(Director of Studies)

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Papers

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List of Abbreviations

aGvHD	Acute Graft versus host disease
AIF	Apoptosis inducing factor
AP-1	Apoptosis protein-1
APAF-1	Apoptosis promoting associated factor-1
APC	Antigen presenting cell
ATP	Adenine-5'-triphosphate
BCB	Buffy coat bag
BH	Bcl-2 homology domain
°C	Degrees Centigrade
cGvHD	Chronic Graft versus host disease
CAD	Caspase-activated deoxyribonuclease
CALLA	Common acute lymphoblastic leukaemia antigen
CARD	Caspase recruitment domain
CD	Cluster differentiation
CDK	Cyclin dependent kinases
CD30-L	CD30-Ligand
CD40-L	CD40-Ligand
cm	Centimetre
CS	Carboxy-SNARF®-1-AM
CTCL	Cutaneous T cell lymphoma
CTL	Cytotoxic T lymphocyte
DED	Death effector domain
$DiOC_6$	3,3' dihexyloxacarbocyanine iodide
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DNA'ase	Deoxyribonucleic acid'ases
ECP	Extracorporeal Photopheresis
FAM-VAD-FMK	Carboxyfluorescein-benzyloxycarbonyl-valine-alanine-aspartic acid-flouromethyl-ketone
Fas-L	Fas-Ligand

FADD	Fas-associated protein with death domain
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FL	Fluorescence
FLICE	Fas-associated ICE-like protease
FS	Forward scatter
g	Gravity
GvHD	Graft versus host disease
H^+	Free hydrogen ion
hct	Haematocrit
HD	Hodgkin's disease
hrs	Hours
HSPs	Heat shock proteins
HTLV-1	Human T cell lymphotropic type 1
IAP	Inhibitors of apoptosis
ICAD	Caspase-activated deoxyribonuclease inhibitor
ICAM-1	Intercellular adhesion molecule-1
IFNα	Interferon alpha
IFNγ	Interferon gamma
IL	Interleukin
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide,
J	Joules
KJ	Kilojoules
LAK	Lymphocyte activation killer
LFA-1	Lymphocyte function-associated antigen-1
LFL	Log fluorescence
m	Meter
*m*CICCP	Carbonyl cyanide-*m*-chlorophenylhydrazone
MF	Mycosis Fungoides
mg	Miligram
MGG	May, Grunwald and Giemsa stain

MHC	Major histocompatability complex
min	Minute
Min HA	Minor histocompatability antigen
ml	Millilitre
mM	Millimolar
µl	Microlitre
µm	Microlitre
µM	Micromolar
8-MOP	8-Methoxypsoralen
MPT	Mitochondrial permeability transition pores
mRNA	Messenger ribonucleic acid
$\Delta\psi_m$	Mitochondrial transmembrane potential
ng	Nanogram
nM	Nanomolar
NK	Natural killer
NHL	Non Hodgkin's lymphoma
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE-CY-5	Phycoerythrin-cyanin-5
%	Percentage
pH	Negative log of hydrogen ion concentration
PI	Propidium Iodide
PMA	Phorbol 12-myristate 13-acetate
PS	Phosphatidylserine
PUVA	Psoralen and Ultraviolet A
RE	Reticuloendothelial
rhIL	Recombinant Interleukin
ROS	Reactive oxygen species
RPMI	Rosweli Park Memorial Institute
SD	Standard deviation
SEM	Standard error of the mean

SS	Side scatter
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl tranferase
Th1	T helper 1
Th2	T helper 2
TNFα	Tumour necrosis factor alpha
TNFR1	Tumour necrosis factor receptor 1
TRAIL	TNF-related apoptosis inducing ligand
TUNEL	Terminal deoxynucleotidetidyl tranferase-mediated dUTP nicked-end labelling
U	Units
UV	Ultraviolet

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Chapter 1: Introduction

1.1. History

1.1.1. Phototherapy

The use of 'phototherapy' as a treatment modality for health restoration was reported in Greek culture three thousand years ago. The 'patients' whole body was exposed to the sun or arenation in specially set aside areas. Termed 'Heliotherapy', the celebrated physician Herodotus, taught the practice in the restoration of health. However, with the advent of Christianity, the use of the sun for healing was considered paganistic and its use diminished (Daniell and Hill, 1991). Only at the start of the nineteenth century was sunlight again documented as a treatment modality. Cauvin reported the beneficial effects of sunlight in the treatment of rickets, rheumatism, paralysis, swellings, dropsy and muscle weakness (Cauvin, 1815). However popularisation of phototherapy is attributable to Niels Finsen, a Danish physician, who received the Nobel Prize in 1903. He pioneered the treatment of lupus vulgaris using carbon arc illumination (Finsen, 1901). In 1896 the Finsen light institution was founded and following the endorsement of the institute by Princess Alexandra, wife of the future Edward VII, phototherapy was being performed in London (Daniell and Hill, 1991). Currently, the treatment of neonatal jaundice is the most popular use of phototherapy (Daniell and Hill, 1991).

1.1.2. Photochemotherapy

In the early twentieth century, the clinical benefits of light on photosensitive chemicals were first demonstrated. Von Tappeiner, a Professor working in Munich, was interested in why the chemical, acridine, was only toxic to protozoan in-vitro, but not in-vivo. One of his medical students, Oscar Raab, identified that acridine toxicity was dependent on exposure to light, or more precisely the fluorescence it induces (Daniell and Hill, 1991). Follow on work saw Von Tappeiner and the Dermatologist, Jesionek, treat skin cancer, lupus of the skin and chondylomata of the female genitalia with the photosensitizer, eosin (Von Tappeiner, 1900). Von Tappeiner was the first to coin the term, 'Photodynamic therapy'. Evolution of photodynamic therapy has seen it utilised

in the treatment of a variety different malignancies ranging from skin cancer to gynaecological cancer (Daniell and Hill, 1991). Photosensitizers utilised for clinical practise have included haematoporphyrin derivatives, non-metal porphyrines and benzoporphyrin-derivatives monoacid ring A (Elmets and Bowen, 1986; Musser and Fiel, 1991; Simkin *et al.,* 1997), whilst modern technology has allowed the use of laser light for excitation (Perria *et al.,* 1980).

1.1.3. Psoralen with UVA (PUVA)

The origins of PUVA therapy dates back to 1400BC. Documented in the sacred Indian book 'Atharva Veda' is the use of psoralens, derived from the plant extracts of the plant *Psoralea corylifolia,* in the treatment of pigmentary disorders (Daniell and Hill, 1991). Around the 12th century AD the Egyptians obtained psoralen from another plant *Anmi majus* and used them in the treatment of leucoderma (Fitzpatrick and Pathak, 1959). The active ingredient of *Amni majus;* 8 Methoxypsoralen (8-MOP), was isolated and characterised in 1947 (Fitzpatrick and Pathak, 1984). Aaron Lerner was fascinated by the transformation of 8-MOP from a naturally occurring inert substance to a potent photosensitizer by the action of sunlight (Gasparro *et al.,* 1985). In 1953 Lerner *et al* were the first to use the combination of 8-MOP and sun light exposure as an effective treatment modality (Lerner *et al.,* 1953). The mutagenic properties of 8-MOP were first identified in 1955 (Musajo, 1955). Musajo later demonstrated that irradiated 8-MOP could react with DNA (Musajo *et al,* 1965). Subsequent research discovered that 8-MOP intercalates into the DNA double helix by binding to pyrimidine bases. Following exposure to UVA light, the psoralen forms covalent bonds with the DNA and the resultant photoadduct is able to disrupt DNA replication and the viability of the effected cell (Cole, 1970; Dan'Acqua *et al.,* 1970).

However it was not until the advent of artificial, high intensity UVA light sources in the early 1970's that the full benefits of 8-MOP and UVA therapy were realised (Levin and Parrish, 1975). The first studies on the use of psoralen and UVA for psoriasis involved the use of topical psoralen followed by UVA

exposure (Walter and Voorhees, 1973; Weber, 1974). However this treatment modality was unpredictable and UVA dosimetry was difficult to establish (Hönigsmann *et al.,* 1993). In 1974 it was shown that the combination of orally administered psoralen and UVA was more predictive and proved to be highly effective in the treatment of psoriasis (Parrish *et al.*, 1974). Parrish *et al.* coined the acronym, PUVA for the treatment using psoralen and UVA (Parrish *et al.*, 1974). Other alternatives used PUVA-bath therapy, which employed whole body immersion in 8-MOP baths followed by total body irradiation (Lowe, *et al.,* 1986).

Second only to Psoriasis, PUVA is used extensively in the treatment of cutaneous T cell lymphoma (CTCL). PUVA therapy was dramatically effective in early stage disease (stage IA, IB), in which complete and long lasting remissions were induced (Gilchrest *et al.,* 1976). However patients with the tumour stage CTCL exhibit a high rate of early occurrence following PUVA therapy (Hönigsmann *et al.,* 1984). Although some extension of remission has been achieved using UVA/8-MOP in conjunction with retinoids (Thomsen *et al.,* 1989) and interferon alpha 2a (Roenigk *et al.,* 1990). More recently, the use of PUVA to the skin, has been shown to be effective in the treatment of chronic Graft versus host disease (cGvHD) (Volc-Platzer *et al.,* 1990; Jampel *et al.,* 1991). Following PUVA treatment, the skin lesions of chronic GvHD patients improved and reduced the need for immunosuppressive therapy. In addition, no flare of acute GvHD (aGvHD) was noted, although PUVA demonstrated no improvement in the cGvHD contained within the liver (Volc-Platzer *et al.,* 1990). However, the long-term exposure of the skin to PUVA carries potential risks. Most serious of which is the associated increased risk of squamous cell carcinoma, especially in the immunosuppressed (Stern *et al.,* 1984 Altman and Alder, 1994).

In animal studies, the infusion of specific autoreactive T cell clones induces encephalomyelitis (Cohen, 1984). However Perez *et al* demonstrated that if these T cell clones were lethally damaged with direct 8-MOP/UVA treatment

prior to re-infusion, subsequent infusion of autoreactive clones do not induce the pathological disease (Perez *et al.,* 1989). The initial observations by Perez (Perez *et al.,* 1986) prompted the first clinical trial of Extracorporeal Photopheresis on CTCL patients, whereby leucocytes separated by leucopheresis were exposed to 8-MOP/UVA and subsequently re-infused (Edelson *et al.,* 1987).

1.1.4. Extracorporeal Photopheresis

In 1987 Edelson *et al* reported the therapeutic use of Extracorporeal Photopheresis (ECP) in the treatment of erythrodermic CTCL; 83% of patients with disease resistant to a variety of conventional therapies demonstrated improvements within the skin. The extent of skin lesions improved by better than 75% in 13% of patients and a 50-75% improvement in skin score was observed in 35% of patients (Edelson *et al.,* 1987). ECP subsequently received FDA-approval in 1987, as the first selective immunotherapy for the treatment of cancer (Knobler and Girardi, 2001). Subsequent trials of ECP in North America and Europe, for the treatment of CTCL, demonstrated overall response rates of between 31% and 80% and complete response of between 14% and 59% (Heald et al., 1989; Vonderheid et al., 1998, Jiang et al., 1999; Wollina et al., 2000). Figures 1.1.4.1 and 1.1.4.2 demonstrate the clinical response typically observed in a CTCL 'responder' receiving ECP therapy. Expansion of its clinical benefit has seen ECP used to treat acute and chronic GvHD (Rossetti et al., 1995. Richter et al., 1997; Taylor 2000), Systemic Sclerosis (Scleroderma) (Rook *et al.,* 1992), rejection post organ transplant (Barr *et al.,* 1998) and several different autoimmune conditions; including Pemphigus Vulgaris (Rook *et al.,* 1990) and SLE (Knobler *et al.,* 1992). ECP gained popularity because of the remarkably low toxicity, when compared to more conventional treatment modalities (Knobler and Girardi, 2001). The lymphocytes containing 8-MOP are activated in-situ by the action of the artificial UVA light source. Because photoactivated 8-MOP has such a short half-life all the toxicity is lost by the time the blood is returned to the patient, thus avoiding toxic side effects to other organs. In addition, the returned non-nucleated blood components, namely red

cells, platelet and plasma proteins are not significantly affected by this treatment (Gasparro *et al.*, 1985).

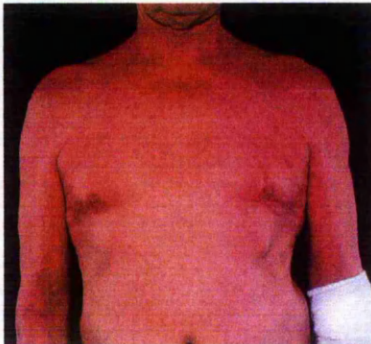


Figure 1.1.4.1. The Erythrodermatous skin of a CTCL patient prior to ECP

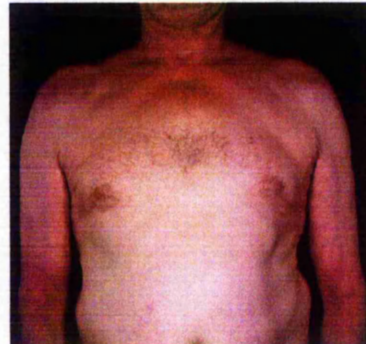


Figure 1.1.4.2. The same CTCL patients following 6 months of ECP therapy

Figures 1.1.4.1 and 1.1.4.2 demonstrate the clinical response observed in a CTCL 'responder' treated at the Photopheresis department, Rotherham General Hospital.

Photographs kindly provided by the Medical Illustration Department, Rotherham General Hospital, following consent by patient.

1.2. Clinical Conditions treated using ECP

1.2.1. Cutaneous T cell Lymphoma

The cutaneous T-cell lymphomas (CTCL) are the most frequently occurring lymphomas involving the skin (Edelson, 1980). The CTCLs are a clonally derived malignant proliferation of skin-invasive CD4+ T lymphocytes (Weiss *et al.*, 1989; Diamandidou *et al.*, 1996). CTCL commonly pass sequentially through three important stages. In the early mycosis fungoides (MF) stage, the malignant T cells are only identifiable in the skin. Further progression involves the migration and detection of the malignancy within lymph nodes and blood. The last stage is one in which dissemination leads to a dangerous systemic phenomena, termed Sezary syndrome (Edelson, 2001). The initial skin lesions

appear as asymptomatic thin red patches, which can increase in area and/or thickness as the disease progresses (Heald, 2001). Expansion of the malignant clone can lead to a compromise in normal T cell function rendering the patient susceptible to infection and further malignancy (Hoppe *et al.,* 1995). Patients with CTCL may have an extremely long natural history of disease, with a medium 6-year duration from the onset of skin symptoms to diagnosis of MF (Hoppe, 1991). However the onset of Sezary syndrome carries a poor prognosis, with a median survival of 31 months and a 5-year survival rate of 33.5% (Bernengo *et al.,* 1998).

1.2.1.1. Aetiology of CTCL

A retroviral trigger for CTCL was initially suggested because the malignant cells demonstrated 'viral-like' particles, whilst CTCL cell cultures demonstrated syncytia and became immortalised; processes not observed in healthy subjects (Zucker-Franklin, 2001). Although initial reports demonstrated human T cell lymphotropic type I (HTLV-I) in some cases of CTCL, the vast majority of CTCLs are negative for antibodies to the structural proteins of HTLV-I (Poiesz *et al.,* 1980; Uchiyama, 1997; Zucker-Franklin, 2001). However, most CTCL patients demonstrate both HTLV- proviral DNA in their lymphocytes and antibodies to the Tax sequence of HTLV-I, without being effected by the whole virus. This posed the suggestion that the presence of Tax-HTLV-I may, following numerous antigenic or inflammatory stimuli, lead to the neoplastic transformation (Zucker-Franklin *et al*, 1997; Zucker-Franklin, 2001). Alternatively, the poor proliferative response of CTCL cells to the TCR/CD3 receptor complex may suggest CTCL progression may be a disorder of apoptosis rather than an advantage of proliferation (Golstein *et al.,* 1986; Varashachary, *et al.,* 1997; Meech, *et al.,* 2001). Reduced levels of CD95 on CD4+ T cells in CTCL may contribute to the defective apoptosis proposed (Dereure *et al.,* 2000)

1.2.1.2. Treatment of CTCL

The treatment of the early mycosis fungoides stage of CTCL using a variety of therapies, including electron beam radiation, topical mechlorethamine and PUVA, yield a high rate of initial response in regard to skin disease (Thomsen *et al.,* 1989; Vonderheid *et al.,* 1998). However these treatment regimens and the recommended multidrug chemotherapy did not prolong survival in the later stage Sezary syndrome patients. Furthermore these therapies were associated with a substantial degree of morbidity (Kay *et al.,* 1989). The use of ECP to treat CTCL, in a multicentre trial in 1987, yielded excellent beneficial results with very few side effects (Edelson *et al.,* 1987). Follow on studies reaffirmed these results and led to the extensive use of ECP for erythrodermic CTCL (Knobler and Girardi, 2001). To utilise ECP therapy in the immunocompromised patient, combination therapies with other biological modifiers, such as IFN-α and retinoids have shown promising results (Wilson *et al.,* 1995; Dippel *et al.,* 1997)

1.2.2. Graft versus Host Disease

Allogeneic and autologous bone marrow transplantation have been used for the treatment of a number of malignant and non-malignant diseases, such as acute and chronic leukeamias, myelomas, lymphomas, aplastic anaemias and severe immuno deficiencies for several years (Krenger and Ferrara, 1996). More recently, histocompatible haematopoietic stem cell transplantation is the treatment of choice for haematological, immunological and genetic conditions (Remberger *et al,* 2001). However, less than 20% of patients have histocompatible donors and thus unrelated bone marrow, mobilised peripheral blood cells and cord cells have to be used for the transplant procedure (Anasetti and Hansen, 1993). The transplantation of genetically non-identical cells does carry significant risk, the most common complication being the development of Graft versus host disease (GvHD) (Ferrara and Deeg, 1991). A complex condition in which specific host tissue, including the skin, liver, lung, gastrointestinal tract and the immune system itself are severely damaged (Krenger and Ferrara, 1996). Acute GvHD (aGvHD) occurs 1-2 months post

transplant and is characterised by erythematous dermatitis, an elevation of hepatocellular enzymes and diarrhoea. The manifestation of chronic GvHD (cGvHD), on the other hand, are sclerodermatous skin changes, malabsorption and obstructive hepatitis. Patients also demonstrate marked collagen deposition in target organs and pulmonary fibrosis (Parkman, 1998).

1.2.2.1. Aetiology of GvHD.

Post Transplant GvHD is thought to be mediated by alloreactive donor T cells recognising major histocompatibility complex (MHC) antigens and/or minor histocompatibility antigens (Minor HA) of the host (Krenger and Ferrara, 1996). Depletion of the donor T cells prior to transplantation have been effective in reducing GvHD, but presents other complications, including graft rejection, increased leukaemic relapse and impaired immunological reconstitution (Poynton, 1988; Keever *et al.,* 1989). More recently there is evidence to implicate the inappropriate production of cytokines as a primary cause in the induction and maintenance of experimental and clinical GvHD (Antin and Ferrara, 1992; Ferrara, 1993).

1.2.2.1.1. Acute GvHD.

The aggressive conditioning regimens (irradiation and/or chemotherapy) prior to transplantation, used to remove the existing haematological stem cells, also leads to damage to host tissue, including intestinal mucosa, liver and other tissues. The activated host cells subsequently produce the inflammatory cytokines, tumour necrosis factor alpha (TNFα) and IL1 (Xun *et al.,* 1994). These events enhance MHC and minor HA antigen expression and allow rapid recognition by donor T cells following transplantation. The production of Interleukin-2 (IL2) and Interferon gamma (IFN γ) by the type 1 donor cells further exacerbates this mechanism by inducing further T cell expansion and priming additional mononuclear phagocytes to produce IL1 and TNFα (Krenger and Ferrara, 1996).

1.2.2.1.2. Chronic GvHD

GvHD detected over 100 days post treatment is described as chronic GvHD (cGvHD) (Ratanatharathorn *et al.,* 2001). Although often thought of as a separate disease distinct from aGvHD, 90% of patients with cGvHD have had prior aGvHD (Parkman, 1998). The dose of donor T lymphocytes transplanted seems a significant parameter in determining whether acute or chronic GvHD is induced. In the mouse model, higher numbers of donor T cells produce aGVHD, whilst lower doses induce features characteristic of cGVHD. The pathogenesis of cGVHD resembles several autoimmune disorders (Ferrara and Deeg, 1991). Autoreactive T lymphocytes are non-cytolytic and secrete cytokines, including IFNγ, that can stimulate fibroblast collagen production (Parkman, 1986). The lack of regulation of the cytokine network has been shown to be a primary cause for induction and maintenance of cGvHD (Barak *et al.,* 1995). Elevation of Th2 cytokines (namely IL4 and IL10) have been observed in the mouse model of cGVHD and proposed as mediators of cGVHD (De Wit *et al.,* 1993; Garlisi *et al.,* 1993). However, clinical samples from human cGvHD sufferers demonstrate an elevation of the same cytokines which are associated with aGVHD. Enhanced levels of IL2, TNFα and IFNγ have all been detected in chronic form of GvHD (Ochs *et al.,* 1996). The predominant pro-inflammatory cytokine identified is IFNγ, the source of which may be donor Th1 T cells, CD8+ T cells, monocytes, macrophages, host endothelial cells or fibroblasts (Ochs *et al.,* 1996). Like aGvHD, the production of these pro-inflammatory cytokines induces further T cell expansion and primes additional mononuclear phagocytes to produce IL1 and TNFα (Krenger and Ferrara, 1996).

1.2.2.2. Treatment of GvHD

Combination immunosuppression has been shown to be superior to single agents for prophylaxis against the development of aGVHD (Storb *et al.,* 1986). Corticosteroids and immunosuppressive agents are initially used to treat cGVHD, however they have demonstrated limited efficacy in patients with extensive cGVHD (Sullivan *et al.,* 1981; Vogelsang *et al.,* 1992; Foss *et al.,*

2002). In many cases even high doses of prednisolone, cyclosporine and/or azathioprine fail to suppress the development of, or improve lichenoid skin eruptions and mucous membrane lesions (Volc-Platzer *et al.,* 1990). Extensive immunosuppression, in addition to the GvHD, can render the patients susceptible to infections (Sullivan *et al.,* 1981; Vogelsang *et al.,* 1992). More recently, PUVA has been successfully used for the treatment of cGVHD (Volc-Platzer, 1990). Promising results, which lead to effective use of ECP in the treatment of both chronic and acute GvHD (Owsianowski *et al.,* 1994: Richter *et al.,* 1997).

1.2.3. Scleredema

Scleredema is a systemic condition characterised by a stiffening of the skin, usually extending from the neck and spreading throughout the body (Buschke, 1902). As the condition progresses movement in the neck and arms becomes more restricted (Stables *et al.*, 2000).

1.2.3.1. Aetiology of Scleredema

Classically, Scleredema naturally resolves in less than 18 months. However, for more than 50% of sufferers, the symptoms persists for more than 2 years and in 16.5% of cases, symptoms remain for more than 10 years (Leinwand, 1951; Sansom *et al.,* 1994). An association with many conditions has been observed and include; diabetes, rheumatoid arthritis, multiple myeloma and primary hyperparathyroidism (Venencie *et al.,* 1984; Berk and Lorincz, 1988; Hodak *et al.,* 1988). Histologically, Scleredema shows normal epidermis, but up to a three times the normal thickening of the dermis (Cole and Winkelmann, 1990). Within the affected area an increase in glycosaminoglycan and collagen are observed (Roupe *et al.*, 1987). The role of the raised paraprotein, observed in some patients is unclear, but has been shown to increase synthesis of collagen from autologous and normal fibroblasts (Ohta *et al.,* 1987).

1.2.3.2. Treatment of Scleredema

Many different treatment regimens have been tried for Scleredema and include thyroid hormones, corticosteroids and antifibrotic therapy (Stables *et al.,* 2000). Recently, the use of 8-MOP and UVA has been shown to be beneficial, initially using a bath PUVA system (Hager *et al.,* 1998) and more recently using ECP (Stables *et al.,* 2000).

1.3. ECP procedure

ECP therapy is performed using either the UVAR® or XTS™ photopheresis system. Using the UVAR® system, white cells are separated, using leucopheresis, and stored in the buffy coat bag. Six collection cycles are performed, excess red cells and plasma are returned to the patient following each collection. During the procedure, approximately 240ml of leucocyte-rich blood is mixed with 300ml of patients plasma and 200ml of sterile saline. Once completed, the buffy coat bag contains 25-50% of the total peripheral mononuclear cell compartment (Wolfe *et al.,* 1994). The haematocrit (hct) should ideally be between 2.5 and 7%. Following the collection of the first buffy coat, the separated cells are passed through a sterile plastic cassette as a 1mm film. Surrounded by ultraviolet A (UVA) emitting bulbs the cells are exposed to 2J/cm^2 UVA per lymphocyte. The UVA exposure is 180 minutes; however, the UVA exposure only commenses after the final collection of white cells. The treated cells are subsequently re-infused. This process is repeated on a consecutive day, patients returning for treatment either two or four weekly (Edelson *et al.,* 1987)

The XTS™ photopheresis system differs from that of the UVAR® system in that the harvesting of leucocytes (buffy coat) uses a 'collect and elutriation' 6 cycle apheresis system. Following the collection of all six cycles, the collected cells are mixed with saline and placed in the leucopheresis bowl. This further concentrates the buffy coat and removes excess red cell. The concentrated cells are subsequently exposed to a 1.5J/cm^2/cell UVA radiation source, in a plastic photocell, as a film 1mm thick. For the XTS™ system, the exposure time is

calculated by assessment of the volume and hct of the buffy coat. Exposure to UVA is usually calculated to be between 15 and 50 minutes. However longer incubation times can occur if the collection bag contains either a high volume or increased numbers of contaminating red cells.

1.4. ECP's proposed mechanism of action

ECP's mechanism of action is thought to involve the modulation of the immune system (Knobler and Girardi, 2001). Effective treatment using ECP is best achieved in patients who demonstrate immunocompetence. Good CTCL 'responders' typically demonstrate significantly higher baseline absolute CD8+ T cells than 'non-responders' and display normal natural killer cell activity (Gottlieb *et al.,* 1996; Zouboulis *et al.,* 1998; Rook *et al.,* 1999), whilst enhanced response rates are achieved when immune enhancing agents, such as IFNα, rather than potentially immunosuppressive drugs, are used to supplement ECP (Wolfe *et al.,* 1994). In addition, patients exposed to extensive immunosuppressive and chemotherapeutic regimens, prior to ECP, do less well than comparative groups with a low or moderate immunosuppresive history (Wolfe *et al.,* 1994; Rook *et al.,* 1999). Further evidence for the utilisation of the immune system follows the observation that; although ECP therapy is only performed monthly and only exposes 3-5% of the tumour load on any given treatment day (Duvic *et al.,* 1996; Gottlieb *et al.,* 1996), complete responses are observed in 'responders' within 12 months of commencing treatment (Di Renzo *et al* 1997, Zouboulis *et al.,* 1998). Referred to previously as an anti-clonotypic response, ECP is thought capable of inducing a response whereby non treated, but clonal cells are removed (Edelson *et al.,* 1994; Rook *et al.*, 1999)

For CTCL, the presence of the malignant clone within the periphery is essential for effective treatment using ECP. No patients with plaque disease covering greater than 10% of the skin surface area, in the absence of peripheral blood involvement have responded to photopheresis (Wolfe *et al.,* 1994). These observations indicate that direct modification to treated cells as paramount in ECP's mechanism of action.

Following ECP, several cellular changes have been observed in treated mononuclear cells, including induction of apoptosis in treated lymphocytes, secretion of pro-inflammatory cytokines and activation of monocytes (Marks and Fox, 1991, Vowels *et al.,* 1992, Yoo *et el.,* 1996).

1.5. Apoptosis

1.5.1. General features

Apoptosis is a form of cell death distinct from necrosis, where cellular demise occurs without invoking an inflammatory response (Wyllie *et al.,* 1980). In necrosis, the organelles swell and loose the ability to make sufficient ATP. Subsequently the necrotic cell's ability to regulate osmotic pressure is lost and the cell swells and lyses (Cohen *et al.,* 1991). Following lysis, the chemotactic cell content, released by the necrotic cell, attracts a swift inflammatory reaction to clear the debris (Cohen *et al.,* 1991). Apoptosis, conversely involves a loss of cell volume, membrane blebbing and the collapse of the nucleus into very dense chromatin 'beads' or 'apoptotic bodies' (Wyllie, 1988). Because apoptotic cells remain in tact or break up into membrane bound apoptotic bodies the acute inflammatory response is not initiated. Instead, the cells are phagocytosed by the nearest cell capable of it; a swift process which often precedes the death of a cell (detected by vital dyes) (Kerr *et al.,* 1993). The breakdown of the DNA within the apoptotic and necrotic cell highlights the very different nature of these cell-killing processes. Apoptotic cells demonstrate a double-stranded cleavage at the linker regions between nucleosomes, leading to the formation of fragments that are multiples of units comprising 180-200 base pairs, whilst necrotic cells demonstrate a non-selective, random cleavage of the DNA (Afanas'ev *et al.,* 1986; Duvall and Wyllie, 1986)

Apoptosis is an important mechanism in many normal cellular systems. It plays a key role in tissue and organ development during embryogenesis, as well as in adult tissues during cell turnover (Cohen, 1991). Apoptosis has been also observed in some pathological conditions, including virtually all untreated tumours (Wyllie, 1985) and the brains of Alzheimer patients (Cotman and

Anderson, 1995). Necrosis however, is always pathological, being the outcome of catastrophic injury to the cell (Wyllie *et al.,* 1980). Apoptosis is central to the maintenance of lymphocyte homeostasis. Apoptosis provides the mechanism for the deletion of both autoreactive T cells in the thymus (Mac Donald and Lees, 1990) and low responsive B cells in the germinal centre (Liu, *et al,* 1989). Cytotoxic T lymphocytes and NK cells induce apoptosis in cells targeted (Cohen, 1991; Arends and Wyllie, 1991). However apoptosis can be induced by many varied external sources including radiation, cancer chemotherapeutic agents, hyperthermia and cytokine (TNFα) and hormone withdrawal, to name but a few (Kerr *et al.,* 1993). The cellular responses that initiate and drive the cell to an apoptotic death are varied. Depending on the apoptotic trigger and the cell type these pathways can include disruption to mitochondrial function, activation of the death receptors, such as the CD95 (Fas) system and changes in the expression of the pro- and anti-apoptotic genes, such as p53, Bcl-2 and Bax. Changes which ultimately result in activation of the down-stream effectors of death, such as the caspases (Adam and Cory, 1998; Ashkenazai and Dixit, 1998; Evans and Littlewood, 1998; Green and Reed, 1998; Thornberry and Lazebnik, 1998).

1.5.2. Apoptosis induced by death receptors

The cell surface receptors termed 'death receptors' play a central role in instructive apoptosis. Following binding of the appropriate ligand, apoptotic demise is initiated promptly (Ashkenazi and Dixit, 1998). Death receptors belong to the tumour necrosis factor (TNF) receptor gene superfamily, identifiable by the similarity of cysteine-rich extracellular domains (Smith *et al.,* 1994; Gruss and Dower, 1995). Many death receptors have been described and include TNRF1 (Tumour necrosis factor receptor-1), death receptor 3 (DR3) (also called Apo-3), DR4 and DR5 (also called Apo-2). Each death receptor has an appropriate ligand responsible for activation of its respective receptor. Tumour necrosis factor (TNF) and lymphotoxin α bind to TNFR1, whilst Apo3L binds to DR3 and Apo2L (also called TNF-related apoptosis inducing ligand (TRAIL)) binds to DR4 and DR5 (Ashkenazi and Dixit, 1998).

1.5.2.1. CD95 (Fas) Pathway

1.5.2.1.1. CD95's mechanism of action

The best characterised and most researched death receptor is CD95 (Fas, APO-1) (Gruss and Dower, 1995). CD95 is a type I transmembrane glycoprotein belonging to the nerve growth factor/TNF receptor superfamily (Nagata, 1996). The Fas gene was cloned by Yonehara *et al,* as a cell surface molecule that directly mediates apoptosis (Yonehara *et al.,* 1989). A similar gene, termed APO-1, was identified by another group, which was subsequently found to be an identical molecule to the Fas gene (Trauth *et al.,* 1989).

CD95 is expressed on a number of haemopoietic cells, including normal activated T and B cells (Nagata, 1996), thymocytes (Zhou *et al.,* 1997) and malignant T and B cells (Peter *et al.,* 1995). CD95 is also expressed on numerous cell types outside of the immune system, including liver, lung and kidney cells (Watanabe-Fukunage *et al.,* 1992). CD95 is weakly expressed on non-stimulated peripheral blood lymphocytes (Yoshino *et al.,* 1994).

When CD95 (Fas) is crosslinked by Fas-L, CD95 receptor 'death domains' begin to cluster at the receptor site (Huang *et al.,* 1996). An adapter protein called FADD (Fas-associated death domain) binds to the CD95 death domains, through its own death domains (Chinnaiyan *et al.,* 1995). FADD contains 'death effector domains' which cluster with procaspase 8 and lead to the activation to caspase 8 (FLICE) through autoproteloysis by neighbouring caspase 8 molecules (Chinnaiyan *et al.,* 1995). Caspase 8 subsequently triggers downstream activation of the caspase cascade (Boldin et al.,1996; Muzio et al., 1998). (See Figure 1.5.2.1.1.1). The caspases are amongst the most specific proteases, capable of inducing irreversible apoptosis (Allen *et al.,* 1998; Thornberry and Lazebnik, 1998). Caspases contribute to apoptosis by direct disassembly of cell structure and induction of display signals that mark a cell for phagocytosis (See Section 1.5.4) (Thornberry and Lazebnik, 1998). The activated caspase 8 also processes the Bcl-2 family member BID to a form which translocates to the mitochondria, where it promotes dissociation of

cytochrome c (See Figure 1.5.2.1.1.2) (Li *et al.,* 1998). The release of cytochrome c permitting the association of APAF-1 and caspase 9, leading to apoptosis induction (See Section 1.5.3.2) (Li *et al.,* 1997)

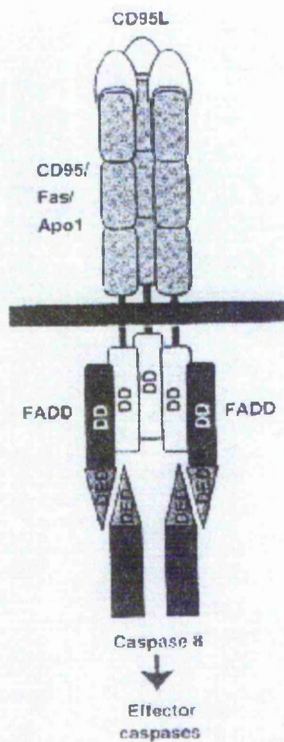


Figure 1.5.2.1.1.1. The induction of apoptosis through CD95 ligation.

Following ligation of CD95, associated 'death domains' (DD) bind to the DD of FADD. The 'death effector domains' (DED) of FADD subsequently activate caspase 8 and lead to apoptosis through a caspase-dependent pathway (Ashkenazai and Dixit, 1998)

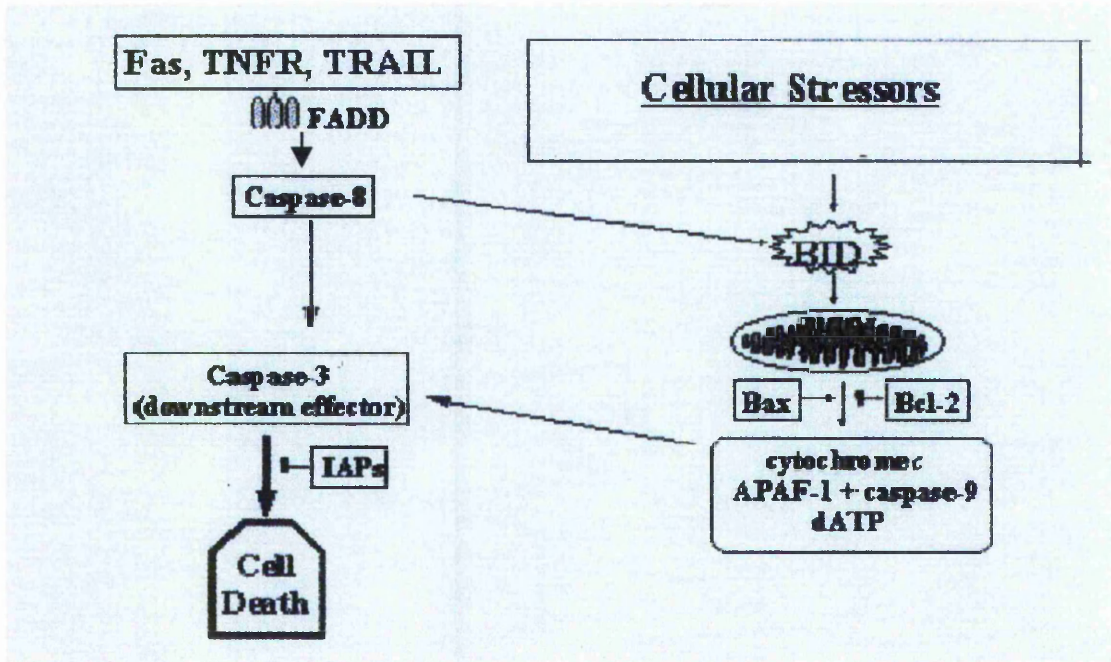


Figure 1.5.2.1.1.2. The interaction of death receptors and the mitochondria in the activation of caspases.

Following ligation of the appropriate death effector (ie Fas-L on CD95), apoptosis is induced as indicated in Figure 1.5.2.1.1.1. Cellular stress can also lead to the induction of apoptosis through the release of cytochrome c from the mitochondria (See Figure 1.5.3.2.1). However, the autoproteolysis of caspase 8 also processes a Bcl-2 family member BID to a form which translocates to the mitochondria, where it promotes the dissociation of cytochrome c. (www.scripss.edu/mem/hema/gottlieb/)

1.5.2.1.2. Activation of the CD95 signalling pathway

The cross-linking of CD95 by Fas-L on target cells can occur by transfer from adjacent activated cytotoxic T cells or by expression on the target cell membrane itself (cell suicide) (Dhein *et al.,* 1995). The CD95 system is critical for growth control of T cells (Dhein *et al.,* 1995). The Fas system is normally involved in both the clonal deletion of autoreactive T cells in peripheral lymphoid organs (Lynch *et al.,* 1997) and the elimination of activated T cells after they have responded to foreign antigens (Nagata and Golstein, 1995). Dysregulation of the Fas/Fas-L system has been implicated in malignant lymphoid conditions, including multiple myeloma (Landowski *et al.,* 1997), non Hodgkins lymphoma (Gronbaek *et al.,* 1998) and acute lymphoblastic leukaemia (Beltinger *et al.,* 1998). In CTCL, peripheral blood CD4+ T lymphocytes demonstrate a decreased expression of CD95, suggesting its progression may due to defective apoptosis, rather than true proliferation (Dereure *et al.,* 2000).

Cellular stress-inducing agents are capable of activating the CD95 ligand/receptor interaction, leading to apoptosis (Friesen *et al.,* 1996). The CD95/Fas-L complex has been implicated in UVB induced apoptosis of lymphocytes. Partial blocking of UVB-induced apoptosis has been achieved using a neutralising anti Fas-L and reduced levels of apoptosis have been observed in a variant cell line resistant to Fas-L following UVB exposure (Caricchio *et al.,* 1998). In addition, Scleroderma patients have demonstrated an enhanced CD95 expression in the CD4+ T lymphocytes population 24 hours post ECP treatment (Aringer *et al.,* 1997).

1.5.3. Mitochondria

1.5.3.1. Mitochondria and apoptosis

The mitochondria are responsible for the bulk of the cell's energy production (Thress *et al.,* 1999). Any event that disrupts mitochondrial function will inevitably lead to cell death (Thress *et.,* 1999). Apoptotic thymocytes demonstrate profound alterations to mitochondria ultrastructure, including

condensed chromatin patterns (Petit *et al.,* 1995). During apoptosis the function of the mitochondria becomes compromised. The apoptotic cell demonstrates an uncoupling of electron transport from ATP production, generation of reactive oxygen species (ROS) and the opening of the mitochondria permeability transition pores (MPT), leading to a disruption of the mitochondrial inner transmembrane potential ($\Delta\psi_m$) (Zamzami *et al.,* 1995; Kroemer *et al.*, 1997; Tada-Oikawa *et al.,* 1998). However, some evidence indicates that ROS production and ATP depletion may reflect a final stage of apoptotic cellular degeneration, rather than an initiating process (Thress *et al.,* 1999). In some cell systems, ATP production can occur late in the apoptotic process, whilst other cells require ATP for the propagation of an apoptotic death signal (Eguchi *et al.,* 1997). In addition, although in some types of apoptotic cell death ROS is increased (Bredesen, 1995) and antioxidants have been demonstrated to inhibit or delay apoptosis (Greenlund *et al.,* 1995), some apoptotic stimuli function in the near absence of oxygen (Jacobson and Raff, 1995), whilst rises in ROS accompanying apoptosis have been observed at a late stage (Kroemer *et al.,* 1995). Although the roles of ROS and electron transport in apoptosis are still unclear, there is little question that the mitochondria do participate actively in the apoptotic cell death through the release of pro-apoptotic factors (Thress *et al.,* 1999).

1.5.3.2. Mitochondria and cytochrome c release

A very important component in the apoptotic process mechanism is the pro-apoptotic factor, cytochrome c. The release of cytochrome c by the mitochondria of apoptotic cells binds to the apoptogenic factor; apoptosis promoting associated factor –1 (APAF-1). This union leads to the activation of caspase 9 and cell death via a caspase cascade dependent process (Li *et al.,* 1997) (See Figure 1.5.4.2.1). However activation of caspase 8 can also lead to the release of cytochrome c from the mitochondria through the cleavage of the Bcl-2 family member, Bid (Li *et al.,* 1998). A mechanism which would lead to the amplification of the apoptotic signal through enhanced caspase activation (Thress *et al.,* 1999). Significantly, caspase inhibitors do not prevent

cytochrome c release induced by several apoptogenic agents including UV irradiation, Staurosporine and over expression of Bax (Bossy-Wetzel *et al.*, 1998; Vander Heiden *et al.*, 1997; Jurgensmeier *et al.*, 1998). Once cytochrome c is released, the cell is destined to die, either by a rapid caspase-dependent mechanism, or a slower necrotic-like process. During the necrotic-like process, the loss of cytochrome c from the mitochondria leads to a collapse of electron transport through the generation of oxygen free radicals and depletion of ATP (Green and Reed, 1998).

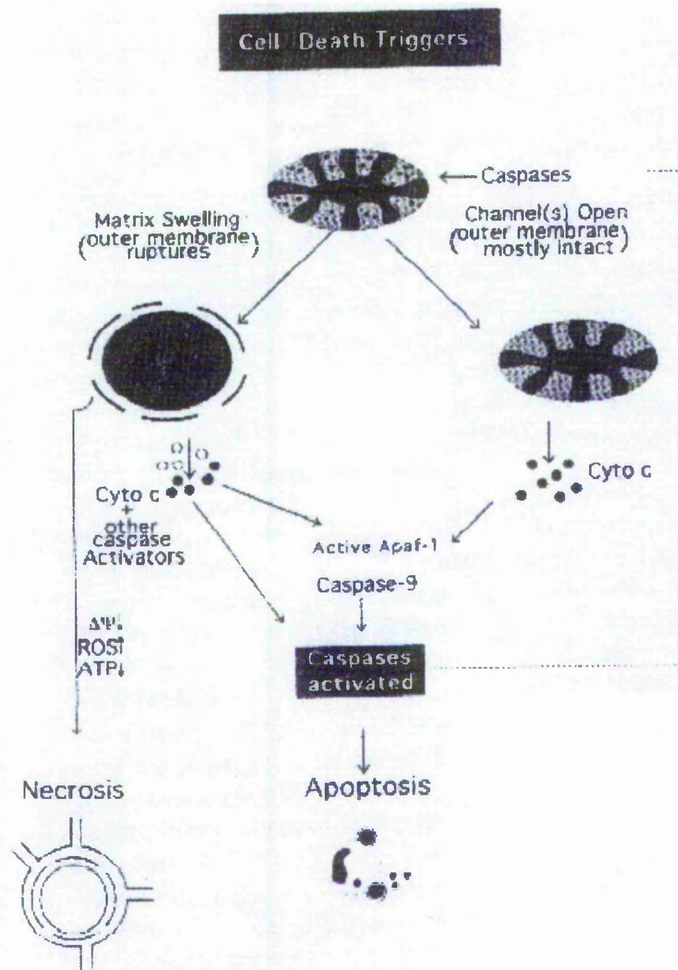


Figure 1.5.3.2.1. The mitochondria and apoptosis induction

Following exposure to cell death triggers, the mitochondria releases cytochrome c; either through the expansion of the matrix and subsequent membrane rupture or by opening of channels in the outer membrane. The release of cytochrome c causes association between APAF-1 and caspase 9, inducing caspase-dependent apoptosis. Alternately mitochondrial rupture could induce a slower non-apoptotic cell death, because of the loss of the electrochemical gradient, increase in ROS, or decline in ATP production (Green and Reed, 1998).

1.5.3.3. Mitochondrial transmembrane potential ($\Delta\psi_m$).

Normally within a cell, a H^+ gradient across the inner mitochondrial membrane maintains a transmembrane potential ($\Delta\psi_m$). Different apoptotic signals can disrupt this potential by initiating the opening of large conductive channels, known as the mitochondrial permeability transition pores (MPT) (Petit *et al.*, 1996; Zamzami *et al.*, 1995). The opening of these pores leads to the osmotic expansion of the mitochondria matrix, leading eventually to membrane rupture and subsequent release of apoptogenic molecules such as cytochrome c (Petit *et al.*, 1998). Inhibiting MPT pore formation appears to block apoptosis, providing support for the important role of MPT formation in the apoptosis process (Zamzami *et al.*, 1996). Many of the Bcl-2 family members reside or are translocated to the mitochondria membrane (Adam and Cory, 1998). Bcl-2 and Bcl-xl has been shown to exert their anti-apoptotic effects, at least in part through inhibition of cytochrome c. A process which may rely on the prevention of MPT formation (Yang *et al.*, 1997; Kluck *et al.*, 1997; Zamzami *et al.*, 1996). Another Bcl-2 family member, Bax, induces both apoptosis and the formation of MPT pores (Zamzami *et al.*, 1996b). Bax can associate with adenine nucleotide translocator (ANT); co-operation between these two proteins can cause channel formation in artificial membranes (Marzo *et al.*, 1998). Of further interest, some pro-apoptotic proteins, such as Bax, can induce mitochondrial damage and subsequent cell death, by apoptosis, even when caspases are inactivated (Xiang *et al.*, 1996). *In-vivo* the presence of a reduced $\Delta\psi_m$ denotes an irreversible stage of pre programmed lymphocyte death (Zamzami *et al.*, 1995). The early detection of a reduced $\Delta\psi_m$ in some cells may indicate that the mitochondria are the primary target during apoptosis (Vayssière *et al.*, 1994).

1.5.3.4. Mitochondrial changes post UV exposure

The exposure of cells to short wavelength form of UVA, termed UVA-1, induces an immediate change in mitochondrial function. UVA-1 exposure causes an early opening of the MPT and subsequent depolarisation of the $\Delta\psi_m$: a process which is thought to be triggered by the damage caused by singlet-

oxygen generation (Godar, 1999). Conversely the delayed apoptotic process, induced by PUVA treatment, is a consequence of the DNA-damage inflicted (Marks and Fox, 1991; Yoo *et al.,* 1996). The DNA damage causes activation of the transcription factor, Apoptosis Protein-1 (AP-1), increasing either expression of Fas, or p53 which indirectly causes mitochondrial pore formation (Godar, 1999).

1.5.4. Caspases

1.5.4.1. Caspases and apoptosis

Caspases are amongst the most specific proteases. The strict specificity of caspases is consistent with the observation that apoptosis is not accompanied by indiscriminate protein digestion, rather a select set of proteins are cleaved in a co-ordinate manner, resulting in a loss or change of function (Thornberry and Lazebnik, 1998). The caspases are a family of proteases whose initial activation triggers a cascade effect, where further caspases are activated, leading ultimately to the death of the cell (Thress *et al.,* 1999; Allen *et al.,* 1998). caspase activation seems to be a common intracellular effector pathway to which different apoptotic systems are in contact. Once activated, the caspase cascade is capable of opposing cellular attempts to stop apoptosis (Allen *et al.,* 1998). Caspases contribute to apoptosis by direct dissemble of cell structure. They cut off contacts with surrounding cells, reorganise the cytoskelton, shut down DNA replication and repair, interrupt splicing, destroy DNA, disrupt the nuclear structure, induce the cell to display signals that mark it for phagocytosis and disintegrate the cell into apoptotic bodies (Thornberry and Lazebnik, 1998).

1.5.4.2. Induction of apoptosis through caspase activation

Induction of the caspase cascade is through one of two distinct mechanisms. Activation of the initiator caspase; procaspase 8 (FLICE) requires association with the cofactor FADD (Fas-associated protein with death domain) through the DED (death effector domain) (Boldin *et al.,* 1996; Muzio *et al.,* 1996). Alternatively procaspase 9 activation involves the cofactor APAF-1 acting on the caspase recruitment domain (CARD) (Li *et al.,* 1997). Cytochrome c and

deoxyadenosine triphosphate are required for the activation of caspase 9. These differences may highlight the necessity of multiple co-factors for the activation of the caspase pathway (Thornberry and Lazebnik, 1998).

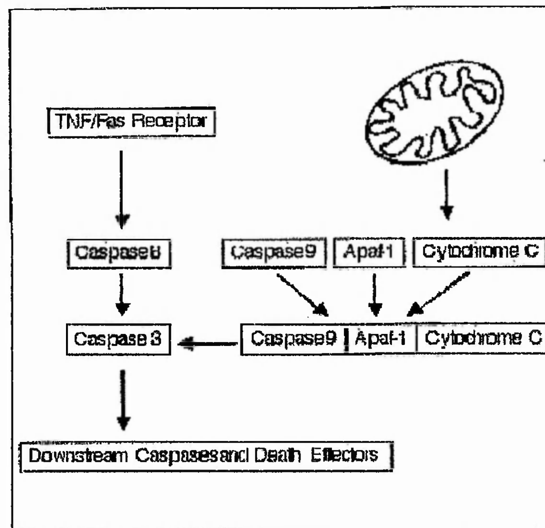


Figure 1.5.4.2.1. Activation of the Caspase cascade

Cytochrome c released from the mitochondria (See Section 1.5.3.2) forms a union with APAF-1 and Caspase 9. This combined unit activates the caspase cascade, including caspase 3, resulting in the fragmentation of DNA in the nucleoli. Alternately, the TNF/Fas receptors activate caspase 8, which bypasses mitochondrial control, to induce apoptotic changes through the activation of downstream caspases, including caspase 3 (Scaffidi et al., 1999).

The caspase family has been proposed to be under the control of the inhibitors of apoptosis (IAP) family of proteins (Roy *et al.,* 1997). The IAP family member, XIAP, inhibits the caspases high in the cascade, whilst c-IAP-1 and c-IAP-2 specifically bind to the death effector proteases; caspase-3 and caspase-6 (Roy *et al.,* 1997).

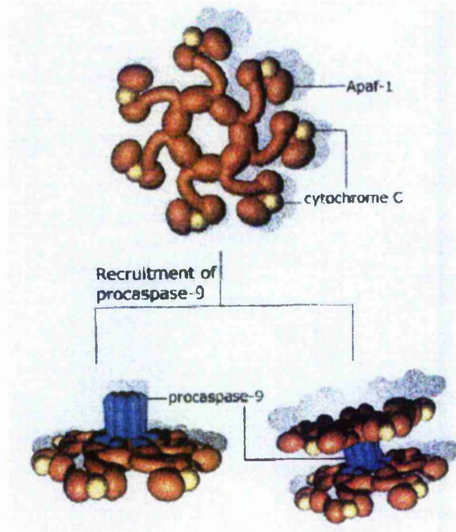


Figure 1.5.4.2.2. Activation of Caspase 9
Cytochrome c released from the mitochondria (See Section 1.5.3.2) binds to APAF-1. Conformational changes to APAF-1 allow other APAF-1 to associate with each other. The wheel-like structure, termed 'apoptosome', binds 7 molecules of procaspase 9. Procaspase 9 is subsequently activated either through the binding process or by the action of neighbouring apoptosomes. (www. sghms.ac.uk)

1.5.4.3. Caspase-independent apoptosis

Apoptosis has been described in the absence of caspase activation. The early apoptosis, as a result of different chemotherapy regimens, was observed in some patients in the presence of the caspase inhibitor (FAM-VAD-FMK). A process which occurred with the simultaneous over-expression of Bax (Stahnke *et al.,* 2001). Bax, a member of the Bcl-2 family, is capable of inducing apoptosis in the presence of caspase inhibition (Xiang *et al.,* 1996). The induction of apoptosis in the absence of caspase activation is most likely to involve the mitochondria (Green and Reed, 1998), possible mediated through the activation of other cysteine proteases, possible calpains (Denecker *et al.,* 2001).

1.5.5. Apoptotic proteins

1.5.5.1. p53

The p53 tumour suppressor gene plays a pre-eminent role in protecting cells from malignant transformation following DNA damage (Moll and Schramm, 1998). p53 acts as the mediator of genomic stability by regulating the G1/S – phase checkpoint during the cell cycle (Yeargin and Haas, 1995). An increase in wild-type p53 protein induces G1 phase cell cycle arrest in some cell types (Kuerbitz *et al.,* 1992). Alternately, in other cell types an elevation in p53 levels causes a cell to die by apoptosis (Lowe *et al.,* 1993). Following DNA damage, wild type p53 transcriptionally stimulates genes acting in cell cycle arrest and senescence, such as $p21^{Waf1/Cip1}$. p21 inhibits both the cyclin dependent kinases (CDK'S) that govern cell cycle progression and PCNS, a processitivity co factor of DNA polymerases δ and ε. (El-Diery *et al.,* 1993; Harper *et al.,* 1993; Xiong *et al.,* 1993). However the p21 mediated mechanism is not involved in apoptosis (Brugarolas *et al.,* 1995, Deng *et al.,* 1995). The apoptosis promoting function of p53 can be mediated by repressing transcription of a broad range of growth and survival genes (Sands *et al*., 1994). p53 can down-regulate the expression of the apoptosis-inhibiting gene, Bcl-2 (Miyashita and Reed, 1995), whilst other targets involved in p53 induced apoptosis include: insulin-like growth factor-I (IGF-I) receptor (Ohlsson, *et al.,* 1989), IL6 (Sands *et* al., 1994), components of the renin-angiotensin system (Pierzchalski *et al.,* 1997) and Bax (Miyashita and Reed, 1995).

In most normal cells, wild type p53 levels are extremely low due to the short half-life (20-30 min) (Moll and Schramm, 1998). Irradiation of cells with ionising radiation or UV light induces high levels of nuclear p53, concomitant with a decrease in replicate DNA (Hall *et al.,* 1993; Kastan *et al.,* 1991). For cells with a high intrinsic proliferation rate, such as cells from haemopoietic or lymphoid lineages, p53 mediated growth control is through programmed cell death or apoptosis (Moll and Schramm, 1998). Exposure to stimuli which increase p53 concentrations result in apoptosis of normal human peripheral blood lymphocytes (Yeargin and Haas, 1995).

Following UVB exposure, cells demonstrate a substantial increase in p53 protein levels, at 24 hours. (Wang *et al.,* 1998), Whilst the early apoptosis induced by UVA-1 is independent of p53 expression (Godar, 1999). The DNA damage caused by PUVA treatment causes an accumulation of p53 and a delayed-type apoptotic process (Godar, 1999).

1.5.5.2. The Bcl2 family

The Bcl-2 gene was initially found in a proportion of B-cell follicular lymphomas (Tsujimoto, 1984). Since then, at least 15 Bcl-2 family members have been identified in mammalian cells (Chao and Korsmeyer, 1998), some of which promote apoptosis and others which inhibit apoptosis (Allen *et al.,* 1998). All Bcl-2 family members' posses at least one of four Bcl-2 homology domains, named BH1 to BH4. The members most similar to Bcl-2 contain all four, although a requirement of BH1 and BH2 is sufficient to provide pro-survival characteristics (Adams and Cory, 1998). The 'BH3 domain' proteins may well represent the antagonists of the pro-survival proteins. Pro and anti-apoptotic family members can heterodimerise with each other and thus influence one another's function. Mechanisms that act as a rheostat for cell death and survival (Oltvai *et al.,* 1993).

<u>*1.5.5.2.1. Bcl-2*</u>

Bcl-2 can inhibit apoptosis induced by a variety of stimuli and in many cell types, including those of the haemopoietic lineage. (Vaux *et al.,* 1988; Nunez *et al.,* 1990). Initially, Bcl-2 was thought capable of protecting a cell from apoptosis by scavenging free radicals following cellular or oxidative damage (Hockenbery *et al.,* 1993). However the subsequent discovery that Bcl-2 can inhibit apoptosis in nearly anaerobic conditions, may indicate that this process may be a down-stream consequence, rather than a primary function of Bcl-2 (Jacobson and Raff, 1995; White, 1996). Bcl-2 has also been demonstrated to preserve mitochondrial potential and block calcium movement into the cytoplasm; two mechanism which can perpetuate a cell to death by apoptosis (Lam *et al.,* 1994; Zamzami *et al.,* 1995). Bcl-2 can directly or indirectly inhibit

the release of cytochrome c from the mitochondria (Zamzami *et al,* 1998); a process which would prevent the activation of caspase 9 through APAF-1 association with CARD (Caspase recruitment domain) (Li *et al.,* 1997). However the inhibition of cytochrome c release is unlikely to be an isolated mechanism utilised by Bcl-2, because Bcl-2 can protect some cells after cytochrome c release or after microinjection of cytochrome c. (Green and Reed, 1998). Current theories for the regulation of apoptosis by Bcl-2 family members rely heavily on the ratio of death promoter to death inhibitor (Oltvai *et al.,* 1993; Boyd *et al.,* 1995, White, 1996). Bax, another member of the Bcl-2 family is associated with apoptosis induction (White, 1996).

1.5.5.2.2. Bax

Bax promotes apoptosis by causing MPT pore formation and the subsequent release of cytochrome c leading ultimately to activation of caspase-3 (Rossé *et al.,* 1998). The protein product of Bcl-2 and Bax are intimately linked, able to homo-and heterodimerise with each other. An excess of Bcl-2/Bax heterodimers inhibits apoptosis whilst Bax/Bax homodimers enhance the apoptotic process (Oltvai *et al.,* 1993). The ratio of Bcl-2 to Bax provides an index of how sensitive some cells are to apoptosis following stimulation (Van der Vliet, *et al.,* 1997). The treatment of mycosis fungiodes cells with a Jak kinase inhibitor Ag490 induced apoptosis in conjunction with a 54% decrease in Bcl-2 and a 128% increase in Bax expression (Nielson *et al.,* 1999), whilst a marked increase in Bax expression has accompanied the very early apoptotic lymphoid cells observed post chemotherapy exposure (Stahnke *et al.,* 2001). The over-expression of Bax is capable of inducing apoptosis in a caspase independent manner (Xiang *et al.* 1996). Alternatively Bax and Bax-like protein, including Bcl-x, cause death through the promotion of mitochondrial permeability transition (Green and Reed, 1998). Exposure of cells to UVA radiation has induced an 'immediate-type' apoptotic response. These cells also demonstrated a concomitant down-regulation of Bcl-2; Bcl-2 expression reduced by 40% at 4 hours post UVA exposure (Wang *et al.,* 1998).

1.6. ECP induced lymphoid apoptosis and immunomodulation

1.6.1. ECP induced apoptosis

The ability of ECP to induce apoptosis in lymphoid cells was first identified by Marks and Fox in 1991 who reported distinctive apoptotic cell morphology and DNA fragmentation in cultured cells exposed to *in-vitro* ECP-like conditions (Marks and Fox, 1991). Further evaluation of lymphocytes, following *in-vivo* ECP or after exposure to ECP-like condition *in-vitro*, have confirmed the presence of apoptosis (Yoo *et al.,* 1996). Histological examination of skin biopsies, from ECP treated mycosis fungoides patients, have also demonstrated a significant increase in apoptosis following treatment (Mirraco *et al.,* 1997). All reports demonstrate the presence of apoptotic lymphocytes some 24 hours following UVA/8MOP exposure; their numbers increasing significantly further after 48 hours in culture (Marks and Fox, 1991; Yoo *et al.,* 1996; Aringer *et al.,* 1997). However no evidence of apoptosis was detected in ECP treated lymphocytes tested immediately after the procedure (Aringer *et al.* 1997; Enomoto *et al.* 1997) or 6 hours post ECP (Yoo *et al.* 1996). If these cells were still viable for some days, they would remain undetected by the reticuloendothelial (RE) system and have the capacity to modulate the immune system through release of cell signalling protein (Enomoto *et al.* 1997). In addition, because normal cells are replaced at a more rapid rate than the malignant cells of CTCL patients migrate to the periphery, removal of the malignant clone by ECP may depend on the repeated treatment cycles (Yoo *et al.,* 1996).

1.6.2. Immunomodulation post ECP

Following ECP, phagocytosed apoptotic lymphocytes have been observed within the ECP treated monocyte/macrophages and monocyte-derived dendritic cells (Yoo *et al.,* 1996; Berger *et al.,* 2001). Phagocytosis by APCs is a natural process for the removal of unwanted apoptotic cells. The processing and presentation can lead to the generation of an anti-clonotypic cytotoxic T cell clone, capable of removing the untreated, but clonal, malignant cells (Liu *et al.,* 1992; Albert *et al.,* 1998; Henry *et al.,* 1999). A response supported by the

immunocompetent profile required of ECP 'responders' (Gottlieb *et al.*, 1996; Zouboulis *et al.*, 1998; Rook *et al.*, 1999)

1.7. CD10 expression in apoptosis

Peripheral lymphocyte expression of membrane CD10 is most usually associated with cells demonstrating the common acute lymphoblastic leukaemia antigen (CALLA) phenotype (Shipp *et al.*, 1989). However more recently, induction of CD10 expression on lymphocytes has been linked to growth arrest (Mari *et al.*, 1997) and apoptosis (Cutrona *et al.*, 1999). The presence of CD10, on an apoptotic lymphocytes, *in vivo*, may promote the cleavage of inflammatory or pro-inflammatory mediators released by the dying cell, limiting the level of inflammation at the site, or act as a target antigen for macrophage recognition and phagocytosis (Cutrona *et al.*, 1999). ECP induces lymphoid apoptosis (Yoo *et al.*, 1996) and up-regulation of some pro-inflammatory cytokines, particularly IL1 (Wolfe *et al.*, 1994) IL6 and TNFα (Vowels *et al.*, 1992). The expression of CD10, on the re-infused dying lymphocytes, may therefore have a direct influence on the *in-vivo* ECP process.

1.8. Heat shock proteins

1.8.1. Heat shock proteins in apoptosis and immunomodulation

Heat shock proteins perform an important protective role, both in cells growing under optimal conditions and those exposed to less favourable environments. The role of HSPs include the prevention of protein aggregation, protection of proteins from denaturisation by stress-inducing agents and participation in the repair or degradation of polypeptides that have become denatured under stress (Schlesinger, 1990; Craig *et al.*, 1994). HSPs are intracellular proteins and most of their known functions are connected to intracellular processes (Sapozhnikov *et al.*, 1999). However HSPs have been observed on the plasma membrane of both normal and tumour cells (Erkeller-Yeksel *et al.*, 1992; Ferrarini *et al.*, 1992). Although their role here is unclear there is evidence they can stabilise lipid membrane and prevent denaturation of membrane-localized proteins during stress (Török *et al.*, 1997). Recently a positive relationship between the

level of HSP expression and the onset of apoptosis has been described (Chant *et al.*, 1996). However the role of HSPs as protectors or assistants of apoptosis is still unclear. HSPs may provide some protection at the early stages of stress induced damage, but are unable to provide complete protection once the apoptotic cascade is initiated (Samali and Cotter, 1966).

Alternatively HSPs may play a role in the immunmodulatory role of apoptosis. Recently it has been demonstrated that the immunogenicity of apoptotic leukaemia cells is enhanced by the expression of HSPs. Apoptotic cells expressing HSPs induced a significantly more effective anti-tumour response, than apoptotic cells without up-regulated HSPs (Feng *et al.,* 2001). The presence of HSP 72 on the surface of tumour cells may act in an MHC – unrestricted manner, as a tumour specific recognition structure for distinct NK cell populations (Multhoff *et al.,* 1997).

1.8.2. Heat shock proteins and cytokine secretion

Monocytes exposed to HSP 70 up-regulate production of some pro-inflammatory cytokines, including TNFα and IL6, suggesting that the intracellular production of HSP 70 in monocytes would promote the same effect (Asea *et al.*, 2000). Enhanced expression of TNFα has anti-tumour activity, which can be directed against leukaemia and lymphoma cells (Fransen *et al.*, 1986).

Following ECP, lymphocytes become apoptotic (Marks and Fox, 1991; Yoo *et al.*, 1996) and monocytes up-regulate secretion of IL6 and TNFα (Vowels *et al.*, 1992). Two prominent mechanism thought to be involved in the mechanism leading to modulation of the immune system and the removal of untreated, clonal T cells (Hanlon *et al.*, 1998; Berger *et al.*, 2001; Rook *et al.*, 1999)

1.9. Antigen presentation

The processing of antigens by APCs ultimately leads to the presentation to T cells and immune recognition (Liu *et al.*, 1992). When dendritic cells acquire

antigens from apoptotic cells a cytotoxic response against similar antigens is induced (Albert *et al.,* 1998), whilst processing of apoptotic cells, by activated monocytes, involves antigen presentation to T cells, inducing immune recognition of similar antigens (Henry *et al.,* 1999). However to fully activate and induce proliferation, the presentation of antigens to T cells requires the presence of co-stimulatory signals. In the absence of co-stimulatory signals T cells presented with an antigen may enter a state of unresponsiveness called anergy (Rattis *et al.,* 1998). Interactions between ICAM-1 (CD54) and its ligand lymphocyte function-associated antigen (LFA-1) on leucocytes constitutes an important stage in T cell activation and the generation of an immune response (Boyd *et al.,* 1988). ICAM-1 expression on monocytes is inducible by activation and adherence and there is evidence to suggest that this may play a role in antigen presentation (Dougherty *et al.,* 1988). Transfection studies have, similarly, identified CD80 and CD86 as distinct co-stimulatory ligands incorporated in T-cell proliferation and the subsequent generation of cytotoxic T cell (Lanier *et al.*, 1995). CD80 and CD86 are thought capable of activating Th1 and Th2 cells respectively (Freeman *et al.,* 1995; Ranger *et al.,* 1996). Blocking experiments using monoclonal antibodies to CD86 interfere with a prostaglandin driven dendritic-T-cell interaction that ultimately leads to a Th2 like reaction (Ullrich *et al.,* 1998). In addition co-stimulation by CD86 enhances the production of serum IL-10, thus suppressing Th-1 cell-mediated immune reactions (Ullrich *et al.,* 1998) and blocking anti CD80 interaction, during T cell activation, induces functional inactivation or anergy (Chen and Nabavi *et al.,* 1994). An autoimmune condition, experimental allergic encephalomy (EAE) in a mouse model is induced by Th1 cells, but disease prevention can be achieved by Th2 cells. Using anti CD80 and anti CD86 in the EAE model, the suppression of CD80 reduces the incidence of the disease and is associated with T cells that produce 4-5 fold more IL4 than PBS. Whilst T cells derived after prolonged exposure to anti CD86 demonstrate an increased severity of EAE and a 2-3 fold upregulation of IFNγ secretion (Kuchroo *et al.,* 1995).

Exposure of monocytes to UV irradiation inhibits the levels of ICAM-1 and the subsequent ability of the monocyte to stimulate T cells (De Luca *et al.,* 1997). Whilst the up-regulation of CD80 and CD86 following allogeneic mixed lymphocyte reaction experiments was inhibited by prior exposure to UV-B exposure (Fujihara *et al.,* 1996). Processes that may account for some of the immunosuppressive responses observed in APCs following UV therapy.

1.10. The role of monocytes in ECP

Monocytes are resistant to apoptosis induced by ECP (Yoo *et al.,* 1996; Tambur *et al.,* 2000). Fas and Fas-L expression in treated monocytes remains unchanged 16 hours post ECP (Tambur *et al.,* 2000). Paradoxically monocytes are activated by ECP, demonstrating enhanced secretion of TNFα and up-regulation of CD36 (Vowels *et al.,* 1992; Fimiani *et al.,* 1997). TNFα is capable of direct anti-tumour activity, which can be directed against leukaemia and lymphoma cells (Fransen *et al.,* 1986). Enhanced CD36 expression on APCs has been implicated in the recognition process for apoptotic cells (Savill *et al.,* 1992). APCs expressing CD36 are capable of activating autologous T cells, mainly CD8+T cells (Shen *et al.,* 1983; Pimpinelli *et al.,* 1991). ECP-treated monocytes/macrophages/dendritic cells also demonstrate an increased ability to phagocytose apoptotic T cells (Yoo *et al.,* 1996, Berger *et al.,* 2001). These changes may indicate that treated monocytes have a role in the immunomodulation process, proposed for ECP. The processing of antigens by APCs ultimately leads to the presentation to T cells and immune recognition (Lui, 1992). When APCs acquire antigens from apoptotic cells a cytotoxic response against similar antigens is induced (Albert *et al.,* 1998; Henry *et al.,* 1999). Monocyte-derived dendritic cells, observed following ECP, demonstrate a significant alloreactive lymphocyte proliferative response and enhanced expression of membrane MHC class II molecules. Processes which may lead to the anti-clonotypic responses associated with ECP (Rook *et al.,* 1999; Berger *et al.,* 2001).

1.11. Cytokines

Cytokines are secreted regulatory proteins that control the survival, growth and differentiation and effector function of tissue cells. Cytokines encompass those families of regulators variously known as growth factors, colony-stimulating factors, interleukins, lymphokines, monokines and inteferons. Using secreted cytokines the producer cell transmit appropriate 'messages' to the recipient cell, where the cytokine is received by appropriate receptors (Nicola, 1994). Cytokines are the major modulators of immune response and inflammatory process in human disease (Dinarello and Mier, 1987; Beutler and Cerami, 1989)

1.11.1. Tumour necrosis factor alpha (TNFα)

TNFα is a multifunctional cytokine that modulates inflammatory and immunological processes important in host defence and can provide protection against infections (William *et al.,* 1999). TNFα belongs to a whole family of related polypeptides that includes Fas-L, lymphotoxin, CD27-L, CD30-L, CD40-L and TNF related apoptosis inducing ligand (TRAIL) (Nagata and Golstein. 1995). TNFα is produced by monocytes or by T and B cells and has pro-inflammatory activity (Vassalli, 1992). TNFα mediates its cytolytic action by high-affinity binding to TNF receptors (TNFR) that are expressed on target cells (Williams *et al.,*1999). Binding of TNFα to TNFR leads to apoptosis (Tartaglia *et al.,* 1993). TNFα can induce apoptosis in human leukaemic U937 cells (Wright *et al.,* 1992). TNFα recruits both the caspase cascade to promote apoptosis as well as generating ceramide, which also promotes apoptosis (Williams *et al.,* 1999).

TNFα has been shown to produce tissue damage in a skin explant model of acute GvHD and is directly related to the level of histological damage (Dickinson *et al.,* 1991). Macrophages in animals with GvHD are primed to release TNFα after stimulation with LPS (Nestel *et al.,* 1992). Whilst the pre-transplant conditioning regimen in clinical bone marrow transplantation (BMT), often total body irradiation (TBI) or chemotherapy or both, damage the tissues,

causing the production of TNFα (Xun *et al.,* 1994). In addition, injury to the bowel may have enabled bacterial breakdown products like LPS to enter the circulation and reach immune compartments and induce further TNFα release (Krenger and Ferrara, 1996). TNFα has also been implicated in the development of chronic GvHD (Barak *et al.,* 1995)

1.11.2. Interleukin 6 (IL6)

Interleukin-6 (IL6) is a pleiotropic cytokine that acts on a wide variety of cell types (Horn *et al.,* 2000). IL-6 is a major mediator of acute phase responses, leading to the production of the acute phase proteins. In addition, IL-6 is involved in the regulation of differentiation, proliferation and survival of many cells, including those of the lymphoid series (Hirano, 1998). Following ECP, IL6 in addition to TNFα has been upregulated in monocytes (Vowels *et al.,* 1992). A mechanism which may add to the febrile response sometimes observed post ECP (Vowels *et al.,* 1992). IL-6 has been described as a good marker for the acute GvHD severity or for the occurrence of transplant related complications, particularly severe infectious complications (Abdullah *et al.,* 1997).

1.11.3. Interleukin 12 (IL12)

IL12 is a cytokine produced by monocyte-macrophages, dendritic cells and other APCs (D'Andrea *et al.,* 1993). IL12 is a powerful inducer of IFNγ production (Trinchieri, 1998). IL12 augments natural killer cytotoxicity (Kobayashi *et al.,* 1989) and cytotoxic T cell proliferation and function (Chouaib *et al.,* 1994). IL12 has significant anti-tumour effect *in-vivo* against transplantable and primary tumours. Further more, mice cured of tumours by IL12 treatment can reject re-challenges with the same tumour cells and acquire tumour specific protective immunity (Nishimura *et al.,* 1996). Following stem cells transplantation, IL12 has been demonstrated to have the potential to mediate anti-tumour effects against residual lymphoma without compromising the lymphohaemopoietic recovery (Verbik *et al.,* 1996).

The peripheral blood cells of patients with advanced CTCL demonstrate a deficiency of IL12 (Rook *et al.,* 1995). The addition of IL12 to the PBMCs of Sezary syndrome patients resulted in enhanced NK activity (Rook *et al.,* 1995). Subsequent clinical trials using recombinant IL12 has been demonstrated to induce lesion regression in CTCL patients, a treatment process well tolerated and associated with anti-tumour cytotoxic T-cell responses (Rook *et al.,* 1999b).

Treatment of BMT recipient mice with IL12 for five days on the day of transplant lead to a marked protection against chronic GvHD (Via *et al.,* 1994). However treatment of IL12 for a longer period, starting the day before BMT converted the chronic GvHD to a lethal acute GvHD-like syndrome (Williamson *et al.,* 1996). IL12 inhibitory effect on GvHD is thought to be due to the inhibition of the activation and expansion of donor T cells that are normally seen in untreated GvHD mice (Sykes *et al.,* 1995; Dey *et al.,* 1998). Fas–mediated apoptosis of donor T cells are thought to play role in the GvHD protection by IL12 (Dey *et al.,* 1998).

1.11.4. Interferon gamma (IFNγ)

PBMCs from Sezary syndrome patients demonstrate depressed IFNγ and IL2 production (Rook *et al.,* 1995). The malignant clone typically demonstrates a Th2 type cytokine profile (Vowels *et al.,* 1994). Th2 cytokines production has the effect of down-regulating the release of Th1 cytokines, including IFNγ (Rook *et al.,* 1995). Down-regulation of IFNγ suppresses anti-tumour responses, such as cell-mediated cytotoxicity, Natural Killer (NK) and Lymphokine Activation Killer (LAK) cell activation. (Gajewski and Fitch, 1988; Peleman *et al.,* 1989). IFNγ also plays an important role in enhancing the tumouricidal-mediated cytotoxicity role of monocytes (Philip and Epstein, 1986).

IFNγ produced by activated alloreactive T cells plays an important role in the induction of acute GvHD (Allen *et al.,* 1993). High levels of both IFNγ and

TNFα correlate with the most intense cellular damage associated with aGvHD (Dickinson *et al.,* 1991). The addition of an anti-IFNγ monoclonal antibody prevented the intestinal damage associated with GvHD (Mowat, 1989). IFNγ release is also thought to prime the macrophages to produce TNFα, following stimulation with LPS, following the conditioning process prior to transplant (Gifford and Lohmann-Matthes, 1987), whilst the frequent transcription of IFNγ in cutaneous chronic GvHD supports its potential role in mediating the associated tissue injury (Ochs *et al.,* 1996). Such that IFNγ is the predominant cytokine demonstrated in patients with cGvHD and in mice cGvHD models. (Parkman, 1998).

1.12. Cytokine modulation following ECP

In early stage CTCL, the lymphocytes demonstrate no polarisation toward a Th1 or Th2 cytokine profile. However as the disease progresses toward the systemic leukaemic phase, termed Sezary syndrome, the number of IFNγ- producing T cells decreases (Rook *et al.,* 1993). The predominance of a Th2 cell phenotype in CTCL perpetuates the conditions. The proliferating T cells production of Th2 cytokines inhibit the release of the Th1 cytokines, IL2, IFNγ (Rook *et al.,* 1995) and IL12 (D'Andrea *et al.,* 1993). IL4's inhibition of IL2 and IFNγ, directly suppressed cell-mediated cytotoxicity, NK and LAK cell activation (Gajewski and Fitch, 1988; Peleman *et al.,* 1989). The Th1 cytokine, IL10 inhibits Th1 cytokines in two ways; indirect, by reducing IL12 levels, a known potent inducer of IFNγ production and direct as IL12 can augment NK activity directly (Kobayashi *et al.,* 1989).

The modification to cytokine production following exposure to 8-MOP and UVA is controversial. PBMCs treated with *in-vivo* and *in-vitro* PUVA and subsequently stimulated with lipopolysaccharide have demonstrated suppression of TNFα and IL6. Northern blot analysis revealed a down-regulation of mRNA encoding for IL6 and TNFα (Neuner *et al* 1994). However, the assessment of ECP treated monocytes, with and without IFNγ stimulation, demonstrate an

enhanced production of both IL6 and TNFα (Vowels *et al.,* 1992). Similar results are also observed with IFNγ. PBMCs exposed to UVA/8MOP and subsequently stimulated with Con A demonstrate a skewing toward Th1 cytokine production (Tokura *et al.,* 1999), whilst PBMCs, pre-stimulated with anti CD3, rhIL-2 and rhIL4 prior to 8MOP/UVA, and subsequently stimulated with PMA, ionomycin and monensin demonstrated an opposite shift from Th1 to Th2 in the CD4+ T cells (Klosner *et al.,* 2001). Regression of Cutaneous T cell lymphoma lesions has been achieved using recombinant IL12 (Rook *et al.,* 1999b).

1.13. Aim of the project

This study's aim was to try to further understand the mechanisms, which are responsible for the positive clinical outcomes of patients treated using ECP. A deeper understanding of these mechanisms may help in the development of new technologies based on the ECP theory; technologies that may improve the morbidity and mortality of patients currently receiving ECP treatment or increase the repertoire of diseases which may benefit from ECP.

With the aid of prior knowledge of ECP-induced lymphoid apoptosis, the studies initial goals were to determine:

i) The onset and extent of apoptosis induction post ECP, and

ii) The cellular processes responsible for ECP-induced apoptosis.

Using the acquired knowledge about the apoptotic processes induced by ECP, to further evaluate and understand what influence ECP may have on cell signalling and immunomodulatory processes, by assessing:

The effect of ECP on the cytokine secretion patterns of treated cells.

The influence ECP may have on the antigen-presenting processes of monocytes.

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Clinical chemicals

Crawford Pharmaceuticals, Milton Keynes, Buckinghamshire, UK

Oral 8-Methoxypsoralen (8-MOP)

Ben Venue Laboratories, Bedford, Ohio, USA

Liquid 8-MOP (UVADEX)

CP Pharmaceuticals Ltd., Wrexham, Clwyd, UK

Heparin infusion

2.1.2. General chemicals

Sigma-Aldrich Chemical Company, Poole, Dorset, UK.

Phosphate buffered saline (PBS) sachets, Phorbol 12-Myristate 13-Acetate (PMA), Ionomycin, Brefeldin A, Rhodamine 123, Carbonyl cyanide m-chlorophenlyhydrazone (mC1CCP), Dimethyl Sulphoxide (DMSO)

Molecular Probes, Eugene, Oregon, USA

Carboxy-SNARF-1-AM (126208-13-17 Spirol [7H-benzo[c]xanthene-7,1'(3H)-isobenzofuran]-ar'-carboxylic acid,3-(acetyloxy)-10-(dimethylamino)-3'-oxo, (acetyloxy)methyl ester, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide)

Fresenius Kabi Ltd., Warrington, Cheshire, UK

0.9% Saline

Nycomed, Oslo, Norway

Lymphoprep density gradient.

Merck, West Drayton, Middlesex, UK

Glutaldehyde, 0.12M Millonigs Phosphate Buffer (MPB), Agar, Osmium Tetroxide, Gelatin, Sodium Tretraborate, 1% Toluidine Blue, Xylene, Reynolds Alkaline Lead Citrate, Uranyl Acetate

Genta, Tockwith, North Yorkshire, UK

Absolute alcohol

Taab Laboratories Equipment, Aldermaston, Berkshire, UK.
EMIX epoxyresin, EMIX medium hardener, BDMA accelerator:

2.1.3. Flow cytometry stains/antibodies

DAKO Ltd, High Wycombe, Buckinghamshire, UK
PE-CY-5-conjugated anti CD4, PE-CY-5-conjugated anti CD8, FITC-conjugated anti CD10, PE-conjugated anti CD14, FITC-conjugated anti CD45, FITC-conjugated anti CD95, FITC-conjugated anti CD86, FITC-conjugated anti p53, FITC-conjugated anti Bcl-2
Appropriate isotype controls were also purchased

Immunotech, High Wycombe, Buckinghamshire, UK
FITC-conjugated anti CD3, PE-conjugated anti CD14, FITC-conjugated anti CD45, FITC-conjugated anti CD54, FITC-conjugated anti CD80, unconjugated anti Bax. Annexin V/Propidium Iodide apoptosis detection kit
Appropriate isotype controls were also purchased

Pharmingen, Becton & Dickinson, Cowley, Oxfordshire, UK
Unconjugated anti Fas-L
An appropriate isotype control was also purchased

Caltag Medsystems Ltd, Towcester, North Hampshire, UK
FITC-conjugated goat anti-mouse antibody
An appropriate isotype control was also purchased

Ylem, Gramsci 56-00197, Roma, Italy
Apoptest™ apoptosis detection kit.

Stratech Scientific, Luton, Bedfordshire, UK
Unconjugated anti heat shock protein (HSP) 70(72)
An appropriate isotype control was also purchased

R & D systems, Abingdon, Oxfordshire, UK
rhIFNγ, FITC-conjugated anti TNFα, FITC-conjugated anti IL6, FITC-conjugated anti IL12 and FITC-conjugated anti IFNγ.
Appropriate isotype controls were also purchased.

Oxford Biotechnology, Kidlington, Oxford, Oxfordshire, UK
Cell permeabilisation kit

Intergen Company, Oxford, Oxfordshire, UK

CaspaTag™ Fluorescein Caspase activity kit

2.1.4. Cell culture chemicals

Biowhittaker Ltd, Wokingham, Berkshire, UK

RPMI cell culture medium, Glutamine, Fetal calf serum (FCS)

Lilly, Basingstoke, Hampshire, UK

Vancomycin

Evans, Leatherhead, Kent, UK

Streptomycin

2.1.5. Other consumables

All distilled water was obtained from the Elgar water purification system (Vivendi, Wirksworth, Derbyshire, UK)

Becton & Dickinson, Cowley, Oxfordshire, UK

4.5ml heparin vacutainer® sample tubes, syringes and needles

DAKO Ltd, High Wycombe, Buckinghamshire, UK

Plastic 3.5ml (55mm x 12mm) flow cytometry tubes, hydrophobic pen

Sarstedt, Beaumont Leys, Leicestershire, UK

Plastic 5ml (75mm x 12mm) test tubes, plastic 7ml (50mm x 16mm) cell culture tubes and lids

L.I.P., Shipley, West Yorkshire, UK

Plastic 20ml (110mm x 16mm) centrifugation tubes, 1-5ml pipette tips

Taab Laboratories Equipment, Aldermaston, Berkshire, UK.

Heavy duty single edged blade, Plastic gelatin 'capsules'

VWR International Ltd, Lutterworth, Leicestershire, UK.

50 ml Volumetric tripour polythene beaker

Alpha, Eastleigh, Hampshire, UK

10-200μl pipette tips

Elkay, Basingstoke, Hampshire, UK

200-1000μl pipette tips

Laboratory Sales, Rochdale, Lancashire, UK

Glass slides

Thermo life Science, Basingstoke, Hampshire, UK

1-10μl pipette tips

Bayer Diagnostics, Newbury, Berkshire, UK

Cytospin filter paper and plastic cytospin holders

Seton healthcare gp. plc. Knutsford, Cheshire, UK

Aseptic cleaning Sterets

Minnisart, Gottingen, Germany

Syringe and filters for cell culture prep

2.1.6. Equipment

2.1.6.1. Clinical equipment

Therakos Europe, Bracknell, Berkshire, UK

UVAR® and XTS™ ECP systems

2.1.6.2. Laboratory equipment

Beckman-Coulter, High Wycombe, Buckinghamshire, UK

EPICS® Flow cytometer

DAKO Ltd, High Wycombe, Buckinghamshire, UK

Galaxy® flow cytometer

Bayer Diagnostics, Newbury, Berkshire, UK

Automated cell counter (Advia 120™), Cyto-tek® cytospin centrifuge

Sorvall Hareus, Bishop Stortford, Hertfordshire, UK

Centrifuge

Grant instruments (Cambridge) Ltd., Shepreth, Cambridgeshire, UK.

Waterbath

Olympus Diagnostics Systems, London, UK.

Olympus BH2 light microscope, Olympus BX41 Fluorescent microscope, Olympus Camedia Digital Camera.

Spot, Sterling Heights, Michigan, USA

Microscope photographic equipment

Annachem Ltd., Luton, Bedfordshire, UK

Gilson pipettes

Ohaus UK Ltd., Beaumont Leys, Leicestershire, UK.

Weighing scale

FEI Company, Eidhoven, The Netherlands.

Phillips 400T electron microscope:

Leica Microsystems Ltd (UK), Milton Keynes, Buckinghamshire, UK.

Ultracut E ultramicrotome:

Falc, Treviglio (BG), Monzio Compagnoni, Italy.

Hot plate

Taab Laboratories Equipment, Aldermaston, Berkshire, UK.

Oven

2.2. Methods

2.2.1. Photopheresis equipment, sampling and sample preparation

2.2.1.1. Clinical and normal samples.

Samples were obtained from many patients receiving extracorporeal photopheresis for several conditions including Cutaneous T cell lymphoma (CTCL), Graft versus host disease (GvHD) and Scleredema. Diagnosis for each condition was based on established histological and immunological criteria. CTCL patients were staged according to the Bunn and Lambert Staging system. Using this system each stage of CTCL is identified as outlined below:

Stage IA: Eczematous patches, papules or limited plaques covering less than 10% or more of the skin surface.
No clinically abnormal peripheral lymph nodes and pathology for CTCL is negative.
No involvement of visceral organs.

Stage IB: As for Stage IA, except erythematous patches, papules, or generalised plaques cover 10% or more of skin surface.

Stage IIA: As for stage I, except clinically abnormal peripheral lymph nodes are visible; pathology of which is negative for CTCL.

Stage IIB: As for stage I, except one or tumours are present. Abnormal peripheral lymph nodes may be present, but pathology for CTCL is negative.

Stage III: As for stage I, except a generalised erythroderma is present. Abnormal peripheral lymph nodes may be present, but pathology for CTCL is negative.

Stage IVA: The skin involvement can present as any prior stage. Abnormal peripheral lymph nodes may be present, but pathology for CTCL is positive. No involvement of visceral organs.

Stage IVB: The skin involvement can present as any prior stage. Abnormal peripheral lymph nodes may be present and pathology for CTCL can be positive or negative. Viseral involvment of organs is confirmed by pathology.

Each chapter will identify the cohort of patients tested. Where normal controls were tested they were age and sex matched, with no known haematological or inflammatory conditions. No controls had recently taken any anti-inflammatory medication. Informed consent was obtained from all patients and controls. Local ethical approval was granted for the study.

2.2.1.2. Photopheresis treatment

Photopheresis was performed using the UVAR®or XTS™ ECP systems. Which machine was used for each study will be stated at the beginning of the chapter.

2.2.1.2.1. Photopheresis using the UVAR® ECP system

Following cannulation, a continuous infusion of 15,000U of unfractionated heparin is mixed with whole blood and dispensed into a leucopheresis bowl, spinning at 4800 rpm ± 5%. Using centrifugal separation, the white cells (buffy coat) is fractionated out and dispensed into a 'buffy coat collection bag'. Following each collection cycle, red cells and excess plasma are returned to the

patient. Each collection cycle takes between 10 and 20min to harvest. This process is repeated six times to dispense approximately 240ml of buffy coat and 300ml of plasma in the 'buffy coat bag' (BCB) which contains 200ml of saline solution. Immediately following the first collection cycle, the cells contained in the BCB are exposed to a UVA radiation source (2 Jcm^2/cell). Using a plastic photocell, the cells of the BCB are passed through a UVA bank of lights, as a film only 1mm thick. Once all six cycles are complete, a 180min incubation time begins. At the end of the incubation, the BCB is immediately re-infused to the patient. This whole process is repeated on the following day, patients returning either 2 or 4 weekly for treatment.

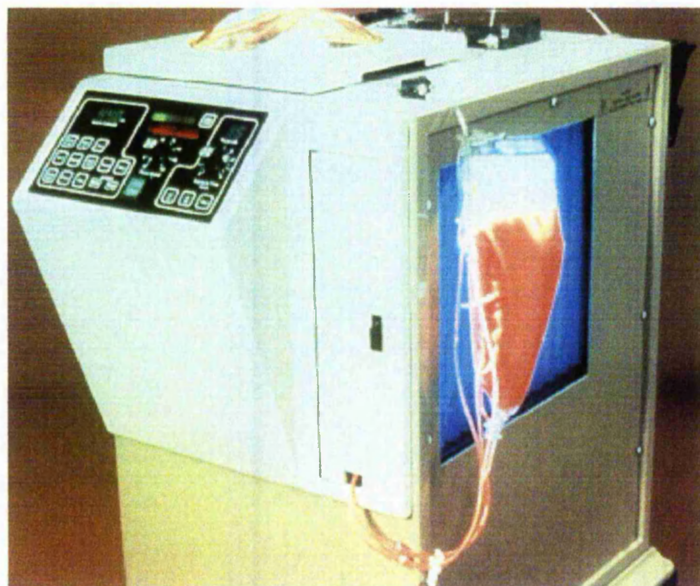


Figure 2.2.1.2.1.1 The UVAR® ECP System

2.2.1.2.2. Photopheresis treatment using the XTS™ ECP system

Using the XTS™ system, ECP therapy harvests the buffy coat using a 'collect and elutriation' 6 cycle apheresis system, which includes a final 'concentration' step. Following all six cycles of leucopheresis the XTS™ system draws the contents of the buffy coat bag back into the centrifuge bowl to further concentrate the buffy coat. Excess red cells and plasma are returned after each cycle. The cells collected are exposed to a 1.5J/cm^2/cell UVA radiation source, through a plastic photocell, as a film 1mm thick. The exposure time is calculated by the XTS™ system and is dependent on the volume and haematocrit (hct) of the buffy coat (approx. 15-50min.) The UVA exposure time begins after all 6 buffy coat collections are complete. After irradiation the treated cells are re-infused. This process is repeated on the following day, patients returning either 2 or 4 weekly.

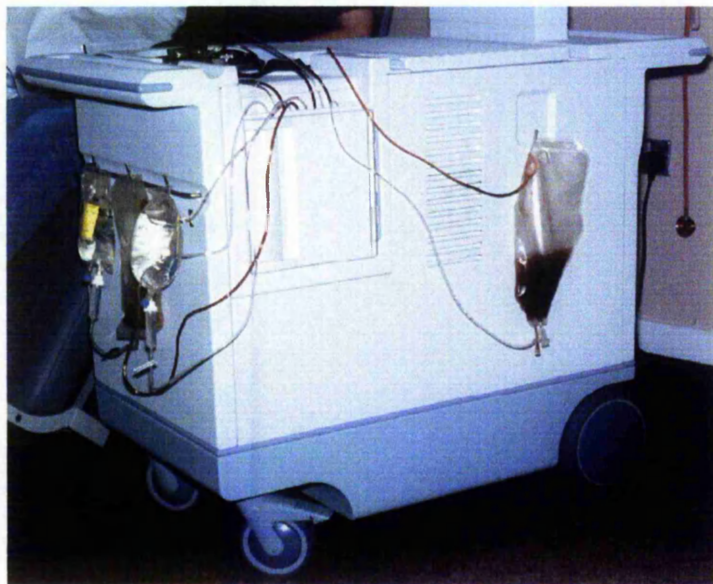


Figure 2.2.1.2.2.1. The XTS™ ECP System

2.2.1.3. 8-Methoxpsoralen (8-MOP)

For experiments in Chapter 3-5, the CTCL and Scleredema patients took the 8-MOP as an oral preparation 90min before the start of the ECP. For GvHD patients the liquid 8-MOP (UVADEX) is injected directly into the BCB immediately after the collection of the first buffy coat. For chapters 3-5, buffy coat 8-MOP levels were tested in the CTCL and Scleredema patients to ensure all patients had at least 100ng/ml. Recommended treatment regimes altered during the course of this study, such that in Chapters 6-9 8-MOP (UVADEX) was injected directly into the BCB for all patients.

2.2.1.4. Sampling

At several stages of the ECP process, blood was taken into heparin. Samples taken pre ECP were obtained immediately following cannulation and before the heparin infusion. The 'first buffy coat' samples were obtained from the BCB immediately following the first collection cycle, prior to UVA exposure. Post ECP (pre re-infusion) samples were taken from the BCB, following full UVA/8MOP exposure, just prior to re-infusion (i.e. less than 1 min of incubation left). Samples taken from the BCB were taken aseptically with a needle and syringe through a designated port on the BCB. Samples were always taken into sterile 4.5ml heparin vacutainer® sample bottles. The time point for sampling, for each study, will be stated at the beginning of the chapter.

2.2.1.5. Preparation of peripheral blood mononuclear cells (PBMCs)

Using a 20ml-centrifugation tube, the 4.5ml heparinsed sample were immediately mixed with a 4.5ml of isotonic saline. The resultant 9ml mixture was carefully layered onto 9ml density gradient (Lymphoprep). Following a 20min centrifugation at 765g, the isolated PBMCs were identified as a white monolayer lying on the density gradient. Using careful pipetting, the PBMCs were removed and dispensed into another 20ml centrifugation tube. Eight ml of 'warm' phosphate buffered saline (PBS) was immediately added and the tube gently mixed. The cells were subsequently centrifuged at 340g for a further 5min. For immediate testing the washed PBMCs were added to the respective

test buffer to give an appropriate cell count for testing, usually between 0.5 and 2.0 x 10^6/ml. For cell cultivation, PBMCs were added to a RPMI medium to give a final count of 1.0 to 2.0 x 10^6/ml. Before testing cultured cells were washed in PBS by centrifugation at 340g.

2.2.1.6. Cell cultivation

Cells for cultivation were added to an appropriate RPMI cell medium, following washing with PBS, to give a final cell count of 1.0 to 2.0 x 10^6/ml. The RPMI medium was supplemented with 10% fetal calf serum (FCS), 140µg/ml Streptomycin, 50µg/ml Vancomycin and 1% Glutamine. Each supplement was added aseptically through a syringe and filter, within a laminar flow cabinet. The medium was checked daily and discarded when two weeks old or when deterioration had been observed. Before testing, cultured cells were mixed gently and centrifuged at 340g. Following the careful removal of the supernatant, warm PBS was added to the 'button' of cells. The tube was subsequently gently mixed to re-suspend the cells. The tube was centrifuged again at 340g and the supernatant removed. The cells were then added to their respective test buffer to give the optimal test concentration.

2.2.1.6.1. Stimulation for cytokine expression

For the investigation of cytokine expression; immediately following the addition of washed cells to the cell culture medium, 30ng/ml Phorbol 12-Myristate 13-Acetate (PMA), 1ug/ml Ionomycin and 10µg/ml Brefeldin A was added. PMA acts to mimic T-cell activation through the activation of Protein Kinase C and subsequent release and mobilsation of Ca^{++}. Ionomycin is a mobile ion carrier for Ca^{++}. Brefeldin A is a ionophor which is known to interrupt subcellular transport of secretory cytokines, such that the arrested cytokines accumulate in the Golgi Stack and can be easily accessed using a 'fix and perm' permeabilisation procedure (see Section 2.2.1.7). Following 6 hr of stimulation, the cells were centrifuged at 340g. The 'button' of cells was subsequently washed in 'warm' PBS, as before, before addition to the test buffer.

2.2.1.7. Cell fixation and permeabilisation.

To allow access to the intracellular compartment, cells had to be fixed and permeabilised. Permeabilisation makes 'holes' in the cell membrane through which appropriate detection monoclonal antibodies may flow. For each test, which involved cell permeabilisation, 50µl of a 1.0 to 2.0 x 10^6/ml cell suspension was added to a labelled tube. To each tube was added 2 drops of fixation media ('reagent a'), (a reagent which contains formaldehyde, but remains undisclosed due to manufacturers patent) mixed and incubated at room temperature, in the dark, for 15min. The cells were washed once using 4.5ml of PBS and centrifuging at 340g. Following removal of the supernatant, 2 drops of permeabilisation media ('reagent b') (a reagent which remains undisclosed due to manufacturers patent) was added to the cell pellet and mixed. The appropriate test antibody (vol of which is identified in each relevant section) was immediately added, the cells were mixed once more and incubated in the dark at room temperature for 15min. The cells were washed again, using 4.5ml of PBS and the supernatant removed. For conjugated antibodies, 500µl of PBS was directly added to the tube and the cells processed. For unconjugated antibodies 100µl of PBS was added followed by the secondary antibody and incubated in the dark, at room temperature, for 15min The cells were washed again using 4.5ml PBS. The pellet of cells was subsequently re-suspended in 500µl of PBS, and then processed.

2.2.2. Cell identification

2.2.2.1. Morphology

<u>*2.2.2.1.1. Cytospin Preparation*</u>

Cytospins were performed on the 6, 24 and 48 hr post ECP cell cultures of Chapter 5. Each cell suspension was diluted in culture medium to give a WBC of 0.2 x 10^6/ml. Five hundred µl of this diluted cell suspension was added to two prepared cytospin chambers. The chambers were spun at 30g for 10min in a Cyto-Tek®cytospin centrifuge. The cytospin for staining with May, Grumwald and Giemsa (MGG) stain were marked with a 'diamond pen' and allowed to

dry, whilst those for staining by florescent antibody were marked with a 'hydrophobic pen'.

2.2.2.1.2. Cytospin staining using May. Grumwald and Giemsa (MGG) stain

The cytospin preparations were fixed for 15min in methanol and allowed to dry. The cytospins were subsequently flooded with a 1 in 2 diluted May and Grumwald (MG) stain for 10min. The MG stain was tipped off and the cytospins were immediately flooded with a 1 in 10 diluted Giemsa stain. Following a 5min incubation, the stain was tipped off and the stains flooded with pH 7.2 PBS for 5min. When the cytospins were dry, the slides were viewed through a x 400 magnification microscope and cells of interest were photographed (See Figure 5.4.7.1, 5.4.7.2 and 9.4.3.1)

2.2.2.1.3. Preparation for electron microscopy

2.2.2.1.3.1. Preparation of sample

A pre ECP and 24hr post ECP cell culture sample were centrifuged at 340g for 10min and the cell medium carefully removed. For fixation, 5ml of 8% glutaraldehyde (in 0.12M Millonigs Phosphate Buffer (MPB)) was dispensed into the tube and the 'cell pellet' re-suspended. Following a 2hr incubation, the cell suspension was centrifuged at 340g for 10min. Molten agar, heated to 105°C, was pipetted onto the subsequent cell pellet and the mixture mixed. Quickly, the agar/cell solution was centrifuged at 2100g for 10min and allowed to cool. The bottom of the solidified agar/cell block, which contained the cells, was chopped into 1mm^3 cubes using a heavy duty single edge blade. Lipid fixation involved, incubating the chopped cubes into 1% aqueous Osmium Tetroxide for 40 to 120min, followed by two washes in distilled water. The cubes were subsequently dehydrated, by sequentially placing in increasing concentrations (70%, 95% and 99%) of absolute alcohol. Finally the cubes were further dehydrated using a 30min incubation in Propylene Oxide.

2.2.2.1.3.2. Embedding sample

Resin was prepared by mixing 15ml of EMIX epoxyresin and 15 EMIX medium hardener and 15 drops of BDMA accelerator in a 50ml volumetric tripour polythene beaker. The cubes prepared, as described in 2.2.2.1.3.1, were impregnated into a 50/50 mixture of propylene oxide and resin and placed at 37°C for 30 minutes. The cubes were carefully removed and placed in warm (37°C) neat resin. The neat resin/cell suspension was dispensed into plastic gelatin 'capsules' and place at 65°C overnight to polymerise. The following morning the gelatin, encapsulating the resin block, was removed by soaking in boiling water to reveal the resin blocks.

2.2.2.1.4. Staining for Electron microscopy

'Thick' sections of the resin block, containing the cells for EM, were cut using the glass knife on the ultramicrotome, layered onto a glass slide and placed on a hot plate for 5 minutes. The sections were stained with filtered, 1% hot Sodium Tretraborate in 1% Toluidine Blue until the stain turned black, when they were rinsed with hot water, layered onto a dry slide and placed on a hot plate briefly. When cool the sections were dipped in Xylene and a coverslip applied. Following identification of the 'best areas' 'thin' sections were cut using the diamond knife on the ultramicrotome. Further staining involved the addition of 50% alcohol saturated uranyl acetate for 5-10 min, followed by washing in 90% absolute alcohol and 2 subsequent washes in distilled water. The final stain was the addition of Reynolds alkaline lead citrate for 2-5 min, which was followed by 2 washes in distilled water. Each section was viewed using standard transmission electron microscopy and cells of interest were photographed (see Figures 5.4.7.4 and 9.4.3.2).

2.2.2.2. Flow cytometry

Cells were processed through either a Coulter EPICS® Profile II or DAKO Galaxy® flow cytometer. Alignment and fluorescence were standardised using Alignment beads and Fluorescent beads.

2.2.2.2.1. Identification of lymphocytes

Isolated PBMCs were stained with 10μl of RPE-conjugated anti CD14 and 10μl of FITC- conjugated anti CD45. Using flow cytometry, the lymphocytes can be identified using a forward scatter (FS) vs. side scatter (SS) 'dot plot' (see Figure 2.2.2.2.1.1 and Figure 2.2.2.2.1.2). Expression of 90% or greater CD45 ensured the cells were white cells and not debris or electronic 'noise', whilst lack of CD14 (<2%) excluded potentially contaminating monocytes (See Figure 2.2.2.2.1.3). Relevant isotype controls were tested to establish the background level of florescence (See Section 2.2.2.2.6.1).

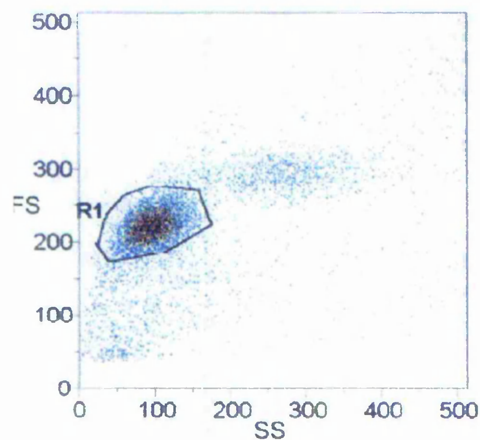


Figure 2.2.2.2.1.1. Lymphocytes identified using a forward scatter (FS) and side (SS) 'dot plot' on the DAKO Galaxy® flow cytometer.

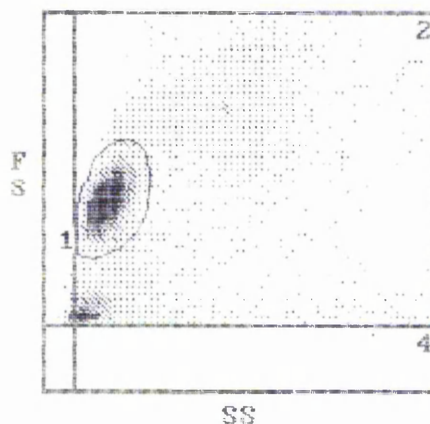


Figure 2.2.2.2.1.2. Lymphocytes identified using a forward scatter (FS) and side (SS) 'dot plot' on the EPICS® Profile II flow cytometer.

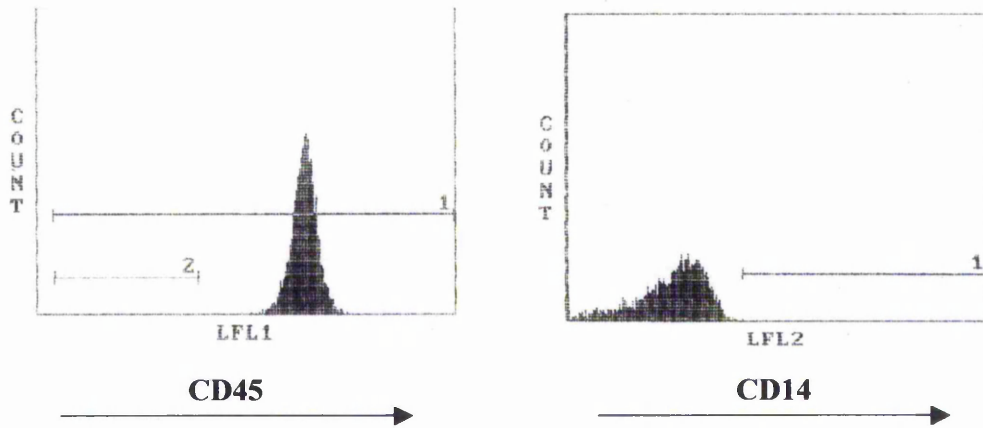


Figure 2.2.2.2.1.3. Following identification of lymphocytes using FS and SS (See Figure 2.2.2.2.1.1. and 2.2.2.2.1.2), CD45 expression >90% and CD14 expression <2% excluded debris and monocytes respectively. Graphs taken from EPICS® Profile II flow cytometer.

2.2.2.2.2. Identification of T lymphocytes

To identify T cells, PBMCs were stained using 10μl PE-CY5-conjugated anti CD3. Using flow cytometry the T cells were identified on the 'dot plot' of anti CD3 (PE-CY5) (FL-III) and SS (See Figure 2.2.2.2.2.1). Relevant isotype controls determined background fluorescence (See Section 2.2.2.2.6.1).

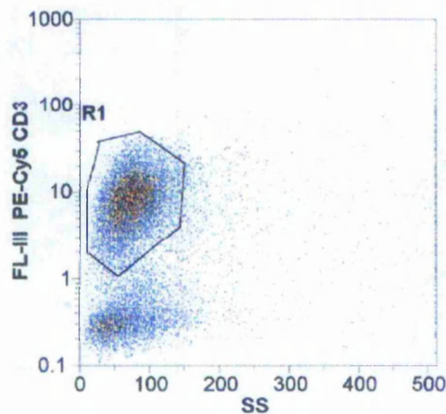


Figure 2.2.2.2.2.1. Identification of T cells using anti CD3 and SS

2.2.2.2.3. Identification of T helper (CD4+) lymphocytes

T helper cells were identified by staining with 10µl PE-CY5-conjugated anti CD4. Identification used a 'dot plot' of anti CD4 (PE-CY5) (FL-III) and SS (see Figure 2.2.2.2.3.1). Relevant isotype controls were performed to determine background fluorescence (See Section 2.2.2.2.6.1).

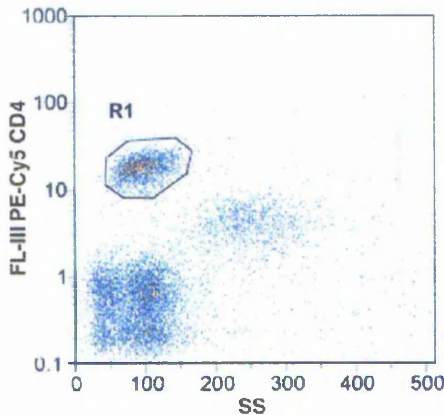


Figure 2.2.2.2.3.1.

Identification of T helper cells using anti CD4 and SS

2.2.2.2.4. Identification of T cytotoxic (CD8+) lymphocytes

T cytotoxic cells were identified by staining with 10µl PE-CY5-conjugated anti CD8. Identification used a 'dot plot' of anti CD8 (PE-CY5) (FL-III) and SS (See Figure 2.2.2.2.4.1). Background fluorescence was detected using relevant isotype controls (See Section 2.2.2.2.6.1).

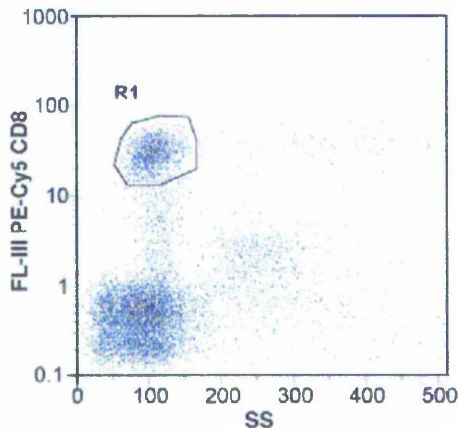


Figure 2.2.2.2.4.1.

Identification of cytotoxic T cells using anti CD8 and SS

2.2.2.2.5. *Identification of monocytes*

The monocytes, contained in the PBMC fraction, were identified using 10µl PE-conjugated anti CD14. Using a 'dot plot' of anti CD14 (PE) (FL-II) and SS, the monocytes could be bitmapped (See Figure 2.2.2.2.5.1). Relevant isotype controls were performed to detect the background fluorescence (See Section 2.2.2.2.6.1).

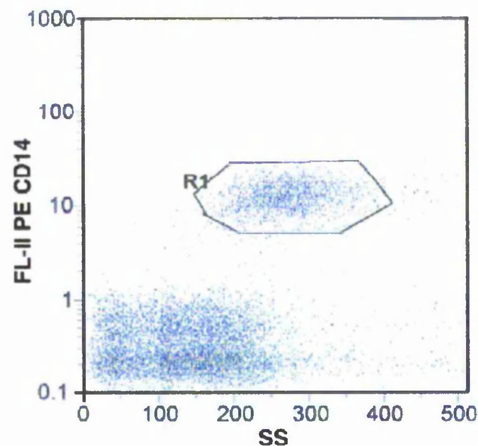


Figure 2.2.2.2.5.1. Identification of monocytes using anti CD14 and SS

2.2.2.2.6. *Identifying positive cells using flow cytometry*

2.2.2.2.6.1. *Setting 'region gates' using relevant isotype controls*

When using flow cytometry, there is always some background fluorescence from the equipment used, some non-specific binding on the cell population and from the auto-fluorescence of some cell types. To ensure these factors do not influence the results obtained using the specific test monoclonal antibodies, the levels of the background fluorescence are assessed using relevant isotype controls. The isotype controls are antibodies to target antigens not present on the cell population. Isotype controls are of the same antibody class, same fluorochrome and tested at the same concentration as the corresponding test antibody. The isotype control is tested with the same method as the test antibody.

2.2.2.2.6.1.1. Setting 'region gates' using a single histogram plot.

If only one test antibody is to be used, the isotype can be assessed as a single 'histogram'. The 'region gates' (RN1) are placed to begin to the immediate right of the bottom of the histogram (See Figure 2.2.2.2.6.1.1.1). When using the corresponding test monoclonal antibody, cells which demonstrate fluorescence above the right hand marker of the 'region gate' (RN1) are deemed positive and are included in the enumeration (See Figure 2.2.2.2.6.1.1.2)

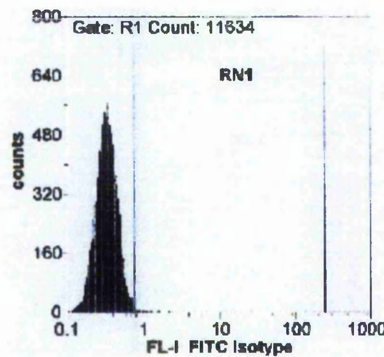


Figure 2.2.2.2.6.1.1.1. Setting of the 'region gate' (RN1) using a relevant isotype control on a single histogram plot.

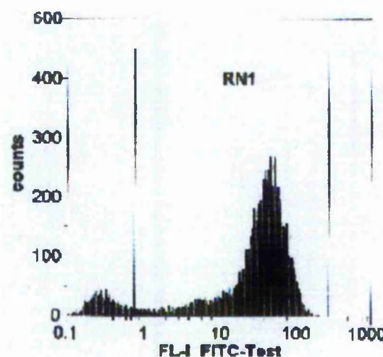


Figure 2.2.2.2.6.1.1.2. Using the region gates set, using a relevant isotype control, the percentage number of cells positive using the test monoclonal antibody can be enumerated in region RN1.

2.2.2.2.6.1.2. Setting 'quadrant gates' using a 'dot plot'.

When performing multi-colour analysis, it may be desirable to assess two test antibodies together (i.e. to observe double positivity). Using a two colour 'dot plot' quadrant gates are set on the fluorescence of the isotype control, such that >99% of events are contained within the Q3 quadrant (see Figure 2.2.2.2.6.1.2.1). When using the corresponding test monoclonal antibodies, cells which demonstrate fluorescence for either or both test antibodies can be enumerated by the quadrant they are contained within, either Q1, Q2 or Q4 (See Figure 2.2.2.2.6.1.2.2).

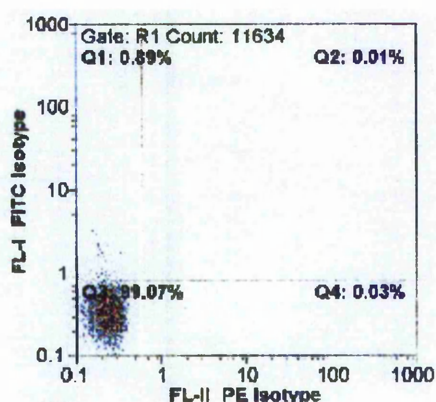


Figure 2.2.2.2.6.1.2.1. 'Quadrant gates' are set on the isotype control for both colours (i.e. FL-I FITC and FL-II PE).

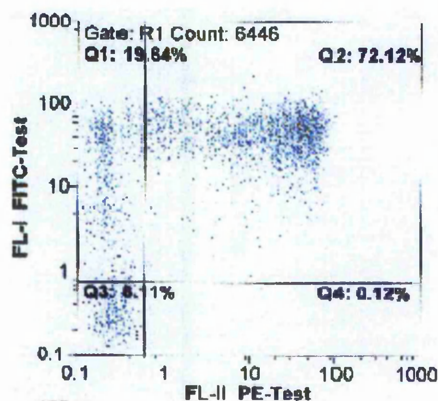


Figure 2.2.2.2.6.1.2.2. Using the quadrant gates, set from the isotype control, the percentage of cells positive, for either or both antibodies, can be enumerated.

2.2.2.2.7. Mean Fluorescence Intensity (MFI) assessment, using flow cytometry

Some tests involve assessing if the level of a marker on a cell is increased. Because test monoclonal antibodies are added in excess, the level of fluorescence emitted by each cell is limited by the level of the target site on the cell. The MFI is a measure of the mean level of fluorescence within the test cell population and is increased if the target marker is upregulated. To assess the MFI of a test involves initially determining the MFI of a relevant isotype control (See Figure 2.2.2.2.7.1). This value is then subtracted from that calculated for the test MFI (See Figure 2.2.2.2.7.2) to give the final MFI.

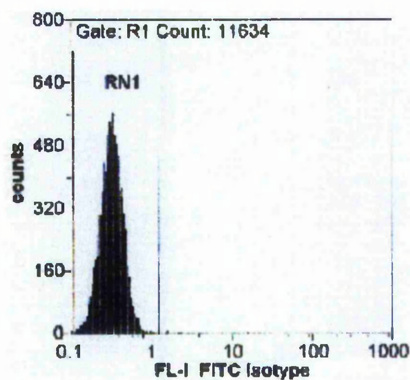


Figure 2.2.2.2.7.1. By setting 'region gates' (RN1) before and after the fluorescence 'peak', the MFI value within this gate can be determined.

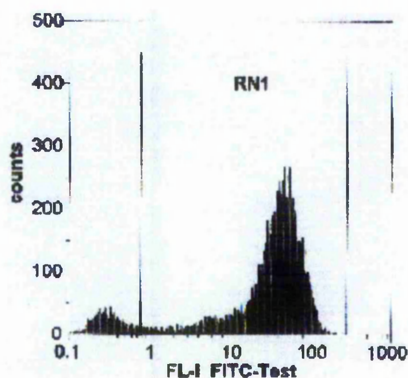


Figure 2.2.2.2.7.2. Using the region gates set by the isotype control (See Figure 2.2.2.2.6.1.1.1), the MFI of a test population can be assessed.

2.2.3. Apoptosis determination

2.2.3.1. Annexin V/ Propidium Iodide

Annexin V/Propidium Iodide determination was as per manufacturers recommendations. Immediately prior to testing stock binding buffer was diluted 1 in 10 with distilled water to achieve a 'working strength' solution. Only sufficient working strength buffer was made for the immediate tests. Propidium Iodide (PI) powder was reconstituted with 1ml of working strength binding buffer to achieve a stock concentration of 250µg/ml. The stock (250µg/ml) Annexin V was diluted 1 in 10 with working strength binding buffer prior to testing. Only sufficient working strength Annexin V was made for the immediate tests. Both working strength binding buffer and Annexin V were immediately placed on ice. Washed cells, for testing, were added to 490µl of cold working strength binding buffer to give a count of 0.5 to 1.0 x 10^6/ml and placed on ice. Five µl of working strength Annexin V and 5ul of PI were then added and incubated for 10 min in the dark. The cells were immediately processed through the flow cytometer.

Lymphocytes were bitmapped by their respective low forward and side scatter (See Figures 2.2.2.2.1.1. and 2.2.2.2.1.2). Exclusion of debris was achieved by ensuring above 90% CD45 positivity. Exclusion of monocytes using CD14 ensured 2% or less contamination (See Figure 2.2.2.2.1.3). Using the same bitmap at least 5000 events were gathered. Data for Annexin V and PI were gathered on a dot plot of fluorescence I (FL-I) and log fluorescence II (FL-II) respectively (See Figures 3.4.1.1.2 and 5.4.2.2). The FL-I gathered Annexin V events and FL-II identified any necrosis using PI. Gates were set at the edge of the dot plot cluster on the pre sample of each patient; these same gates were used to assess cells at the other stages of the ECP cycle (i.e. first buffy coat, pre-infusion samples, culture samples).

2.2.3.2. Apoptest™

For Apoptest™ determination, washed cells were added to 400µl of PBS to give a count of 0.5 to 1.0 x 10^6/ml in the total volume (i.e. 500µl). 100µl of neat

Apoptest™ reagent was then added and incubated at room temperature, in the dark for 20min. The cells were immediately processed through the flow cytometer.

Following the identification of the lymphocyte population (See Figures 2.2.2.2.1.1 and 2.2.2.2.1.2), data for the Apoptest™ were gathered using a dot plot of FS and fluorescence III (FL-III) (See Figure 3.4.1.3.2). The FL-III gathered Apoptest™ events. Gates were set at the edge of the dot plot cluster on the pre sample of each patient; these same gates were used to assess cells at the other stages of the ECP cycle (i.e. first buffy coat and pre-infusion samples).

2.2.3.3. Carboxy-SNARF®-1-AM (CS)

The Carboxy-SNARF®-1-AM (CS) was reconstituted with Dimethyl Sulphoxide (DMSO) to give 1mM. This stock solution was subsequently aliquoted into 2ml polypropylene vials and stored at –70°C. Immediately before testing, the CS stock solution was diluted 1 in 100 in PBS to give a working strength of 10µM. Only sufficient 10µM working strength CS was diluted for the immediate tests. Washed cells were added to the 1ml of 10µM working strength CS to give a count of 0.5 to 1.0 x 10^6/ml and incubated in the dark at 37°C for 30min. The cells were then washed once by adding 8ml of PBS and centrifuging at 340g. The supernatant was removed and 500µl of PBS added to re-suspend the cells. The cells were immediately processed through the flow cytometer.

Following the identification of the lymphocyte population (See Figures 2.2.2.2.1.1 and 2.2.2.2.1.2), the Carboxy-SNARF-1-AM (CS) data were collected as a ratio of FL-II/FL-III. When excited at 488nm, the acidic form of CS fluoresces at 575nm (FL-II) and the basic form fluoresces at 635nm (FL-III). Using a ratio of both, an indication of pH can be achieved. The results were represented as a dot plot, gating again involved setting on the pre ECP sample and using the same gates for other subsequent stages of ECP cycle for testing

(See Figure 3.4.1.4.2). Any increase in acidity causes an increase in the FL2/FL3 ratio.

2.2.4. Intracellular apoptotic protein evaluation

2.2.4.1. p53 evaluation

For p53 evaluation the cells were initially 'fixed and permeabilised' (See section 2.2.1.7). During permeabilisation, 10µl of FITC- conjugated anti p53 antibody were added to respective tubes containing a cell count of 1.0 to 2.0 x 10^6/ml. Relevant isotype controls were tested by the same method. All tubes were incubated in the dark, at room temperature, for 15min. The cells were washed once with 8ml PBS, by centrifuging at 340g. The supernatant was removed and 500µl of PBS added to re-suspend the cells. The cells were immediately processed through the flow cytometer.

Following the identification of the lymphocyte population (See Figures 2.2.2.2.1.1 and 2.2.2.2.1.2), FL-I channel gathered events for the p53 isotype control as a histogram. Gates were set at the edge of the histogram for the isotype control (See Figure 2.2.2.2.6.1.1.1), from which p53 positive cells within the lymphocyte bitmap were enumerated (See Figure 2.2.2.2.6.1.1.2).

2.2.4.2. Bcl-2 identification

Separated cells were initially 'fixed and permeabilised' (See Section 2.2.1.7). During permeabilisation, 10µl of FITC- conjugated anti Bcl-2 antibody was added to respective tubes containing a cell count of 1.0 to 2.0 x 10^6/ml. Isotype controls were tested by the same method. All tubes were incubated in the dark, at room temperature, for 15min. The cells were washed once with 8ml PBS, by centrifuging at 340g. The supernatant was removed and 500µl of PBS added to re-suspend the cells. The cells were immediately processed through the flow cytometer.

Following the identification of the lymphocyte population (See Figures 2.2.2.2.1.1 and 2.2.2.2.1.2), the MFI of the relevant isotype control (See Figure

2.2.2.2.7.1) and Bcl-2 test were determined from the FL-I histogram (See Figure 2.2.2.2.7.2). To calculate the final Bcl-2 MFI, the MFI of the isotype control was subtracted from that of the test MFI.

2.2.4.3. Bax evaluation

For Bax determination 0.1mg of lyophilised anti Bax was reconstituted with 100µl of distilled water to give 1mg/ml stock solution. This stock solution was subsequently aliquoted into 2ml polypropylene vials and stored at -70°C. After the addition of the permeabilisation solution (See Section 2.2.1.7), 2µl of 1mg/ml anti Bax was added to the test tube containing a cell count of 1.0 to 2.0 x 10^6/ml. Following a 15min incubation, in the dark, at room temperature, the cells were washed once with 8ml PBS, by centrifuging at 340g. One hundred µl of PBS was subsequently added to re-suspend the cells. Ten µl of a secondary FITC- conjugated goat, anti mouse antibody, previously diluted 1 in 20 with PBS, was added and the cells incubated for a further 15min in the dark, at room temperature. The cells were washed in PBS again and following the removal of the supernatant, 500µl of PBS added. A relevant isotype control was tested in the same way. The cells were immediately processed through the flow cytometer.

Following the identification of the lymphocyte population (See Figures 2.2.2.2.1.1 and 2.2.2.2.1.2), the MFI of the relevant isotype control (See Figure 2.2.2.2.7.1) and Bax test (See Figure 2.2.2.2.7.2) were determined from the relevant FL-I histograms. To calculate the final Bax MFI, the MFI of the isotype control was subtracted from that of the test MFI.

2.2.5. Mitochondrial transmembrane potential ($\Delta\psi_m$) analysis

2.2.5.1. Rhodamine 123.

Rhodamine 123 powder was reconstituted with PBS to give a 50µg/ml stock solution. The stock solution was aliquoted into 2ml polypropylene vials and stored at -70°C. One µl of the stock solution of was added to 1ml of PBS, to

give a final Rhodamine 123 concentration of 50ng/ml. Washed cells were added to the working strength solution to give a count of 0.5 to 1.0 x 10^6/ml. The tube was then sealed and incubated in the dark, for 15min at 37°C. The cells were then washed once by adding 8ml of PBS and centrifuging at 340g. Following removal of the supernatant, the washed cells were subsequently re-suspended in 1ml PBS and analysed on the flow cytometer. Controls cells were incubated in 5μM carbonyl cyanide m-chlorophenlyhydrazone (mC1CCP), an uncoupling agent that abolishes the $\Delta\psi_m$.

Following the identification of the lymphocyte population (See Figures 2.2.2.2.1.1 and 2.2.2.2.1.2), region gates were set using the pre sample and control experiments using mC1CCP (Carbonyl Cyanide m-Chlorophenylhydrazone). These gates were subsequently used to assess the post ECP samples. Rhodamine 123 expression was analysed on the flow cytometer, as a histogram, using the FL-I channel. A fall in $\Delta\psi_m$ was enumerated using the pre set region gates (See Figure 5.4.4.2). This process was repeated to assess the further samples incubated in cell culture medium.

2.2.5.2. JC-1

A 10mM stock solution of JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide) was prepared by reconstituted JC-1 powder in Dimethyl Sulphoxide (DMSO). The stock solution was aliquoted into 2ml polypropylene vials and stored at -70°C. One μl of stock JC-1 was added to 1ml of PBS to give a final concentration of 10μM. Washed cells were added to the 10μM working strength JC-1 to give a count of 0.5 to 1.0 x 10^6/ml. The tube was then sealed and incubated in the dark for 15min at 37°C. The cells were then washed once by adding 8ml of PBS and centrifuging at 340g. Following removal of the supernatant, the cells were re-suspended in 1ml PBS and analysed on the flow cytometer. As with Rhodamine 123, control experiments were performed using the cells diluted in 5μM carbonyl cyanide m-chlorophenlyhydrazone (mC1CCP).

Following the identification of the lymphocyte population (See Figures 2.2.2.2.1.1 and 2.2.2.2.1.2), region gates were set using the pre sample and control experiments using mC1CCP (Carbonyl Cyanide m-Chlorophenylhydrazone). These gates were used to assess the post ECP samples. For JC-1, control 'quadrant gates' set using the pre sample and control experiments, determined the transition from red (FL-II) J-aggregates to green (FL-III) monomers, as the $\Delta\psi_{m}$, became depolarised (See Figure 5.4.5.2). This process was repeated to assess the further samples incubated in cell culture medium.

2.2.6. Caspase activity assay

Caspase activity was determined using the Caspatag™ Fluorescein caspase activity kit The system involves the detection of several activated caspases, including Caspase 1, 3, 4, 5, 7, 8 and 9, using a FITC conjugated activated caspases inhibitor; FAM-VAD-FMK (Carboxyfluorescein-benzyloxycarbonyl-valine-alanine-aspartic acid-flouromethyl-ketone). Determination of caspase activation was as per manufacturers recommendations. The FITC-conjugated FAM-VAD-FMK reagent was reconstituted with 50µl of DMSO to give a 150x concentrate. The reagent was aliquoted into 2ml polypropylene vials and stored at –70°C. Washed cells were diluted in 300µl of RPMI medium to give a cell count of 0.5 to 1.0 x 10^{6}/ml. Following thawing, 8µl of PBS was added to 2µl of the 150x stock solution of FAM-VAD-FMK and the resultant 10µl of 30x stock solution added to the cell suspension. The tube was sealed and incubated in the dark, at 37°C for 1hr. The cell suspension was washed twice with 2ml of 1x working dilution wash buffer by centrifuging at 340g. Following removal of the supernatant, the cell pellet was then re-suspended in 1ml of 1x working dilution wash buffer and processed through the flow cytometer.

Following the identification of the lymphocyte population (See Figures 2.2.2.2.1.1 and 2.2.2.2.1.2), region gates (RN1) were set on a FL-I histogram of the pre ECP sample. Using these region gates, the number of cells

demonstrating Caspase activation was determined in subsequent pre and post samples (See Figure 5.4.6.2). This process was repeated to assess the further samples incubated in cell culture medium.

2.2.7. Cell membrane associated antigens

2.2.7.1. CD10 identification.

To 0.5 to 1.0 x 10^6/ml of washed cells in 1ml of PBS was added 10μl of FITC-anti CD10. The tubes were incubated in the dark for 15 min and processed immediately through the flow cytometer. Relevant isotype controls were tested in the same way.

Following the identification of the lymphocyte population (See Figures 2.2.2.2.1.1 and 2.2.2.2.1.2), the FL-I gathered events for the CD10 isotype control as a histogram (See Figure 2.2.2.2.6.1.1.1). Gates were set at the edge of the dot plot for the isotype control, from which CD10 positive cells were enumerated (See Figure 2.2.2.2.6.1.1.2). This process was repeated to assess the further samples incubated in cell culture medium.

2.2.7.2. CD95 identification

To 0.5 to 1.0 x 10^6/ml of washed cells in 1ml of PBS was added 10μl of either PE-CY5 conjugated anti CD4 or anti CD8. Ten μl of FITC-conjugated anti CD95 was subsequently added and the cells incubated in the dark, at room temperature, for 20min. Relevant isotype controls were tested in the same way. The tubes were then processed immediately through the flow cytometer.

CD4+ or CD8+ T cells were identified by their PE-CY5 (FL-III) expression and SS (See Figure 2.2.2.2.3.1 and 2.2.2.2.4.1 respectively). This region was 'bitmapped' and from this bitmap CD95 expression was determined. Using an appropriate isotype controls 'region gates' were set on the two dimensional 'dot plot' of PE-CY5 (FLIII) and FITC (FL-I) (See Figure 2.2.2.2.6.1.2.1). These 'gates' then determined the percentage of T cell expressing CD95 (See Figure 2.2.2.2.6.1.2.2). This process was repeated to assess the further samples

incubated in cell culture medium. The MFI of the CD95 expressing cells was also evaluated. The total MFI value was calculated by subtracting the MFI value of the appropriate isotype control (See Figure 2.2.2.2.7.1) from that of the test MFI (2.2.2.2.7.2).

2.2.7.3. Fas-Ligand identification

2 μl of unconjugated anti Fas-L was added to 1ml of PBS containing a cell count of 0.5 to 1.0 x 10^6/ml. The tube was then incubated in the dark, at room temperature, for 20 min. The cells were then washed in PBS by centrifuging at 340g, and re-suspended in 1ml PBS. 10μl of a previously 1 in 20 diluted secondary FITC-conjugated anti mouse antibody was subsequently added. Following a 20min, room temperature incubation in the dark, the tubes were again washed in PBS. The final stage involved re-suspending the cells in 1ml PBS and adding either 10μl of PE-CY5-conjugated anti CD4 or PE-CY5-conjugated anti CD8. The tubes were again incubated for 20 min in the dark at room temperature. The tubes were then processed immediately through the flow cytometer. Relevant isotype controls were tested in the same way.

Following the identification of CD4+ or CD8+ T cells (See Figure 2.2.2.2.3.1 and 2.2.2.2.4.1 respectively), an appropriate isotype controls 'region gates' were set on the two dimensional 'dot plot' of PE-CY5 (FLIII) and FITC (FL-I) (see Figure 2.2.2.2.6.1.2.1). These 'gates' then determined the percentage of T cell expressing either Fas-L (See Figure 2.2.2.2.6.1.2.2). This process was repeated to assess the further samples incubated in cell culture medium (See Figure 6.4.3.2).

2.2.8. Heat shock 70(72) Determination

Upon receipt of anti HSP 70(72), the liquid antibody was aliquoted into 2ml polypropylene vials and stored at -70°C.

2.2.8.1. Membrane associated HSP 70(72) determination

For cell membrane evaluation of HSP 70(72), 2μl of anti HSP 70(72) was added to 1ml PBS containing a cell count of 0.5 to 1.0 x 10^6/ml. The cells were incubated for 20min, in the dark, at room temperature. The cells were subsequently washed by centrifuging at 340g. The supernatant was removed and the cell pellet was re-suspended in 1ml PBS. Ten μl of a previously 1 in 20 diluted secondary FITC-conjugated anti mouse antibody was subsequently added. Following a 20min, room temperature incubation in the dark, the tubes were again washed in PBS and 1ml of PBS added. The final stage involved the addition of 10μl of PE-CY-5 conjugated anti CD3 and 10μl of PE-conjugated anti CD14. The cells were incubated, in the dark at room temperature, for a further 15min. Control cells were placed in cell culture medium and incubated at 42°C for 1hr. Following a further 6hr incubation at 37°C, the control cells were stained for HSP 70(72) in the same way. The cells were immediately processed through the flow cytometer.

T cells and monocytes were identified using anti CD3 (FL-III) and SS and anti CD14 (FL-II) and SS respectively (See Figures 2.2.2.2.2.1. and 2.2.2.2.5.1 respectively). The appropriate regions were bitmapped and used to assess HSP expression. Using a histogram of FLI expression, 'region gates' were set using control cells exposed to 42°C heat source. Using these gates, pre and post ECP samples were tested after 0, 6 and 24 hr of cultivation. The number of cells expressing membrane HSP 70(72) was determined using the FL-I channel.

2.2.8.2. Determination of total HSP 70(72)

For the determination of total HSP 70(72), including intracellular HSP 70(72), a cell count of 1.0 to 2.0 x 10^6/ml was used. Following fixation and at the permeabilsation stage (See section 2.2.1.7), 2μl anti HSP 70(72) was added. The cells were incubated, in the dark at room temperature, for 15min. The cells were subsequently washed and 10μl of a previously 1 in 20 diluted secondary FITC-conjugated anti mouse antibody added. The cells were subsequently

incubated for a further 15min in the dark at room temperature. Following further washing, the cells were re-suspended in 1ml PBS. Finally, 10μl of PE-CY-5 conjugated anti CD3 and 10μl of PE-conjugated anti CD14 were added. The cells were incubated, in the dark at room temperature, for 15min. The cells were processed immediately through the flow cytometer. Control experiments were performed by heating 1ml RPMI, containing a cell count of 1.0 to 2.0 x 10^6/ml cells, to 42°C in the dark, for one hr. The cells were then removed from the 42°C heat and incubated at 37°C for a further 6hr. The cells were washed and tested as before.

T cells and monocytes were identified as outlined earlier (See Figures 2.2.2.2.2.1. and 2.2.2.2.5.1 respectively). Using appropriate 'region gates' set using control cells heated at 42°C pre and post ECP samples were tested after 0, 6 and 24 hr of cultivation. The number of cells expressing total HSP 70(72) was determined using the FL-I channel (See Figure 7.4.2.1).

2.2.9. Co- stimulatory molecules evaluation

2.2.9.1. CD54 expression

10 μl of anti CD14 and 10μl of FITC-conjugated anti CD54 was added to 1ml PBS with a cell count of 0.5 to 1.0 x 10^6/ml. The cells were incubated in the dark, at room temperature, for 20min. The tubes were then processed immediately through the flow cytometer. Relevant isotype controls were tested in the same way.

Monocytes were identified using anti CD14 (FL-II) and SS respectively (See Figure 2.2.2.2.5.1). The appropriate regions were bitmapped and used to assess CD54 expression. Using a histogram of FL-I expression, 'region gates' determined the MFI of an appropriate isotype control (See Figure 2.2.2.2.7.1) and that of the anti CD54 using the FL-I channel (See Figure 2.2.2.2.7.2). The final MFI was calculated by subtracting the MFI of the isotype control from that

of the test MFI. This process was repeated to include all pre and post ECP testing stages (See Figure 9.4.1.2)

2.2.9.2. CD86 identification

10 μl of anti CD14 and 10μl of FITC-conjugated anti CD86 was added to 1ml PBS with a cell count of 0.5 to 1.0 x 10^6/ml. The cells were incubated in the dark, at room temperature, for 20min. The tubes were then processed immediately through the flow cytometer. Relevant isotype controls were tested in the same way.

Following the identification of monocytes (See Figure 2.2.2.2.5.1) and the setting of appropriate 'regions gates' (See Figure 2.2.2.2.7.1) the MFI of CD86 was determined using the technique outlined in the CD54 methodology.

2.2.9.3. CD80 identification

10 μl of anti CD14 and 10μl of FITC-conjugated anti CD80 was added to 1ml PBS with a cell count of 0.5 to 1.0 x 10^6/ml. The cells were incubated in the dark, at room temperature, for 20 min. The tubes were then processed immediately through the flow cytometer. Relevant isotype controls were tested in the same way.

Following the identification of monocytes (See Figure 2.2.2.2.5.1), appropriate 'region gates' were set using an isotype control (See Figure 2.2.2.2.6.1.1.1). Using these region gates the percentage of CD80-expressing monocytes was determined using the FL-I channel (See Figure 2.2.2.2.6.1.1.2). This process was repeated to include all pre and post ECP testing stages.

2.2.9.4. CD80 MFI evaluation following IFNγ stimulation

Pre and post ECP PBMCs, suspended in RPMI medium (See Section 2.2.1.5.) were stimulated with IFNγ (500 U/ml) for 24 hr. The cells were then washed in PBS by centrifuging at 340g and the count adjusted to 0.5 to 1.0 x 10^6/ml. The

cells were stained with anti CD14 and anti CD80, as outlined earlier (See Section 2.2.9.3). Relevant isotype controls were tested in the same way.

Following the identification of monocytes (See Figure 2.2.2.2.5.1) and the setting of appropriate 'regions gates' (See Figure 2.2.2.2.7.1) the MFI of CD80 was evaluated using the FL-I channel (See Figure 2.2.2.2.7.2). The final MFI was calculated by subtracting the MFI of the isotype control from that of the test MFI. This process was repeated to include all pre and post ECP testing stages.

2.2.10. Identification of intracellular cytokines

2.2.10.1. Interleukin-6 (IL6) evaluation

Following stimulation and washing of PBMCs for cytokine expression (See Section 2.2.1.6.1), 10μl of PE-conjugated anti CD14 was added to a tube containing 1ml PBS and a cell count of 1.0 to 2.0 x 10^6/ml. The cells were washed by the addition of 8ml PBS and centrifuging at 340g. The cells were then re-suspended in 50μl PBS. Following 'fixing and at the permeabilisation stage (See Section 2.2.1.7), 20μl of FITC-conjugated anti IL6 was added. After a 15min, room temperature incubation in the dark, the cells were washed in PBS and processed. Appropriate isotype controls were performed.

Following the identification of monocytes (See Figure 2.2.2.2.5.1) 'region gates' were set on the two dimensional 'dot plot' of PE (FL-II) and FITC (FL-I) using an appropriate isotype control (See Figure 2.2.2.2.6.1.2.1). These 'gates' then determined the percentage of T cell expressing IL6 (See Figure 2.2.2.2.6.1.2.2). This process was repeated to assess the post ECP sample.

2.2.10.2. Interleukin-12 (IL12) evaluation

Using the methodology outined for Interleukin 6 (See Section 2.2.10.1) PBMC's were stained for CD14 and intracellular IL12.

Following the identification of monocytes (See Figure 2.2.2.2.5.1) and the setting of appropriate 'regions gates' (See Section 2.2.2.2.6.1) the percentage of

cells demonstrating IL12 was determined using the technique outlined in the IL6 methodology.

2.2.10.3. Tumour necrosis factor alpha (TNFα)

Using the methodology outined for Interleukin 6 (See Section 2.2.10.1) PBMC's were stained for CD14 and intracellular TNFα.

Following the identification of monocytes (See Figure 2.2.2.2.5.1) and the setting of appropriate 'regions gates' (See Section 2.2.2.2.6.1) the percentage of cells demonstrating TNFα was determined using the technique outlined in the IL6 methodology.

2.2.10.4. Interferon gamma (IFNγ) evaluation

Using the methodology outined for Interleukin 6 (See Section 2.2.10.1) PBMC's were stained for CD14 and intracellular IFNγ.

Following the identification of monocytes (See Figure 2.2.2.2.5.1) and the setting of appropriate 'regions gates' (See Section 2.2.2.2.6.1) the percentage of cells demonstrating IFNγ was determined using the technique outlined in the IL6 methodology.

2.2.11. Statistical analysis

Statistical analysis was performed using paired t-tests. A p value lower than 0.05 was regarded as significant

Chapter 3: Extracorporeal photopheresis induces apoptosis in the lymphocytes of cutaneous T cell lymphoma and graft versus host disease patients.

3.1. Introduction

Apoptosis, unlike necrosis, is a natural form of cell death that does not normally evoke an inflammatory response (Wyllie *et al.,* 1980). Within lymphoid haemopoiesis, apoptosis is responsible for the removal of autoreactive T cells in the thymus (MacDonald and Lees, 1990) and activated T cells, when the response to foreign antigens is complete (Nagata and Golstein, 1995). Histological examination of skin biopsies, from ECP treated mycosis fungoides patients, have demonstrated a significant increase in apoptosis following ECP treatment (Miracco *et al.*, 1997). Apoptotic markers have been detected on lymphocytes cultured from samples taken immediately after ECP exposure (Yoo *et al.*, 1996; Aringer *et al.,* 1997; Enomoto *et al.*, 1997) and *in vitro* exposure of lymphocytes to 8MOP/UVA has demonstrated significant levels of apoptosis (Marks and Fox, 1991; Yoo *et al.,* 1996).

Because normal T cells are replaced at a more rapid rate than those of the malignant clone, the removal of the unwanted T cells, as observed in ECP treated patients, may depend on repeated treatment ECP cycles to gradually replace malignant T cells with normal T cells (Yoo *et al.,* 1996). Cell cultures demonstrate the occurrence of apoptotic markers in treated lymphocytes at 24 hours post ECP. Testing *ex-vivo* (Aringer *et al.,* 1997; Enomoto *et al.*, 1997) and 6 hours post ECP demonstrate no significant evidence of lymphoid apoptosis induction (Yoo *et al.* 1996).

On re-infusion into the peripheral circulation any apoptotic lymphocytes would be rapidly removed by phagocytosis (Fadok *et al.,* 1992). However, if these cells were still viable for some days, they would remain undetected by the

reticuloendothelial (RE) system and have the capacity to modulate the immune system through release of cell signalling proteins (Enomoto *et al.*, 1997).

CTCL is a malignant expansion of the CD4+ T cells (Haynes *et al.*, 1981). Advanced CTCL or Sezary syndrome demonstrate a high CD4/CD8 ratio in the peripheral blood (Rook *et al.*, 1993; Zouboulis *et al.*, 1998). ECP reverses the CD4/CD8 ratio back to normal, in 'responders' within 12 months of treatment. (Zouboulis *et al.*, 1998). Using a very sensitive Southern blot method, the removal of the malignant clone is complete in those patients where remission is achieved using ECP (Wolfe *et al.*, 1994). CTCL, in the absence of the malignant clone in the peripheral blood, do not respond to ECP (Wolfe *et al.*, 1994). Changes, which indicate that direct exposure of the malignant cells to ECP, are essential in inducing a clinical response.

3.2. Aim

The aim of this chapter was to investigate if treated lymphocytes tested immediately after ECP were apoptotic prior to their re-infusion. Testing cells for positive markers of apoptosis, following leucocyte collection, but prior to UV exposure would identify the apoptotic effects of the white cell isolation procedure, namely peristalsis pumping and centrifugation. Testing *ex-vivo*, immediately before re-infusion would evaluate the apoptotic effect of the whole ECP procedure. In addition, because CD4/CD8 ratios are normalised in 'responders', evaluation of apoptosis induction in the lymphocyte subsets would determine if the CD4+ T cells were more sensitive to ECP induced-apoptosis.

3.3. Specific patients and methods

The specific cohort of patients, ECP technology and analysis methods pertaining to this chapter are detailed below. For a full explanation, please see Materials and Methods chapter.

3.3.1. Lymphocyte apoptosis

3.3.1.1.Patients. Samples were obtained from six CTCL patients (mean age ± S.D; 66.3 ± 7.4 years), four GvHD patients (37.3 ± 5.7) and one Scleredema patient (54).

3.3.1.2. Photopheresis treatment.

Extracorporeal photopheresis cycles were performed using the UVAR®system. See section 2.2.1.2.1. of the Materials and Methods chapter.

3.3.1.3. Sampling.

Heparinised blood was taken from patients; immediately before ECP, from the first buffy coat collected and from the incubated BCB just prior to re-infusion.

3.3.1.4. Cell identification.

Lymphocytes were identified using a FS vs SS dot plot. Within the bitmapped population, CD45 and CD14 expression excluded debris and monocytes respectively. See section 2.2.2.2.1. of the Materials and Methods chapter.

3.3.1.5. Apoptosis determination.

Apoptosis was determined using Annexin V, Apoptest™ and Carboxy-SNARF®-1-AM. See Sections 2.2.3.1, 2.2.3.2 and 2.2.3.3 of the Materials and Methods chapter.

3.3.2. T helper and T cytotoxic apoptosis

3.3.2.1. Patients.

Samples were obtained from 6 CTCL patients (mean age ± S.D; 62.3 ± 8.0 years), 5 GvHD patients (36.0 ± 7.3) and one Scleredema patient (56).

3.3.2.2. Photopheresis treatment

Extracorporeal photopheresis cycles were performed using the UVAR® system. See Section 2.2.1.2.1. of the Materials and Methods chapter.

3.3.2.3. Sampling.

Heparinised blood was taken from patients pre ECP and from the incubated BCB just prior to re-infusion.

3.3.2.4. Cell identification.

Helper T cells and cytotoxic T cells were identified using FITC-conjugated anti CD4 and SS and FITC-conjugated anti CD8 and SS respectively. See Sections 2.2.2.2.4 and 2.2.2.2.5 of the Materials and Methods chapter

3.3.2.5. Apoptosis determination.

Apoptosis was determined using Annexin V. See section 2.2.3.1 of the Materials and Methods chapter.

3.4. Results

3.4.1. Lymphocyte apoptosis

Following isolation and 'bitmapping', using their distinctive low forward and side scatter, conformation of the location of lymphocytes were determined using anti CD45 and CD14 antibodies. This ensured the population did not include unwanted debris and monocytes respectively (See Section 2.2.2.2.1). For all the apoptotic assays, as only one Scleredema patient was tested, no statistical increase could be determined between the different stages of ECP treatment for this patient group. However the levels of apoptosis observed were similar to those of the CTCL patients

3.4.1.1. Annexin V

Annexin V attaches to the phosphatidylserine (PS) residues on lymphocytes, which are 'flip-flopped' from the inner to outer cell membrane, as a consequence of early apoptosis (Verms *et al.,* 1995). By conjugating the Annexin V to a FITC fluorochrome, cells demonstrating externalisation of PS can be detected using flow cytometry or fluorescent microscopy. Figure 3.4.1.1.1 demonstrates how staining with Annexin V was significantly increased following leucocyte isolation in the first buffy coat collected and further increased when lymphocytes were exposed to the full ECP treatment, namely 8-MOP and UVA.

Using flow cytometry, the increase in Annexin V positive lymphocytes can be easily identified and enumerated using 'quadrant gates' set on the pre ECP sample (See Figure 3.4.1.1.2). Although all patient groups demonstrate a significant increase in apoptosis, as detected by Annexin V, post ECP, the GvHD patients demonstrate the highest levels. Observations that may indicate that the lymphocytes of GvHD patients are particularly sensitive to ECP-induced apoptosis. Comparison of the two day treatment cycle demonstrate no significant difference in the levels of Annexin V positive lymphocytes (See Figure 3.4.1.5).

Figure 3.4.1.1.1. ECP induces a significant increase in the number of treated lymphocytes positive for Annexin V

*Samples were taken pre ECP, from the 1st buffy coat collected and immediately post ECP, prior to re-infusion. PBMCs were isolated by density gradient and the cell count adjusted to 0.5 to 1.0 x 10^6/ml (See Section 2.2.1.5). Lymphocytes were stained for PS externalisation using Annexin V (See Section 2.2.3.1). Positive cells were identified using flow cytometry (See Figure 3.4.1.1.2). The data represents the mean ±SD obtained from each patient group, * denotes $p < 0.05$, ** denotes $p<0.01$ and *** denotes $p < 0.001$. No statistics were available on the Scleredema patient group, as only one patient was tested.*

Figure 3.4.1.1.2. Flow cytometric identification of Annexin V positive lymphocytes pre and post ECP.

Using flow cytometry, 'quadrant gates' were set on the pre ECP sample. Post ECP, an increase in Annexin V positive lymphocytes were determined using these gates.

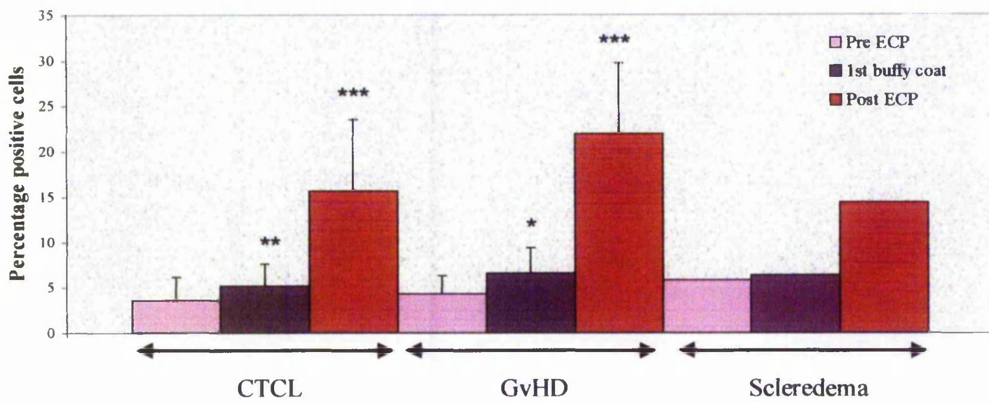


Figure 3.4.1.1.1. ECP induces a significant increase in the number of treated lymphocytes positive for Annexin V

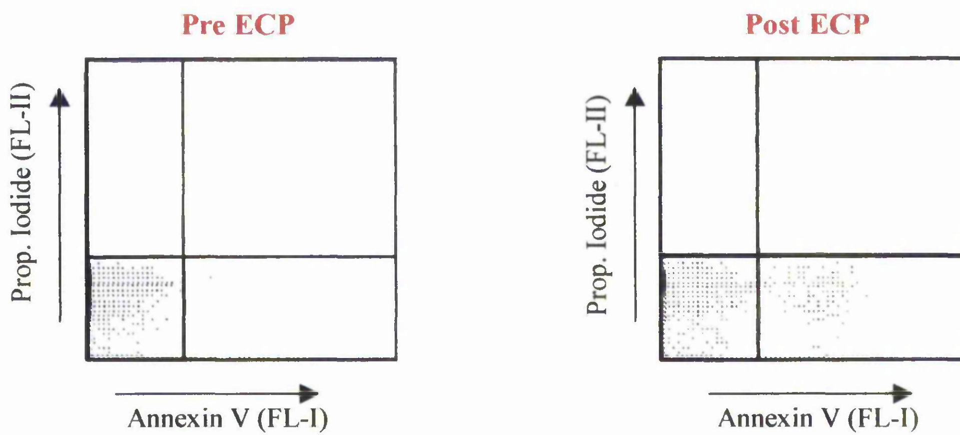


Figure 3.4.1.1.2. Flow cytometric identification of Annexin V positive lymphocytes pre and post ECP.

3.4.1.2 Propidium Iodide

Propidium Iodide (PI) is a stain normally excluded by viable cells, however PI is absorbed by necrotic cells and stains the DNA. The subsequent fluorescence emitted by positive cells can be detected using the FL-II (PE) channel of the floe cytometer. When PI is used in combination with Annexin V, necrotic cells and apoptotic cells can be distinguished. All samples tested, for all patients, had less than 1% of lymphocytes stained positive for PI (Data not shown). These results clearly demonstrate that ECP induces apoptosis and not a necrotic state, when tested *ex-vivo.*

3.4.1.3. Apoptest™

The Apoptest™ detects the transient changes in chromatin texture associated with apoptosis (Ferlini *et al.,* 1997). Figure 3.4.1.3.1 demonstrates the marked increase in apoptosis, detected using Apoptest™, in the lymphocyte population immediately post ECP.

By setting a discrimination 'gate' on the pre ECP, the number of lymphocytes crossing the 'gate' at each of the two later testing points were enumerated (See Figure 3.4.1.3.2). Apoptest™ demonstrated the highest level of apoptosis for all three patient groups, this could be a consequence of the combined effect of apoptosis induction and chromatin damage induced by UVA exposure. Apoptest™, like Annexin V, indicates the highest level of apoptosis to be present in GvHD patient group. The GvHD patients also show the highest level of apoptosis detected using Apoptest™, in lymphocytes prior to UV and 8-MOP exposure (first buffy coat). Results that reinforce the effect leucocyte isolation has on apoptosis induction. Both day 1 and day 2 of the ECP treatment regimen demonstrated similar levels of apoptosis, as detected by Apoptest™ (See Figure 3.4.1.5).

Figure 3.4.1.3.1 ECP induces a significant increase in the number of treated lymphocytes positive for Apoptest™

*Samples were taken pre ECP, from the 1st buffy coat collected and immediately post ECP, prior to re-infusion. PBMCs were isolated by density gradient and the cell count adjusted to 0.5 to 1.0 x 10^6/ml (See Section 2.2.1.5). Following staining with Apoptest™ (See Section 2.2.3.2), the positive cells were enumerated using flow cytometry (See Figure 3.4.1.3.2). The data represents the mean ±SD obtained from each patient group, * denotes $p < 0.05$ and *** denotes $p < 0.001$. No statistics were available on the Scleredema patient group, as only one patient was tested.*

Figure 3.4.1.3.2. Flow cytometric identification of Apoptest™ positive lymphocytes pre and post ECP.

Using flow cytometry, region gates were set on the pre ECP sample. Post ECP, the increased number of Apoptest™ positive lymphocytes were enumerated using these gates.

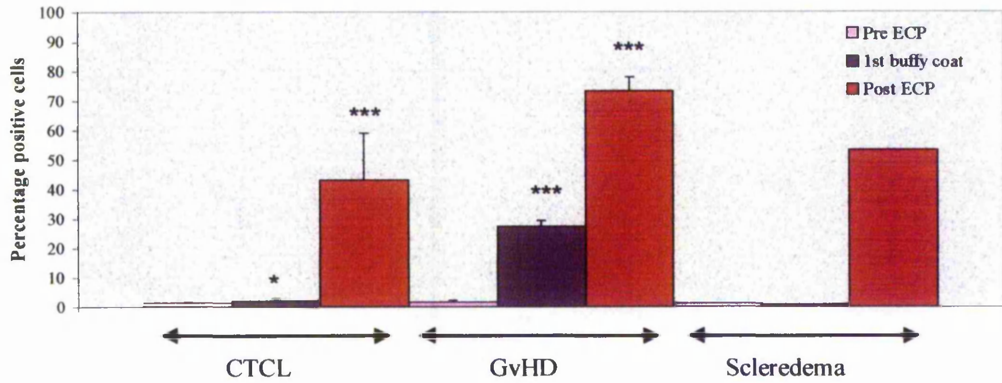


Figure 3.4.1.3.1 ECP induces a significant increase in the number of treated lymphocytes positive for Apoptest™

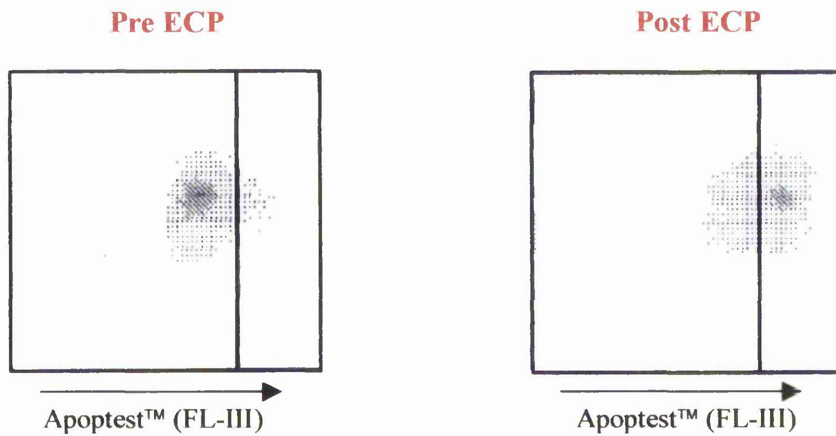


Figure 3.4.1.3.2. Flow cytometric identification of Apoptest™ positive lymphocytes pre and post ECP.

3.4.1.4. Carboxy-SNARF®-1-AM.

When cells become apoptotic, the intracellular compartment becomes more acidic (Ishaque *et al.*, 1997). Carboxy-SNARF®-1-AM (CS) is a dye which, through its fluorescence pattern, indicates the intracellular acidity of cells (Weider *et al.* 1993; Ishaque *et al.* 1997). When a cell has a basic intracellular pH, CS fluoresces in the FL-III channel of the flow cytometer, whilst an acid pH within a cell causes fluorescence which can be observed using the FL-II detection unit. Comparison of both the FL-II and FL-III and the calculation of their FL-II/FL-III ratio can track the intracellular pH of cells following ECP exposure. Discrimination 'gates', set on the pre ECP sample, were used in the evaluation of the samples taken later in the ECP cycle. Figure 3.4.1.4.1 demonstrated the levels of cells positive for staining with CS, whilst the flow cytographs observed pre and post ECP are demonstrated in Figures 3.4.1.4.2.

Figure 3.4.1.4.1 ECP induces a significant increase in the number of treated lymphocytes positive for Carboxy-SNARF®-1-AM

*Samples were taken pre ECP, from the 1st buffy coat collected and immediately post ECP, prior to re-infusion. PBMCs were isolated by density gradient and the cell count adjusted to 0.5 to 1.0 x 10^6/ml (See Section 2.2.1.5). Following staining with Carboxy-SNARF®-1-AM (See Section 2.2.3.3), the positive cells were enumerated using flow cytometry (See Figure 3.4.1.4.2). The data represents the mean ± SD obtained from each patient group, * denotes $p < 0.05$ and *** denotes $p < 0.001$. No statistics were available on the Scleredema patient group, as only one patient was tested.*

Figure 3.4.1.4.2. Flow cytometric identification of Carboxy-SNARF®-1-AM positive cells pre and post ECP.

Gates were set on the pre ECP sample. Lymphocytes demonstrating the increased intracellular acidity, associated with apoptosis, were identified using these gates. Post ECP, the number of positive cells increased significantly

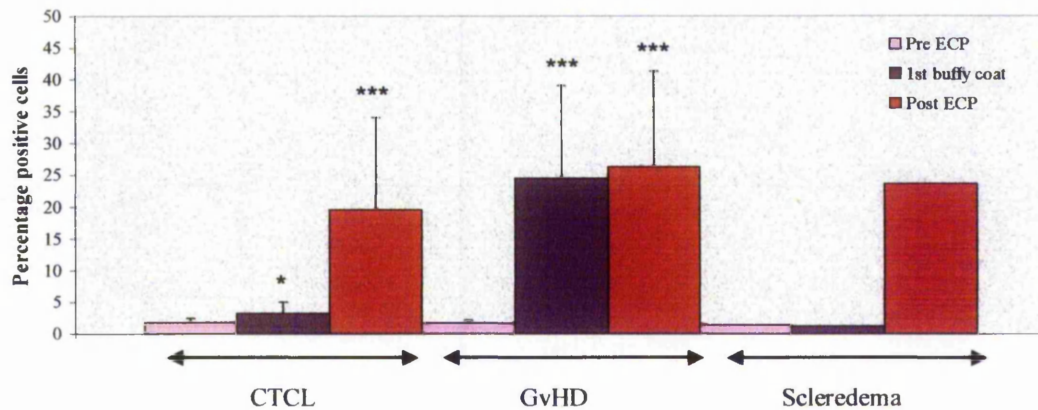


Figure 3.4.1.4.1 ECP induces a significant increase in the number of treated lymphocytes positive for Carboxy-SNARF®-1-AM

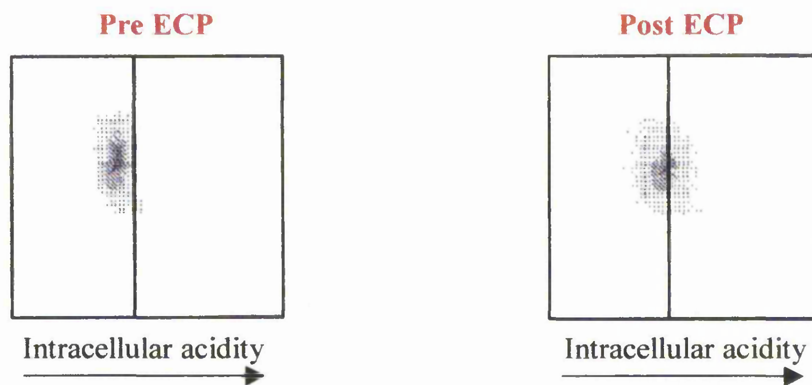


Figure 3.4.1.4.2. Flow cytometric identification of Carboxy-SNARF®-1-AM positive cells pre and post ECP.

All groups demonstrate a marked increase in apoptosis as a consequence of ECP treatment. Of interest is the high level of lymphocytes demonstrating CS positivity in the first buffy coat bag of the GvHD patients, indicating that these patients may be very sensitive to leucocyte collection processes, with a subsequent and rapid change in internal pH. However, the CS test involved a long incubation time at 37°C and a washing step in its procedure. These factors may have contributed to the slightly conflicting results observed. The CS testing group also demonstrates the only difference in apoptosis levels on day one and day two. Lymphocytes from the GvHD patients demonstrated higher levels of apoptosis, using CS, on day two. However no such response is observed for the CTCL patients.

Figure 3.4.1.5. No significant increase in Annexin V or Apoptest™ positivity was observed on day 2 of treatment. However the GvHD patients demonstrated an increase in CS positivity on the second day of ECP.

*The increase in number of lymphocytes positive for Annexin V, Apoptest™ and CS were calculated by subtracting the number of positive cells pre ECP from the number observed immediately post ECP. The increase in lymphocytes positive for each apoptotic marker for day one and day two were compared. The data represents the mean ±SD obtained from each patient group. * denotes a p value of <0.05. No statistics were available on the Scleredema patient group as only one patient was tested.*

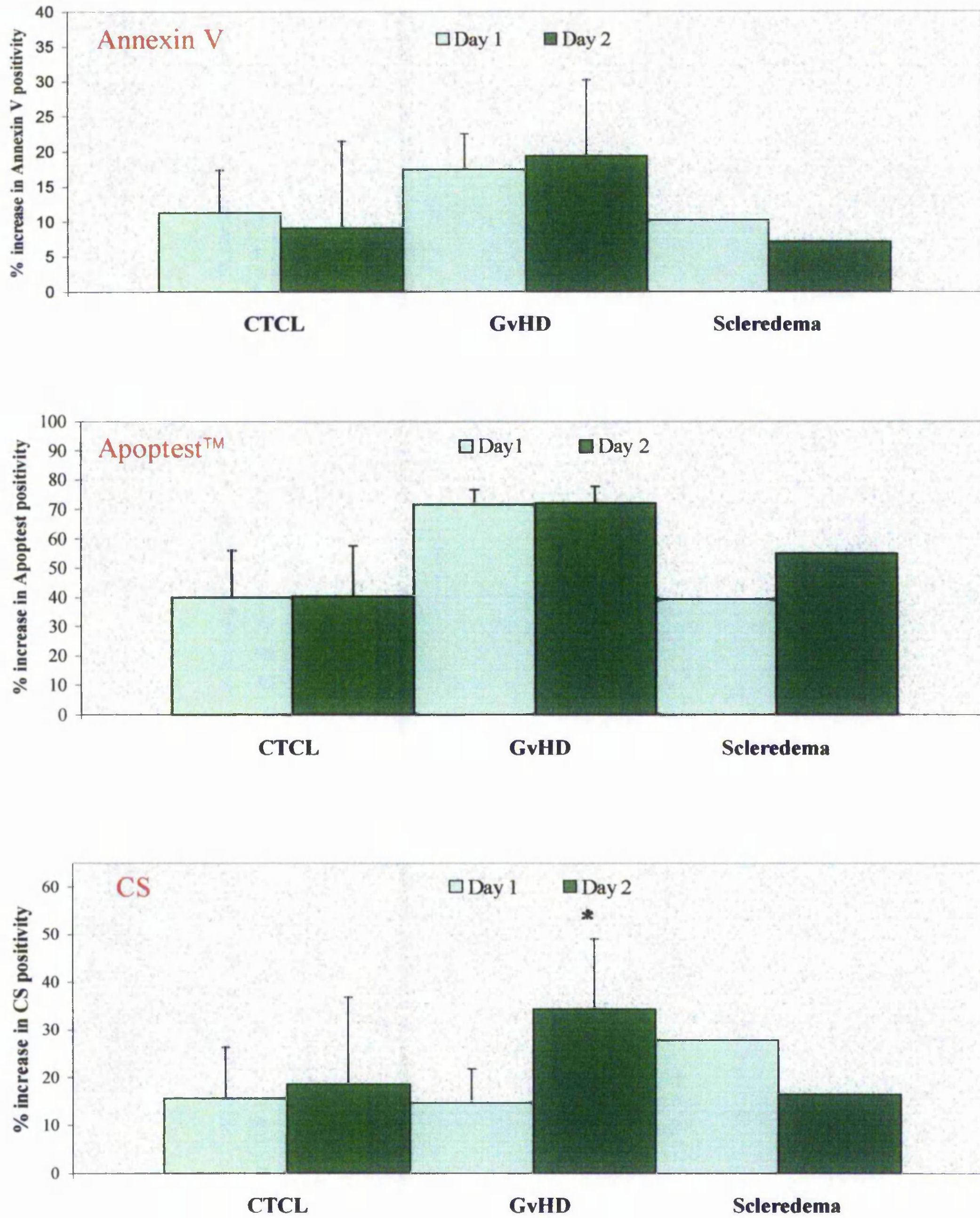


Figure 3.4.1.5. When comparing the increase in apoptotic lymphocytes (post ECP – pre ECP), no significant increase in Annexin V or Apoptest ™ positivity was observed on day 2 of treatment. However the GvHD patients demonstrated an increase in CS positivity on the second day of ECP.

3.4.2. T helper and T cytotoxic apoptosis

Because ECP 'responders' demonstrate a reduction in CD4+ T cell numbers after several rounds of treatment, the CD4+ and CD8+ T cells were individually evaluated for apoptosis induction following ECP. Annexin V was chosen as the apoptosis detection markers, due its well recognised and robust methodology. Each CD4+ and CD8+ T cell subset was identified using FITC-conjugated anti CD4 and anti CD8 and SS respectively. Section 2.2.2.2.3 and 2.2.2.2.4 demonstrates how each respective CD4+ and CD8+ T cell subset was observed and 'bitmapped' using flow cytometry. The level of apoptosis induced in each T cell subset, at re-infusion is represented in Figure 3.4.2.1. As only one Scleredema patient was tested, no statistical increase in apoptosis was detected between the different stages of ECP treatment, however the levels of apoptosis observed were similar to those of the CTCL patients. For both CTCL and GvHD patient groups, significant levels of apoptosis were detected at re-infusion. However, comparisons of CD4+ and CD8+ T cell subsets demonstrated no significant difference in apoptotic numbers at re-infusion. As before, when comparing lymphocyte apoptosis numbers for the CTCL and GvHD patients, the GvHD group demonstrated a significant higher number of apoptotic lymphocytes at re-infusion.

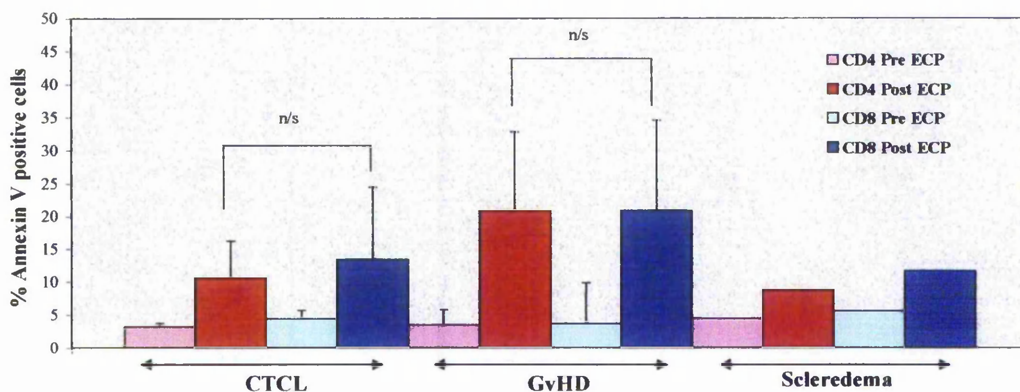


Figure 3.4.2.1 ECP does not preferentially induce apoptosis in the CD4+ or CD8+ T cell subset

Samples were taken immediately pre and post ECP. PBMCs were separated by density gradient centrifugation and the cell count adjusted to 0.5 to 1.0 x 10^6/ml (See Section 2.2.1.5). The cells were stained using either PE-CY-5 conjugated anti CD4 or anti CD8 (See Sections 2.2.2.2.3 and 2.2.2.2.4) and Annexin V (See Section 2.2.3.1). The CD4+ or CD8+ T cells were identified using flow cytometry and bitmapped (See Figures 2.2.2.2.3.1 and 2.2.2.2.4.1). From this bitmap the number of Annexin V positive cells was enumerated. The data represents the mean ± SD obtained from each patient group; n/s denotes a non-significant p value. The Scleredema patient group represents the results of only one patient

3.5. Discussion

Using a panel of tests, which determine different cellular expressions of early apoptosis, significant levels of lymphoid apoptosis were determined in the buffy coat bag *ex-vivo*. Previous *in-vitro* studies have demonstrated that 8-MOP alone does not induce apoptosis (Yoo *et al.,* 1996). However, the apoptotic effects, attributable to the mechanics of cell separation, i.e. cannulation, peristalsis pumping and centrifugation are unknown. To determine this effect would have involved testing cells after ECP treatment without exposure to UVA irradiation. This was however considered unethical, as the patients treatment would not be in accordance with current guidelines and as such its benefits may be compromised. Samples were therefore taken pre ECP and from the first buffy coat collected, prior to any UVA irradiation. Additional samples were taken just prior to re-infusion to assess the apoptotic effect of both the cellular manipulation plus the exposure of the buffy coat to 8-MOP and UVA irradiation.

Analysis of the CTCL and GvHD patients demonstrated a significant increase in apoptosis in the pre infusion bag, using Annexin V, Apoptest™, and Carboxy-SNARF®-1-AM (CS). The Apoptest™, associated with very early apoptosis detection (Ferlini *et al.,* 1997), demonstrated the most significant increase. This higher value could be because Apoptest™ was detecting chromatin texture changes as a consequence UVA damage, in addition to changes induced by apoptosis. These results indicate that the ECP therapy induces apoptosis in a significant numbers of lymphocytes before the cells are re-infused.

Previous investigations of ECP did not detect significant levels of lymphocyte apoptosis when tested immediately after the procedure (Aringer *et al.,* 1997; Enomoto *et al*., 1997) or 6 hours post ECP (Yoo *et al*., 1996). Determination of apoptosis in these experiments included the use of PI and the TUNEL technique (Aringer *et al.,* 1997), gel electrophoresis and in situ nick translation of DNA fragmentation (Enomoto *et al*., 1997) and detection of DNA strand breaks and changes in cells size and density (Yoo *et al.,* 1996). All of these techniques are

associated with a relatively 'later' stage of apoptosis. For the determination of apoptosis in this study, markers associated with early apoptosis; Annexin V (Vermes *et al.,* 1995), Apoptest™ (Ferlini *et al.*, 1997) and CS (Ishaque *et al.*, 1997) were selected. Annexin V has demonstrated significant levels of apoptosis in the T lymphoblastoid cell line CCRF-HSB-2, 4 hours after induction with 8 Gy irradiation (Vermes *et al.,* 1995). Apoptest™ has determined apoptosis in PBMCs 4 hours after 8 Gy irradiation (Ferlini *et al.*, 1997). Apoptosis has been observed in hybridoma cells 3 hours after exposure to campothecin, staurosporine and lutamine and oxygen deprivation using CS (Ishaque *et al.,*1997). The time difference in expression of apoptotic markers, between this and previous studies may therefore most likely be due to the methodology chosen for apoptosis determination.

Apoptotic lymphocytes expressing phosphatidylserine on their membrane are rapidly removed by phagocytosis (Fadok *et al.,* 1992). Apoptotic lymphocytes expressing increased Apoptest™ uptake start to become positive for Annexin V expression soon after (Ferlini *et al.,* 1997). It therefore seems likely that a significant number of ECP treated lymphocytes would be promptly removed, *in-vivo*, after their re-infusion. The unsuccessful attempt to identify apoptotic cells, *in-vivo*, at 1, 6 and 24 hours (Yoo *et al.,* 1996) and 24 hours (Aringer *et al.,* 1997) post re-infusion seems to confirm this. Results reinforced by the lack of an increase in apoptotic cells in the pre ECP sample on day 2 compared to day 1, some 19 hours post re-infusion. Previously cells in a pre-apoptotic state, post ECP, have been linked to immune cell signalling processes prior to the start of their apoptotic demise, some 24 hours and 48 hours post re-infusion (Enomoto *et al.* 1997). The prompt removal of apoptotic lymphocytes, by phagocytosis, would indicate that this process may be less pronounced that previously thought. More likely is that an immunomodulatory response is initiated by the processing of the apoptotic lymphocytes by immunocompetent APCs (Edelson *et al.,* 1994, Rook *et al.,* 1999).

In CTCL 'responders', ECP restores the CD4/CD8 imbalance after 12 months of treatment (Zouboulis *et al.,* 1998). Following ECP induced apoptosis, normal T lymphocytes are replaced at a rate greater than malignant cells migrate to the peripheral blood (Yoo *et al.,* 1996). However the success of ECP is not due to the selective induction of apoptosis in the CD4+ population. For both CTCL and GvHD patients groups, no significant difference in lymphoid apoptosis, induced in each T cell subset, was detected at re-infusion. However, this is not a surprising; although ECP exposes 25-50% of the peripheral nucleated cell count during a treatment cycle (Wolfe *et al.,* 1994), this still only accounts for exposing 3-5% of the tumour load (Gottlieb *et al.,* 1996; Hanlon *et al.,* 1998). The removal of the clonal T cells is more likely to be as a result of the induction of immunomodulatory process, whereby non-treated, but clonal T cells are targeted (Perez *et al.,* 1989; Edelson *et al.,* 1994). A theory strengthened by the phenotype of ECP 'responders'. ECP 'responders' demonstrate immune competency, with near normal absolute CD8+ and normal natural killer (NK) activity (Gottlieb *et al.,* 1996; Zouboulis *et al.,* 1998; Rook *et al.,* 1999). In addition, the malignant clone has to be present in the periphery for ECP response; no patient with plaque disease covering greater than 10% of the skin surface area, in the absence of peripheral blood involvement, have responded to photopheresis (Wolfe *et al.,* 1994). Observations, which highlight the importance of ECP modification to treated lymphocytes, but just as important is the role of the immune system in dealing with these apoptotic cells.

The process of apoptosis induction appears to begin immediately, CTCL, Scleredema and GvHD patients demonstrated enhanced apoptosis in the cells collected in the first buffy coat. The greatest increases were seen in the GvHD patients using the Apoptest™ and CS, with mean increases of over 20%. Interestingly this sample was taken before the 8-MOP had been injected into the collection bag. Greater levels of apoptosis at re-infusion demonstrate the addition effect of 8 MOP and UVA irradiation. However it is with interest to note that exposure to the mechanics of ECP and an *ex vivo* state seems able to induce some apoptosis of ECP treated lymphocytes, to which the GvHD

patients seemed most sensitive. Recently we observed a similar pre UV induction of apoptosis with the updated 'XTS™' ECP machine for CTCL and Scleredema patients. This fully automated machine 'concentrates' the buffy coat by enhanced cellular manipulation before the cells are photoactivated. This enhanced manipulation seems able to induce significant levels of apoptosis in the lymphocytes of these individuals before the cells are irradiated (Bladon and Taylor, 1999). At re-infusion, the GvHD group demonstrate significantly higher levels of lymphocyte apoptosis. A result that is mirrored with the apoptosis levels determined in both the CD4+ and CD8+ T cell subsets.

The levels of apoptosis induction on day one and day two of treatment were compared. Only the lymphocytes from GvHD patients demonstrated a significant increase on the second day of treatment ($p = 0.016$). This seemed to indicate that the recommended two day treatment may be intentioned to expose a large cell numbers to the ECP therapy rather than priming the cells in some way on day one.

These results indicate that the ECP therapy causes a significant degree of apoptosis induction in the lymphocytes of treated patients prior to re-infusion. This response, along with the other immunological changes, seen after ECP, may contribute to the observed clinical effect.

Exposure of lymphocytes to UVA-1, induces apoptosis predominately of the 'immediate-type', whilst PUVA induces a delayed typed apoptosis (Godar, 1999). The early apoptotic process is associated with a drop in Bcl-2 whilst delayed apoptosis involves up-regulation of the tumour suppresser gene p53 (Wang *et al.,* 1998). To further understand the mechanism responsible for this, previously undescribed early apoptosis induced by ECP, various apoptotic proteins were measured in the lymphocytes immediately following ECP.

CHAPTER 4: Lymphocytes treated by Extracorporeal Photopheresis (ECP) demonstrate a drop in Bcl-2/Bax Ratio. A possible mechanism involved in ECP induced apoptosis.

4.1. Introduction

Several genes have been identified with the regulation of apoptosis, through either promotion of cell death or extension of cell survival (William and Smith, 1993). The tumour suppresser gene p53 regulates transition through the G1/S-phase of the cell cycle (Yeargin and Haas, 1995). When DNA damage is detected, p53 protein levels increase leading to either cell cycle arrest and repair, or induction of apoptosis (Kuerbitz *et al.,* 1992; Lowe *et al.,* 1993). In this way p53 acts to maintain the integrity of the genome, preventing mutagenesis and tumour development (Moll and Schramm, 1998).

The Bcl-2 family of genes are key regulators of apoptosis (Williams, 1993) with over a dozen members identified (Allen *et al.,* 1998). Bcl-2 and Bax are two genes within the Bcl-2 family, which have been very widely researched and have been shown to influence the survival of many cell types, including those of the haematopoietic system (Van der Vliet *et al.,* 1997; Allen *et al.,* 1998). The protein products of Bcl-2 and Bax are intimately linked, the ratio of one to the other influencing cell survival (Van der Vliet *et al.,*1997). Bcl-2 and Bax are able to homo-and heterodimerise with each other, an excess of Bcl-2/Bax inhibiting apoptosis whilst Bax/Bax homodimers enhance the apoptotic process (Oltvai *et al.,* 1993).

The exposure of lymphocytes to UVA/8MOP has been demonstrated to cause DNA damage (Parrish *et al.,* 1974). Exposure of UVA/8MOP induces delayed apoptosis in treated lymphocytes 24 hours post ECP (Mark and Fox, 1991; Yoo *et al.,* 1996, Enomoto *et al.,* 1997). The expression of Bcl-2 protein in the apoptotic lymphocytes of Scleroderma patients 24 and 48 hours post ECP remained unchanged (Aringer *et al.,* 1997), while enhanced expression of p53

was observed in non-lesional skin of psoriasis following PUVA therapy (Hannuksela-Svahn *et al.,* 1999). However, recently ECP has been demonstrated to induce apoptosis in significant numbers of lymphocytes immediately post ECP (Chapter 3, Bladon and Taylor, 1999b).

4.2. Aim

This chapter aims to establish if the early apoptosis, observed immediately following ECP, was associated with an increased expression of the p53 protein or dysregulation in the ratio of the intracellular levels of Bcl-2 proteins to Bax proteins.

4.3. Specific materials and methods

The specific cohort of patients, ECP technology and analysis methods pertaining to this chapter are detailed below. For a full explanation, please see the Materials and Methods chapter.

4.3.1. Patients

Samples were obtained from nine CTCL patients, 5 stage III and 4 stage IVA (mean age ± S.D; 66.8 ± 5.8 years), four GvHD patients (31.3 ± 8.7) and one Scleredema patient (56).

4.3.2. Photopheresis treatment

Extracorporeal photopheresis cycles were performed using the UVAR® system. See Section 2.2.1.2.1 of the Materials and Methods chapter.

4.3.3. Sampling

Heparinised blood was taken from patients immediately before ECP and from the incubated BCB just prior to re-infusion.

4.3.4. Cell identification.

Lymphocytes were identified using FS vs SS. Confirmation of the cell type utilised anti CD45 and anti CD14. See Section 2.2.2.2.1 of the Materials and Methods chapter.

4.4. Results

The discovery of significant levels of apoptosis in lymphocytes very soon after exposure to ECP prompted evaluation of several proteins linked directly to apoptotic mechanisms. p53, Bcl-2 and Bax were evaluated in the lymphocytes of ECP-treated patients immediately pre-ECP and in the buffy coat bag prior to re-infusion. Lymphocytes were identified and isolated using the characteristic low forward and side scatter patterns they possess (See Figures 2.2.2.2.1.1 and 2.2.2.2.1.2). The additional staining, using CD45 expression and CD14 confirmed this population as being clear of significant monocyte and debris infiltration (See Figure 2.2.2.2.1.3.). Because the p53, Bcl-2 and Bax proteins are retained within lymphocytes, cells were fixed and permeabilised (See Section 2.2.1.7). Using this technique allowed access of the appropriate antibody to the intracellular compartment. Because this method did not depend on estimation of total protein quantification, post cell lysis, evaluation of each cell on an individual basis could be achieved.

4.4.1. p53 evaluation

Staining using an isotype control allowed for the setting of 'region gates' on a single histogram of FL-I (FITC) expression (See Figure 2.2.2.2.6.1.1.1). Following staining with FITC-conjugated anti p53, this region gate was used to enumerate the percentage of lymphocytes expressing p53 at each testing stage (See Section 2.2.2.2.6.1.1.2).

For both the CTCL and GvHD patient groups, no significant increase in p53 expression was observed before re-infusion. No patient demonstrated more than a 2% increase in lymphocytes with a positive p53 expression (Data not shown). Results, which definitively rule out the involvement of the p53 protein in the mechanism responsible for the high levels of lymphoid apoptosis, induced by ECP immediately prior to re-infusion

4.4.2. Bcl2 and Bax evaluation

Because the interaction of Bcl-2 and Bax has a pronounced effect on the induction of cell to apoptosis, the levels of each apoptotic protein were evaluated and a ratio of Bcl-2 to Bax calculated. To test the sensitivity of Bcl-2 and Bax, to the effects of ECP, lymphocytes were again evaluated immediately pre and post ECP. The intracellular content of Bcl-2 and Bax were derived from the intensity of signal emitted, following binding of respective FITC-conjugated antibodies. The resultant final mean fluorescent intensity (MFI) for the Bcl-2 and Bax from lymphocytes pre ECP and pre re-infusion were calculated by subtracting the MFI of the appropriate isotype control from the MFI of the test (See Section 2.2.2.2.7).

The Bcl-2/Bax ratio was calculated by dividing the final MFI of Bcl-2 by the final MFI of Bax lymphocyte apoptosis. Each patient tested demonstrated a fall in the Bcl2/Bax ratio between each stage of ECP. Figure 4.4.2.1 to 4.4.2.3 demonstrates the final MFI's for both Bcl-2 and Bax pre and post ECP, for each patient group, whilst the drop in the Bcl-2/Bax ratio are shown in Figures 4.4.2.4 to 4.4.2.6.

As only one Scleredema patient was tested, no statistical increase in apoptosis was detected between the different stages of ECP treatment. However the levels of apoptosis observed were similar to those of the GvHD and CTCL patients

Figure 4.4.2.1. Changes in Bcl-2 and Bax levels in the lymphocytes of CTCL patients pre and post ECP

Samples were taken immediately pre and post ECP. PBMCs were isolated by density gradient and the cell count adjusted to 1.0 to 2.0 x 10^6*/ml (See Section 2.2.1.5). Using flow cytometry the lymphocytes were bitmapped and examined for intracellular Bcl-2 and Bax expression (See Sections 2.2.4.2 and 2.2.4.3). The mean and SEM of the final MFI values for Bcl-2 and Bax are displayed for 9 CTCL patients.*

Figure 4.4.2.2. Changes in Bcl-2 and Bax levels in the lymphocytes of GvHD patients pre and post ECP

Samples were prepared as identified in Figure 4.4.2.1. The mean and SEM of the final MFI values for Bcl-2 and Bax are displayed for 3 GvHD patients.

Figure 4.4.2.3. Changes in Bcl-2 and Bax levels in the lymphocytes of a Scleredema patient pre and post ECP

Samples were prepared as indicated in Figure 4.4.2.1. The final MFI values for Bcl-2 and Bax are displayed. As only one patients was tested no SEM value was calculated

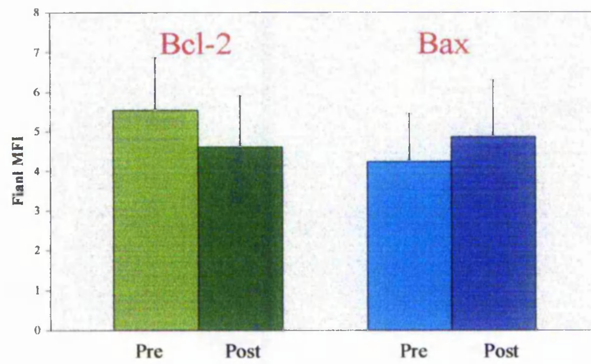


Figure 4.4.2.1. Changes in Bcl-2 and Bax levels in the lymphocytes of CTCL patients pre and post ECP

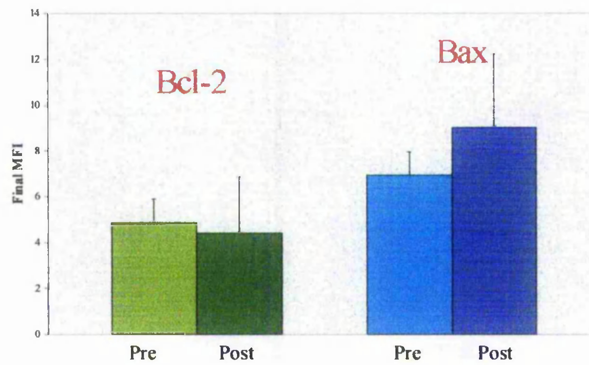


Figure 4.4.2.2. Changes in Bcl-2 and Bax levels in the lymphocytes of GvHD patients pre and post ECP

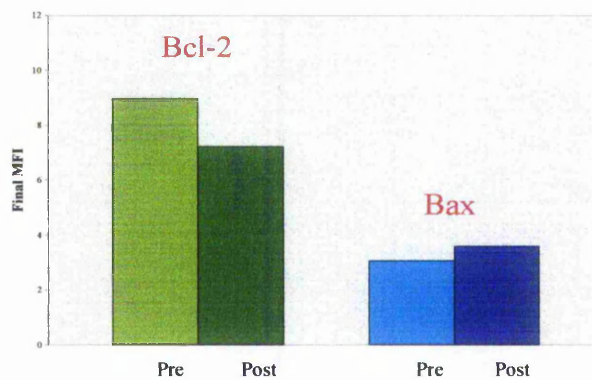


Figure 4.4.2.3. Changes in Bcl-2 and Bax levels in the lymphocytes of a Scleredema patient pre and post ECP

4.4.2.4. The Bcl-2/Bax ratio in the lymphocytes of CTCL patients are reduced by ECP exposure

Samples were taken immediately pre and post ECP. PBMCs were isolated by density gradient and the cell count adjusted to 1.0 to 2.0 x 10^6/ml (See Section 2.2.1.5). Using flow cytometry the lymphocytes were bitmapped and examined for intracellular Bcl-2 and Bax expression (See Sections 2.2.4.2 and 2.2.4.3). The final MFI values for Bcl-2 and Bax were calculated by subtracting the MFI of the isotype control from the test MFI. The subsequent Bcl-2 MFI was divided by that of Bax to give the Bcl-2/Bax ratio. The data represents the Bcl-2/Bax ratio of 9 different CTCL patients tested.

Figure 4.4.2.5. The Bcl-2/Bax ratio in the lymphocytes of GvHD patients are reduced by ECP exposure

Samples were prepared as identified in Figure 4.4.2.4. The data represents the Bcl-2/Bax ratio of 3 different GvHD patients tested

Figure 4.4.2.6. The Bcl-2/Bax ratio in the lymphocytes of a Scleredema patient is reduced by ECP exposure

Samples were prepared as indicated in Figure 4.4.2.4. The data represents the Bcl-2/Bax ratio for a Scleredema patient tested

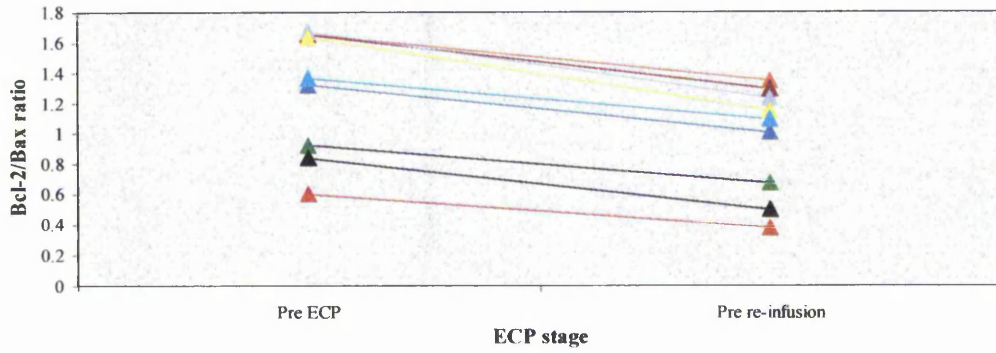


Figure 4.4.2.4. The Bcl-2/Bax ratio in the lymphocytes of CTCL patients are reduced by ECP exposure

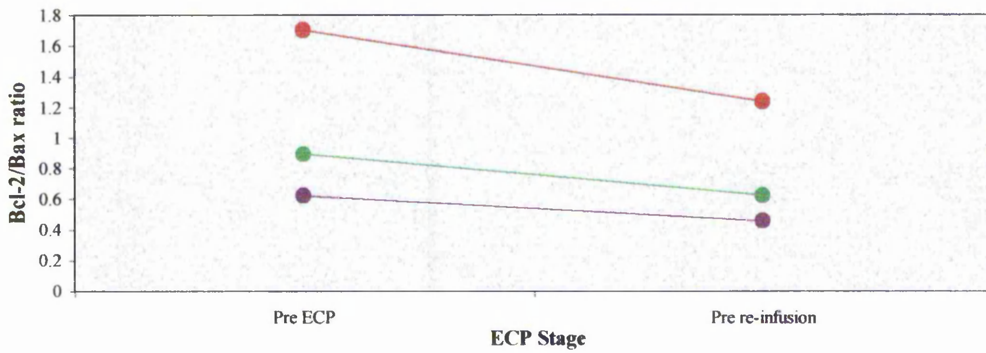


Figure 4.4.2.5. The Bcl-2/Bax ratio in the lymphocytes of GvHD patients are reduced by ECP exposure

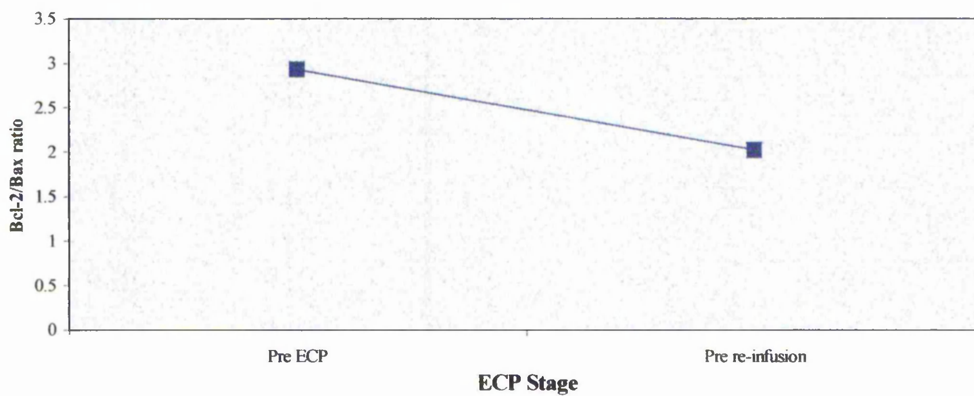


Figure 4.4.2.6. The Bcl-2/Bax ratio in the lymphocytes of a Scleredema patient is reduced by ECP exposure

Lymphocytes from ECP treated CTCL and GvHD patients demonstrate a moderate, but significant decrease in intracellular Bcl2/Bax protein ratio before re-infusion ($p<0.05$). CTCL patients demonstrated a mean fall of 27.08%. The lymphocytes of GvHD patients showed a mean fall of 27.93%. The Scleredema patient demonstrated the largest drop, with a 31.1% decrease in Bcl2/Bax ratio at re-infusion. These results indicate that ECP may influence the production and subsequent interaction of these two apoptotic proteins. Further evaluation, by comparing the drop in Bcl-2 /Bax ratio with levels of apoptosis, as detected using Annexin V, confirms a close relationship. A correlation coefficient of 0.91 and 0.99 were determined for CTCL and GvHD patients respectively.

Comparison of the CTCL and GvHD patients demonstrated no significant difference between the two patient groups in the fall in Bcl-2/Bax ratio immediately post ECP. These results were in conflict with previous findings, in which the GvHD patients demonstrated higher levels of apoptosis than those observed for CTCL and Scleredema (Chapter 3, Bladon and Taylor, 1999b). However, further analysis of apoptosis between 0 and 48 hours following ECP (Chapter 5, Bladon and Taylor, 2002) demonstrates no significant difference in the levels of apoptosis observed between the CTCL and GvHD patient groups.

4.5. Discussion

Significant levels of apoptotic lymphocytes have been observed immediately post ECP, prior to the re-infusion (Chapter 3, Bladon and Taylor, 1999b) and one hour post ECP (Gerber *et al.,* 2000). The aim of this chapter was to investigate if this very early induction of apoptosis involved expression or dysregulation of the known apoptotic associated genes p53, Bcl-2 and Bax.

The gene p53 has been closely linked to the induction of apoptosis. Upon detection of DNA damage, protein levels of the tumour suppresser gene p53 increase, leading to either cell cycle arrest and repair or induction of apoptosis (Kuerbitz *et al.,* 1992; Lowe *et al.,* 1993) thus preventing mutagenesis and tumour development (Moll and Schramm, 1998). Increased wild-type p53 has been elicited in response to DNA damage induced by ultraviolet light (Wang *et al.,* 1998). Elevated p53 concentrations, resulting in apoptosis in normal human peripheral blood lymphocytes, have also been detected (Yeargin and Haas, 1995). The lymphocytes tested immediately post ECP did not demonstrate any increase in p53, but did however demonstrate a significant fall in the ratio of the Bcl-2/Bax MFI. An interesting finding, as a decrease in the expression of Bcl-2, whilst simultaneously enhancing the gene Bax has been described as under the influence of p53 (Miyashita *et al.,* 1994).

The Bcl-2 family of genes are key regulators of apoptosis (Williams, 1993) with over a dozen members identified (Allen *et al.,* 1998). The gene Bcl-2 was initially found in a proportion of B-cell follicular lymphoma's (Tsujimoto, 1985). The presence of Bcl-2 inhibits apoptosis induced by a variety of stimuli (Vaux *et al.,* 1988; Nunez *et al.,* 1990). Bcl-2 exerts its anti apoptotic effect through several different processes including antioxidant activity, preservation of mitochondrial potential and blockage of calcium movement into the cytoplasm (Hockenbery *et al.,* 1993; Lam *et al.,* 1994; Zamzami *et al.,* 1995). Bax, conversely promotes apoptosis by inducing the release of cytochrome c from the mitochondria leading to activation of caspase-3 (Rossé *et al.,* 1998). The translocation of Bax from the cytosol to the mitochondria initiating

mitochondrial disruption, with the subsequent release of cytochrome c. (Giorgi *et al.,* 2002). The protein products of Bcl-2 and Bax are intimately linked, able to homo-and heterodimerise with each other. An excess of Bcl-2/Bax heterodimers inhibits apoptosis whilst Bax/Bax homodimers enhance the apoptotic process (Oltvai *et al.,* 1993). The ratio of Bcl-2 to Bax provides an index of how sensitive some cells are to apoptosis following induction (Van der Vliet *et al.,* 1997; Allen *et al.,* 1998). Cell types showing a low Bcl-2/Bax ratio show the greatest susceptibility to anti-Fas-induced apoptosis (Van der Vliet *et al.,* 1997). Additionally, dysregulation of these genes, as a consequence of stimulation can influence their expression within the cell, influencing survival. Xia *et al* demonstrated a significant fall in the peripheral lymphocyte expression of Bcl-2 and c-myc, in addition to enhanced anti-Fas expression, in cells induced into apoptosis by tricyclic antidepressants (Xia *et al.,* 1997). While treatment of mycosis fungoides cells with a Jak kinase inhibitor Ag490 induced apoptosis in conjunction with a 54% decrease in Bcl-2 and a 128% increase in Bax expression (Nielson *et al.,* 1999).

In this chapter, a significant fall in the ratio of Bcl-2/Bax in the lymphocytes tested immediately post ECP was observed. The drop in Bcl-2/Bax ratio occurs very rapidly, detectable before the cells are re-infused. Studies of *in-vitro* UVA exposure on breast cancer epithelial cells have also shown the onset of apoptosis to occur rapidly, with 50% TdT positivity in cells 4 hours after irradiation (Wang *et al.,* 1998). Wang also noted that the UVA induced apoptosis was associated with no increase in p53 expression, but a dose related drop in Bcl-2 expression (Wang *et al.,* 1998). Other researchers have demonstrated that the induction of apoptosis following photodynamic therapy is due to the selective destruction of Bcl-2, leaving Bax unaffected (Choi Kim *et al.,* 1999; Isoherranen *et al.,* 1999). Termed 'immediate type apoptosis', early apoptosis induced by UVA involves inducing oxidative stress, cell membrane damage and mitochondrial membrane depolarisation, but no p53 enhancement (Wang *et al.,* 1998; Godar, 1999). In this chapter, the early apoptosis induced by ECP was p53-independent. The early apoptosis, induced by ECP, has also been

demonstrated to give rise to the externalisation of phosphatidylserine ('flip-flop) on the cells membrane (Chapter 3, Bladon and Taylor, 1999b) and caused the early depolarisation of the $\Delta\psi_m$ (Chapter 5, Bladon and Taylor, 2002). Bcl-2 preserves the $\Delta\psi_m$, whilst Bax is capable of triggering pore-forming activity in the mitochondrial membrane, releasing cytochrome c, a process Bcl-2 can only antagonise if present in sufficient quantities (Rossé *et al.,* 1998). Correlation coefficient values for the Bcl-2/Bax ratio reduction and apoptosis induction (detected by Annexin V) for CTCL and GvHD patients were 0.91 and 0.99 respectively. For CTCL patients this correlation was very significant ($p<0.005$), however for GvHD no p value was determined, due to insufficient data points. Using florescence microscopy, an investigation of Bax translocation from the cytosol to mitochondria could have been observed on cytospin preparations. As Bax transloaction is essential for apoptosis induction involving Bax, these experiments would have helped in identifying the role Bax played in early ECP-induced apoptosis.

ECP treatment has previously been associated with delayed apoptosis (Marks and Fox, 1991; Yoo *et al.,* 1996; Enomoto *et al.,* 1997), by a mechanism which usually depends on the accumulation of p53 (Wang *et al.,* 1998). This evidence emanating from the absence of established apoptotic indicators in lymphocytes sampled immediately before re-infusion and tested *ex-vivo* and 6 hours post ECP (Yoo *et al.,* 1996, Enomoto *et al.,* 1997), but present in cultures 24 and 48 hours after ECP treatment (Marks and Fox, 1991; Yoo *et al.,* 1996; Enomoto *et al.,* 1997). However, the relatively early apoptosis determined, *ex vivo*, after ECP (Chapter 3, Bladon and Taylor, 1999b) coupled with dysregulation of the Bcl-2 pathway, may indicate that ECP may evoke, to some extent, an early and immediate apoptotic process.

Previous studies of Bcl-2 in ECP induced apoptosis noted no significant change in Bcl-2 expression after ECP treatment (Aringer *et al.,* 1997). However, because of the hetero- and homodimers formed between Bcl-2 and Bax proteins, measurement of their ratio is considered a better indicator of the cells fate than

expression of either protein in isolation (Oltvai *et al.,* 1993). This result could also be because what Aringer was measuring at 24 and 48 hour cultures (Aringer *et al.,* 1997), was a delayed type apoptosis involving a pathway associated with an increase in p53 expression (Wang *et al.,* 1998). This suggestion is supported by the up-regulation of the p53 gene detected in cultured ECP treated lymphocytes (Yoo *et al.,* 1996)

If ECP does induce an immediate onset of apoptosis in some treated lymphocytes, these cells would be phagocytosed soon after re-infusion. Their demise *in-vivo* possibly due to their engulfment by macrophages, which detect the translocation of phosphatidylserine groups (detected by Annexin V) from the internal to external membrane (flip-flop) (Chapter 3, Bladon and Taylor, 1999b). The processing of apoptotic cells in this way may also help explain the clone specific immunity associated with ECP. The process of phagocytosis involving antigen uptake, processing and presentation (Yoo *et al.,* 1996).

The induction of immediate apoptosis in ECP treated lymphocytes may serve to complement the apoptosis detected at a later stage, detected 24 and 48 hours post ECP (Marks and Fox, 1991; Yoo *et al.,* 1996; Enomoto *et al.,* 1997). Apoptosis detected later may involve invoking an up-regulation of the p53 gene and possible activation of the CD95 pathway (Aringer *et al.,* 1997)

This study demonstrates that the lymphocyte apoptosis detected immediately prior to re-infusion in ECP treated cells involves the dysregulation of the Bcl-2 and Bax proteins, but does not involve enhancement of p53 protein expression. The early apoptosis may target the rapidly proliferating lymphocyte in a process similar to that previously termed immediate-type apoptosis (Wang *et al.,* 1998; Godar, 1999). The early apoptosis may compliment the apoptosis induced in the delayed type process, previously described (Marks and Fox, 1991; Yoo *et al.,* 1996; Enomoto *et al.,* 1997). An important ECP mechanism may therefore be the immunomodulation induced by an immediate and sustained exposure of apoptotic lymphocytes.

The discord between the extent of apoptosis detected *ex-vivo* (Chapter 3, Bladon and Taylor, 1999b) and that detected after 24 hours in culture (Marks and Fox, 1991; Yoo *et al.,* 1996; Enomoto *et al.,* 1997) can probably be attributed to the test method chosen for apoptosis detection. However Wang's demonstration of immediate type apoptosis 4 hours after UVA radiation used the detection of DNA fragmentation as an assessment method, a method also used by Yoo *et al,* 1996, who did not detect any apoptosis when testing ECP treated lymphocytes, *ex-vivo* pre re-infusion. This difference could however be explained by the dose of UVA radiation used. Wang used a 150 KJ UVA per m^2 radiation source (Wang *et al.,* 1998), whilst Yoo used a conventional ECP source of 2J UVA per cm^2 (Yoo *et al.,* 1996).

The different apoptosis related proteins and their involvement in the induction of early and late apoptosis induced by ECP might indicate the utilisation of two apoptotic pathways in the ECP treatment modality. By inducing an immediate and progressive challenge to the immune system following re-infusion, ECP may increase the chances of evoking a strong immunomodulatory response. To further understand the mechanism leading to the early apoptosis observed following ECP, two systems with a strong association with the progression of apoptosis following induction were investigated. Changes to the mitochondria and caspase cascade have a pronounced effect on apoptosis progression and have definitive links to the externalisation of PS. In addition recently the membrane antigen CD10 has been cited as a macrophage target antigen, which may have a down-regulatory influence on pro-inflammatory cytokines.

CHAPTER 5: Extracorporeal photopheresis, in cutaneous T cell lymphoma and graft versus host disease, induces both immediate and progressive apoptotic processes

5.1. Introduction

ECP induces apoptosis prior to re-infusion (Chapter 3, Bladon and Taylor, 1999b). The speed and mechanisms responsible for carrying out this process, following re-infusion, may determine the extent of immunomodulation.

The mitochondria have long been considered responsible for the bulk of the cell's energy production (Thress *et al.,* 1999). Although necrotic cells demonstrate swollen mitochondria, early reports of apoptosis observed no marked changes to mitochondria morphology (Darzynkiewicz *et al.,* 1992). More recently, electron microscopy has demonstrated profound changes in the mitochondria morphology of apoptotic thymocytes (Petit *et al.,* 1995), while changes to the mitochondrial function of the apoptotic cell is now known to include disruption in the mitochondrial transmembrane potential ($\Delta\psi_m$) (Petit *et al.,*1995; Zamzami *et al.,* 1995; Tada-Oikawa *et al.,* 1998).

Caspases (cysteine aspartate-specific proteases) are a family of related proteases synthesised as latent zymogens (Procaspases) (Hug *et al.,* 1999). In mammals at least 14 different caspases have been described (Hu *et al.,* 1998). After apoptosis induction, activation of 'early' caspases triggers further activation of 'down stream' caspases, the resultant cascade effect leading to amplification of the apoptotic signal (Allen, *et al.,*1998; Thress *et al.,* 1999). The caspases are capable of opposing cellular attempts to stop apoptosis and ultimately act as the final executioner (Allen, *et al.,* 1998).

Under most circumstances, the reduction in $\Delta\psi_m$ and the activation of the caspases are irreversible processes and indicative of certain cellular demise (Zamzami *et al.,* 1995, Hug *et al.,* 1999). They are also thought to be amongst

the earliest indicators of the apoptotic process, preceding the point at which cells are recognised *in-vivo* as apoptotic by external phosphatidylserine expression and phagocytosis (Martin *et al.*,1996; Castedo *et al.*, 1996)

Peripheral lymphocyte expression of membrane CD10 is most usually associated with cells demonstrating the common acute lymphoblastic leukaemia antigen (CALLA) phenotype (Shipp *et al.*, 1989). However more recently, induction of CD10 expression on lymphocytes has been linked to growth arrest (Mari *et al.*, 1997) and apoptosis (Cutrona *et al.*, 1999) Although the function of the CD10 expressed by the apoptotic lymphoid cells is not fully understood, it has been postulated that its presence, *in vivo*, may be to limit the level of inflammation at the site of apoptosis. CD10, selectively expressed during apoptosis, may cleave the proteins with inflammatory or proinflammatory activity released by the dying cells. Alternatively CD10 may act as a macrophage target (Cutrona *et al.*, 1999).

5.2. Aim

This aim of this chapter was to determine:

i) The speed and extent of ECP-induced apoptosis in treated lymphocytes.
ii) The apoptotic mechanisms involve in the induction of apoptosis in lymphocytes exposed to ECP.

5.3. Specific patients and methods

The specific cohort of patients, ECP technology and analysis methods pertaining to this chapter are detailed below. For a full explanation, please see the Materials and Methods chapter.

5.3.1. Patients

Samples were obtained from 12 patients receiving ECP treatment 6 CTCL (mean age ± S.D; 69.8 ± 10.3 years), 5 GvHD patients (36.2 ± 8.8) and one Scleredema patient (57).

5.3.2. Photopheresis treatment

Extracorporeal photopheresis cycles were performed using the XTS™ ECP system. See Section 2.2.1.2.2 of the Materials and Methods chapter.

5.3.3. Sampling

PBMCs were immediately separated pre and post ECP. Cells for immediate testing were added to their appropriate test buffer. Cells for testing at 6, 24 and 48 hours were added to RPMI medium (See Section 2.2.1.6)

5.3.4. Cell identification

Lymphocytes identification used a FS vs SS plot. Staining with CD14 and CD45 excluded monocytes and debris respectively. See Section 2.2.2.2.1 of Materials and Methods chapter.

5.3.5. Apoptosis and secondary necrosis identification

Apoptosis and secondary necrosis were determined using Annexin V/ PI (see Section 2.2.3.1)

5.4. Results

Using markers, which are indicators of a number of processes, which can ultimately lead to the demise of a lymphocyte by apoptosis, this chapter was designed to try to establish the mechanisms responsible for the apoptosis observed in ECP-treated lymphocytes. By observing cells over a longer time frame, namely immediately prior to re-infusion through to 48 hours post ECP, it was also the intention to try to gain a deeper insight into the cellular processes which would occur *in-vivo*, following re-infusion of the ECP-treated cells.

Statistical analysis on the Scleredema patient was not possible because only one patient was tested. However, because the previous study of apoptosis induction by ECP therapy demonstrated similar findings for the CTCL and Scleredema lymphocytes (Chapter 3, Bladon and Taylor, 1999b), the data for the Scleredema patient was assessed with the CTCL results. The mean percentage and SEM of lymphocytes demonstrating a positive expression of Annexin V, Propidium Iodide, CD10, Rhodamine 123, JC-1 and activated caspases for both patient groups, namely CTCL & Scleredema and GvHD, are demonstrated in Figures 5.4.1.1, 5.4.2.1, 5.4.3.1, 5.4.4.2, 5.4.5.1 and 5.4.6.1 respectively

5.4.1. Annexin V

Annexin V identifies the externalisation of phosphatidylserine (PS) residues, changes indicative of early apoptosis. For both patient groups and at each testing stage, the post ECP treated cells show significantly higher levels of apoptosis, than those within the comparable pre ECP group (See Figure 5.4.1.1). Apoptosis induction was rapid, with significant levels of apoptosis detected in the sample tested *ex-vivo* from the buffy coat bag. The levels of apoptotic lymphocytes post ECP increased rapidly and included the majority of treated cells by 48 hours post ECP. However it was interesting to note that the increase in the levels of apoptosis from 0 to 6 hours was very small, for both groups, and not significant.

5.4.2. Propidium Iodide

Propidium iodide (PI), absorbed by necrotic cells, can be easily identified by the FL-II (PE) channel of the flow cytometer. For both patient groups, the onset of necrosis, within the lymphoid population treated using ECP, was not observed until 24 hours post ECP (See Figure 5.4.2.1). The combined staining using Annexin V and PI demonstrated the onset of secondary necrosis, following the initial expression of Annexin V. Figure 5.4.2.2 shows the Annexin V/ PI expression of lymphocytes pre ECP and post ECP (ex-vivo and 48 hours post ECP), as observed using the flow cytometer.

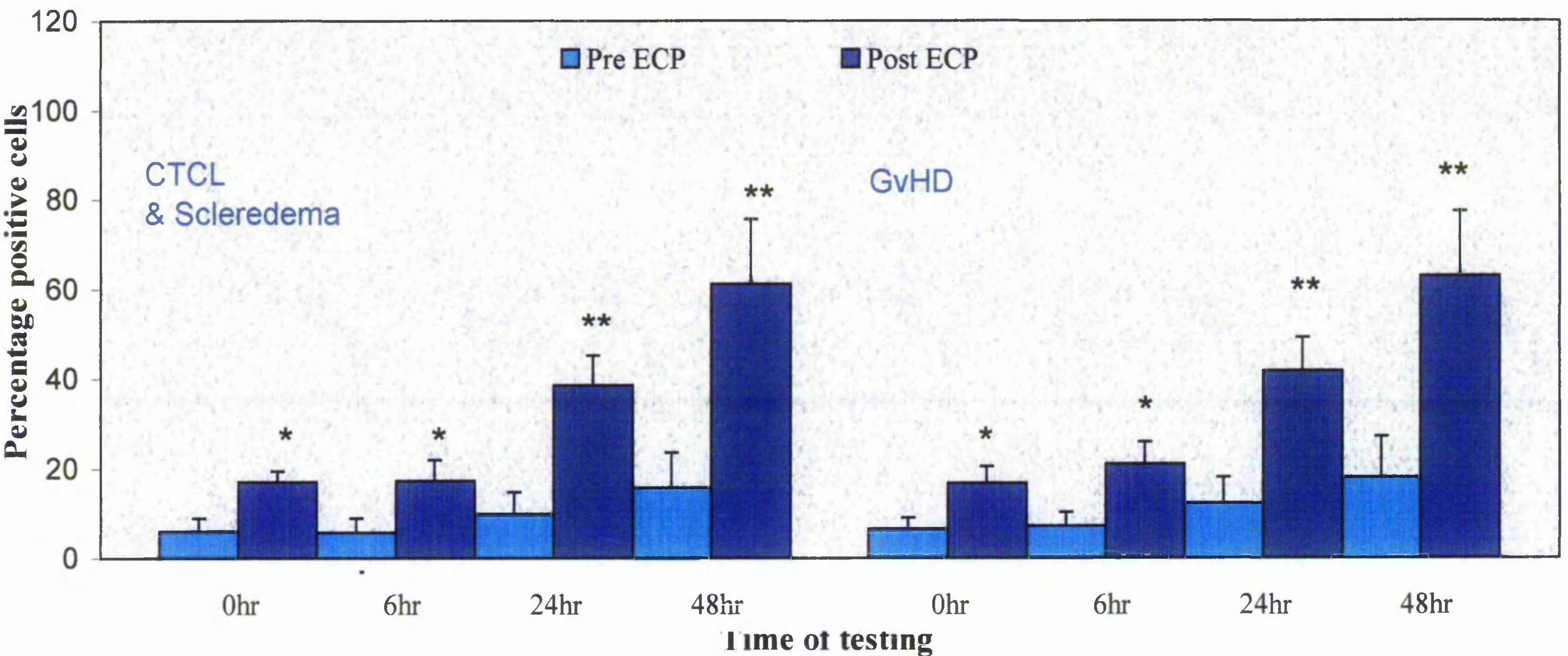


Figure 5.4.1.1 ECP induces an immediate and progressive increase in Annexin V positivity on treated lymphocytes

*Samples were taken immediately pre and post ECP and the PBMCs were isolated by density gradient (See Section 2.2.1.5). The pre- and post-ECP PBMCs were either tested immediately or added to cell culture (See Section 2.2.1.6). Lymphocytes were stained for phosphatidylserine (PS) externalisation, using FITC- conjugated Annexin V, after 0, 6, 24 and 48 hours in cell culture (See Section 2.2.3.1). The data represents the mean ± SEM of lymphocytes positive for PS externalisation obtained from each patient group. Statistical analysis compared the pre- and post-ECP results at each testing stage. * denotes $p < 0.05$ and ** denotes $p < 0.005$. An increase in Annexin V positivity was first observed immediately post ECP*

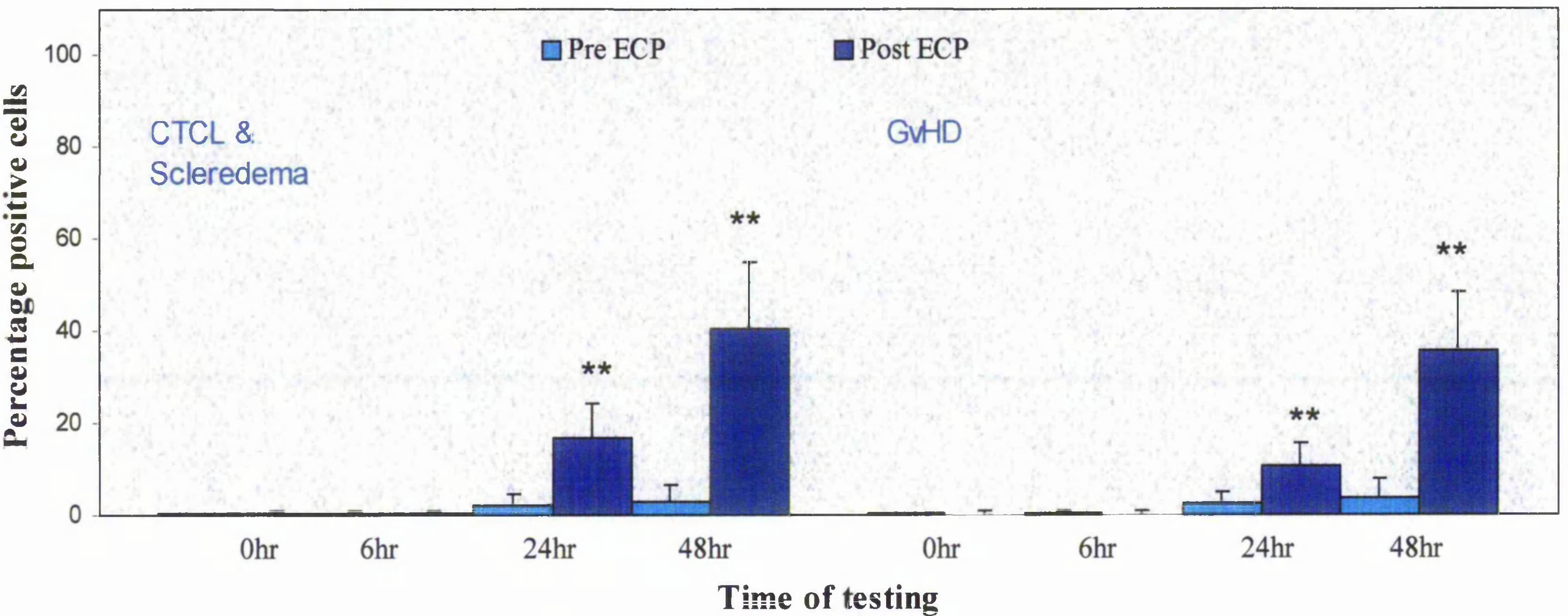


Figure 5.4.2.1 Lymphocytes treated by ECP do not stain positive for Propidium Iodide (PI) until 24 hours post exposure

*Samples were taken immediately pre and post ECP and the PBMCs were isolated by density gradient (See Section 2.2.1.5). The pre- and post-ECP PBMCs were either tested immediately or added to cell culture (See Section 2.2.1.6). Lymphocytes were stained for PI positivity after 0, 6, 24 and 48 hours in cell culture (See Section 2.2.3.1). The data represents the mean ± SEM of lymphocytes positive for PI obtained from each patient group. Statistical analysis compared the pre- and post-ECP results at each testing stage. * denotes $p < 0.05$ and ** denotes $p < 0.005$. PI positivity was observed later in the ECP process, at 24 hours post treatment.*

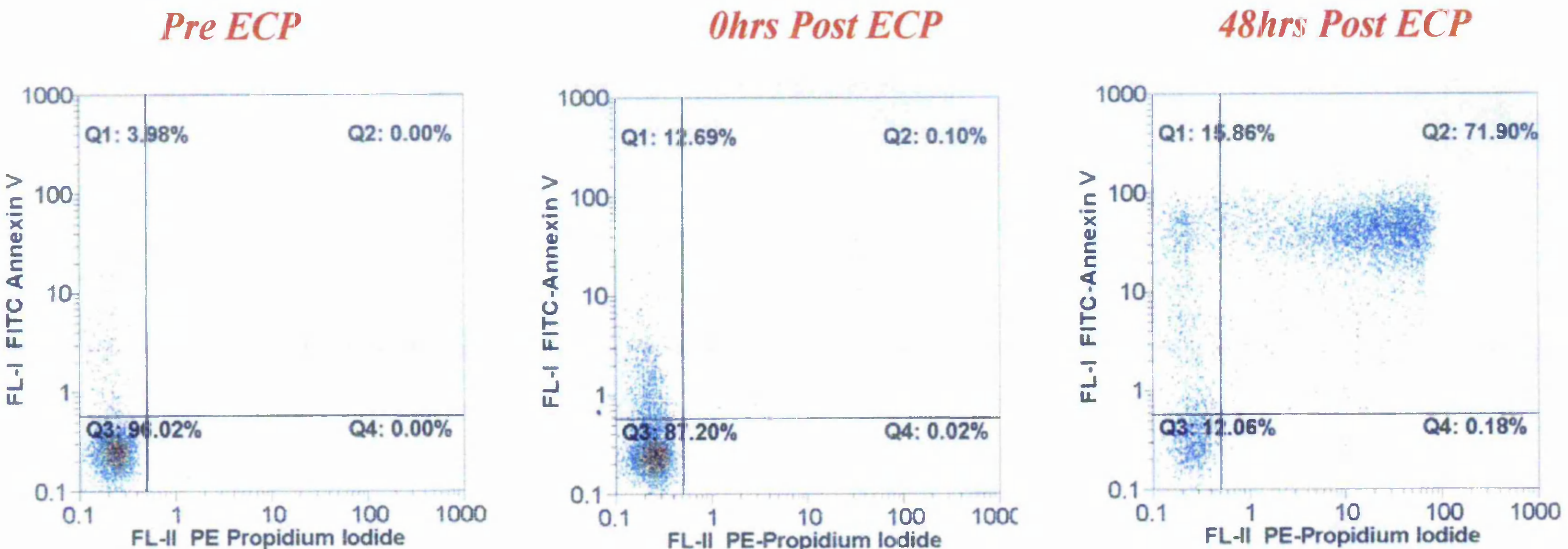


Figure 5.4.2.2 Flow cytometric representation of Annexin V/ Propidium iodide staining pre ECP and 0 and 48 hours post ECP.

Quadrant gates were set on the pre ECP sample. At subsequent testing stages Annexin V positive and PI positive lymphocytes were enumerated using these gates. Annexin V positive lymphocytes were identified in quadrant 'Q1', whilst secondary necrotic cells, demonstrating dual positivity for Annexin V and PI, are identified in quadrant Q2.

5.4.3. CD10 expression

Recently it has been proposed that the expression of CD10 on apoptotic lymphoid cells may cleave the proteins with inflammatory or proinflammatory activity that are released during apoptosis. Alternatively CD10 may act as a macrophage target. CD10 expression was observed on the lymphocytes dying by apoptosis at 24 and 48 hours post ECP (See Figure 5.4.3.1). However the expression was very weak, with florescence peaks which displayed only a slight shift from the isotype control. Of all markers tested, CD10 was observed in the smallest population of lymphocytes.

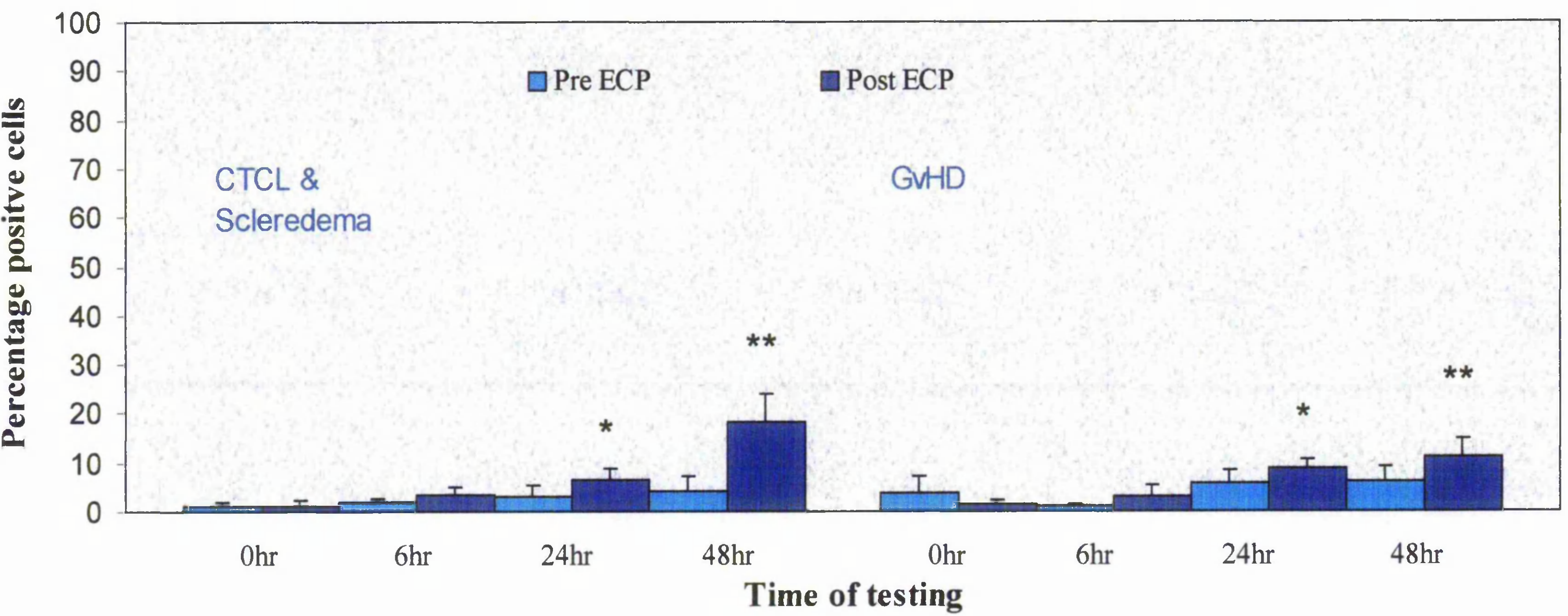


Figure 5.4..3.1 ECP induces a 'weak' and 'late' expression of CD10 on treated lymphocytes

*Samples were taken immediately pre and post ECP and the PBMCs were isolated by density gradient (See Section 2.2.1.5). The pre- and post-ECP PBMCs were either tested immediately or added to cell culture (See Section 2.2.1.6). Lymphocytes were stained for CD10 expression after 0, 6, 24 and 48 hours in cell culture (See Section 2.2.3.1). The data represents the mean ± SEM of lymphocytes positive for CD10, obtained from each patient group. Statistical analysis compared the pre- and post-ECP results at each testing stage. * denotes $p < 0.05$ and ** denotes $p < 0.005$. Although CD10 expression was first observed at 24 hours post ECP, expression was 'very weak'.*

5.4.4. Rhodamine 123

Rhodamine 123 is a dye, which is effective in detecting disruptions in the $\Delta\psi_m$ of mitochondria. Rhodamine 123 accumulates in the mitochondrial matrix under the influence of the $\Delta\psi_m$. Disruption of the $\Delta\psi_m$ is detected by a reduction in uptake of the stain and a subsequent lower level of fluorescence, detectable using the FL-I (FITC) channel (See Figure 5.4.4.1).

For comparative pre and post ECP samples, a significant disruption of the $\Delta\psi_m$, detected by Rhodamine 123, was observed from 6 hours post ECP onward (See Figure 5.4.4.2). Both the CTCL and Scleredema and the GvHD patients showed similar results.

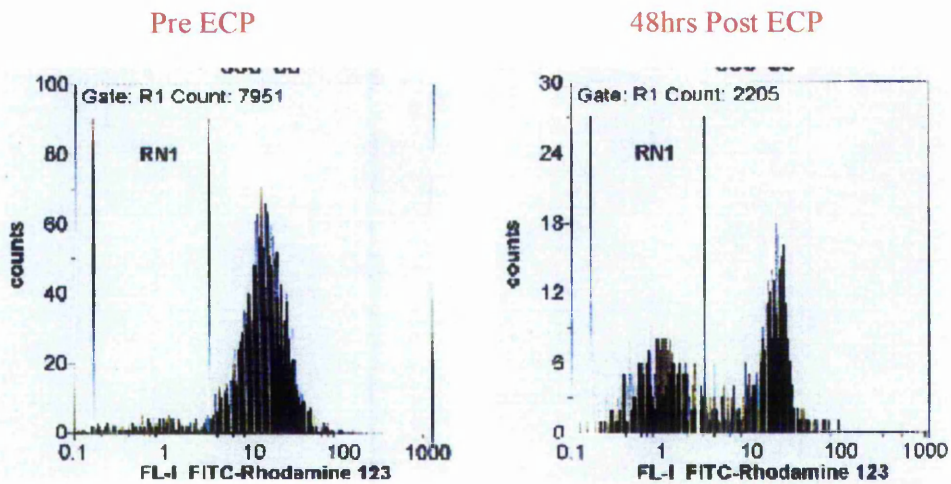


Figure 5.4.4.1 Flow cytometric representation of lymphocytes stained using Rhodamine 123 pre ECP and 48 hours post ECP.

Region gates were set on the FL-1 histogram using the pre ECP sample and control experiments using mClCCP (RN1). Subsequent samples, which demonstrate a reduction in the $\Delta\psi_m$, are detected within the RN1 region gate, as florescence diminishes. At 48 hours post ECP, a marked increase in cells demonstrating a reduced $\Delta\psi_m$ is clearly visible.

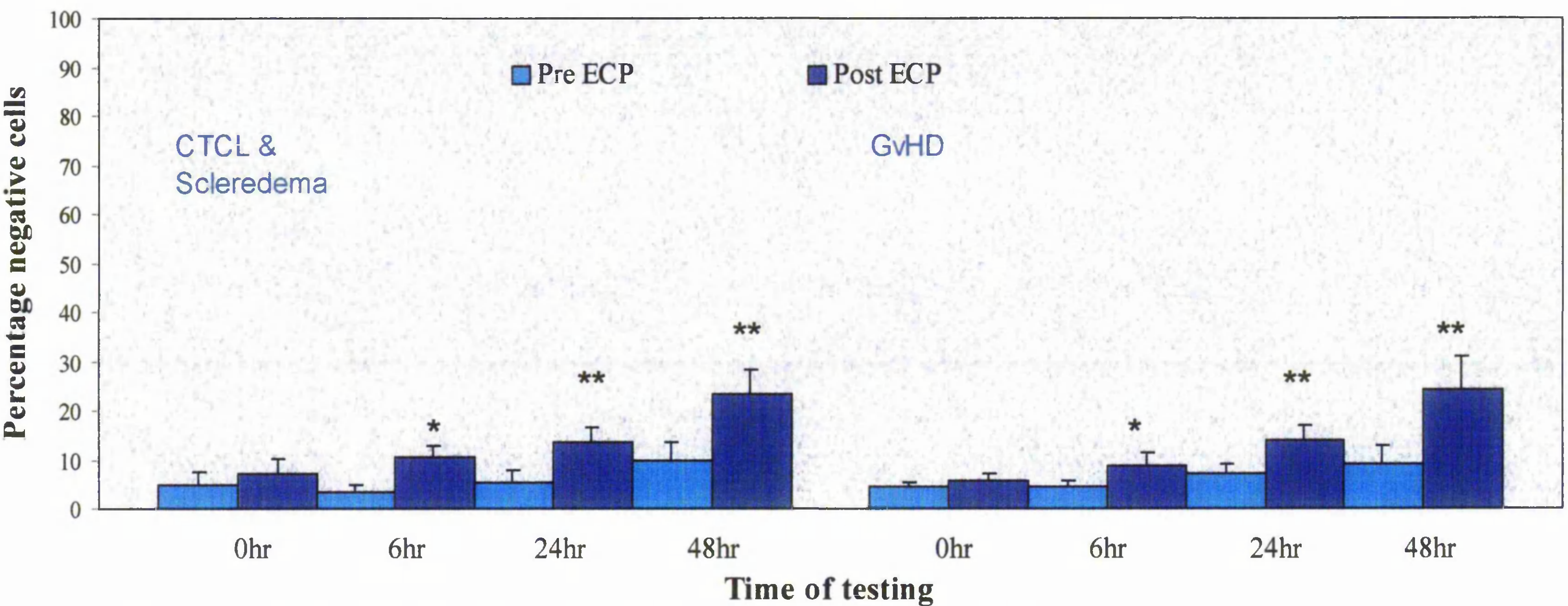


Figure 5.4.4.2 ECP induces a drop in mitochondrial membrane potential, as detected by Rhodamine 123

*Samples were taken immediately pre and post ECP and the PBMCs were isolated by density gradient (See Section 2.2.1.5). The pre- and post-ECP PBMCs were either tested immediately or added to cell culture (See Section 2.2.1.6). Lymphocytes were stained for Rhodamine 123 after 0, 6, 24 and 48 hours in cell culture (See Section 2.2.3.1). The data represents the mean $\pm$ SEM of lymphocytes negative for Rhodamine 123, obtained from each patient group. Statistical analysis compared the pre- and post-ECP results at each testing stage. * denotes $p < 0.05$ and ** denotes $p < 0.005$. A fall in Rhodamine 123 staining, for both patient groups was first observed at 6 hours post ECP, however Rhodamine 123 is not as sensitive to changes in mitochondrial membrane potential as JC-1.*

5.4.5. JC-1

In normal cells, the high $\Delta\psi_m$ causes the stain JC-1 to forms J-aggregates, which fluoresces red and can be detected in the FL-II (PE) channel of the flow cytometer. However when the $\Delta\psi_m$ becomes depolarised the stain preferentially forms the monomeric type, which fluoresces green and can be observed using the FL-I (FITC) channel. By analysing cells on a 'dot plot' of both FL-II and FL-I, tracking of the status of the $\Delta\psi_m$ could be achieved. Depolarisation of the $\Delta\psi_m$ was observed very early in the ECP treatment cycle. A significant increase in cells demonstrating a positive green (FL-I) fluorescence was observed immediately prior to re-infusion. This transformation from red to green fluorescence continued, in both patient groups, to include the majority of cells by 48 hours post ECP (See Figure 5.4.5.1). The flow cytometric representation of this shift from the J-aggregates to monomers is demonstrated in Figure 5.4.5.2.

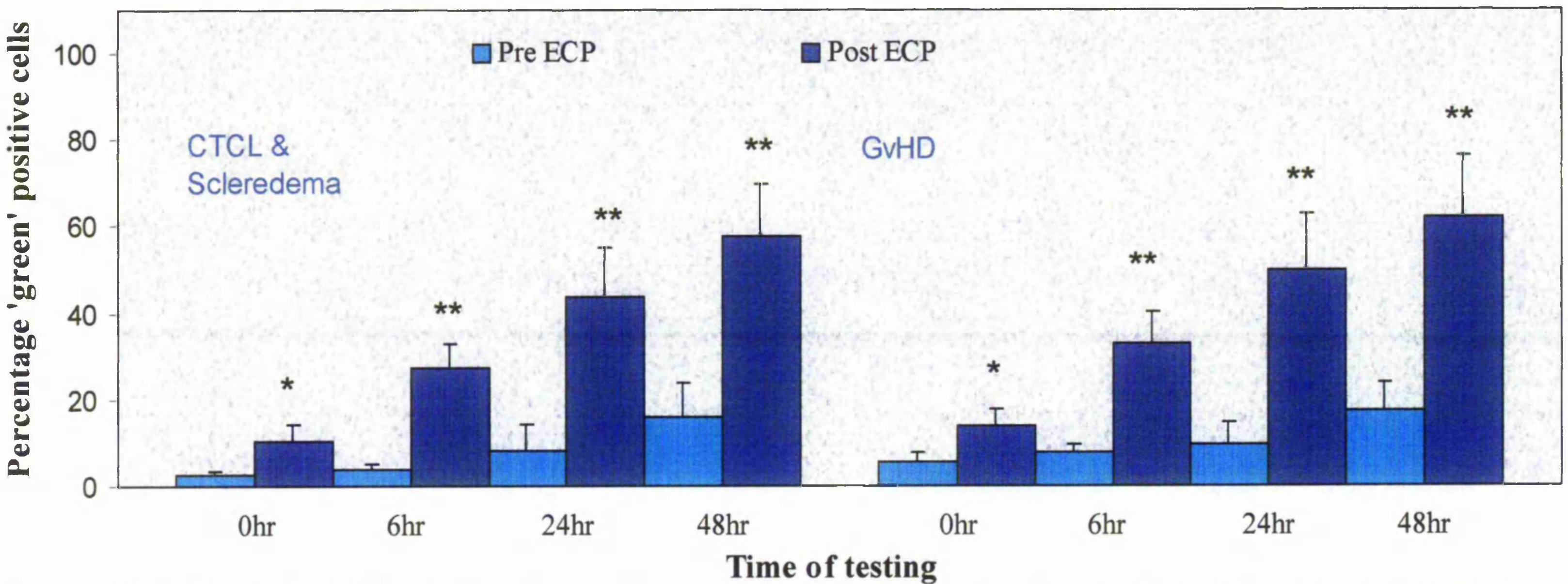


Figure 5.4.5.1 Using JC-1, ECP treated lymphocytes demonstrate an early and sustained drop in mitochondrial membrane potential

*Samples were taken immediately pre and post ECP and the PBMCs were isolated by density gradient (See Section 2.2.1.5). The pre- and post-ECP PBMCs were either tested immediately or added to cell culture (See Section 2.2.1.6). Lymphocytes were stained for JC-1 after 0, 6, 24 and 48 hours in cell culture (See Section 2.2.3.1). The data represents the mean $\pm$ SEM of lymphocytes demonstrating a positive 'green' signal, obtained from each patient group. Statistical analysis compared the pre- and post-ECP results at each testing stage. * denotes $p < 0.05$ and ** denotes $p < 0.005$. Using JC-1, a fall in mitochondrial membrane potential is first observed at 0 hours post ECP, increasing to include the majority of lymphocytes by 48 hours post ECP*

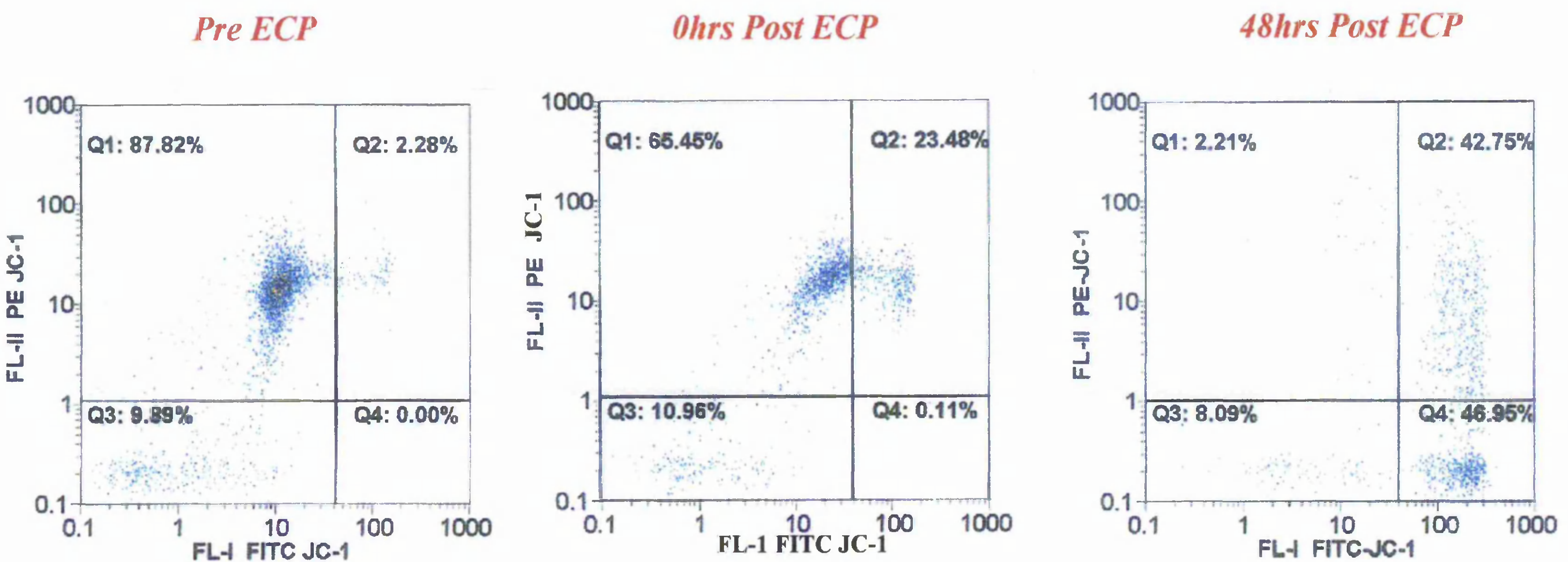


Figure 5.4.5.2 Flow cytometric representation of lymphocyte stained using JC-1; pre ECP and 0 and 48 hours post ECP

Quadrant gates were set using the pre ECP sample and control experiments using mCICCP. Subsequent samples, at 0 hours post ECP, demonstrate an increase in FL-I (green) fluoresecense, as depolarisation of the mitochondrial membrane causes an increase in the number of cells demonstrating the monomeric form of JC-1. At 48 hours post ECP the majority of cells now demonstrate a complete lack of the red (FL-II) JC-1 aggregates, in preference to the monomer form, as the mitochondrial membranes become depolarised.

5.4.6. Caspases

Activation of the caspase cascade leads to amplification of the apoptotic signal and the ultimate demise of the cell. The Caspases consists of many family members which, following activation, trigger the down-stream caspases. The ensuing cascade effect leads to the apoptotic death of the cells. Using the Caspatag™ florescence caspase activity kit, several activated caspases are detected simultaneously. An increase in caspase activity was first detected in the 6 hour sample and a substantial further increase in activation of the caspases was observed from 24 hours post ECP (See Figure 5.4.6.1). At 48 hours post ECP, the majority of ECP-treated lymphocytes demonstrated caspase activation. In Figure 5.4.6.2 a single histogram plot demonstrates the dramatic increase in caspase activity observed late in the ECP cycle.

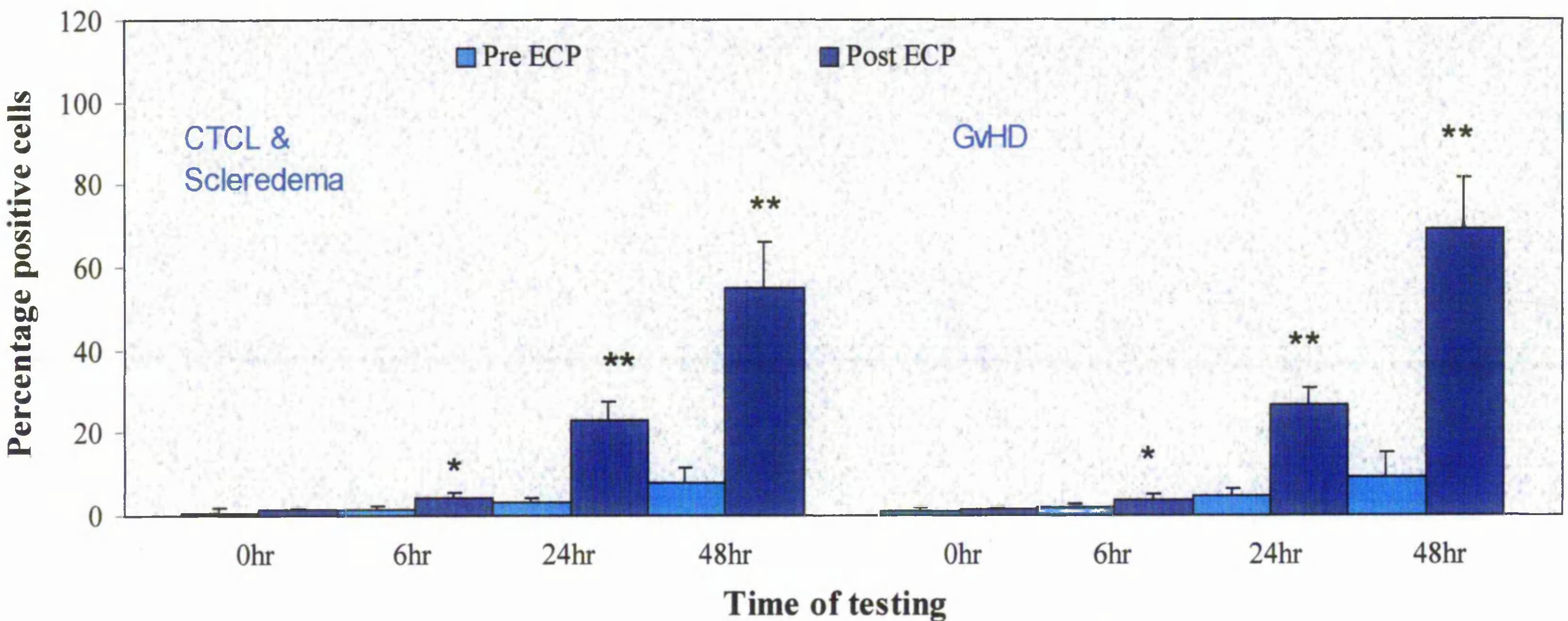


Figure 5.4.6.1 A marked increase in activated caspases was observed from 24 hours post ECP

*Samples were taken immediately pre and post ECP and the PBMCs were isolated by density gradient (See Section 2.2.1.5). The pre- and post-ECP PBMCs were either tested immediately or added to cell culture (See Section 2.2.1.6). Lymphocytes were stained for activated caspase after 0, 6, 24 and 48 hours in cell culture (See Section 2.2.3.1). The data represents the mean ± SEM of lymphocytes positive for activated caspase, obtained from each patient group. Statistical analysis compared the pre- and post-ECP results at each testing stage. * denotes $p < 0.05$ and ** denotes $p < 0.005$. A marked increase in the detection of activated caspases was first observed at 6 hours post ECP, increasing substantially from 24 hours post ECP.*

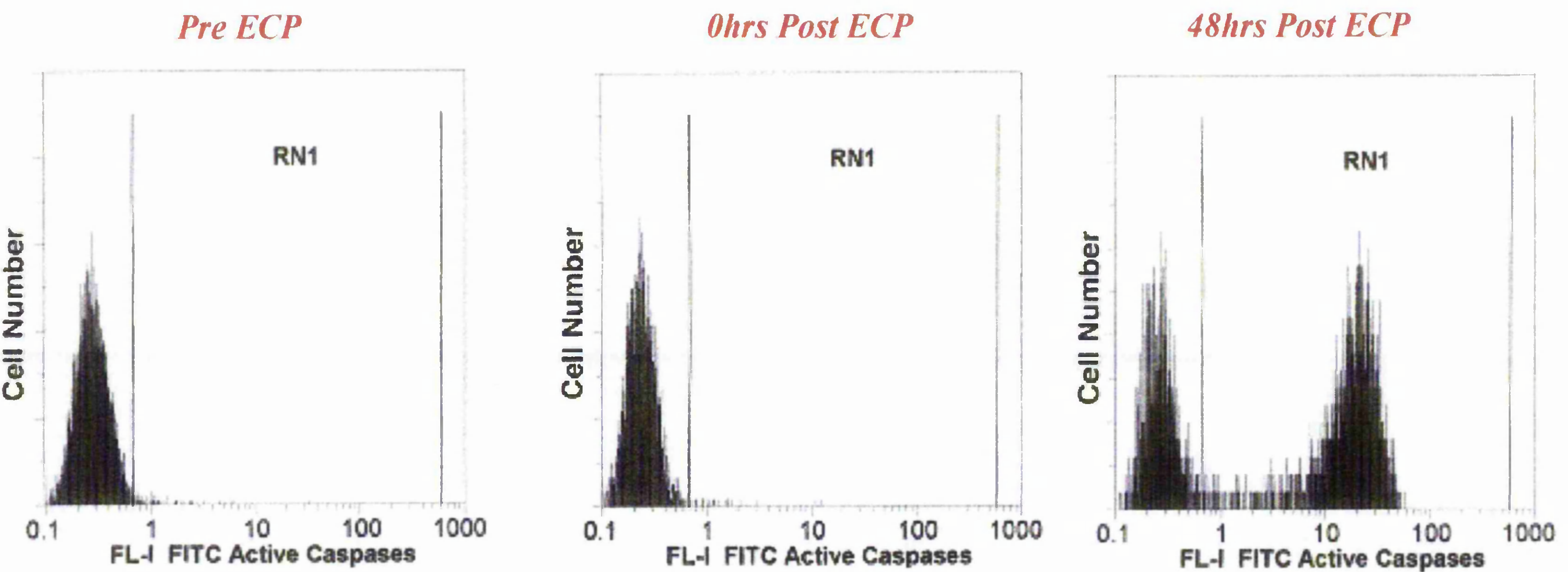


Figure 5.4.6.2 Flow cytometric representation of lymphocytes positive for activated Caspases pre ECP and 0 and 48 hours post ECP

Region gates (RN1) were set on the Fl-I signal of the pre ECP sample. Subsequent samples were enumerated for cells positive for activated caspases using these gates. At 0 hours post ECP, no marked increase in the number of lymphocytes expressing activated caspases was observed. However in the 48 hours post ECP sample a marked increase in cells positive for activated caspases was demonstrated, detectable by the large peak in the RN1 region.

5.4.7. Morphological examination.

Cytospin preparations of the 6, 24 and 48 hours post ECP stages, stained with May-Grunwald Giemsa (See Section 2.2.2.1.2), demonstrated the presence of some early apoptotic morphological features in the 6 and 24 hours post ECP sample (See Figure 5.4.7.1). The number of lymphocytes demonstrating apoptotic features increased in the 48 hour sample, with some lymphocytes demonstrated morphological changes consistent with 'late' apoptosis (See Figure. 5.4.7.2). Using an electron microscope, the classical features of apoptosis could be observed. These included the 'crescent' shaped nucleus and a lack of active mitochondria. Figure 5.4.7.4 demonstrates the marked morphological differences, observed using electron microscopy seen between the normal lymphocytes pre ECP and the apoptotic lymphocytes observed post ECP.

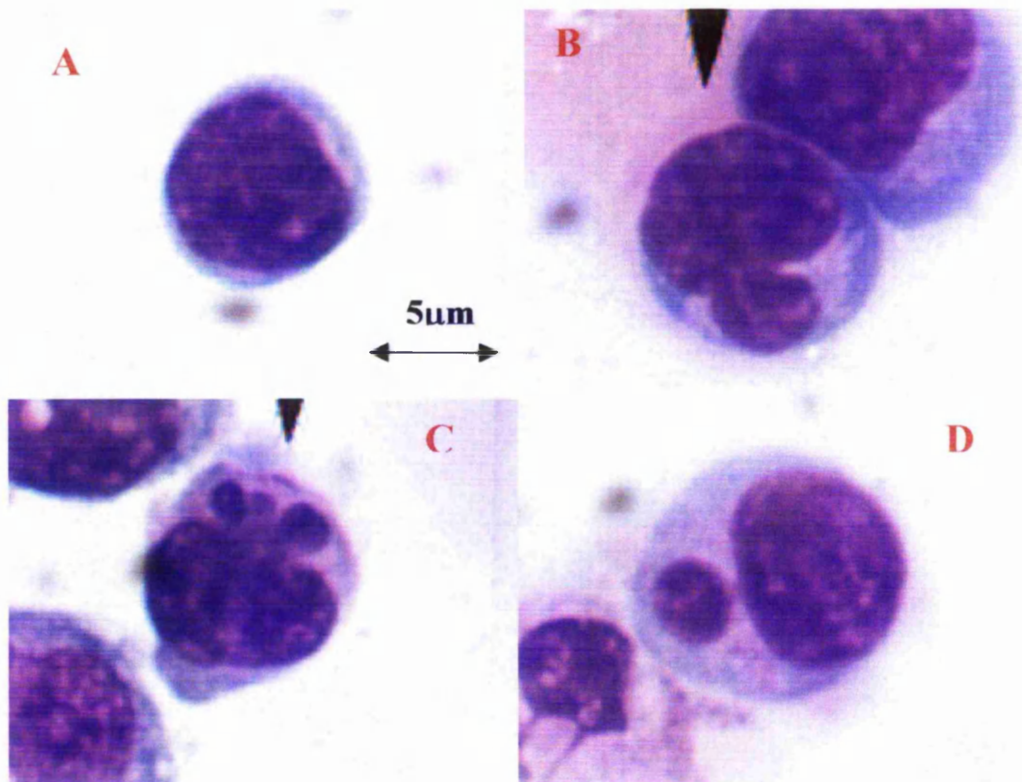


Figure 5.4.7.1. Photomicrographs of normal lymphocytes pre ECP and early apoptotic lymphocytes at 6 and 24 hours post ECP, stained with MGG.

PBMCs were separated by density gradient (See Section 2.2.1.5) and placed in cell culture (See Section 2.2.1.6). Cytospins were prepared and stained as indicated in Sections 2.2.2.1.1 and 2.2.2.1.2. Panel A demonstrates a normal lymphocyte with regular shaped nucleus. Panel B-D demonstrate the early morphological changes seen in lymphocytes observed in cytospins prepared from the 6 hours post ECP cell culture. The apoptotic changes include the initial formation of 'apoptotic bodies'

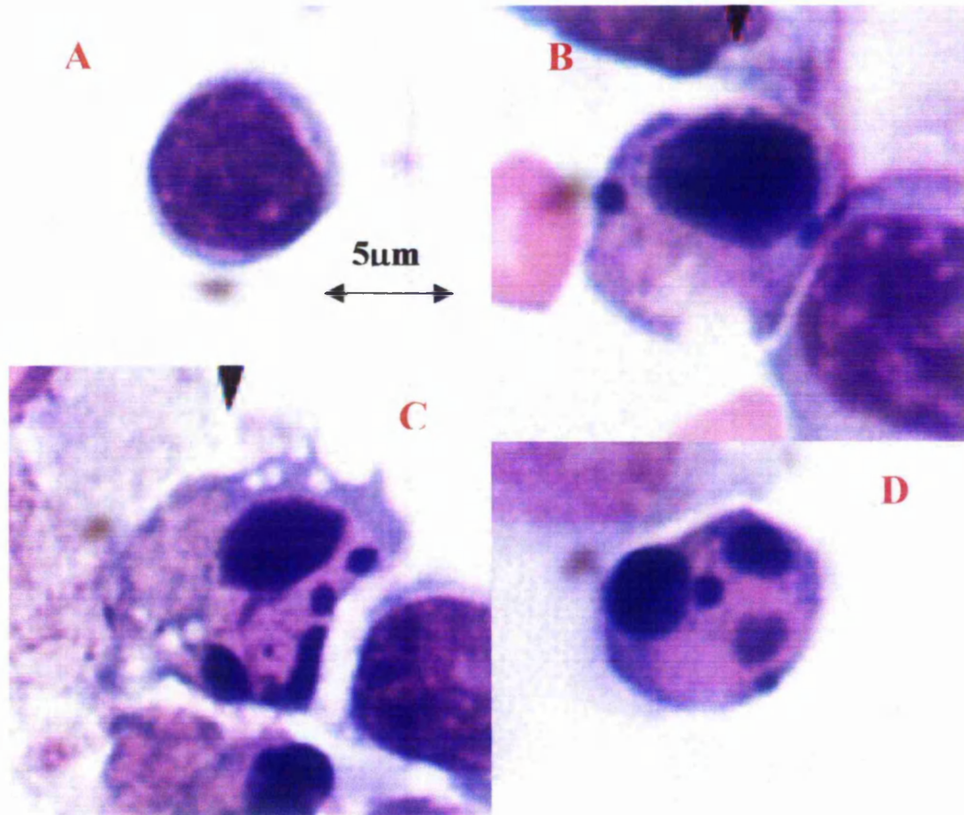


Figure 5.4.7.2 Photomicrographs of normal lymphocytes pre ECP and 'late' apoptotic lymphocytes observed 48 hours post ECP

PBMCs were separated by density gradient (See Section 2.2.1.5) and placed in cell culture (See Section 2.2.1.6). Cytospins were prepared and stained as indicated in Sections 2.2.2.1.1. and 2.2.2.1.2. Panel A demonstrates a normal lymphocyte with regular shaped nucleus. Panels B-D show the 'late' morphological changes seen in apoptotic cells 48 hours post ECP. The apoptotic lymphocytes demonstrate considerable apoptotic body formation and 'blebbing' of the cell membrane

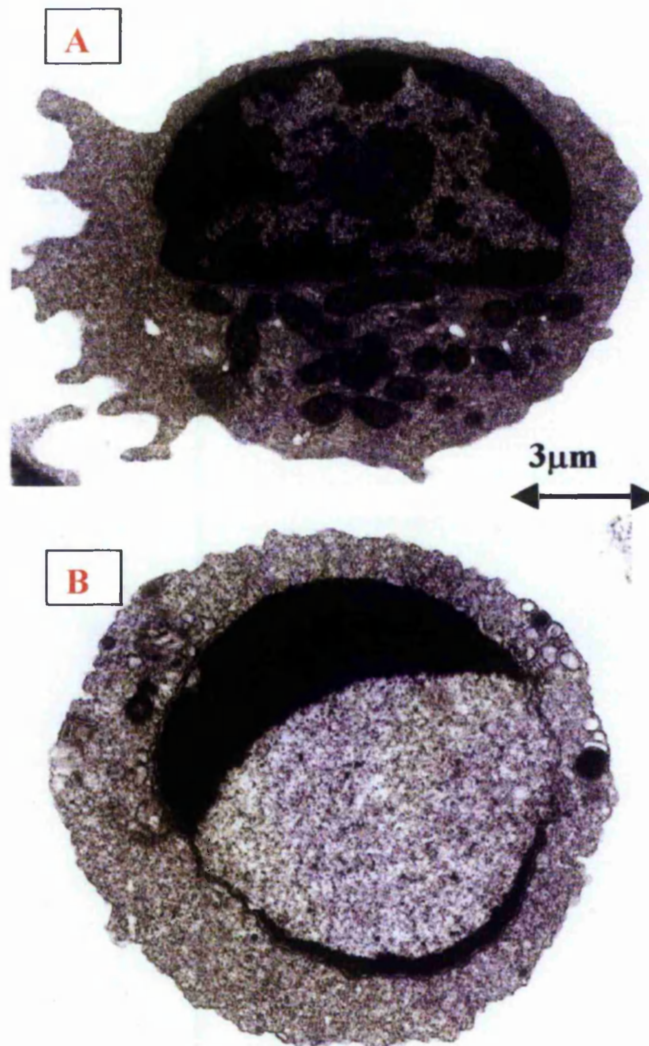


Figure 5.4.7.4 Electron micrograph of normal lymphocyte pre ECP and apoptotic lymphocyte 24 hours post ECP

Samples were taken immediately pre ECP and post ECP. The PBMCs were separated and placed in cell culture (See Section 2.2.1.5 and 2.2.1.6). Following 24 hours of cultivation, the lymphocytes were examined using electron microscopy (See Section 2.2.2.1.3 and 2.2.2.1.4). Panel A demonstrates the normal lymphocytes pre ECP, the lymphocyte have a normal appearance, with active mitochondria and a prominent nucleoli. Panel B shows the marked changes of an apoptotic lymphocyte post ECP. The lymphocyte demonstrates the characteristic 'crescent-shaped' nucleus and no presence of active mitochondria.

Photomicrographs kindly prepared by Bart Wagner, Histopathology department, Northern General Hospital, Sheffield

5.5. Discussion

The enigma of ECP therapy is how damage to a small proportion of abnormal lymphocytes, circulating in the peripheral circulation, induces a distant response in untreated cutaneous lymphoma cells. Edelson has suggested that the effect of ECP on malignant cells in the treated buffy coat, subsequently modulates the immune system to recognise non-treated, but clonal disease (Edelson *et al.*, 1994). Investigations into the process, by which cells are modified by ECP, has identified apoptosis induction in directly treated lymphocytes. Originally this process was described as a 'delayed-type' mechanism (Yoo *et al.* 1996; Aringer *et al.*, 1997; Enomoto *et al.*, 1997), while more recently apoptotic lymphocytes have been detected immediately prior to re-infusion (Chapter 3, Bladon and Taylor, 1999b). As one marker for the aetiology of CTCL is defective apoptosis rather than true proliferation (Dereure *et al.*, 2000) this process may have a direct association. Experiments were therefore designed to identify the apoptotic processes occurring at and after re-infusion. Previous work has highlighted the importance of the mitochondria and caspase cascade in the regulation and execution of apoptosis (Zamzami *et al.*, 1995, Hug *et al.*, 1999) and more recently a functional role for CD10 has been proposed for apoptotic lymphoid cells in vivo (Cutrona *et al.*, 1999).

Recently Annexin V, in conjunction with other apoptosis determining systems, has identified significant levels of apoptosis in ECP treated lymphocytes prior to re-infusion (Chapter 3, Bladon and Taylor, 1999b). Using Annexin V/PI in a cell culture system, investigations were designed to determine the speed and level of lymphocyte apoptosis, occurring *in-vivo*, following re-infusion.

The progressive exposure of PS on the membrane of the ECP treated lymphocytes is particularly significant, as the presence of PS on the membrane of an apoptotic lymphocyte, *in-vivo,* acts as a target for recognition (Fadok *et al.*, 1992; Falasca *et al.*, 1996). Phagocytosis of PS-expressing apoptotic lymphocytes precedes loss of membrane integrity (Verhoven *et al.*, 1999). Loss of membrane integrity allows the infusion and subsequent positive staining of

PI by cells. The onset of PI positivity was not observed until 24 hours following ECP exposure. It is therefore highly likely that the ECP treated lymphocytes would be promptly processed *in-vivo*, the process beginning immediately following re-infusion. In the cell culture system, many of the lymphocytes from the 24 and 48 hour post ECP cultures demonstrate Annexin V/PI positivity. However its is unlikely that this phenotype is present *in vivo*. The externalisation of PS is sufficient to cause swift phagocytosis and processing by APCs (Fadok *et al.,* 1992). The high lymphocytes to APC ratio within the *in-vitro* cell culture system allows the apoptotic lymphocytes to remain unprocessed and proceed to secondary necrosis. The processing of early apoptotic lymphocytes is probably enhanced by the observation that ECP treated monocytes do not become apoptotic (Yoo *et al.,* 1996; Tambur *et al.,* 2000). On the contrary, photopheresis treated monocytes demonstrate the membrane markers of an activated APC and an increased ability to phagocytose apoptotic T cells (Yoo *et al.,* 1996; Fimiani *et al.,* 1997). This mechanism is further supported by the lack of detectable apoptotic lymphocytes, *in-vivo,* in patients tested at 1, 6, 19 and 24 hours post re-infusion (Yoo *et al.,* 1996; Enomoto *et al.,* 1997, (Chapter 3, Bladon and Taylor, 1999b)).

Initial reports on mitochondrial changes during apoptosis noted no significant change to morphology or function (Darzynkiewicz *et al.,* 1992). However, recently profound alterations of mitochondria ultrastructure have been observed (Petit *et al.,* 1995). Functional changes to the mitochondria of apoptotic cells have now also been described and include; the uncoupling of electron transport from ATP production, a reduced rate of mitochondrial protein transportation, and disruption of the mitochondrial inner transmembrane potential ($\Delta\psi_m$) (Petit *et al.,* 1995; Zamzami *et al.,* 1995, Tada-Oikawa S *et al.,* 1998; Kroemer *et al.,* 1997). Different apoptotic signals can disrupt the $\Delta\psi_m$ by initiating the opening of mitochondrial permeability transition pores (MPT), leading to the osmotic expansion of the mitochondria matrix, eventual membrane rupture and subsequent release of apoptogenic molecules such as cytochrome c (Petit *et al.,* 1998; Thress *et al.,* 1999). *In-vivo* the presence of a reduced $\Delta\psi_m$ denotes an

irreversible stage of pre programmed lymphocyte death (Zamzami *et al.,* 1995). The early detection of a reduced $\Delta\psi_m$ in some cells may indicate that the mitochondria are the primary target during apoptosis (Vayssière *et al.,* 1994). Several stains have been utilised for the determination of a cells $\Delta\psi_m$ and include $DiOC_6$, Rhodamine 123 and JC-1 (Petit *et al.,* 1995; Zamzami *et al.,* 1995, Tada-Oikawa S *et al.,* 1998). In this study, evaluation using Rhodamine 123 and JC-1 demonstrated a disruption to the $\Delta\psi_m$ in the ECP treated cells. Although the levels detected using Rhodamine 123 were lower, this may be explained by the differing sensitivity of the stains. Rhodamine 123 shows a lower sensitivity to changes in $\Delta\psi_m$, possible due to the presence of energy-independent Rhodamine 123 binding sites. JC-1 conversely does not seem to be affected in this way (Salvioli *et al.,* 1997). Using JC-1, significant levels of lymphocytes demonstrating a depolarised $\Delta\psi_m$ were detected very early, prior to re-infusion.

Previous categorisation of UVA induced apoptosis has termed the rapid induction of apoptosis as 'immediate type' apoptosis. A process associated with perturbation of mitochondrial membrane permeability (Godar, 1999). Previously early apoptosis observed in ECP treated lymphocytes demonstrate a reduction in the intracellular Bcl-2/Bax protein ratio (Chapter 4, Bladon and Taylor, 2002b). Of interest, Bcl-2 has been demonstrated to inhibit MPT in isolated mitochondria and in cells (Zamzami *et al.,* 1996b), whilst a reduction in Bcl-2 expression in apoptotic lymphocytes is more usually associated with cells induced in an 'immediate-type' apoptotic process (Wang *et al.,* 1998). Also co-operation between Bax and a component of the MPT, adenine nucleotide translocator, can cause channel formation in artificial membranes (Marzo *et al.,* 1998).

Disruption to the $\Delta\psi_m$ and the exposure of PS appear to be intimately linked and constitute a central event in early apoptosis (Castedo *et al.,* 1996). Several speculatory theories link the process of PS exposure and a reduction in $\Delta\psi_m$.

The rapid cessation of ATP synthesis by MPT can induce PS exposure, whilst the release of a soluble protein(s) by the mitochondria undergoing MPT may activate the lipid Scramblase, which is responsible for the 'flip-flop' and subsequent exposure of PS (Castedo *et al.,* 1996). At the very early pre re-infusion stage, the numbers of cells demonstrating Annexin V and JC-1 were very similar suggesting a possible link between these two cellular events.

The caspases are a family of proteases whose initial activation triggers a cascade of further caspase activation leading ultimately to the death of the cell (Allen *et al.,* 1998; Thress *et al.,* 1999). Caspase activation seems to be a common intracellular effector pathway to which different apoptotic systems are in contact. Their activation is capable of opposing cellular attempts to stop apoptosis (Allen *et al.,* 1998). Measurement of several activated Caspases was performed using the Caspatag™ system. The system uses a FITC-conjugated caspase inhibitor (FAM-VAD-FMK) to bind to several activated caspase members. Using flow cytometry, an increase in caspase activation was first observed at 6 hours post ECP, increasing substantially 24 hours following ECP exposure. The early changes observed post ECP may represent the very early stages of apoptosis preceding the point at which caspase activation reaches detection. However, ECP has been demonstrated to increase the expression of CD95 at 24 hours post ECP in the $CD4^+$ lymphocytes of systemic sclerosis patients (Aringer *et al.,* 1997). Activation of the CD95 pathway initiating the activation of caspase cascade through FLICE (Caspase 8) (Medema *et al.,* 1997) Some of the apoptosis observed at 24 hours post ECP may therefore involve some activation of the caspase 8 pathway, through Fas activation.

In Haemopoietic cells, the CD10 was originally discovered and most commonly associated with the common acute lymphoblastic leukaemia antigen (CALLA) of the CALLA acute leukaemia phenotype (Shipp *et al.,* 1989). The functional role of CD10 in the expressing lymphoid cells of the haematapoietic system is, however, unclear (Cutrona *et al.,* 1999). Recently CD10 has been shown to influence lymphoid cell proliferation and apoptosis (Mari *et al.,* 1997; Cutrona

et al., 1999). It has been proposed that the presence of CD10 on apoptosing lymphoid cells promotes the cleavage of inflammatory or pro-inflammatory mediators released by the dying cell, limiting the level of inflammation at the site, or acts as a target antigen for macrophage recognition and phagocytosis (Cutrona *et al.,* 1999). As ECP's mechanism of action includes apoptosis and up-regulation of some cytokines, particularly IL1 (Wolfe *et al.,* 1994) IL6 and TNFα (Vowels *et al.,* 1992) it is possible that the expression of CD10, on the re-infused dying lymphocytes, may have a direct influence on the *in-vivo* ECP process. Apoptotic cells induced by ECP do express CD10, however the number of CD10 expressing cells are relatively low and appear very later in the ECP process. Because CD10 expression appears after strong PS expression, phagocytosis and processing of the apoptotic cell would occur long before the emergence of CD10 could have any influence on any *in-vivo* inflammatory response (Bladon and Taylor, 2000). It therefore seems highly improbable that the expression of CD10 would have any influence on the mechanism of action utilised by ECP.

These results seem to indicate a progressive process of lymphocyte apoptosis post ECP, which is initiated immediately after the exposure of the separated cells to the UVA light. Apoptotic lymphocytes demonstrate concurrent expression of PS and $\Delta\psi_m$ disruption, followed by substantial caspase activation, but only relatively low levels of cells expressing very weak CD10 expression. The rapidly proliferating cell may represent the lymphocytes which demonstrate very early apoptosis following ECP exposure.

Of greater interest is the effect of this immediate and sustained exposure of apoptotic lymphocytes to the immune system. For CTCL, perhaps the immediate and progressive processing of these apoptotic cells by the immune system is capable of reversing the defective apoptotic processes associated with this condition. A process complimented by the effect of ECP treatment on monocytes. ECP increases the numbers of circulating CD36$^+$ monocytes and

enhances the production of TNFα, both processes associated with anti-tumour cytotoxicity (Vowels *et al.,* 1992; Fimiani *et al.,* 1997).

An increase in CD95 expression has been observed on the CD4+ T cell numbers of Scleroderma patients 24 hours following ECP exposure (Aringer *et al.,* 1997). Previously, no differential sensitivity of CD4+ and CD8+ T cells to ECP induced apoptosis has been observed (Bladon and Taylor, 2000b). In addition recent data indicate that CTCL lymphocytes may demonstrate selective resistant to apoptosis due to a reduced expression of CD95 (Dereure *et al.,* 2000). It therefore seems very relevant to establish, at the early and late stages of the ECP cycle, to what extent the CD95 pathway is involved.

Chapter 6: Treatment of Cutaneous T cell lymphoma with extracorporeal photopheresis induces Fas-Ligand expression on treated T cells

6.1. Introduction

CTCL patients present with a malignant expansion of CD4+ T cells, which are predominantly of the Th2 cytokine profile (Haynes *et al.*, 1981, Vowels *et al.*, 1994). Rather than a true proliferation, CTCL may occur as a consequence of defective lymphocyte apoptosis (Dereure *et al.*, 2000). ECP therapy reverses the CD4+ expansion and Th1/Th2 imbalance back to normal in 'responders' within 1 year of commencing treatment (Di Renzo *et al.*, 1997, Zouboulis *et al.*, 1998). Several processes have been linked to this anti-tumour mechanism, including the induction of apoptosis in treated lymphocytes (Marks and Fox, 1991; Yoo *et al.*, 1996, Aringer *et al.*, 1997; (Chapter 3, Bladon and Taylor, 1999b).

An important mechanism in lymphoid apoptosis is the CD95 (Fas) pathway. Fas, a member of the tumour necrosis factor receptor family, induces apoptosis when crosslinked with Fas-Ligand (Fas-L) (Juo *et al.*, 1998). Fas-L activates the caspase cascade via activation of caspase 8 (FLICE). (Juo *et al.*, 1998). Activation of the caspases lead to the amplification of the apoptotic signal and ultimately act as the final executioner (Allen *et al.*, 1998, Thress *et al.*, 1999). The CD95 system is an important mechanism in controlling T cell expansion (Dhein *et al.*, 1995). However, some cellular stress-inducing agents utilise the activation of the CD95 ligand/receptor interaction to induce apoptosis (Friesen *et al.*, 1996).

Reduced levels of CD95 on CD4+ T cells in CTCL are thought to contribute to the defective apoptosis hypothesis. Thus allowing the retention of malignant cells normally removed by a natural apoptotic process (Dereure *et al.*, 2000). ECP has previously been demonstrated to enhance CD95 expression on the CD4+ T lymphocytes of Systemic Sclerosis patients 24 hours post ECP

(Aringer *et al.,* 1997), whilst the number of T cells expressing CD95 and Fas-L increases 16 hours post ECP (Tambur *et al.*, 2000).

6.2. Aim

The aim of this chapter was to determine the CD95 expression on both the CD4+ and CD8+ T cell subsets of the early apoptotic lymphocytes observed pre re-infusion and the later apoptotic cells observed 24 hours later. Subsequent investigations involved assessing the expression of Fas-L on both the CD4+ and CD8+ T cells at 0, 12 and 24 hours pre and post ECP.

6.3. Specific patients and methods

The specific cohort of patients, ECP technology and analysis methods pertaining to this chapter are detailed below. For a full explanation, please see the Materials and Methods chapter.

6.3.1. Patients

Samples were obtained from 9 Cutaneous T cell lymphoma (CTCL) patients (mean age ± S.D; 62.0 ± 16.7 years) receiving ECP treatment, 2 stage IIA, 5 stage III and 2 stage IVA.

6.3.2. Photopheresis treatment

Extracorporeal photopheresis cycles were performed using the XTS™ ECP system. See Section 2.2.1.2.2 of the Materials and Methods chapter.

6.3.3. Sampling

Samples were taken pre ECP and immediately post ECP. CD95 expression was evaluated at 0, 2, 4 and 24 hours pre and post ECP (See Section 2.2.7.2). Fas-L expression was determined at 0, 12 and 24 hours pre and post ECP (See Section 2.2.7.3).

6.3.4. Cell identification

T helper cells were identified using anti CD4 and SS. T cytotoxic cells were identified using anti CD8 and SS (See Sections 2.2.2.2.3 and 2.2.2.2.4)

6.4. Results

6.4.1 CD95 expression

In order to identify the number of CD95 expressing cells within both the CD4+ and CD8+ T subset, cells were dual stained with either PE-CY5-conjugated anti CD4 or CD8 and FITC-conjugated CD95 (See Section 2.2.7.2). Using a 'dot plot' of FL-III (PE-CY5) and SS, the CD4+ T cells were bitmapped (See Figure 2.2.2.2.3.1). Isotype controls were used to set the 'region gates' on a histogram plot (See Figure 2.2.2.2.6.1.1.1), from which the number of CD95-expressing cells were enumerated (See Figure 2.2.2.2.6.1.1.2). Following the isolation of the CD8+ T cells (See Figure 2.2.2.2.4.1), the number of CD8+/CD95+ T cells were observed using the same flow cytometric procedure.

The effects of ECP therapy on the CD95 expression of treated CD4+ and CD8+ T lymphocytes are demonstrated in Figures 6.4.1.1 and 6.4.1.2 respectively. Statistical analysis compared the pre ECP and post ECP cells at the same testing stage i.e. 0, 2, 4 and 24 hours. Within the CD4+ T cell population, no significant difference was observed between each corresponding testing stage. The CD8+ T cells only demonstrated a slight increase in CD8+/CD95+ T cells at 24 hours post ECP ($p<0.05$).

Figure 6.4.1.1. The percentage of CD4+/CD95+ T cells is not increased by ECP treatment.

Samples were taken immediately pre and post ECP and the PBMCs were isolated by density gradient (See Section 2.2.1.5). Cell for testing were adjusted to a count of 0.5 to 1.0 x 10^6/ml, whilst those added to cell culture were adjusted to 1.0 to 2.0 x 10^6/ml (See Section 2.2.1.6). The cells were stained using anti CD4 and anti CD95 (See Section 2.2.7.2). The CD4+ T cells were identified using flow cytometry and bitmapped (See Figure 2.2.2.2.3.1). From the bitmap, region gates were set on the isotype control, from which the number of CD95 positive cells was enumerated (See Section 2.2.2.2.6.1.1). The data represents the mean percentage ±SD of CD4+/CD95+ T cells pre and post ECP at each testing point.

Figure 6.4.1.2. The percentage of CD8+/CD95+ T cells numbers is increased 24 hours post ECP

*Following PBMC isolation and cell cultivation (See Sections 2.2.1.5 and 2.2.1.6), the cells were stained using anti CD8 and anti CD95 (See Figure 2.2.7.2). The CD8+ T cells were identified using flow cytometry and bitmapped (See Figure 2.2.2.2.4.1). From the bitmap, region gates were set on the isotype control, from which the number of CD95 positive cells was enumerated (See Section 2.2.2.2.6.1.1). The data represents the mean percentage ±SD of CD8+/CD95+ T cells pre and post ECP at each testing point. * denotes a p value of <0.05.*

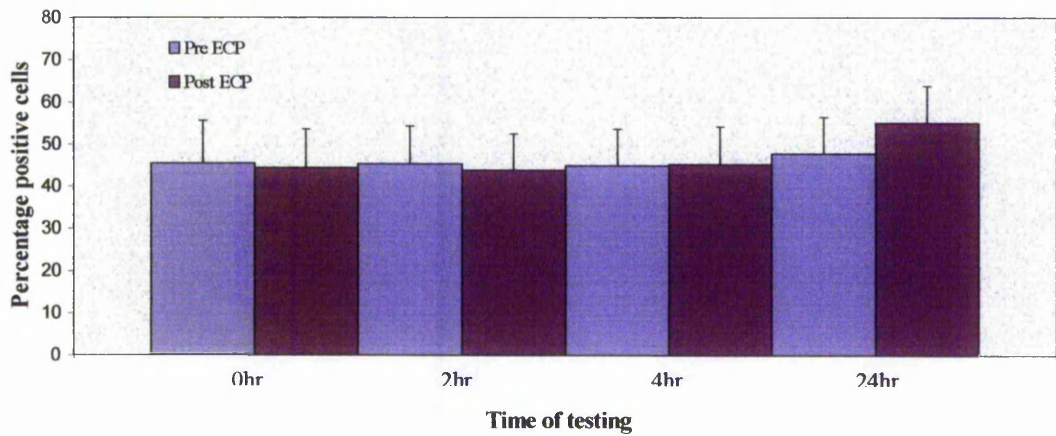


Figure 6.4.1.1. The percentage of CD4+/CD95+ T cells is not increased by ECP treatment.

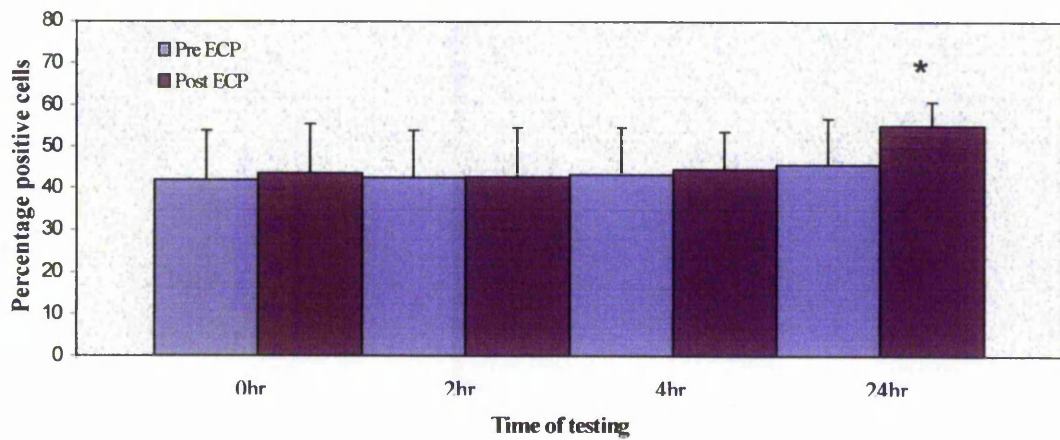


Figure 6.4.1.2. The percentage of CD8+/CD95+ T cells numbers is increased 24 hours post ECP

6.4.2. CD95 MFI expression

Although no change or very little change was observed in the number of T cells expressing CD95 post ECP, it was important to establish if T cells already expressing CD95 responded post ECP by increasing the number of CD95 expressing sites. Using the mean fluorescence intensity (MFI) a guide to the density of CD95 expression on both CD4+/CD95+ and CD8+/CD95 could be assessed (See Section 2.2.2.2.7). The MFI for CD95 expression was assessed at each testing stage, namely 0, 2, 4 and 24 hours pre and post ECP and displayed in Figure 6.4.2.1 For both T cell subsets, the MFI intensity of CD95 expression post ECP was not significantly different to that observed pre ECP. Thus indicating that the CD95 expressing cells did not up-regulate CD95 expression as a consequence of ECP therapy.

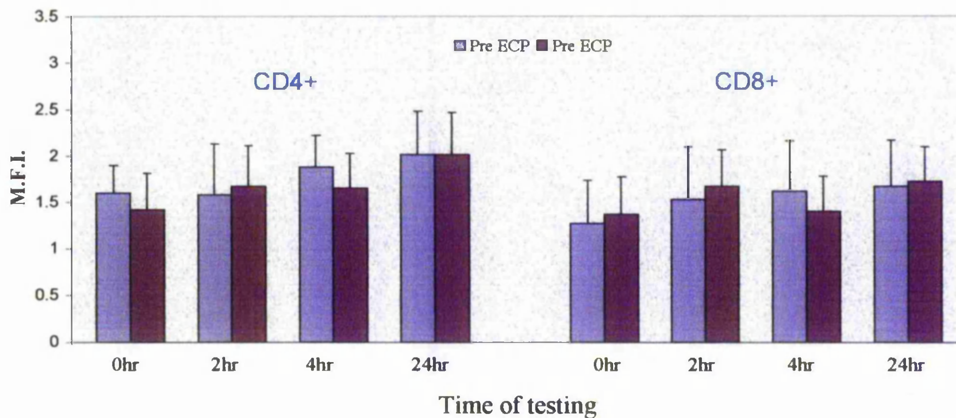


Figure 6.4.2.1. The MFI of CD95 expression on T lymphocytes is not increased by ECP treatment

Samples were taken immediately pre and post ECP and the PBMCs were isolated by density gradient (See Section 2.2.1.5). Cells for testing were adjusted to a count of 0.5 to 1.0 x 10^6/ml, whilst those added to cell culture were adjusted to 1.0 to 2.0 x 10^6/ml (See Section 2.2.1.6). The cells were stained using anti CD4/anti CD8 and anti CD95 (See Section 2.2.7.2). The CD4/8+ T cells were identified using flow cytometry and bitmapped (See Figures 2.2.2.2.3.1 and 2.2.2.4.1). From this bitmap the MFI of the CD95 positive cells was enumerated (See Section 2.2.2.2.7). The data represents the mean ± SD of pre and post ECP samples at each testing point.

6.4.3. Fas-L expression

Subsequent testing for Fas-L expression on the CD4+ and CD8+ T cell populations was achieved using a two-stage binding method. Cells were tested with a mouse unconjugated anti Fas-L, followed by a goat FITC-conjugated anti-mouse antibody. To identify the Fas-L expression on each T cells subset, the cells were dual stained with either anti CD4 or anti CD8 (See Section 2.2.7.3). Initially cells were tested at 24 hours post ECP, however to determine if the expression of Fas-L also occurred at the earlier apoptotic stage post ECP, T cells were also tested at 0 and 12 hours pre and post ECP. The extent of Fas-L expression in the CD4+ and CD8+ T cells are demonstrated in Figures 6.4.3.1 and 6.4.3.2 respectively. A very significant increase in Fas-L expression was observed in both the CD4+ and CD8+ T cells at 24 hours post ECP. Figure 6.4.3.2 demonstrates how the Fas-L expressing cells were determined using flow cytometry. However at 0 and 12 hours post ECP no significant increase was observed. When comparing CD4+ T cell expression of Fas-L with the levels of Fas-L expressed on CD8+ T cells, at the same testing stage, no significant difference was detected. Results that demonstrate the later involvement of Fas-L in apoptosis induction is not selective of either T cell subset.

Figure 6.4.3.1. The percentage of CD4+/Fas-L+ is significantly increased 24 hours following ECP exposure

*Samples were taken immediately pre and post ECP and the PBMCs were isolated by density gradient (See Section 2.2.1.5). Cell for testing were adjusted to a count of 0.5 to 1.0 x 10^6/ml, whilst those added to cell culture were adjusted to 1.0 to 2.0 x 10^6/ml (See Section 2.2.1.6). The cells were stained using anti CD4 and anti Fas-L (See section 2.2.7.3). The CD4+ were identified using flow cytometry and bitmapped (See Figure 2.2.2.2.2.1). From the bitmap, 'quadrant gates', set using the isotype control, were used to determine the number of Fas-L positive cells (See Section 2.2.2.2.6.1.2). The data represents the mean percentage $\pm$SD of CD4+/Fas-L+ T cells pre and post ECP at each testing point. ** denotes a p value of <0.01.*

Figure 6.4.3.2. The percentage of CD8+/Fas-L+ is significantly increased 24 hours following ECP exposure

*Following PBMC isolation and cell cultivation (See Sections 2.2.1.5 and 2.2.1.6), the cells were stained using anti CD8 and anti Fas-L (See section 2.2.7.3). The CD8+ were identified using flow cytometry and bitmapped (See Figure 2.2.2.2.4.1). From the bitmap, 'quadrant gates', set using the isotype control, were used to determine the number of Fas-L positive cells (See Section 2.2.2.2.6.1.2). The data represents the mean percentage $\pm$SD of CD8+/Fas-L+ T cells pre and post ECP at each testing point. ** denotes a p value of <0.01.*

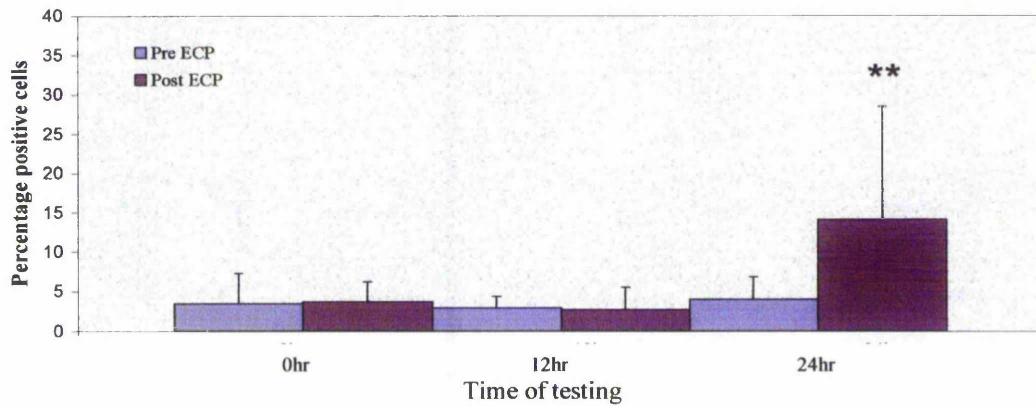


Figure 6.4.3.1. The percentage of CD4+/Fas-L+ is significantly increased 24 hours following ECP exposure

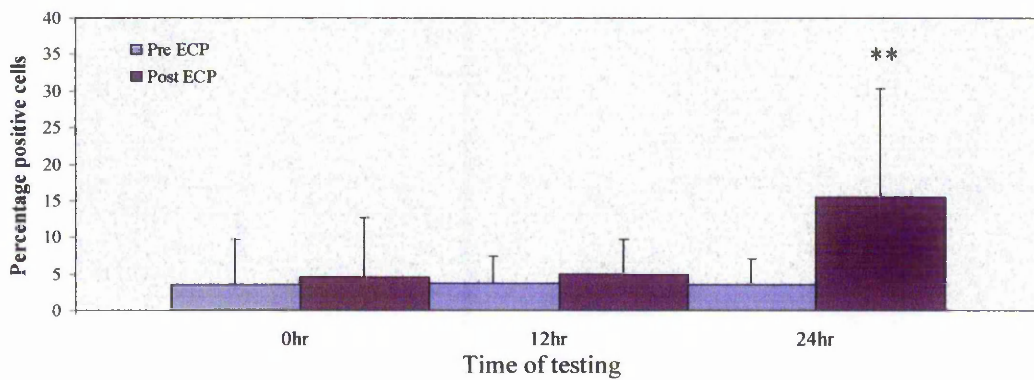
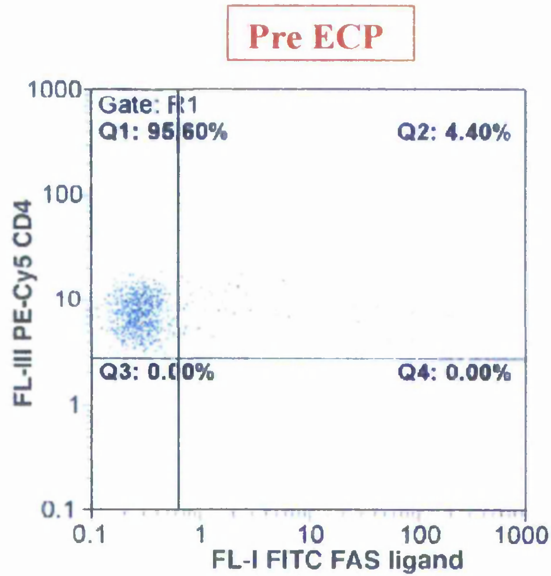


Figure 6.4.3.2. The percentage of CD8+/Fas-L+ is significantly increased 24 hours following ECP exposure



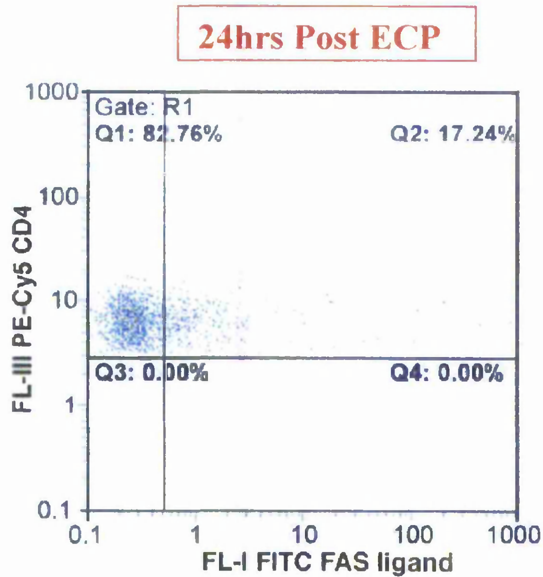


Figure 6.4.3.3. Flow cytometric representation of Fas-L expression pre ECP and 24 hours post ECP on CD4+ T cells

Quadrant gates were set using an appropriate isotype control (See Section 2.2.2.2.6.1.2). These gates were subsequently used to enumerate Fas-L expressing T cells. At 24 hours post ECP, a significant increase in the number of lymphocytes demonstrate Fas-L expression was observed.

6.5. Discussion

CD95 (Fas) expression is weakly expressed on non-stimulated peripheral blood lymphocytes (Yoshino *et al.,* 1994). When Fas is crosslinked by Fas-L, caspase 8 (FLICE) is activated, which consequently triggers downstream activation of the caspase cascade (Medema *et al.,* 1997; Juo *et al.,* 1998). The caspase cascade is capable of inducing irreversible apoptosis (Allen *et al.,* 1998). Fas based killing if through an autocrine mechanism. The cross-linking of Fas by Fas-L on target cells can occur by transfer from an adjacent activated cytotoxic T cells or by expression of Fas and Fas-L on the target cell membrane itself, coming together possible by membrane folding (cell suicide) (Dhein *et al.,* 1995; Nagata and Golstein, 1995). The CD95 system is critical for growth control of T cells (Dhein *et al.,* 1995). Activation of the Fas system is required for the removal of autoreactive T cells, excess activated T cells following a response to foreign antigens and low response B cells within the germinal centre (Lynch *et al.,* 1997; Nagata and Golstein, 1995). However cellular stress-inducing agents can also activate the CD95 ligand/receptor interaction (Allen *et al.,* 1998).

In CTCL, peripheral blood CD4+ T lymphocytes demonstrate decreased expression of CD95, suggesting its progression may be due to defective apoptosis, rather than true proliferation (Dereure *et al.,* 2000). In Scleroderma patients, enhanced CD95 expression has been observed on CD4+ T lymphocytes 24 hours post ECP (Aringer *et al.,* 1997). Experiments were designed to establish if ECP enhanced CD95 expression in CTCL lymphocytes and also if the CD95 pathway was responsible for the early apoptosis observed immediately post ECP (Chapter 3, Bladon and Taylor, 1999b).

Only a slight increase in CD95 expression in the CD8+ T cells ($p<0.05$) at 24 hours post ECP was observed. However the MFI of CD95 expression on both the CD4+ and CD8+ cells demonstrated no significance difference at each testing stage. This seemed to indicate that the ECP did not up-regulate the expression of CD95 on T cells. The increase in CD8+/CD95+ T cells observed

at 24 hours constituted the new expression of CD95 on T cells previously negative for CD95 expression. Subsequent testing for Fas-L expression noted a very significant increase in Fas-L in both the CD4+ and CD8+ T cells at 24 hours post ECP ($p<0.005$). To determine if this process was responsible for the early apoptosis induction observed immediately prior to re-infusion, Fas-L expression was also determined at 0 and 12 hours post ECP. At these stages, however, no enhanced expression was detected. This may indicate that the later stage of apoptosis induction, i.e. 24 hours and later involves Fas-L expression. This was consistent with the later Fas-L expression observed in post ECP T cells by Tambur (Tambur *et al.,* 2000). This process, in part, is probably responsible for the later induction of the caspase cascade, observed at 24 and 48 hours post ECP (Chapter 5, Bladon and Taylor, 2002). It was also noted that the levels of CD4+ T cell expressing Fas-L at 24 hours post ECP was not significantly different to the numbers of CD8+ T cells expressing Fas-L at the same time point. An observation in agreement with previous observations that ECP does not preferentially induce apoptosis in CD4+ T cells (Chapter 3, Bladon and Taylor, 2000b). Reduction in CD4+ T cells numbers by 'responders' (Zouboulis *et al.,* 1998, Taylor, 2003) is therefore unlikely to be due to the selective removal of the malignant cells by the direct killing using ECP. More likely is the modulation of the immune system to identify and remove other malignant cells of the same clone (Edelson *et al.,* 1994).

To determine if the activation of the CD95 pathway by ECP was limited by the decreased CD95 expression of the CTCL CD4+ T cells, two patients receiving ECP therapy for Graft versus Host Disease (GvHD) were tested. Both patients demonstrated similar results to those seen for the CTCL patients (Data not shown). This seems to indicate that ECP induction of Fas-L expression on treated T cells is not specific to CTCL. However with only slight increases in levels of CD95 expression on treated T cells post ECP, the apoptotic process induced by the Fas pathway would only include cells already expressing Fas. To identify if the enhanced Fas-L was involved in the apoptotic process, the 'later' apoptotic cells were isolated by their low FS and high SS in the 24 hours post

ECP sample and Fas-L expression assessed. Within this population the Fas-L expression was substantially higher than in the whole population, indicating the participation of Fas-L in the induction of apoptosis (Data not shown)

In conclusion, activation of the CD95 pathway is non-selective for a T cell subgroup, but may, in part, explain the some of the late apoptosis observed and caspase activation found at 24-48 hours post ECP.

Chapter 7: Mononuclear cells treated by Extracorporeal Photopheresis do not express Heat Shock Protein 70(72)

7.1. Introduction

The role of heat shock proteins (HSPs) within a cell, includes the prevention of protein aggregation, protection of proteins from denaturalisation by stress-inducing agents and participation in the repair or degradation of polypeptides that have become denatured under stress (Schlesinger, 1990; Craig *et al.*, 1994). The HSPs perform an important protective role, both in cells growing under optimal conditions and those exposed to less favourable environments (Sapozhnikov *et al.*, 1999). HSPs were initially identified as intracellular proteins and as such most of their known functions are connected to intracellular processes (Sapozhnikov *et al.*, 1999). However HSPs have also been observed on the plasma membrane of both normal and tumour cells (Erkeller-Yeksel *et al.*, 1992; Ferrarini *et al.*, 1992). Their role here is unclear, but there is evidence that they can stabilise lipid membrane and prevent denaturation of membrane-localised proteins during stress (Török *et al.*, 1997).

Recently a positive relationship between the level of HSP expression and the onset of apoptosis has been described and there is some evidence which indicates that HSPs may play a role in the modulation of the apoptotic process (Chant *et al.*, 1996; Sapozhnikov *et al.*, 1999). In addition, it has been demonstrated that the immunogenicity of apoptotic leukaemia cells is enhanced by the expression of heat shock proteins. Apoptotic cells expressing HSPs induced a significantly more effective anti-tumour response, than apoptotic cells without up-regulated HSPs (Feng *et al.*, 2001). The presence of HSP 72 on the surface of tumour cells may act in an MHC–unrestricted manner as a tumour specific recognition structure for distinct NK cell population (Multhoff *et al.*, 1997).

Monocytes that are exposed to HSP 70 up-regulate the production of some pro-inflammatory cytokines, including TNFα and IL6 (Asea *et al.,* 2000). Asea *et al* also suggests that intracellular production of HSP 70 within the monocytes would promote the same effect (Asea *et al.,* 2000). Enhanced expression of TNFα has anti-tumour activity, which can be directed against leukaemia and lymphoma cells (Fransen *et al.,* 1986). Following ECP, lymphocytes become apoptotic (Marks and Fox, 1991; Yoo *et al.,* 1996) and monocytes up-regulate secretion of IL6 and TNFα (Vowels *et al.,* 1992). These are two prominent mechanisms thought to be involved in the process leading to modulation of the immune system and the removal of untreated, clonal T cells (Vowels *et al.,* 1992; Hanlon *et al.,* 1998; Rook *et al.,* 1999; Berger *et al.,* 2001).

7.2. Aim

This chapter aimed to determine the cellular content of intracellular and membrane HSP 70 (72) at various points following ECP, with a view to establishing if HSPs play a role in the anti-tumour mechanisms of ECP, through either the immunogenesis of membrane bound HSP on treated lymphocytes or cytokine mediated signalling from ECP-exposed monocytes. The assessment of intracellular HSPs in lymphocytes at various time points post ECP would also ascertain if HSPs play a role in perpetuating the apoptotic process.

7.3. Specific patients and methods

The specific cohort of patients, ECP technology and analysis methods pertaining to this chapter are detailed below. For a full explanation, please see the Materials and Methods chapter.

7.3.1. Patients

Samples were obtained from 9 patients receiving ECP treatment. Six CTCL CTCL patients (mean age ± S.D; 60.7 ± 13.4 years) and 3 GvHD patients (39.2 ± 8.6).

7.3.2. Photopheresis treatment

Extracorporeal photopheresis cycles were performed using the XTS™ ECP system. See section 2.2.1.2.2 of the Materials and Methods.

7.3.3. Sampling

PBMCs were immediately separated pre and post ECP. Cells were tested after 6, 24 and 48 hours of cultivation.

7.3.4. Cell identification

T cells were identified using anti CD3 and SS. (See Section 2.2.2.2.2).

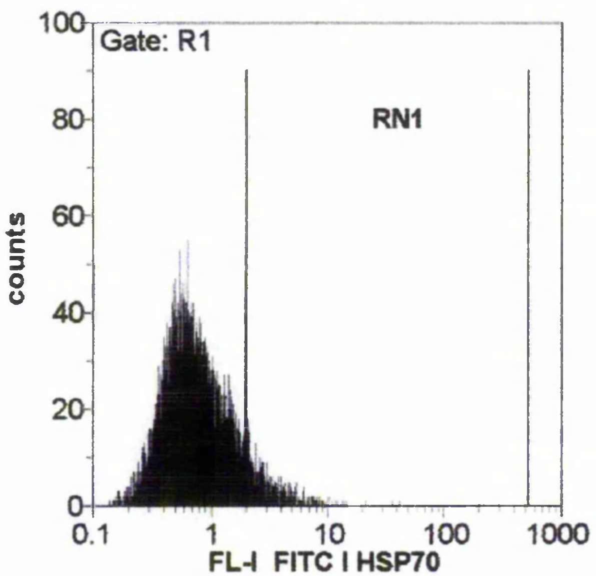
Monocytes were identified using anti CD14 and SS. (See Section 2.2.2.2.4)

7.4. Results

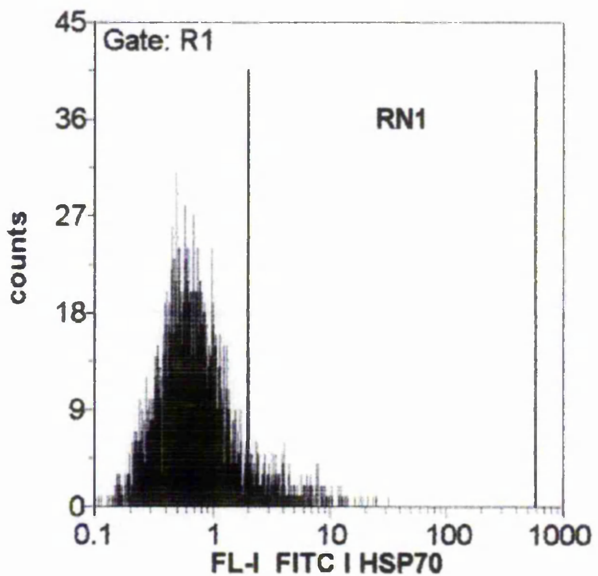
7.4.1. HSP and T cells

The expression of HSP 72 was determined on T cells following their isolation and 'bitmapping' using FITC-conjugated anti CD3 and SS, as demonstrated in Section 2.2.2.2.2 of the Materials and Methods chapter. By heating separated PBMCs, from the pre ECP sample, to 42°C for one hour provided a control sample and clearly demonstrated an increase in HSP 72. Using a histogram plot, region gates (RN1) were set on these control cells to assess HSP 72 in the pre and post ECP samples. The surface expression of HSP 72 was determined at all the testing stages, namely 6, 24 and 48 hours pre and post ECP. Six hours was chosen as the start point, because most data indicate this as a minimum time for HSP expression by cells post exposure to stimuli (Lamb *et al.,* 1989; Heufelder *et al.,* 1992). No significant increase in HSP 72 was determined at any of these testing stages. To establish if no surface expression of HSP 72 was due to a lack of production within the cells or because the protein had not migrated to the membrane, total HSP 72 levels within the cell were determined in the pre and post ECP sample. Using a 'fix and perm' kit (See Section 2.2.1.7), the total levels, including the intracellular compartment of the lymphocytes, were assessed for HSP 72. Like surface testing, no increase in HSP 72 was observed, at each testing stage, indicating no enhancement of HSP 72 production within the ECP-treated lymphocyte. Figure 7.4.1.1 demonstrate the flow cytometric representation of HSP expression pre ECP, 48 hours post ECP and in the control cells heated to 42°C.

Pre ECP



48hrs Post ECP



Control (42°C)

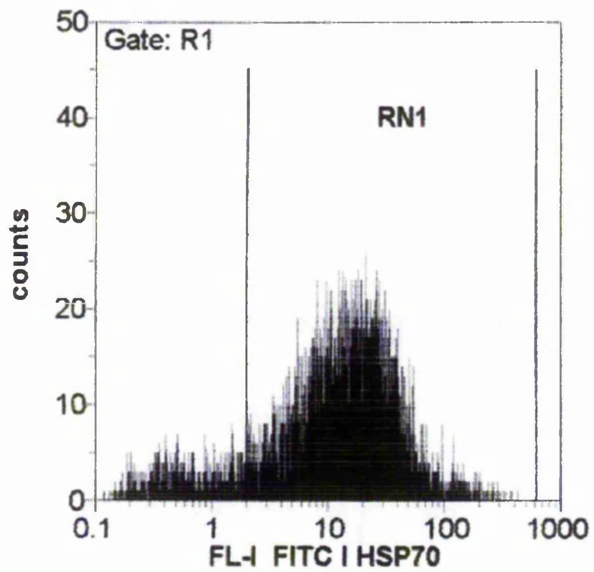


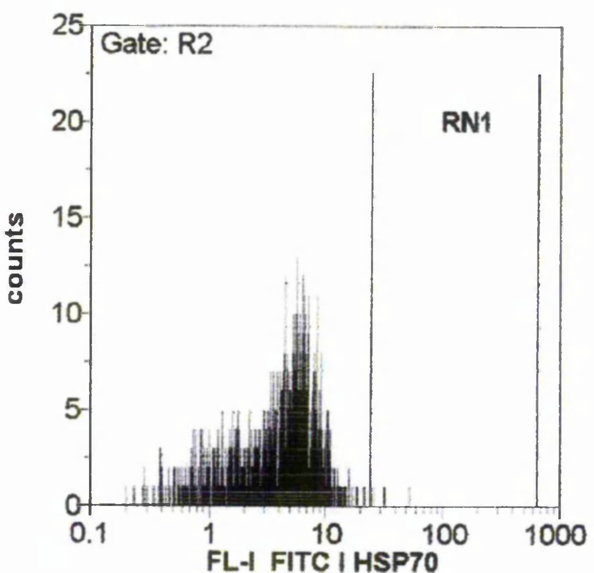
Figure 7.4.1.1 Flow cytometric representation of intracellular HSP 70 (72) expression in lymphocytes pre ECP, 48 hours post ECP and in the control cells heated to 42°C

Region gates (RN1) were set on the FL-I signal of the bitmapped T cells of the pre ECP sample (See Section 2.2.2.2.2). The location of the gate was confirmed by the control cells heated to 42°C. The number of lymphocytes demonstrating HSP 70 (72) was enumerated using these gates, at each subsequent testing stage.

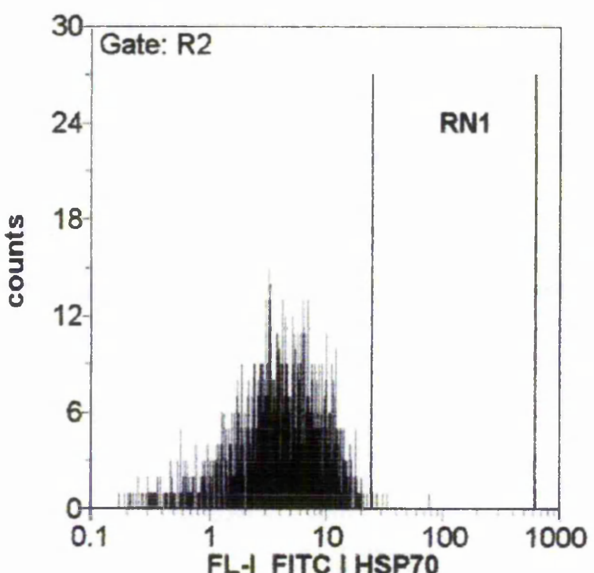
7.4.2. HSP and monocytes

The simultaneous evaluation of the monocyte population at 6, 24 and 48 hours post ECP was possible by 'bitmapping' cells with positive CD14 expression and high SS (See Section 2.2.2.2.5). Using the monocyte 'bitmap' HSP 70 was evaluated initially on the membrane pre and post ECP. Total HSP 70(72), including the intracellular compartment of monocyte, were subsequently tested pre and post ECP. For both patient groups, no significant increase in either membrane bound or total HSP 70(72) was determined at each of testing stages. As before, control experiments, where cells were heated to 42°C for one hour, demonstrated dramatic increases in both membrane and intracellular HSP 70. Figure 7.4.2.1 clearly demonstrates the profound changes observed in the control cell group, whilst demonstrating the lack of HSP72 expressed in the monocytes of patients exposed to ECP.

Pre ECP



48hrs Post ECP



Control (42 ℃)

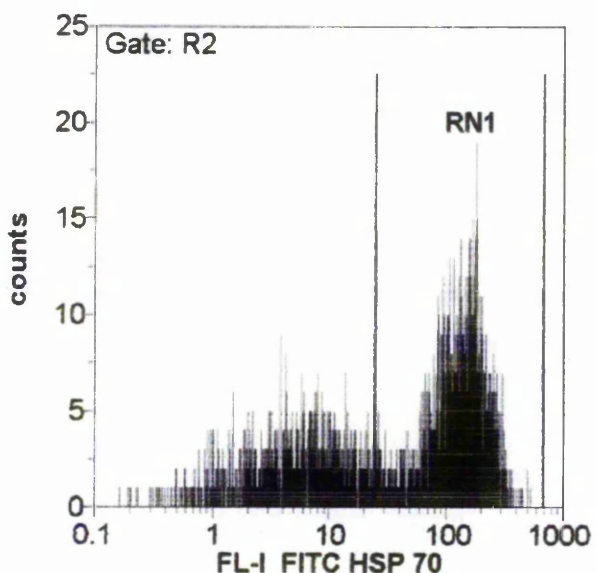


Figure 7.4.2.1 Flow cytometric representation of intracellular HSP 70 (72) expression in monocytes pre ECP, 48 hours post ECP and in the control cells heated to 42 ℃

Region gates (RN1) were set on the FL-I signal of the bitmapped monocytes of the pre ECP sample (See Section 2.2.2.2.5). The location of the gate was confirmed by the control cells heated to 42 ℃. The number of monocytes demonstrating HSP 70 (72) was enumerated using these gates, at each subsequent testing stage.

7.5. Discussion

During a treatment cycle, ECP only exposes between 2 and 5% of the total tumour load (Gottlieb *et al.,* 1996, Hanlon *et al.,* 1998). However, CTCL 'responders' demonstrate a reversal of the CD4/CD8 back to normal within 12 months of starting treatment (Zouboulis *et al.,* 1998). ECP 'responders' are immunocompetent, with near normal absolute CD8+ numbers and normal NK activity (Gottlieb *et al.,* 1996; Zouboulis *et al.,* 1998, Rook *et al.,* 1999). ECP's mechanism of action is thought to involve some modulation of the immune system (Perez *et al.,* 1989, Edelson *et al.,* 1994). Lymphocytes exposed to ECP become apoptotic (Marks and Fox, 1991, Yoo *et al,* 1996, Enemoto *et al.,* 1997; (Chapter 3, Bladon and Taylor 1999b). Processing of the apoptotic malignant/autoreactive clonal lymphocytes leads to an acquired immunomodulatory process capable of identifying and removing untreated, but clonal disease cells (Albert *et al,*. 1998. Hanlon *et al.,* 1998, Rook *et al.,* 1999). However apoptotic cells are thought not to be immunogenic. The initiation of an immune response is only induced when a cell dies by necrosis (Sauter *et al.,* 2000). Monocytes do not become apoptotic post ECP (Yoo *et al.,* 1996, Tambur *et al.,* 2000). Paradoxically they become activated and demonstrate increased secretion of IL6 and TNFα (Vowel *et al.,* 1992). TNFα is capable of inducing an anti-tumour responses (Fransen *et al.,* 1986; Wright *et al.,* 1992).

HSPs are thought to perform an important role in protection of cells exposed to stress-inducing agents or environments, such as hyperthermia, hyperoxia, hypoxia and other perturbations which alter protein synthesis (Moseley, 2000). There many tasks include the prevention of protein aggregation and denaturation and the repair or degradation of polypeptides. (Schlesinger, 1990; Craig *et al.,* 1994). The up-regulation of HSPs following stress is thought to provide stability within the cell, up until a critical point, from which the cell triggers an apoptotic cascade (Samali and Cotter, 1996). However evidence now indicates that HSPs may play a role in the modulation of the apoptotic process (Sapozhnikov *et al.,* 1999). HSP 60 can increase the vulnerability of pro-caspase-3 to activation by upstream protease activators (Xanthoudakis *et al.,*

1999). The presence of HSP 72 on the surface of tumour cells is also thought to be highly immunostimulatory (Lamb *et al.*, 1989; Heufelder *et al.*, 1992), possibly through a MHC–unrestricted manner, as a tumour specific recognition structure for a distinct NK cell population (Multhoff *et al.*, 1997). Recently, the re-infusion of apoptotic and viable tumour cells to immunocompetent mice only induced a significant anti-tumour response if the apoptotic cells were pre-stressed. The stressing process induced the expression of HSP 70 (72) on the plasma membrane (Feng *et al.*, 2001). In addition, following apoptosis *in vivo* the presence of the pro-inflammatory cytokines; IL12, IFNγ and TNFα are thought to aid the responses believed responsible for the immunogenic response (Feng *et al.*, 2001). ECP induces the expression of TNFα and IL6 by treated monocytes (Vowels *et al.*, 1992). The enhanced expression of TNFα, following ECP, is capable of direct anti-tumour activity, which can be directed against leukaemia and lymphoma cells (Fransen *et al.*, 1986; Wright *et al.*, 1992). TNFα can also induce apoptosis in the human leukaemia U937 cell line (Wright *et al.*, 1992). In addition, the combination of IFNγ and TNFα is capable of enhanced macrophage cytotoxicity (Hori *et al.*, 1987). IFNγ and IL12 can increase the anti-tumour effect of cytotoxic T cells (Seo *et al.*, 1998), whilst rhIL12 has been demonstrated to be effective in reducing CTCL lesion (Rook *et al.*, 1999b). Monocytes exposed to HSP 70 up-regulate production of some pro-inflammatory cytokines, including TNFα and IL6. Suggesting intracellular production of HSP 70 in monocytes would promote the same effect. (Asea *et al.*, 2000). Tumour lysates containing HSP 70 can also stimulate immature dendritic cells to increase phagocytosis *in vitro*, whilst attracting macrophages and dendritic cells into the tumour environment (Todryk *et al.*, 1999).

The examination of lymphocytes and monocytes at 6, 24 and 48 hours pre and post ECP demonstrated no significant increase in either intracellular or membrane bound HSP 70(72). This is in contrast to that of organ-cultured normal human skin and human fibrosarcoma cell line, where HSP 72 was detected following exposure to PUVA and UVA respectively (Muramatsu *et al.*,

1993; Trautinger *et al.,* 1999). The discrepancy between these results is probable attributable to the differences in cell types. The expression of surface HSP expression can be varied within a sub population. The mouse EL-4 mouse lymphoma cell expresses HSP 72, whilst mouse thymocytes and mouse CTLL-2 T cells demonstrating no membrane HSP 72 (Sapozhnikov *et al.,* 1999). The immunomodulatory responses observed following ECP are therefore not attributable to the HSP system. Of significant interest is the observation that immediately following ECP, the results of this thesis has subsequently noted a reduction in the number of TNFα-expressing T cells and TNFα-expressing monocytes (Chapter 8, Bladon and Taylor, 2002e). Findings consistent with other the findings of other researchers, where TNFα down-regulation followed exposure of PBMCs to UVA/8MOP (Neuner *et al.,* 1994).

Although the apoptotic lymphocytes do not express HSP, the phagocytosis of apoptotic lymphocytes by APCs is apparent following ECP (Yoo *et al.,* 1996; Berger *et al.,* 2001). A process also observed, in this thesis in the post ECP stages (See Figures 9.4.3.1 and 9.4.3.2). One prominent membrane macrophage recognition marker, phosphatidylserine (PS), is present on significant numbers of lymphocytes immediately post ECP (Chapter 3, Bladon and Taylor, 1999b). The number of PS-expressing cells increases post ECP, to include the majority of lymphocytes by 48 hours (Chapter 5, Bladon and Taylor, 2002). PS externalisation is not only necessary, but sufficient to induce macrophage recognition and engulfment (Verhoven *et al.,* 1999). In addition, the monocytes treated by ECP do not become apoptotic (Yoo *et al.,* 1996, Tambur *et al.,* 2000). Paradoxically they demonstrate enhanced avidity for T cell phagocytosis and demonstrate activation markers (Yoo *et al.,* 1996, Fimiani *et al.,* 1997).

Tumour cells exposed to UVA/8-MOP lymphocytes demonstrate a 2-3 fold increase in cell surface expression of MHC class 1 molecules. A process which may also induce a 2-3 fold parallel increase in tumour antigens (Hanlon *et al.,* 1998). Processing of apoptotic lymphocytes displaying enhanced levels of tumour antigens may lead to more effective antigen recognition by selective

CD8+ T cells, leading to an anti-clonotypic mechanism capable of removing non-ECP treated cells (Albert *et al.*, 1998; Hanlon *et al.*, 1998; Rook *et al.*, 1999). Therefore in the setting of ECP, induction of apoptosis and not necrosis may be more beneficial. The phagocytosis and the subsequent anti-clonotypic response suggest that apoptosis is sufficient mechanism to initiate an immune response leading to the removal of untreated clonal cells.

Following exposure to 8-MOP and UVA, cytokine production remains a contentious issue. Enhanced TNFα secretion has been linked to anti tumour responses observed post ECP (Vowels *et al.*, 1992), whilst the transient up-regulation of IFNγ, observed in UVA/8MOP treated lymphocytes (Tokura *et al.*, 1999) may enhance the tumoricidal function of CD8+ cytotoxic cells (Seo *et al.*, 1998). These processes may account partly for the normalisation of the Th1/Th2 imbalance, observed in CTCL patients treated by ECP (Di Renzo *et al.*, 1997). However 8-MOP/UVA is effective in conditions, such as GvHD and psoriasis, in which TNFα and Th1 mediated cytokines are directly involved in the pathology. Any cytokine signalling which originates from the ECP treated monocyte population would not involve the enhancement in HSP expression.

The lack of any HSP expression in and on lymphocytes post ECP has also identified that HSP appear to play no role in the apoptotic process. What effect the exposure of heat shock, in addition to UVA/8-MOP might have remains unknown. The MHC-independent nature of the HSP response is at odds with the immunostimluatory theory processed for ECP (Hanlon *et al.*, 1998). However the mouse model seems to provide compelling evidence of the immunogenicity of lymphocytes expressing HSPs (Feng *et al.*, 2001). To establish this effect, in a clinical setting, would involve many ethical and technical considerations to ensure the safe and efficacious re-infusion of treated cells.

Chapter 8: Extracorporeal photopheresis reduces the number of mononuclear cells that produce pro-inflammatory cytokines, when tested ex-vivo.

8.1. Introduction

Cytokines are secreted regulatory proteins that control the survival, growth and differentiation and effector function of tissue cells (Nicola, 1994). Major modulation of immune responses and inflammatory processes in human disease are directed by cytokines (Dinarello and Mier, 1987). The malignant CD4+ T cells in Sezary syndrome are of the Th2 subclass, expressing predominately IL4, IL5 and IL10 (Vowels *et al.,* 1994). The secretion of Th2 cytokines enhances the production of more cytokines of the Th2 subclass through the direct suppression of Th1 cytokine production (Rook *et al.,* 1995). Patients who respond to ECP demonstrate a reduction in this Th2 subclass, in favour of a normalisation in Th1 cell numbers (Di Renzo *et al.,* 1997). However, paradoxically the GvHD patients, who benefit from ECP therapy, demonstrate an over-expression of the Th1 cytokine IFNγ (Ochs *et al.,* 1996, Parkman, 1998).

The mechanism of action of 8-MOP and UVA is thought to involve the induction of apoptosis in treated lymphocytes (Yoo *et al.,* 1996; Chapter 3, Bladon & Taylor 1999b) and the up-regulation of several pro-inflammatory cytokines, including TNFα, IL6 and IFNγ (Vowels *et al* 1992; Tokura *et al* 1999). TNFα is capable of exerting anti-tumour responses toward leukaemias and lymphomas (Fransen *et al.,* 1986), whilst IFNγ, in combination with TNFα, increases the cytotoxicity of macrophages (Hori *et al.,* 1987) and IFNγ and IL12 increase the anti-tumour effect of cytotoxic T cells (Seo *et al.,* 1998). RhIL12 has also been demonstrated to be effective in reducing CTCL lesions (Rook *et al.,* 1999b).

Early lymphoid apoptosis has been detected in the buffy coat bag, immediately post ECP, when tested *ex vivo* (Chapter 3, Bladon & Taylor 1999b).

8.2 Aim

In this chapter, experiments were designed to establish the immediate influence of ECP on pro-inflammatory cytokine secretion patterns at the very early pre re-infusion stage. The levels of monocytes and T cells expressing the different intracellular cytokines was enumerated immediately before ECP and post ECP, prior to re-infusion.

8.3. Specific patients and methods

The specific cohort of patients, ECP technology and analysis methods pertaining to this chapter are detailed below. For a full explanation, please see the Materials and Methods chapter.

8.3.1. Patients and Normal controls

Samples were obtained from 11 patients receiving ECP treatment (6 CTCL (mean age ± S.D; 58.7 ± 17.2 years) and 5 GvHD (41.4 ± 8.2). All CTCL patients were stage III and had evidence of clonal disease in the peripheral blood. Samples were also taken from 11 age and sex matched normal healthy controls.

8.3.2. Photopheresis treatment

Extracorporeal photopheresis cycles were performed using the XTS™ system. See section 2.2.1.2.2 of the Materials and Methods chapter.

8.3.3. Sampling

Samples were taken immediately pre and post ECP.

8.3.4. Cell identification

T cells were identified using anti CD3 and SS (See Section 2.2.2.2.2). Monocyte identification used anti CD14 and SS (See Section 2.2.2.2.5)

8.4. Results

8.4.1. T cells

To assess cytokine expression in T cells, the PBMCs were stimulated for 6 hours with PMA and Ionomycin. Brefaldin A was also added during the 6 hour stimulation stage. PMA and Iononmycin mimic T-cell activation, resulting in a cascade of cellular responses, including cytokine secretion, through release and transportation of Ca^{++} respectively. Brefaldin A is an ionophor, which is known to interrupt subcellular transport of secretory proteins, which are arrested and then subsequently accumulate in the Golgi Stack. To gain intracellular access to these cytokines cells are exposed to a 'fix and perm' method (See Section 2.2.1.7). Following the identification of T cells using PE-CY-5-conjugated anti CD3 (See Figure 2.2.2.2.2.1), 'quadrant gates' were set using an isotype control, from which the number of TNFα- and IFNγ-secreting T cells were enumerated (See Section 2.2.2.2.6.1.2).

Figure 8.4.1.1 demonstrates the number of T cells positive for TNFα expression pre and post ECP and in the normal age/sex matched controls. The levels of T cells expressing TNFα in the CTCL patients were the same as the normal pre ECP. However, levels of TNFα-secreting T cells in the GvHD patient group were significantly higher than the normals. ECP induced a significant fall in the levels of T cells positive for TNFα ($p<0.05$), both patient groups demonstrated similar reductions. Using flow cytometry the reduction in the number of TNFα positive T cells can clearly be observed (See Figure 8.4.1.2)

Figure 8.4.1.1. The number of T cells expressing TNFα is significantly reduced immediately following ECP treatment

*Samples were taken immediately pre and post ECP. PBMCs were isolated by density gradient and the cell count adjusted to 1.0 to 2.0 x 10^6/ml (See Section 2.2.1.5). The cells were subsequently placed in cell culture medium and stimulated for 6 hours (See Section 2.2.1.6.1) Using flow cytometry, the T cells were bitmapped and examined for intracellular TNFα expression (See Section 2.2.10.3). The data represent the mean $\pm$SEM of normals and CTCL and GvHD patients tested pre and post ECP. * denotes a p value of <0.05.*

Figure 8.4.1.2. Flow cytometric representation of TNFα expressing T cells pre and post ECP

Quadrant gates were set using appropriate isotype controls (See Section 2.2.2.2.6.1.2). Following bitmapping, the number of T cells expressing TNFα was determined using these quadrant gates (Q2). Post ECP, the number of TNFα-expressing T cells dropped significantly.

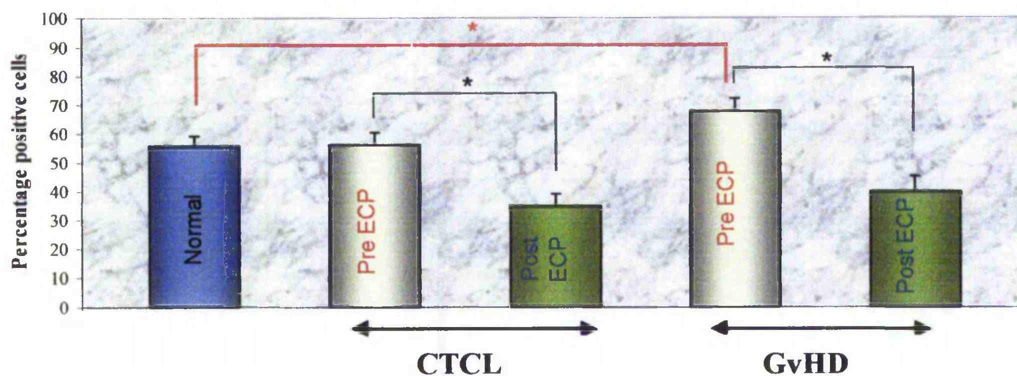


Figure 8.4.1.1. The number of T cells expressing TNFα is significantly reduced reduced immediately following ECP treatment

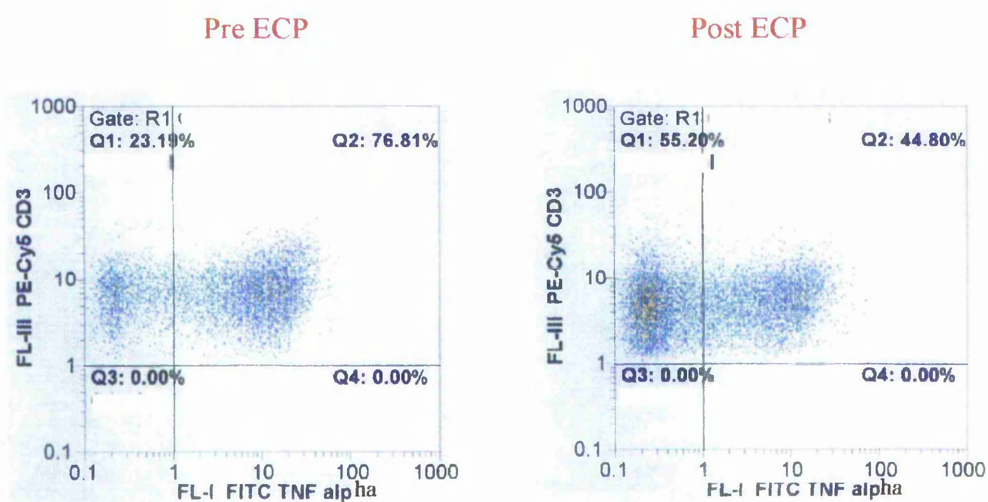


Figure 8.4.1.2. Flow cytometric representation of TNFα expressing T cells pre and post ECP

In addition to the fall in TNFα-expressing T cells, ECP also induces a reduction in the number of T cells secreting IFNγ. The mean number ± SEM of IFNγ-secreting T is demonstrated in Figure 8.4.1.3. Levels are shown for both normal age/sex matched controls and the CTCL and GvHD patients tested pre and post ECP. Pre ECP, the number of IFNγ-expressing T cells were lower than normal in the CTCL population, in keeping with the known expansion of the Th2 subtype in this condition (Vowels *et al.,* 1994). In the GvHD patient group, T cells showed higher numbers of T cells expressing IFNγ, suggestive of a Th1 proliferation. ECP reduced the levels of IFNγ-expressing T cells at re-infusion. GvHD levels falling by a mean of 10%, whilst CTCL patients fell by a mean of 6.5%. Figure 8.4.1.4 demonstrates how the observed fall in IFNγ-expressing T cells was observed using flow cytometry.

Figure 8.4.1.3. The number of T cells expressing IFNγ is significantly reduced immediately following ECP treatment

*Samples were taken immediately pre and post ECP. PBMCs were isolated by density gradient and the cell count adjusted to1.0 to 2.0 x 10^6/ml (See Section 2.2.1.5). The cells were subsequently placed in cell culture medium and stimulated for 6 hours (See Section 2.2.1.6.1). Using flow cytometry, the T cells were bitmapped and examined for intracellular IFNγ-expression (See Section 2.2.10.4). The data represent the mean ± SEM of normals and CTCL and GvHD patients tested pre and post ECP. * denotes a p value of <0.05 and ** denotes a p value of <0.01*

Figure 8.4.1.4. Flow cytometric representation of IFNγ-expressing T cells pre and post ECP

Quadrant gates were set using appropriate isotype controls. Following bitmapping, the number of T cells expressing IFNγ was determined using these quadrant gates (Q2) (See Section 2.2.2.2.6.1.2). Post ECP the number of IFNγ-expressing T cells dropped significantly (Q2).

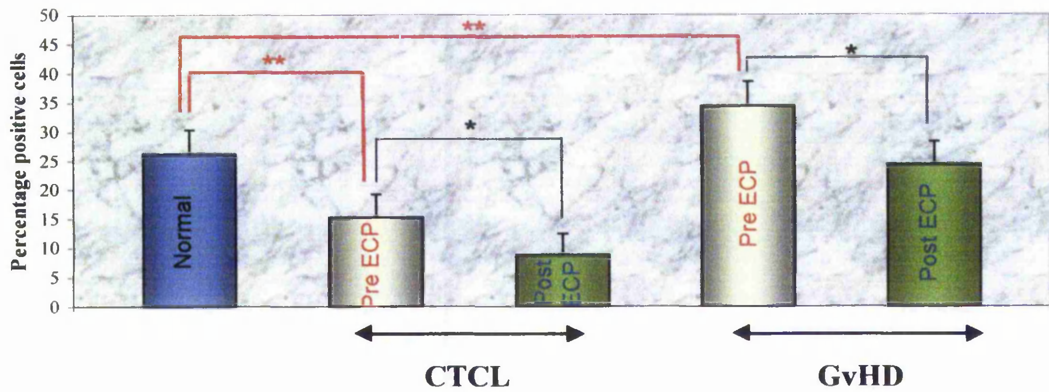


Figure 8.4.1.3. The number of T cells expressing IFNγ is significantly reduced immediately following ECP treatment

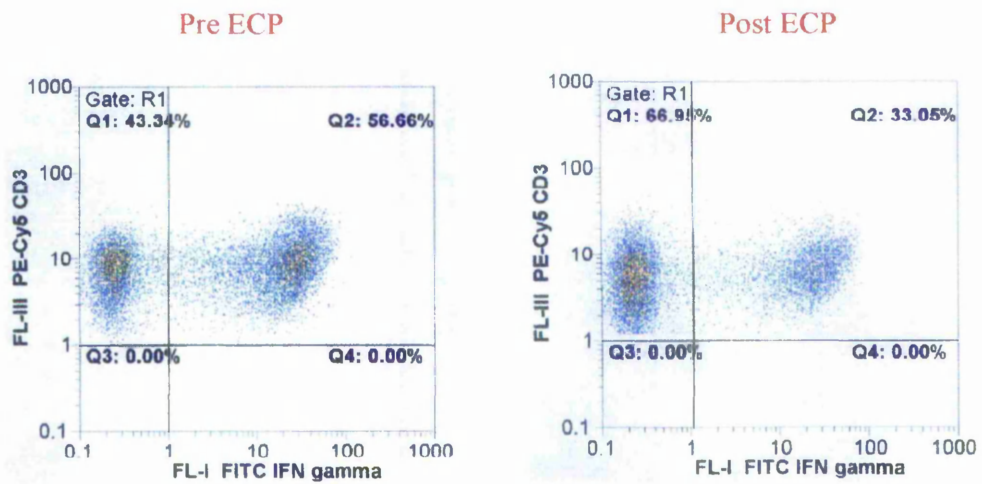


Figure 8.4.1.4. Flow cytometric representation of IFNγ-expressing T cells pre and post ECP

8.4.2. Monocytes

To try to represent the conditions present in the buffy coat collection bag prior to re-infusion, all the separated PBMCs were co-cultured. Hence monocytes were simultaneously stimulated with PMA and Ionomycin, whilst Brefaldin A aided in the accumulation of intracellular cytokines for assessment. Like T cells, monocytes expression of cytokines were evaluated through a 'fix and perm' methodology (See Section 2.2.1.7). Monocytes were identified by CD14 and high SS expression (See Section 2.2.2.2.5). Pre ECP, the number of TNFα-expressing monocytes, for both patient groups, was not significantly different to normals. However, at re-infusion the percentage of TNFα positive monocytes dropped significantly for both patient groups (see Figure 8.4.2.1).

Figure 8.4.2.2 demonstrate the number of monocytes positive for IL6, IFNγ and IL12 in the CTCL and GvHD patient groups pre and post ECP. No significant difference to the normals was observed at either testing stage.

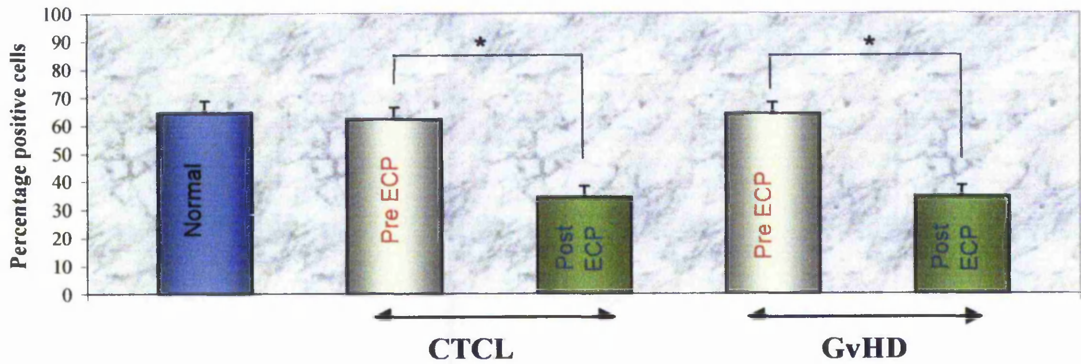


Figure 8.4.2.1. The number of monocytes expressing TNF α is significantly reduced immediately following ECP treatment

*Samples were taken immediately pre and post ECP. PBMCs were isolated by density gradient and the cell count adjusted to 1.0 to 2.0 x 10^6/ml (See Section 2.2.1.5). The cells were subsequently placed in a cell culture medium and stimulated for 6 hours (See Section 2.2.1.6.1). Using flow cytometry the monocytes were bitmapped and examined for intracellular TNFα expression (See Section 2.2.10.3). The data represent the mean $\pm$SEM of normals and CTCL and GvHD patients tested pre and post ECP. * denotes a p value of <0.05*

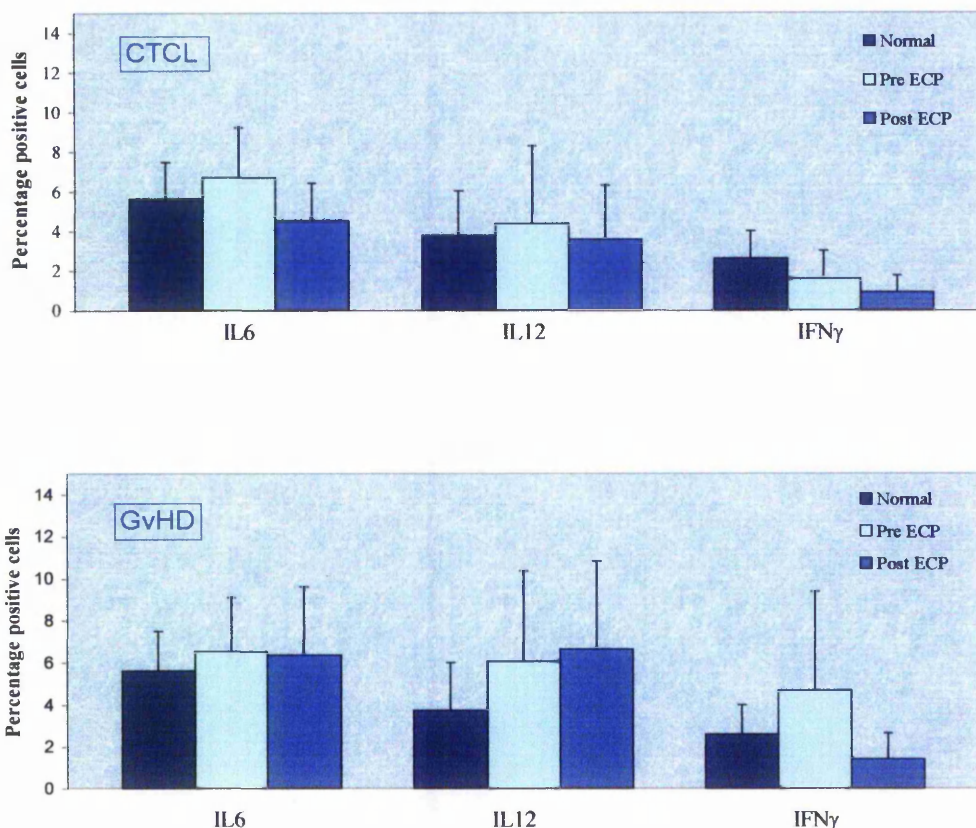


Figure 8.4.2.2. The number of monocytes, from CTCL and GvHD patients, expressing IL6, IL12 and IFNγ, at re-infusion, is unaffected by ECP treatment

Samples were taken immediately pre and post ECP. PBMCs were isolated by density gradient and the cell count adjusted to 1.0 to 2.0 x 10^6/ml (See Section 2.2.1.5). The cells were subsequently placed in a cell culture medium and stimulated for 6 hours (See Section 2.2.1.6.1). Using flow cytometry, the monocytes were bitmapped and examined for intracellular IL6, IL12 and IFN γ expression (See Sections 2.2.10.1, 2.2.10.2 and 2.2.10.3). The data represent the mean ± SD of normals and CTCL patients tested pre and post ECP.

8.5. Discussion

The pattern of cytokine production following exposure to 8-MOP and UVA is controversial. PBMCs treated with *in-vivo* and *in-vitro* PUVA and subsequently stimulated with lipopolysaccharide have demonstrated suppression of TNFα and IL6. Northern blot analysis revealed a down-regulation of mRNA encoding for IL6 and TNFα (Neuner *et al.,* 1994). However, the assessment of ECP treated monocytes, with and without IFNγ stimulation, demonstrate an enhanced production of both IL6 and TNFα (Vowels *et al.,* 1992). Similar results are also observed with IFNγ. PBMCs exposed to UVA/8MOP and subsequently stimulated with Con A demonstrate a skewing toward Th1 cytokine production (Tokura *et al.,* 1999). In contrast, PBMCs, pre-stimulated with anti CD3, rhIL2 and rhIL4 prior to 8MOP/UVA and subsequently stimulated with PMA, ionomycin and monensin demonstrate an opposite shift from Th1 to Th2 in the CD4+ T cells (Klosner *et al.,* 2001).

Many of the previous studies have involved the measurement of total cytokine levels in 8-MOP/UVA exposed cell cultures, after prolonged incubations (Vowels *et al.,* 1992; Tokura *et al.,* 1999; Klosner *et al.,* 2001). The aim of this section of work was to observe what influence ECP had on the cytokine secretion patterns of clinical samples very early in the ECP cycle, testing cells immediately post ECP, prior to re-infusion. At this early point, significant levels of apoptotic lymphocyte has been previously been identified (Chapter 3, Bladon & Taylor 1999b). This aim of this chapter was also to determine at the single cell level if cells demonstrated induction or suppression of cytokine production. To closely represent the environmental conditions present in the buffy coat bag prior to re-infusion, the T cells and monocytes were not isolated prior to culture.

Assessment *ex-vivo* demonstrated a fall in the number of TNFα-secreting monocytes at re-infusion, whilst the number of TNFα- and IFNγ-secreting T cells was also reduced following ECP. The levels of IFNγ, IL6 and IL12 expression remained unchanged for the monocyte population.

For CTCL patients the enhanced expression of TNFα, following ECP, is capable of direct anti-tumour activity, which can be directed against leukaemia and lymphoma cells (Fransen *et al.,* 1986). When combined with IFNγ, TNFα is capable of enhanced macrophage cytotoxicity (Hori *et al.,* 1987), whilst IFNγ and IL12 are capable of enhancing the tumouricidal effect of cytotoxic T cells (Seo *et al.,* 1998). Therefore an increase in the secretion of these cytokines, post UVA/8MOP may play a part in the removal of the CTCL malignant cells, as previously observed following several rounds of ECP therapy (Zouboulis *et al* 1998; Taylor, 2002 (unpublished observations)). However, TNFα, IL6 and IFNγ have also been implicated in the pathology of GvHD. In acute GvHD (aGvHD), TNFα, IL6 and IFNγ levels are all increased, whilst elevated levels of TNFα and IL6 are observed in chronic GvHD (cGvHD) (Imamura *et al.,* 1994). IFNγ has also been demonstrated in the skin biopsies of patients with cGvHD (Ochs *et al.,* 1996), prompting the suggestion that in cGvHD, IFNγ is the predominant cytokine (Parkman, 1998). Suppression of these inflammatory cytokines has been effective in the treatment of aGvHD and cGvHD. Anti TNFα antibodies have been used in the prevention of cutaneous and gastrointestinal lesions during aGvHD (Piguet *et al.,* 1987), whilst transplanted mice whose cGvHD was tolerated demonstrated statistical reductions in TNFα and IFNγ levels (Nagler *et al.,* 2000).

In this study, the levels of TNFα- and IFNγ-secreting T cells was higher in the GvHD patients pre ECP. This indicates that these two cytokines may have a prominent role in the aetiology of chronic GvHD. The early reduction in TNFα and IFNγ secreting mononuclear cells following ECP may have a direct role in reducing the pathologic progress of cGvHD. In 22 cases of steroid refractory chronic GvHD, there was statistically significant and progressive reduction in cutaneous disease at 3, 6 and 9 months with a concurrent significant reduction in steroid usage (Taylor, 2003)

The reduction in these cytokines seem to preferentially suit the treatment of GvHD, however ECP is a very effective treatment in the removal of the malignant cells responsible for CTCL. Following ECP, treated lymphocytes become apoptotic (Yoo *et al.,* 1996; Chapter 3, Bladon & Taylor 1999b). 8MOP/UVA also increases the expression of MHC class I molecules on lymphocytes, a process which may also induce a 2-3 fold parallel increase in tumour antigens (Hanlon *et al.,* 1998). Monocytes treated by ECP demonstrate a rapid conversion to dendritic cells and avidly phagocytose the apoptotic malignant T cells (Berger *et al.,* 2001). This process which leads to the presentation of antigens to cytotoxic CD8+ T cells and a subsequent anti-clonotypic immunity capable of removing non-ECP treated cells (Albert *et al.,* 1998, Hanlon *et al.,* 1998, Rook *et al.,* 1999). Of significant interest is that this immunomodulatory process is possible without the requirement for exogenous cytokines (Berger *et al.,* 2001). The removal of the malignant Th2 T cells by this mechanism may explain the long term restoration of the Th1/Th2 imbalance, as observed by Di Renzo *et al* (Di Renzo *et al.,* 1997)

These results suggest that, *ex-vivo*, UVA/8MOP exposure down-regulates pro-inflammatory cytokine responses. In GvHD and Psoriasis, in which excessive Th1 cytokine secretion patterns are observed, these responses may, in part, be responsible for the clinical responses observed following UVA/8MOP treatment.

For monocytes and their derivatives to assist in the anti-clonotypic response, thought responsible for the removal of malignant or autoreactive cells, it is essential that mechanism responsible for antigen presentation are unaffected by ECP exposure. The co-stimulatory molecules that present antigens to cytotoxic T cells have previously been down-regulated when APCs are exposed to UV. As a consequence it was necessary to establish if ECP effected the same response.

Chapter 9: ECP does not suppress the expression of co-stimulatory molecule on monocytes.

9.1. Introduction

The processing of antigens by antigen-presenting cells (APCs) ultimately leads to the presentation to T cells and immune recognition (Liu, 1992). ECP is thought to induce and anti –clonotypic cytotoxic T cell response following the phagocytosis of apoptotic T cells (Edelson *et al.,* 1994; Berger *et al.,* 2001). APCs exposed to ECP demonstrate an avidity for the phagocytosis of apoptotic T cells (Yoo *et al.,* 1996; Berger *et al.,* 2001). Induction and activation of T lymphocytes require two signals from the APC. The first signal requires the binding of the T cell receptor (TCR) to its antigen-MHC ligand, whilst the second signal is provided by the co-stimulatory molecules expressed on the APC (Kuchroo *et al.,* 1995). When APCs acquire antigens from apoptotic cells, a cytotoxic response against similar antigens is induced (Albert *et al.,* 1998; Henry *et al.,* 1999). The processing of antigens on the malignant apoptotic T cells post ECP, by APCs, may trigger the immune recognition of other non-treated malignant T cells of the same clone (Rook *et al.,* 1999; Berger *et al.,* 2001). When antigens are processed by APCs, in the absence of co-stimulatory signals, a state of T cell unresponsiveness called anergy can ensue (Rattis *et al.,* 1998). CD54 (ICAM-1), CD80 and CD86 are important co-stimulatory molecule present on the membrane of APCs (Iwai *et al.,* 1999; Hubbard *et al.,* 2000). The ICAM-1 /LFA-1 (lymphocyte function associated antigen-1) system involves the activation of T-cell mediated cytotoxicity (Boyd *et al.,* 1988), whilst transfection studies have demonstrated the important role of CD80 and CD86 in T cell proliferation (Lanier *et al.,* 1995). CD80 and CD86 are important molecules in polarising an immune response through the specific activation of Th1 and Th2 cells respectively (Kuchroo *et al.,* 1995).

Previously the treatment of monocytes by UV has demonstrated a marked suppression of CD54 expression and reduced inducible expression of CD80 and

CD86 (Hertl *et al.,* 1991; Fujihara *et al.,* 1996; De Luca *et al.,* 1997). Processes that would impair the APC properties of monocytes and add to an immunosuppressive state (Fujihara *et al.,* 1996).

9.2. Aim

By testing monocytes exposed to ECP, the experiments in this chapter were designed to determine if the co-stimulatory molecules, closely linked to antigen presentation, were suppressed by ECP treatment. The ability of the ECP-treated monocytes to phagocytosis the apoptotic lymphocytes was observed using morphological techniques.

9.3. Specific patients and methods

The specific cohort of patients, ECP technology and analysis methods pertaining to this chapter are detailed below. For a full explanation, please see the Materials and Methods chapter.

9.3.1. Patients

Samples were obtained from 9 Cutaneous T cell lymphoma (CTCL) patients (mean age ± S.D; 62.0 ± 16.7 years) receiving ECP treatment, 2 stage IIA, 5 stage III and 2 stage IVA.

9.3.2. Photopheresis treatment

Extracorporeal photopheresis cycles were performed using the XTS™ system. See section 2.2.1.2.2 of the Materials and Methods chapter.

9.3.3. Sampling

Samples were taken pre ECP and post ECP, immediate prior to re-infusion

9.3.4 Cell identification

T cells were identified using anti CD3 and SS (See section 2.2.2.2.2). Monocyte identification used anti CD14 and SS (See section 2.2.2.2.5)

9.4. Results

9.4.1. CD54 and CD86 expression

Pre and post ECP-treated monocytes were tested at 0, 2, 4 and 24 hours post sampling intervals. Monocytes were identified and 'bitmapped' using a PE-conjugated anti CD14 and high SS (See Section 2.2.2.2.5). The mean fluorescence intensity (MFI), which is indicative of the levels of antigen expressed, of CD54 and CD86 on monocytes placed in culture medium increases over time. To evaluate the MFI of CD54 and CD86, an initial MFI of an isotype control was determined using a single histogram plot (See Figure 2.2.2.2.7.1). This value was then subtracted from the MFI value obtained when testing was performed using either the anti CD54 or anti CD86 monoclonal antibody (See Figure 2.2.2.2.7.2) The MFI of CD54 expression on monocytes tested after 0, 2, 4 and 24 hours pre and post ECP are demonstrated in Figure 9.4.1.1. An example of the histogram plots of CD54 expression at all stages pre and post ECP are displayed in Figure 9.4.1.2. Comparisons of each corresponding testing stage demonstrated no significant difference in CD54 expression on monocytes post ECP to those pre ECP. Similar results were observed for the expression of CD86 on monocytes following ECP. The levels for the MFI of CD86 on the bitmapped monocytes following ECP were not significantly different to the comparative stage pre ECP (See Figure 9.4.1.3).

Figure 9.4.1.1 Exposure to ECP does not suppress the expression of CD54 on monocytes

Samples were taken immediately pre and post ECP and the PBMCs were isolated by density gradient (See Section 2.2.1.5). Cells for testing were adjusted to a count of 0.5 to 1.0 x 10^6/ml, whilst those added to cell culture were adjusted to 1.0 to 2.0 x 10^6/ml (See Section 2.2.1.6). Following bitmapping (See Figure 2.2.2.2.5.1), the monocytes were evaluated for CD54 expression (see Section 2.2.9.1). The data represents the mean ±SD for the MFI of CD54. No significant difference between the pre and post ECP expression of CD54 was observed at each testing stage

Figure 9.4.1.2. ICAM-1 (CD54) expression observed on monocytes at all testing stages pre and post ECP

At all testing stages, post ECP MFI values were not significantly different to those at the corresponding pre ECP stage. 0hrs (Blue), 2hrs (green), 4hrs (red) and 48hrs (purple)

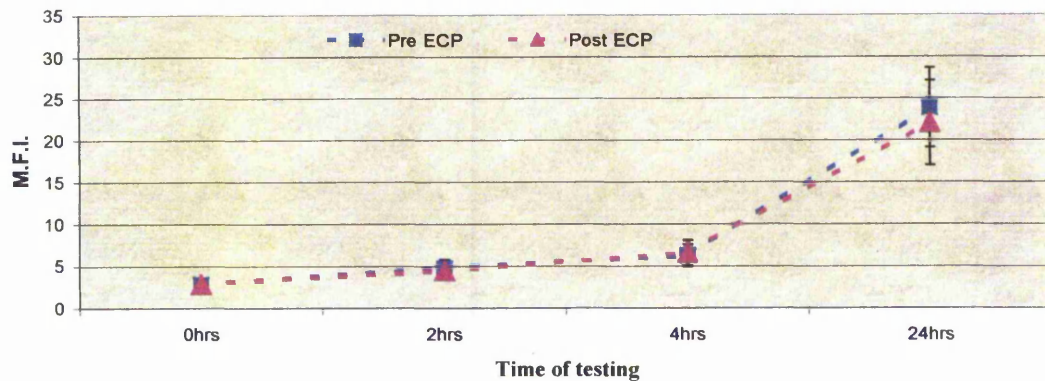


Figure 9.4.1.1 Exposure to ECP does not suppress the expression of CD54 on monocytes

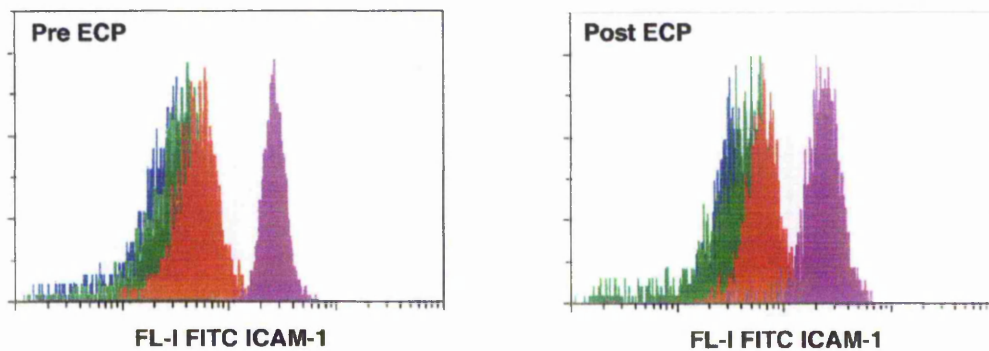


Figure 9.4.1.2. ICAM-1 (CD54) expression observed on monocytes at all testing stages pre and post ECP

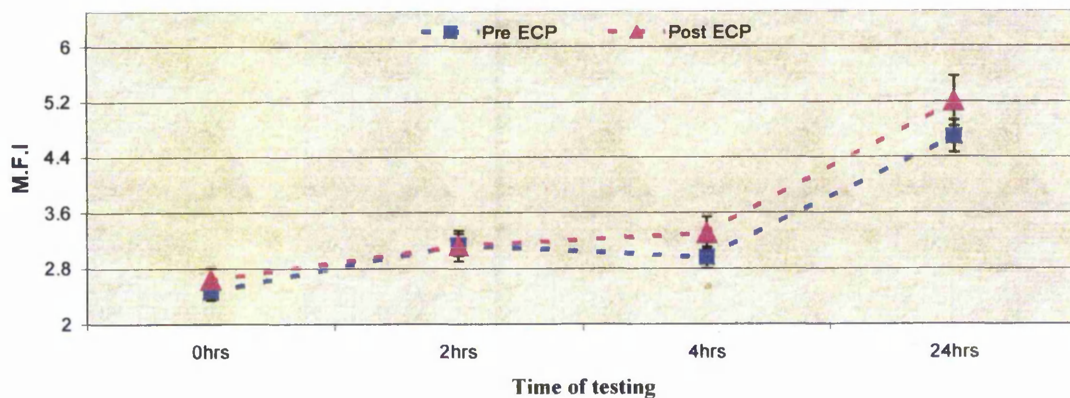


Figure 9.4.1.3. Exposure to ECP does not suppress the expression of CD86 on monocytes

Samples were taken immediately pre and post ECP and the PBMCs were isolated by density gradient (See Section 2.2.1.5). Cells for testing were adjusted to a count of 0.5 to 1.0 x 10^6/ml, whilst those added to cell culture were adjusted to 1.0 to 2.0 x 10^6/ml (See Section 2.2.1.6). Following bitmapping (See Section 2.2.2.5.1), the monocytes were evaluated for CD86 expression (See Section 2.2.9.3). The data represents the mean $\pm$ SD for the MFI of CD86. No significant difference between the pre and post ECP expression of CD86 was observed at each testing stage

9.4.2. CD80 expression

For the evaluation of CD80, the relatively low number of monocytes expressing CD80, without stimulation, meant CD80-expressing monocyte were assessed using 'region gates' set on isotype controls (See Section 2.2.2.2.6.1.1). At each of the testing stages post ECP the number of monocytes expressing CD80 remained unaltered by ECP (See Figure 9.4.2.1). Using rhIFNγ, CD80 expression increases on monocytes to a level easily measured using a MFI. Previously, prior exposure of monocytes to UV has suppressed the enhanced expression of CD80, normally observed following stimulation using rhIFNγ. However, monocytes exposed to ECP demonstrated no significant difference in CD80 up-regulation, post rhIFNγ stimulation, than monocytes tested pre ECP (See Figure 9.4.2.2).

Figure 9.4.2.1. The levels of CD80-expressing monocytes is not altered by ECP exposure

Samples were taken immediately pre and post ECP and the PBMCs were isolated by density gradient (See Section 2.2.1.5). Cells for testing were adjusted to a count of 0.5 to 1.0 x 10^6*/ml, whilst those added to cell culture were adjusted to 1.0 to 2.0 x* 10^6*/ml (See Section 2.2.1.6). Following bitmapping (See Figure 2.2.2.2.5.1), the number of CD80-expressing monocytes were enumerated (See Section 2.2.9.2). The data represents the mean ±SD. No significant difference between the pre and post ECP expression of CD80 was observed at each testing stage*

Figure 9.4.2.2. IFN γ stimulation of CD80 expression is not suppressed by prior ECP treatment

Separated cells were immediately stimulated with 500U/ml IFN γ (See Section 2.2.9.4). Following a 24 hour incubation, the monocytes were bitmapped and evaluated for CD80 expression (See Section 2.2.9.2). The data represents the mean ±SD for the MFI of CD80. No significant difference between the pre and post ECP expression of CD80 was observed

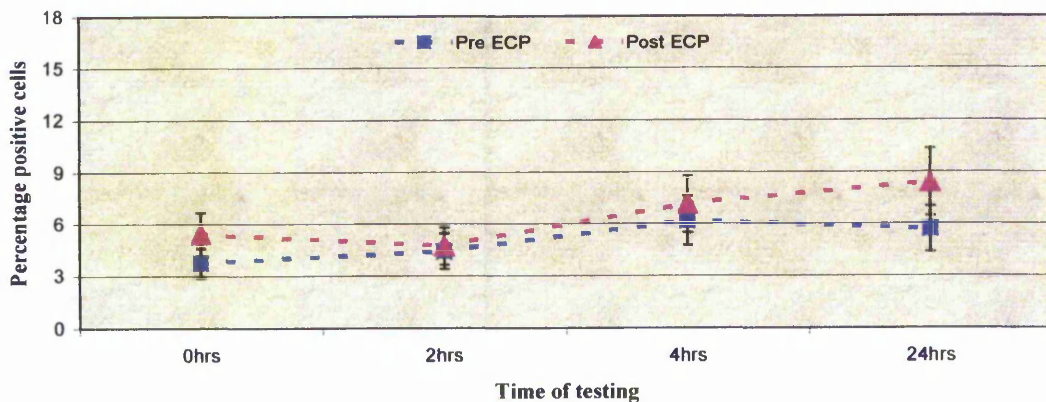


Figure 9.4.2.1. The levels of CD80-expressing monocytes is not altered by ECP exposure

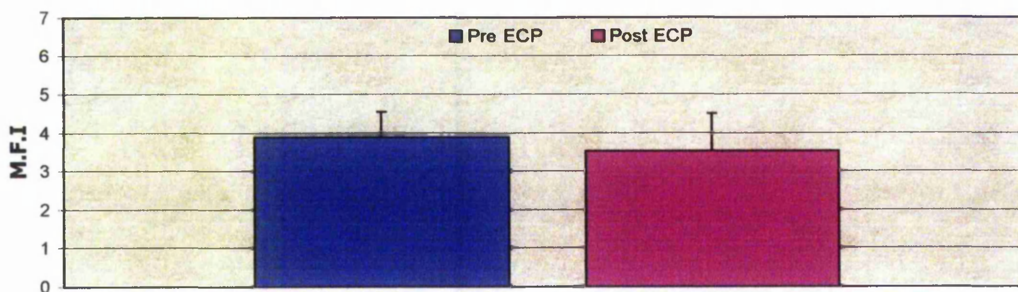


Figure 9.4.2.2. IFN γ stimulation of CD80 expression is not suppressed by prior ECP treatment

9.4.3. Visualisation of phagocytosis

Suitable morphological preparations, made from post ECP samples, were stained for light and electron microscopy (See Sections 2.2.2.1). Following ECP, the ECP-treated monocytes avidly phagocytosed the apoptotic lymphocytes induced by ECP. Using light microscopy, Figure 9.4.3.1 demonstrates the inclusion of apoptotic lymphocytes or apoptotic bodies, within monocytes. Using electron microscopy, the phagocytosis process is more easily visualised, many monocytes demonstrating multiple ingestions (See Figure 9.4.3.2). Within the 24 hour post ECP sample, the mean and SD of monocytes demonstrating lymphoid inclusions was 21.9 ± 10.3. The phagocytosis of apoptotic T cells is thought to initiate the anti-clonotypic response proposed for ECP.

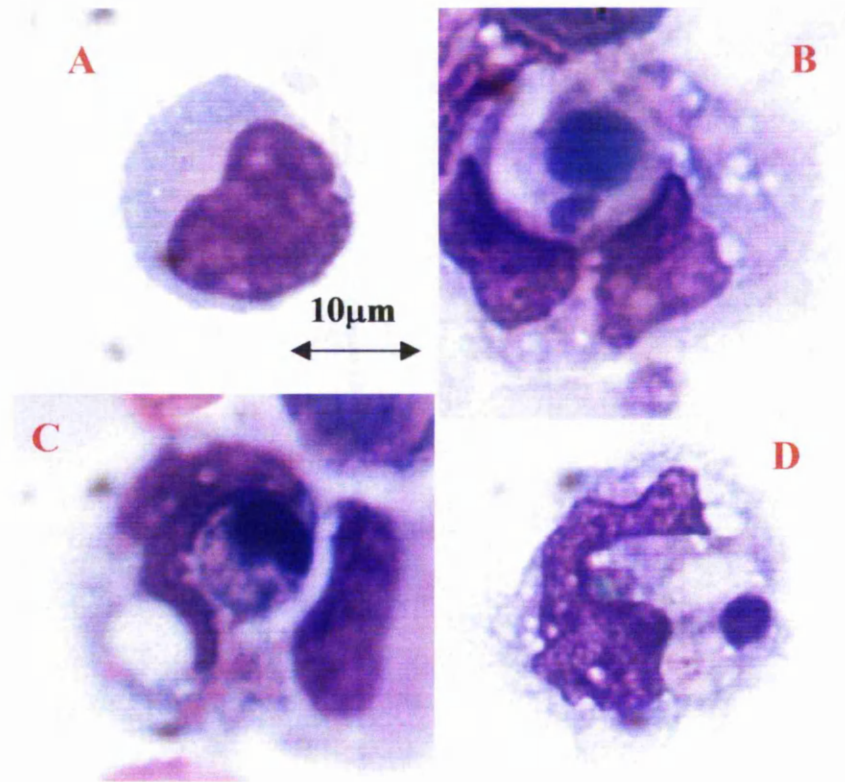


Figure 9.4.3.1. Photomicrograph of normal monocyte pre ECP and monocytes with engulfed apoptotic cells 24 hours post ECP

PBMCs were separated by density gradient (See Section 2.2.1.5) and placed in cell culture (See Section 2.2.1.6). Cytospins were prepared and stained, as outlines in Sections 2.2.2.1.1 and 2.2.2.1.2). Panel A shows a normal monocyte, observed pre ECP. Panel B-D demonstrate monocytes/dendritic cells 24 hours post ECP. Many of the monocytes now contain apoptotic lymphocytes, or the apoptotic bodies derived from apoptosis, within the cytoplasm, following phagocytosis

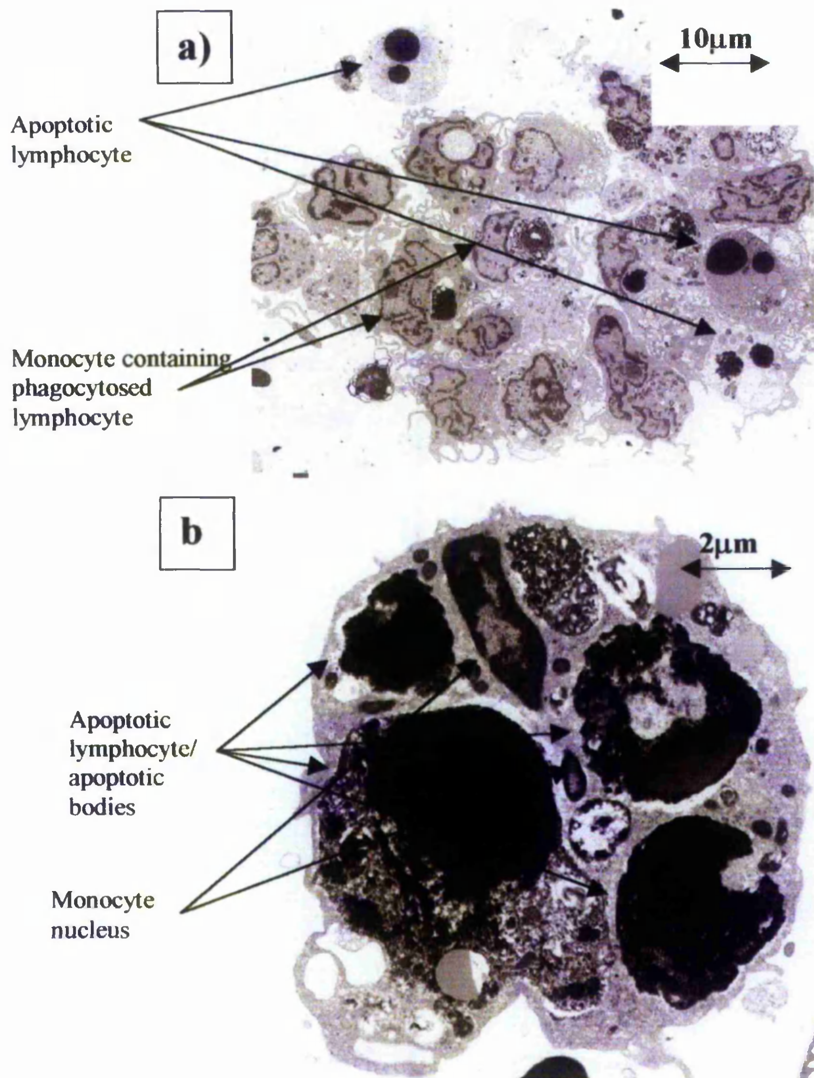


Figure 9.4.3.2. Electron micrograph of monocytes containing apoptotic lymphocytes at two magnification

Samples were taken immediately pre ECP and post ECP. The PBMCs were separated and placed in cell culture (See Section 2.2.1.5 and 2.2.1.6). Following 24 hours of cultivation, the lymphocytes were examined using electron microscopy (See Section 2.2.2.1.3 and 2.2.2.1.4). Panel a) demonstrates apoptotic lymphocytes with nuclear 'blebbing' and monocytes containing the phagocytosed apoptotic lymphocytes. Panel b), taken at higher magnification, clearly shows a monocytoid cell containing phagocytosed apoptotic cells/ apoptotic bodies.

Photomicrographs kindly prepared by Bart Wagner, Histopathology department, Northern General Hospital, Sheffield

9.5. Discussion

Monocytes are resistant to apoptosis induced by ECP (Yoo *et al.,* 1996; Tambur *et al.,* 200). Paradoxically, monocytes are activated by ECP, demonstrating enhanced secretion of TNFα and up-regulation of CD36 (Vowels *et al.,* 1992; Fimiani *et al.,* 1997). ECP-treated monocytes also demonstrate an increased ability to phagocytose apoptotic T cells (Yoo *et al.,* 1996). In this chapter cytospin preparations from the 24 hour post ECP cell cultures demonstrated extensive phagocytosis of apoptotic lymphocytes by co-cultured monocytes. These changes may indicate that treated monocytes have a role in the immunomodulation process, proposed for ECP.

The processing of antigens by APCs ultimately leads to the presentation to T cells and immune recognition (Liu, 1992). When dendritic cells acquire antigens from apoptotic cells, a cytotoxic response against similar antigens is induced (Albert *et al.,* 1998). The processing of apoptotic cells, by activated monocytes, involves antigen presentation to T cells, inducing immune recognition of similar antigens (Henry *et al.,* 1999). However to fully activate and induce proliferation, the presentation of antigens to T cells requires the presence of co-stimulatory signals (Rattis *et al.,* 1998). CD54, CD80 and CD86 are important co-stimulatory molecule in this process (Iwai *et al.,* 1999; Hubbard *et al.,* 2000). CD80 and CD86 are thought to activate Th1 and Th2 cells respectively (Kuchroo *et al.,* 1995). However, previous *in-vitro* data demonstrate a loss of CD54 expression in monocytes exposed to UVB radiation (Fujihara *et al.,* 1996; De Luca *et al.,* 1997) and inducible expression of CD80 and CD86 is decreased following UVB (De Luca *et al.,* 1997).

Comparative studies of pre- and post-ECP monocytes, tested at 0, 2, 4 and 24 hours post ECP, show no significant difference in CD54 expression at each testing stage. CD86 and IFNγ-induced CD80 expression also remains unchanged following ECP. These results indicate that ECP treated monocytes are not compromised in the antigen presentation processes dependent on these co-stimulatory mechanisms. In addition, the Th1/ Th2 responses observed post

ECP are not attributable to preferential changes in either CD80 or CD86 expression on treated monocytes.

The discrepancy between the findings of this thesis and that of previous workers may, in part, be attributed to the type and strength of UV used. Loss of CD54 on treated monocytes has been observed following exposure to UVB radiation (De Luca *et al.,* 1997). UVB also induces the loss of inducible CD80 and CD86 expression (Fujihara *et al.,* 1996) and apoptosis induction in APCs (Rattis *et al.,* 1998). ECP treated monocytes are resistant to all these processes and as such the maintenance of the co-stimulatory antigens may be a consequence of the differing effects of UVB. UVA irradiation reduces the ability of Langerhans cells to present antigens to T cells, a process associated with a loss of CD54, CD80 and CD86 expression (Iwai *et al.,* 1999). However this effect is seen at UVA doses in excess of those used for ECP. These factors may indicate that ECP's effect on monocytes is more subtle, leading to fundamentally different response to that observed for other UV therapies.

Berger *et al* determined that the maturing DCs, detected post ECP, demonstrated increased expression of MHC class II and an enhanced ability to produce alloreactive lymphocytes in a mixed lymphocyte reaction (MLR) (Berger *et al.,* 2001). Conversely, APCs exposed to other photodynamic therapies exhibit a loss of MHC class II and demonstrate an impaired capacity to stimulate the proliferation of alloreactive T cells (Gruner *et al.,* 1986; King *et al.,* 1999). ECP therefore, seems to induce a response in the APC compartment, whereby exposed cells are stimulated. In contrast to other UV or photodynamic therapies, which depend on a suppressing the normal role of APCs.

The manipulation of the immune system by ECP may involve inducing effects in both the mononuclear cells present in the periphery. The apoptotic lymphocytes induced by ECP may, in part, be processed by the activated and immunologically competent monocytes. A procedure, which may lead to the alloreactive T cells, thought to be responsible for the immunomodulatory effect

of ECP (Perez *et al.*, 1989; Edelson *et al.*, 1994). This mechanism would require monocytes that retain the relevant capabilities to perform this task. The retention of co-stimulatory molecules, in addition to a resistance to apoptosis induction, an active phenotype and release of anti-tumour cytokines, indicates this process is possible. Recently this thesis has determined that, *ex-vivo*, the number of monocytes expressing TNFα are reduced (Chapter 8, Bladon and Taylor, 2003). However, the increased expression of MHC class II and an enhanced ability to produce alloreactive lymphocytes in a mixed lymphocyte reaction (MLR), by DCs post ECP is observed in the absence of exogenous cytokines (Berger *et al.*, 2001).

Chapter 10: General discussion

10.1. Apoptosis induction in ECP

Many cellular responses have been observed following treatment with 8MOP/UVA. One prominent response observed has been the induction of apoptosis in the treated lymphocytes. Initially, exposure to *in-vitro* PUVA was demonstrated to induce apoptosis treated lymphocytes some 24 or 48 hours post exposure (Mark and Fox, 1991; Yoo *et al.,* 1996). Analysis of clinical samples, following ECP therapy, demonstrated a similar response, with no significant increase in apoptosis detectable *ex-vivo* and 6 hours following exposure to 8MOP/UVA (Yoo *et al.*, 1996; Aringer *et al.*, 1997; Enomoto *et al.,* 1997). This observation prompted the suggestion that the pre-apoptotic lymphocytes, returned immediately following ECP, would remain undetected by the reticuloendothelial (RE) system and have the capacity to modulate the immune system through release of cell signalling protein (Enomoto *et al.,* 1997). However, determination of apoptosis in these experiments included the use of Propidium Iodide and the TUNEL technique (Aringer *et al.,* 1997), gel electrophoresis and in situ nick translation of DNA fragmentation (Enomoto *et al*., 1997) and detection of DNA strand breaks and changes in cells size and density (Yoo *et al.,* 1996). All of these techniques are associated with markers present at a relatively 'late' stage of apoptosis.

10.2. Detection of early lymphoid apoptosis post ECP

Using early markers of apoptosis, namely Annexin V, Apoptest ™ and Carboxy-SNARF®-1-AM, apoptotic lymphocytes have been detected much earlier in the ECP process, with significant numbers detectable in the buffy coat bag prior to re-infusion (Chapter 3, Bladon and Taylor, 1999b). Of the early apoptotic detection systems, Annexin V was of the greatest interest because of its *in-vivo* significance. Annexin V detects the externalised expression of phosphatidylserine (PS) on the membrane of the apoptotic cell (Verms *et al.,* 1995). Apoptotic lymphocytes expressing PS on their membrane are targeted and swiftly removed by phagocytosis (Fadok *et al.* 1992; Falasca *et al.,* 1996).

The apoptotic lymphocytes which demonstrate increased Apoptest™ uptake, start to become positive for Annexin V expression soon after (Ferlini *et al.*, 1997). The expression of PS on ECP-treated lymphocytes is markedly increased at 24 hours post ECP and by 48 hours post ECP the majority of lymphocytes demonstrate Annexin V positivity (Chapter 5, Bladon and Taylor, 2002). The early apoptosis induced by ECP was confirmed by Gerber *et al*, who determined a significant increase in Annexin V expression of ECP treated lymphocytes at 1 hour post ECP (Gerber *et al.*, 2000). They also demonstrated a marked, further increase in Annexin V expression at 20 hours post ECP (Gerber *et al.*, 2000). The apoptotic cells at this later stage of apoptosis also begin to demonstrate the simultaneous uptake of Propidium Iodide (PI). (Gerber *et al.*, 2000; Chapter 5, Bladon and Taylor, 2002). PI uptake reflects the early changes of treated lymphocytes from early apoptotic to secondary necrosis (Verms *et al.*, 1995). However, *in vivo* many of the apoptotic cells would not reach this stage, instead they would be rapidly removed by APCs. This process has been observed in *in-vitro* 24 and 48 hour cell culture samples, where phagocytosis of apoptotic lymphocytes by monocyte/macrophages/dendritic cells has been observed (Yoo *et al.*, 1996; Berger *et al.*, 2001; Chapter 9, Bladon and Taylor, 2003b). Within the cell culture system many apoptotic/secondary necrotic lymphocytes persist and remain unprocessed, due to the greater ratio of lymphocytes to monocytes/dendritic cells/macrophages.

Historically, ECP has been performed on a two day treatment regimen (Edelson *et al.*,1987). No clear evidence is present for why this technique is retained; other than for the convenience of patients who attend the often sparsely distributed ECP centres. Assessment of apoptotic levels of day 1 and day 2 of ECP therapy was performed and the results of this thesis and Gerber *et al* results differ with regard to the levels of apoptosis noted on both days of treatment. The results of this thesis demonstrated no significant difference in the number of apoptotic lymphocytes on day one and two, whilst Gerber *et al* observed a slightly higher level on the second day (Chapter 3, Bladon and Taylor, 1999b; Gerber *et al.*, 2000). However, evaluation of apoptosis in this thesis used a

panel of early apoptosis detection systems, namely Annexin V, Apoptest™ and Carboxy-SNARF®-1-AM (Chapter 3, Bladon and Taylor, 1999b). Gerber *et al,* however relied on Annexin V only for the evaluation of the early apoptosis (Gerber *et al.,* 2000). The data presented in this thesis would indicate that the second day of treatment is primarily to attain twice the number of apoptotic lymphocytes, rather than prime the lymphocytes for a greater yield on the second day. The intention of the two day treatment is probable to impact as big an immunological challenge as possible (Chapter 3, Bladon and Taylor, 1999b)

When comparing the 6 hour post ECP sample to that of the 0 hour post ECP, it is interesting to note that the increase in the number of lymphocytes positive for Annexin V expression is slight and not significant (Chapter 5, Bladon and Taylor, 2002). These results are reinforced by observations made at 2 and 4 hours post ECP, where Annexin V positive lymphocyte levels were not significantly higher than those at one hour post ECP (Gerber *et al.,* 2000). This may indicate that the early apoptosis, observed immediately post ECP (Chapter 3, Bladon and Taylor 1999b), may represent a separate cohort of rapidly proliferating lymphocytes sensitive to the ECP process (Chapter 5, Bladon and Taylor 2002). The induction of apoptosis in 8-MOP/UVA treated lymphocytes has been linked to the DNA damage caused. UV activation of the 8-MOP intercalated into the DNA causes inhibition of the DNA replication stage and hence blocks cell survival (Marks and Fox, 1991; Godar, 1999). This process would selectively induce apoptosis in T cells where cell replication was high.

10.3. The mechanisms responsible for ECP-induced apoptosis

The early induction of apoptosis has been linked with other different cellular events, including changes observed in the mitochondria (Zamzami *et al.,* 1995). In some cell types the mitochondria is thought to be the primary target for apoptotic induction (Vayssière *et al.,* 1994). Functional changes to the mitochondria of apoptotic cells include; the uncoupling of electron transport from ATP production, a reduced rate of mitochondrial protein transportation, and disruption of the mitochondrial inner transmembrane potential ($\Delta\psi_m$) (Petit

et al., 1995; Zamzami *et al.,* 1995; Tada-Oikawa *et al.,* 1996; Kroemer *et al.,* 1997). The presence of a reduced $\Delta\psi_m$, *in-vivo*, denotes an irreversible stage of pre programmed lymphocyte death (Zamzami *et al.,* 1995). Apoptotic signals can disrupt the $\Delta\psi_m$ by initiating the opening of mitochondrial permeability transition pores (MPT), leading to the osmotic expansion of the mitochondria matrix, eventual membrane rupture and subsequent release of apoptogenic molecules such as cytochrome c (Petit *et al.,* 1998; Thress *et al.,* 1999) Once released from the mitochondria, cytochrome c binds to the apoptosis inducing factor (AIF), which subsequently leads to caspase- 8 (FLICE) activation (Li *et al.,* 1997). Evaluation of ECP-treated lymphocytes, using Rhodamine 123 and JC-1, demonstrated an early disruption to the $\Delta\psi_m$. Using the intracellular stain, JC-1, a significant number of lymphocytes demonstrating a depolarisation of the $\Delta\psi_m$ were detected very early in the ECP process; prior to re-infusion (Chapter 5, Bladon and Taylor, 2002).

Previously, the categorisation of rapid induction of apoptosis by UVA irradiation has been associated with a perturbation of mitochondrial membrane permeability and release of apoptosis inducing factor (AIF) (Godar, 1999). Early UVA induced apoptosis is also associated with no accumulation of p53 (Wang *et al.,* 1998). Following ECP, the early apoptosis, induced immediate prior to re-infusion, also demonstrated no increase in p53 expression (Chapter 4, Bladon and Taylor, 2002b). In contrast to the apoptosis observed in ECP treated lymphocytes at 24 hours post ECP where enhanced expression of p53 has been documented (Yoo *et al.,* 1996). P53 is an apoptogenic protein that protects the genome from mutagenesis (Moll and Schramm, 1998). It is well characterised that p53 up-regulation is observed following DNA damage leading to either cell cycle arrest or apoptosis, depending on the cell type (Kuerbitz *et al.,* 1992; Lowe *et al.,* 1993). Within the lymphoid haemopioesis lineage, p53 mediated growth control is through the induction of apoptosis (Moll and Schramm, 1998; Yeargin and Haas, 1995). The accumulation of p53 has been reported following exposure to UVB and PUVA, although no increase in p53 expression is observed after irradiation with UVA-1 (Wang *et al.,* 1998; Godar, 1999;

Hannuksela-Svahn *et al.,* 1999). The UVA of a UVA-1 source selectively emits UVA of the shorter wavelenght and induces apoptosis very rapidly (Godar, 1999), whilst UVB and PUVA induce apoptosis are dependent on p53 upregulation following DNA damage (Wang *et al.,* 1998; Godar, 1999). Apoptosis induced by UVA-1 is linked to oxidative stress and the down-regulation of the apoptosis inhibiting protein Bcl-2 (Wang *et al.,* 1998).

Immediately prior to re-infusion, ECP treated lymphocytes demonstrate a reduction in the intracellular Bcl-2/Bax protein ratio. Lymphocytes demonstrated a fall of between 20 and 30% at re-infusion (Chapter 4, Bladon and Taylor, 2002b). In addition to its association with immediate type apoptosis (Wang *et al.,* 1998), Bcl-2 has been demonstrated to inhibit MPT in isolated mitochondria and in cells (Zamzami *et al.,* 1995). Bcl-2 protects the cells from apoptosis by stabilising the $\Delta\psi_m$. In transfected cells, over-expression of Bcl-2 prevents both a decrease in $\Delta\psi_m$, as well as apoptosis by oxidative stress (Dispersyn *et al.,* 1999). The immediate apoptosis, induced by UVA-1, demonstrates the presence of reactive oxygen species (ROS) and a disruption to the $\Delta\psi_{m.}$ (Godar, 1999). The Bcl-2 family member, Bax, co-operates with a component of the MPT, adenine nucleotide translocator, leading to channel formation in artificial membranes (Marzo *et al.,* 1998). Bax acts at the level of the mitochondrial membrane to promote the exit of cytochrome c into the cytoplasm (Jürgensmeier *et al.,* 1998). A process which can be antagonised by Bcl-2, in present in sufficient quantity (Zamzami *et al,* 1998).

The very early induction of apoptosis in lymphocytes by various chemotherapeutic agents has been associated with a lack of p53 expression and an increase in Bax (Stahnke *et al.,* 2001). These observation which may indicate a common link between the early apoptotic mechanism (Bladon and Taylor, 2002d).

As previously mentioned, the release of cytochrome c leads to activation of the caspase cascade and the ultimate demise of the cell by apoptosis (Li *et al.,*

1997). Activation of the caspase cascade was first detected at 6 hour post ECP, with a substantial increase in caspase detection at 24 hours post ECP (Chapter 5, Bladon and Taylor, 2002). The use of chemotherapeutic agents induce a similar early stage apoptosis, associated with a six fold increases in Bax expression with little or no caspase activation (Stahnke *et al.,* 2001). The apoptosis induced in the early phase of ECP may therefore represent a mechanism or cell type (high replication rate) distinct from that observed at the later stage (Chapter 5, Bladon and Taylor, 2002). Even in the presence of a caspase inhibitor, over expression of Bax-like proteins, or their enforced dimerization, kills mammalian cells provoking DNA condensation and membrane alterations without caspase activation or DNA degradation (Xiang *et al.* 1996). Bax and Bax-like proteins might mediate caspase-independent death via channel activity, which could promote the mitochondrial permeability transition or puncture the mitochondrial outer membrane (Green and Reed, 1998). However, the presence of a caspase-independent apoptotic pathway following ECP treatment remains unproven and the delay in caspase activation observed may represent a delay in detection only.

Disruption to the $\Delta\psi_m$ and the exposure of PS appear to be intimately linked and constitute a central event in early apoptosis (Zamzami *et al.,* 1995). Several speculatory theories link the process of PS exposure and a reduction in $\Delta\psi_m$. The rapid cessation of ATP synthesis by MPT can induce PS exposure, whilst the release of a soluble protein(s) by the mitochondria undergoing MPT may activate the lipid Scramblase. Scramblase is a lipid and is responsible for the 'flip-flop' and subsequent exposure of PS (Castedo *et al.,* 1996). The failure of the caspase inhibitor zVAD, to prevent externalisation of PS on cells exposed to glucocorticoids demonstrates that PS redistribution is not dependent on the activation of caspases cascade (Verhoven *et al.,* 1999).

The caspases are a family of proteases whose initial activation triggers a cascade of further caspase activation leading ultimately to the death of the cell (Thress *et al.,* 1999; Allen *et al.,* 1998). The caspase system is activated through

the caspase 8 dependent or caspase 9 dependent pathways (Boldin *et al.,* 1996; Muzio *et al.,* 1996; Li *et al.,* 1997). Once activated, irreversible apoptosis ensues through a system of very well organised processes to stop DNA replication and destroy DNA (Thornberry and Lazebnik, 1998). Expression of caspase activation, in the ECP system, was determined using the Caspatag™ system, which identifies the presence of several activated caspases including caspase 1, 3, 4, 5, 7, 8 and 9 (Chapter 5, Bladon and Taylor, 2002). Caspase activation was first detected at 6 hours post ECP, increasing significantly 24 and 48 hours post ECP (Chapter 5, Bladon and Taylor, 2002). Previous studies of UVA induced apoptosis have demonstrated similar findings, with the activation of the caspases thought to be triggered directly by the change in $\Delta\psi_m$ (Tada-Oikawa *et al.,* 1998). Although the early apoptosis detected immediately post ECP demonstrated a delay in caspase detection, the subsequent marked increase in caspase activation parallels the rapid rise in apoptosis observed from 24 to 48 hours post ECP (Bladon and Taylor, 2002). Further activation of the caspase cascade may be, in part, activated by the CD95 and p53 dependent mechanism.

ECP has been demonstrated to increase the number of $CD95^+/CD4^+$ lymphocytes of Systemic Sclerosis patients, significant numbers detectable 24 hours following ECP (Aringer *et al.,* 1997). In addition, peripheral blood lymphocytes exposed to UVB increase expression of Fas-L, while some UVB exposed lymphocyte cell lines also release soluble Fas-L (Caricchio *et al.,* 1998). However only a slight elevation in CD95 expressing CD8+ T cells was observed at 24 hours post ECP. Subsequent testing revealed a significant increase in the number of ECP treated T cells expressing Fas-L at the same 'late' testing stage, although no Fas-L expression was observed at the earlier 0 and 12 hours post ECP stages (Chapter 6, Bladon and Taylor, 2003). Activation of the CD95 pathway initiates the activation of the caspase cascade through FLICE (caspase 8) (Medema *et al.,* 1997). When Fas is cross-linked by Fas-L, caspase 8 (FLICE) is activated, which subsequently triggers downstream activation of the caspase cascade (Chinnaiyan *et al.,* 1995; Muzio *et al.,* 1998). The caspase cascade, once activated, is capable of inducing irreversible

apoptosis (Allen *et al.,* 1998). It is well established that apoptosis induced by the CD95 system is critical for growth control of T cells, eliminating autoreactive T cells and responsible for the removal of activated T cells after they have responded to foreign antigens (Nagata and Golstein, 1995; Lynch *et al.,* 1997). However, cellular stress-inducing agents can also activate the CD95 ligand/receptor interaction, leading to apoptosis of the cell exposed (Friesen *et al.,* 1996). The cross-linking of Fas by Fas-L on target cells can occur by transfer from adjacent activated cytotoxic T cells or by expression on the target cell membrane itself (cell suicide) (Dhein *et al.,* 1995). In CTCL, peripheral blood CD4+ T lymphocytes demonstrate decreased expression of CD95. An observation which suggests that CTCL progression may due to defective apoptosis, rather than true proliferation (Dereure *et al.,* 2000). DNA damage leads to the induction of Fas-L expression; a process which involves the binding of the transcription factor AP-1 to the Fas-L promoter, thus increasing Fas-L mRNA and subsequently Fas-L protein expression, which is translocated to the plasma membrane (Kasibhatla *et al.,* 1998). Alternately, DNA-damage may involve the transcription factor p53 inducing cell-cycle arrest or apoptosis (Wang *et al.,* 1998). Following UV induced DNA damage, Fas and Fas-L receptors have been up-regulated, partly through a p53 dependent pathway (Fulda *et al.,* 1997; Aragene *et al.,* 1998). The significant elevation of Fas-L, and not CD95, 24 hours post ECP may only be responsible for the induction of cells already expressing CD95. In addition, the MFI of CD95 expression on ECP treated T cells remains unchanged, indicating that T cells already expressing CD95 do not upregulate CD95 expression. However the number of apoptotic lymphocytes detected 24 hours and 48 hours post expression is higher than the number of cells expressing either CD95 of Fas-L. An observation, which indicates that the involvement of CD95 in the orchestrated killing, performed by ECP, constitutes only a minor role. It is possible that the discrepancy may be attributable to a CD95 independent delayed type process. (Chapter 6, Bladon and Taylor, 2003). This mechanism may be linked to the enhanced expression of p53, observed at the later apoptotic stage of ECP (Yoo *et al.,* 1996).

Because the death receptors, such as CD95 system, are dependent on caspase 8, whilst the mitochondrial release of cytochrome c utilises caspase 9 activation, identification of the relevant pathways utilised by ECP could be determined using either caspase 8 or caspase 9 specific inhibitors.

10.4. Processing of apoptotic lymphocytes post ECP

The targeting, ingestion and processing of apoptotic cells by antigen presenting cells are intended to prevent the detrimental effects of inflammation. (Kerr *et al.*, 1993). The phagocytosis of apoptotic cells is mediated by several different cell surface mechanisms. Several sites have been isolated including the $\alpha_v\beta_3$- 'vitronectin receptor', a heterodimer belonging to the β_3 or cyto-adhesion family of intergrins, which binds to the bridging thrombospondin binding moiety of the apoptotic cells (Savill *et al.*, 1992). The recognition by phagocyte lectins is dependent on putative changes in cell surface sugars consequent to apoptosis (Duvall *et al.*, 1985). PS is a well characterised target marker for apoptotic phagocytosis following externalisation (Fadok *et al.*, 1992). The externalisation of PS occurs very early following apoptosis induction (Vermes *et al.*, 1995; Krahling *et al.*, 1999; Chapter 3, Bladon and Taylor, 1999b, Chapter 5, Bladon and Taylor, 2002). The PS normally present on the internal membrane flips over to be exposed on the external membrane surface very early in the apoptotic process (Fadok *et al.*, 1992; Krahling *et al.*, 1999). For apoptotic T cells, parallel kinetics of PS externalisation and the development of PS-sensitive phagocytosis by macrophages suggest that PS expression is not only necessary but sufficient for to generate a recognition signal (Verhoven *et al.*, 1999). The large influx of apoptotic lymphocytes (25-50% of the peripheral count) (Wolfe *et al.*, 1994) would constitute a very large immunological challenge following re-infusion. The replenishment of normal T cells into the periphery, at a rate more rapid than that of malignant cells may indicate a gradual mechanism for ECP. A process of attrition that would account for the numerous treatment cycles required to induce remission (Yoo *et al.*, 1996). However, although a large percentage of peripheral cells are treated, this only accounts for 2-5% of the tumour load, with regard to CTCL (Duvic *et al.*, 1996;

Gottlieb *et al.,* 1996). A more likely explanation is the generation of an immunomodulatory process that would target and remove non-treated but clonal T cells (Edelson *et al.,* 1994). In animal studies, the infusion of specific autoreactive T cell clones induces encephalomyelitis. Perez *et al* demonstrated that if these T cell clones were lethally damaged with direct 8-MOP/UVA, treatment prior to re-infusion, subsequent infusion of autoreactive clones did not induce the pathological disease (Perez *et al.,* 1989). Conceivable the processing of apoptotic T cells demonstrating clonality, such as the malignant lymphocytes of CTCL or the autoreactive T cells of GvHD, would generate CD8+ cytolytic T cells capable of inducing and removing intact clonal cells. Data supported by the immunocompetence profile of responders. Good ECP 'responders' typically demonstrate significantly higher baseline absolute CD8+ T cells than 'non-responders' and display normal natural killer cell activity (Gottlieb *et al.,* 1996; Zouboulis *et al.,* 1998; Rook *et al.,* 1999).

CTCL lymphocytes are thought to proliferate because of a diminished expression of tumour antigens and/or a failure of the immune system to contain and destroy the malignant cells (Dereure *et al.,* 2000). However, CTCL lymphocytes do express tumour specific antigens at the cell surface with class I major histocompatabilty molecules (Berger *et al.,* 1996). Lymphocytes treated using 8MOP/UVA demonstrate a 200-300% increase in MHC class I expression on treated T cells. A modification which may induce a parallel increase in display of tumour antigens (Hanlon *et al.,* 1998). Processing of apoptotic clonal T cells, demonstrating increased expression of specific clonal antigens, may generate the specific CD8+ cytolytic T cells capable of inducing and removing intact clonal cells (Hanlon *et al.,* 1998; Rook *et al.,* 1999; Berger *et al.,* 2000). Treated lymphocytes demonstrate the maximal expression of MHC class I at 20 hours, coinciding with the onset of delayed type apoptosis (Moor *et al.,* 1995; Hanlon *et al.,* 1998). However the increase in MHC class I expression is gradual and up-regulation in MHC class I expression has been observed 4 hours following the exposure of cells to 8MOP and UVA doses used clinically (Hanlon *et al.,* 1998). Therefore, it is conceivable that the enhanced MHC class

I, and thus tumour antigens, would begin with the simultaneous induction of early apoptosis, beginning very promptly following re-infusion. A dramatic increase in recognition and phagocytosis of apoptotic T cells by macrophages occurs 4 hours following externalisation of PS (Verhoven *et al.,* 1999). Hence, the early apoptotic cells would be processed when MHC class I, and thus tumour antigens are beginning to increase. Apoptotic T cells have been observed within the monocytes/macrophages and the derived dendritic cells following ECP (Yoo *et al.,* 1996, Berger *et al.,* 2001). A mechanism observed in morphological preparation post ECP (Chapter 9, Bladon and Taylor, 2003b).

Following ECP, the number of monocytes expressing CD36 increase, a process which occurs very promptly, prior to re-infusion (Fimiani *et al.,* 1997). Using monoclonal antibodies to CD36, the ability of monocyte/macrophages to take up apoptotic lymphocytes has been inhibited. Indicating the role of CD36 as an important phagocyte recognition antigen (Gregory, 1995). Further to these observations Berger *at al* have incubated the whole treated buffy coat bag for a further 24 hours following completion of the treatment cycle. They observed significant levels of phagocytosis of apoptotic T cells by monocyte-derived dendritic cells within the bag after 24 hours incubation. Berger *et al* suggested that the 'loaded dendritic' cells would evoke the immunomodulatory response, observed in ECP 'responders' (Berger *et al.,* 2001). The presence of the early apoptotic cells, following ECP, would indicate that this process would begin promptly *in-vivo* in suitable APCs contained within the tissues (Chapter 3, Bladon and Taylor 1999b). The stimulation of the immune system through APC presentation would be sustained by the progressive induction of apoptosis at 24 and 48 hours (Chapter 5, Bladon and Taylor, 2002). The second day treatment cycle furthering this process to provide a substantial immunological challenge (Chapter 3, Bladon and Taylor, 1999b). Of interest was the observation that only apoptotic cells and not necrotic cells were capable of charging dendritic cells with antigen (Albert *et al.,* 1998). Further to this process, the activated re-infused monocytes and subsequent derivatives would represent a very active and capable APC group.

Recently it has been suggested that CD10 may act as a target recognition site for the macrophage phagocytosis of apoptotic T cells (Cutrona *et al.,* 1999). CD10 is most usually associated with cells demonstrating the common acute lymphoblastic leukaemia antigen (CALLA) phenotype (Shipp *et al.,* 1989) However lymphocyte expression of CD10 has also been linked to growth arrest (Mari *et al.,* 1997) and apoptosis (Cutrona *et al.,* 1999). The ECP treated lymphocytes did demonstrate a 'weak' CD10 at 24 and 48 hours post ECP. However the CD10 expression followed a very strong Annexin V and PI signal. PS externalisation alone is sufficient to induce recognition and removal by APCs prior to a loss of membrane integrity, as determined by PI absorption (Verhoven *et al.,* 1999, Chapter 5, Bladon and Taylor, 2002) The swift processing, *in-vivo*, of PS expressing apoptotic lymphocytes means it is very unlikely that cells would remain unprocessed sufficiently long enough to demonstrate CD10 (Bladon and Taylor, 2000).

10.5. The role of heat shock proteins in ECP

Recently it has been demonstrated that the immunogenicity of apoptotic leukaemia cells is enhanced by the expression of heat shock proteins (HSPs) (Feng *et al.,* 2001). In addition, HSPs have also been linked to the modulation of the apoptosis process itself (Sapozhnikov *et al.,* 1999; Xanthoudakis *et al.,* 1999). The presence of HSP 72 on the surface of tumour cells may act in an MHC –unrestricted manner as a tumour specific recognition structure for distinct NK cell population (Multhoff *et al.,* 1997). Apoptotic cells expressing HSPs induced a significantly more effective anti-tumour response, than apoptotic cells without up-regulated HSPs (Feng *et al.,* 2001). However, the role of HSPs as protectors or assistants of apoptosis is still unclear. HSPs may provide some protection at the early stages of stress induced damage, but are unable to provide complete protection once the apoptotic cascade is initiated (Samali and Cotter, 1996). Following ECP, both monocytes and T cells were evaluated for the total expression of HSP 70 (72) present within the cell and on the membrane. At 6, 24 and 48 hours post ECP, no significant increase in HSPs

was demonstrated. The expression of HSPs could easily be induced by the prior incubation of the cells at 42°C. Therefore it could be concluded that the apoptotic processes, which follow ECP, do not involve up-regulation of HSP 70(72). In addition, the immunogenicity of ECP treated cells was not dependent on the membrane expression of HSP 70(72) (Chapter 7, Bladon and Taylor, 2002c). However, the apoptotic lymphocytes do express PS (Chapter 3, Bladon and Taylor, 1999b) and phagocytosed apoptotic T cells have been observed in cell cultures following ECP (Yoo *et al.,* 1996; Berger *et al.,* 2001, Chapter 9, Bladon and Taylor, 2003b). Two processes which demonstrate the immunogenicity of the ECP-treated apoptotic lymphocytes. However, to what extent the ECP-induced immunogenic process could be enhanced by stressing lymphocytes prior to UVA exposure and apoptosis remains unknown. Considerable thought would have to be made to the purification of the white cell collection process. The plasma and red cells would have to be completely removed by a washing process. Heating to 42°C would lead to the destruction of some plasma proteins and the lysis of red cells. Re-infusion of any products, which contain lytic red cells, is very dangerous and thus any product derived in this way would have to have stringent safety procedures. However it is interesting to note heat and cold shock do not seem as effective as 8MOP/UVA in increasing the expression of MHC-1 molecules (Hanlon *et al.,* 1998). The increase in MHC-1 expression and parallel increase in tumour antigens thought to be a major contributing factor in the immungenicity of ECP treated lymphocytes (Hanlon *et al.,* 1998).

The role HSP proteins as cytokine mediators have also been described. Monocytes exposed to HSP70 up-regulate production of some pro-inflammatory cytokines, including TNFα and IL6, suggesting intracellular production of HSP 70 in monocytes would promote the same effect (Asea *et al.,* 2000). TNFα is capable of direct anti-tumour activity, which can be directed against leukaemia and lymphoma cells (Fransen *et al.,* 1986). When combined with IFNγ, TNFα is capable of enhanced macrophage cytotoxicity (Hori *et al.,*

1987), whilst TNFα has linked to the induction of apoptosis in U937 leukaemia cells (Wright *et al.,* 1992). The presence of HSP 70 in tumour lysates can stimulate immature dendritic cells to increase phagocytosis, whilst attracting macrophages and dendritic cells into the tumour environment (Todryk *et al.,* 1999). In addition, the immunogenic response to apoptotic lymphocytes, *in-vivo,* is enhanced by the presence of the pro-inflammatory cytokines; IL12, IFNγ and TNFα (Feng *et al.,* 2001). All of these processes have a very significant association in the ECP process. TNFα and IL6 up-regulation have been described in ECP treated monocytes, with and without IFNγ stimulation (Vowels *et al.,* 1992). Monocytes have demonstrated an enhanced avidity for the phagocytosis of T cells following ECP (Yoo *et al.,* 1996). Infusion of rhIL12 has caused a significant reduction in CTCL lesions (Rook *et al.,* 1999b). However monocytes exposed to ECP do not demonstrate an enhancement in the intracellular or membrane bound HSP 70 expression. Results, which indicate that the proposed cytokine responses observed post ECP, are not as a consequence of a HSP response (Chapter 7, Bladon and Taylor 2002c). However, results of this thesis demonstrate a down-regulation in cytokine expression post ECP (See Section 10.6) (Chapter 8, Bladon and Taylor, 2002e)

10.6. Cytokine patterns post ECP

T cells are sub-classified according to the cytokine profiles they secrete when stimulated. T cells that preferentially secrete IL2 and IFNγ are deemed Th1, while Th2 cells preferentially secrete IL4, IL5 and IL10 (Mosmann and Coffman, 1989). The pattern of cytokine production following exposure to 8-MOP and UVA is controversial. PBMCs treated with *in-vivo* and *in-vitro* PUVA and subsequently stimulated with lipopolysaccharide have demonstrated suppression of TNFα and IL6. Northern blot analysis revealed a down-regulation of mRNA encoding for IL6 and TNFα (Neuner *et al.,* 1994). However, the assessment of ECP treated monocytes, with and without IFNγ stimulation, demonstrate an enhanced production of both IL6 and TNFα (Vowels *et al.,* 1992). Similarly PBMCs exposed to UVA/8MOP and

subsequently stimulated with Con A demonstrate a skewing toward Th1 cytokine production (Tokura *et al.*, 1999), whilst PBMCs, pre-stimulated with anti CD3, rhIL2 and rhIL4 prior to 8MOP/UVA, and subsequently stimulated with PMA, ionomycin and monensin demonstrated an opposite shift from Th1 to Th2 in CD4+ T cells (Klosner *et al.*, 2001). Testing at the early stage of ECP, immediately prior to re-infusion, this thesis sought to determine the cytokine profile of cells, where early apoptosis had previously been observed (Chapter 3, Bladon and Taylor, 1999b). To closely represent the environmental conditions present in the buffy coat bag prior to re-infusion, the T cells and monocytes were not isolated prior to culture. The pro-inflammatory cytokines; IL6, IL12, IFNγ and TNFα were chosen for evaluation. IL6, IFNγ and TNFα because of their close association with the immunmodulatory processes observed post ECP and their conflicting reports in the literature. IL12 was chosen because recently regression of CTCL lesion has been achieved using rhIL12 (Rook *et al.*, 1999b). In addition, IL12 has been closely linked to the progression of GvHD, possibly as a consequence of the action of IL12 on IFNγ (Yang, 2000).

ECP had no immediate effect on IL6, IL12 or IFNγ secretion from monocytes (Chapter 8, Bladon and Taylor, 2002e). Although IL6 has been linked to the severity of acute GvHD, the presence of similar IL6 levels in transplant with and without GvHD implies that IL6 does not directly mediate the tissue damage seen in this pathology (Abdullah *et al.*, 1997). *Ex-vivo* assessment of ECP treated monocytes did, however, demonstrated a fall in the number of TNFα secreting monocytes prior to re-infusion (Chapter 8, Bladon and Taylor, 2002e). Results which are not in conflict to those of HSP expression (Chapter 7, Bladon and Taylor, 2002c). Of further interest, the number of TNFα secreting T cells was also reduced following ECP when tested *ex-vivo* (Chapter 8, Bladon and Taylor, 2002e) The down-regulation of TNFα expression seems to indicate that TNFα, at this early stage, is not responsible to the removal of CTCL lymphoma cells as suggested by Vowel (Vowels *et al.*, 1992). TNFα has been observed as a barometer of response to IL2 immunotherapy in malignant lymphomas. Both

HD and NHL patients demonstrate higher TNFα than normals. More over, clinical stage III and IV demonstrate TNFα levels higher than those of stage I-II. Using IL2 immunotherapy, stabilising of the conditions was associated with a reduction in TNFα, whilst TNFα levels increased in patients whose disease progressed (Lissoni *et al.,* 1999). The enhancement of the anti-tumour response by the apparent release of IFNγ (Hori *et al.,* 1987) is also questionable because immediately post ECP, IFNγ-secreting T cells are reduced in number (Bladon and Taylor 2002e). The skewing toward a Th1 type cytokine profile following ECP is thought responsible for the reversal of the Th1/Th2 ratio, as observed in ECP 'responders' (Tokura *et al.,* 1999). However excessive TNFα and IFNγ have also been implicated in the pathology of other conditions treated using 8MOP/UVA (Ochs *et al.,* 1996). In addition to a Th1 skewing cytokine profile, Psoriasis patients demonstrate overproduction of cytokines by peripheral blood monocytes, including TNFα (Okubo and Koga, 1998). In acute GvHD, TNFα and IFNγ levels are all increased, whilst elevated levels of TNFα are also observed in chronic GvHD (Imamura *et al.,* 1994). IFNγ has been demonstrated in the skin biopsies of patients with cGvHD (Ochs *et al.,* 1996), prompting the suggestion that in cGvHD IFNγ is the predominant cytokine (Parkman, 1998). Suppression of these inflammatory cytokines has been effective in the treatment of aGvHD and cGvHD. Anti TNFα antibodies have been used in the prevention of cutaneous and gastrointestinal lesions during aGvHD (Piguet *et al* 1987), whilst transplanted mice whose cGvHD was tolerated demonstrated statistical reductions in TNFα and IFNγ levels (Nagler *et al.,* 2000). Of further interest this thesis noted how the levels of TNFα and IFNγ secreting T cells were higher in the GvHD patients pre ECP, indicating that these two cytokines may have a prominent role in the aetiology of chronic GvHD (Chapter 8, Bladon and Taylor, 2002e). The early reduction in TNFα and IFNγ secreting mononuclear cells following ECP may have a direct role in reducing the pathologic progress of cGvHD. In 22 cases of steroid refractory chronic GvHD, there was statistically significant and progressive reduction in cutaneous disease at 3, 6 and 9 months with a concurrent significant reduction in steroid usage (Taylor,

2003). UVA-1 therapy has been associated with a marked reduction in skin-infiltrating T cells and down-regulation of IFNγ in lesional atopic skin (Grewe *et al.,* 1998). UVA-1 therapy, where the shorter wavelength of UVA is emitted, has also been demonstrated to very effective in inducing immediate type apoptosis (Godar, 1999). It is interesting to speculate whether the cohort of early apoptotic lymphocytes and IFNγ reduction observed very early in the ECP process is as a consequence of UVA-1 like mechanisms. ECP therapy may therefore utilise the processing of apoptotic cells to remove malignant and autoreactive T cells of CTCL and GvHD respectively, whilst simultaneously reducing the levels of pro-inflammatory mediators linked primarily to the pathology of GvHD, but also linked to malignant lymphoma prognosis.

In addition to their proposed role at macrophage recognition sites, apoptotic cells expressing CD10 are thought to aid in limiting potential inflammatory responses induced by the apoptotic cell (Cutrona *et al.,* 1999). CD10 is able to hydrolyse a variety of biologically active peptides, including growth and chemotactic factors (Mumford *et al.,* 1981; Connelly *et al.,* 1985). Germinal centre B cells upregulate CD10 on apoptosis induction (Ingvarsson *et al.,* 1999.), whilst apoptotic T cells *in-vivo* demonstrate CD10 (Cutrona *et al.,* 1999). However the expression of CD10 by apoptotic lymphocytes occurs 'late' in the apoptotic process with very 'weak' expression (Bladon and Taylor, 2000). It seems unlikely that CD10 plays any role in the cytokine processes that are linked to ECP.

10.7. ECP induced immunomodulation

The processing of antigens by APCs ultimately leads to the presentation to T cells and immune recognition (Liu, 1992). When APCs acquire antigens from apoptotic cells a cytotoxic response against similar antigens is induced (Albert *et al.,* 1998; Henry *et al.,* 1999). Unlike ECP-treated T cells, exposure of monocytes to ECP does not induce apoptosis (Yoo *et al.,* 1996 Tambur *et al.,* 2000). Conversely they demonstrate an increased avidity for the phagocytosis of apoptotic T cells (Yoo *et al.,* 1996). A cellular process observed in cytospin

prepared from *in-vitro* cell cultures 24 hours post ECP (Chapter 9, Bladon and Taylor 2003b). Following ECP, the monocytes demonstrate activation markers, the number of CD36 expressing monocytes increasing significantly (Fimiani *et al.,* 1997). CD36 has been implicated in the recognition process for apoptotic cells (Savill *et al.,* 1993). Monocytes/macrophages/dendritic cells expressing CD36 are capable of activating autologous T cells, mainly CD8+T cells (Shen *et al.,* 1983; Pimpinelli *et al.,* 1991). Of further interest, the enhanced number of CD36 expressing cells is detectable during the ECP process, indicating the immediate nature of this response (Fimiani *et al.,* 1997). The processing of antigens on the malignant apoptotic T cells post ECP, by APCs, may trigger the immune recognition of other non-treated malignant T cells of the same clone (Rook *et al.,* 1999; Berger *et al.,* 2001). The very early increase in CD36 expressing cells may indicate that this process may parallel the very early induction of apoptotic lymphocytes, detectable prior to re-infusion (Chapter 3, Bladon and Taylor, 1999b). Phagocytosis of apoptotic cells by monocytes accelerates the monocyte maturation process (Rovere *et al.,* 1998). Indicating the rapid conversion of monocytes to professional APCs, following ECP exposure and apoptotic cell uptake. A process observed by Berger *et al*, in the buffy coat collection bags stored for 24 hours (Berger *et al.,* 2001). These mechanism would lead to a significant expansion of CD8+ T cells and clinical relevant anti tumour responses (Heald *et al.,* 1992).

Further evidence for the immunomodulatory mechanism of ECP is highlighted by the different effects observed following the early apoptosis induced in lymphocytes exposed to ECP and some chemotherapy regimens (Bladon and Taylor, 2002d). Both ECP and chemotherapy induce a very early induction of lymphoid apoptosis that demonstrate very similar characteristics. Both treatment modalities induce apoptosis independent of p53 and involve dysregulation of Bax and pronounced changes to mitochondrial function (Chapter 3, Bladon and Taylor, 1999b; Stahnke *et al.,* 2001; Chapter 5, Bladon and Taylor, 2002). However in contrast to ECP, where CTCL ‘responders’ demonstrate a specific reduction in the malignant CD4+ clone (Zouboulis *et al.,*

1998, Taylor, 2003), chemotherapy induces a pan deletion of T cells (Stahnke *et al.*, 2001). This difference may be a consequence of the anti-clonal immunity, thought to be responsible for the removal of clonal T cells, without direct ECP exposure (Edelson *et al.*, 1994; Rook *et al.*, 1999). A mechanism which may be dependent on the processing and presentation of ECP-induced apoptotic lymphocytes by APCs *in-vivo* (Hanlon *et al.*, 1998). Supplementation of this mechanism may involve the processing of apoptotic lymphocytes by monocytes/dendritic cells activated by ECP exposure. A role which Berger *et al* speculate could be further harnessed by delaying the re-infusion of the ECP exposed leucocytes and hence allowing the processing of the apoptotic cells by the activated monocytes to begin prior to re-infusion (Berger *et al.*, 2001).

When antigens are processed by APCs, the presence of co-stimulatory signals on the APCs is required to avoid specific T cell unresponsiveness called anergy (Rattis *et al.*, 1998). CD54 (ICAM-1), CD80 and CD86, are important co-stimulatory molecule in this process (Iwai *et al.*, 1999; Hubbard *et al.*, 2000). CD80 and CD86 modulate the activation of Th1 and Th2 cells respectively (Kuchroo *et al.*, 1995) Previously the treatment of monocytes by UV has demonstrated a marked suppression of CD54 expression and reduced inducible expression of CD80 and CD86 (Hertl *et al.*, 1991; Fujihara *et al.*, 1996; De Luca *et al.*, 1997). Comparative studies of pre- and post-ECP monocytes, tested at 0, 2, 4 and 24 hours post ECP, show no significant difference in CD54 expression at each testing stage. CD86 and IFNγ-induced CD80 expression also remains unchanged following ECP. These results indicate that ECP treated monocytes are not compromised in the antigen presentation processes dependent on these co-stimulatory mechanisms. Retaining the antigens necessary for the processing of apoptotic cells and the induction of a related immunomodulatory response (Chapter 9, Bladon and Taylor 2003). The discrepancy between this thesis and that of previous workers may, in part, be attributed to the type and strength of UV used. Loss of CD54 on treated monocytes has been observed following exposure to UVB radiation (De Luca *et al.*, 1997). UVB also induces the loss of inducible CD80 and CD86 expression

(Fujihara *et al.,* 1996) and apoptosis induction in APCs (Rattis *et al.,* 1998). ECP-treated monocytes are resistant to all these processes and as such the maintenance of the co-stimulatory antigens may be a consequence of the differing effects of UVB. UVA irradiation reduces the ability of Langerhans cells to present antigens to T cells. A process associated with a loss of CD54, CD80 and CD86 expression (Iwai *et al.,* 1999). However this effect is seen at UVA doses in excess of those used for ECP. In addition, the ability of ECP treated APCs to increase MHC class II expression and to induce proliferation of alloreactive lymphocytes (Berger *et al.,* 2001) is the opposite of responses observed in APCs following other photodynamic therapies (Gruner *et al.,* 1986; King *et al.,* 1999). These observations may indicate that ECP's effect on monocytes is more subtle, leading to fundamentally different response to that observed for other UV therapies.

The manipulation of the immune system by ECP may involve inducing effects in both the mononuclear cells present in the peripheral circulation. The apoptotic lymphocytes induced by ECP may, in part, be processed by the activated and immunologically competent monocytes. A procedure, which may lead to the alloreactive T cells, thought to be responsible for the immunomodulatory effect of ECP (Perez *et al.,* 1989; Edelson *et al.,* 1994). This mechanism would require monocytes that retain the relevant capabilities to perform this task. The retention of co-stimulatory molecules, in addition to a resistance to apoptosis induction and an active phenotype indicates this process is possible.

10.8. Summary

In conclusion, ECP induces extensive apoptosis in treated lymphocytes. Early apoptosis is observed immediately post ECP. The majority of exposed lymphocytes are apoptotic by 48 hours post ECP. Up-regulation of tumour antigens and the immediate and sustained nature of the immunological challenge may lead to the anti-clonotypic response observed. Further to these observations, down-regulation of pro-inflammatory cytokines may be beneficial

in stabilising some of the inflammatory pathology often observed in some conditions, treated using ECP, such as GvHD. The additional effects of ECP on monocytes may direct the design of the next generation of ECP machines. ECP activates monocytes and subsequently assists in the conversion toward competent APCs capable of processing the apoptotic T cells induced by the ECP system. Increasing the number of cells taking part in this reaction, prior to re-infusion, may enhance the immunomodulatory process of ECP. HSPs may also play a part in ECP's future. Although no enhancement of HSPs was observed in treated cells, the HSPs may act as carrier proteins for tumour antigens within the cell. An extension of the ECP process may involve utilising this process by exposing the collected cells to a short hyperthermic shock prior to apoptosis induction and re-infusion. However this process would involve several modifications to the ECP system to ensure safety and efficacy. Alternative groups have also considered to the use of UVA-1 as the UV source within the ECP system (Krutmann and Morita, 1999). This would have the effect of inducing almost complete apoptosis of the lymphocyte population prior to re-infusion. The effect this would have on monocytes is, however, unknown.

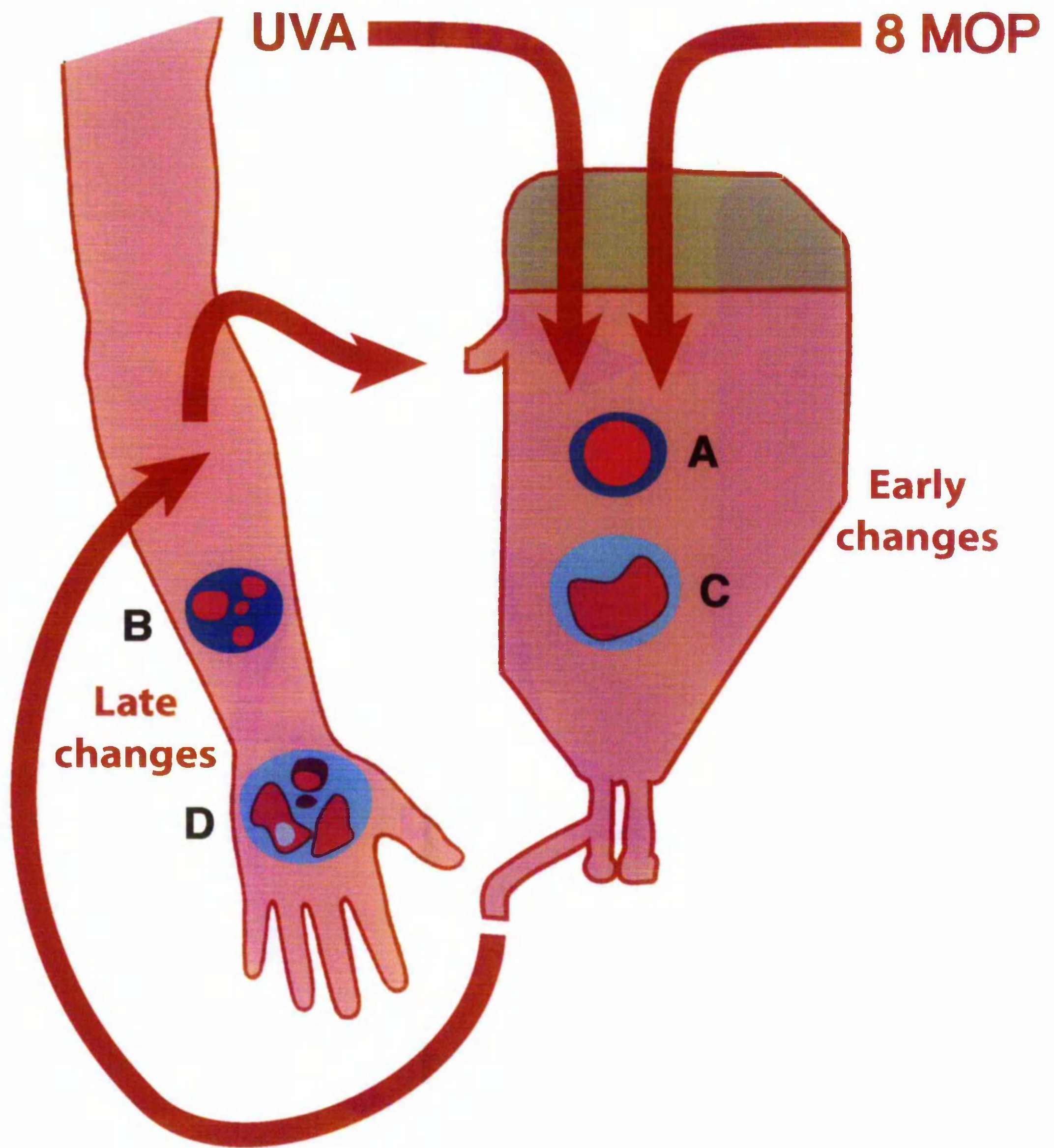
Figure 10.8.1 Early and late cellular changes observed following extracoporeal photopheresis

A) Following exposure to UVA/8-MOP, the lymphocytes begin to demonstrate early apoptotic changes, including a depolarisation of the mitochondrial transmembrane potential and a fall in the Bcl-2/Bax ratio. Changes, which may be responsible for the apoptotic markers observed, most important of which is phosphatidlyserine (PS) externalisation. PS exposure acts as a target antigen for macrophages. During the early stage post ECP, T cells down-regulate expression of pro-inflammatory cytokine.

B) In cell cultures 24 and 48 hours post ECP the lymphocytes begin to demonstrate morphological changes, including apoptotic body formation. However, *in-vivo* this change may never be observed due to earlier phagocytosis by antigen-presenting cells (See D). Marked caspase activation and significant levels of Fas ligation are detectable at this later testing stages.

C) Monocytes are resistant to ECP-induced apoptosis and unlike other UV therapies, retain the co-stimulatory markers required for presenting processed antigens. TNFα expression, within the monocyte population, is reduced in the pre re-infusion period following ECP.

D) Phagocytosis of apoptotic lymphocytes is observed as early as 6 hours post ECP, when co-culturing ECP treated lymphocytes and monocytes. However a marked increase in phagocytosis is observed 24 and 48 hours post ECP. The processing of apoptotic T cells, is able to induce an anti-clonal immune response, capable of removing non-treated T cells of the same clone.


UVA
8 MOP
A
Early changes
C
B
Late changes
D

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Cutaneous Biology

Extracorporeal photopheresis in cutaneous T-cell lymphoma and graft-versus-host disease induces both immediate and progressive apoptotic processes

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Summary

Background Extracorporeal photopheresis (ECP) therapy is used in the treatment of many T-cell-mediated conditions including cutaneous T-cell lymphoma and graft-versus-host disease and involves the reinfusion of a patient's own white cells following exposure to 8-methoxypsoralen and ultraviolet A. ECP has been demonstrated to induce significant levels of apoptosis in treated lymphocytes. Previous work has highlighted the importance of mitochondria and the caspase cascade in the regulation and execution of apoptosis and, more recently, a functional role for CD10 has been proposed for apoptotic lymphoid cells *in vivo*.

Objectives To determine the effect of ECP on phosphatidylserine (PS) exposure, mitochondrial function, caspase activation and CD10 expression of treated lymphocytes.

Methods Lymphocytes were tested pre-ECP and at several stages post-ECP for changes to PS, mitochondrial transmembrane potential ($\Delta\psi_m$), activated caspases and CD10 expression.

Results Early apoptosis induced a disruption in $\Delta\psi_m$, while caspase activation was not observed until 24 h post-ECP. CD10 expression was very weak and 'late' in the apoptotic process.

Conclusions The early induction of apoptosis by ECP involves mitochondrial dysfunction, while later apoptosis is associated with the activation of caspases. CD10 expression was very weak and 'late', preceded by a strong PS exposure. These apoptotic processes, *in vivo*, would induce the immediate and progressive phagocytosis of the majority of ECP-treated lymphocytes within 48 h.

Key words: apoptosis, caspases, CD10, mitochondrial transmembrane potential, photopheresis

Extracorporeal photopheresis (ECP) is a treatment by which leucopheresed cells are exposed to 8-methoxypsoralen (8-MOP) and ultraviolet (UV) A radiation, followed by reinfusion.[1] Primarily utilized for the treatment of cutaneous T-cell lymphoma (CTCL),[1] ECP has also been shown to be of clinical benefit in the treatment of other T-cell-mediated conditions, including graft-versus-host disease (GVHD).[2,3] Although the mechanism of action of ECP has not been fully elucidated, ECP has been demonstrated to induce apoptosis in treated lymphocytes,[4,5] with significant numbers identified *ex vivo*, before reinfusion.[6] Because ECP-treated cells are reinfused immediately after exposure, we were interested to identify the apoptotic processes at and after this reinfusion process.

The mitochondria have long been considered to be responsible for the bulk of the cells' energy production.[7] Although necrotic cells demonstrate swollen mitochondria, initial studies of early apoptosis reported no marked changes to the morphology of mitochondria.[8] However, more recently, electron microscopy has demonstrated profound changes in the morphology of mitochondria in apoptotic thymocytes,[9] while changes to the mitochondrial function of the apoptotic cell have

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included a disruption in the mitochondrial transmembrane potential ($\Delta\psi_m$).[9–11]

Caspases (cysteine aspartate-specific proteinases) are a family of related proteinases synthesized as latent zymogens (procaspases).[12] At least 14 different caspases have been described in mammals.[13] After induction of apoptosis, activation of 'early' caspases triggers further activation of 'downstream' caspases, the resultant cascade effect leading to amplification of the apoptotic signal.[7,14] The caspases are capable of opposing cellular attempts to stop apoptosis and ultimately act as the final executioner.[14]

The reduction in $\Delta\psi_m$ and the activation of the caspases are irreversible processes and indicative of certain cellular demise.[11,12] They are also thought to be among the earliest indicators of the apoptotic process, preceding the point at which cells are recognized *in vivo* as apoptotic by external phosphatidylserine (PS) expression and are phagocytosed.[15,16]

Peripheral lymphocyte expression of membrane CD10 is most usually associated with cells demonstrating the common acute lymphoblastic leukaemia antigen (CALLA) phenotype.[17] However, more recently, induction of CD10 expression on lymphocytes has been linked to growth arrest[18] and apoptosis.[19] Although the function of the CD10 expressed by the apoptotic lymphoid cells is not fully understood, it has been postulated that its presence, *in vivo*, may be to limit the level of inflammation at the site of apoptosis, or to act as a macrophage target antigen.[19]

This study involved testing lymphocytes from various stages of the ECP process for annexin V/propidium iodide (PI), $\Delta\psi_m$, activated caspases and CD10 expression, to determine if these processes are involved in ECP-induced apoptosis and, if so, at what stage.

Patients and methods

Patients

Samples were obtained from 12 patients receiving ECP treatment (six with CTCL, five with GVHD and one with scleroedema). Diagnosis of each condition was based on established histological and immunological criteria. Informed consent was obtained from all patients and local ethical approval was granted for the study.

Photopheresis treatment

ECP cycles were performed using the XTS (Therakos Europe, Bracknell, Berks, U.K.). The treatment involves harvesting leucocytes (buffy coat) using a 'collect and elutriation' six-cycle apheresis system, which includes a final 'concentration' step. This system optimizes the haematocrit (Hct) and increases the white cell/volume ratio in the buffy coat bag (BCB). The cells collected are exposed to a 1·5-J cm^{-2} UVA radiation source, commencing from the end of the concentration step. The exposure time is dependent on the volume and Hct of the buffy coat (~15–60 min). After irradiation, the treated cells are reinfused. This process in then repeated on the following day, patients returning either 2- or 4-weekly. For patients with CTCL and scleroedema the 8-MOP is taken orally 90 min before the start of the ECP. For patients with GVHD the 8-MOP (Uvadex; Ben Venue Laboratories, Bedford, OH, U.S.A.) is injected directly into the BCB immediately prior to UV exposure. Buffy coat 8-MOP levels were tested in the patients with CTCL and scleroedema to ensure that they all achieved a concentration of 100 ng mL^{-1}.

Sampling and preparation of lymphocytes

Heparinized blood (4·5 mL) was taken from patients immediately before ECP and from the incubated BCB just prior to reinfusion. The peripheral blood mononuclear cells were immediately separated by adding 4·5 mL of isotonic saline and centrifuging on a density gradient (Lymphoprep; Nycomed, Oslo, Norway). The isolated cells were then washed once with 8 mL of phosphate-buffered saline (PBS). The pre-ECP and post-ECP cells for immediate testing were added to their appropriate test buffer to give a final cell count of 0·5–1·0 × 10^6 mL^{-1}. Pre-ECP and post-ECP cells for testing at 6, 24 and 48 h were added to RPMI medium containing 10% fetal calf serum, 0·14 mg mL^{-1} streptomycin, 50 μg mL^{-1} vancomycin and 1% glutamine to give a final count of 1·0–2·0 × 10^6 mL^{-1}. Before testing, cultured cells were washed in PBS.

Lymphocyte identification

Using cells added to 1·0 mL PBS, identification of lymphocytes used a forward scatter (FS) vs. side scatter (SS) plot. Allowances were made for the reduced FS and increased SS seen in the 'late apoptotic' lymphocytes. Staining with 10 μL of R-phycoerythrin-conjugated CD14 (Dako, Ely, Cambs, U.K.) and 10 μL of fluorescein isothiocyanate (FITC)-conjugated CD45 (Dako) excluded monocytes and debris, respectively. Relevant isotype controls (Dako) were used for both anti-CD14 and anti-CD45.

Identification of apoptosis and secondary necrosis

Apoptosis and secondary necrosis were determined using annexin V/PI (Immunotech, High Wycombe, Bucks, U.K.). Annexin V detects the externalization of PS groups ('flip-flop') associated with apoptosis, while PI identifies secondary necrotic cells whose membrane integrity is lost, allowing PI to enter the cell. Annexin V/PI determination was according to the manufacturer's recommendations. The washed cells were added to 980 μL of cold 'working strength' binding buffer to give a count of $0{\cdot}5$–$1{\cdot}0 \times 10^6$ mL^{-1} and placed on ice; 10 μL of 'working strength' annexin V and 10 μL of PI were then added, and incubation was for 10 min in the dark. Cells were processed immediately.

Analysis of mitochondrial transmembrane potential

$\Delta\psi_m$ was determined using two different methods: rhodamine 123 (Sigma, Aldrich Chemical Co., Gillingham, Dorset, U.K.) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1; Cambridge Bioscience, Cambridge, U.K.). Rhodamine 123 accumulates in the mitochondrial matrix under the influence of the $\Delta\psi_m$. A loss in the membrane potential-related fluorescence is recorded using the FL-1 channel of the flow cytometer. JC-1, however, has a unique property of forming J-aggregates under high $\Delta\psi_m$, and these fluoresce red (FL-2). When depolarization of the $\Delta\psi_m$ occurs, only the monomeric form, which fluoresces green (FL-1), is present. Using JC-1, depolarization of the $\Delta\psi_m$ can therefore be determined using flow cytometry, by the transition from red to green. For rhodamine 123 determination, cells were diluted in 1 mL PBS to give a count of $0{\cdot}5$–$1{\cdot}0 \times 10^6$ mL^{-1}. Ten microlitres of a stock solution of rhodamine 123 was added to give a final concentration of 50 ng mL^{-1}. The cells were then incubated in a sealed tube, in the dark, for 15 min at 37 °C. The cells were then washed in PBS and analysed on the flow cytometer using the FL-1 channel. For JC-1, cells were again diluted in 1 mL PBS to give a count of $0{\cdot}5$–$1{\cdot}0 \times 10^6$ mL^{-1}. One microlitre of stock JC-1 was added to give a final concentration of 1 μmol L^{-1}. The cells were then incubated in a sealed tube, in the dark, for 15 min at 37 °C. The cells were then washed in PBS and analysed on the flow cytometer, using both the FL-1 and FL-2 channels. Control experiments were performed using cells diluted in 5 μmol L^{-1} carbonyl cyanide *m*-chlorophenylhydrazone (mC1CCP; Sigma), which is an uncoupling agent that abolishes the $\Delta\psi_m$.

Identification of caspase activation

Caspase activity was determined using the Caspatag™ fluorescein caspase activity kit (Intergen Co., Oxford, U.K.). The system involves the detection of several activated caspases using the FITC-conjugated activated caspase inhibitor carboxyfluorescein-benzyloxycarbonyl-valine-alanine-aspartic acid-fluoromethyl-ketone (FAM-VAD-FMK). Determination of caspase activation was as according to the manufacturer's recommendations. Separated cells were diluted in 300 μL of RPMI medium to give a count of $0{\cdot}5$–$1{\cdot}0 \times 10^6$ mL^{-1}. Eight microlitres of PBS was added to 2 μL of a 150 × stock solution of FAM-VAD-FMK and the resultant 10 μL of 30 × stock solution was added to the cell suspension. The tube was sealed and incubated in the dark, at 37 °C for 1 h. The cell suspension was washed twice with 2 mL of 1 × working dilution wash buffer. The cells were then resuspended in 1 mL of 1 × working dilution wash buffer and processed through the flow cytometer. Caspase activity was assessed using the FL-1 channel.

Identification of CD10

In two tubes, cells were diluted in 1 mL of PBS to give a final count of $0{\cdot}5$–$1{\cdot}0 \times 10^6$ mL^{-1}. To tube 1, 10 μL of FITC-conjugated isotype control (Dako) was added. To tube 2, 10 μL of FITC-conjugated anti-CD10 (Dako) was added. The tubes were incubated in the dark for 15 min and processed immediately.

Flow cytometry

Cells were processed through a Dako Galaxy flow cytometer. Alignment and fluorescence were standardized using Alignment Beads (Molecular Probes) and Fluorospheres (Dako). A minimum of 10 000 events was gathered for each test. For determination of apoptosis and secondary necrosis, 'region gates' were set on the 'dot plot' of the pre-ECP sample. These same region gates were then used to assess the further samples processed at the post-ECP stages. The same gating procedure was used in the assessment of activated caspases. For the determination of $\Delta\psi_m$, gates were set using the pre-ECP sample and control experiments using mC1CCP, these same gates assessing the post-ECP samples. For CD10 expression, the 'region gates' were set using the isotype control.

Cytospin preparation

Cytospins were performed on the 6-, 24- and 48-h post-ECP cell cultures. Each cell suspension was diluted in culture medium to give a count of $0{\cdot}2 \times 10^6$ mL^{-1}. This diluted cell suspension (0·5 mL) was added to two prepared cytospin chambers. The chambers were spun at 30 *g* for 10 min in a Cyto-Tek® cytospin centrifuge. The cytospins were allowed to dry, fixed for 15 min in methanol, and stained using May–Grunwald–Giemsa stain.

Statistical analysis

Statistical analysis was performed using paired *t*-tests. $P < 0{\cdot}05$ was regarded as significant.

Results

Statistical analysis on the patient with scleroedema was not possible because only one patient was tested. However, because our previous study of apoptosis induction by ECP therapy demonstrated similar findings for CTCL and scleroedema lymphocytes,[6] the data for the scleroedema patient were assessed with the CTCL results. The mean ± SEM percentage of lymphocytes demonstrating a positive expression of annexin V, PI, CD10, rhodamine 123, JC-1 and activated caspases for both patient groups, namely CTCL and scleroedema, and GVHD, are demonstrated in Figure 1(a–f).

For the post-ECP samples the 24-h and 48-h samples were subdivided into two groups: the post-ECP group refers to the results obtained when the whole lymphocyte bitmap was assessed, while the post-ECP 'late apoptotic' group refers to the lymphocytes demonstrating a reduced FS and increased SS, size and density changes typical of 'late' apoptotic cells (see Fig. 2). The pre-ECP samples were not separated in this way, due to the lack of apoptotic cells that demonstrated this distinctive change in light scattering properties.

At each testing stage the post-ECP cells showed significantly higher levels of apoptosis, as determined by annexin V expression, when compared with the pre-ECP cells ($P < 0{\cdot}05$). The levels of apoptotic lymphocytes post-ECP increased rapidly, to include the majority of treated cells by 48 h post-ECP. Figure 3 shows the annexin V/PI expression of lymphocytes pre-ECP (*ex vivo* and 48 h) and post-ECP (*ex vivo* and in the post-ECP 'late apoptotic' group 48 h post-ECP).

Comparison of all stages in both patient groups pre-ECP and post-ECP also demonstrated a significant disruption in $\Delta\psi_m$, as detected by rhodamine 123 and JC-1, in addition to significant increases in activated caspases and CD10 expression in cells exposed to ECP ($P < 0{\cdot}05$). However, the percentage of lymphocytes demonstrating each of these apoptosis-associated markers varied in terms of number of expressing cells and the stage of ECP cycle when the apoptotic changes occurred. For both patient groups significant increases in annexin V and JC-1 expression were seen at all stages from 0 h post-ECP onwards, while significant increases in rhodamine 123 and activated caspases were detected from 6 h post-ECP and significant levels of PI and CD10 positivity were not observed until 24 h post-ECP.

The number of cells demonstrating a disruption in $\Delta\psi_m$ increased at each stage of testing, but the number of positive cells differed for each of the stains used. Fewer cells were detected using rhodamine 123 than were detected using JC-1, at all the post-ECP stages. The levels of JC-1-positive lymphocytes were comparable with annexin V expression at each stage of the ECP process.

The expression of activated caspases occurred later than both the initial exposure of PS and the disruption in $\Delta\psi_m$. Although some activity was observed in the 6-h post-ECP sample, marked increases in activity were not observed until 24 h post-ECP. The activated caspases appeared at a point after PS exposure, but before initial expression of PI. However, in the 48-h sample, lymphocytes demonstrating activated caspases and PS were comparable in number.

The expression of CD10 by the apoptotic lymphocytes was very weak, and appeared relatively 'late' in the apoptotic process. Only the CD10 expression in the 48-h post-ECP 'late apoptotic' group showed a significant difference between the CTCL/scleroedema and GVHD patient groups.

Cytospin preparations of the 6-, 24- and 48-h post-ECP stages, stained with May–Grunwald–Giemsa, did not demonstrate the presence of apoptotic morphological features until the 24-h post-ECP samples. Their appearance and number increased in the 48-h samples.

Discussion

The enigma of ECP therapy is how damage to a small proportion of abnormal lymphocytes circulating in the peripheral circulation induces a distant response in untreated cutaneous lymphoma cells. Edelson *et al.* suggested that the effect of ECP on malignant cells in the treated buffy coat subsequently modulates the immune system to recognize non-treated, but clonal

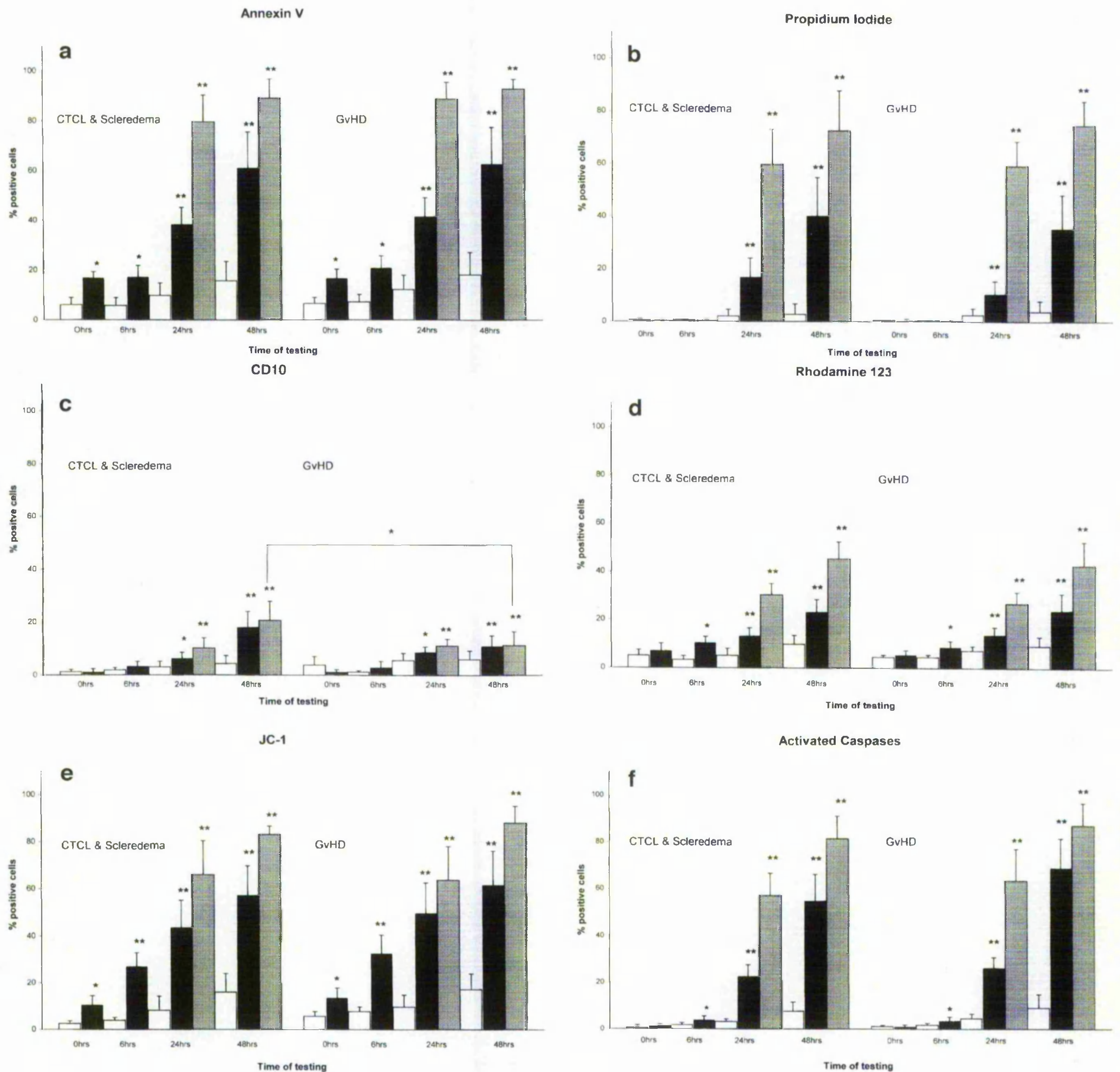

Figure 1. Mean ± SEM lymphocyte expression of (a) annexin V, (b) propidium iodide (PI), (c) CD10, (d) rhodamine 123, (e) 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1) and (f) activated caspases 0, 6, 24 and 48 h after sampling pre-extracorporeal photopheresis (ECP; white bars) and 0, 6, 24 and 48 h after sampling post-ECP (black bars). The post-ECP sample is subdivided at 24 and 48 h; the post-ECP group (black bars) refers to the whole lymphocyte population, while the post-ECP 'late apoptotic' group (spotted bars) refers to the lymphocytes with reduced forward scatter and increased side scatter (see Fig. 2). $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}005$ for each comparative stage pre- and post-ECP. For both patient groups significant increases in annexin V and JC-1 expression were detected immediately post-ECP, rhodamine 123 and activated caspases were observed at 6 h post-ECP, while significant levels of PI and CD10 expression were not detected until 24 h post-ECP. The only significant difference between the patient groups was in the level of CD10 expression in the 'late apoptotic' group 48 h post-ECP. CTCL, cutaneous T-cell lymphoma; GvHD, graft-versus-host disease.

disease.[20] Investigations into the process by which cells are modified by ECP have identified apoptosis induction in directly treated lymphocytes. Originally this process was described as a 'delayed-type' mechanism;[4,5] more recently, apoptotic lymphocytes have been detected immediately prior to reinfusion.[6] As one marker for CTCL is defective apoptosis rather than true proliferation,[21] this process may have a direct association. We

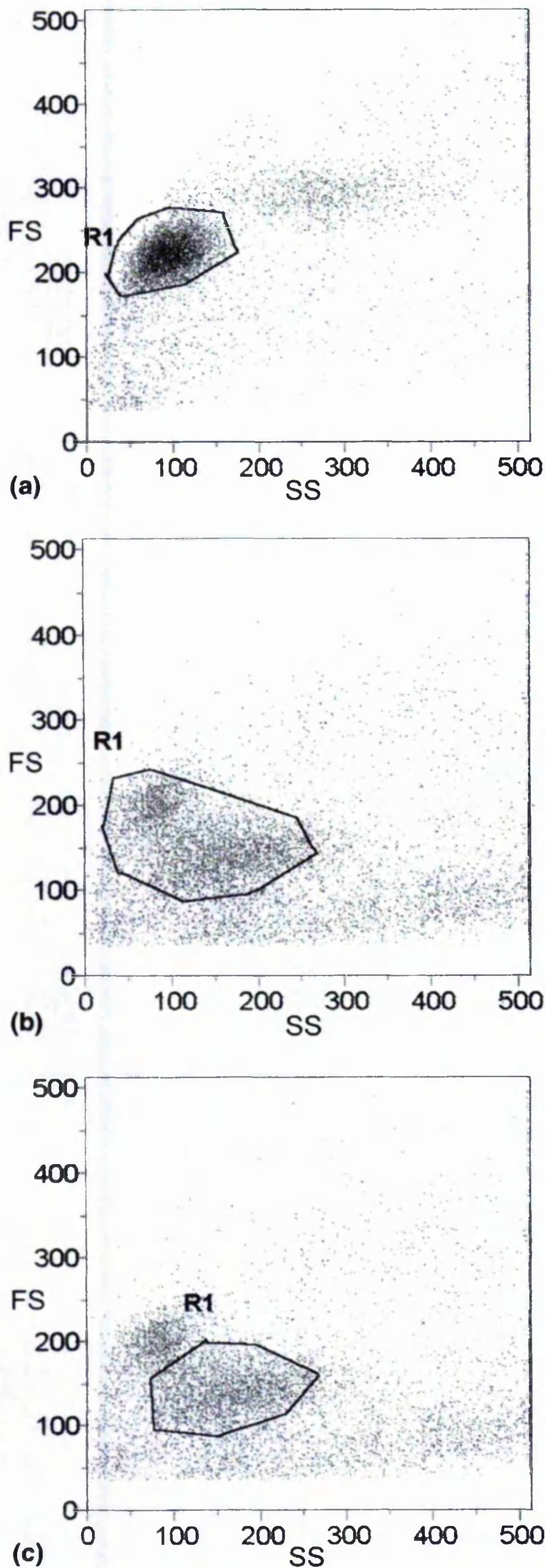

Figure 2. The different forward scatter (FS) and side scatter (SS) properties of lymphocytes treated by extracorporeal photopheresis (ECP). Panel (a) shows the typical small and tight bitmap of the normal and early apoptotic lymphocytes observed in all stages pre-ECP and in the 0- and 6-h post-ECP samples. The 24- and 48-h post-ECP samples, however, demonstrate an additional population showing a reduced FS and an increased SS. This population is referred to in the text and Figure 1 as the 'late apoptotic' group. In Figure 1, for the 24- and 48-h samples the post-ECP group (black bars) refers to all lymphocytes in both populations (panel b), while the post-ECP 'late apoptotic' group (spotted bars) refers only to the lymphocytes with reduced FS and increased SS (panel c).

were interested to identify the apoptotic processes occurring at and after reinfusion. Previous work has highlighted the importance of mitochondria and the caspase cascade in the regulation and execution of apoptosis,[7,14] and more recently a functional role for CD10 has been proposed for apoptotic lymphoid cells *in vivo*.[19]

The use of annexin V, in conjunction with other apoptosis determining systems, has recently identified significant levels of apoptosis in ECP-treated lymphocytes prior to reinfusion.[6] Using annexin V/PI in a cell culture system, we sought to determine the speed and level of lymphocyte apoptosis occurring *in vivo*, following re-infusion. ECP-induced apoptosis in treated lymphocytes was initiated prior to reinfusion, to include the majority of cells by 48 h (Fig. 1a). By 24 h, morphological and light scatter changes (reduced FS, increased SS) were identified, changes typical of 'later' apoptosis[8] (see Fig. 2).

The progressive exposure of PS on the membrane of the ECP-treated lymphocytes is particularly significant, as expression of PS on the membrane of an apoptotic lymphocyte, *in vivo*, acts as a target for recognition.[22,23] Because phagocytosis of PS-expressing apoptotic lymphocytes precedes loss of membrane integrity,[24] it is highly likely that the ECP-treated lymphocytes would be promptly processed *in vivo*, the process beginning immediately following reinfusion. This putative processing of early apoptotic lymphocytes is supported by the observation that ECP-treated monocytes do not undergo apoptosis.[4,25] On the contrary, photopheresis-treated monocytes demonstrate an increased ability to phagocytose apoptotic T cells.[4] This mechanism is further supported by the lack of detectable apoptotic lymphocytes, *in vivo*, in patients tested at 1, 6 and 24 h postreinfusion.[4,5]

Initial reports on mitochondrial changes during apoptosis noted no significant change in morphology or function.[8] However, profound alterations of mito-

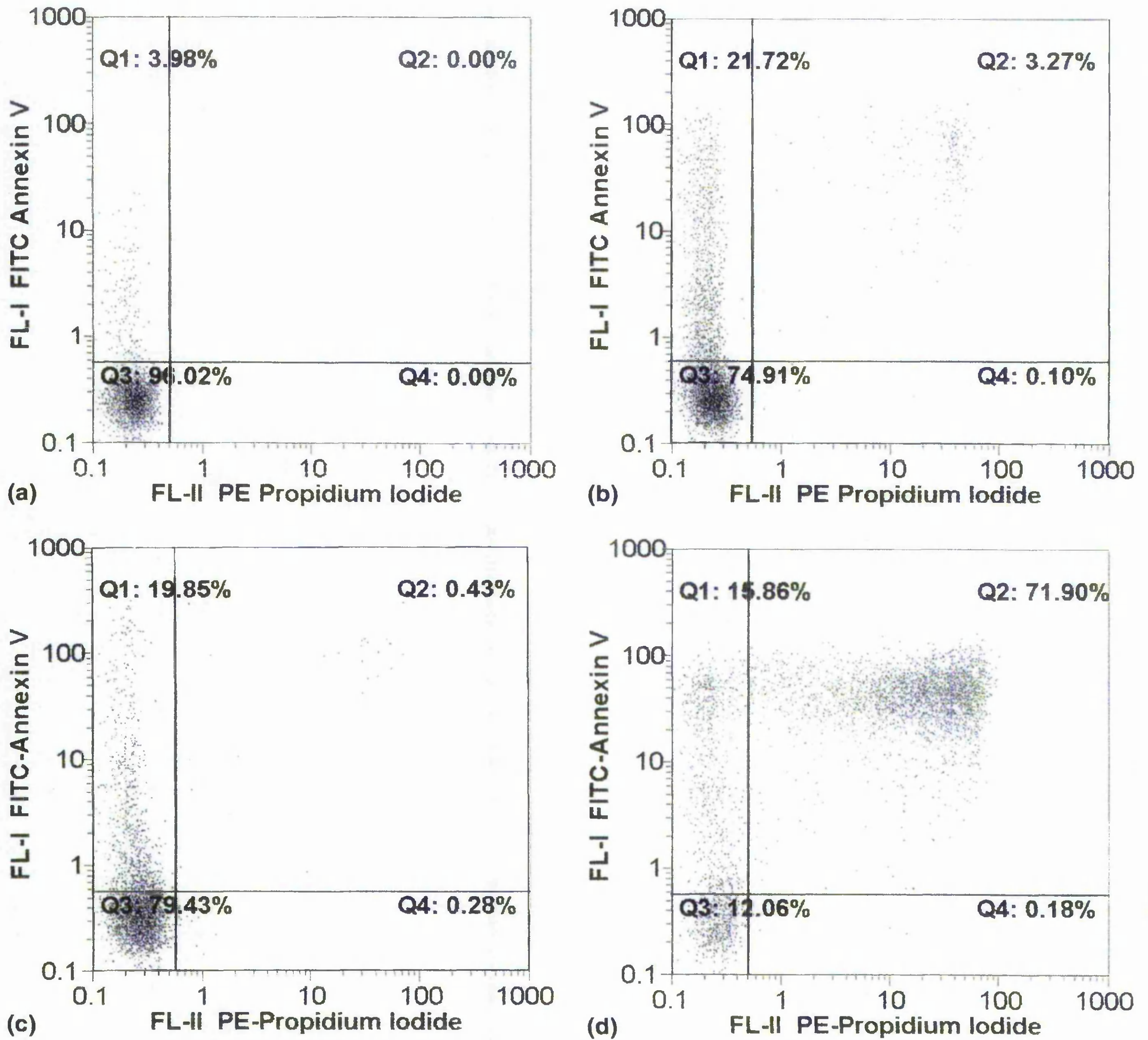

Figure 3. Annexin V/propidium iodide (PI) expression of lymphocytes tested pre-extracorporeal photopheresis (ECP; 0 and 48 h) and post-ECP (0 h and, in the 'late apoptotic' group, 48 h post-ECP). Panel (a), at 0 h pre-ECP, shows a small number of lymphocytes demonstrating exposure of phosphatidylserine (PS), determined by annexin V binding (see quadrant Q1). The level of spontaneous apoptosis in culture is shown in the 48-h pre-ECP sample (panel b). Immediately post-ECP (panel c) the number of PS-expressing lymphocytes increases, as observed in quadrant Q1. At 48 h post-ECP (panel d), most lymphocytes are now annexin V positive and are demonstrating the loss of membrane integrity, as detected by PI absorption, typical of secondary necrosis (see quadrant Q2). FITC, fluorescein isothiocyanate; PE, phycoerythrin.

chondrial ultrastructure have been observed recently.[9] Functional changes to the mitochondria of apoptotic cells have now also been described and include the uncoupling of electron transport from adenosine triphosphate (ATP) production, a reduced rate of mitochondrial protein transportation, and disruption of the mitochondrial inner transmembrane potential ($\Delta\psi_m$).[9–11,26]

Different apoptotic signals can disrupt the $\Delta\psi_m$ by initiating the opening of mitochondrial permeability transition pores (MPT), leading to the osmotic expansion of the mitochondrial matrix, eventual membrane rupture and subsequent release of apoptogenic molecules such as cytochrome *c*.[7,27] *In vivo*, the presence of a reduced $\Delta\psi_m$ denotes an irreversible stage of preprogrammed lymphocyte death.[11] The early detection of a

reduced $\Delta\psi_m$ in some cells may indicate that the mitochondria are the primary target during apoptosis.[28] Several stains have been utilized for the determination of $\Delta\psi_m$, including $DiOC_6$, rhodamine 123 and JC-1.[9–11]

In the present study, evaluation using rhodamine 123 and JC-1 demonstrated a disruption to the $\Delta\psi_m$ in the ECP-treated cells. Although the levels detected using rhodamine 123 were lower, this may be explained by the differing sensitivity of the stains. Rhodamine 123 shows a lower sensitivity to changes in $\Delta\psi_m$, possibly due to the presence of energy-independent rhodamine 123 binding sites. JC-1, conversely, does not seem to be affected in this way.[29] Using JC-1, significant levels of lymphocytes demonstrating a depolarized $\Delta\psi_m$ were detected very early, prior to reinfusion.

Previous categorization of UVA-induced apoptosis has termed the rapid induction of apoptosis as 'immediate type' apoptosis, a process associated with perturbation of mitochondrial membrane permeability.[30] We have previously demonstrated that the early apoptosis observed in ECP-treated lymphocytes demonstrated a reduction in the intracellular Bcl-2/Bax protein ratio.[31] Interestingly, Bcl-2 has been demonstrated to inhibit MPT in isolated mitochondria and in cells,[32] while a reduction in Bcl-2 expression in apoptotic lymphocytes is more usually associated with cells undergoing an 'immediate type' apoptotic process.[33] Also, co-operation between Bax and a component of the MPT, adenine nucleotide translocator, can cause channel formation in artificial membranes.[34]

Disruption to the $\Delta\psi_m$ and the exposure of PS appear to be intimately linked, and constitute a central event in early apoptosis.[16] Several speculatory theories link the process of PS exposure and a reduction in $\Delta\psi_m$. The rapid cessation of ATP synthesis by MPT can induce PS exposure, while the release of soluble protein(s) by the mitochondria undergoing formation of MPT may activate the lipid, scramblase, which is responsible for the 'flip-flop' and subsequent exposure of PS.[16]

The caspases are a family of proteinases whose initial activation triggers a cascade of further caspase activation, leading ultimately to the death of the cell.[7,14] Caspase activation seems to be a common intracellular effector pathway to which different apoptotic systems are in contact. Their activation is capable of opposing cellular attempts to stop apoptosis.[14] Measurement of several activated caspases was performed using the Caspatag™ system. A marked increase in caspase activation was not observed until 24 h post-ECP. However, the number of cells expressing activated caspases from this point rose dramatically, indicating significant caspase activity later in the ECP process. Previous studies of UVA-induced apoptosis have demonstrated similar findings, with the activation of the caspases thought to be triggered directly by the change in $\Delta\psi_m$. However, the time interval between these two events was only 1–4 h,[10] and not the 24 h we observed. This seems to indicate that the mechanism leading to PS exposure early in the ECP process is independent of caspase activation. Interestingly, ECP has been demonstrated to increase the expression of CD95 at 24 h post-ECP in the CD4+ lymphocytes of patients with systemic sclerosis.[5] Activation of the CD95 pathway initiates activation of the caspase cascade through FLICE (caspase 8).[35]

CD10 was originally discovered in haematopoietic cells and is most commonly associated with cells demonstrating the CALLA phenotype.[17] The functional role of CD10 in the expressing lymphoid cells of the haematopoietic system is, however, unclear.[19] CD10 has recently been shown to influence lymphoid cell proliferation and apoptosis.[18,19] It has been proposed that the presence of CD10 on apoptosing lymphoid cells promotes the cleavage of inflammatory or proinflammatory mediators released by the dying cell, limiting the level of inflammation at the site, or that it acts as a target antigen for macrophage recognition and phagocytosis.[19] As the mechanism of action of ECP includes apoptosis and upregulation of some cytokines, particularly interleukin (IL)-1,[2] IL-6 and tumour necrosis factor (TNF)-α,[36] it is possible that the expression of CD10 on the reinfused dying lymphocytes may have a direct influence on the *in vivo* ECP process. Apoptotic cells induced by ECP do express CD10, but the number of CD10-expressing cells is relatively low and they appear much later in the ECP process. Because CD10 expression appears after strong PS expression, phagocytosis and processing of the apoptotic cell would occur long before the emergence of CD10 could have any influence on any *in vivo* inflammatory response.[37]

These results seem to indicate a progressive process of lymphocyte apoptosis post-ECP, which is initiated immediately after the exposure of the separated cells to UVA. The initial apoptosis demonstrates the concurrent expression of PS and $\Delta\psi_m$ disruption, while later apoptosis demonstrates additional pronounced caspase activation, but only relatively low levels of cells showing very weak expression of CD10. Alternatively, ECP may involve the induction of PS exposure via a

dual process, early apoptosis involving disruption of $\Delta\psi_m$ and dysregulation of the Bcl-2/Bax ratio, with later apoptosis mediated by the activation of caspases, possibly involving the CD95 pathway.

Of greater interest is the effect of this immediate and sustained exposure of apoptotic lymphocytes on the immune system. For CTCL, perhaps the immediate and progressive processing of these apoptotic cells by the immune system is capable of reversing the defective apoptotic processes associated with this condition, a process complemented by the effect of ECP treatment on monocytes. ECP increases the numbers of circulating CD36+ monocytes and enhances the production of TNF-α; both processes are associated with antitumour cytotoxicity.[36,38] The induction of T-cell-mediated specific antitumour response by apoptotic leukaemic cells has recently been described.[39] In this model the immunogenicity of tumour cells was dependent upon the upregulation of heat shock proteins on the apoptotic cell membrane. Further work is required to determine whether similar changes to ECP-induced apoptotic cells contribute to the immunomodulatory effect.

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was obtained from all patients and normal controls and local ethical approval was granted for the study.

Photopheresis Treatment

Extracorporeal photopheresis cycles were performed using the XTS™ system (Therakos, UK). The treatment involves harvesting leucocytes (Buffy coat) using a "collect and elutriation" 6-cycle apheresis system, which includes a final "concentration" step. This system optimises the haematocrit (Hct) and increases the white cell/volume ratio in the buffy coat bag (BCB). The cells collected are exposed to 8 Methoxypsoralen (8-MOP) (UVADEX) (Ben Venue Laboratories, Bedford, OH) and a 1.5 J/cm^2 UVA radiation source, commencing from the end of the concentration step. The exposure time is dependent on the volume and Hct of the buffy coat (approximately 15–60 minutes). After irradiation, the treated cells are re-infused. This process in then repeated on the following day, patients returning monthly.

Sampling and Preparation of Peripheral Blood Mononuclear Cells (PBMCs)

Heparinised blood (2 × 4.5 ml) were taken from patients immediately before commencing ECP treatment (pre ECP) and from the incubated BCB (1 × 4.5 ml) just prior to re-infusion (post ECP). The peripheral blood mononuclear cells (PBMCs) were immediately separated by adding 4.5 ml of isotonic saline to each 4.5-ml sample and centrifuging on a density gradient (Lymphoprep, Nycomed, Norway). The isolated cells were then washed with phosphate buffered saline (PBS). The separated cells were added to RPMI medium (Biowhittaker, UK) containing 10% Fetal Calf Serum, 0.14 mg/ml Streptomycin, 50 μg/ml Vancomycin, and 1% Glutamine to give a final count of 1.0 to 2.0 × 10^6/ml and incubated in the dark at 37°C. The cells were immediately stimulated for 6 hours with 30 ng/ml Phorbol 12-Myristate 13-Acetate (PMA), 1 μg/ml Ionomycin, and 10 μg/ml Brefeldin A (Sigma, UK). Following stimulation, the cell culture was washed with PBS prior to testing.

Intracellular Cytokine Expression

To tubes containing 1 ml of PBS and a cell count of 2.0 × 10^6/ml were added 10 μl of PE-CY5-conjugated anti CD3 and 10 μl of PE-conjugated anti CD14 (Immunotech, UK). The cells were incubated in the dark, at room temperature, for 20 minutes. The cells were immediately washed with PBS and subsequently treated with a "fix and perm" commercial kit (Harlan Sera-lab, UK). At the permeabilisation stage, 20 μl of either FITC-conjugated anti IL6, IL12, IFNγ, or TNFα (R & D Systems, UK) was added. After a further 15-min room-temperature incubation in the dark, the cells were washed in PBS and processed. Appropriate isotype controls were performed.

Flow Cytometry

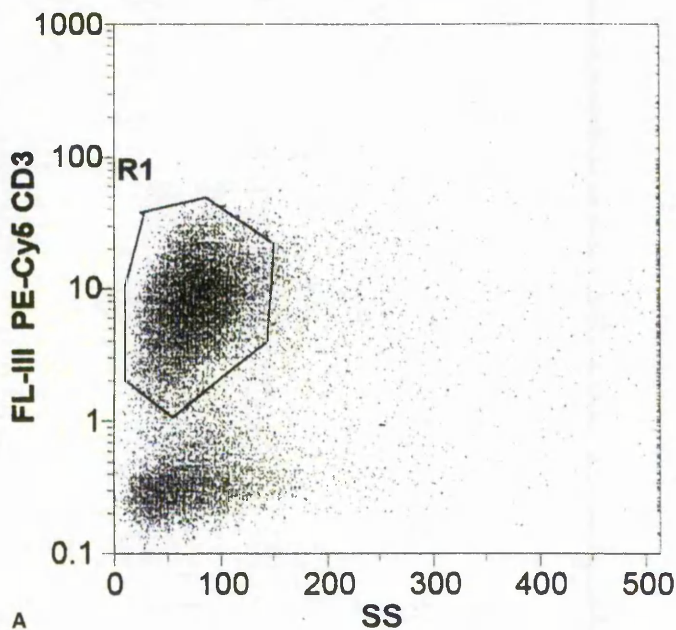
Cells were processed through a DAKO Galaxy flow cytometer. Alignment and fluorescence were standardised using Alignment beads (Molecular Probes, UK) and Flourospheres (DAKO, UK). A minimum of 20,000 events were gathered for each test. T cells were identified by their anti CD3 (PE-CY5, FL-III) expression and side scatter (SS) (see Fig. 1a), whilst monocytes were identified using anti CD14 (PE, FL-II) and SS (see Fig. 1b). The T cell and monocyte regions were "bitmapped" and from this bitmap the number of cytokine-expressing cells were determined. Using isotype controls "quadrant gates" were set on the two-dimensional "dot plot" of PE-CY5 (FLIII) and FITC (FL-I) for T cells and PE (FL-II) and FITC (FL-I) for monocytes. These "gates" then determined the percentage of T cells expressing either IFNγ or TNFα and monocytes expressing either IL6, IL12, IFNγ, or TNFα (see Fig. 2).

RESULTS

The mean number and standard error of the mean (SEM) of IFNγ-secreting T cells, TNFα-secreting T cells, and TNFα-secreting monocytes and are shown in Figure 3. Levels are shown for both normal age/sex matched controls and the CTCL and GvHD patients pre and post ECP. Pre ECP, the number of TNFα-expressing monocytes, for both patient groups, was not significantly different to the normal controls. However, at re-infusion, the percentage of TNFα positive monocytes dropped significantly for both patient groups ($P < 0.05$). IL6, IFNγ, and IL12 expression by monocytes demonstrated no significant difference to normals pre and post exposure to 8-MOP and UVA.

The levels of T cells expressing TNFα in the CTCL patients were not significantly different from the normal controls pre ECP. However, the number of TNFα-secreting T cells in GvHD patients was significantly higher than normal ($P < 0.05$). For both patient groups, a significant fall in the levels of T cells secreting TNFα was observed post ECP ($P < 0.05$).

Pre ECP, the numbers of IFNγ-expressing T cells were lower than normal in the CTCL population, in



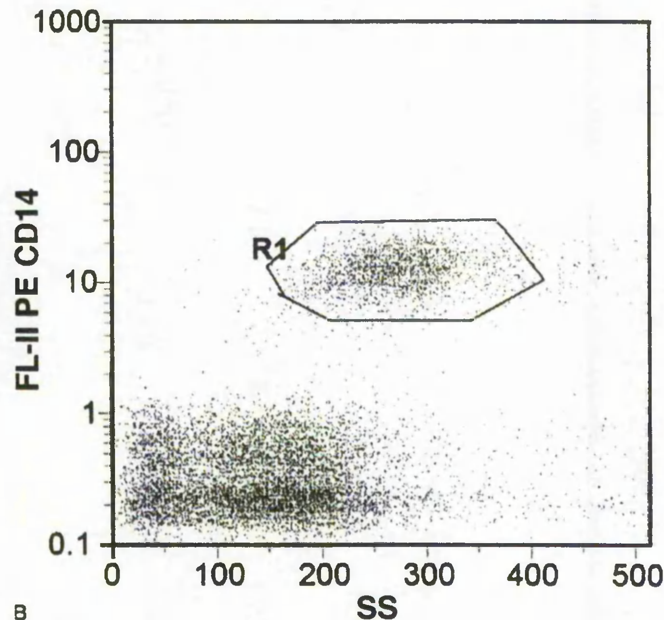
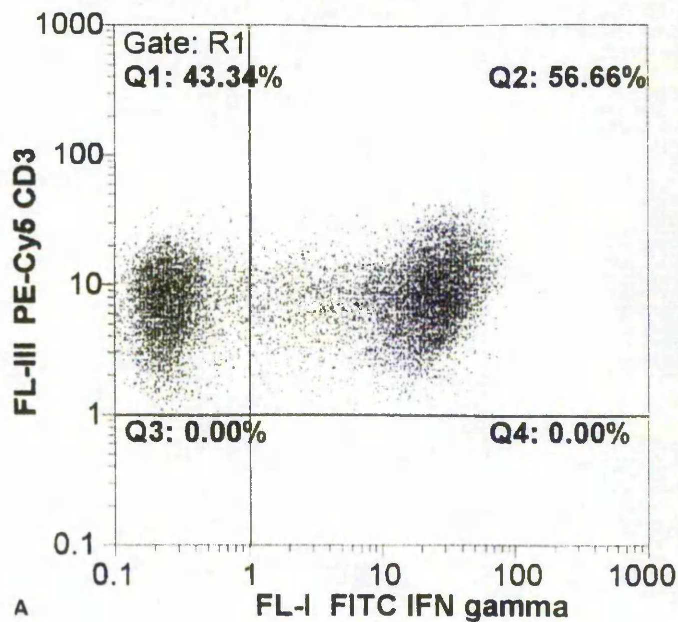


Fig. 1. Identification of T cells and monocytes by flow cytometry. **A:** T cells were identified using PE-CY5-conjugated anti CD3 (FL-III) and SS. **B:** Monocyte identification utilised PE-conjugated anti CD14 (FL-II) and SS. The T cells and monocytes were "bitmapped" (R1). These bitmaps were used to determine the level of cytokine expressing cells in each cell population (see Fig. 2). PE-CY5: Phycoerythrin-cyanin 5; PE: Phycoerythrin; SS: Side scatter.



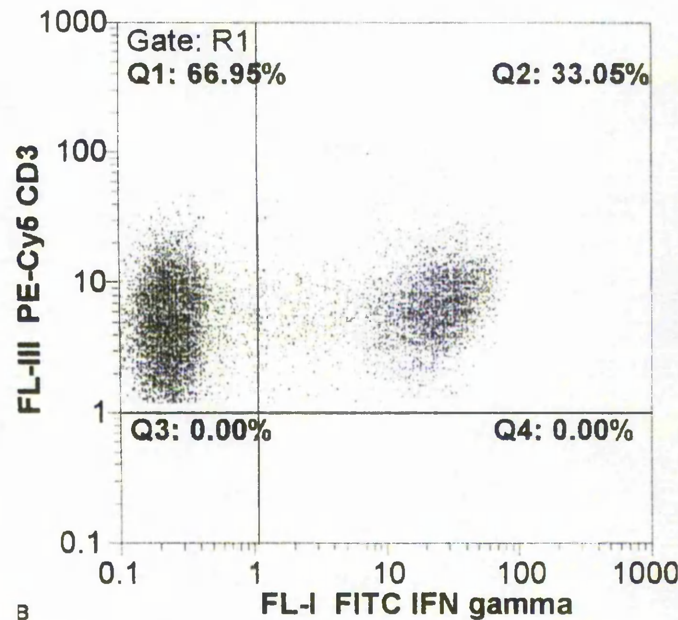


Fig. 2. Intracellular flow cytometric enumeration of IFNγ-secreting T cells. Using the relevant bitmaps (R1), the percentage of IFNγ-expressing T cells was enumerated. Using relevant isotype control, "quadrant gates" were set on a "dot plot" of anti-CD3 (PE-CY5 [FL-III]) and IFNγ (FITC [FL-I]). IFNγ-secreting T cells were identified and enumerated in quadrant Q2. Data presented are the (**A**) pre and (**B**) post ECP results of one patient. PE-CY5: Phycoerythrin-cyanin 5; FITC: Flourescein isothiocyanate.

keeping with the known expansion of the Th2 subtype in this condition [11] ($P < 0.05$). Whilst in the GvHD patient group, T cells showing higher numbers of T cells expressing IFNγ were detected ($P < 0.05$), suggestive of a Th1 proliferation. ECP significantly reduced the levels of IFNγ-expressing T cells at re-infusion. GvHD levels fell by a mean of 10% whilst CTCL patients fell by a mean of 6.5% ($P < 0.05$).

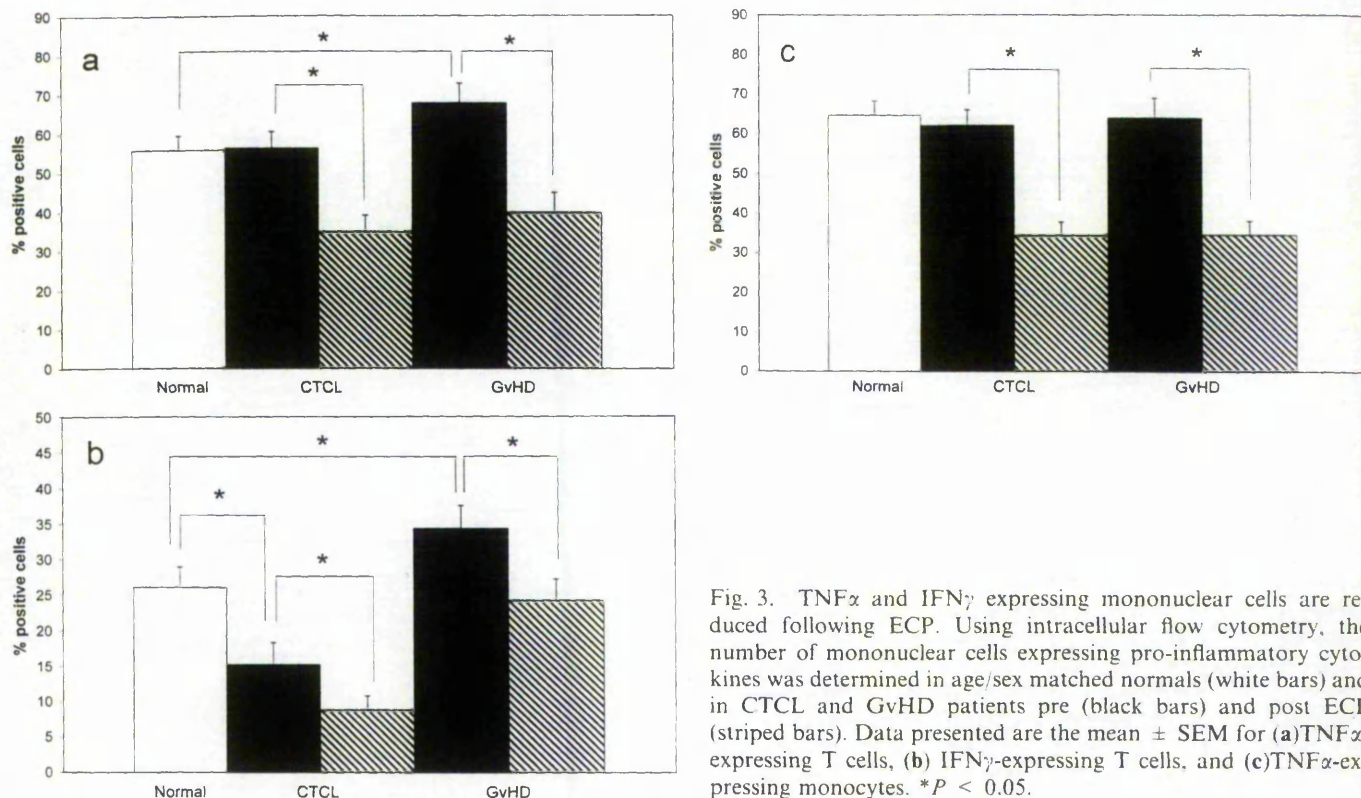


Fig. 3. TNFα and IFNγ expressing mononuclear cells are reduced following ECP. Using intracellular flow cytometry, the number of mononuclear cells expressing pro-inflammatory cytokines was determined in age/sex matched normals (white bars) and in CTCL and GvHD patients pre (black bars) and post ECP (striped bars). Data presented are the mean ± SEM for (**a**)TNFα-expressing T cells, (**b**) IFNγ-expressing T cells, and (**c**)TNFα-expressing monocytes. *$P < 0.05$.

DISCUSSION

The pattern of cytokine production following exposure to 8-MOP and UVA is controversial. PBMCs treated with in vivo and in vitro PUVA and subsequently stimulated with lipopolysaccharide have demonstrated suppression of TNFα and IL6. Northern blot analysis revealed a down-regulation of mRNA encoding for IL6 and TNFα [12]. The assessment of ECP treated monocytes, with and without IFNγ stimulation, demonstrate an enhanced production of both IL6 and TNFα [8]. Similar results are also observed with IFNγ. PBMCs exposed to UVA/8MOP and subsequently stimulated with Con A demonstrate a skewing toward Th1 cytokine production [7]. PBMCs pre-stimulated with anti CD3, rhIL-2, and rhIL4 prior to 8MOP/UVA, and subsequently stimulated with PMA, ionomycin, and monensin demonstrated an opposite shift from Th1 to Th2 in the CD4+ T cells [13].

Many of the previous studies evaluating 8MOP/UVA responses have involved either in vitro 8MOP/UVA exposure of normal PBMCs, measurement of total cytokine levels, or prolonged incubations following exposure [7,8,12,13]. We were interested to observe what influence ECP had on the cytokine secretion patterns of clinical samples very early in the ECP cycle, testing cells immediately post ECP, prior to re-infusion. At this early point, we have previously identified lymphocyte apoptosis, which may be induced by mechanisms different to those responsible for the later apoptosis detected 24 hours post ECP [10]. We also wanted to determine at the single cell level if cells demonstrated induction or suppression of cytokine production. To closely represent the environmental conditions present in the buffy coat bag prior to re-infusion, the T cells and monocytes were not isolated prior to culture.

Ex vivo assessment demonstrated a fall in the number of TNFα-secreting monocytes at re-infusion. However, the number of TNFα- and IFNγ-expressing T cells was reduced following ECP. The levels of IFNγ, IL6, and IL12 expression remained unchanged for the monocytes.

For CTCL patients, the enhanced expression of TNFα, following ECP, is capable of direct anti-tumour activity, which can be directed against leukaemia and lymphoma cells [14]. When combined with IFNγ, TNFα is capable of enhanced macrophage cytotoxicity [15], whilst IFNγ and IL12 are capable of enhancing the tumoricidal effect of cytotoxic T cells [16]. Therefore, an enhanced secretion of these cytokines, post UVA/8MOP, may play a part in the removal of the CTCL malignant cells, as has been observed following several rounds of ECP therapy [17] (Taylor, unpublished data). However, TNFα, IL6, and IFNγ have also been implicated in the pathology of GvHD. In acute GvHD (aGvHD), TNFα, IL6, and IFNγ levels are all increased, as elevated levels of

TNFα and IL6 are also observed in chronic GvHD (cGvHD) [18]. IFNγ has been demonstrated in the skin biopsies of patients with cGvHD [19], prompting the suggestion that in cGvHD IFNγ is the predominant cytokine [20]. Suppression of these inflammatory cytokines has been effective in the treatment of aGvHD and cGvHD. Anti TNFα antibodies have been used in the prevention of cutaneous and gastrointestinal lesions during aGvHD [21]. Transplanted mice, whose cGvHD was tolerated, demonstrated statistical reductions in TNFα and IFNγ levels [22].

In this study, the levels of TNFα- and IFNγ-secreting T cells were higher in the GvHD patients pre ECP, indicating that these two cytokines may have a prominent role in the aetiology of chronic GvHD. The early reduction in TNFα- and IFNγ-secreting mononuclear cells following ECP may have a direct role in reducing the pathologic progress of cGvHD. In 22 cases of steroid refractory chronic GvHD treated by ECP, there was a statistically significant and progressive reduction in cutaneous disease at 3, 6, and 9 months with a concurrent significant reduction in steroid usage (Taylor, unpublished data).

A correlation analysis of the increase in lymphoid apoptosis, as detected by Annexin V and a reduction in the number of IFNγ-expressing T cells at 6 hours post ECP, demonstrated a significant inverse relationship for both patient groups ($P < 0.05$) (data not shown). CTCL and GvHD patients demonstrated a correlation coefficient of −0.81 and −0.86, respectively. However, no significant correlation was observed between apoptosis induction and the down-regulation of TNFα in T cells. It is unlikely that the reduction in TNFα-secreting cells is related to apoptosis induction, as ECP does not induce apoptosis in treated monocytes [6,23]. However, the down-regulation of IFNγ has previously been observed in apoptotic cells [24], whilst enhancement of Th2 cytokines, namely IL4 and IL10, has also been directly observed in cells dying by apoptosis [24,25]. A reduction in IFNγ- and IL2-secreting CD4+ T cells, in conjunction with an increase in IL4-secreting T cells, was observed 5 hours post UVA/8MOP exposure. The shift in cytokine profile and cell viability was not linked, as no significant increase in apoptosis was detected at the time of testing [13]. However, PI was used to determine apoptosis, a technique that detects a relatively late stage of apoptosis [10,13]. Using Annexin V, a significant increase in lymphoid apoptosis was observed ex vivo and 6 hours post ECP [5,10]. The early shift in cytokine pattern from Th1 and Th2 may, therefore, be a simultaneous effect linked to the early apoptosis observed in lymphocytes immediately following ECP treatment.

The reduction in these cytokines seems to preferentially suit the treatment of GvHD. However, ECP is very effective treatment in the removal of the malignant cells responsible for CTCL. Following ECP, treated lymphocytes become apoptotic [5,6,10]. 8MOP/UVA also increases expression of MHC class 1 molecules on lymphocytes, a process that may also induce a 2–3-fold parallel increase in tumour antigens [26]. Monocytes treated by ECP demonstrate a rapid conversion to dendritic cells and avidly phagocytose the apoptotic malignant T cells [27], a process that leads to the presentation of antigens to cytotoxic CD8+ T cells and subsequent anticlonotypic immunity capable of removing non-ECP-treated cells [26,28,29]. Interestingly, this immunomodulatory process is possible without the requirement for exogenous cytokines [27]. The removal of the malignant Th2 T cells in CTCL by this mechanism may explain the long-term restoration of the Th1/Th2 imbalance, as observed by Di Renzo et al. [30].

These results suggest that, ex vivo, UVA/8MOP exposure down-regulates pro-inflammatory cytokine responses. In GvHD and psoriasis, in which excessive Th1 cytokine secretion patterns are observed, these responses may, in part, be responsible for the clinical responses observed following UVA/8MOP treatment. Further analysis, involving the evaluation of several different Th1 and Th2 cytokines, would be needed to determine if the shift in cytokine expression was linked to the early apoptosis determined immediately post ECP.

CONCLUSIONS

The use of 8-MOP/UVA has paradoxically been clinically effective in the treatment of Th1-mediated conditions, such as psoriasis and GvHD, as well as the Th2-mediated condition CTCL. This process may be accounted for partly by a multifaceted mechanism. The immunomodulatory process of lymphoid apoptosis and enhanced MHC expression, followed by phagocytosis, may account for the removal of previously undetectable malignant Th2 cells of CTCL. The immediate down-regulation of TNFα and IFNγ, two cytokines actively involved in the pathology of GvHD, may explain an important mechanism that predominately benefits GvHD. This immunosuppressive response may also explain the beneficial effects of 8MOP/UVA on other Th1-mediated conditions, such as psoriasis.

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Extracorporeal photopheresis induces apoptosis in the lymphocytes of cutaneous T-cell lymphoma and graft-versus-host disease patients

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Summary. Extracorporeal photopheresis (ECP) is used in the treatment of T-cell-mediated disorders. However, the mechanism by which ECP achieves its effect remains illusive. Over recent years the ability of ECP to induce apoptosis has been demonstrated by cell culture experiments and retrospective histological analysis. We investigated if apoptosis could be determined in samples tested *ex vivo* from the UVAR:ECP system. Lymphocytes from 11 patients (six with cutaneous T-cell lymphoma, four with graft-versus-host disease, and one with scleredema) were isolated at three stages of the ECP process: immediately before ECP treatment, from the first buffy coat collected, and post UV irradiation, prior to re-infusion. Using flow cytometry each stage was tested for the early apoptotic markers; Annexin V, Apoptest™ and Carboxy-SNARF-1-AM. Comparisons of the pre-ECP and pre-infusion samples demonstrated a significant increase in apoptotic lymphocytes for all three flow cytometric techniques ($P < 0{\cdot}01$). Increases between the pre-ECP and first buffy coat, used as a measure of the extracorporeal manipulation, were much lower. These results demonstrate that ECP directly induces significant levels of apoptosis in lymphocytes of CTCL, GvHD and scleredema patients. The apoptosis of these lymphocytes may contribute to the ECP effect.

Keywords: photopheresis, cutaneous T-cell lymphoma, chronic graft-versus-host disease, apoptosis, flow cytometry.

Extracorporeal photopheresis (ECP) is a unique approach to the treatment of T-cell-mediated disorders (Rook *et al*, 1993), including cutaneous T-cell lymphomas (CTCL) (Edelson *et al*, 1987), graft-versus-host disease (GvHD) (Bowell *et al*, 1990) and systemic sclerosis (scleroderma) (Rook *et al*, 1992). The technique involves the separation and exposure of leucocytes to UVA irradiation, following the administration of 8-methoxypsoralen (8-MOP). The treated leucocytes are re-infused after a minimum of 90 min exposure. This process is then repeated on a consecutive day, patients returning for treatment either 2- or 4-weekly (Edelson *et al*, 1987).

The ECP system was developed by Edelson *et al* (1987) following the successful use of PUVA for CTCL. Response rates of 60–75% have been reported with the treatment of CTCL patients with ECP, when used alone or in conjunction with interferon-alpha (IFNα) (Wolfe *et al*, 1994). Further encouraging studies led to a recommendation, from an International Consensus conference on staging and treatment for CTCL, that photopheresis be considered as a first line treatment for patients with erythrodermic-stage disease (Lim & Edelson, 1995).

In bone marrow transplantation (BMT) 30–60% of allogeneic BMTs are affected by chronic graft-versus-host disease (GvHD) (Ferrara & Deeg, 1991). Bowell *et al* (1990) initially demonstrated the effectiveness of ECP in cutaneous GvHD with clinical improvements in two of three patients treated. ECP has also been shown to be effective in patients with resistance to conventional GvHD treatment (Dall'Amico *et al*, 1997) or where treatment was poorly tolerated (Rossetti *et al*, 1995).

Rook *et al* (1992) demonstrated significant improvements in the skin of 68% of scleroderma patients post 6 months ECP treatment.

The effectiveness of ECP has been explained, in part, by the induction of apoptosis in the treated lymphocytes (Yoo *et al*, 1996). Apoptosis is a form of cell death distinct from necrosis, where cellular demise occurs without invoking an inflammatory response (Wyllie *et al*, 1980). Histological examination of skin biopsies from ECP-treated mycosis fungoides patients have demonstrated a significant increase in apoptosis following treatment (Miracco *et al*, 1997).

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Apoptotic markers have been detected on lymphocytes cultured from samples taken immediately after ECP exposure (Yoo *et al*, 1996; Enomoto *et al*, 1997; Aringer *et al*, 1997) and after *in vitro* exposure of cells to ECP conditions (Yoo *et al*, 1996; Marks & Fox, 1991). Lymphocytes cultured after ECP treatment demonstrated significant apoptotic markers at 24 h, their numbers increasing after 48 h in culture. However, no evidence of apoptosis was detected in ECP-treated lymphocytes tested immediately after the procedure (Enomoto *et al*, 1997; Aringer *et al*, 1997) or 6 h post ECP (Yoo *et al*, 1996).

On re-infusion into the peripheral circulation any apoptotic lymphocytes would be rapidly removed by phagocytosis (Fadok *et al*, 1992). If these cells were still viable for some days, they would remain undetected by the reticuloendothelial (RE) system and have the capacity to modulate the immune system through release of cell-signalling proteins (Enomoto *et al*, 1997). The aim of this study was to investigate if, by using markers associated with early apoptosis, treated lymphocytes tested immediately after ECP were apoptotic prior to their re-infusion.

MATERIALS AND METHODS

Patients. Samples were obtained from six CTCL patients (mean age ±SD $66{\cdot}3 \pm 7{\cdot}4$ years), four GvHD patients ($37{\cdot}3 \pm 5{\cdot}7$ years) and one scleredema patient (54 years). Diagnosis for each condition was based on established histological and immunological criteria. Informed consent was obtained from all patients and local ethical approval was granted for the study.

Photopheresis treatment. Extracorporeal photopheresis cycles were performed using the Therakos system (Johnson & Johnson). Treatment involved the collection of approximately 240 ml of buffy coat and 300 ml of plasma (from six collection cycles) into a 'buffy coat bag' (BCB) which contained 200 ml of saline solution. Red cells and excess plasma were returned to the patient after each cycle. The BCB was exposed to a UVA radiation source (2 Jcm^2/cell) through a plastic photocell, as a film 1 mm thick, beginning immediately after the first cells were collected. The BCB was incubated for 90 min, the incubation time beginning after the collection of the final buffy coat. At the end of the incubation the BCB was re-infused to the patient. This process was then repeated on the following day, and then either 2- or 4-weekly. For the CTCL and scleredema patients, 8-MOP was taken orally 90 min before the start of the ECP. For GvHD patients 8-MOP (Uvadex) was injected directly into the BCB immediately after the collection of the first buffy coat. Buffy coat 8-MOP levels were tested in the CTCL and scleroderma patients to ensure a concentration of at least 100 ng/ml.

Sampling and preparation of lymphocytes. 4·5 ml of heparinized blood were taken from patients; immediately before ECP, from the first buffy coat collected and from the incubated BCB just prior to re-infusion. The peripheral blood mononuclear cells (PBMCs) were immediately separated by adding 4·5 ml of isotonic saline and centrifuging on a density gradient (Lymphoprep, Nycomed, U.K.). The isolated cells were then washed once with 8 ml of phosphate-buffered saline (PBS) and added to each respective test buffer to give a final count of $0{\cdot}5\text{–}1{\cdot}0 \times 10^6$/ml. Using cells added to 0·5 ml PBS, identification of lymphocytes used a forward scatter (FS) versus side scatter (SS) plot. Staining with 2·5 μl of RPE-conjugated CD14 (DAKO, U.K.) and 5 μl of FITC-conjugated CD45 (Immunotech, U.K.) excluded monocytes and debris respectively. Propidium iodide (PI), used in conjunction with Annexin V, was used to identify any necrotic cells. This process was repeated on the second day of treatment.

Apoptosis determination. Three different techniques for apoptosis determination were used: Annexin V (Immunotech, U.K.), which tests for the externalization of phosphatidylserine groups ('flip-flop'). Apoptest™ (Ylem, Italy), a cell-permeable DNA probe which detects changes in chromatin texture and Carboxy-SNARF-1-AM (Molecular Probes, U.K.) which tests for intracellular pH. Annexin V/PI determination was as per manufacturer's recommendations. The washed cells were added to the 490 μl of cold 'working strength' binding buffer to give $0{\cdot}5\text{–}1{\cdot}0 \times 10^6$/ml and placed on ice, 5 μl of 'working strength' Annexin V and 5 μl of propidium iodide were then added and incubated for 10 min in the dark. Cells were processed immediately. For Apoptest™ determination, cells were added to 400 μl of PBS to give $0{\cdot}5\text{–}1{\cdot}0 \times 10^6$/ml in total volume (i.e. 500 μl), 100 μl of Apoptest™ reagent was then added and incubated at room temperature, in the dark for 20 min. Cells were processed immediately. Carboxy-SNARF-1-AM (CS) was reconstituted with dimethyl sulphoxide (DMSO) to give a final concentration of 1 mM. This stock solution was stored at −20°C. Immediately before testing, the CS stock solution was diluted to 10 μM in PBS. Cells were added to this working strength solution to give $0{\cdot}5\text{–}1{\cdot}0 \times 10^6$/ml and incubated in the dark at 37°C for 30 min. The cells were then washed once in PBS and the supernatant removed, 500 μl of PBS was added to resuspend the cells. The cells were then immediately processed.

Flow cytometry. Cells were processed through a Coulter EPICS Profile II flow cytometer. Alignment and fluorescence were standardized using Coulter beads. Lymphocytes were bitmapped by their respective low forward and side scatter. Exclusion of debris was achieved by ensuring >97% CD45 positivity. Exclusion of monocytes using CD14 ensured <2% contamination. Using the same bit map, 5000 events were gathered for each apoptosis test. Data for Annexin V was gathered on a dot plot of log fluorescence 1 (LFL1) and log fluorescence 2 (LFL2). The LFL1 gathered Annexin V events and LFL2 identified any necrosis using PI. Gates were set at the edge of the dot plot cluster on the 'pre' sample of each patient; these same gates were used to assess cells in the first buffy coat and pre-infusion samples. Data for the Apoptest™ were gathered using a dot plot of FS and fluorescence 3 (FL3). The gating regime was as for Annexin V. The Carboxy-SNARF-1-AM (CS) data were collected as a ratio of FL2/FL3. When excited at 488 nm, the acidic form of CS fluoresces at 575 nm (FL2) and the basic form fluoresces at 635 nm (FL3). Using a ratio of both, an indication of pH could be achieved. The results were represented as a histogram, gating again

Table I. Mean levels of lymphocyte apoptosis and the statistical increases between the different stages of the ECP treatment.

	Mean level of apoptosis (%)			Statistical significance (paired *t* test)		
	Pre-ECP	First buffy coat	Pre-infusion	Pre-ECP to first buffy coat	Pre-ECP to pre-infusion	First buffy coat to pre-infusion
Annexin V						
CTCL ($n = 6$)	3·6	5·1	15·7	<0·01	<0·01	<0·01
GvHD ($n = 4$)	4·3	6·5	21·9	<0·02	<0·01	<0·01
Scleroderma ($n = 1$)	5·8	6·3	14·4	<0·4 ns	<0·08 ns	<0·08 ns
Apoptest™						
CTCL ($n = 6$)	1·3	1·9	43·1	<0·05	<0·01	<0·01
GvHD ($n = 4$)	1·6	27·3	73·3	<0·01	<0·01	<0·01
Scleroderma ($n = 1$)	1·2	0·9	53·3	n/a	<0·08 ns	<0·09 ns
Carboxy-SNARF						
CTCL ($n = 6$)	1·7	3·2	19·5	<0·02	<0·01	<0·01
GvHD ($n = 4$)	1·7	24·4	26·2	<0·01	<0·01	<0·4 ns
Scleroderma ($n = 1$)	1·4	1·2	23·6	<0·3 ns	<0·08 ns	<0·09 ns

ns = not significant.

involved setting on the pre-ECP sample and using the same gates for the subsequent first buffy coat and pre-infusion samples. Any increase in acidity caused an increase in the FL2/FL3 ratio.

Statistical analysis. Statistical analysis was performed using paired *t*-tests. A *P* value $<0{\cdot}05$ was regarded as significant.

RESULTS

All samples showed <1% of lymphocytes expressed the necrotic marker, propidium iodide. The mean values for lymphocyte apoptosis and statistical differences between each stage of ECP for cells are shown in Table I.

As only one scleredema patient was tested, no statistical increase in apoptosis could be detected between the different stages of ECP treatment. However, the levels of apoptosis observed were similar to those of the CTCL patients.

The CTCL and GvHD patients demonstrated significant increases in lymphocyte apoptosis, using all three flow cytometric methods, prior to their re-infusion. The increase in apoptosis at the first buffy coat stage was much lower. Only lymphocytes from GvHD patients tested with CS did not demonstrate a significant increase in apoptosis from the first buffy coat to the re-infusion sample ($P = 0{\cdot}347$).

Levels of apoptosis induction at re-infusion on day 1 and day 2 of treatment were also compared. Only lymphocytes from GvHD patients, tested with CS, showed a significantly higher number of apoptotic lymphocytes at re-infusion on the second day of ECP treatment.

The CS test involved a long incubation time at 37°C and a washing step in its procedure. These factors may have contributed to the slightly conflicting results observed.

DISCUSSION

Several mechanisms have been proposed to explain the action of ECP, one of which is the induction of apoptosis (Yoo *et al*, 1996). The ability of ECP to induce apoptosis in lymphoid cells was first identified by Marks & Fox (1991) who reported distinctive apoptotic cell morphology and DNA fragmentation in cultured cells exposed to *in vitro* ECP-like conditions. More recent papers have identified apoptosis by *in vitro* exposure of cultured cells to ECP conditions (Yoo *et al*, 1996), and by culturing the cells of patients following ECP therapy (Yoo *et al*, 1999; Enomoto *et al*, 1997; Aringer *et al*, 1997). Apoptosis has also been observed in histological examination of skin tissues from ECP-treated patients (Miracco *et al*, 1997). Here we attempted to identify apoptosis of peripheral lymphocytes in the leucocyte collection bag, without the need for culturing. A panel of tests which determine different cellular expressions of early apoptosis was chosen in order to provide supportive evidence for the occurrence of apoptosis. Annexin V identifies the translocation of phosphatidylserine groups from the internal to the external cell membrane ('flip-flop'). When used in conjunction with propidium iodide, which identifies necrosis, apoptotic cells can be identified by their Annexin V+/PI− expression (Verms *et al*, 1995). Apoptest™ is a membrane-permeable DNA dye which detects the transient changes in chromatin texture associated with early apoptosis. When chromatin texture changes, as a consequence of apoptosis, the DNA loop unwinds and accommodates more dye (Ferlini *et al*, 1997). Carboxy-SNARF-1-AM identifies the increase in intracellular acidity associated with apoptosis (Ishaque *et al*, 1997; Weider *et al*, 1993).

Previous *in vitro* studies have demonstrated that 8-MOP alone does not induce apoptosis (Yoo *et al*, 1996). However, the apoptotic effects attributable to the mechanics of cell separation, i.e. cannulation, peristalsis pumping and centrifugation, are unknown. To determine this effect would have involved testing cells after ECP treatment without exposure to UV irradiation. This was considered unethical because the patient's treatment would not be in accordance

with current guidelines and as such its benefits may be compromised. Samples were therefore taken pre-ECP and from the first buffy coat collected, prior to any UVA irradiation. Additional samples were taken just prior to re-infusion, to assess the apoptotic effect of both the cellular manipulation plus the exposure of the buffy coat to 8-MOP and UVA irradiation. Analysis of the CTCL and GvHD patients demonstrated a significant increase in apoptosis in the pre-infusion bag, using all three apoptosis markers. The Apoptest™, associated with very early apoptosis detection (Ferlini *et al*, 1997), demonstrated the most significant increase. Because only one scleredema patient was tested, the statistics showed no significant increase between the different stages of the ECP treatment. However, the increase in expression of apoptotic markers were very similar to those seen for the CTCL patients. These results indicated that the ECP therapy induced apoptosis in a significant numbers of lymphocytes before the cells were re-infused.

Previous investigations of ECP did not detect significant levels of lymphocyte apoptosis when tested immediately after the procedure (Enomoto *et al*, 1997; Aringer *et al*, 1997) or 6 h post ECP (Yoo *et al*, 1996). Determination of apoptosis in these experiments included the use of propidium iodide and the TUNEL technique (Aringer *et al*, 1997), gel electrophoresis and *in situ* nick translation of DNA fragmentation (Enomoto *et al*, 1997), and detection of DNA strand breaks and changes in cells size and density (Yoo *et al*, 1996). All of these techniques are associated with a relatively later stage of apoptosis. For the determination of apoptosis in this study, markers associated with early apoptosis, Annexin V (Vermes *et al*, 1995), Apoptest™ (Ferlini *et al*, 1997) and Carboxy-SNARF-1-AM (Ishaque *et al*, 1997), were selected. Annexin V has demonstrated significant levels of apoptosis in the T-lymphoblastoid cell line CCRF-HSB-2, 4 h after induction with 8 Gy irradiation (Vermes *et al*, 1995). Apoptest™ has determined apoptosis in PBMC 4 h after 8 Gy irradiation (Ferlini *et al*, 1997). Apoptosis has been observed in hybridoma cells 3 h after exposure to campothecin, staurosporine and lutamine and oxygen deprivation using Carboxy-SNARF-1-AM (Ishaque *et al*, 1997). The time difference in expression of apoptotic markers, between this and previous studies, is therefore most likely due to the methodology chosen for apoptosis determination.

Apoptotic lymphocytes expressing phosphatidylserine on their membrane are rapidly removed by phagocytosis (Fadok *et al*, 1992). Apoptotic lymphocytes expressing increased Apoptest™ uptake started to become positive for Annexin V expression soon after (Ferlini *et al*, 1997). It therefore seems likely that a high percentage of ECP-treated lymphocytes are promptly removed, *in vivo*, after their re-infusion. The unsuccessful attempt to identify apoptotic cells, *in vivo*, at 1, 6 and 24 h (unpublished observations in Yoo *et al*, 1996) and 24 h (Aringer *et al*, 1997) post re-infusion seems to confirm this. We also noted no increase in apoptotic cells in the pre-ECP sample on day 2 compared to day 1, some 19 h post re-infusion. Previously cells in a pre-apoptotic state, post ECP, have been linked to immune cell signalling processes prior to their apoptotic demise, some 24 h and 48 h post re-infusion (Enomoto *et al*, 1997). The prompt removal of apoptotic lymphocytes, by phagocytosis, would indicate that this process is less pronounced that previously thought.

Monocytes do not become apoptotic when treated with ECP (Yoo *et al*, 1996), but produce increased levels of TNFα (Vowels *et al*, 1992). TNFα can augment a number of antitumour immune responses, including cytotoxic T-cell responses (Grunfeld & Palladino, 1990). It may be that it is these activated monocytes which are responsible for the greatest degree of immune modulation observed post ECP.

Following ECP-induced apoptosis, normal T lymphocytes are replaced at a rate greater than malignant cells migrate to the peripheral blood (Yoo *et al*, 1996). The combination of lymphocyte apoptosis and monocyte activation may complement each other and contribute significantly to the ECP effect, helping to replace the malignant lymphocytes with normal T-cells.

The process of apoptosis induction appears to begin immediately with CTCL, scleredema and GvHD patients demonstrating enhanced apoptosis in the cells collected in the first buffy coat. The greatest increases were seen in the GvHD patients using the Apoptest™ and Carboxy-SNARF, with mean increases of >20%. Interestingly, this sample was taken before the 8-MOP had been injected into the collection bag. Greater levels of apoptosis at re-infusion demonstrated the addition effect of 8-MOP and UVA irradiation. However, it is of some interest that exposure to the mechanics of ECP and an *ex vivo* state seemed able to induce some apoptosis of ECP-treated lymphocytes, to which the GvHD patients seemed most sensitive. Recently we observed a similar pre UV induction of apoptosis with the updated 'XTS' ECP machine for CTCL and scleredema patients. This fully automated machine 'concentrates' the buffy coat by enhanced cellular manipulation before the cells are photoactivated. This enhanced manipulation seems able to induce significant levels of apoptosis in the lymphocytes of these individuals before the cells are irradiated (Bladon & Taylor, 1999).

The levels of apoptosis induction on day 1 and day 2 of treatment were compared. Only the lymphocytes from GvHD patients demonstrated a significant increase on the second day of treatment ($P = 0{\cdot}016$). This seemed to indicate that the recommended 2 d treatment exposed large cell numbers to the ECP therapy rather than prime the cells in some way on day 1.

These results indicate that ECP therapy caused a significant degree of apoptosis induction in the lymphocytes of treated patients. This response, along with the other immunological changes, seen after ECP, may contribute to the observed clinical effect.

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Treatment of cutaneous T cell lymphoma with extracorporeal photopheresis induces Fas-ligand expression on treated T cells, but does not suppress the expression of co-stimulatory molecules on monocytes

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Abstract

Following extracorporeal photopheresis (ECP), lymphocytes become apoptotic and upregulate class I MHC antigenic peptides. Conversely, ECP treated monocytes demonstrate activation markers and have an increased avidity for the phagocytosis of apoptotic T cells. Processing of apoptotic T cells by monocytes, following ECP, is thought to induce an immunomodulatory response, which targets untreated, but clonal T cells. Recently we detected apoptotic lymphocytes immediately post ECP. Although enhanced CD95 (Fas) expression has been observed 24 h post ECP, CD95 and Fas-ligand (Fas-L) expression have not been determined at this very early apoptotic stage. Exposure of monocytes to UV has previously suppressed expression of the co-stimulatory molecules required for the presentation of processed antigens to T cells. Our data demonstrate no increase in CD95 or Fas-L expression on T cells tested immediately following ECP. However, the number of T cells expressing Fas-L significantly increased 24 h post ECP (P<0.005). The expression of the co-stimulatory molecules, CD54, CD80 and CD86, remained unaltered on monocytes treated by ECP. Although the mechanism responsible for early induction of lymphocyte apoptosis remains unclear, the later apoptosis involves Fas-L expression. The maintenance of co-stimulatory molecules, on treated monocytes, indicates that they retain the ability to induce an immunomodulatory response.

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Keywords: Extracorporeal photopheresis; Cutaneous T cell lymphoma; CD95; Fas-ligand; Co-stimulatory molecules

1. Introduction

The treatment of cutaneous T cell lymphoma (CTCL) by extracorporeal photopheresis (ECP) was originally described 16 years ago. The process involves the separation of white cells by leucopheresis, followed by exposure to 8-methoxypsoralen (8-MOP) and UVA irradiation and subsequent reinfusion [1].

CTCL patients present with a malignant expansion of CD4+ T cells, predominantly of the Th2 subtype [2,3]. Rather than a true proliferation, CTCL may occur as a consequence of defective lymphocyte apoptosis [4]. ECP therapy reverses the CD4+ expansion and Th1/Th2 imbalance back to normal in 'responders' within 1 year of commencing treatment [5,6]. Several processes have been linked to this anti-tumour mechanism, including the induction of apoptosis in treated lymphocytes [7–10] and activation and cytokine production by treated monocytes [11,12]. Th1 and Th2 skewing cytokine patterns have also been observed following 8-MOP–UVA [13,14].

An important mechanism in lymphoid apoptosis is the CD95 (Fas) pathway. Fas, a member of the tumour necrosis factor receptor family, induces apoptosis when crosslinked with Fas-ligand (Fas-L) [15]. Reduced levels of CD95 on CD4+ T cells in CTCL may contribute to the defective apoptosis [4]. Recently we detected apoptotic lymphocytes immediately post ECP, prior to reinfusion [10]. ECP has previously been demonstrated to enhance CD95 expression on the CD4+ T lymphocytes of systemic sclerosis patients 24 h post ECP [9]. However, ECP's effect on CD95 and Fas-L expression on the early apoptotic lymphocytes, observed pre re-infusion, has not been determined.

The processing of antigens by antigen-presenting cells (APCs) ultimately leads to the presentation to T cells and immune recognition [16]. When APCs acquire antigens

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from apoptotic cells, a cytotoxic response against similar antigens is induced [17,18]. The processing of antigens on the malignant apoptotic T cells post ECP, by APCs, may trigger the immune recognition of other nontreated malignant T cells of the same clone [19,20]. When antigens are processed by APCs, the presence of co-stimulatory signals on the APC is required to avoid specific T cell unresponsiveness (anergy) [21]. CD54 (ICAM-1), CD80 and CD86, are important co-stimulatory molecule in this process [22,23]. CD80 and CD86 modulate the activation of Th1 and Th2 cells, respectively [24]. Previously the treatment of monocytes by UV has demonstrated a marked suppression of CD54 expression and reduced the upregulation of CD80 and CD86 following IFNγ stimulation [25–27].

We determined CD95 expression on CD4+ and CD8+ T lymphocytes and CD54, CD80 and CD86 expression on monocytes, at several stages post ECP, to observe the early and late effect of ECP on these antigens.

2. Materials and methods

2.1. Patients

Samples were obtained from nine cutaneous T cell lymphoma (CTCL) patients receiving ECP treatment, two stage IIA, five stage III and two stage IVA. All patients demonstrated the presence of clonal disease in the peripheral blood. Diagnosis for each patient was based on established histological and immunological criteria. Informed consent was obtained from all patients and local ethical approval was granted for the study.

2.2. Photopheresis treatment

Extracorporeal photopheresis cycles were performed using the XTS™ system (Therakos, UK). The treatment involves harvesting leucocytes (buffy coat) using a 'collect and elutriation' six-cycle apheresis system, which includes a final 'concentration' step. This system optimises the haematocrit (Hct) and increases the white cell:volume ratio in the buffy coat bag (BCB). The cells collected are exposed to 8-methoxypsoralen (8-MOP) (Uvadex) (Ben Venue Labs., Bedford, OH, USA) and a 1.5 J/cm^2 UVA radiation source, commencing from the end of the concentration step. The exposure time is dependent on the volume and Hct of the buffy coat (~15–60 min). After irradiation, the treated cells are reinfused. This process in then repeated on the following day, patients returning monthly.

2.3. Sampling and preparation of peripheral blood mononuclear cells (PBMCs)

Heparinised blood was taken from patients immediately before ECP and from the incubated BCB just prior to re-infusion. The peripheral blood mononuclear cells (PBMCs) were immediately separated by adding an equal quantity of isotonic saline and centrifuging on a density gradient (Lymphoprep) (Nycomed, Norway). The isolated cells were then washed once with phosphate buffered saline (PBS). The pre- and post-ECP cells for immediate testing were added to their appropriate test buffer to give a final cell count of 0.5–1.0×10^6/ml. Pre- and post-ECP

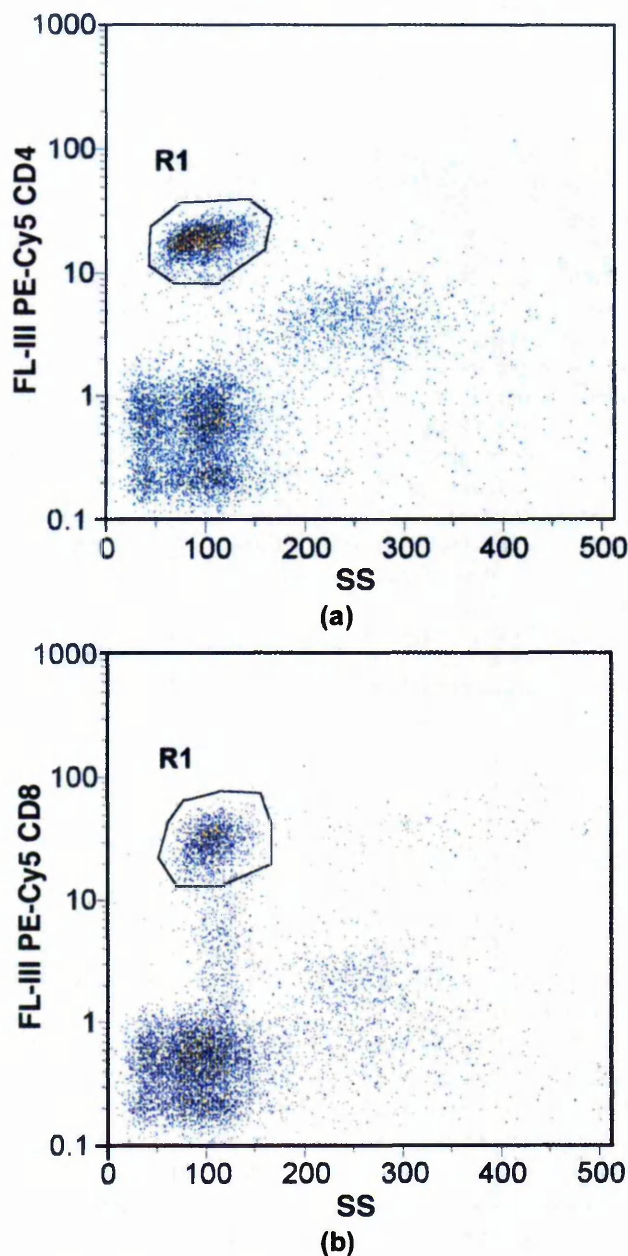
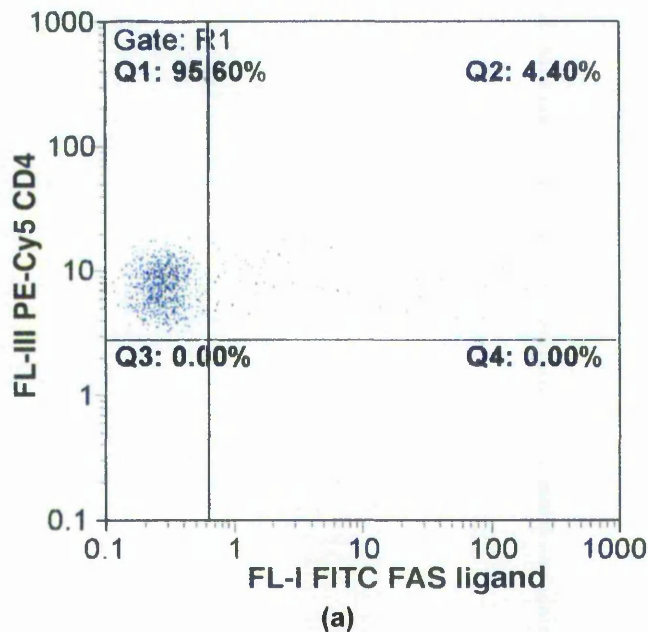


Fig. 1. Identification of CD4+ and CD8+ T cells. (a) Using a two-dimensional dot plot of CD4 (PE-CY5, FLIII) and SS, a bitmap was drawn (R1) around the CD4+ lymphocytes (low SS). From this bitmap the percentage of cells expressing CD95 or Fas-L was determined. (b) CD8+ T cells were determined using the same bitmapping strategy.

cells for testing at later stages were added to RPMI medium (Biowhittaker, UK) containing 10% fetal calf serum, 140 μg/ml streptomycin, 50 μg/ml vancomycin and 1% glutamine to give a final count of 1.0 to 2.0×10^6/ml and incubated in the dark at 37 °C. Before testing cultured cells were washed in PBS.



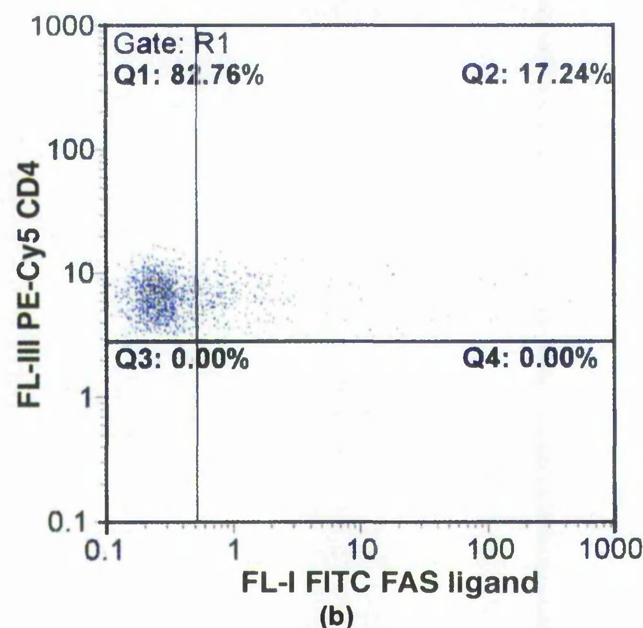


Fig. 2. Fas-L expression on CD4+ T cells 24 h pre and post ECP. Using the bitmapped CD4+ T cells (R1) (see Fig. 1a), a two-dimensional dot plot of CD4 (PE-CY5, FLIII) and Fas-L (FITC, FL-1) was utilised. Quadrant gates were set using relevant isotype controls. The percentage of cells expressing Fas-L was determined in quadrant Q2. (a) 24 h pre-ECP; a low level of CD4+ T cells express Fas-L. This number significantly increases (b) 24 h post-ECP.

2.4. CD95 expression

To tubes containing 1 ml of PBS and a cell count of $0.5–1.0\times10^6$/ml was added 10 μl of either PE-CY5-conjugated anti CD4 or PE-CY5-conjugated anti CD8 (DAKO, UK). A 10-μl volume of FITC-conjugated anti CD95 (Immunotech, UK) was subsequently added and the tubes incubated in the dark, at room temperature, for 20 min. The tubes were then processed immediately. CD95 expression was assessed at 0, 2, 4 and 24 h pre and post ECP. Relevant isotype controls were performed.

2.5. Fas-ligand expression

A 2-μl portion of unconjugated anti Fas-L (Pharmingen, UK) was added to 1 ml of PBS containing a cell count of $0.5–1.0\times10^6$/ml. The tubes were then incubated in the dark, at room temperature, for 20 min. The tubes were then washed in PBS and resuspended in 1 ml PBS. A 10-μl volume of a previously 1 in 20 diluted secondary FITC-conjugated anti-mouse antibody (Caltag, UK) was subsequently added. Following a 20 min, room temperature incubation in the dark, the tubes were again washed in PBS. The final stage involved resuspending the cells in 1 ml PBS and the addition of either 10 μl of PE-CY5-conjugated anti CD4 or 10 μl of PE-CY5-conjugated anti CD8 (DAKO). Following a 20-min incubation in the dark at room temperature, the tubes were immediately processed. Fas-L expression was assessed at 0, 12 and 24 h pre and post ECP. Relevant isotype controls were performed.

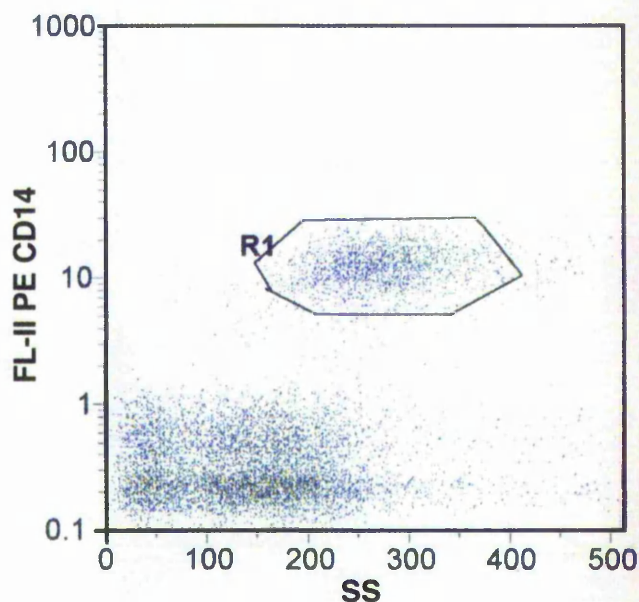


Fig. 3. Identification of monocytes. Using a two-dimensional dot plot of CD14 (PE-FLII) and SS, a bitmap was drawn (R1) around the CD14+ monocytes (high SS). From this bitmap the percentage of cells expressing CD54, CD80 and CD86 was determined.

2.6. CD54, CD80 and CD86 expression

To a cell count of 0.5–1.0×10^6/ml in 1 ml PBS was added 10 μl of PE-conjugated anti CD14 (Immunotech) and either 10 μl of FITC-conjugated anti CD54, anti CD80 (Immunotech) or anti CD86 (DAKO). The tubes were then incubated in the dark, at room temperature, for 20 min. The tubes were then processed immediately. CD54, CD80 and CD86 expression were assessed at 0, 2, 4 and 24 h pre and post ECP. Relevant isotype controls were performed.

2.7. IFNγ stimulation

Pre- and post-ECP PBMCs, suspended in RPMI medium (see Section 2.3) were stimulated with IFNγ (500 U/ml) and incubated in the dark at 37 °C for 24 h. The cells were subsequently washed and the count adjusted to 0.5–1.0× 10^6/ml. The cells were stained with anti CD14 and anti CD80, as outlined earlier.

2.8. Flow cytometry

Cells were processed through a DAKO Galaxy flow cytometer. Alignment and fluorescence being standardised using Alignment beads (Molecular Probes, UK) and Fluorospheres (DAKO). A minimum of 10 000 events were gathered for each test. For the determination of CD95 and Fas-L expression, each subset of T cell, either CD4+ or CD8+, were identified by their PE-CY5 (FL-III) expression and side scatter (SS) (Fig. 1). The relevant CD4+/CD8+ T cells were 'bitmapped' and from this bitmap CD95 and Fas-L expression was determined. Using

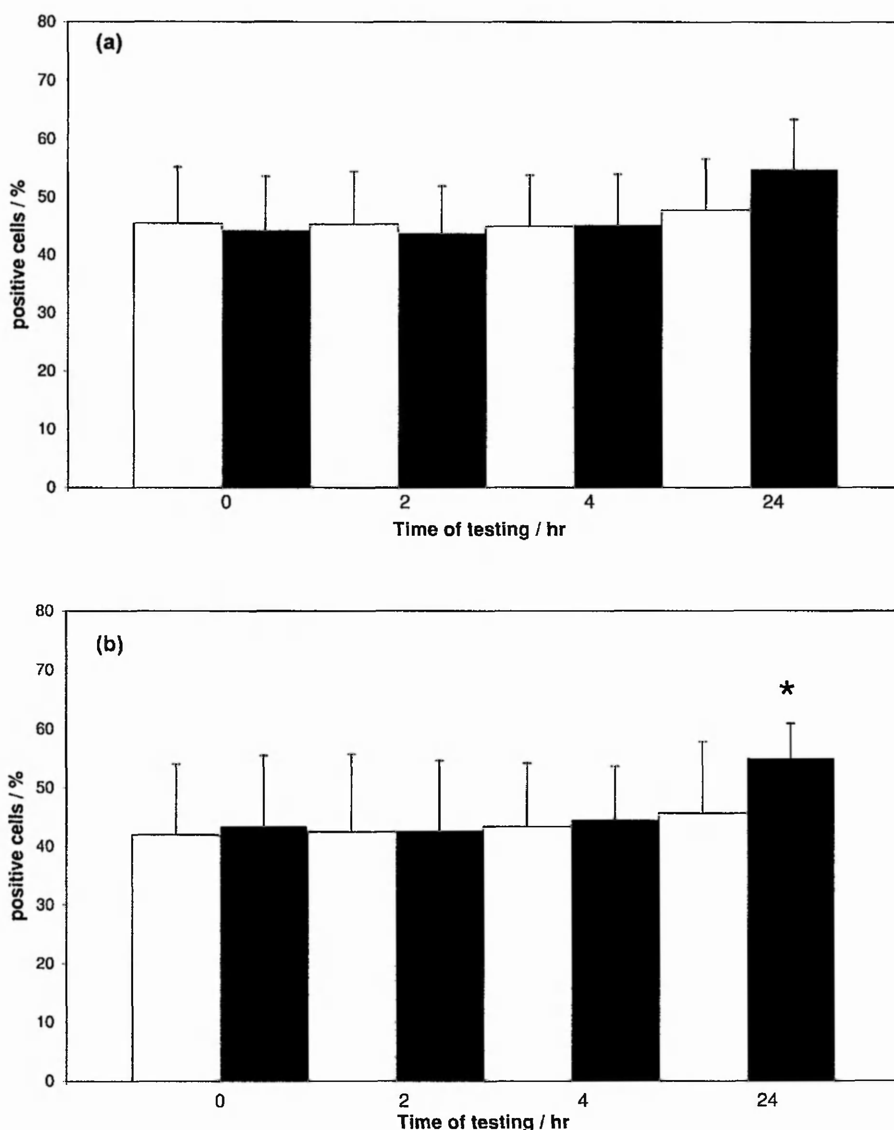


Fig. 4. Mean and standard deviation (S.D.) of (a) CD4+ and (b) CD8+ T cells expressing CD95 after 0, 2, 4 and 24 h pre ECP (white bars) and 0, 2, 4 and 24 h post ECP (black bars). Only in the CD8+ T cell population at 24 h post ECP was a significant increase in CD95 expression detected ($P<0.05$) (denoted by *).

isotype controls 'quadrant gates' were set on the two-dimensional dot plot of PE-CY5 (FLIII) and FITC (FL-I). These 'gates' then determined the percentage of T cell expressing either CD95 or Fas-L (Fig. 2). This process was repeated for samples incubated in cell culture medium. For the determination of CD54, CD80 and CD86 on monocytes, cells were identified by their distinctive CD14 (PE) (FL-II) and SS pattern (Fig. 3). This region was bitmapped and used to identify the CD54, CD80 and CD86 expression. Assessment of background fluorescence was determined with appropriate isotype controls. Using histograms, 'region gates' determined the mean fluorescent intensity (MFI) of CD54 and CD86 expression on the monocytes. For CD80, 'region gates' set using isotype controls determined the percentage of CD80-expressing monocytes. These techniques were repeated to include all pre- and post-testing stages. Following IFNγ stimulation, the MFI of CD80 on pre and post monocytes, was determined using 'region gates' on a histogram plot.

2.9. Statistical analysis

Statistical analysis was performed using paired t-tests. A P value lower than 0.05 was regarded as significant.

3. Results

The effects of ECP therapy on the CD95 expression of treated CD4+ and CD8+ T lymphocytes are demonstrated in Fig. 4. Statistical analysis compared the pre- and post-ECP cells at the same testing stage i.e. 0, 2, 4 and 24 h. Only in the CD8+ T cells at 24 h post ECP was a significant increase in CD95 expression observed ($P<$

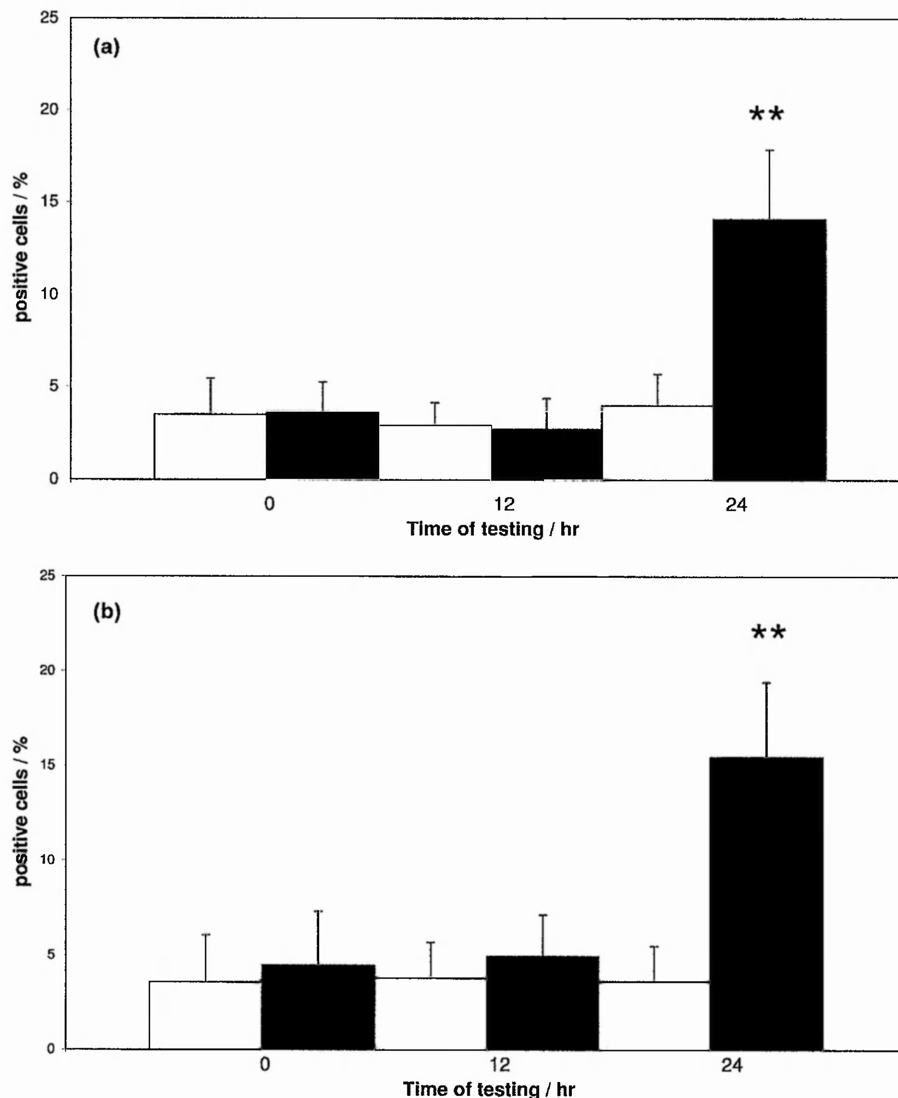


Fig. 5. Mean and S.D. of (a) CD4+ and (b) CD8+ T cells expressing Fas-L after 0, 12 and 24 h pre ECP (white bars) and 0, 12 and 24 h post ECP (black bars). Both T cell populations demonstrate a very significant increase in Fas-L expression 24 h post ECP ($P<0.005$) (denoted by **).

0.05). Subsequent testing for Fas-L expression on the CD4+ and CD8+ T cell populations are demonstrated in Fig. 5. A very significant increase in Fas-L expression on both CD4+ and CD8+ T cells ($P<0.005$) was detected at 24 h post ECP. However, at 0 and 12 h post ECP no significant increase was observed. When comparing CD4+ T cell expression of Fas-L with the levels of Fas-L expressed on CD8+ T cells at the same testing stage no significant difference was detected.

The mean fluorescence intensity (MFI) of CD54 on monocytes placed in culture medium increases over time. The MFI of monocytes tested after 0, 2, 4 and 24 h pre and post ECP are demonstrated in Fig. 6a and displayed as histograms in Fig. 7. Comparisons of each corresponding testing stage demonstrated no significant difference in CD54 expression on monocytes post ECP to those pre ECP. CD86 MFI expression on monocytes post ECP was not significantly different to that on monocytes, at the same testing stage pre ECP (Fig. 6b). The percentage of CD80-expressing monocyte numbers remained unaltered by ECP, for all testing stages (Fig. 8a). Post ECP, inducible CD80 MFI levels, following 24 h of IFNγ stimulation, also showed no significant difference to those of monocytes pre ECP (Fig. 8b).

4. Discussion

Treatment of advanced CTCL or Sezary syndrome using multidrug chemotherapy does not prolong survival and is associated with a substantial degree of morbidity [28]. Initially described by Edelson in 1987 [1], ECP was the first FDA-approved selective immunotherapy for any type of cancer [29]. Treatment of erythrodermic stage CTCL, using ECP, has demonstrated overall response rates between 50 and 65% and complete response rates between 18

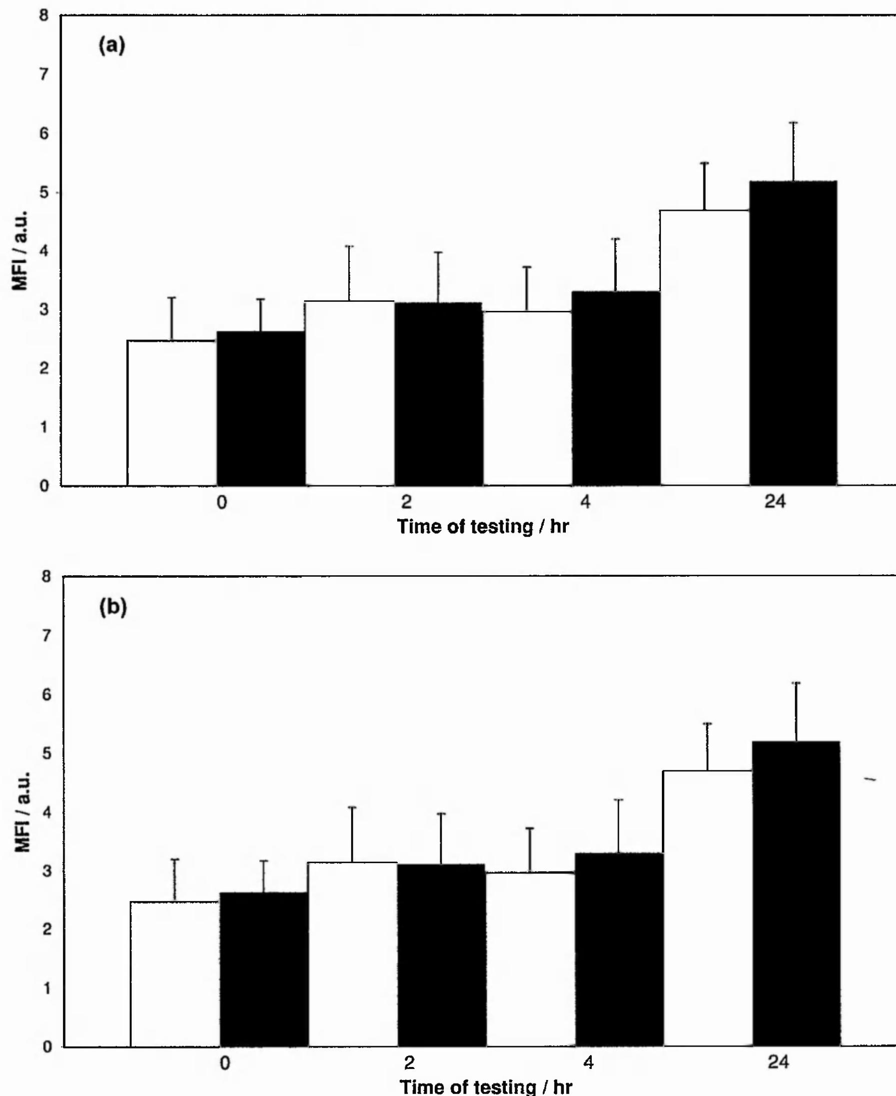


Fig. 6. Mean and S.D. of the mean fluorescence intensity (MFI) (arbitrary units) of (a) CD54 and (b) CD86 on monocytes tested after 0, 2, 4 and 24 h pre ECP (white bars) and 0, 2, 4 and 24 h post ECP (black bars). No significant difference in MFI was observed at each corresponding stage.

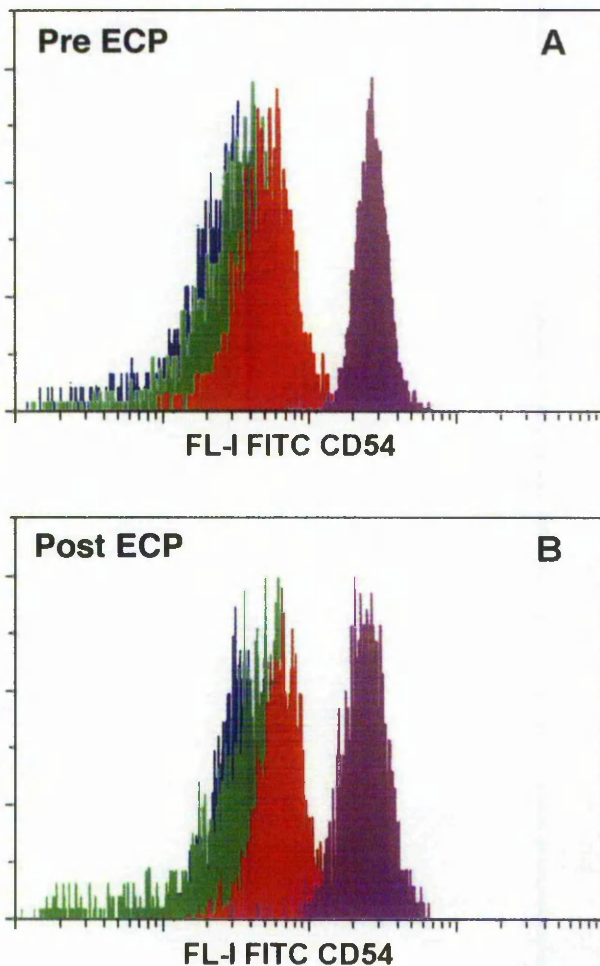


Fig. 7. CD54 expression on monocytes (a) pre and (b) post ECP. Histogram representation demonstrates the increase in CD54 expression over time. At each testing point, post ECP CD54 MFI values were not significantly difference to pre ECP. 0 h (blue), 2 h (green), 4 h (red), 24 h (purple).

and 23% [30,31]. Research indicates an immunomodulatory process for ECP's mechanism of action [29]. Although ECP therapy is only performed monthly and only exposes 3–5% of the tumour load on any given treatment day [30,32], complete responses are observed in 'responders' within 12 months of commencing treatment [5,6]. Good 'responders' demonstrate significantly higher baseline absolute CD8+ T cells and normal natural killer cell activity [5,19,30]. Following ECP, treated lymphocytes become apoptotic [7–10]. However, exposed monocytes are resistant to ECP induced apoptosis [8,33]. ECP treated monocytes demonstrate activation markers, an enhanced avidity for the phagocytosis of apoptotic T cells and secrete cytokines with anti-tumour activity (TNFα) [8,11,12]. Skewing toward either Th1 or Th2 cytokine responses have also been observed following 8-MOP–UVA [13,14,34].

Recently we detected very early induction of apoptotic lymphocytes by ECP, present prior to reinfusion [10]. The majority of treated lymphocytes express apoptotic markers by 48 h post ECP [35]. CD95 (Fas) expression is weakly expressed on nonstimulated peripheral blood lymphocytes [36]. When Fas is crosslinked by Fas-L, caspase 8 (FLICE) is activated, which consequently triggers downstream activation of the caspase cascade [15,37]. The caspase cascade is capable of inducing irreversible apoptosis [38]. The crosslinking of Fas by Fas-L on target cells can occur by transfer from adjacent activated cytotoxic T cells or by expression on the target cell membrane itself (cell suicide) [39]. The CD95 system is critical for growth control of T cells [39]. However, cellular stress-inducing agents can also activate the CD95 ligand–receptor interaction [40]. In CTCL, peripheral blood CD4+ T lymphocytes demonstrate decreased expression of CD95, suggesting disease progression may be due to defective apoptosis, rather than true proliferation [4]. In scleroderma patients, enhanced CD95 expression has been observed on CD4+ T lymphocytes 24 h post ECP [9]. We wanted to establish if ECP enhanced CD95 expression on the lymphocytes of CTCL patients and also if the CD95 pathway was responsible for the early apoptosis observed immediately post ECP [10]. Only a slight increase in CD95 expression on the CD8+ T cells ($P<0.05$) at 24 h post ECP was observed. However, subsequent testing for Fas-L expression noted a very significant increase in Fas-L on both the CD4+ and CD8+ T cells at 24 h post ECP ($P<0.005$). To determine if this process was responsible for the early apoptosis induction observed immediately prior to reinfusion, Fas-L expression was also determined at 0 and 12 h post ECP. At these stages, however, no enhanced expression was detected. This may indicate that the later stage of apoptosis induction, (i.e. 24 h and later) involves Fas-L expression. This was consistent with the later Fas-L expression observed in post ECP T cells by Tambur [33]. Using light scatter to selectively identify 'late' apoptosis (low forward and high side scatter), the number of cells expressing Fas-L were elevated, demonstrating the participation of Fas-L in ECP induced apoptosis (data not shown). The expression of Fas-L, in part, is probably responsible for the later induction of the caspase cascade, observed at 24 and 48 h post ECP [35]. The levels of CD4+ T cells expressing Fas-L at 24 h post ECP was not significantly different to the numbers of CD8+ T cells expressing Fas-L at the same time point. An observation in agreement with our previous observation that the CD4+ T cells of CTCL demonstrate no significant different to CD8+ T cells in susceptibility to apoptosis induced by ECP [41]. Reduction in CD4+ T cells numbers in 'responders' is therefore unlikely to be due to the selective removal of the malignant cells by the direct killing using ECP. More likely is the modulation of the immune system to identify and remove other malignant cells of the same clone [42]. Following exposure to UVA–8-MOP, lymphocytes demonstrate a 2–3-fold increase in cell surface expression of MHC class I molecules. A process that may also induce a

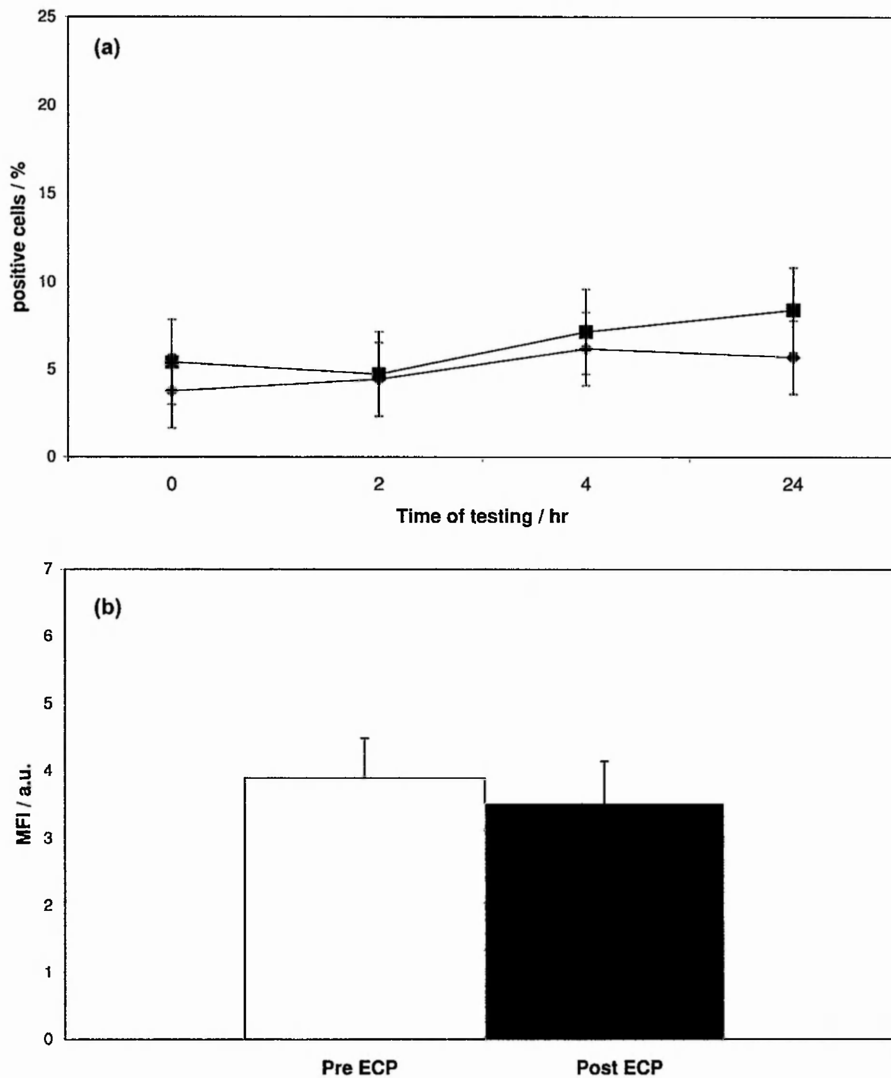


Fig. 8. Percentage of (a) CD80-expressing monocytes at 0, 2, 4 and 24 h pre ECP (black diamonds) and 0, 2, 4 and 24 h post ECP (black squares). No significant difference in expression is observed at each post ECP testing stage. The MFI (arbitrary units) of (b) CD80, following IFNγ stimulation, demonstrates no significant difference between monocytes pre and post ECP.

2–3-fold parallel increase in tumour antigens [32]. Processing of apoptotic lymphocytes displaying enhanced levels of tumour antigens may lead to more effective antigen recognition by selective CD8+ T cells, leading to an anti-clonotypic mechanism capable of removing non-ECP treated cells [17,19,32]. To determine if the activation of the CD95 pathway by ECP was limited by the decreased CD95 expression of the CTCL CD4+ T cells, we tested two patients receiving ECP therapy for graft versus host disease. Both patients demonstrated similar results to those seen for the CTCL patients (data not shown). This finding suggests that ECP induction of Fas-L expression on treated T cells is not specific to CTCL. However with only slight increases in levels of CD95 expression on treated T cells post ECP, the apoptotic process induced by the Fas pathway would only include cells already expressing Fas. Although, using the Vβ family of antibodies to identify clonality, Berger et al. have demonstrated phagocytosed apoptotic CTCL tumour cells within dendritic cells 24 h post ECP [20].

Monocytes are resistant to apoptosis induced by ECP [8,33]; Fas and Fas-L expression on treated monocytes remains unchanged 16 h post ECP [33]. Paradoxically monocytes are activated by ECP, demonstrating enhanced secretion of TNFα and upregulation of CD36 [11,12]. These monocytes also demonstrate an increased ability to phagocytose apoptotic T cells [8]. These changes may indicate that treated monocytes have a role in the immunomodulation process, proposed for ECP. The processing of antigens by APCs ultimately leads to the presentation to T cells and immune recognition [16]. When dendritic cells acquire antigens from apoptotic cells a cytotoxic response against similar antigens is induced [17]. Whilst processing of apoptotic cells by activated monocytes involves antigen presentation to T cells, inducing immune recognition of similar antigens [18]. However, to fully activate and

induce proliferation, the presentation of antigens to T cells requires the presence of co-stimulatory signals [21]. CD54, CD80 and CD86 are important co-stimulatory molecule in this process [22,23]. CD80 and CD86 are thought to activate Th1 and Th2 cells, respectively [24]. However, previous in vitro data demonstrate a loss of CD54 expression in monocytes exposed to UVB radiation [25,26] whilst inducible expression of CD80 and CD86 is decreased following UVB [26]. Comparative studies of pre and post ECP monocytes, tested at 0, 2, 4 and 24 h, show no significant difference in CD54 expression at each testing stage. CD86 and IFNγ induced CD80 expression also remains unchanged following ECP. These results indicate that ECP treated monocytes are not compromised in the antigen presentation processes dependent on these co-stimulatory mechanisms. In addition, the Th1/Th2 responses observed post ECP are not attributable to preferential changes in either CD80 or CD86 expression on treated monocytes.

The discrepancy between our findings and that of other UV treatment modalities may be attributed to the type and strength of UV used. Loss of CD54 on treated monocytes has been observed following exposure to UVB radiation [25]. UVB also induces the loss of inducible CD80 and CD86 expression [26] and apoptosis induction in APCs [21]. ECP-treated monocytes are resistant to all these processes and as such the maintenance of the co-stimulatory antigens may be a consequence of the differing effects of UVB. UVA irradiation reduces the ability of Langerhans cells to present antigens to T cells. A process associated with a loss of CD54, CD80 and CD86 expression [23]. However, this effect is seen at UVA doses in excess of those used for ECP. These factors may indicate that ECP's effect on monocytes is more subtle, leading to fundamentally different response to that observed for other UV therapies.

The manipulation of the immune system by ECP may involve inducing effects in both the mononuclear cells present in the periphery. The apoptotic lymphocytes induced by ECP may, in part, be processed by the activated and immunologically competent monocytes. A procedure, which may lead to the alloreactive T cells, is thought to be responsible for the immunomodulatory effect of ECP [43]. This mechanism would require monocytes that retain the relevant capabilities to perform this task. The retention of co-stimulatory molecules, in addition to a resistance to apoptosis induction, an active phenotype and release of anti-tumour cytokines, indicates that this process is possible.

5. Abbreviations

ECP	Extracorporeal photopheresis
CTCL	Cutaneous T cell lymphoma
8-MOP	8-Methoxypsoralen
UV	Ultraviolet
MHC	Major histocompatibility complex
Th	T helper
TNFα	Tumour necrosis factor α
IFNγ	Interferon γ
Fas-L	Fas-ligand
APCs	Antigen presenting cells
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
FL	Fluorescence
MFI	Mean fluorescence intensity
PE-CY5	Phycoerythrin-cyanin 5
PE	Phycoerythrin
FITC	Fluorescein isothiocyanate
SS	Side scatter
S.D.	Standard deviation

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Lymphocytes Treated by Extracorporeal Photopheresis Demonstrate a Drop in the Bcl-2/Bax Ratio: A Possible Mechanism Involved in Extracorporeal-Photopheresis-Induced Apoptosis

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Key Words
Photopheresis · Bcl-2 · Bax · Apoptosis · Flow cytometry

Abstract
Background: Recently, apoptosis has been identified in treated lymphocytes, prior to their re-infusion, when tested ex vivo. Previous work has demonstrated a close association between the genes p53, Bcl-2 and Bax and apoptosis induced by UV irradiation. ***Objectives:*** We wanted to establish whether the expression of the protein product of these genes was altered in lymphocytes treated with extracorporeal photopheresis (ECP) prior to re-infusion and therefore possibly implicated in the early apoptosis observed. ***Method:*** Lymphocytes were isolated immediately before treatment and immediately prior to re-infusion and tested for intracellular levels of p53, Bcl-2 and Bax proteins. ***Results:*** No increase in p53 expression was observed at re-infusion; however, the mean fluorescent intensity ratio of the apoptotic inhibitor protein Bcl-2 to the apoptosis-inducing protein Bax dropped significantly. ***Conclusion:*** The early apoptosis observed in ECP-treated lymphocytes at re-infusion might be attributed to dysregulation in the expression of the apoptotic genes Bcl-2 and Bax.

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Introduction

Cell death as a consequence of apoptosis differs distinctly from necrosis, as death occurs without the acute inflammatory response [1]. Initially described as a process of cell 'suicide' to maintain cellular homoeostasis within tissues, apoptosis is an essential process for normal development, host defence and oncogenesis [2, 3].

Several genes have been identified in the regulation of apoptosis, through either promotion of cell death or extension of cell survival. The tumour suppresser gene p53 regulates cellular transition through the G_1/S phase. When DNA damage is detected, p53 protein levels increase leading to either cell cycle arrest and repair or apoptosis [4]. In this way, p53 acts to maintain the integrity of the genome, preventing mutagenesis and tumour development [5]. The Bcl-2 family of genes consists of key regulators of apoptosis, with over a dozen members identified [3]. Bcl-2 and Bax are two genes within the Bcl-2 family, which have been very widely researched and have been shown to influence the survival of many cell types, including those of the haematopoietic system [6]. The protein products of Bcl-2 and Bax are able to homo- and heterodimerise with each other, an excess of Bcl-2/Bax heterodimers inhibiting apoptosis whilst Bax/Bax homodimers enhance the apoptosis process [7].

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Extracorporeal photopheresis (ECP) is used in the treatment of conditions mediated by T lymphocytes. ECP treatment involves separating out white cells by leucopheresis and exposing them to 8-methoxypsoralen and UVA irradiation [8]. The exposure of lymphocytes to ECP has been demonstrated to cause DNA damage, inducing delayed apoptosis in treated lymphocytes 24 h after ECP [9–11]. The expression of Bcl-2 protein in the apoptotic lymphocytes of scleroderma patients 24 and 48 h after ECP remained unchanged [11], while enhanced expression of p53 was observed in non-lesional skin of psoriasis patients following PUVA therapy [12]. However, recently ECP has been demonstrated to induce apoptosis in significant numbers of lymphocytes immediately after ECP [13]. We were interested to establish whether this early apoptosis was associated with an increased expression of the p53 protein or dysregulation in the ratio of the intracellular levels of Bcl-2 proteins to Bax proteins. The expressions of the p53, Bcl-2 and Bax proteins were measured using intracellular flow cytometry.

Materials and Methods

Samples were obtained from 9 cutaneous T cell lymphoma (CTCL; 5 stage III and 4 stage IVa), 3 graft-versus-host disease (GvHD) and 1 scleredema patients. Diagnosis for each condition was based on established histological and immunological criteria. All CTCL patients had evidence of clonal disease in the peripheral blood. ECP was performed using the UVAR© (Therakos, UK) according to the standard method reported by Edelson et al. [8]. Peripheral blood samples were taken from the patients immediately before ECP and from the incubated buffy coat bag just prior to re-infusion. Peripheral blood mononuclear cells separated by centrifugation on a density gradient (Lymphoprep, Nycomed, Norway) were washed once with PBS and adjusted to give a final cell count of 1.0–2.0 × 10^6/ml. Fifty microlitres of cells were immediately treated with a 'Fix and Perm' commercial kit (Harlan Sera-lab, UK) and incubated with either 10 μl of relevant isotype controls, FITC-conjugated anti-Bcl-2, FITC- conjugated anti-p53 (Dako, UK) or 2 μg unconjugated anti-Bax (Immunotech, UK) antibodies. The anti-Bax antibody involved a second step using 10 μl of a previously 1:20 diluted FITC-conjugated goat anti-mouse antibody (Immunotech). Each stage involved a 15-min incubation in the dark at room temperature, followed by washing with PBS. Cells were processed through a Coulter Epics Profile II flow cytometer. Lymphocytes were bitmapped using their typical low forward and side scatter. The addition of 5 μl of FITC-anti-CD45 (Dako) and 5 μl RPE-CD14 (Immunotech) to 1 ml of cell suspension verified the position of the bitmap, excluding debris and monocytes, respectively. Using this bitmap 5,000 events were gathered for each test. The mean fluorescent intensities (MFI) of the Bcl-2 and Bax tests were determined using a histogram of log fluorescence 1. The MFI of the relevant isotype control was also validated. The log fluorescence 1 gathered events for the p53 isotype control as a dot plot. Gates were set at the edge of the dot plot for the isotype control from which p53-positive cells were enumerated. Statistical analysis was performed using paired t tests. A p value lower than 0.05 was regarded as significant.

Results

The final MFI for the Bcl-2 and Bax from lymphocytes before ECP and before re-infusion were calculated by subtracting the MFI of the appropriate isotype control from the MFI of the test. The Bcl-2/Bax ratio was calculated by dividing the final MFI of Bcl-2 by the final MFI of Bax. The resultant fall in the Bcl-2/Bax ratio between each stage of ECP for all three patient groups is shown in figure 1a–c.

Statistical analysis on the scleredema patient was not possible as only 1 patient was tested. However, the fall in the Bcl-2/Bax ratio was similar to that observed for CTCL and GvHD.

Lymphocytes from ECP-treated CTCL and GvHD patients demonstrated a moderate but significant decrease in the intracellular Bcl-2/Bax protein ratio before re-infusion ($p < 0.05$), a mean fall of 27.08 and 27.93%, respectively. The scleredema patient demonstrated a 31.1% decrease in the Bcl-2/Bax ratio before re-infusion.

Comparisons of the CTCL and GvHD patients demonstrated no significant difference in the level of Bcl-2/Bax decrease before re-infusion.

For both the CTCL and GvHD patients, no significant increase in p53 expression was observed before re-infusion. No patient demonstrated more than a 2% increase in lymphocytes with a positive p53 expression.

Discussion

ECP has been utilised in many T cell disorders, achieving excellent clinical responses with little or no side-effects [14]. Many processes have been described which may contribute to, what is probable, the multi-factorial process of ECP [for a review, see 15]. One of these processes is the ability of ECP to induce apoptosis in treated lymphocytes [9–11, 13]. Initially described as a delayed-type process, apoptotic lymphocytes have been identified 24 h after ECP [9–11]. However, significant levels of apoptotic lymphocytes have recently been observed immediately after ECP, prior to the re-infusion [13]. We wanted to investigate if this very early induction of apoptosis involved expression or dysregulation of the known apoptosis-associated proteins p53, Bcl-2 and Bax.

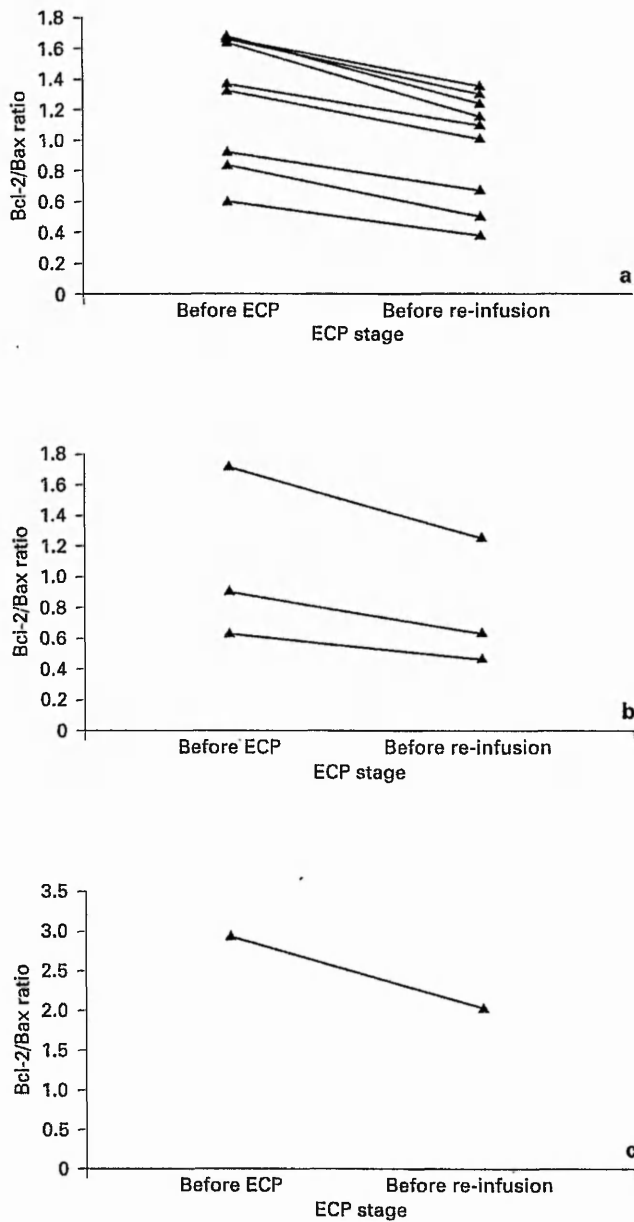

Fig. 1. Demonstrates the Bcl-2/Bax ratio for each patient before ECP and before re-infusion. The line between the points represents the fall in ratio between the two stages of the ECP cycle. **a** contains all the data for the CTCL patients, whilst **b** and **c** contain data for the GvHD and scleredema patients, respectively.

The gene p53 has been closely linked to the induction of apoptosis. Upon detection of DNA damage, protein levels of the tumour suppresser gene p53 increase, leading to either cell cycle arrest and repair or apoptosis, thus preventing mutagenesis and tumour development [4, 5]. Increased wild-type p53 has been elicited in response to DNA damage induced by UV light [16]. Elevated p53 concentrations resulted in apoptosis in normal human peripheral blood lymphocytes [4]. The lymphocytes tested immediately after ECP did not demonstrate any increase in p53, but did however demonstrate a significant fall in the ratio of the Bcl-2/Bax MFI.

The Bcl-2 family of genes consists of key regulators of apoptosis with over a dozen members identified [3]. Bcl-2 exerts its anti-apoptotic effect through several processes including antioxidant activity, preservation of mitochondrial potential and blockage of calcium movement into the cytoplasm [17]. Bax, conversely, promotes apoptosis by inducing the release of cytochrome c from the mitochondria leading to activation of caspase 3 [18]. The protein products of Bcl-2 and Bax are intimately linked, able to homo- and heterodimerise with each other. An excess of Bcl-2/Bax heterodimers inhibits apoptosis whilst Bax/Bax homodimers enhance the apoptosis process [7]. Dysregulation of these genes, as a consequence of stimulation, can influence their expression within the cell, influencing survival [19, 20]. Here we demonstrate a significant fall in the ratio of Bcl-2/Bax in the lymphocytes tested immediately after ECP. Previously UVA-induced apoptosis, associated with no increase in p53 expression but a fall in Bcl-2 expression leaving Bax unaffected, has been termed 'immediate-type apoptosis' [21, 22]. This early apoptosis induced by UVA involves inducing oxidative stress, cell membrane damage and mitochondrial membrane ($\Delta\psi_m$) depolarisation [23]. The externalisation of membrane phosphatidylserine ('flip-flop') [13], in addition to the early depolarisation of the $\Delta\psi_m$, has been detected in ECP-treated lymphocytes immediately before re-infusion (unpublished data). Correlation coefficient values for Bcl-2/Bax ratio reduction and apoptosis induction (detected by annexin V) for CTCL and GvHD patients were 0.91 and 0.99, respectively. For CTCL patients, this correlation was very significant ($p < 0.005$); however, for GvHD, no p value was determined due to insufficient data points.

Previous studies of ECP-induced apoptosis noted no significant change in Bcl-2 expression after ECP treatment [11]. This could be because what Aringer et al. [11] were measuring in 24- and 48-hour cultures was delayed-type apoptosis, a pathway associated with an increase in p53 expression. This suggestion is supported by the up-regulation of the p53 gene detected in cultured ECP-treated lymphocytes (unpublished data taken from [10]).

This study demonstrates that the lymphocyte apoptosis detected immediately prior to re-infusion in ECP-treated cells involves the dysregulation of the Bcl-2 and Bax proteins but no enhancement of p53 protein expres-

sion, a process similar to that previously termed immediate-type apoptosis. Apoptosis induced in this way possibly complements the apoptosis in the delayed-type process, previously described [9–11]. An important ECP mechanism may therefore be the immunomodulation induced by the immediate and sustained exposure of apoptotic lymphocytes.

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