PREVENTION OF SKIN ALLOGRAFT REJECTION BY PHOTODYNAMIC THERAPY (PDT) USING BENZOPORPHYRIN DERIVATIVE MONOACID RING A (BPD) VERTEPORFIN

by

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Abstract

The effect of photodynamic therapy (PDT) using the photosensitizer, benzoporphyrin derivative monoacid ring A (BPD) verteporfin which absorbs strongly at 690 nm, on murine skin allograft rejection was tested using a unique approach. Skin sections (C57BL/6) were exposed *in vitro* to varying doses of BPD (0.125 - 1.0 μg/mL) and 10 J/cm^2 light at a wavelength of 690 nm prior to implantation onto recipients (BALB/c). We found that pretreatment of skin to be grafted with PDT can significantly prolong the survival of allografts from 9.3 (n = 42) $\pm$ 2.2 days (control group) to 16.9 (n = 20) $\pm$ 1.7 days (treated group). Higher doses of BPD did not result in longer survival of the skin allografts. Rather, the most beneficial effects of the treatment were observed at lower doses of BPD (0.25 - 0.5 μg/mL) as opposed to 1.0 μg/mL and light (10 J/cm^2, 690 $\pm$ 10 nm wavelength). Our strategy was, therefore, termed low-dose PDT to reflect the fact that we used low doses of BPD and visible light, doses where, even at the highest level, the histological features of pre-treated donor skins revealed no obvious tissue damage.

In order to identify mechanisms by which pretreatment of donor skin could effect anti-allograft immune responses, we investigated the effect of low-dose PDT on the antigen presenting cells of the epidermis, the Langerhans cells (LC). Using flow cytometry, we found that the levels of expression of the major histocompatibility complex (class I and II) and the costimulatory (B7-1 and B7-2) molecules on LC isolated from treated skin was substantially reduced (60 - 90 % reduction) in comparison to the control preparations. On the contrary, the levels of the leukocyte common antigen (CD45), the adhesion molecule

(ICAM-1), the endocytic receptor (DEC-205), the ectophosphatase intensities on LC, as well as LC viabilities, were unchanged. Furthermore, the ability of LC to stimulate the proliferation of naive or pre-sensitized alloreactive T cells was impaired. Finally, the histology and immunohistology of graft tissues from graft recipients revealed that this treatment kept the levels of inflammatory cellular infiltration into the graft low compared to the control grafts. Our findings suggest that the immunomodulatory effects of low-dose PDT of tissue grafts associated with extended engraftment may depend on a selective effect upon epidermal cells, especially LC and may not require cell depletion in order to permit the acceptance of skin allograft. Since the engagement of T cell receptors (TCR) in the absence of costimulation results in suboptimal activation of T cells and ultimately anergy, it appears that the immunomodulatory effects of low-dose PDT depends, in part, upon decreased expression of MHC and costimulatory molecules.

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APC	antigen presenting cells
BODIPY®	8-chloromethyl-4,4-difluoro-1,3,5,5-tetramethyl-4-bora-3a,4a-diazaindacene
BPD	benzoporphyrin derivative monoacid ring A verteporfin
Con A	concanavalin A
CsA	cyclosporine A
DC	dendritic cells
DETC	Dendritic epidermal TCR-$\gamma\delta^+$ T cells
EC	epidermal cells
FACS	fluorescent activated cell sorter
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HSA	heat stable antigen
ICAM	intercellular adhesion molecule
KC	keratinocyte
LC	Langerhans cells
LED	light emitting diode
MECLR	mixed epidermal cell-lymphocyte reaction
MHC	major histocompatibility complex
MLR	mixed leukocyte response
MTT	3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyl tetrazolium bromide
NLDC	non-lymphoid dendritic cell
PBS	phosphate buffered saline
PDT	photodynamic therapy
PE	R-phycoerytherin
UV	ultraviolet radiation

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Dedication

In loving memory of my father Mr. James U. Obochi,

And to my mother, Mrs Esther O. Obochi,

whose teaching and guidance, sacrifice and love equipped me for a better tomorrow.

"I can do all things through Christ which gives me strength" - Phil. 4:13.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Cutaneous immunology.

Contrary to earlier opinions of the skin as an inert barrier, it is clear now that the skin is a major arm of the immune system (Streilein, 1983; Bos and Kapsenberg, 1993). The skin can initiate immune reactions because of its endowment with different antigen presenting cells (APCs), lymphocytes, cytokines and other inflammatory mediators (McKenzie and Saunder, 1990; Luger and Schwarz, 1990a & b; Opas *et al.*, 1985). As a result the concept of skin immune system has been suggested and defined as the cutaneous complexity of interacting immune response-related cells (Bos and Kapsenberg, 1993). The cellular component of the cutaneous immune system is made up of the Langerhans cells (LC), keratinocytes (KC), dendritic epidermal TCR-$\gamma\delta^+$ T cells, epidermal-homing T cells, melanocytes and fibroblasts.

a. Langerhans cells (LC).

The Langerhans cells are specialized, highly potent antigen-presenting cells (APC) of bone marrow origin (Romani *et al.*, 1990; Katz *et al.*, 1979). They are the skin-specific members of the dendritic cell (DC) family (Steinman, 1991; Stingl *et al.*, 1993). LC are found in the suprabasal layer of the epidermis (Mommaas *et al.*, 1994), where they are normally the only cell type that express the major histocompatibility complex (MHC) class II antigens (Inaba *et al.*, 1990; O'Doherty *et al.*, 1993).

Morphologically, these cells are distinguishable by their dendritic morphology and a unique organelle, the Birbeck granule (Stingl *et al.*, 1980; Streilein and Bergstresser, 1984). They are recognizable by virtue of the intense formalin-resistant adenosine

triphosphatase (ATPase) staining displayed on their cell membrane (Mackenzie and Squier, 1975). ATPase staining has been used extensively and reliably as a histochemical marker for these cells both *in situ* and in suspension (Mackenzie and Squier, 1975; Cormane and Kalsbeek, 1963; Wolff and Winklemann, 1967). The enzyme is located on the plasma membrane with its active site facing the exterior, thus its designation as membrane ATPase (mATPase) and an ectoenzyme (Wolff and Winklemann, 1967). LC lack most B and T cell markers and have none or low levels of macrophage/granulocyte markers (Agger *et al.*, 1990). However, LC express the F4/80 macrophage antigen (McKnight *et al.*, 1996).

As potent APCs, LC express a number of surface proteins that are vital in immune responses. LC constitutively express high levels of MHC class II antigens (previously known in mouse as immune-response-associated (Ia) antigens) unlike monocytes/ macrophages on which the levels can be induced by cytokines such as interferon-gamma (IFN-γ) (Unanue, 1981; Beller *et al.*, 1980; Beller, 1984). Murine epidermal LC express medium to high levels of MHC class I molecules (Sharrow *et al.*, 1994; Rosenberg *et al.*, 1986; Caughman *et al.*, 1986; Witmer-Pack *et al.*, 1988; Lenz *et al.*, 1989). LC also express intergrins such as α^M subunit (CD11b) and adhesion molecules such as intercellular adhesion molecule (ICAM)-1 (CD54) (Cooper *et al.*, 1993) and ICAM-3 (CD50) molecule (Teunissen *et al.*, 1995). In addition, freshly isolated LC express a moderate amount of the B-7 costimulatory molecules (Rattis *et al.*, 1995) whose expression is up-regulated upon a short-term *in vitro* culture or by cytokines (IL-1 and GM-CSF). Moreover, LC have receptors for endocytosis (IgG-Fc receptor and DEC-205 recognized by the non-lymphoid dendritic cell (NLDC)-145 antibody (Cooper *et al.*, 1993). All these surface proteins

contribute to the efficiency of LC in phagocytosis, processing and presentation of antigens to both naive and activated T cells.

LC are considered precursors of dendritic cells which are found in the draining lymph nodes (Heufler *et al.*, 1992). LC mature into DC during migration from the epidermis to the draining lymph nodes or in short term *in vitro* cultures. Following skin sensitization, epidermal LC are stimulated to migrate via the afferent lymphatics to the draining lymph nodes (Kripke *et al.*, 1990). While in transit, LC acquire the characteristics of mature dendritic cells expressing increased levels of ICAM-1 (Cumberbatch *et al.*, 1992), MHC class I and II antigens (Shimada *et al.*, 1987) B7-1 and B7-2 expression (Inaba *et al.*, 1994), and heightened ability to activate naive T cells (Aiba and Katz, 1990; Romani *et al.*, 1989). During short term (2 - 3 days) *in vitro* culture, murine LC develop a marked dendritic morphology, exhibit enhanced surface expression of MHC molecules (Shimada *et al.*, 1987), B-7 molecules (Rattis *et al.*, 1995) and ICAM-1 (Tang and Udey, 1991) and acquire enhanced immunostimulatory function (Shimada *et al.*, 1987; Schuler and Steinman, 1985; Aiba and Katz, 1990). The maturation of LC, mimicked in short-term *in vitro* cultures, presumably is as a result of the production of lymphokines necessary for LC maturation (i.e. IL-1 and GM-CSF) (Heufler *et al.*, 1988; Steinman, 1991; Aiba and Katz, 1990).

The functional capabilities of LC vary with their "maturation state" (Streilein and Grammer, 1989; Pure *et al.*, 1990). Freshly isolated LC have modest phagocytic activity but can effectively process and present peptide antigens. They have a moderate capacity to activate naive T cells and profound ability to activate primed T cells (Xu *et al.*, 1995c).

During their maturation in short-term cultures, LC lose the capacity to process protein antigen but become potent activators of naive allospecific T cells. LC as well as DC have been shown to induce allogeneic T cell proliferation and antigen-specific T cell activation, clustering efficiently with T cells (Girolomoni *et al.*, 1990; Greenberg *et al.*, 1985; Stingl *et al.*, 1980; Pehamberger *et al.*, 1983; Young and Steinman, 1990). This property is attributable, at least in part, to the constitutive expression of high levels of MHC and various accessory molecules (Paglia *et al.*, 1993). It seems that these changes may be necessary adaptations for their *in vivo* function - naive LC in the skin encounter new protein antigens that they process and present on their cell surfaces; they then mature and traffic to a regional draining lymph node. There, they maintain the processed protein antigen on their cell surfaces while screening large numbers of naive T cells for any that are reactive against the original protein antigen (Larsen *et al.*, 1990a; Kripke *et al.*, 1990; Bergstresser *et al.*, 1992). LC, though they represent a minor (3-6 %) cell population of the epidermis (Mommaas *et al.*, 1994), are critical for both the afferent and efferent limbs of contact hypersensitivity and transplantation rejection (Aiba and Katz, 1990; Cruz *et al.*, 1990; Kripke *et al.*, 1990).

b. Keratinocytes.

Keratinocytes (KC) comprise over 95 % of the cell population in the epidermis (Nickoloff and Turka, 1993). KC, while seen historically as structural cells, are now known to play important roles in cutaneous immune function (Nickoloff and Turka, 1993; Nickoloff, 1995).

KC produce pro-inflammatory and immunoregulatory cytokines which can be

released in response to a number of insults including injury, microbial attack or ultraviolet (UV) radiation (Matsue *et al.*, 1992; Schwartz and Luger, 1989). KC have the ability to release quite a number of cytokines including interleukin (IL)-1, IL-3, IL-6, IL-7, IL-8, IL-10, growth factors, including tumor necrosis factor-alpha (TNF-α), colony-stimulating factors (CSF), including granulocyte-macrophage colony-stimulating factor (GM-CSF), and chemotactic factors, including leukotriene B_4 (LTB_4) (Grabbe *et al.*, 1984; Kupper, 1989; Sauder, 1990; Schwartz and Luger, 1992). Murine KC do not normally produce IL-12 (Nickoloff *et al.*, 1995) but produce and secrete IL-12 after treatment with allergens (Aragne *et al.*, 1994; Muller *et al.*, 1994). However, IL-12 is expressed and released by human KC (Aragne *et al.*, 1994). The released cytokines are vital in communication between local and systemic immune responses such as the recruitment and activation of the effector cells of the immune response (Kupper, 1993).

KC cannot process antigens and usually they do not express MHC class II molecules. But, relatively low levels of class II molecules can be induced after IFN-γ treatment *in vitro* (Wikner *et al.*, 1989; Nickoloff *et al.*, 1986) or during inflammatory states *in vivo* (Nickoloff and Turka, 1994). Similarly, cytokine treatment can induce KC to express ICAM-1 but not B7-1 and B7-2 (Nickoloff and Turka, 1994; Nickoloff *et al.*, 1995). Thus, it appears that certain conditions may equip KC to function as accessory cells and to regulate T cells. Three distinct outcomes, depending on the nature of the stimulus, have been identified (Nickoloff *et al.*, 1995). In the presence of alloantigen, it seems that a "null" event takes place between T cells and KC (Nickoloff *et al.*, 1995). There is neither activation nor induction of tolerance. Using allergens, KC induce antigen specific tolerance

(Nickoloff *et al.*, 1995). In contrast, in the presence of bacterial-derived superantigens, phytohemagglutinin or immobilized CD3 monoclonal antibody, KC can significantly activate resting autologous T-cell proliferation and cytokine release (Nickoloff *et al.*, 1995). KC can therefore directly or indirectly regulate resting T-cell proliferation and activation, with both positive and negative consequences and thus, play a significant role in skin allograft rejection.

c. Dendritic epidermal TCR-$\gamma\delta^+$ T cells (DETC).

DETC, identified in the last decade as macrophages (Smolle *et al.*, 1985), are moderately dendritic cells with a CD45-positive phenotype. DETC neither express CD4 nor CD8 surface molecule (Nixon-Fulton *et al.*, 1988). Furthermore, these cells are characteristically very homogeneous with a vast majority expressing Vγ3/δ3 TCR with no junctional diversity (Bergstresser *et al.*, 1993). Functionally, they have been shown to proliferate *in vitro* in response to T cell mitogens, including Con A, anti-CD3 monoclonal antibody and phorbol ester (Takashima *et al.*, 1988; Nixon-Fulton *et al.*, 1988; Havran *et al.*, 1989). It has been demonstrated that DETC produce a variety of cytokines, including keratinocyte-specific growth factor (Boismenu and Havran, 1994), upon activation (Matsue *et al.*, 1993). Several groups have reported that DETC may have a non-MHC-restricted cytotoxicity against tumor targets (Nixon-Fulton *et al.*, 1988; Okamoto *et al.*, 1988; Havran *et al.*, 1989). Furthermore, DETC were found to be stimulated by contact with keratinocytes (Havran *et al.*, 1991) or by contact with stressed epidermal cells (EC) *in vitro* (Lewis and Tigelaar, 1991). Topical application of a chemical irritant was shown to increase the density of DETC *in situ* (Lewis and Tigelaar, 1991) and increased proliferation *in vitro* in response

to IL-2 (Kaminski *et al.*, 1993). The implication of these findings may be that stimuli that produce epidermal stress may induce relevant ligand(s) on neighbouring keratinocytes; thus leading to the activation of DETC. This would point to a role for DETC as effector cells in the immune surveillance of the skin. The fact that they can produce a variety of cytokines (Matsue *et al.*, 1993) suggests that DETC may exhibit immunomodulatory activity. Similarly, DETC may be involved in wound healing since they produce keratinocyte-specific growth factor which selectively induces keratinocyte proliferation and differentiation in tissue culture (Pierce *et al.*, 1994).

d. Other cells.

Other cells that may play a role in the immune function of the skin include the melanocytes, which produce melanin; the fibroblasts, in the dermis, which have a role in extracelluar matrix synthesis; the dermal endothelium; and the epidermal-homing T cells. Both melanocytes and fibroblasts can synthesize and secrete cytokines, and thereby play a role in immune function (McKenzie and Saunder, 1994). Dermal endothelium is another cell type that may play a role in the skin immune function. Adhesion molecules are induced on many cell types including endothelial cell surfaces when these cells are stimulated by cytokines, mainly interleukins (IL-1, IL-6) and interferon-gamma (IFN-γ) (Albelda and Buck, 1990). Adhesion molecules play a key role in the trafficking pattern of APC and leukocytes into damaged tissues because they allow circulating leukocytes to adhere to the endothelial wall and diapedese to the injured area. TCR-$\alpha\beta^+$ T-cells play a significant role in the skin immune system. They circulate through the epidermis and home in on areas of antigenic stimulation by a combination of chemotactic gradients and transient attachments

to adhesion molecules on endothelial cells and KC in inflamed areas (Nickoloff *et al.*, 1990; Swerlick and Lawley, 1993).

1.2 Transplantation immunology.

Organ and tissue transplantation have come a long way from the uncertain and often unsuccessful attempts in the 1950s and early 1960s to become a major form of definitive treatment for many kinds of organ failure. Skin autografts are routinely used to cover injured tissues in burn patients, and corneal transplantation has restored sight to thousands of persons. Heart and kidney transplantation are no longer considered experimental procedures, and transplantation of bone and connective tissues is routinely performed to correct orthopedic deformities. Bone marrow transplantation is a common treatment for aplastic anemia and leukaemia and is often beneficial in patients with certain immune deficiency syndromes (Fischer *et al.*, 1990). Successful liver transplantation has been beneficial in patients with a variety of parenchymal and congenital diseases of the liver and has been used to correct a number of metabolic diseases (Ascher, 1988; Starzl *et al.*, 1989). Transplantation is at a threshold of routine treatments such as lung or heart-lung transplantation for interstitial lung diseases or emphysema (Grossmann *et al.*, 1990; Theodore and Lewiston, 1990) and pancreas or islet-cell transplantation for diabetes mellitus (Bilous *et al.*, 1989; Lancet Editorial, 1990).

a. Transplantation nomenclature.

Based on the genetic relationship between the donor and the recipient, four types of

grafts are defined.

i. Autograft. This is a graft from one part of an animal's or patient's body to another. Skin grafts from healthy sites are often autografted in burn victims, and autogeneic bone marrow is often collected while leukaemia patients are in remission and then infused to rescue the patient from aplasia after irradiation or chemotherapy of a relapse. Coronary patients may benefit from bypassing a partially blocked artery with a section taken from one of the leg veins.

ii. Syngraft (isograft). This is a graft exchanged between genetically identical subjects, such as monozygotic twins or inbred laboratory animals. Except for the genetic differences associated with sex, the members of an inbred strain have identical genomes, and grafts between them are successful. There are a large number of inbred mouse strains available for experimental work.

iii. Allograft (homograft). This is a graft between genetically non-identical (non-syngeneic) members of the same species. With the rare exception of grafts between monozygotic twins, virtually all clinical transplantation involves allografts. The success of allografting rests upon preventing (with immunosuppressive agents) the immune system of the recipient from rejecting the graft it recognizes as non-self.

iv. Xenograft (heterograft). This is a graft of tissue or organs between members of different species. It has been known since early in the century that xenografts will rapidly and inexorably fail due to hyperacute rejection (Giles *et al.*, 1970a; Auchincloss, 1988). The xenogeneic transplantation have proved extremely difficult due to the rapidity with which xenografts are rejected. However, there is still interest in the

possibility that xenogeneic tissues and organs may help relieve the chronic shortages of organs for clinical transplantation. The clinical experience in xenografts to date has involved primarily renal (Reemtsma, 1968) and to some extent liver (Starzl, 1993; Starzl *et al.*, 1993a; Starzl *et al.*, 1974; Starzl, 1969; Porter, 1969; Giles *et al.*, 1970b), heart (Hardy and Chavez, 1968; Barnard, 1975; Jonasson and Hardy, 1985; Girstenbrey, 1984) and bone marrow transplants (Ildstad, 1996; Fricker, 1996; Taylor, 1996; Steele, 1996, Anonymous (Nature), 1996) from chimpanzee and baboon donors. These studies demonstrated clearly that the transplanted organ was capable of function in the human recipient; however, the patient and graft survival in general have been poor (Deodhar , 1986).

b. Transplantation antigens.

When a tissue or an organ is grafted from one individual to another, there are two possible outcomes. In some cases, the grafted tissue survives and functions normally. In others, the immune system mounts an anti-graft immune response and subsequently destroys the graft. This process of anti-graft immune response is called rejection (Abbas *et al.*, 1991). Rejection has remained a major hurdle in clinical transplantation despite the development of various immunosuppressive therapies. The success or rejection of a transplanted tissue or organ by an immunologically competent recipient is determined by the degree of disparities amongst the glycoproteins encoded by genes of the MHC (Bradley *et al.*, 1992). The MHC, discovered in the 1940s, is a large genetic region of highly polymorphic genes whose products are expressed on the surfaces of most cells (Abbas *et al.*, 1991). This genetic region is located at position p21 of chromosome 6 in humans and on chromosome 17 in mice (Rammensee *et al.*, 1993). The proteins encoded by these genes,

commonly referred to as "MHC molecules" or "MHC antigens", are essential for host defense mechanisms and immune competence. They are the principal determinants of graft rejection. Thus, individuals who express the same MHC molecules may accept tissue grafts from one another (syngraft), and individuals who differ at their MHC loci vigorously reject such grafts (allograft).

The loci of the MHC (important in alloresponses) fall into one of two classes, Class I or Class II, based on their tissue distribution, the structure of the expressed antigens, and their functions. Class I antigens are expressed on virtually all nucleated cells. They are composed of two polypeptide chains designated α (heavy) which is polymorphic and a non-polymorphic β_2-microglobulin (Bjorkman *et al.*, 1987). Class I antigens are known as HLA-A, -B, -C, (HLA-E, -F, and -G whose significance in transplantation immunology is currently unknown) in humans and as *H*2K, *H*2D, and *H*2L in mice (Geraghty, 1993; Ting and Baldwin, 1993). Class I molecules serve as recognition units which present peptide antigens to antigen-responsive T lymphocytes ($CD8^+$ T cells) (Ting and Baldwin, 1993). They determine the specificity of attack on target cells, such as virus-infected, tumor, and allogeneic cells by these T cells.

Class II antigens are encoded by multiple genes in the HLA-D region of the MHC in humans and the I (I-A and I-E) loci of mouse *H*2 region (Monaco, 1993). They are typically expressed only by antigen-presenting cells (monocytes/macrophages, Langerhans cells of the epidermis, and dendritic cells of lymphoid organs) B lymphocytes, and thymic epithelial cells (Schwiebert *et al.*, 1995). Other cells (T lymphocytes, airway epithelial cells, fibroblasts and vascular endothelial cells) may express MHC-II molecules when activated

by cytokines or certain infectious agents (Londei *et al.*, 184). However, such induced expression of MHC class II molecules may allow these cells to present antigens and, thereby, permit the generation and exacerbation of a variety of immune and inflammatory disorders (Schwiebert *et al.*, 1995). Class II antigens serve as "restriction elements" in the interaction between APC and antigen-responsive T cells ($CD4^+$ T cells) (Mengle-Gaw and McDevitt, 1985). Furthermore, they mediate a wide variety of immunological phenomena, including activation of lymphocytes which leads to the production of lymphokines, expression of delayed-type hypersensitivity (DTH), and T lymphocyte "help" that is necessary for optimal antibody production by B lymphocytes and the proliferation of effector T cells. Similarly, they stimulate allogeneic T lymphocytes to proliferate in mixed leukocyte reaction (MLR). There is increasing evidence (Shokes and Wood, 1994; Auchincloss and Sachs, 1989) that both class I and II antigens can be processed by antigen presenting cells and presented as allo-peptides during rejection reactions.

c. Mechanisms of allorecognition.

Although it has been fairly well established that T lymphocytes recognize nominal antigens as processed peptides presented by self MHC molecules (MHC restriction), the exact nature of the alloreactive T cell response is not yet clearly understood (De Palma and Gorski, 1995). Over the past few years, knowledge about alloantigen recognition and the activation of alloreactive T lymphocytes has increased remarkably. Presently, there is evidence of two pathways of allorecognition (Auchincloss and Sachs, 1989; Shokes and Wood, 1994; Benham *et al.*, 1995; Gallon *et al.*, 1995).

i. Direct pathway. In the direct pathway of allorecognition, T cells

recognize intact allogeneic MHC molecules on the surface of target cells (Eckels, 1990). This form of antigen recognition may be responsible for the strong proliferative response to allo-antigens seen *in vitro* and the events leading to the early acute rejection of MHC-mismatched skin grafts (Shokes and Wood, 1994). *In vitro*, DC are unique in their capacity to trigger the proliferation of T cells in primary mixed lymphocyte cultures (Mason *et al.*, 1981). Rat renal allografts, rendered less immunogenic by depletion of the passenger leukocytes, could be rapidly rejected after the administration of donor strain DC (Lechler and Batchelor, 1982). The administered donor strain DC are potent APC with high expression of MHC molecules and T cell costimulatory molecules (Austyn, 1992; Larsen *et al.*, 1992). Furthermore, Braun *et al.*(1993) have shown that T cells that recognize donor MHC molecules by the direct route can initiate early acute rejection of newly transplanted rat renal allografts, but not the rejection of kidneys that have survived for more than 50 days before T cells are administered.

ii. Indirect pathway. A basic premise for indirect presentation of allo-peptides as a mechanism for the initiation and/or amplification of rejection process is that donor MHC and minor histocompatibility antigens are shed from the graft and taken up by recipient APC. This pathway is identical to the route taken by conventional antigens, i.e., cell fragments are phagocytosed, processed into small peptide fragments and presented by the recipients' own antigen presenting cells (self MHC-restriction). Thus, it is generally thought that T cells recognize MHC allo-peptides that have been processed and presented by self APCs (Benichou *et al.*, 1992; Fangmann *et al.*, 1992; Lui *et al.* 1992) and may lead to the activation of T helper (Th) cells. The activated Th cells then secrete cytokines and

provide the necessary signals for the growth and maturation of effector cytotoxic T lymphocytes (CTL) leading to allograft rejection (Watschinger *et al.*, 1994; Benham *et al.*, 1995). This has been shown to occur *in vitro* for both allogeneic MHC class I and class II molecules (Emerson and Cone, 1982) and *in vivo* using synthetic class II MHC allo-peptides (Benichou *et al.* (1992).

d. Effector mechanism of allograft rejection.

i. Effector T cells. The polymorphic forms of the individual loci of the MHC have been recognized by antibodies and by various *in vitro* techniques that measure T lymphocyte recognition. These responses, mediated by the recipient's recognition of polymorphism in the donor, correlate with the strong rejection reactions that take place *in vivo*. The major effector mechanism of allograft rejection has antigen-specific and antigen-nonspecific components (Rosenberg and Singer, 1992). Although the details of the effector mechanisms mediating allograft rejection remain controversial (Hall and Dorsch, 1984; Mason *et al.*, 1984; Steinmuller, 1985; Mason and Morris, 1986; Auchincloss and Sachs, 1989; Hall, 1991), cytotoxic $CD8^+$ T cells likely perform the antigen-specific arm while inflammatory cells or their secretory products are responsible for the antigen-nonspecific component. Investigation into the cellular basis of graft rejection, using both *in vitro* and *in vivo* studies, has revealed that both class I and II antigens can directly act as stimuli for rejection reactions such that both $CD4^+$ and $CD8^+$ T cells can mediate graft rejection alone or in concert (Auchincloss and Sachs, 1989).

Examples of effector function by $CD4^+$ T cells include cytotoxic activity against target cells bearing MHC class II antigens *in vitro* (Jacobson *et al.*, 1984; Golding *et al.*,

1985; Lancki *et al.*, 1991) and triggering allograft rejection (Rosenberg *et al.*, 1986; Hao *et al.*, 1987; Gill *et al.*, 1989) and lethal graft versus host disease (GVHD) *in vivo* (Korngold and Sprent, 1985; Carmody *et al.*, 1987). Conversely, there are a number of examples of the effector functions by $CD8^+$ T cells. Studies from a number of laboratories have shown that purified $CD8^+$ T cells can proliferate autonomously to allogeneic stimuli both *in vitro* (Sprent *et al.*, 1986; Inaba *et al.*, 1987) and *in vivo* (Sprent *et al.*, 1986). $CD8^+$ T cells also have been shown to be capable of synthesizing and releasing a number of cytokines, including interleukin (IL)-2 (Andrus *et al.*, 1981; Kelso and Glasebrook, 1984; Gill *et al.*, 1987; Rosengerg *et al.*, 1988). Further, $CD8^+$ T cells have been shown to be sufficient for the activation and expression of skin-allograft rejection (Rosenberg *et al.*, 1986; Rosenberg and Singer, 1992), lethal GVHD (Korngold and Sprent, 1985) and viral immunity (Doherty *et al.*, 1992) *in vivo*. During skin graft rejection solely due to class I disparity, only $CD8^+$ T cells were observed to infiltrate the graft epithelial tissue (Kobayashi and Fujiwara, 1992). Furthermore, selective depletion of each cell type with monoclonal antibodies suggested the exclusive involvement of $CD8^+$ in the rejection of class I disparate skin grafts (Wheelahan and McKenzie, 1987; Smith *et al.*, 1988; Hasenkrug *et al.*, 1992).

Nevertheless, many investigators still argue that help may be generated predominantly by the $CD4^+$ population whereas the majority of the cytolytic activity resides within $CD8^+$ cells (Pilarski, 1977; Keene and Forman, 1982; Raulet and Bevan, 1982; Wagner *et al.*, 1982). Unfortunately, it is difficult to differentiate T-helper (Th) from T-cytotoxic (Tc) cells since both $CD4^+$ and $CD8^+$ T-cell populations may contain different proportions of Th and Tc (Golding and Singer, 1985; Golding *et al.*, 1987). Taken

together, these studies indicate that $CD4^+$ and $CD8^+$ T cells share overlapping functional capacities (Widmer and Bach, 1981; Roopenian *et al.*, 1983; Rosenberg and Singer, 1992). However, it is possible that there are a number of different mechanisms that contribute to the rejection of fully-incompatible skin grafts and they may contribute to differing degrees, depending upon the circumstances. For example, depletion of $CD4^+$ or $CD8^+$ T cells with monoclonal antibodies is not successful in prolonging the survival of grafts with class I plus class II incompatibilities or with *H2* plus non-*H2* incompatibilities (Wheelahan and McKenzie, 1987). But, nude mice reject grafts expressing all types of antigens if reconstituted with $CD4^+$ or $CD8^+$ cells alone as well as with both cell types together (Rosenberg *et al.*, 1987). Similarly, $CD4^+$ or $CD8^+$ cells adoptively transferred into severe combined immunodeficiency (scid) mice mediate rejection of allografts with full mismatch (incompatibilities in all types of antigens) (Shelton *et al.*, 1992).

ii. Cytokines and regulation of T-cell responses. Another important area of consideration in effector mechanisms of allograft rejection relates to the role of cytokines in regulating immune responses. One could infer from the studies of effector T cell in allograft rejection that the T helper cells drive the all-or-none response of cytotoxic T cells, which then mediate graft rejection. However, increasing understanding of the cytokine network requires that we consider both quantitative and qualitative properties of immune responses. The variable cytokine-producing potential of activated T cells (both $CD4^+$ and $CD8^+$), as demonstrated by T helper 1 (type 1 or Th1) versus T helper 2 (type 2 or Th2), heavily influences the quality of the immune responses (inflammatory versus humoral) (Mosmann *et al.*, 1991; Salgame *et al.*, 1991; Romagnani *et al.*, 1993; Subash *et al.*,

1995). Th1 cells secrete interleukin (IL)-2, IL-12 and interferon-gamma (IFN-γ) which help cell-mediated immune reactions and help B cells produce complement-fixing antibodies (IgG2a and IgG3 in the mouse). Th2 cells, on the other hand, secrete IL-4, IL-5, IL-6 and IL-10 which help B cells produce non-complement fixing antibodies (IgG1) and are involved with immune reactions generally associated with allergy (IgE production, activation of mast cells and eosinophils). In addition, the cytokines produced by each subset of T cells can regulate the function and activity of the other set. For example, the IL-10 produced by Th2 cells suppresses the secretion of IFN-γ by Th1 cells (Fiorentino *et al.*, 1989), whereas IL-12 interferes with the secretion of IL-10 (Tripp *et al.*, 1993). Similarly, IFN-γ and IL-12 promote the differentiation of Th1 cells (Manetti *et al.*, 1993; Hsieh *et al.*, 1993), and IL-12 has been reported to interfere with the differentiation of Th2 cells (McKnight *et al.*, 1994), whereas IL-4 promotes the differentiation of Th2 cells (Hsieh *et al.*, 1992).

The impact of cytokine production on the outcome of immune responses has been well documented (Coffman *et al.*, 1991). In *Leishmania major* infections, protective immunity correlates with a Th1-like cytokine profile, whereas progressive, non-healing infections correlate with a Th2-like profile. Importantly, the nature of the response can be manipulated by perturbing the cytokine network (Hsieh *et al.*, 1993; Mosmann *et al.*, 1991; Coffman *et al.*, 1991). The nature of allograft immunity is also greatly influenced by cytokines present during the response (Dallman, 1992; Hodgkin *et al.*, 1985; Hao *et al.*, 1990; Takeuchi *et al.*, 1992). This is also true for skin allograft rejection across a MHC class II barrier (Rosenberg *et al.*, 1990). This indicates that factors that influence the effector function of primed T cells will have a major impact on the outcome of the response.

e. Mechanisms underlying skin graft rejection.

The rejection of skin grafts is primarily studied as a model of acute graft rejection. As the tissue is not a primary vascularized organ, hyperacute rejection, a rapidly-occurring reaction involving antibody responses in blood vessels (Scoazec and Leseche, 1993), does not occur. It is thought that the main target cells in graft rejection, including skin grafts, may be the endothelial cells (Guillen *et al.*, 1986; Scoazec and Leseche, 1993). This may be true for hyperacute, acute and chronic rejection, even though the mechanisms underlying each of these rejection processes may be different.

Acute rejection is an antigen-specific, T cell-dependent response (Rosenberg and singer, 1988; Rosenberg *et al.*, 1989; Bradley and Bolton, 1992). In the skin, it is effectively a T cell response involving different proportions of the $CD4^+$ and $CD8^+$ sub-populations depending on the nature of the antigen (Rosenberg and Singer, 1992). It has been shown that athymic nude mice do not develop T cells and do not reject even strongly incompatible skin grafts. However, if these mice are reconstituted by adoptive transfer of T cells they are fully capable of rejecting the grafts (Rosenberg and Singer, 1988, 1992; Rosenberg *et al.*, 1986, 1987). Furthermore, mice with severe combined immunodeficiency (scid) lack T and B cell activity but can reject skin grafts following adoptive transfer of functional $CD4^+$ and $CD8^+$ T cells (Shelton *et al.*, 1992).

It is believed that a significant component of allograft rejection is the consequence of recipient T lymphocyte recognition of alloantigens expressed by non-parenchymal cells, the antigen presenting cells, present within the grafted tissue (Mason and Morris, 1986). The "passenger leukocyte" hypothesis suggests that immunostimulatory leukocytes (the

dendritic cell family), migrate from the allograft to regional lymph nodes and sensitize alloreactive T lymphocytes, which ultimately cause the destruction of the graft (Lechler and Batchelor, 1982). Most of what is known about the importance of dendritic cells (DC) in initiating graft rejection responses *in vivo* came initially from studies with endocrine organ allografts. Lafferty *et al.* (1983) and Faustman *et al.* (1984) observed that donor organ allograft survival could be prolonged by depleting dendritic cells from such organs. However, subsequent injection of the engrafted animal with minimal numbers of DC, even at distal sites from the allograft, stimulated rapid rejection of the organ graft.

In the skin, dendritic cells are known as Langerhans cells (LC), and the immunogenicity of skin allografts correlates directly with the density of LC they contain (Mathieson *et al.*, 1975; Sena *et al.*, 1976; Chen and Silvers, 1983). That is, the rejection rate of histoincompatible skins obtained from different sites on an individual donor animal correlates with their density of LC (Bergstresser *et al.*, 1980a & b): trunk and ear skin express the highest density of LC and are most rapidly rejected, whereas tail skin with relatively few LC is rejected less rapidly. For skin allograft rejection, responses are felt to be initiated from the response of host T cells to Langerhans cells that have migrated out of the skin graft to the draining lymph nodes (central sensitization), and not by donor LC remaining within the graft or by host LC or DC migrating into the skin graft (peripheral sensitization) (Rosenberg and Singer, 1992). The consensus on central sensitization is based on the studies of Barker and Billingham (1968) and Tilney and Gowans (1970) which showed that severance of lymphatic connections to skin grafts resulted in graft acceptance or in delayed rejection. Similarly, tissues that naturally lack lymphatic drainage sustained

allografts for prolonged periods of time (Billingham and Silvers, 1962; Barker and Billingham, 1977). Using skin grafts and skin organ culture, Larsen *et al.* (1990b) have further supported the concept of central sensitization in that LC were observed to migrate out of the epidermis into dermal lymphatic channels and subsequently out of the skin graft. The reciprocal migration of dendritic cells from host blood into skin grafts has been diligently sought but never found (Larsen *et al.*, 1990a). Following LC migration and subsequent allospecific-stimulation, sensitized host helper T (Th) cells secrete cytokines that activate the host cytokine-dependent cytotoxic T (Tc) cells. The Th and Tc cells may be distinct populations or can be a single population of T cells with helper and cytolytic properties (Rosenberg and Singer, 1992). Ultimately, allografted skin is characteristically rejected via the cytolytic activity of the Tc cells by 7-12 days post grafting.

1.3 Prevention of allograft rejection.

Although current day immunosuppressive treatments have led to success in a number of clinical transplant situations, the conundrum of modern transplantation remains the need to overcome the anti-graft immune response without compromising the entire immune system of the recipient (Trucco and Stassi, 1996). Attempts to prolong the survival of allografts and xenografts in recipients, both in experimental and clinical transplantation, have centred mainly on the suppression of the immune apparatus of the recipient.

Several immunosuppressive agents have been used to treat the rejection of different tissue or organ allografts. In skin allografts, the donor skin for reasons not fully understood,

is very antigenic and the skin allograft rejection is a strong immune response. For example, the number of functional $CD4^+$ cells required to induce skin allograft rejection in the rat is much less than that required to initiate internal organ allograft rejection (Morton *et al.*, 1993). Consequently, the rejection of skin tends to be more difficult to prevent with therapy than most other grafts in the same species and under the same treatment. Vascularized organ grafts can be protected more effectively by immunosuppressive drugs or monoclonal antibodies than can skin grafts (Green, 1986; Mason *et al.*, 1988; Morris *et al.*, 1991; Cai *et al.*, 1992; Chen *et al.*, 1992; Pearson *et al.*, 1992; Qian *et al.*, 1992; Ochiai *et al.*, 1993).

a. First generation immunosuppressive therapies.

These are drugs, including azathioprine, demethasone, and corticosteroids (e.g. prednisolone), which were available prior to the discovery of cyclosporine A (CsA). Even with their relatively non-specific immunosuppressive action compared to CsA, they are still being used in combination therapy because low doses of all the drugs is associated with reduced side effects (Morris, 1991; Kahan, 1992). In general, they show only weak protection of skin allografts against acute rejection in rodents when used as the sole treatment. In mice, dematha sone (Becker *et al.*, 1990, 1991) and azathioprine (Borel *et al.*, 1976; Tomioka *et al.*, 1989) induced only relatively small increases in skin graft survival.

b. Cyclosporine A and analogues.

Cyclosporine A (CsA) is now established in clinical transplantation for the prevention of acute rejection of a wide range of organ and tissue allografts (Morris, 1991; Kahan, 1992). CsA acts by inhibiting the phosphatase enzyme calcineurin through its initial binding

to an immunophillin-chaperone molecule, cyclophilin (Bierer *et al.*, 1993; Schreier, 1993; Schreiber and Gabtree, 1992; Lui, 1993). Subsequently, the transcription of several genes, including that for interleukin (IL)-2, is inhibited. As a consequence, CsA particularly inhibits the functions of T cells which release IL-2 as a major stimulus to cell proliferation and thus clonal expansion (Lui, 1993).

In the early studies CsA was shown to prolong skin allograft survival in several species including mice, rats and rabbits. However, the grafts were inevitably rejected after the treatment was discontinued (Green, 1986; Borel, 1989). CsA induced dose-related increases in survival of strongly-incompatible skin grafts of up to 7.5 days when it was administered intra peritoneally (i.p.) at doses of 2 - 64 mg/kg on days 0 - 5 (Lagodzinski *et al.*, 1990). At relatively low doses of 5, 10 and 15 mg/kg, CsA induced only small increases in strongly-incompatible graft survival but the grafts were rejected despite the continued treatment with the drug (Alfrey *et al.*, 1992). Longer survival times but not tolerance were observed during long term treatment with 75 mg/kg (Lems *et al.*, 1980). However, CsA was capable of inducing long term survival of heart grafts in mice when studied in direct comparison with skin grafts (Mason *et al.*, 1988).

c. Tacrolimus (FK506).

FK506 is a cyclic macrolide which, like CsA, inhibits calcineurin as its major mode of action. It also acts by inhibiting calcineurin through its initial binding to an immunophillin binding protein, FKBP. The cyclic macrolide is an order of magnitude more potent than CsA and encouraging pharmacological studies in graft rejection rapidly led to its clinical use (Morris, 1991; Kahan, 1992; Kaufmann *et al.*, 1992).

In strongly-incompatible mouse strain combination, long term treatment with FK506 (1-10 mg/kg intra muscularly, daily from day 0 for 4 weeks) resulted in the protection of most (1 mg/kg) or all grafts (10 mg/kg) while the treatment was continued. The skin grafts eventually rejected after the treatment was stopped (Wada *et al.*, 1991). The increase in skin allograft survival was 52.1 days with 10 mg/kg. However, the graft rejected 24 days after the treatment was discontinued (Wada *et al.*, 1991). In another study, FK506 was shown to induce moderate dose-related increases in skin allograft survival in the dose range 0.05 - 1.0 mg/kg (given intra peritoneally on days 0 - 5) with the maximum increase of 8.5 days at the highest dose (Lagodzinski *et al.*, 1990). CsA was similarly effective but at much higher doses (1.75 - 35 mg/kg) (Lagodzinski *et al.*, 1990). As with CsA, lower doses of FK506 were effective in protecting heart or liver grafts for significantly longer periods of time when studied in direct comparison with skin grafts (Cai *et al.*, 1992).

d. Rapamycin.

This is a macrolide antibiotic isolated from *Streptomyces* with a potent immunosuppressant properties (Morris, 1992). It is known to interact with some immunophillins but does not inhibit the synthesis of IL-2. However, it markedly inhibits the cell proliferation induced by IL-2 or IL-4 (Morris, 1991; Schreiber, 1992). The main mode of action is thought to be the inhibition of the p70 S6 kinase which is important in signal transduction in cytokine-activated cells (Schreiber, 1992; Bierer *et al.*, 1993; Thomson and Starzl, 1993). In mice with multiple incompatible minor antigens, rapamycin at 4 or 8 mg/kg/day i.p. for 6 days (days 1 - 6) induced 4 -5 days increase in graft survival times whereas CsA induced only about 2 days increase at the same doses (Eng *et al.*, 1991).

e. Ultraviolet (UV) light.

UV light, particularly UVB (290 - 320 nm) has been shown to modify the immune system (Vermeer and Hurks, 1994). It induces changes in many mammalian cells including increases in nuclear transcription factors and damage to DNA (Devary *et al.*, 1993). UVB exposure has been shown to induce inflammation (Katiyar *et al.*, 1995), alter cutaneous immune cells and immunosuppress skin responses such as contact and delayed-type hypersensitivity responses (Kripke, 1983; Kripke, 1984; Toews *et al.*, 1980; Noonan *et al.*, 1981a and b; Mommaas *et al.*, 1993; Strickland, *et al.*, 1994; Katiyar *et al.*, 1995). Treatment of corneal grafts with UVB resulted in corneal allografts tolerogenic for allospecific delayed hypersensitivity responses and acceptance of the graft (Niederkorn and Mayhew, 1993).

UVB immunosuppression is thought to involve a number of possible mechanisms: a). depletion and/or inactivation of epidermal Langerhans cells and thus impairment of antigen presenting functions (Noonan and de Fabo, 1992, Burnham *et al.*, 1993; Mommaas *et al.*, 1993). Specifically, UV radiation has been shown to induce clonal anergy in T-helper type 1 (Th1) cells (Cooper *et al.*, 1992; Noonan and de Fabo, 1992; Simon *et al.*, 1990, 1991). Noonan *et al.* showed that ultraviolet radiation suppressed contact hypersensitivity in mice at the level of antigen presentation (Noonan *et al.*, 1981b); b). infiltration into the skin of macrophages that preferentially activate naive Th2 cells (Baadsgaard *et al.*, 1987; Baadsgaard *et al.*,1988; Cooper *et al.*, 1986). c). direct effect on T cells including decreased viability, proliferative responses and associated cytokine production (Teunissen *et al.*, 1993); d). induction of KC to secrete a wide variety of cytokines (Luger and

Schwarz, 1994), many of which have been shown to modulate APC function (Ullrich, 1994; Rivas and Ullrich, 1994).

The *in vivo* immunosuppressant effect of UVB does not appear, at least in skin allograft rejection, to be a general phenomenon. It appears to depend on the genetic make up of the recipient mice. For instance, exposure of mouse tail skin grafts to UVB prior to implantation to the flank of MHC class II incompatible mice resulted in an increased graft survival in only one strain combination (B10.AQR to B10.T(6R) but not with the reverse combination (Vermeer *et al.*, 1988). However, UVB irradiation of bone marrow cells before transplantation into lethally gamma-irradiated allogeneic rats prevented graft-versus-host disease and induced a stable complete lymphohematopoietic chimerism (Oluwole *et al.*, 1993). Furthermore, UV irradiation has been shown to prevent alloimmunization by inactivating contaminating leukocytes in animals receiving platelet transfusion (Slichter *et al.*, 1987). Similarly, histoincompatible rat pancreatic islets of Langerhans irradiated with 900 J/m^2 of UVB and transplanted into diabetic Lewis rat recipients were permanently accepted (Hardy *et al.*, 1984).

f. Other immunosuppressant therapies.

A few of the available immunosuppressive agents have been discussed for the purposes of understanding their efficacy and mechanisms in suppressing allograft rejection. However, there are numerous agents for immunosuppressant treatments that have been developed for use in preventing allograft rejection which have not been discussed. Examples include cyclophosphamide (Piekoszewski *et al.*, 1994); methotrexate (Hadden and Smith, 1992); antilymphocyte antibody; monoclonal anti-T cell antibodies (e.g. anti-CD3, soluble

$CTLA_4$, $CTLA_4$-Ig fusion protein) and other monoclonal antibodies with varying specificities (Fung *et al*, 1992); Leflunomide (HWA486) (Kuchle *et al.*, 1991; Schlorlemmer *et al.*, 1993); purine and pyrimidine inhibitors (mycophenolate mofetil (RS-61443), mizoribine (bredinin), brequinar sodium) (Turka *et al.*, 1991; Allison *et al.*, 1993; Allison and Eugui, 1993; Makowka *et al.*, 1993); 15-Deoxyspergualin (Morris, 1991; Kaufmann *et al.*, 1992; Thomson and Starzl, 1993); Didemnins A and B (Alfrey *et al.*, 1992); Vitamin D analogues (1,25-dihidroxy vitamin D_3, CB 966, KH 1060) (Veyron *et al.*, 1993); Adriamycin (doxorubicin) (Eckert *et al.*, 1989); Triptolide (Yang *et al.*, 1992); Prostaglandins (Becker *et al.*, 1990).

In spite of the numerous types of agents available for experimental transplantation, only a handful of them are well established for clinical use. CsA still remains the primary drug in most immunosuppressant treatment regimens in clinical transplantation. But, although CsA and other agents may be effective, they are not without significant side effects. Most of these agents are associated with significant toxicities (Fung *et al.*, 1992). In general, severe suppression of the immune system is necessary to maintain graft survival, thus leaving the recipients at high risk of developing unusual neoplasms such as B-cell lymphomas and rampant and potentially deadly opportunistic infections (Trucco and Stassi, 1996) which is the leading cause of death in human transplant patients (Jarowenko *et al.*, 1986). The representative side effects (Fung *et al.*, 1992; Hadden and Smith, 1992; Guymer and Mandel, 1993) of some of the immunosuppressive agents are outlined in table 1.3.1.

Table 1.3.1 Representative side effects of some immunosuppressive agents (Fung *et al.*, 1992; Hadden and Smith, 1992; Guymer and Mandel, 1993).

AGENTS	REPRESENTATIVE SIDE EFFECTS
Antilymphocyte antibody	Fever, chills, gastrointestinal (GI) distress, myalgias, arthralgias, anaphylactoid reactions, serum sickness, thrombocytopenia, anemia, leucopenia
Azathioprine	Myelosuppression, GI symptoms, hepatoxicity
Corticosteroids	Mood swings, weight gain, hypertension, diabetes, ulcerogenesis, osteoporosis, acne, growth retardation in children, glaucoma, cataracts
Cyclosporine	Nephrotoxicity, hypertension, hyperkalemia, hirsutism, tremors, gingival hypertrophy, hepatoxicity, neurotoxicity
Tacrolimus (FK 506)	Nephrotoxicity, neurotoxicity (predominantly in liver transplant recipients), glucose intolerance, hyperkalemia
Ultraviolet light	Marked increase progression of UV-induced tumors, inflammation

In view of thiese complications, a number of other compounds or agents are under investigation as potential additions to immunosuppressant regimens for transplant patients. It is very likely that new agents may become part of combination therapy rather than being used alone. Rather than focus on treating the rejection process itself, transplantation biologists have sought methods to suppress immune responsiveness in an antigen-specific manner so that only the response to the donor alloantigen would be lost. The ultimate goal

of this immunomodulation process is to achieve donor-specific tolerance without alteration in immunity to other antigens (Fung *et al.*, 1992). Such specific immunosuppression may be achieved by modifying either the antigenicity of the tissue to be grafted or the specific cells capable of mediating rejection. Since it has been suggested (Mason and Morris, 1986) that a significant component of allograft rejection is the consequence of recipient T lymphocyte recognition of alloantigens expressed by immunostimulatory APCs present within the grafted tissue, it seems logical to speculate that anti-rejection strategies focused on the modification and/or elimination of these MHC-bearing "passenger leukocytes", may be a more selective and less toxic approach to prevent allograft rejection. Such treatment may result in the reduction or modification of active passenger lymphoid cells; thus affecting the stimulator cell population necessary for tissue immunogenicity. Initial attempts to remove passenger leukocytes from organs prior to transplantation were not successful (Lafferty *et al.*, 1986). These attempts involved the induction of leukopenia in the tissue donor by procedures such as whole body irradiation, cyclophosphamide pretreatment, or treatment with antilymphocyte serum. At best, only marginal effects were observed with kidney transplants across MHC barriers and heart allografts transplanted across multiple minor differences.

Since the elucidation of the requirement for donor strain dendritic cells in renal allograft rejection (Lechler and Bachelor, 1982), several attempts to diminish the antigenicity of donor tissues prior to transplantation have been made. The effect of organ culture on the immunogenicity of MHC-incompatible allografts has been studied. Extended time culturing of the donor tissues (Lafferty *et al.*, 1975; Lafferty *et al.*, 1976) led to the

prolongation of graft acceptance across MHC barriers. When skin, fetal pancreas, fetal proislets, adult islets and thyroid were transplanted across minor histocompatibility differences, skin and fetal pancreas were rejected much more acutely than the later tissues, which survived indefinitely (Lafferty *et al.*, 1986). Similarly, it has been shown that the depletion of the potent APC from pancreatic islet (Faustman *et al.*, 1984) or thyroid (Iwai *et al.*, 1989) allografts with monoclonal antibodies directed against donor MHC permitted prolonged acceptance when these tissues were transplanted across mouse MHC. UV irradiation has been shown to have profound immunomodulatory effects on dendritic cells (Pamphilon *et al.*, 1991). Irradiation of donor tissues with UV prevented host-versus-graft responses, prolonged allograft survival in animal models of bone marrow, pancreatic islet and cardiac transplantation (Deeg, 1988; Hardy *et al.*, 1986; Hardy *et al.*, 1988). Furthermore, donor tissues have been treated with a wide variety of substances, such as the topical application of cyclosporine A (Black *et al.*, 1988; Zhao *et al.*, 1988; Llull *et al.*, 1995) and FK506 (Yuzawa *et al.*, 1996) ointments to the donor skin prior to transplantation. While topical FK506 was able to prolong skin graft survival in primary skin grafting (Yuzawa *et al.*, 1996), the CsA ointment could only prolong graft survival after an initial systemic administration of CsA or prednisolone (Black *et al.*, 1988; Llull *et al.*, 1995).

In general, variable results were obtained by these methods and long term donor-specific tolerance is far from being achieved. Above all, none of these treatments has been shown to have any potential clinical significance. Thus, there is still a need to develop more selective and less toxic immunosuppressive regimens for reducing the antigenicity of donor tissues prior to engraftment. Ideally, such pretreatment regimens should modulate rather

than deplete the donor-derived antigen presenting cells and should not induce any inflammatory response. One such new approach for reducing the antigenicity of donor tissue is photodynamic therapy (PDT).

1.4 Photodynamic therapy (PDT).

PDT is a proven method for eradicating certain tumor types and is now an approved treatment for cancers of the oesophagus, lung, and bladder in many countries including Canada, the USA, Japan, France, and the Netherlands (Brown, 1996). Also, PDT has potential for use in the treatment of a number of non-oncologic conditions such as age-related macular degeneration (AMD) (Richter *et al.*, 1994a), psoriasis (Richter *et al.*, 1994a; Levy *et al.*, 1994b), atherosclerotic plaque and restenosis (Richter *et al.*, 1994a), bone marrow purging for treatment of leukaemias with autologous bone marrow transplantation (Richter *et al.*, 1994b), inactivation of viruses in blood or blood products (Richter *et al.*, 1994b; North *et al.*,1993), and several autoimmune conditions, including rheumatoid arthritis (Richter *et al.*, 1994a & b; Ratkay *et al.*, 1994b). This treatment modality selectively destroys malignant cells by an interaction between absorbed visible light and tissue-retained photosensitizing agents (Manyak *et al.*, 1988). PDT is non-invasive, safe and has few side effects. It can be used in conjunction with chemotherapy, radiotherapy or surgery (Dougherty *et al.*, 1990; Moan and Berg, 1992).

a. Historical background.

Photodynamic principles were established a long time ago. Phototherapy is attributed

to the ancient Egyptians (Dougherty, 1990) and to the ancient cultures of India and China (Spikes and Straight, 1990). However, the term "photodynamic effect" was first used by Oscar Raab in 1900, when he demonstrated that a combination of acridine orange and visible light could be used to kill *paramecia* (Raab, 1900). In 1903, von Tappenier and Jesionek treated skin cancer with eosin and sunlight (von Tappenier and Jesionek, 1903). In the 1940's, it was discovered that hematoporphyrin is preferentially retained in malignant tissues as compared with normal tissues (Auler and Banzer, 1942; Figge *et al.*, 1948). In the 1960's Lipson used hematoporphyrin derivative (HpD) to detect and treat metastatic chest wall breast cancer (Lipson *et al.*, 1961, Lipson, 1966). The recent catalyst for the use of PDT in clinical research began in the 1970's with pioneering works of Dougherty and Diamond (Diamond, 1972; Dougherty, 1974).

b. Mechanisms of photodynamic action.

Many different chemical photosensitizers have been developed for experiments in photochemical cytotoxicity but porphyrin derivatives are the most widely used in clinical research (Spikes and Straight, 1987). Clinical PDT is a two-step process (Fig. 1.4.1). The first step consists of the intravenous administration of photosensitizer while the second step is a direct activation of the sensitizer with visible light (Gomer, 1989; Dougherty *et al.*, 1990; Marcus, 1990) at a wavelength of light where the sensitizer has a peak of absorption. So far, several different types of light sources has been used in PDT including, incandescent bulbs, fluorescent bulbs, xenon-arc lamps, quartz-halogen lamps, light-emitting diodes (LEDs) and lasers.

The excitation of the sensitizer in the presence of oxygen leads to the production of

cytotoxic agents and a subsequent oxidative destruction of cellular components. Singlet oxygen, a highly reactive and short-lived excited state of oxygen produced following irradiation of photosensitizers, is believed to be the main component responsible for the cytotoxic changes (Langlois *et al.*, 1986). Singlet oxygen can react directly with proteins, lipids, and nucleic acids, generating products that can subsequently initiate the production of free radicals leading to auto-oxidation and further cellular damage.

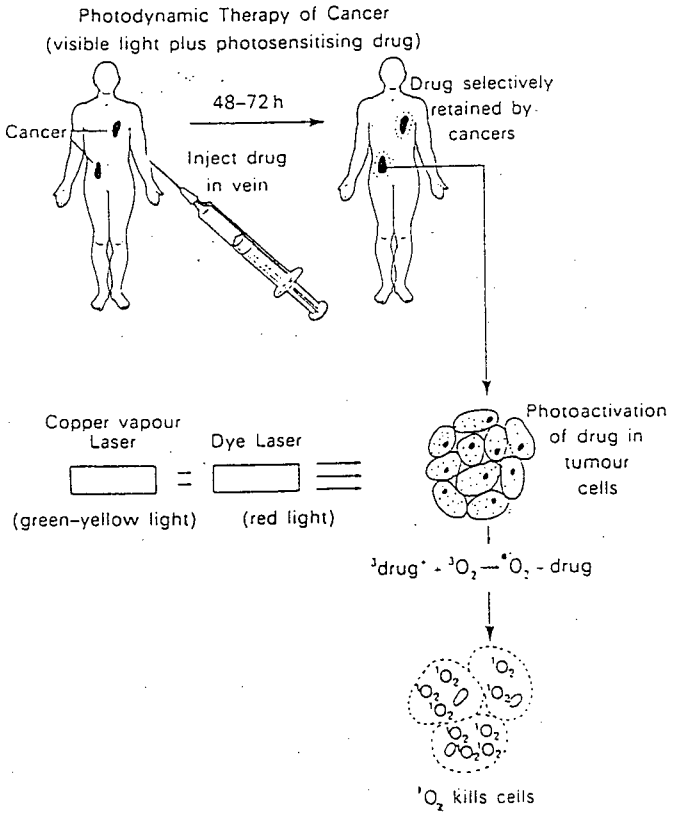


Fig. 1.4.1. Schematic diagram illustrating the main steps involved in PDT (Adapted from the cover page of British Journal of Cancer, Volume 57 Number 5, May 1988).

The mechanism of PDT-induced tumor destruction, though not fully understood, may be quite complex. It may depend on a number of factors including the nature of the photosensitizer, the time of irradiation following photosensitizer administration and the doses of photosensitizer and light used. In general, the mechanism of PDT-induced tumor destruction is thought to include direct damage to the neoplastic cells and disruption of tumor microvasculature and non-vascular stroma, resulting in ischemic necrosis of the tumor (Zhou, 1989; Richter *et al.*, 1991). Sub-cellular targets involved with PDT mediated oxidative injury include the plasma membrane, mitochondria, DNA, and cytosolic enzymes (Kessel, 1986; Moan and Berg, 1992). Fingar *et al.* (1991), showed that PDT-induced tumour necrosis may be due to an acute inflammatory reaction and that the overall damaging process is further enhanced by the release of some vasoactive or tissue-lysing substances from photodamaged mast cells and neutrophils. There is increasing evidence that PDT may also induce rapid cell death by apoptosis (Agarwal *et al.*, 1991).

c. Benzoporphyrin derivative monoacid ring A (BPD) verteporfin.

The first generation photosensitizer preparation (Photofrin®) widely used in clinical trials of PDT of cancer consists of a complex mixture of hematoporphyrin derivative (HpD) dimers, trimers and oligomers; thus making characterization of the active components difficult (Dougherty, 1987). Furthermore, two problems are inherent with Photofrin® therapy. One is its weak absorption above 600 nm (van Lier, 1990) (Fig. 1.4.2), the part of the spectrum where light penetrates more deeply into living tissues (Wilson *et al.*, 1984; Smith, 1977). Second, there is prolonged skin photosensitization since this preparation is retained in the skin for as long as 4-6 weeks post PDT (Dougherty *et al.*, 1984). As a

result, a number of porphyrin and porphin analogues with improved photophysical properties within the therapeutic light range have been advanced over the years (van Lier *et al.* 1988; Spikes, 1990; Rosenthal, 1991; Pass, 1993). BPD (QLT PhotoTherapeutics Inc., Vancouver) (Fig. 1.4.3) is one of the products of this advancement.

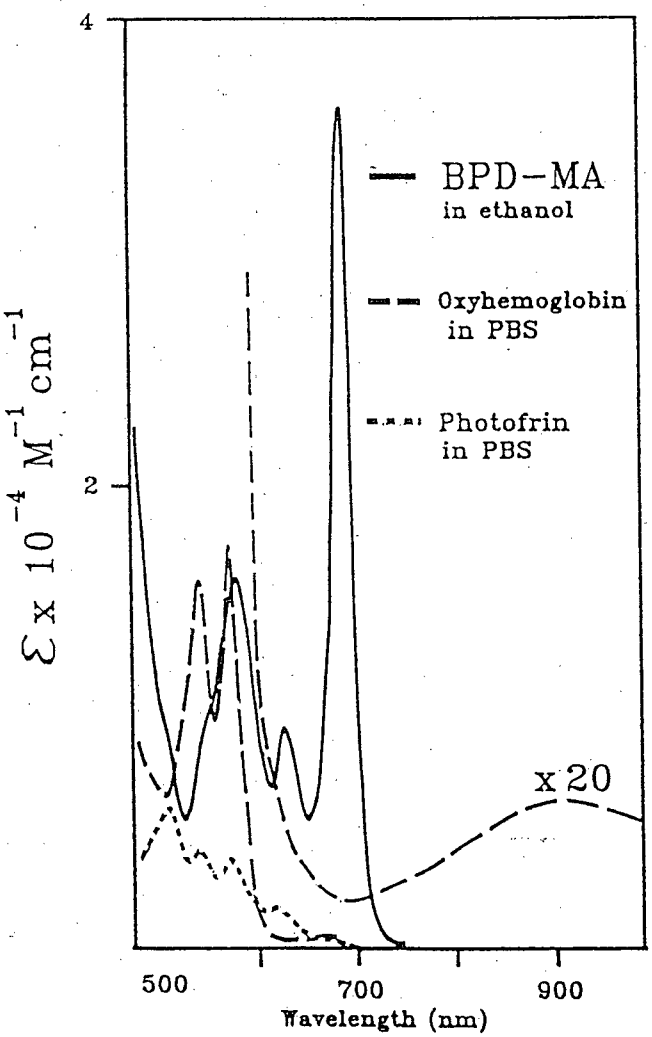
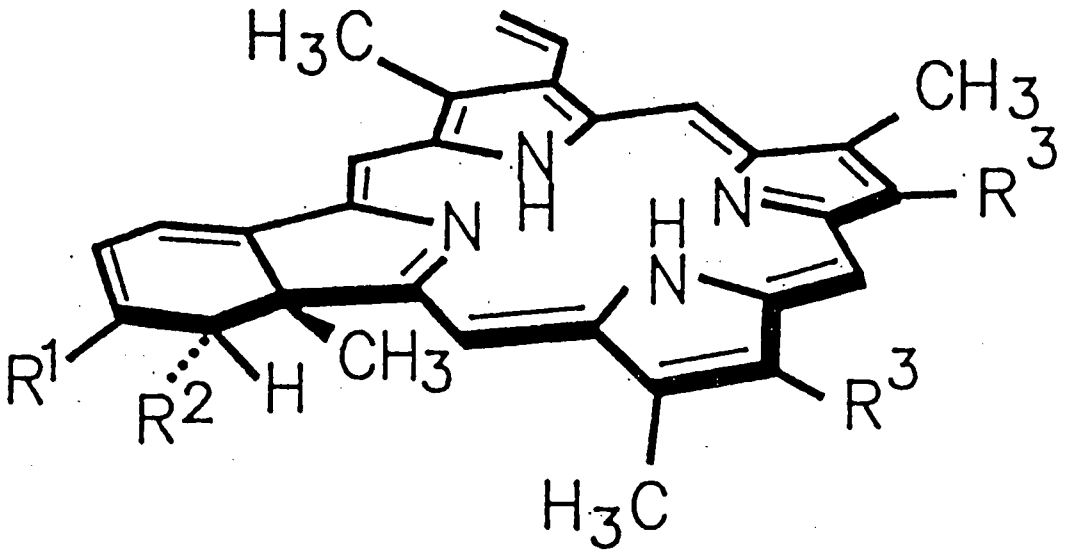


Fig. 1.4.2. Absorption spectrum of BPD (solid line), oxyhemoglobin (broken line), and Photofrin® (dotted line).



$$R^1 = R^2 = CO_2Me$$

$$R^3 = (CH_2)_2CO_2Me$$

or

$$(CH_2)_2CO_2H$$

Fig. 1.4.3. Structure of benzoporphyrin derivative (BPD) monoacid ring A verteporfin. Formula: $C_{41}H_{42}N_4O_8$. Molecular weight: 718.81.

BPD (Fig. 1.4.3) is a hydrophobic, chlorin-like porphyrin derivative that is effective in PDT. BPD is a chemically homogeneous preparation (Richter *et al.*, 1987) and absorbs light maximally at 690 nm (Fig. 1.4.2), a wavelength of visible light at which hemoglobin absorbs minimally (Levy, 1994) and which penetrates living tissue well (Wilson *et al.*, 1984; Smith, 1977). It is rapidly cleared from the body, thus causing minimal normal vascular or skin damage (Richter *et al.*, 1994b). Skin photosensitivity does not extend beyond a few days (Levy *et al.*, 1994b). *In vitro* studies have shown that BPD is rapidly taken up by target cells and is 10 times more cytotoxic on adherent cell lines and 10 -70 times more cytotoxic towards non-adherent cell lines than Photofrin® (Richter *et al.*, 1987; Allison, 1992). BPD is currently in clinical trials for both oncological and non-oncological indications including non-melanoma skin cancer, psoriasis, AMD and endometrial ablation (Levy *et al.*, 1994a).

d. PDT and immunity.

We studied the feasibility of using BPD-mediated PDT to modulate the immune system because of a series of observations which indicate that PDT, depending on the photosensitizer, may exert immunomodulatory effects. It has been shown that exposure of murine peritoneal cells to Photofrin® and light can affect T cell responses inducing systemic immunosuppression of contact sensitivity of mice to an administered hapten (Elmets and Bowen, 1986). Lynch *et al.* showed that this immunosuppression can be adoptively transferred with macrophages (Lynch *et al.*, 1989). Furthermore, treatment of mice with PDT was shown to reversibly inhibit the ability of lymphocytes to proliferate in response to mitogens (Canti *et al.*, 1981; Kol *et al.*, 1986; 1989), stimulate a mixed leukocyte

reaction (Lynch *et al.*, 1989; Canti *et al.*, 1981), and to mediate graft-versus-host disease (Canti *et al.*, 1981). Similarly, PDT has also been reported to inhibit the activity of cytotoxic lymphocytes (Franco *et al.*, 1983). Furthermore, Qin *et al.* showed that intraperitoneal photosensitization induced a transient systemic cellular immunosuppression by depleting peritoneal lymphocytes and activating the peritoneal macrophages (Qin *et al.*, 1993); thus permitting prolonged survival of skin allografts. Also, photosensitization using Photofrin® was shown to inhibit the high affinity Fc receptor ($Fc_\gamma RI$) on human monocytes (Krutmann *et al.*, 1989). Additionally, porphyrin-mediated PDT have been shown to induce the release of immunosuppressive agents such as prostaglandin E_2 (Henderson and Donovan, 1989). The inflammatory and immunosuppressive responses observed during *in vivo* PDT (Fingar *et al.*, 1990) may be associated with prostaglandin and/or thromboxane release. Likewise, porphyrins without direct exposure to visible light have been shown to be hematostimulatory (Hunt *et al.*, 1994) and to have anti-allergic and anti-inflammatory properties (Nagai *et al.*, 1992). At a molecular level, PDT was shown to enhance the translation of stress genes (heat shock proteins, glucose regulated proteins and heme oxygenase) (Gomer *et al.*, 1988; 1989; 1991, Curry and Levy, 1993) as well as early response genes such as c-fos, c-jun, c-myc and erg-1 that encode for proteins that regulate gene expression at the level of transcription (Luna *et al.*, 1994).

The exposure of freshly isolated murine Langerhans cells (LC) to *in vitro* PDT using psoralen and UVA radiation (PUVA) inhibited the ability of Langerhans cells to stimulate T cell proliferation (Tang and Udey, 1991). This was attributed to the reduced expression of the intercellular adhesion molecule-1 (ICAM-1) (Tang and Udey, 1991) but not Ia

expression (Tang and Udey, 1991; Aberer, 1982) or epidermal cell viability (Aberer, 1982). In clinical studies, diseases such as cutaneous T cell lymphoma (CTCL) (Edelson *et al.*, 1987), rheumatoid arthritis (Malawista *et al.*, 1991), systemic sclerosis (Rook *et al.*, 1992), systemic lupus erythematosus (Knobler *et al.*, 1992), and AIDS-related complex (Bisaccia *et al.*, 1990) responded positively to photopheresis using 8-methoxypsoralen and UVA. Similarly, skin (Perez *et al.*, 1991) and cardiac allografts (Constanzo-Nordin *et al.*, 1992) were prolonged in experimental animals with photopheresis.

i. Transdermal PDT. Studies in our laboratories have shown that in comparison to normal murine splenocytes, mitogen-activated spleen cells accumulate up to four- to five-fold higher concentrations of BPD within 30 min of incubation (Richter *et al.* 1994b). This suggested that immunologically activated lymphocytes were more likely to be eliminated upon exposure to light, as established in *in vitro* cytotoxicity studies using splenocytes (Obochi *et al.*, 1995) and peritoneal macrophages (Hunt *et al.*, 1995). Experiments using whole blood have also shown that activated human leukocytes expressing high levels of IL-2 receptor (IL-2R) and HLA-DR antigens were selectively depleted from a heterogenous mixture by BPD and light (North *et al.*, 1993). PDT, therefore, allows activated cells to be destroyed with some selectivity. The strong absorption band of BPD at 690 nm allows maximal transmission through tissue with minimal attenuation by blood pigments (North *et al.*, 1992). Thus, this might enable BPD associated with leukocytes in the circulation to be photoinactivated transcutaneously.

On that basis, a new approach for PDT as an immunomodulatory technology was developed in our laboratory whereby photosensitizers were activated in the blood by whole

body illumination with visible light without causing photosensitivity (Richter *et al.*, 1994b). This procedure was termed transdermal photodynamic therapy to reflect the fact that BPD was activated in circulation by light that was being delivered transcutaneously. A treatment window for the exposure of experimental animals to red light (15 J/cm^2 at a wavelength of 690 $\pm$ 10 nm) was established to be 1 h following intravenous administration of BPD at doses up to 1.0 mg/kg. This allowed sufficient time for differential distribution of the sensitizer between tissues, but skin photosensitivity was very low and insignificant during the time frame in question (Richter *et al.*, 1994b). The potential for transdermal PDT in the immunomodulatory technology has been explored in different animal models. We (Chowdhary *et al.*, 1994) showed that transdermal PDT using BPD may inhibit disease onset in adjuvant-enhanced arthritis in MRL-*lpr* mice by selectively eliminating the adjuvant-activated lymphocytes in the circulation and/or joints of mice in this model. Similarly, Hunt *et al.* showed that transdermal PDT delayed the onset of paralysis of mice in an experimental allergic encephalomyelitis (EAE) model (Hunt *et al.*, 1994). Recently, we (Simkin *et al.*, 1995) showed that transdermal PDT using BPD significantly inhibited the development of contact hypersensitivity (CHS) response against the hapten dinitrofluorobenzene (DNFB). The cellular targets and the molecular mechanisms of transdermal PDT are not fully understood. However, at the molecular level, PDT with BPD was shown to stimulate the stress-activated c-Jun protein kinase (SAPK) and p38 HOG1 mitogen activated protein (MAP) kinases in murine keratinocytes (Tao *et al.*, 1996) and to induce stress protein expression in both tumor cells and tissues (Curry and Levy, 1993).

1.5 Objectives and rationale.

In this thesis, we used a murine skin allograft model because the rejection of skin allografts has been one of the most potent and convenient experimental models for the study of immunologically mediated tissue destruction *in vivo* (Rosenberg and Singer, 1992). Skin is the favoured tissue for experimental transplantation because grafts can be performed and observed with ease. Skin grafting is a relatively simple and very convenient experimental technique with an unambiguous end point. The status of grafts can be examined visually or microscopically on a regular basis. In addition, skin transplant rejection has another advantage as an indicator of immune competence as it is a strong immune response and may therefore be a sensitive test for the immunosuppressive effects of experimental treatments. The mouse is the most widely used rodent in this type of study because the major transplantation antigens, MHC antigens (encoded by the *H2* genes in mouse), are better characterized in comparison with, for example the equivalent RT-1 antigens in rats.

This thesis is based on the premise that antigen presenting cells, which play significant role in the initiation of allograft rejection, are required for the control of rejection and, ultimately, graft acceptance. We hypothesize that the modulation, rather than total depletion, of donor-derived APC within donor tissues may be enough to prevent the rejection of allografts. All previous attempts to prevent the rejection of allografts, focused on the depletion of donor-derived APC. Results have shown that the depletion of APC was not sufficient to sustain allograft survival. On that basis, we propose the following objectives:

1. **To establish whether PDT can prevent murine skin allograft rejection.**

   a). To determine whether the exposure of donor skin to low doses of BPD and light (low-dose PDT) can modulate epidermal Langerhans cells and thus prolong skin allograft survival.

   b). To evaluate whether the selective elimination of activated effector T cells with BPD and red light delivered transcutaneously (transdermal PDT) post engraftment can prolong skin allograft survival.

2. **To identify mechanisms by which PDT could effect anti-allograft immune responses.**

   a). To evaluate the effect of low-dose PDT on the allo-reactivity of LC.

   b). To establish the effect of low-dose PDT on the viability and surface molecules on LC.

   c). To determine the fate and activity of LC after low-dose PDT.

   d). To evaluate the effect of low-dose PDT on the infiltration of cells into grafts during rejection.

It is hoped that this approach of treating donor skin tissues prior to engraftment may be beneficial (subject to clinical trails) to burn patients requiring large areas of skin grafts. It is also possible that this treatment regimen could be adapted to vascularized internal organ transplants such as pancreatic islets, heart, and intestinal tissue allografts in experimental transplantation. Furthermore, the attenuation of the antigen presentation function of Langerhans cells with low-dose PDT may advance the understanding of immunobiologists in the apparent role of these cells in health and in disease. Lastly, our findings may have

application beyond that of tissue transplantation and may be effective for the treatment of a variety of diseases where dendritic or Langerhans cells are implicated, including atopic dermatitis, leishmaniasis, and psoriasis.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Benzoporphyrin derivative and analogues.

a. Benzoporphyrin derivative mono acid ring A (BPD) verteporfin.

A lyophilized powder of liposomally formulated BPD was obtained from QLT PhotoTherapeutics Inc. (Vancouver, British Columbia). Aliquots were reconstituted with distilled water at a concentration of 2 mg/mL and stored, protected from light at 4 °C, for not longer than 2 weeks. Further dilutions were carried out immediately prior to experiments using culture medium or serum-free electrolyte solution (5 % Dextrose Injection, USP, pH 7.4, Baxter Corporation, Toronto, Ontario).

b. Structural analogues of BPD verteporfin.

Dry powders of BPD-monoacid, ring A (BPD) and ring B (BPD-MB), and BPD-diacid, ring A (BPD-DA) and ring B, (BPD-DB) (Fig. 2.1) were supplied by Dr. Ethan Sternberg (Department of Chemistry, University of British Columbia, Vancouver, B.C.). All four analogues were maintained in 100 % tissue culture grade dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO) at a concentration of 2 mg/mL and stored, protected from light, at -20 °C. Further dilutions were carried out immediately prior to each experiment using culture medium such that the final solution contained no more than 0.01 % DMSO.

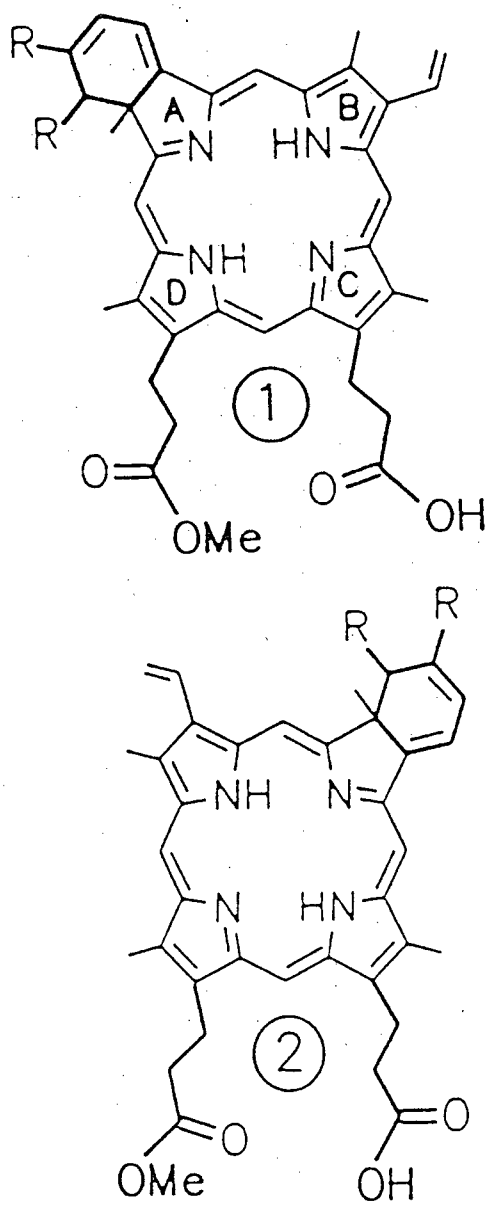


Fig. 2.1.1. Structure of BPD (1) monoacid, ring A analogue, (2) monoacid ring B analogue. R = CO_2Me. Diacid analogues differ from monoacids only in that they have the ester group replaced with acid groups; therefore, they have two acid groups at C and D rings of the porphyrin. The four BPD (monoacid and diacid) analogues have similar absorption spectra (Richter *et al.*, 1991).

2.2 Experimental animals.

Adult female BALB/c ($H2^d$) and C57BL/6 ($H2^b$) mice and male DBA/2 ($H2^d$) were supplied by Charles River Breeding Laboratories Canada (Montreal, Quebec). Animals were maintained under pathogen-free conditions at the animal unit (core facility) of QLT PhotoTherapeutics, Inc. (Vancouver, B.C.) under a 12 h light / 12 h dark cycle. The mice were kept on a standard laboratory rodent diet (Ralston Purina) with acidified water *ad libitum*. The colony was routinely screened for viruses (Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, reovirus type 3, lymphocytic choriomeningitis virus, epizootic diarrhea of infant mice virus, lactate dehydrogenase virus) using the Murine ImmunoComb test (Charles River Laboratories, MA). No detectable antibody against any of the viruses tested was observed. The protocol for the surgical procedure was approved by the Animal Care Committee of QLT PhotoTherapeutics Inc. (Vancouver, B.C.) where all the experiments were conducted. Animals were 8 - 12 weeks of age when used for experiments.

2.3 Culture media.

Unless otherwise stated, the majority of the cell culture experiments described in this thesis were performed in RPMI-1640 medium (Gibco BRL Life Tech. Inc., Grand Island, N.Y.) supplemented with 10 % pretested heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS) (Gibco), 2-mercaptoethanol (2×10^{-5} M; Sigma), antibiotics {penicillin (100

U/mL), streptomycin (100 U/mL), fungizone (1μg/mL)} (Gibco), HEPES (20 mM; Sigma) and L-glutamine (1 mM; Gibco). The RPMI-1640 medium supplemented in this manner was called complete culture medium (RPMI-10), the name used through out this thesis, and stored at 4 °C before use.

2.4 P815 cell line.

The murine tumor cell line P815 obtained from the American Type Culture collections (ATCC, Rockville, Maryland) (Lundak and Raidt, 1973; Plaut *et al.*, 1973) was used. The cell line is syngeneic for DBA/2 mice. Cells were maintained in Dulbecco's modified Eagles medium (DMEM) (Gibco BRL Life Tech. Inc., Grand Island, N.Y.) supplemented with 10 % pretested heat inactivated fetal bovine serum (FBS) (Gibco), antibiotics {penicillin (100 U/mL), streptomycin (100 μg/mL)} (Gibco), HEPES (20mM; Sigma), L-glutamine (1 mM; Gibco) and sodium pyruvate (1 mM; Gibco) in a 37 °C, dark humidified incubator containing 5 % carbon dioxide (CO_2) (Forma Scientific, Marietta, Ohio).

2.5 Monoclonal antibodies used for the study.

We used commercially available monoclonal antibodies to mouse cell surface antigens for our study (Table 2.5.1). The antibodies were maintained, protected from light, at 4 °C in buffered saline containing 0.1 % sodium azide (Sigma Chemical Company, St.

Louis, MO) and bovine serum albumin (BSA; Sigma) as a stabilizing protein. 1μg of monoclonal antibody/10^6 cells was used to stain cells both for flow cytometric analysis and the immunobead separation technology (materials and methods section 2.7E), while 10 μg/mL of monoclonal antibody was used in the immunohistology studies (materials and methods section 2.18).

Table 2.5.1 List of anti-mouse monoclonal antibodies used in FACS analysis, immunohistology and immunobead separation technology.

Specificity	Antibody	Clone	Isotype	Form*	Source†/ Catalogue Number
T cells	$\alpha\beta$ TCR	H57-597	Hamster IgG	Purified	Pharmingen 01301D
	T3 complex (CD3)ϵ	145-2C11	Hamster IgG	Purified	Cedarlane CL7202F
	L3/T4 (CD4)	YTS 191.1.2	Rat IgG2b	Phyco-erytherin	Cedarlane CL012PE
	L3/T4 (CD4)	RM4-5	Rat IgG2a,κ	FITC and Biotin	Pharmingen 01064D 01062D
	Ly-2 (CD8a)	YTS 169.4	Rat IgG2b	Phyco-erytherin	Cedarlane CL169PE
	Ly-2 (CD8a)	53-6.7	Rat IgG2a,κ	FITC and Biotin	Pharmingen 01044D 01042D
B cells	IgM	II/41	Rat IgG2a	FITC	Pharmingen 02204D
	CD45R (B220)	RA3-6B2	Rat IgG2a,κ	Phyco-erytherin	Pharmingen 01125B
Activated cells	IL-2R (CD25)	3C7	Rat IgG2b,κ	Phyco-erytherin	Pharmingen 01105A
	Transferrin receptor (CD71)	C2	Rat IgG1,,κ	Phyco-erytherin	Pharmingen 01595A

* FITC, fluorescein isothiocyanate

† Cedarlane Laboratories (Hornby, Ontario); Pharmingen (San Diego, California); Serotec (Toronto, Ontario).

Table 2.5.1(contd) List of anti-mouse monoclonal antibodies used in FACS analysis, immunohistology and immunobead separation technology.

Specificity	Antibody	Clone	Isotype	Form*	Source†/ Catalogue Number
Macrophages	Pan macrophages and monocytes	Moma-2	Rat IgG2b	Purified	Serotec MCA 519
	Mac-1 (CD 11b)	M1/70	Rat IgG2b	Purified	Serotec MCA 74
Langerhans cells	DEC-205R	NLDC-145	Rat IgG2a	Purified	Serotec MCA 949
	Heat Stable Antigen (CD24)	J11d	Rat IgM,κ	Purified	Pharmingen 01251D
	MHC class II (I-A^d)	AMS-32.1	SJL IgG2b,κ	Biotin	Pharmingen 06032D
	MHC class II (I-A^b)	MRC OX-3	Mouse IgG1	FITC	Serotec MCA45B
	MHC class I (*H2*^{b})	KH95	BALB/c IgG2b,κ	Phyco-erytherin and Biotin	Pharmingen 06115A 06112D
	MHC class I (*H2*^{d})	34-2-12	C3H IgG2a,κ	Biotin	Pharmingen 06132D
	ICAM-1 (CD54)	3E2	Hamster IgG	FITC and Biotin	Pharmingen 01544D 01542D
	B7.1 (CD80)	1G10	Rat IgG2a,κ	FITC	Pharmingen 01944D
	B7.2 (CD86)	GL1	Rat IgG2a	FITC	Pharmingen 09274D
	Leukocyte common antigen (CD45)	30F11.1	Mouse IgG2b,κ	Purified	Pharmingen 01111D
	F4/80 Antigen	C1:A3-1	Rat IgG2b	Purified	Serotec MCA 497B

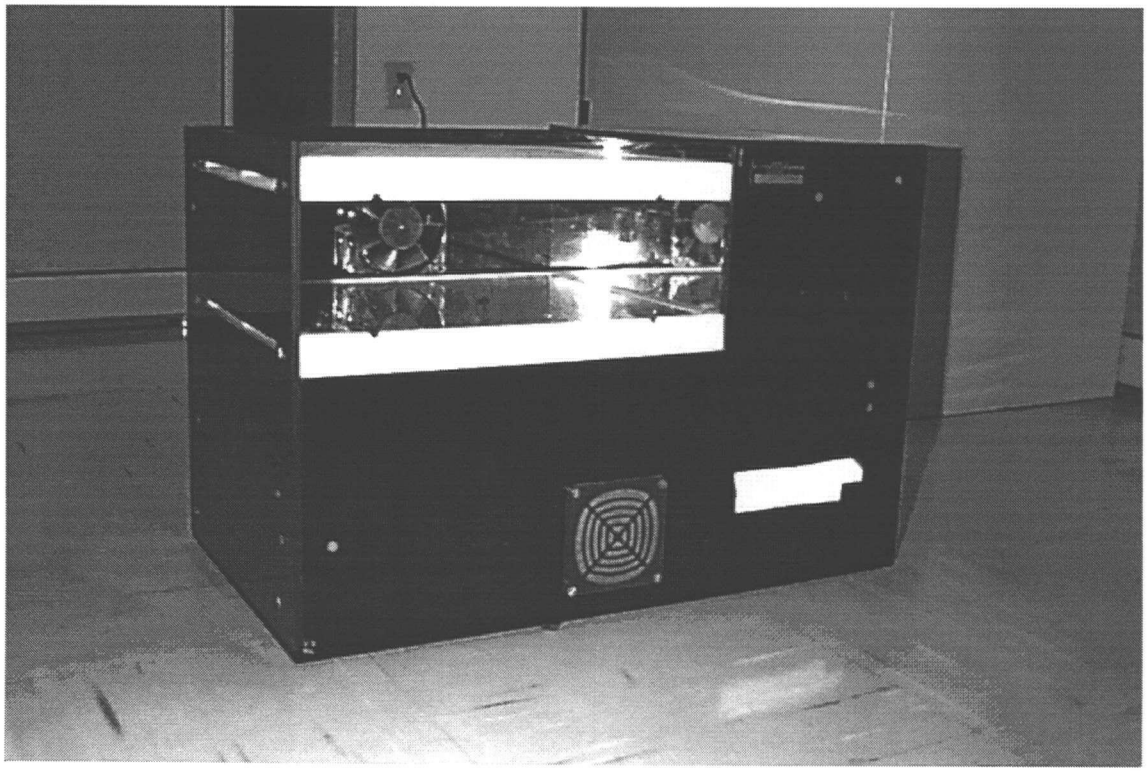
* FITC, fluorescein isothiocyanate

† Cedarlane Laboratories (Hornby, Ontario); Pharmingen (San Diego, California); Serotec (Toronto, Ontario).

2.6 Light source.

The light source was an light emitting diode (LED) Box VER.2 (QLT PhotoTherapeutics Inc., Vancouver, B.C.) consisting of 2 panels of light emitting diodes, each composed of 1280 LED (Hewlett Packard) and separated by a distance of 8 cm (Fig. 2.6.1) The peak wavelength was 696 nm and the full width of the spectrum, at ½ maximum, was 25 nm (Fig. 2.6.2). The intensity of each panel was checked with a photometer (IL 1400A Radiometer/Photometer, International Light Inc., Newburyport, MA) before each experiment and was adjusted to 50 mW/cm^2.

Fig. 2.6.1. The light emitting diode (LED) Box VER.2 (QLT PhotoTherapeutics Inc., Vancouver, B.C.). The light box (25 x 18 cm) consisting of 2 panels of light emitting diodes, each composed of 1280 LEDs (Hewlett Packard) and separated by a distance of 8 cm.



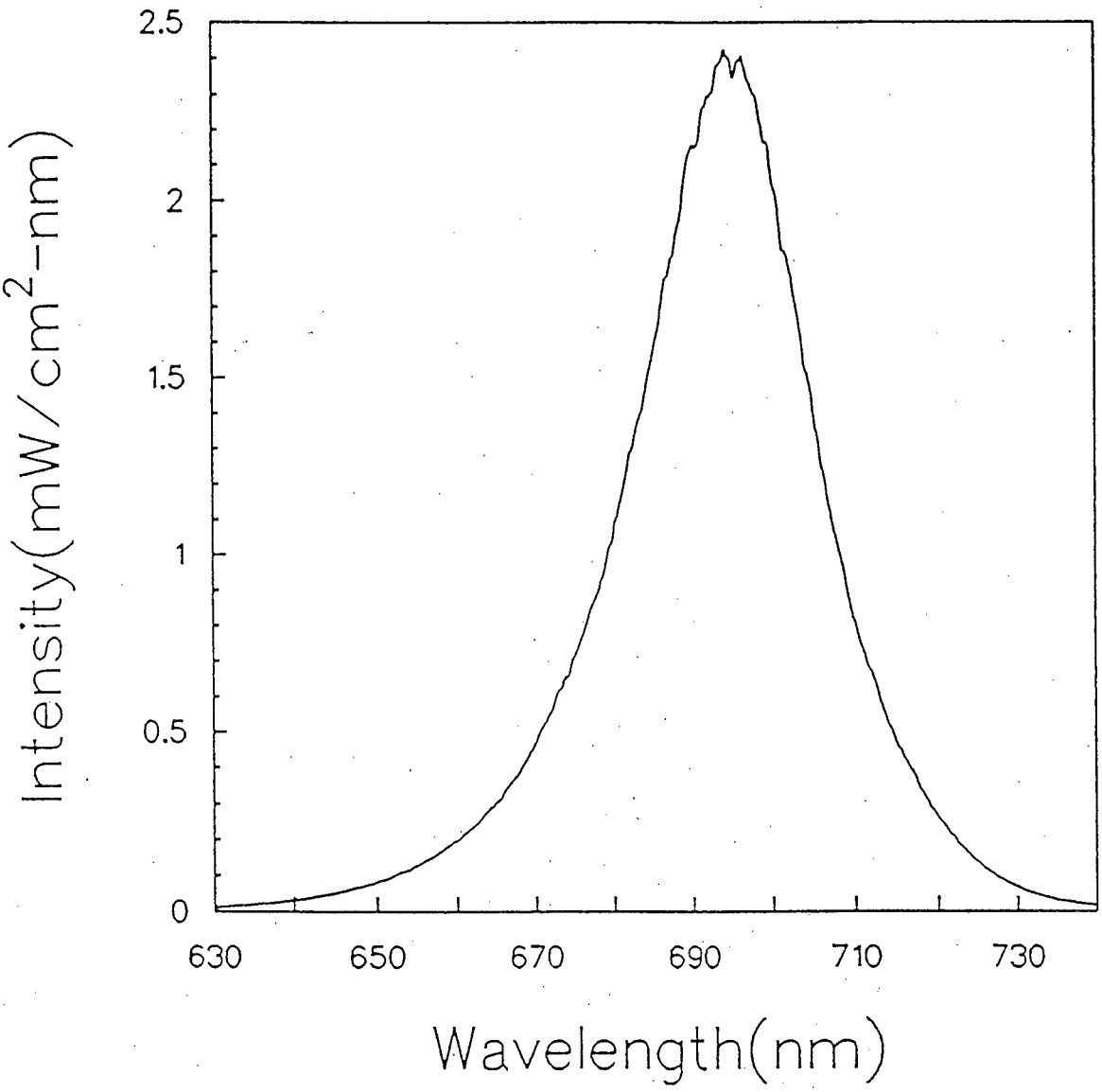


Fig. 2.6.2. The spectrum of the LED panels of the LED light box. The peak wavelength was 696 nm and the full width of the spectrum, at ½ maximum, was 25 nm.

2.7 Cell preparations.

a. Splenocytes.

Spleen cells were prepared as described by Richter *et al.* (1994b). Normal murine (male DBA/2, Charles River Labs, St. Constant, Quebec) spleen cells were obtained aseptically by preparing a single cell suspension from whole spleens. The tissue was pressed through a wire mesh (Fisher Scientific, Ottawa, ON) into a complete culture medium (RPMI-10, Gibco). Splenocytes were washed via centrifugation (1000 rpm x 5 min x 4 °C) and erythrocytes lysed in 0.83% Tris-buffered Ammonium chloride (Sigma), pH 7.2. Later, cells were washed by centrifugation (1000 rpm x 5 min x 4 °C), resuspended in complete culture medium (RPMI-10, Gibco) and cell viability was determined by trypan blue exclusion.

b. Lymph node cells.

Lymph node (superficial inguinal and axillary) cell suspension were prepared with a similar protocol to that described above for splenocytes except that the lysis step with Tris-buffered Ammonium chloride (Sigma) was omitted.

c. Enriched T cells.

T cells were enriched from cell suspensions obtained from murine spleen and lymph nodes using the nylon wool columns according to the standard protocol of Julius *et al.* (1973) as described elsewhere (Hathcock *et al.*, 1991).

i. Preparation of sterilized nylon wool column. Nylon wool columns were assembled using ready-to-use scrubbed and dried nylon wool (Type 200L, Cat. #: NCC-600,

Du Pont Biotech. Boston, MA), and sterilized according to standard protocol (Hathcock *et al.*, 1994). Briefly, the scrubbed nylon wool was fluffed by combing between canine grooming brushes until nylon was free of knots and trebled in volume. Fluffed nylon wool (2.0 g) was inserted and compacted into a 20-ml disposable syringe (Becton Dickinson and Co., Rutherford, NJ) using the plunger from the syringe following which the columns were packaged in an autoclave plastic bag (Baxter Health Care Corp., Deerfield, IL) for autoclaving. The columns were autoclaved for 15 min at 110 °C on a slow exhaust (no dry cycle) (Market Forge Co, New York) and subsequently stored at room temperature until used.

ii. T cell enrichment using nylon wool columns. This was performed according to a standard protocol of Julius *et al.* (1973) as described by (Hathcock *et al.*, 1994). Briefly, an infusion set with a 19-G needle (E-Z set®, Becton Dickinson, Sandy, Utah) was attached to the sterilized nylon wool column clamped to a ring stand (Fisher Scientific) in a laminar flow hood (Nuaire, Plymouth, MN). The column was equilibrated by running 37 °C complete culture medium (25 - 50 mL) (RPMI-10, Gibco) through it. Trapped air bubbles were removed by firmly tapping on the sides of the column until no white (dry) areas were visible followed by tamping down with a sterile pipet to compact the nylon and extrude any additional trapped air. The infusion set was closed and the nylon wool covered with 2 to 3 ml of 37 °C complete culture medium (RPMI-10, Gibco) to prevent dryness. To maintain sterility, the needle was capped and the column covered with the top of a 50-ml conical tube (Falcon). The column was incubated in an upright position for 1 h in a 37 °C, 5% CO_2 humidified incubator (Forma Scientific, Marietta, OH)

following which the medium was allowed to drain completely. The splenic and lymph node single cell suspension (7.5 x 10^7 cells/ml) in complete medium (4 mL) (RPMI-10, Gibco) were added to the column and likewise allowed to drain completely. 1 ml of 37 °C complete medium (RPMI-10, Gibco) was added to ensure that all cells penetrated the column. The infusion set was closed and 2 to 3 ml of 37 °C complete RPMI-10 was added to the column to prevent dryness. With the column covered to maintain sterility, it was incubated for 45 min in an upright position in a 37 °C, 5% CO_2 humidified incubator (Forma). Later, the column was removed from the incubator and clamped to the ring stand. The 19-G needle was replaced with a 23-G needle and the column was filled with 37 °C complete RPMI-10. Immediately, the first 15 mL of the nonadherent, effluent cells were collected in a graduated 50-ml conical tube (Falcon), centrifuged (Beckmann, Palo Alto, CA) for 10 min at 1000 rpm (200 x g) and resuspended in complete RPMI-10. Viable cell yield, assessed routinely using trypan blue exclusion, was > 95 %.

d. Epidermal cell (EC) suspension.

Truncal skin that had been previously exposed *in vitro* to BPD (0 to 1.0 μg/mL) and light (10 J/cm^2 at 690 nm wavelength) was floated dermal side down on 2.4 U/mL neutral protease from Bacillus polymyxa (Dispase type II, Boehringer Mannheim Biochemica, Laval, Quebec) in phosphate buffered-saline (PBS) (Gibco) for 1.5 to 2 h at 37 °C. Epidermal sheets were collected and dissociated by pressing them through a wire mesh (Fisher) into a complete culture medium (RPMI-10, Gibco). The resulting epidermal cell suspension was carefully pipetted and washed three times via centrifugation (1000 rpm x 10 min x 4 °C) and supernatant disposal using 10 ml of the supplemented culture medium

(RPMI-1640, Gibco) for each washing step. Later, cells were resuspended in 2 mL of complete culture medium (RPMI-10, Gibco).

Dead cells and cell debris were removed by passing the cell suspension over a loosely packed sterile glass wool (Corning, # 3950, Corning, NY) column using a modified protocol based on that described by Mishell *et al.* (1980). Briefly, the glass wool columns were prepared by loosely packing small amounts (20 - 50 mg) of glass wool into 3 ml disposable syringes such that fluid flowed through the columns rapidly. The columns were autoclaved for 15 min at 110°C on a slow exhaust (no dry cycle) (Market Forge Co, New York). Upon cooling, the columns were rinsed thoroughly with the complete culture medium (RPMI-10, Gibco) making sure that medium flowed through the columns rapidly. The single epidermal cell suspension in culture medium was passed through the column followed by a rinse step in which complete culture medium of equal volume was also passed through the column. Cell suspension was centrifuged for 10 min at 1000 rpm (200 x g) (Beckmann) and resuspended in complete RPMI-10 medium until used.

e. Dynal magnetic beads-purified Langerhans cells (LC).

The method for enriching Langerhans cells using the magnetic bead separation technology was based on the modification of the protocols described elsewhere (Leclercq *et al.* 1991; Elbe *et al.*, 1992) and that on the manufacturer's instruction guide (Dynal, Inc., New York). Briefly, dynal beads with chemically bound streptavidin (Dynabeads® M-280 Streptavidin, Dynal, Inc., New York), were washed three times in phosphate buffered-saline (PBS) (Gibco) supplemented with 1 % heat-inactivated FBS (Gibco) using the Dynal Magnetic Particle Concentrator (MPC) following which they were resuspended (2 x 10^8

beads/mL) in PBS (Gibco) containing 1 % heat-inactivated FBS. Epidermal cells prepared as described (section 2.7D) were adjusted to 3-5 x 10^6/mL in PBS (Gibco) containing 1 % heat-inactivated FBS and incubated on ice for 30 min with biotin-conjugated mouse macrophage/Langerhans cell monoclonal antibody, F4/80 (1 μg/10^6 cells) (Serotec, Toronto, Ontario). Immediately following incubation, cells were washed twice (1000 rpm x 10 min x 4 °C) with ice cold PBS containing 1 % heat-inactivated FBS. Subsequently, epidermal cells were resuspended at 5 x 10^7/mL in PBS containing 1 % heat inactivated FBS and 25 μL of Dynabeads® M-280 Streptavidin (2 x 10^8/mL of EC) and incubated for 30 min on ice with gentle rotation. The cells bound to beads were separated using the Dynal MPC, washed twice (1000 rpm x 10 min x 4°C) and resuspended in complete culture medium until used. The yield was 2 - 5 % of the starting numbers with viability, assessed by trypan blue exclusion, of > 98 % in both treated and control samples. The purified epidermal cells routinely contained > 90 % Langerhans cells, as assessed by flow cytometry based on cells that stained positive for the anti-non-lymphoid DC (NLDC)-145 (LC-specific) and anti-MHC class II (I-A^b) monoclonal antibodies.

f. Two-step density gradient-enriched LC.

The method used for the enrichment of Langerhans cells using the step-wise density gradient centrifugation was based on the modification of a protocol described by Zambruno *et al.* (1995). Briefly, 1 mL of epidermal cell suspension (1-5 x 10^7 /mL) in complete medium, isolated from treated or untreated donor skin (section 2.7D), was carefully overlaid on 10 mL Percoll solution(1.07 g/mL; Pharmacia Biotechnology Inc., Quebec) and centrifuged at 2000 rpm for 30 min at 18 °C. The medium-Percoll interface cells were

harvested, centrifuged twice (1000 rpm x 10 x 18 °C) and resuspended in 1 mL culture medium. Cells were overlaid on 10 mL Ficoll-Paque (1.064 g/mL; Pharmacia Biotechnology Inc., Quebec) solution and centrifuged at 2000 rpm for 30 min at 18 °C following which interface cells (enriched Langerhans cells) were harvested, washed thrice via centrifugation (1000 rpm x 10 x 18 °C) in complete medium and counted. The yield was 2 - 3 % of the starting numbers and the cell viability, assessed by trypan blue exclusion, was routinely $\geq$ 75 % in both treated and control samples. These interface epidermal cells routinely contained 60 - 70 % Langerhans cells, as assessed by flow cytometry on the basis of cells that stained positive staining for the anti-non-lymphoid DC (NLDC)-145 (LC-specific) and anti-MHC class II (I-A^b) monoclonal antibodies.

2.8 Uptake studies via fluorescence measurements of BPD and analogues.

We used *in vitro* and *in vivo* mitogen stimulated murine splenic lymphocytes for this assay. The mitogen used was a lectin from *Canavalia ensiformis* (concanavalin (Con A), Sigma C-5275), a known stimulator of T lymphocytes (Anaclerio *et al.*, 1974). *In vitro* stimulation of cells (2 x 10^6/mL) was carried out in medium containing 10 % FBS and Con A (2.5 μg/mL; Sigma) for 0-72 h following which they were used for BPD uptake and photocytotoxicity experiments (section 2.9). T cell enriched splenocyte populations which had been cultured for 24 h in the complete RPMI-10 medium, were used as controls. For *in vivo* stimulation, mice (male DBA/2) were injected intravenously (i.v.) with 120 μg/mouse Con A 24 h prior to sacrifice and removal of splenic lymphocytes. Control cells

were splenocytes removed from litter mates that did not receive Con A. Experiments were carried out in the complete RPMI-10 medium (section 2.3).

The method used for the uptake studies was based upon a modification of a protocol described by Richter *et al.* (1994b). Briefly, cells (2 x 10^6/mL) were incubated at 37 °C in RPMI 1640 medium containing 10 % heat inactivated and pre-tested FBS and BPD (2.0 μg/mL) for 0-60 min. At various times, aliquots were removed and washed twice by centrifugation (1000 rpm x 10 min) and supernatant disposal. Cells (2 x 10^6/mL) were lysed by means of freeze-thawing thrice in 2 % Triton X-100 (Sigma) in phosphate buffered saline (PBS) (Gibco). Lysed samples were analyzed immediately by fluorescence measurements. Standards were prepared by adding known concentrations of BPD to equivalent cell lysates unexposed to BPD.

The amount of BPD and analogues in cell lysate was quantified by fluorescence measurements using a spectrofluorometer (Jasco Model FP-770, Japan Spectroscopic Co., Tokyo, Japan) and a 1 ml-microcuvette (Far UV Quartz cell, Starna Cells, California). The excitation and emission wavelengths were set at 439 and 699 nm respectively at which no photoadducts of BPD absorb light. Cell lysates were diluted in PBS (Gibco) containing 1 % Triton X-100 immediately before measurement. Concentrations were determined from a standard curve and were expressed as ng BPD/mg cell protein. Protein content was assessed using Folin phenol reagent after alkaline copper treatment as described in the Lowry method (Lowry *et al.*, 1951).

2.9 Photocytotoxicity assay.

P815 cells and mitogen-stimulated or unstimulated splenocytes (1.5 x 10^6/mL) were incubated in the dark at 37 °C in complete culture medium (RPMI-10) and BPD or analogues (0-50 ng/mL) for 30 min. Control cells were incubated without BPD. Following exposure to BPD or analogues, 200 μl of cells (1.5 x 10^6/mL) were washed twice by centrifugation (1000 rpm x 10 min), dispensed into 96-well flat bottom microtitre plates (Falcon 3077, Becton Dickinson Labware, New Jersey) in quadruplicate and exposed to light (7.2 J/cm^2) at a wavelength of 690 nm. Cells (1.5 x 10^6/mL) were re-stimulated overnight with Con A (2.5 μg/mL) (Sigma) and cell viabilities were determined by the MTT (3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyl tetrazolium bromide) (Sigma) assay (section 2.10). Cell survival was expressed as a percent of the result for control cells incubated without BPD.

2.10 The MTT assay.

The MTT assay was performed according to established protocol (Mosmann, 1983; Blackman *et al.*, 1990; Chen *et al.*, 1990; Wooley *et al.*, 1993). Briefly, MTT (3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyl tetrazolium bromide) (Sigma) was dissolved in PBS (Gibco) at 5 mg/mL and filtered for sterility. At various times, stock MTT solution (10 μL per 200 μL medium or cell suspension) was added to the flat bottom assay wells containing 200 μL of medium and the 96-well flat bottom microtitre plates (Falcon 3077, Becton Dickinson Labware, New Jersey) were incubated at 37 °C for 3 h. Wells were centrifuged

(model TJ-6 centrifuge, Beckmann) for 10 min at 2300 rpm using a rotor equipped with 96-well holder (TH-4 rotor, Beckmann) and the supernatants decanted to eliminate excessive serum in the culture medium. 50 μL PBS and 150 μL of acid-alcohol (1.67 mL concentrated HCl in 500 mL 2-isopropanol) (BDH Inc., Toronto ON) were added to all wells and incubated protected from light for 10 min at room temperature. The contents of each well were mixed thoroughly to dissolve the dark blue crystals by repeated up and down pipetting using a digital multichannel pipette (Eflab, Finlab). The plates were read within 30 min of adding the acid-alcohol solution on a Dynatech MR5000 microelisa plate reader (Dynatech Laboratories, Chantilly, VA), using a wavelength of 590 nm.

2.11 Responses to mitogens.

Single cell suspensions were prepared from spleens and draining lymph nodes (superficial inguinal and axillary) of 8 - 10 week old BALB/c graft recipients or DBA/2 mice of similar age as described (section 2.7A and B). Following erythrocyte lysis (for splenocytes only) with 0.83% Tris-buffered ammonium chloride (pH 7.2) (Sigma), cells were washed thrice in culture medium via centrifugation (1000 rpm x 10 min), counted by trypan blue exclusion and cell density adjusted to 2 x 10^6/mL in complete culture medium (RPMI-10, Gibco). Cells were seeded into 96-well flat bottom microtitre plates (Falcon 3077, Becton Dickinson Labware, New Jersey) in 200 μL (2 x 10^5 cells/well) and incubated with either Con A (Sigma), at a final concentration of 2.5 μg/mL) or lipopolysaccharide from E. coli 055B5 (Sigma) (LPS, at a final concentration of 12.5 μg/mL) for 72 h at 37

°C. Cell proliferation was determined at the end of the incubation using the MTT colorimetric assay (section 2.9) (Blackman *et al.*, 1990; Chen *et al.*, 1990; Wooley *et al.*, 1993).

2.12 Cell analysis by fluorescence-activated cell sorter (FACS).

a. Lymphocytes.

Mitogen-stimulated or unstimulated spleen cells (10^6 cells/mL) were incubated in the absence or presence of BPD (2 μg/mL) in the dark at 37 °C in RPMI 1640 medium containing 10 % FBS for 30 min. Cells were washed twice in culture medium by centrifugation (10 min at 1000 rpm) and resuspended in PBS containing 1 % FBS and 0.1 % sodium azide. Cell surface antigens were labelled for flow cytometric studies by a protocol based on that of Starkey *et al.* (1988). Briefly, cells (10^6/mL) were incubated on ice with a panel of FITC-conjugated (CD8, IgM) or PE-conjugated [CD4, B220, IL-2R (CD25), transferrin receptor (CD71)] or unconjugated ($\alpha\beta$ TCR, CD3ϵ, Mac-1, Moma-2) anti-mouse monoclonal antibodies (Table 2.5.1) for 30 min following which cells were washed twice via centrifugation (1000 rpm x 10 min x 4 °C) with ice cold PBS (Gibco). Cells that were incubated with unconjugated monoclonal antibodies were further incubated on ice with FITC-conjugated anti-IgG (secondary antibody) for 30 min followed by a washing step (1000 rpm x 10 min x 4 °C). Later, cells were analyzed with the EPICS XL® flow cytometry system (Coulter Corp., Miami, FL) for surface antigen alone (quantitative single parameter FACS analysis) or BPD fluorescence and surface antigen (dual parameter

FACS analysis). The excitation wavelength employed for FACS analysis involving BPD was 488 nm while a 690 nm emission (longpass) filter was utilized to detect BPD (red) fluorescence. Also, the excitation wavelength used for cells incubated with FITC- or PE-conjugated monoclonal antibodies was 488 nm while 525 and 575 nm emission (longpass) filters were used to detect FITC and PE fluorescence respectively. To eliminate non-specific binding, gates and quadrants were drawn based on cell samples that were unstained (negative controls) or stained with isotype-matched control antibodies (isotypic controls). Ten thousand cells were analyzed for each histogram.

b. Langerhans cells.

Langerhans cells were purified using either immunomagnetic separation technology or the stepwise density gradient centrifugation described (sections 2.7E and F). Cells (2 x 10^5/mL) were washed twice (1000 rpm x 10 min x 4 °C) and resuspended in PBS (Gibco) containing 1 % heat-inactivated FBS (Gibco) and 0.1 % sodium azide (Sigma). Cell surface antigens were labelled for flow cytometric studies as described above (see section 2.10A) using anti-mouse FITC-conjugated ICAM-1, MHC class II (I-Ab), B7.1 and B7.2 monoclonal antibodies; purified Leukocyte Common Antigen (CD45) and Biotin- or PE-conjugated MHC class I ($H2^b$).

2.13 Skin grafting.

Allogeneic skin transplants from donor C57BL/6 ($H2^b$) to recipient BALB/c ($H2^d$) mice were performed according to established procedures (Billingham and Medawar, 1951).

Briefly, truncal skin of C57BL/6 mice was carefully shaved and used to obtain full thickness skin (about 1 cm x 1 cm). BALB/c mice were shaved and anaesthetized by intra-peritoneal injection of 0.1 ml of a mixture containing ketamine hydrochloride (100 mg/mL; Ayerst Laboratories, Montreal QC), xylasine (20 mg/mL; Chemagro Ltd, Bayvet Division, Etobicoke ON) and PBS (Gibco) combined in a ratio of 2 : 1 : 7. The graft bed was prepared on the right lateral thoracic wall by a careful dissection of the truncal skin (about 1 cm x 1 cm) from each recipient, taking care to maintain the integrity of the panniculus carnosus. Grafts were applied and held with Vetbond tissue adhesive (3M Animal Care Products, St. Paul, MN) applied to the interface of the graft and graft bed. The graft was pressed down with vaseline- (Chesebrough-Ponds Canada, Markham ON) coated sterile gauze sponges (Code 63096, Kendal Canada Inc., Peterborough ON) and the graft and gauze were held in place with Vetrap bandaging tape (3M Animal Care Products, St. Paul, MN) which encompassed the body. The reliability of the skin preparation and grafting techniques were confirmed by performing syngeneic transplants. The success rate for long term ($>$ 120 days) syngrafts exceeded 90 %. Allografts were considered rejected in most instances when necrotic patches were first observed within the grafted tissue, i.e., the onset of rejection. In the earliest experiments (Table 4.3.1), however, allograft rejection time was determined as the time at which $\geq$ 80 % of the graft had become necrotic. This assessment procedure was replaced with the above stated end point since this method was considered to be more objective. Graft survival was expressed as mean $\pm$ standard deviation survival time (days) or percent prolongation relative to control allografts.

2.14 Transdermal PDT of skin grafts.

a. Transdermal PDT of recipient mice.

Transdermal PDT using liposomal BPD verteporfin (section 2.1) was performed at various times (0 - 7 days post grafting) using a standard procedure based upon a previously described technique (Richter *et al.*, 1994b). Briefly, animals received BPD (1.0 mg/kg) intravenously and were kept in the dark for ½ h. Animals were then placed in clear plexiglass containers and exposed to light (15 J/cm^2) at a wavelength of 690 nm. The light was delivered from the light emitting diodes (LED) at a wavelength of 690 nm $\pm$ 10 nm. Treatment time was 15 min. This regimen was previously shown to produce no detectable skin photosensitivity or systemic toxicity (Richter *et al.*, 1994b). Animals were monitored daily in a "blinded" manner by trained personnel via visual and tactile examination for signs of tissue rejection from the 6th day following transplantation.

b. Transdermal PDT of donor skin.

Skin sections were incubated in the dark at 37 °C in a serum-free electrolyte solution (Plasmalyte A, pH 7.4, Baxter Corp., Toronto, Ontario) containing BPD (0 to 1.0 μg/mL) for 30 min. Immediately thereafter, the tissues were exposed to light (10 J/cm^2) from the light emitting diode (LED) source (λ = 690 nm $\pm$ 10 nm) and transplanted onto recipients as described above (section 2.14) (Fig. 2.14.1). Bandages were removed after six days and the skin graft survival was subsequently scored daily in a "blinded" manner by trained personnel through visual and tactile examination for signs of tissue rejection.

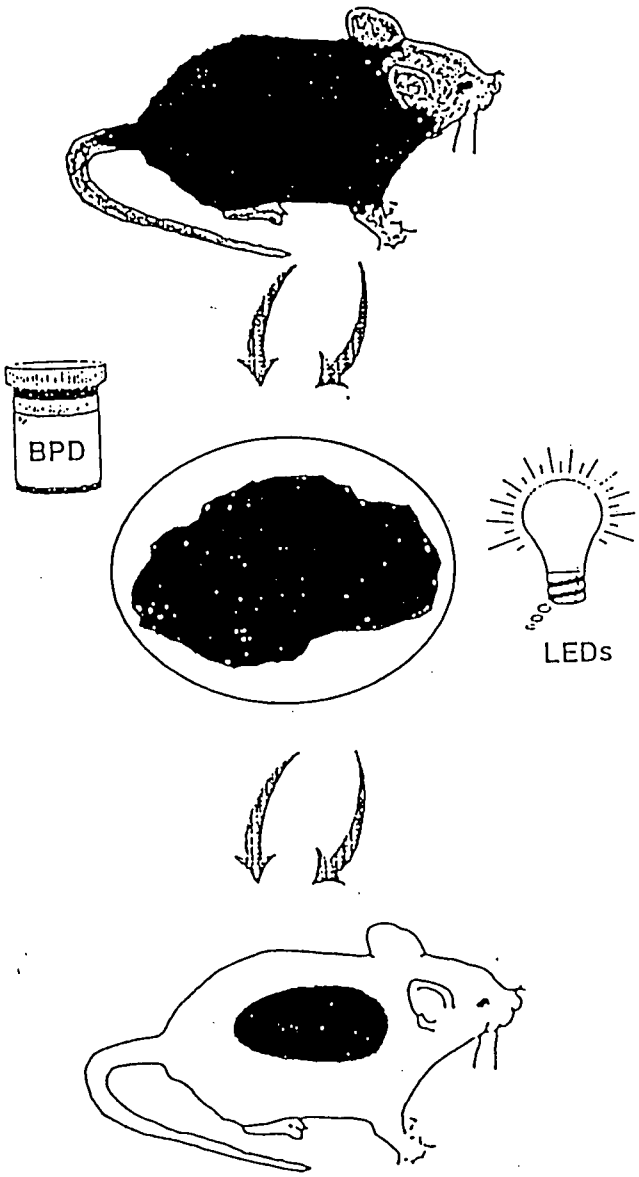


Fig. 2.14.1 Schematic diagram illustrating the transdermal PDT of donor skin.

2.15 Fate of Langerhans cells following transdermal PDT.

Initially, the fate of epidermal LC in response to a contact sensitizer, FITC, following application of transdermal PDT was investigated. Briefly, untreated or mice given transdermal PDT (1.0 mg/Kg and 10 J/cm^2 LED light) were topically painted on the right lateral thoracic wall with FITC (400 μl) (1 mg/mL) in acetone immediately following transdermal PDT. 24 h post treatment, we obtained lymph node cells by preparing single cell suspensions from the draining lymph nodes (superficial inguinal and axillary) of these mice and analyzed them for differences in mean fluorescence intensities of surface antigens characteristic of antigen presenting cells (I-A^d (MHC Class II), ICAM-1 and B7.1) or surface antigen found specifically on LC (DEC-205, recognized by the non-lymphoid dendritic cell (NLDC)-145 monoclonal antibody) and an antigen found on LC as well as lymph node B cells (heat stable antigen (HSA) (section 2.12). We compared results obtained from mice given transdermal PDT and FITC to those which received FITC or PDT alone or none of the treatments.

Alternatively, we investigated the fate of epidermal LC in response to another contact sensitizer known for long-term tracing of living cells (Cumberledge and Krasnow, 1993), Cell Tracker™ Green BODIPY® (8-chloromethyl-4,4-difluoro-1,3,5,5-tetramethyl-4-bora-3a,4a-diazaindacene; Molecular Probes Inc, Eugene OR), following application of transdermal PDT. Untreated or mice given transdermal PDT (0.25, 0.5 and 1.0 mg/Kg and 10 J/cm^2 LED light) were topically painted on the right lateral thoracic wall with the Green BODIPY® (400 μl of 1 mg/mL) in dimethyl sulfoxide (DMSO) and acetone solution

immediately following transdermal PDT. 24 h later, single cell suspensions from the draining lymph nodes (superficial inguinal and axillary) of these mice were analyzed for differences in mean fluorescence intensity of Green BODIPY®. The FITC channel (FL1) of the XL® flow cytometer was used since the maximum λ_{EX} and λ_{EM} for Green BODIPY® were 522 and 528 nm respectively. Data were expressed as mean fluorescence intensity of Green BODIPY® ± standard deviation. We compared results obtained from mice given transdermal PDT and Green BODIPY® to those which received Green BODIPY® (positive control) or none of the treatments (negative control).

2.16 Mixed epidermal cell-lymphocyte reaction (MECLR).

Allogeneic T cells were enriched from cervical and inguinal lymph nodes by passage over a nylon wool column as described (section 2.7C), washed three times (1000 rpm x 10 min) and resuspended in complete culture medium (RPMI-10, Gibco). The epidermal cells (accessory/stimulator cells) were pre-treated with the anti-proliferative agent mitomycin C (Sigma) at 100 μg/mL, 37 °C for 30 min, washed thrice in culture medium via centrifugation (1000 rpm x 10 min) and supernatant disposal and resuspended in complete culture medium. The mitomycin C-treated stimulator epidermal cells (3 x 10^5/well) and the responder enriched allogeneic T cells (4 x 10^5/well) were co-cultured in a 96-well Falcon round-bottomed microtitre plates (0.2 mL/well) (Falcon 3077, Becton Dickinson Labware, New Jersey) containing complete medium (RPMI-10, Gibco). The microtitre plates were incubated for 120 h in a humidified 5 % CO_2 atmosphere at 37 °C. Cell proliferation was

quantified by the non-radioactive 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (section 2.10). The contents of each well were transferred to a 96-well Falcon flat-bottomed microtitre plate and read on a microelisa plate recorder (Dynatech MR5000, Dynatech Laboratories). Results were expressed as percentages of the results obtained with epidermal cells isolated from untreated skin samples, taken as 100 % response.

In experiments where enriched Langerhans cells (LC) were used, varying doses of the viable stimulator epidermal LC (5×10^3 - 4×10^5 LC/well) were co-cultured (0.2 mL/well) with 4×10^5 responder enriched allogeneic T cells/well in replicates of 4 - 8 wells (96-well Falcon round-bottomed microtitre plates). We obtained optimal allogeneic T cell proliferation at LC to T cell ratio of 1:4 i.e. at 1×10^5 LC/well. Thus, all subsequent assays were performed at this cell concentration.

2.17 Histological evaluation of skin sections.

In order to evaluate whether PDT of donor skin produced any histopathologic changes, skin samples were treated with low-dose PDT as described (section 2.14B). Tissues were maintained in electrolyte solution (Plasmalyte A, pH 7.4, Baxter Corp., Toronto, Ontario) for an additional 3 - 24 h at 37 °C following which they were fixed in 10 % formalin. Serial 3 - 5 μm thick sections were stained with haematoxylin and eosin. Microscopic (Axiovert 35, Carl Zeiss Inc., Germany) assessment of the haematoxylin and eosin slides were performed "double blind" by a board certified veterinary pathologist, Dr.

P.N. Nation (Veterinary Pathology Laboratory, Edmonton, Alberta) and Dr. L.G. Ratkay (QLT PhotoTherapeutics Inc., Vancouver) who assessed each anatomic layer and assigned scores to pathologic changes that reflected the degree of inflammation and vascular change. Scores were assigned using a set criteria in which 0 = absence of inflammatory infiltrates, 1 = minimal infiltrating cells, 2 = mild infiltration, 3 = mild to moderate infiltration, 4 = moderate infiltration, 5 = moderate to marked infiltration, and 6 = marked infiltration.

2.18 Immunohistological evaluation of skin sections.

In order to identify the effect of low-dose PDT on the surface antigens of LC within donor skin or on the subpopulation of cells infiltrating the graft post transplantation, skin graft samples and lymph nodes (superficial inguinal and axillary) were collected from donor mice or graft recipients on various times (4 - 8 days post days post engraftment). Tissues were blotted, immersed into Histo Prep™ (Fisher, Fair Lawn, NJ), then snap frozen in liquid nitrogen-chilled isopentane. 8-10 μm thick sections of tissue blocks were cut on a cryostat (2800 Frigocut-N, Reichert-Jung) at -25 °C and collected on electrocoated precleaned glass slides (Fisher). Sections were immunostained with an alkaline-phosphatase streptavidin-biotin method as described elsewhere (Ratkay *et al.*, 1994a) applying commercially available biotinylated monoclonal antibodies directed against mouse cell surface antigens: MHC class II (I-A^d) and ICAM-1 antigens (LC, macrophages), CD4 (T helper) and CD8 (T cytotoxic) (Table 2.1). Following staining, the slides were treated with

New Fuchsin containing Tris buffered saline (pH 7.6) supplemented with 0.1 % Lavamisol (Sigma, St. Louis, MO). The slides were developed under the microscope and counterstained with Mayers's haematoxylin (Sigma), covered with Crystal Mount (Biomeda, Foster City, CA) overnight at room temperature, and permanently mounted with Entellan Merck, Darmstadt, Germany). The slides were later evaluated "blind" under a light microscope (Axiovert 35, Carl Zeiss Inc., Germany) by Dr. L.G. Ratkay (QLT Phototherapeutics Inc, Vancouver) and J. Ratkay (Vancouver). Scores were assigned reflecting the intensity of each stain using set scoring criteria such that 0 = absence of stain, 1 = minimal stain at some areas, 2 = mild stain, 3 = mild to moderate areas of stain, 4 = moderate areas of stain, moderate to marked areas of stain, and 6 = areas of marked staining intensity.

2.19 ATPase staining of epidermal Langerhans cells.

Adenosine triphosphatase-positive cells were identified in enriched epidermal Langerhans cell suspension as described elsewhere (Mackenzie and Squier, 1975; Girolomoni *et al.*, 1993). Briefly, desegregated epidermal cells were obtained from treated or untreated donor mice truncal skin. Langerhans cells were enriched via a single step Ficoll-Paque density gradient centrifugation as described (sections 2.7F). Cells (1 x 10^6/mL) were cytocentrifuged onto clean microscope slides (Fisherbrand Superfrost/Plus; Fisher Scientific, Vancouver) using a standard protocol (Chen-Woan *et al.*, 1996). Briefly, a filter card (SCA-005, Shandon, Sewickley, PA) was assembled between the sample chamber and

a microscopic slide according to the manufacturer's instruction. Cell suspension in complete medium (0.1 mL), diluted in PBS (0.4 mL, Gibco) was added to the sample chamber of the assembled cytospin unit (filter paper-sample chamber-microscopic slide). The cytospin units were loaded to the cytocentrifuge (Cytospin 2, Shandon Southern Products Ltd., Cheshire, England) and spun for 5 min at 700 rpm. Specimens were air dried, fixed in ice cold cocadylate buffered formaldehyde {4% formaldehyde (BDH Chemicals, Toronto ON), 0.8 M sodium cocadylate (Sigma) and 0.2 M sucrose (BDH)} for 20 min, and washed in 3 changes of 40 mL ice cold Trismal buffer (0.2 M Trismal maleate (Sigma), 0.2 M Sucrose (BDH), pH 7.3). They were incubated at 37 °C for 30 min in freshly prepared substrate solution containing 10 mg ATP (Sigma), 5 mL of 5 % magnesium sulphate (Sigma), 3 mL of 2 % lead nitrate (Fisher) in 42 mL Trismal buffer. After 3 washes (40 mL) in Trismal buffer, the specimens were immersed in 0.5 % ammonium sulphide (Sigma) solution for 5 min at room temperature, washed 4 times in 40 mL of distilled water, and mounted in a mounting medium {90 % glycerol (BDH) with 10 % PBS (Gibco)} for microscopic observation.

2.20 Micrographs.

Immunohistology, histology, ATPase slides or cell cultures were observed under a Zeiss inverted microscope (Axiovert 35, Carl Zeiss Inc., Germany) using a transmitted light mode. The light microscope was equipped for a 35-mm camera attachment. Pictures of slides as well as that of graft recipients were taken with Nikon camera (Nikon Japan) using

Fuji color films (ASA 100 and 400, Fuji Japan).

2.21 Statistical analysis.

Treated or untreated group means were compared by the Student's *t*-test using paired sample for means and the regular analysis of variance (ANOVA). Semi-quantitative data obtained using a ranking measurements (ordinal scale) were analyzed using the non-parametric statistic, Kolmogorov-Smirnov test for different distributions. All statistical analysis were performed with the term significant at alpha (ρ-value) = 0.05. Intra-group differences were compared with the Bonferroni (all-pairwise) multiple comparison procedure. Experimental results are presented as the arithmetic means plus or minus the standard deviations (SD) of the mean for each group.

CHAPTER THREE

TARGETING ACTIVATED LYMPHOCYTES WITH PHOTODYNAMIC THERAPY: SUSCEPTIBILITY OF MITOGEN-STIMULATED SPLENIC LYMPHOCYTES TO BPD PHOTOSENSITIZATION

3.1 Abstract.

BPD or its analogues (BPD monoacid ring B, and BPD diacid ring A and ring B) may have potential for both oncologic and non-oncologic applications in PDT. To study the influence of cellular characteristics on the uptake of BPD or analogues , the murine tumor cell line (P815), and *in vitro* and *in vivo* Concanavalin A (Con A)-stimulated and unstimulated murine splenic lymphocytes were incubated with 2 μg/mL BPD or analogues at 37 °C for 0 - 60 min. At various times, cells were lysed and the amount of BPD or analogues taken up by the cells was quantified by fluorescence measurements. Furthermore, the subsets of cells taking up BPD were analyzed using a panel of monoclonal antibodies and the Coulter XL® FACS. Subsequently, Con A-stimulated and unstimulated spleen cells were incubated with 0 - 50 ng/mL of BPD for 1 h prior to exposure to light (7.2 J/cm^2, λ = 690 $\pm$ 10 nm). Cell survival 24 h post PDT was measured by the MTT assay. We found that the rapidly dividing tumor cell line and mitogen stimulated murine T cells (mainly $CD4^+$/IL-$2R^+$) took up significantly more BPD (5-10 fold) than do unstimulated splenic lymphocytes. Increased BPD uptake correlated with greater cytotoxicity when these cells were exposed to light at a wavelength of 690 nm. These findings suggest that activated and rapidly dividing cells may be targets for photoinactivation by BPD.

3.2 Introduction.

Immunologically activated T cells are implicated in the rejection of skin (Rosenberg and Singer, 1992; Shelton *et al.*, 1992) and other organ allografts as well as in the pathology of a majority of autoimmune conditions including; rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and insulin-dependent diabetes. Most of the immunosuppressive agents, including cyclosporine A (CsA) which is currently used to treat rejection or autoimmune diseases, inhibit Ca^{2+}-dependent activation of lymphokine gene transcription, an essential step in T cell activation (Fung, *et al.*, 1992; Lui, 1993; Fruman *et al.*, 1994). However, the interference in T-cell proliferation is non-specific. As a result, most of the agents induce significant adverse reactions and is generally immunosuppressive. Consequently, this leaves the patients at risk of developing potentially deadly opportunistic infections (Trucco and Stassi, 1996). Therefore, the exploration of new, less toxic, methods of immunosuppressive therapy appears necessary.

In this chapter we studied the feasibility of targeting activated cells with PDT because of a series of observations which indicate that PDT alters the outcome of immunological phenomena including mixed leukocyte reaction, response to mitogens and hypersensitivity reaction. It is envisaged that PDT, unlike CsA, might be more specific such that activated cells implicated in the rejection of skin allografts and in the pathogenesis of autoimmune conditions are targeted while the general immune system is spared. Interestingly, many photosensitizers used in PDT appear to be taken up rapidly and selectively by rapidly proliferating tissues and cells. Jamieson *et al.* (1990) demonstrated

that leukaemic cells and cell lines took up more BPD than did normal bone marrow cells and peripheral blood leukocytes. Experiments using whole blood have also shown that activated human leukocytes expressing high levels of IL-2 receptor (IL-2R) and HLA-DR antigens were selectively depleted from a heterogenous mixture by BPD and light (North *et al.*, 1993). Thus, it appears that PDT may allow activated cells to be destroyed with some selectivity. Furthermore, the strong absorption band of BPD at 690 nm allows maximal transmission through tissue with minimal attenuation by blood pigments (North *et al.*, 1992) and may enable BPD associated with leukocytes in the circulation to be selectively photoinactivated. On that basis, a new approach for PDT as an immunomodulatory technology was developed in our laboratory whereby photosensitizers were activated in the blood by whole body illumination with visible light without causing photosensitivity (Richter *et al.*, 1994b). This procedure was termed transdermal photodynamic therapy to reflect the fact that BPD was activated in circulation by light that was being delivered through the skin. A treatment window for the exposure of experimental animals to red light (15 J/cm^2 at a wavelength of 690 $\pm$ 10 nm) was established to be 1 h following intravenous administration of BPD at doses up to 1.0 mg/kg. The potential of transdermal PDT has been explored and found to be effective in different animal models. We showed that transdermal PDT using BPD may inhibit disease onset in adjuvant-enhanced arthritis in MRL-lpr mice by selectively eliminating the adjuvant-activated lymphocytes in the circulation and/or joints of mice in this model (Chowdhary *et al.*, 1994). Similarly, Hunt *et al.* showed that transdermal PDT delayed the onset of paralysis of mice in an experimental allergic encephalomyelitis (EAE) model (Hunt *et al.*, 1994). Recently, we (Simkin *et al.*, 1995) showed that transdermal PDT

using BPD significantly inhibited the development of contact hypersensitivity (CHS) response against the hapten dinitrofluorobenzene (DNFB). Similar immunosuppressive actions of PDT had also been observed using Photofrin® and light (Elmets and Bowen, 1986; Qin *et al.*, 1993). Specifically, Photofrin® and light had been reported to inhibit the activity of cytotoxic lymphocytes (Franco *et al.*, 1983) and to reversibly inhibit the ability of lymphocytes to proliferate in response to mitogens (Canti *et al.*, 1981; Kol *et al.*, 1986; 1989) or stimulate a mixed leukocyte reaction (Lynch *et al.*, 1989; Canti *et al.*, 1981).

In this chapter, we performed uptake and photoinactivation experiments to establish a correlation between uptake and phototoxicity in resting (normal) and activated hemopoietic cells. Subsequently, we used monoclonal antibodies and flow cytometry to characterize the surface antigens of the subsets of T cells taking up BPD. We have demonstrated a solid correlation between uptake and phototoxicity in resting and activated hemopoietic cells and our findings strongly suggest that immunologically activated cells may be a selective target for BPD photosensitization.

3.3 Results.

a. Uptake and Photodynamic activity of BPD analogues.

Experiments were performed to determine the kinetics of uptake of the four structural analogues of BPD (BPD, BPD-MB, BPD-DA, BPD-DB) by murine mastocytoma (P815) cells (ATCC Rockville, Maryland) under standard incubation conditions in order to determine whether a correlation existed between uptake and phototoxicity. We found that

P815 cells took up significantly greater amounts (2-5 fold) of the monoacids (BPD, BPD-MB) than the diacids (BPD-DA, BPD-DB) (Fig. 3.3.1).

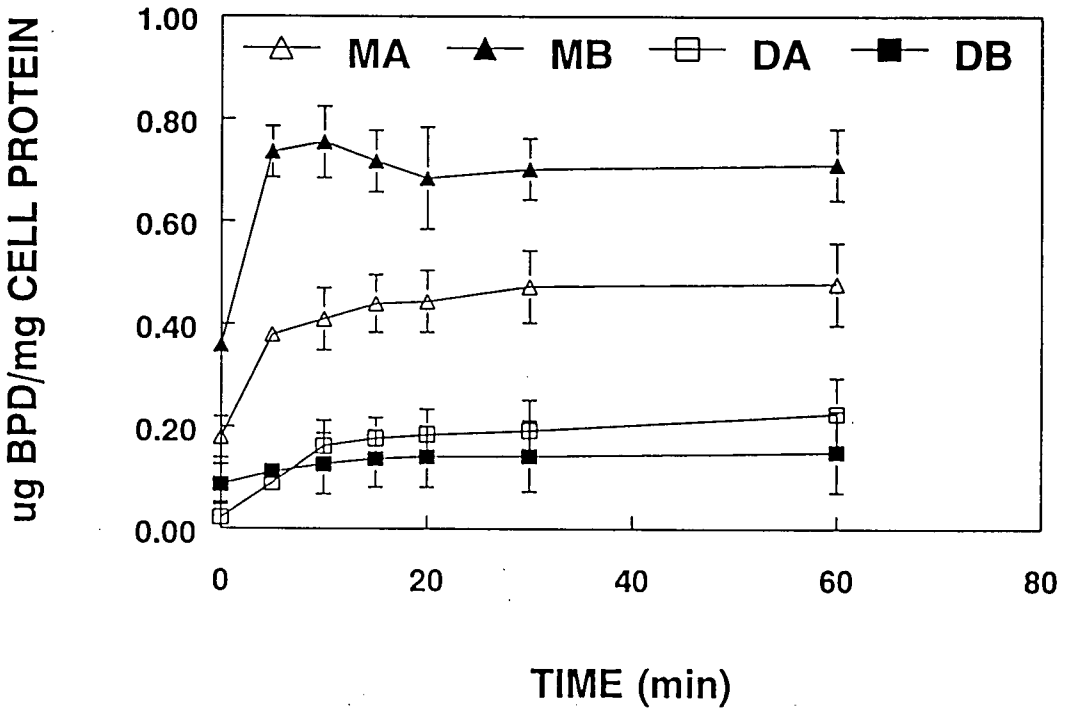


Fig. 3.3.1. Uptake of BPD and structural analogues by P815 cells following 1 - 60 min incubation *in vitro* at 2 μg/mL. The concentration of BPD and analogues was determined by fluorescence (excitation 439 nm, emission 699 nm) in cell lysates in PBS containing 1 % Triton X-100. Each value represents the mean (n = 4) $\pm$ standard deviation.

Because of these structure-dependent differences in uptake, we evaluated whether the differential uptake would translate to a quantitative difference in photodynamic efficiencies by comparing the photosensitizing activity of the four analogues of BPD using P815 cells. Following incubation with the BPD analogues and a washing step (1000 rpm x 10 min), cells were exposed to red light. The results indicate that the susceptibility of P815 cells to cytotoxicity mediated by the four structural analogues differed, especially between monoacids and diacid analogues. The monoacids were at least 5-6 times more potent than the diacids (Fig. 3.3.2). The concentrations of BPD, BPD-MB, -DA and -DB required to kill 50 % of cells (LD_{50}) were 1.2, 1.4, 11.5, and 12.5 ng/mL respectively. This confirmed earlier findings in our laboratory, using M1 cells, that the presence of one or two acids in the porphyrin macrocycle has a major effect on the photosensitizing activity of this family of molecules (Richter *et al.*, 1990b).

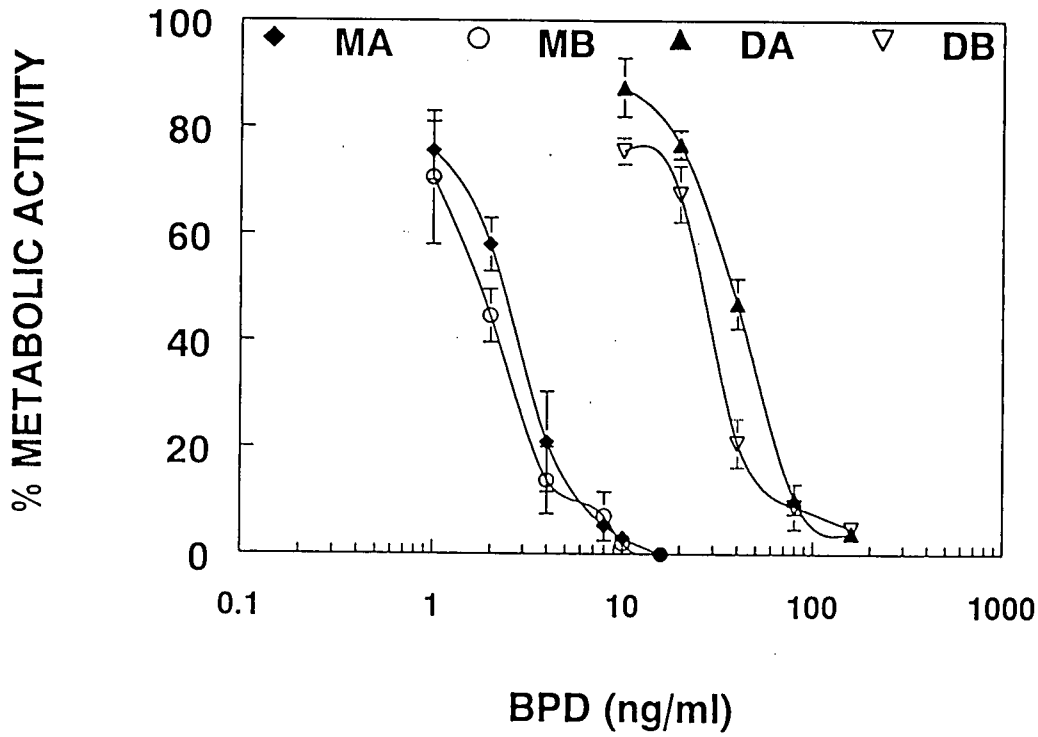


Fig. 3.3.2. Comparison of photosensitizing activity of BPD and structural analogues on P815 cells as tested in an *in vitro* photocytotoxicity assay. Cells (1.5 x 10^6/mL) were incubated for 30 min with photosensitizers, washed with PBS and then exposed to red light (7.2 J/cm^2). Cell viabilities were determined by the MTT assay. Cell survival (determined by metabolic activity using MTT) are presented as the mean of percentages of control cells treated with light only, taken as 100% response. Data are expressed as % metabolic activity (n=3) $\pm$ SD.

In our subsequent experiments, we used the liposomal formulation of BPD monoacid ring A obtained from QLT PhotoTherapeutics Inc. (Vancouver, B.C.). We evaluated whether the kinetics of BPD uptake by mitogen-activated lymphocytes differed from the uptake by non-activated resting cells. We found that uptake of liposomal formulation of BPD into the hemopoietic cells (activated or resting) was very rapid, with maximum concentrations reached within 30 min. Under these experimental conditions, mitogen-activated lymphocytes took up significantly more BPD (5-10 fold) than did resting cells (Fig. 3.3.3).

b. Susceptibility of mitogen-activated and resting lymphocytes to BPD.

Because of the above finding which suggested that rapidly dividing and mitogen-activated lymphocytes took up more BPD (5-10 fold) than did resting cells (Fig. 3.3.3), we further evaluated the susceptibility of mitogen-activated and resting lymphocytes to BPD. Splenocytes taken from mice given Con A 24 h earlier were exposed to BPD and compared to normal splenocytes for susceptibility to PDT. The results show that cells taken from Con A-treated mice were more susceptible to PDT than cells taken from control animals (Fig. 3.3.4). The LD_{50} for cells taken from Con A-treated and untreated litter mates were 1.6 and 2.4 ng/mL respectively. Similarly, splenocytes stimulated *in vitro* with Con A were more susceptible to PDT than unstimulated cells, their LD_{50} being 0.3 and 3.2 ng/mL respectively (Fig. 3.3.5). These results correlated with the data obtained from our uptake studies and suggest that activated splenocytes may be more susceptible to PDT than resting cells.

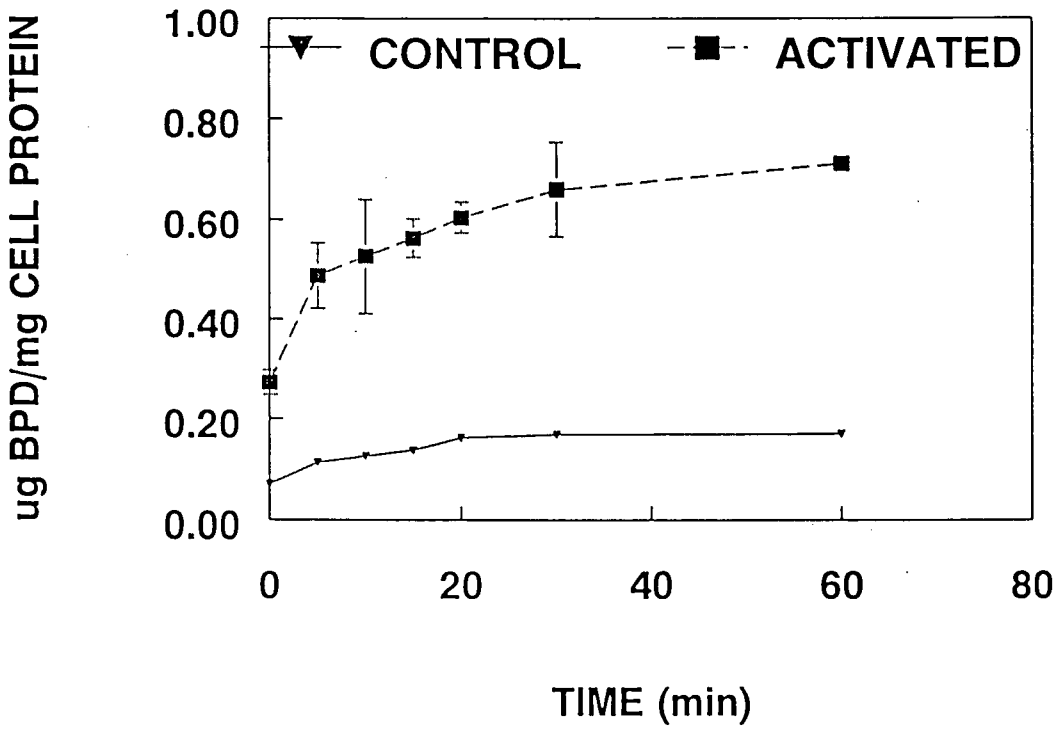


Fig. 3.3.3. Uptake of BPD by normal and *in vitro* Con A-activated mouse splenocytes. Cells (2 x 10^6/mL) were incubated at 37 °C in medium containing 10 % FBS and BPD (2.0 μg/mL) for 0 - 60 min. Splenocytes were activated by culturing with Con A (2.5 μg/mL) for 72 h prior to uptake experiments. Control cells were normal, non-activated splenocytes which were enriched for T cells by 24 h culturing with medium containing 10 % FBS. The concentration of BPD was determined by fluorescence (excitation 439 nm, emission 699 nm) using cell lysates in PBS containing 1 % Triton X-100, and related to the amount of cellular protein. Each value represents mean (n = 4) $\pm$ SD.

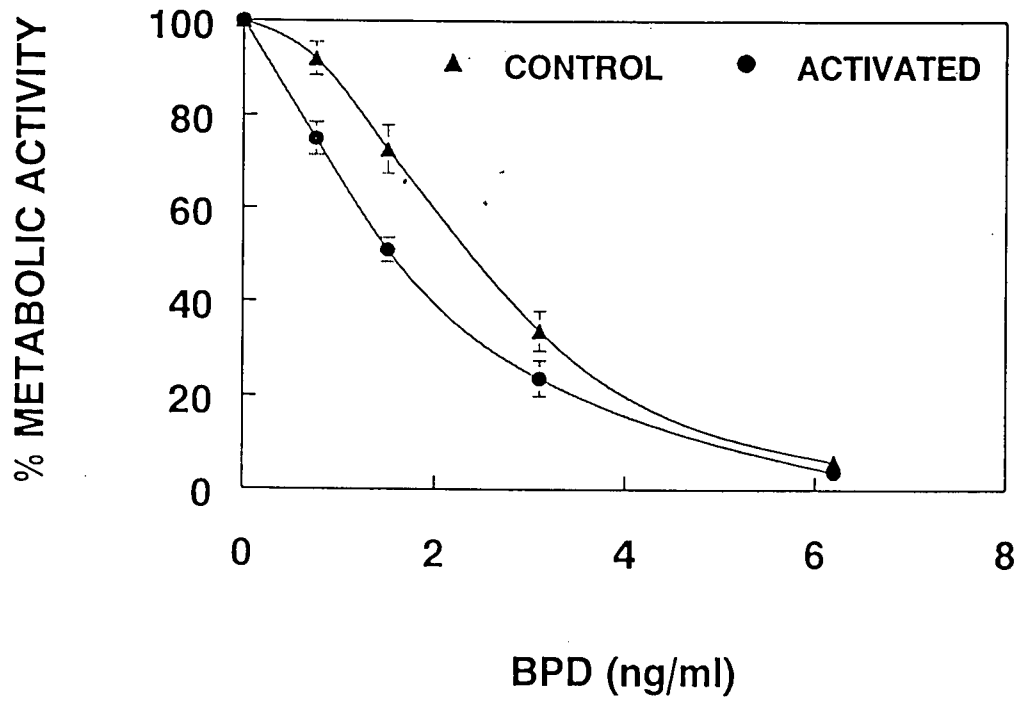


Fig. 3.3.4. *In vitro* photocytotoxicity of BPD on *in vivo* Con A-activated and non-activated splenic lymphocytes. Cells were obtained from mice injected iv with 120 μg/mouse Con A 24 h earlier and from litter mates that did not receive Con A. Cells (1.5 x 10^6/mL) were incubated with BPD for 30 min, washed with PBS and then exposed to red light (7.2 J/cm^2). Cell viabilities were determined by the MTT assay and presented as the mean of percentages of control cells treated with light only, taken as 100% response. Data are expressed as % metabolic activity (n=3) $\pm$ SD.

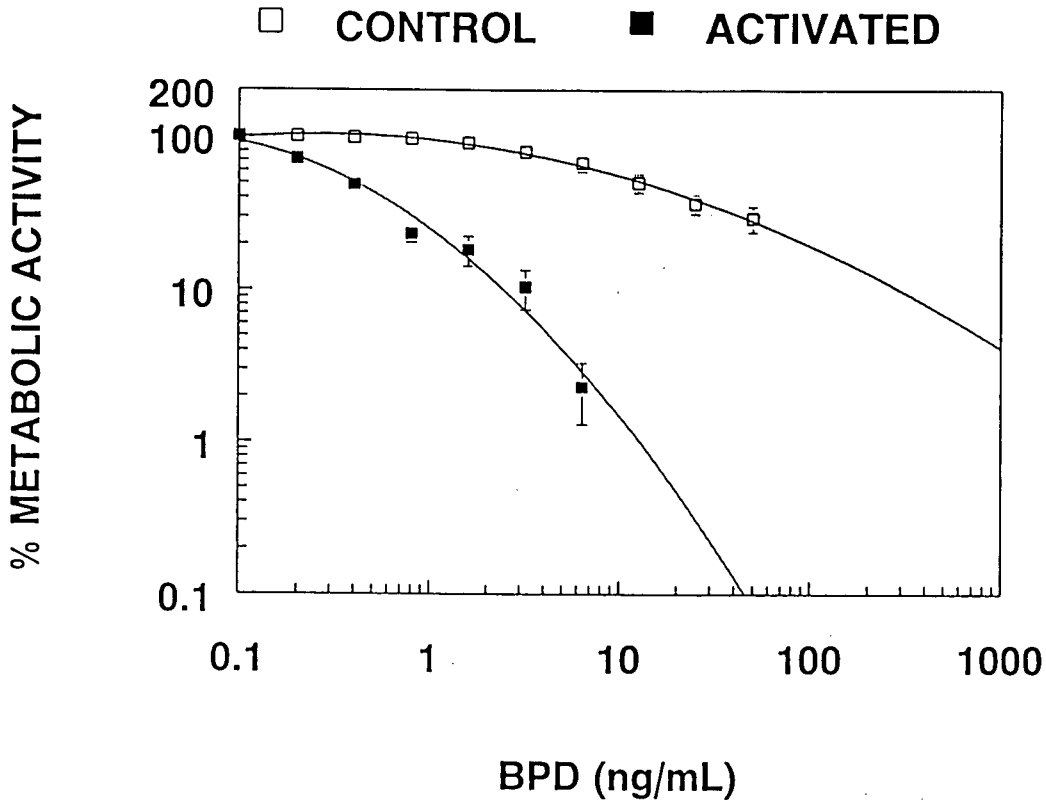


Fig. 3.3.5. Susceptibility of *in vitro* Con A-activated and unactivated murine splenocytes to PDT following 30 min incubation *in vitro* with BPD (0-50 ng/mL) in the presence of 10 % FBS and exposed to red light (7.2 J/cm^2). Cell density was 1.5 x 10^6 cells/mL. Splenocytes were activated by culturing with Con A at 2.5 μg/mL for 72 h prior to photocytotoxicity experiment. Normal, nonactivated splenocytes were enriched for T cells by 24 h culturing in medium containing 10 % FBS. Cell viabilities were determined by the MTT assay and presented as the mean of percentage of control cells treated with light only, taken as 100% response. Data are expressed as % metabolic activity (n=3) $\pm$ SD.

c. Flow cytometric analysis of hemopoietic cells.

To evaluate the proportion of $CD4^+$ T cells that was activated by administering Con A *in vivo*, we analyzed cells taken from Con A-treated mice and compared them to cells taken from control mice for cell surface antigen expression using monoclonal antibodies and flow cytometry. Cells were stained with a panel of rat anti-mouse monoclonal antibodies (materials and method, section 2.12) and analyzed with EPICS XL® FACS machine. We found that the T, but not B, cell pool was activated 24 h following administration of Con A as anticipated. About 10-25% of T cells showed increased levels of expression of activation markers (interleukin (IL)-2 and transferrin (Tr) receptors) (Fig. 3.3.6). In contrast, a majority of the T cells stimulated *in vitro* with Con A were activated; 70 - 80% of the cells being positive for the interleukin-2 receptor ($IL\text{-}2R^+$) (Fig. 3.3.7). Since Con A is known to activate $CD4^+$ T cells, it was not surprising to observe that cells prepared from mice given Con A 24 h earlier, and splenocytes that were stimulated in vitro showed a massive proliferation and/or activation in the $CD4^+$ T cell compartment.

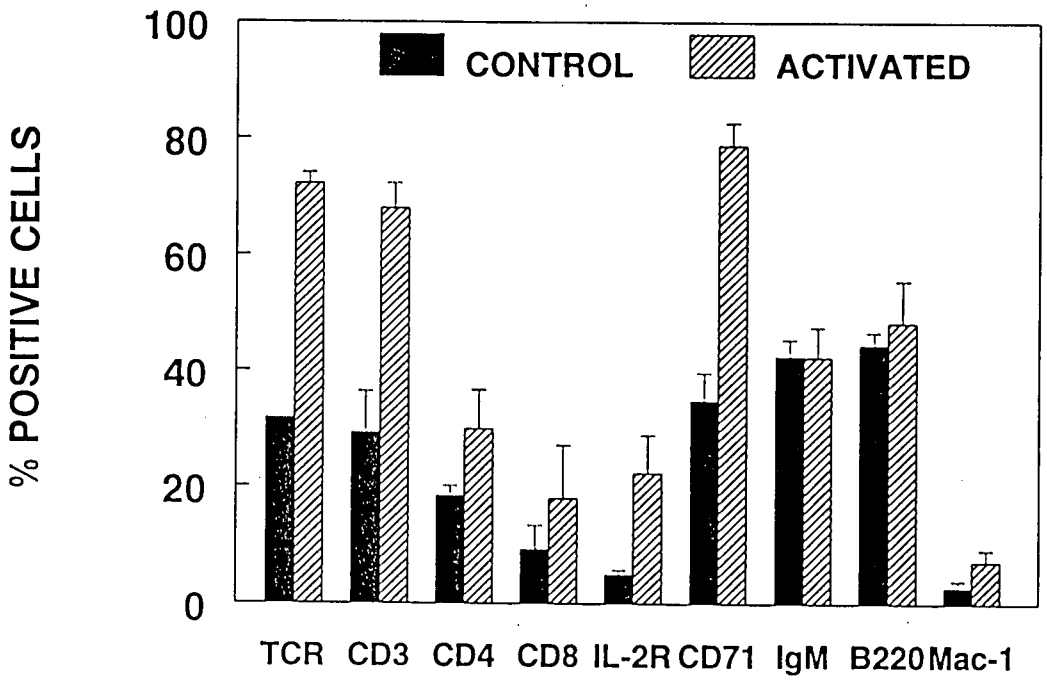


Fig. 3.3.6. Flow cytometric analysis of murine spleen cell surface antigens. Splenic lymphocytes (10^6/mL) from mice injected with Con A 24 h earlier and untreated litter mates were stained with a panel of FITC- or PE-conjugated monoclonal antibodies and analyzed with EPICS XL® flow cytometer. Non-specific binding was eliminated by gating cells based on negative and isotypic control cells. Cells positive for each surface antigen were expressed as mean (n=3) ± SD.

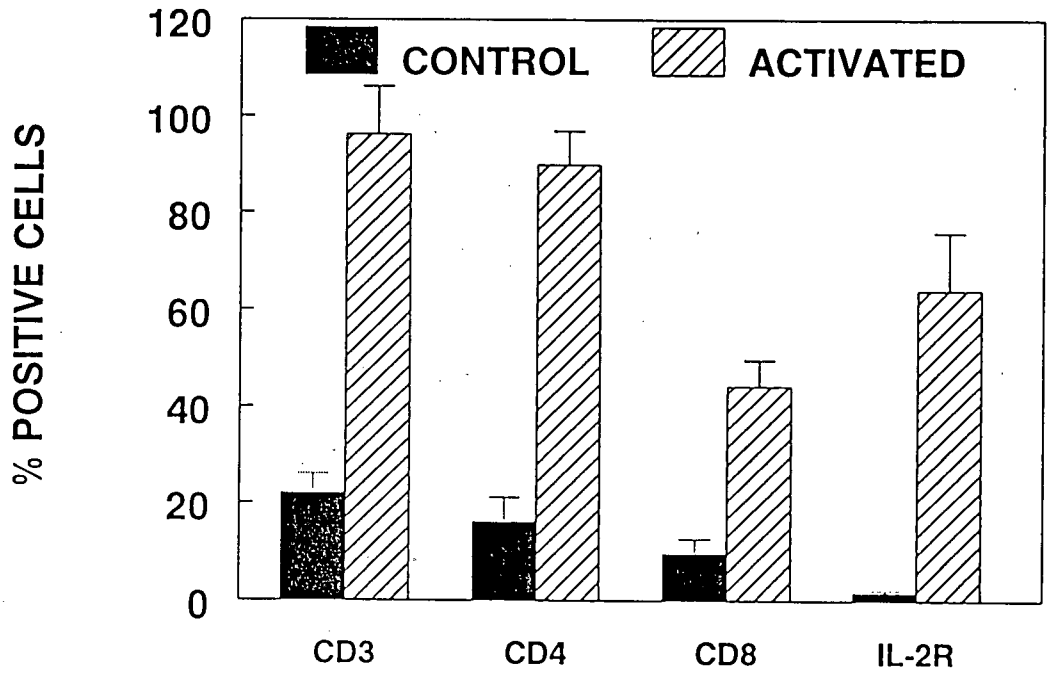


Fig. 3.3.7. FACS analysis of *in vitro* Con A-activated and unactivated murine splenocytes following incubation with anti-mouse T cell monoclonal antibodies (CD3, CD4, CD8 and IL-2R). Splenocytes were activated by culturing them with Con A at 2.5 μg/mL for 72 h prior to photocytotoxicity experiments. Normal, nonactivated splenocytes were used as controls. Cells were analyzed with an EPICS XL® flow cytometer. Non-specific binding was eliminated by gating cells based on negative and isotypic control cells. Cells positive for each surface antigen were expressed as mean (n=3) $\pm$ SD.

Using the EPICS XL® flow cytometer, we evaluated the fluorescence of unactivated and *in vitro* Con A-activated splenic lymphocytes following incubation with BPD. We found that > 80 % of Con A-activated splenic lymphocytes were positive for BPD fluorescence while only 40 - 60 % of unactivated cells were positive for BPD fluorescence (Fig. 3.3.8) when equal number of cells were analyzed. This implies that more of the activated cells took up more BPD in comparison to the unactivated cells. These data confirmed our observation in the uptake studies (Fig. 3.3.3) that Con A-activated splenic lymphocytes took up more BPD than unactivated cells.

Furthermore, we evaluated the subset of T cells that contributed to increased uptake of BPD by Con A activated cells. We exposed *in vitro* Con A-stimulated cells to BPD and FITC-conjugated rat anti-mouse CD4, CD8 and IL-2R following which we performed a dual parameter FACS analysis for T cell surface antigens (CD4, CD8 and IL-2R) and BPD fluorescence. We gated only the cell populations that were highly fluorescent for BPD and used them to evaluate the contribution of different T cell subsets to BPD uptake. The results show that 84.3 % of the cells that took up BPD were $CD4^+$ T cells compared to 34.6 % that were $CD8^+$ T cells (Fig. 3.3.9). 66.3 % of the cells that took up BPD were also positive for IL-2R.

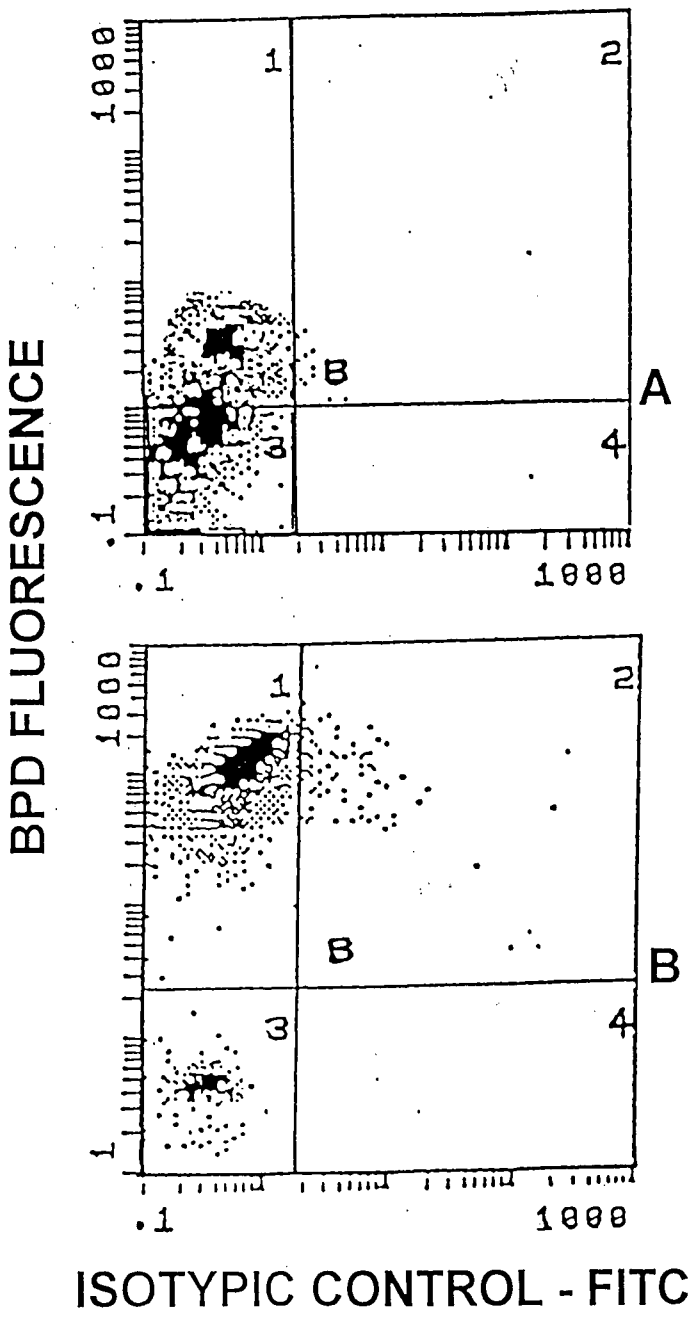


Fig. 3.3.8. BPD fluorescence intensity of a) normal, unstimulated, and b) *in vitro* Con A-stimulated murine splenic lymphocytes by FACS following exposure to BPD. Splenocytes were activated by culturing them with Con A at 2.5 μg/mL for 72 h. Activated and non-activated splenocytes (10^6/mL) were incubated with BPD (2 μg/mL) for 30 min and washed twice in ice cold PBS. Cells were analyzed with EPICS XL® flow cytometer using dual color protocol (BPD fluorescence *vs* Isotypic control FITC). Scatter plot is a representative of 3 independent experiments.

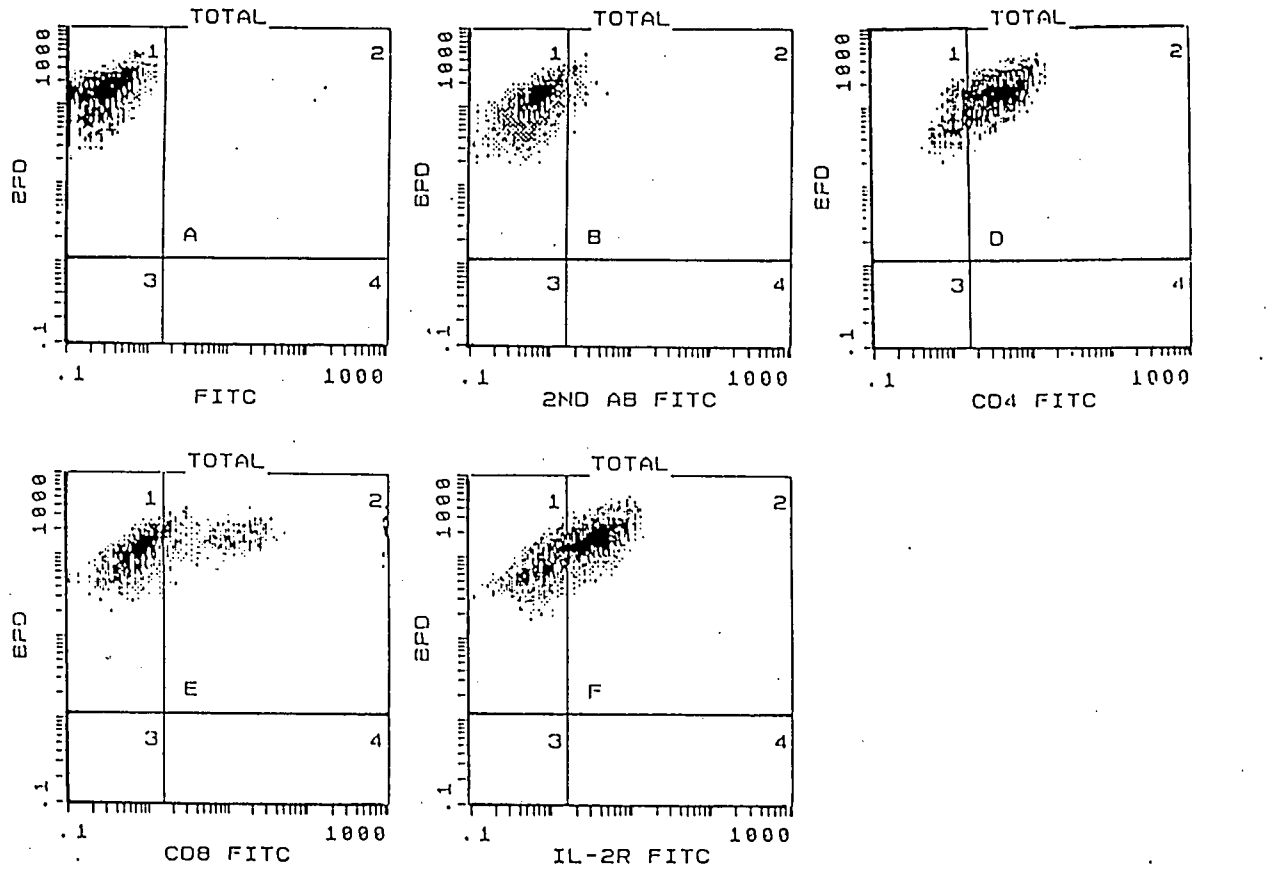


Fig. 3.3.9. Dual color FACS analysis of *in vitro* Con A-activated murine splenic lymphocytes following exposure to BPD. Splenocytes were activated by culturing them with Con A at 2.5 μg/mL for 72 h. Cells (10^6/mL) were incubated with BPD (2 μg/mL) for 30 min and washed twice in ice cold PBS. Cells were incubated with unconjugated CD4, CD8 and IL-2R monoclonal antibodies, washed and stained with FITC-conjugated second antibody. Only cells highly fluorescent for BPD were gated and used for the dual parameter FACS analysis. Scatter plot is representative of 3 independent experiments.

3.4 Discussion.

These studies were undertaken to evaluate the susceptibility of mitogen-stimulated lymphocytes to BPD photosensitization relative to unactivated controls. It is hoped that this model will be beneficial in determining whether this technology might have application for selective immuno-therapy in diseases where activated lymphocytes are implicated. Our initial experiments using a murine tumor hemopoietic cell line, P815 cells, compared the four structural analogues of BPD in uptake and photocytotoxicity studies in order to determine whether a correlation existed between uptake and phototoxicity. The results show that all the four analogues had very similar uptake kinetics. Uptake of BPD by P815 cells was very rapid with maximum levels being reached within 5-20 min. However, BPD monoacids were taken up to much higher levels (2 - 5 fold) by P815 cells than were the diacid forms (Fig. 3.3.1). In general, the monoacid ring B form was taken up much more (up to 2 folds) by P815 cells than the monoacid ring A analogue. Kessel (1989) reported previously that BPD monoacid ring A was more readily taken up by L1210 cells than was BPD diacid ring A. Because of these structure-dependent differences in uptake, we evaluated whether the differential uptake would translate to a quantitative difference in photodynamic efficiencies by comparing the photosensitizing activity of the four analogues of BPD using P815 cells. The results indicate that the susceptibility of P815 cells to cytotoxicity mediated by the four structural analogues differed, especially between monoacids and diacid analogues. The monoacids were at least 5-6 times more potent than the diacids (Fig. 3.3.2). The correlation of uptake to increased phototoxic potency of the

structural analogues suggests the existence of a structure-activity relationship with BPD analogues. This confirms earlier reports from our laboratory that the monoacids were more efficient than the diacids in both *in vitro* and *in vivo* systems (Richter *et al.*, 1990b; Richter *et al.*, 1991). BPD diacids are less hydrophobic, more negatively charged and much less effective as a photosensitizer than the monoacids (Richter *et al.*, 1991). It is possible that these either reduced hydrophobicity or increased negative charge, or both, are most likely responsible for the observed difference in uptake and photosensitizing activity between monoacid and diacid analogues. Furthermore, the differences in photosensitizing potency might be attributable to differences in binding properties at the level of cellular or subcellular membranes. At this level, it is envisaged that small structural differences between these molecules might dictate the subcellular partitioning of the analogues (Richter *et al.*, 1991). The monoacids might partition to and inactivate sensitive cellular targets essential for the survival of the cells. The cellular localization of BPD and analogues is unknown and is a subject of the ongoing studies in our laboratory.

The rationale for studying the feasibility of targeting mitogen-activated splenic lymphocytes with BPD was based on our previous observation that BPD uptake is enhanced in cells that are activated or in cycle in comparison to resting cells (Jamieson *et al.*, 1990; Richter *et al.*, 1994a & b). We confirmed by using spectrofluorimetry and flow cytometry that activated lymphocytes take up significantly more BPD than do resting cells (Fig. 3.3.3 and 3.3.8). The fact that mitogen-activated cells had selectivity for BPD uptake, therefore, raised the question as to whether increased uptake also correlated with photodynamic cell kill. We used both *in vitro* and *in vivo* Con A activated cells for our photocytotoxicity

study. The results showed that mitogen-stimulated cells were more susceptible to BPD photosensitization than unstimulated controls, thus correlating with the results of the uptake studies. It therefore, appears that selective uptake may be a basis upon which to explain the selectivity of mitogen-stimulated lymphocytes to PDT. The mechanism by which activated cells take up more BPD than resting cells is not understood at this time and is currently under study.

We analyzed the surface antigens expressed by Con A-stimulated and control cells. Con A is known to activate $CD4^+$ T cells. Thus, it was not surprising to observe that cells prepared from mice given Con A 24 h earlier, and splenocytes that were stimulated in vitro showed a massive proliferation and/or activation in the $CD4^+$ T cell compartment. Using dual parameter FACS analysis, we confirmed that activated T cells were responsible for the observed increased uptake of BPD by Con-A stimulated cells. The data indicate that IL-$2R^+$ T cells (mainly $CD4^+$ and a few $CD8^+$) may be responsible for the increased uptake of BPD by the activated cell population. Since *in vitro* stimulated splenocytes showed a massive proliferation in the $CD4^+$ T cell compartment, it therefore, appears that increased numbers of IL-$2R^+$ T cells (mainly $CD4^+$ and a few $CD8^+$) within the activated splenic lymphocytes accounted for the greater amount of BPD taken up by activated cells relative to untreated controls. Since uptake correlated with cell killing, it is likely that the selectivity observed in activated splenocytes following PDT were a direct elimination of these activated T cell subsets. This finding is in agreement with the findings of North *et al.* (1993) that BPD and light were selectively killing lymphocytes that bore the activated markers, IL-2R and high levels of human lymphocytes antigen-DR (HLA-DR) in the blood of patients infected with

the virus causing acquired immuno-deficiency syndrome (AIDS).

The studies reported here have shown a new potential for PDT. We (Richter *et al.*, 1994b) have shown that BPD could be activated in the circulation of mice without apparent vascular or skin photosensitivity. Similarly, Chowdhary *et al.* have shown the effectiveness of transdermal PDT by preventing adjuvant-enhanced arthritis in MRL/lpr mice (Chowdhary *et al.*, 1994). We have shown, in this chapter, that activated hemopoietic cells which took up significantly more BPD than resting cells, were more susceptible to PDT-mediated cell death. Since activated cells are implicated in a number of human autoimmune diseases including rheumatoid arthritis, multiple sclerosis and AIDS as well as in the rejection of organ or tissue transplants, it may be possible to target these activated cells using PDT, thus suggesting a possible application for PDT in autoimmune diseases and for the treatment of transplantation rejections. Accordingly, we evaluated the feasibility of using transdermal PDT to prevent skin allograft rejection. This was the subject of chapter four of this thesis.

CHAPTER FOUR

TRANSDERMAL PHOTODYNAMIC THERAPY (PDT) AND SKIN ALLOGRAFT SURVIVAL

4.1 Abstract.

The effect of PDT using BPD on the length of engraftment in skin allograft was tested by two distinct protocols. In the first, transdermal PDT was applied to the grafted area of the skin transplant recipient at various times (0-7 days post grafting). In the second procedure, skin sections (C57BL/6) were exposed *in vitro* to varying doses of BPD (0.125 - 1.0 μg/mL) and light (10 J/cm^2) at a wavelength of 690 nm before implantation onto recipients (BALB/c). We have termed this treatment regimen low-dose PDT to reflect the fact that we used doses of BPD and light that did not cause any significant photodynamic cell damage. We found that application of transdermal PDT at the area of skin allograft did not affect the rate of graft rejection. However, we found that there was a significant increase in the survival of the skin allografts in animals given pretreated donor skin tissues in comparison to the untreated controls depending on the dose of BPD used. Higher doses of BPD did not necessarily translate to the optimal prolongation of the skin allograft survival. Rather, the most beneficial effects of the treatment were observed at lower doses of BPD (0.25 - 0.5 μg/mL) and light (10 J/cm^2, 690 $\pm$ 10 nm wavelength), the treatment at 0.25 μg/mL of BPD and light being optimal. The pretreatment of skin to be grafted with PDT (0.25 μg/mL of BPD and 10 J/cm^2 of visible light at a wavelength of 690 nm) significantly ($p < 0.0001$ by the Bonferroni multiple comparison test) prolonged the survival of allografts from 9.3 (n = 42) $\pm$ 2.2 days (control group) to 16.9 (n = 20) $\pm$ 1.7 days (treated group). The exposure of the tissues to be grafted to BPD or light alone did not prolong the graft survival. These findings suggest that graft enhancement may result

from immunomodulatory effects upon immune cell populations in the skin rather than on any kind of selective cell depletion. The mechanisms by which low-dose PDT prolonged skin allograft survival is the subject of chapters 5, 6 and 7 of this thesis.

4.2 Introduction.

With the introduction of immunosuppressive agents in transplantation biology, several attempts have been made both in experimental models and clinical situations to prolong the survival of allografts and xenografts following transplantation. Major successes in a number of clinical transplant situations have been achieved by treating graft rejection with immunosuppressive agents such as corticosteroids, rapamycin, cyclosporine A, and tacrolimus (FK 506). These treatments centred mainly on the suppression of the immune apparatus of the recipient. However, prolonged usage of the agents and severe suppression of the immune system is necessary to obtain any significant result (Trucco and Stassi, 1996). This makes transplant patients vulnerable to opportunistic infections which have been shown to be the leading cause of death in human transplant patients (Jarowenko *et al.*, 1986). Similarly, most of these agents have inherent toxicities (Fung *et al.*, 1992; Hadden and Smith, 1992).

As a result, researchers in organ transplant experiments have been seeking for ways of achieving donor-specific tolerance while maintaining normal immunity to other antigens (Fung *et al.*, 1992). One way of achieving such specific immunosuppression may be to modify either the antigenicity of the donor tissue or the specific recipient cells capable of

mediating rejection. Since the decisive elucidation by Lechler and Batchelor (1982) that donor strain dendritic cells were required to initiate rejection in renal allograft, several attempts to reduce the antigenicity of donor tissues have been made. Examples inlude organ culture (Lafferty *et al.*, 1975; Lafferty *et al.*, 1976; Bowen *et al.*, 1979), pretreatment of donor tissue with monoclonal antibodies (Faustman *et al.*, 1984; Iwai *et al.*, 1989; Lafferty *et al.*, 1983; 1986), a combination of ultraviolet (UV) A light (320 - 400 nm) and 8 methoxy-psoralen (PUVA) (Gruner *et al.*, 1984, Morison *et al.*, 1981) and a combination of Photofrin® and light (Gruner *et al.*, 1985). Several of these attempts have focused mainly on the depletion of the dendritic cells within the donor tissue. Most of them have led to a prolongation of graft acceptance across MHC barriers in skin, pancreatic islets, and thyroid allografts. However, none of the pretreatment regimens have produced long term donor-specific tolerance in the recipient animal. Furthermore, based on the recent observation that microchimerism can exist for many years in the tissues of human solid organ allograft recipients (Starzl *et al.*, 1993) it has been hypothesized, albeit with a lot of controversy, that microchimerism leads to a state of donor-specific tolerance (Starzl *et al.*, 1996). Since the migratory donor cells required to achieve microchimerism appeared to be the bone marrow-derived dendritic cells (Thomson *et al.*, 1995), one can therefore infer that the total depletion of donor-derived DC may not be the best way to achieve the much desired donor-specific tolerance in cell, tissue or organ transplantation. In fact, this is in agreement with the findings of Rouabhia et al. (Rouabhia *et al.*, 1993) which suggested that the depletion of Langerhans cells (LC) may not be sufficient to sustain skin and epidermal sheet allograft survival. Thus, an anti-rejection strategy focused on the attenuation or modulation rather

than depletion of these MHC-bearing "passenger leukocytes" may be a more selective and less toxic approach for the prevention of allograft rejection. An approach which may be useful in modulating donor-derived dendritic cells is low-dose PDT.

Findings by a number of researchers have indicated that PDT, utilizing porphyrin photosensitizers and visible light, may produce transient suppression of immune responsiveness in the mouse. A dampening effect on the development of the contact hypersensitivity ear swelling response following sensitization and challenge with the sensitizing agent dinitrofluorobenzene (DNFB) was noted in mice treated with Photofrin® (Elmets and Bowen, 1986; Lynch *et al.*, 1989; Musser and Fiel, 1991) and light at an appropriate wavelength. No comprehensive interpretation for the inhibition of this response in PDT-treated mice has been provided, although an immunosuppressive role for splenic adherent cells (presumably macrophages) was proposed (Lynch *et al.*, 1989). Furthermore, the capacity of PDT to modify cellular immune responses was utilized to significantly prolong skin allograft acceptance in recipient mice treated with Photofrin® and intra-peritoneal PDT (Qin *et al.*, 1993). This study indicated that the prolonged graft acceptance observed was a consequence of both the lymphocyte depletion and macrophage activation within the peritoneum of graft recipients (Qin *et al.*, 1993). Similarly, Gruner *et al.* (1985) while working with Photofrin®, pretreated donor mouse tail skin grafts with Photofrin® and light. The skin grafts showed a prolonged survival time on allogeneic recipients; higher doses of Photofrin® and light being most beneficial. The irradiation of Photofrin®-injected mice with light led to a depletion of ATPase-positive epidermal Langerhans cells in the skin.

In our laboratory, working with BPD, we (Richter *et al.*, 1994b) demonstrated that

this photosensitizer could be activated in the blood by whole body illumination with red light, without causing photosensitivity. We have termed this procedure transdermal PDT. Further, we have shown that such treatment could modulate immune responses in certain disease models such as adjuvant arthritis in MRL-*lpr* mice (Chowdhary *et al.*, 1994; Ratkay *et al.*, 1994b) and experimental allergic encephalomyelitis (EAE) in SLJ mice (Hunt *et al.*, 1994). Recently, we (Simkin *et al.*, 1995) showed that transdermal PDT profoundly suppressed contact hypersensitivity (CHS) response to dinitrofluorobenzene (DNFB) in DBA/2 mice.

In this chapter, we report the results obtained on the effect of transdermal PDT on graft rejection reactions using two distinct protocols. In the first, transdermal PDT was applied to the grafted area of skin transplant recipients at various times (0 - 7 days post grafting). In the second procedure, skin allografts were pre-treated with PDT *in vitro* with low doses of BPD and light (a procedure we termed low-dose PDT) and then applied to the recipient mice. This unique approach represents a novel method for reducing the immunogenicity of a donor tissue prior to its introduction to the transplant recipient. Low-dose PDT offers a desirable alternative for the enhancement of graft acceptance and could conceivably minimize the current requirement for the long term treatment of the host with relatively toxic immunosuppressive agents.

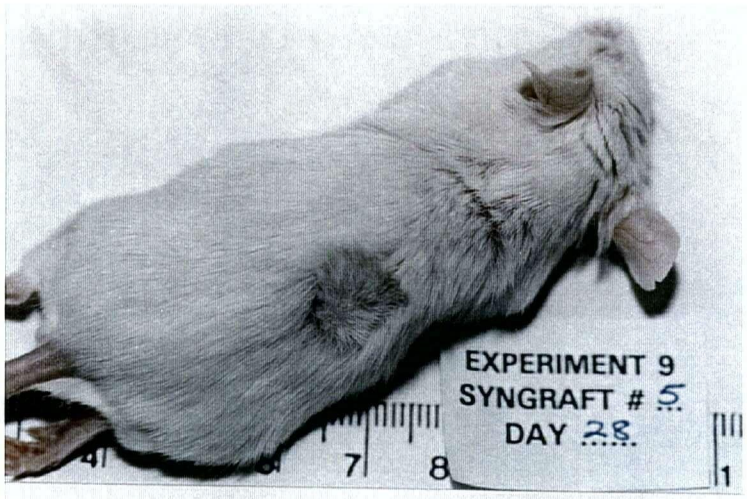
4.3 Results.

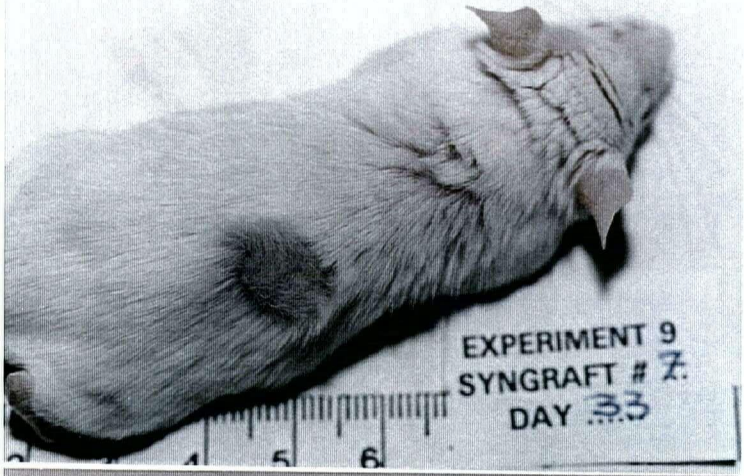
Initially, we tested the reliability of the donor skin preparation and grafting techniques by performing syngeneic transplants. The success rate for long term (> 120 days) syngrafts exceeded 90 %. Figure 4.3.1 shows representative pictures of the indefinitely surviving syngeneic transplants (BALB/c to BALB/c).

a. Transdermal PDT of Recipient mice.

On the basis of our findings which suggest that activated cells of the immune system may be targets for photoinactivation by BPD (chapter 3), we sought to prolong skin allograft survival in mice using transdermal PDT of recipient mice. The skin allograft rejection was considered complete when necrosis within the graft tissue affected $\geq$ 80 % of the transplant surface area. We found that the skin grafts on untreated mice survived for 11.1 $\pm$ 1.9 days. Similarly, when transdermal PDT was used to treat skin transplant areas on recipient animals at various times following skin transfer, we observed that the time of rejection was not significantly different from that observed in the allograft control mice (Table 4.3.1). Furthermore, a few PDT-treated grafts, especially those given transdermal PDT at 4 - 6 days post engraftment, appeared somewhat more vulnerable to necrosis than the untreated controls. Taken together the results indicate that transdermal PDT of recipient mice failed to prolong skin allograft acceptance in our model.

Fig. 4.3.1. Indefinitely (> 120 days post grafting) surviving syngeneic transplants (BALB/c to BALB/c). The progression of graft survival was monitored regularly. Pictures were taken on different days post grafting. Typical pictures are shown for 28, 33 and 120 days post grafting.


EXPERIMENT 9
SYNGRAFT # 5
DAY 28


EXPERIMENT 9
SYNGRAFT # 7
DAY 33

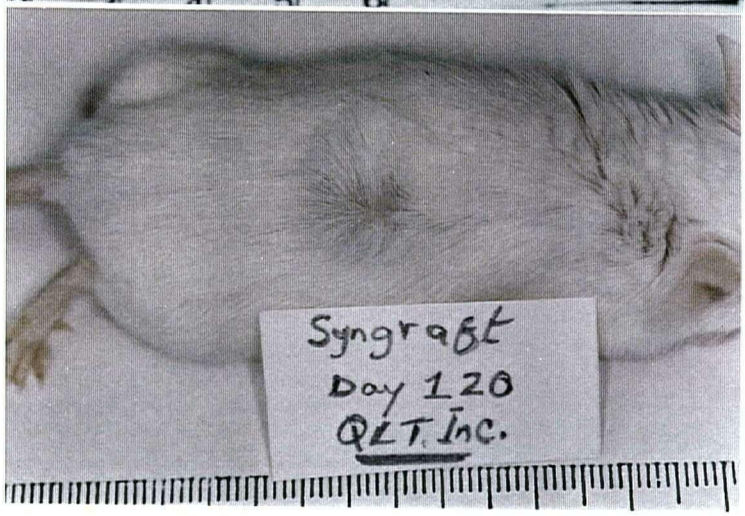

Syngraft
Day 120
QLT Inc.

Table 4.3.1 Skin graft survival in BALB/c recipient mice left untreated or given PDT at various times post-transplant.

Treatment Group	Mean Allograft Survival Time Days ± Standard Deviation
Control	
Untreated (n = 16)	11.1 ± 1.9
Transdermal PDT* (1 mg/Kg BPD, 15 J/cm^2 LED)	
Day 1 post Skin graft (n = 3)	13.3 ± 4.0
Day 3 post Skin graft (n = 5)	13.8 ± 2.8
Day 4 post Skin graft (n = 5)	11.0 ± 2.1
Day 6 post Skin graft (n = 4)	11.0 ± 2.2
Day 7 post Skin graft (n = 4)	11.5 ± 1.7

* Graft survival on mice given transdermal PDT post engraftment was not significantly ($p > 0.05$) different by the Student's *t*-test from untreated control. Similarly, there were no intra-group significant differences within the treated group either by the regular analysis of variance (ANOVA) or by the Bonferroni multiple comparison analysis.

b. Transdermal PDT of donor skin.

The next series of experiments evaluated the effectiveness of pre-treating the graft tissue in isolation rather than treating the graft *in situ*. In our preliminary experiments, skin tissues were incubated in a serum-free electrolyte solution (Plasmalyte A, Baxter Corp., Toronto ON) containing BPD (1 μg/mL) and subsequently irradiated with light (10 J/cm^2,

690 ± 10 nm wavelength) from a light-emitting diode (LED) source, a light dose somewhat less than that used for transdermal PDT of animals, prior to transplantation. We used a similar end point as was used earlier in the transdermal PDT of recipient mice. Thus, allograft rejection were considered complete when necrosis within the graft tissue affected ≥ 80 % of the transplant surface area. We found that grafts given to BALB/c mice which were untreated survived for 11 ± 1.9 days while the skin grafts on mice given pretreated donor skin (1 μg/mL of BPD and 10 J/cm^2 red light at 690 ± 10 nm wavelength) survived for 18.5 ± 2.1 days ($p < 0.001$ by the Student's *t*-test). These preliminary results demonstrated that the treatment of the donor graft tissues with BPD and visible light (at 690 nm wavelength) produced significant prolongation of graft acceptance (Table 4.3.2).

Table 4.3.2 Allograft survival in BALB/c recipient mice given allogeneic skin pre-treated *in vitro* with BPD and light at a wavelength of 690 nm.

Treatment Group	**Mean Allograft Survival Time Days ± Standard Deviation**
Control	
Syngraft (n = 10)	Indefinite
Untreated (n = 16)	11.1 ± 1.9
PDT of Donor Skin	
1.0 μg/mL BPD, 10 J/cm^2 LED (n = 4)	18.5 ± 2.1*

* $p < 0.001$ by Student's *t*-test relative to the untreated control.

Subsequently, we evaluated in greater detail, the effect of BPD concentrations on this effect. Experiments were undertaken to assess the effects of light exposure alone or varying doses of BPD with or without light exposure prior to engraftment on skin allograft survival. Donor skin tissue was incubated in a serum-free electrolyte solution (Plasmalyte A, Baxter Corp., Toronto ON) containing BPD (0.125 - 1.0 μg/mL) and subsequently exposed to light (10 J/cm^2) at a wavelength of 690 nm prior to transplantation. Control groups included animals that received untreated, light only or BPD only-treated donor skins. The end point for rejection time was taken as the point at which necrotic areas were first observed within the graft tissue. This end point was deemed to be more accurate and objective than that used earlier (Tables 4.3.1 and 4.3.2).

Skin grafts on mice given untreated donor tissue survived for 9.3 $\pm$ 2.2 days. Similarly, incubation of tissue grafts with BPD without light exposure or pretreatment of tissue grafts with light in the absence of BPD gave comparable mean survival times and thus, had no significant effect on allograft survival (Table 4.3.3). However, the survival of grafts on mice given skin tissues that were pretreated with BPD (0.125 - 1.0 μg/mL) and light (10 J/cm^2 at a wavelength of 690 nm) prior to implantation were prolonged significantly depending on the dose of BPD (Table 4.3.3). Low-dose PDT at BPD dose of 0.25 μg/mL and light gave the optimum prolongation (82.9 % of control; $p < 0.0001$ by ANOVA) of the skin allograft in our model. The treatment prolonged the survival of the skin allografts from 9.3 $\pm$ 2.2 days (control group) to 16.9 $\pm$ 1.7 days (treated group). Furthermore, at BPD doses of 0.5 or 0.125 μg/mL and light, the survival of the allograft was prolonged from 9.3 $\pm$ 2.2 days (control group) to 15.0 $\pm$ 1.4 and 14.2 $\pm$ 2.5 days

respectively. On the contrary, at BPD dose of 1.0 μg/mL, low-dose PDT prolonged the survival of skin allograft from 9.3 $\pm$ 2.2 days (control group) to 11.2 $\pm$ 0.4 days (treated group) (Table 4.3.3); although, this was not significantly different from the untreated control by the Student's *t*-test or by the Bonferroni multiple comparison analysis. Analysis of the results using the regular analysis of variance showed that the prolongation was significantly different ($p < 0.0001$) from the untreated control and graft treated with 1.0 μg/mL of BPD and light; thus indicating that an escalation of the concentration of the BPD concentration did not lengthen the term of engraftment.

The Bonferroni multiple comparison analysis was performed in order to evaluate possible significant differences within the treated groups. The result of our analysis showed that there were significant differences in the survival of allograft obtained when different doses of BPD were used in the low-dose PDT treatment of donor tissues prior to engraftment. Specifically, the prolongation of graft survival on mice given donor tissue which were pretreated with either 0.5 or 0.25 μg/mL of BPD and light were significantly different from those that were pretreated with either 1.0 or 0.125 μg/mL of BPD and light prior to engraftment. However, mean survival times of grafts obtained when tissue donors were pretreated with 0.5 or 0.25 μg/mL of BPD and light was not significantly different from each other. A similar finding was obtained with donor tissues that were pretreated with 1.0 or 0.125 μg/mL of BPD and light before implantation onto recipient mice.

Typical skin graft survival of BALB/c mice transplanted with C57BL/6 skin 14 days after grafting is shown on figure 4.3.2. The untreated donor skin was necrotic and rejected by BALB/c mice (Fig. 4.3.2A) while the donor skin pretreated with the optimal BPD dose

(0.25 μg/mL) was intact and no sign of rejection was observed (Fig. 4.3.2B). However, the pretreated graft was eventually rejected by BALB/c (Fig. 4.3.2C).

Table 4.3.3. Allograft survival in BALB/c recipient mice given allogeneic skin pre-treated *in vitro* with BPD (0.125, 0.25, 0.5 or 1.0 μg/mL) and light (10 J/cm^2; 690 ± 10 nm wavelength).

Treatment Group	**MST Days ± Stdev***	**Percent Prolongation†**
Control		
Untreated (n = 42)‡	9.3 ± 2.2	--
10 J/cm^2 LED light only (n = 15)	8.4 ± 2.1	-9.7
1.0 μg/mL BPD only (n = 5)	10.2 ± 0.4	9.7
0.25 μg/mL BPD only (n = 5)	9.8 ± 0.4	5.4
PDT of Donor Skin (μg/mL BPD +10 J/cm^2 LED)#		
1.0 μg/mL BPD (n = 5)	11.2 ± 0.4	20.4
0.5 μg/mL BPD (n = 10)	15.0 ± 1.4§	61.3
0.25 μg/mL BPD (n = 20)	16.9 ± 1.7¶	82.9
0.125 μg/mL BPD (n = 10)	14.2 ± 2.5§	52.7

* Mean Allograft Survival Time Days ± Standard Deviation. Allograft rejection was scored at the onset of necrosis within the grafted tissue.

† Percent prolongation of allograft was calculated relative to the untreated group.

‡ n, the number of animals, is listed in parenthesis.

§ $p < 0.001$, and ¶ $p < 0.0001$ by Student's *t*-test relative to the untreated group.

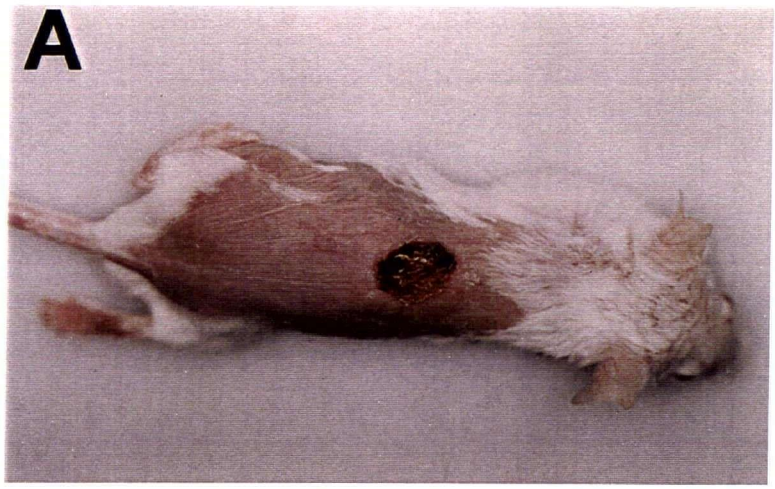
# $p < 0.0001$ by ANOVA at alpha = 0.05. The Bonferroni (all-pairs) multiple comparison analysis was performed after analysis by regular ANOVA to find out possible intra-group significant differences among the treated and untreated groups. The result of this analysis is shown on Table 4.3.4.

Table 4.3.4 Bonferroni (all-pairwise) multiple comparison analysis on the prolongation of allograft following low-dose PDT data shown on table 4.3.3.

	Untreated Control	Light 10 J/cm^2	BPD (μg/mL)		PDT (μg/mL BPD + 10 J/cm^2 LEDs)			
			1.0	0.25	1.0	0.5	0.25	0.125
GROUP*	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1						++	++	++
2						++	++	++
3						++	++	++
4						++	++	++
5						++	++	
6	++	++	++	++	++			++
7	++	++	++	++	++			++
8	++	++	++	++			++	

* The untreated and treated groups (one to eight) are arranged vertically and horizontally such that vertical group 1 is same as horizontal group 1 and so on. The summary of the Bonferroni multiple comparison analysis is read by considering the cell at which the vertically arranged groups intersect with the horizontally arranged groups. The plus sign (++) within shaded cell (intersecting cell) indicates an intra-group significant difference between the vertical and the intersecting horizontal group.

Fig. 4.3.2. Typical skin graft survival of BALB/c mice transplanted with C57BL/6 skin 14 days after grafting. The untreated donor skin was necrotic and rejected by BALB/c mice (A) while the donor skin pretreated with the optimal BPD dose (0.25 μg/mL) and light was intact and no sign of rejection was observed (B). However, the pretreated graft was eventually rejected by BALB/c (C).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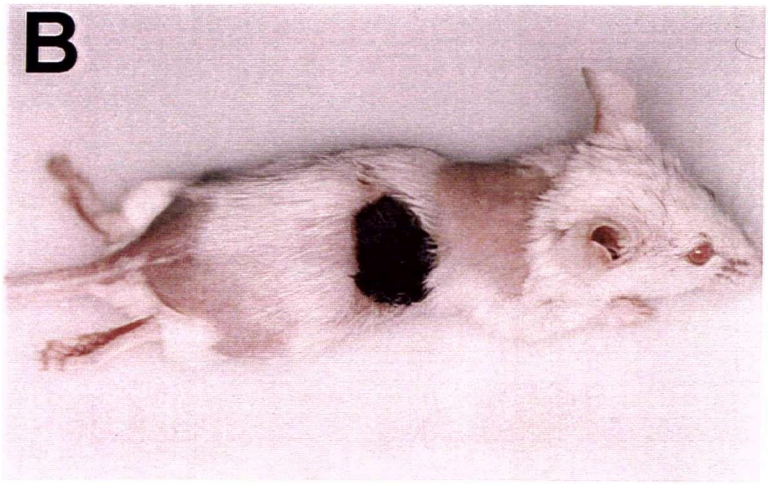
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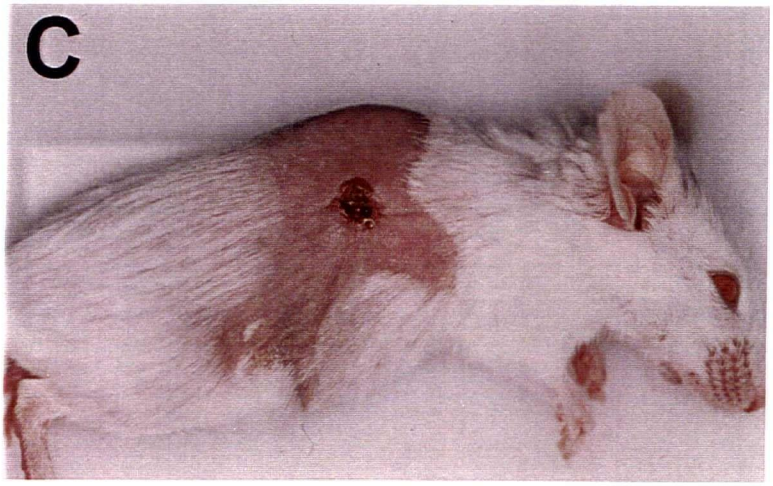

C

4.4 Discussion.

Transdermal PDT involves the intravenous administration of BPD at 1.0 mg/kg, followed 60 minutes later by whole body irradiation with visible light (15 J/cm^2) at a wavelength of 690 nm. These conditions have been shown to be well tolerated by experimental animals and not to cause skin photosensitivity (Richter *et al.*, 1994b). Our laboratory has shown that transdermal PDT with BPD may have significant immunomodulatory effects on the development of contact hypersensitivity response (Simkin *et al.*, 1995), adjuvant enhanced arthritis in MRL-*lpr* mice (Chowdhary *et al.*, 1994) and on the severity of transferred experimental allergic encephalomyelitis (Hunt *et al.*, 1994). In the skin allograft model described in this chapter, we assessed whether transdermal PDT might prolong graft acceptance in MHC-incompatible skin allografts in mice. We used two distinct protocols of transdermal PDT. On the one hand, transdermal PDT was applied to the grafted area of skin transplant recipients at various times post grafting. On the other hand, donor skin tissues were pretreated with PDT in vitro with low doses of BPD and light prior to implantation onto the recipient mice. The latter was termed low-dose PDT to reflect the fact that we used doses of BPD and light that did not cause any significant photodynamic cell damage to effect the anti-graft immune responses in the skin allograft model.

Transdermal PDT of recipient mice failed to induce a significant increase of skin allograft survival in our model. Rather, some grafts on mice given transdermal PDT especially on days 4 - 6 post transplantation seemed more vulnerable to necrosis in comparison with the untreated control animals. This observation is likely a consequence of

the destruction of the new blood vessels formed within the graft tissue. It widely accepted that skin graft survival is dependent on the formation of a microcirculation network at the dermal-epidermal junction of the engrafting skin tissue. Indeed, it has been shown that new vessels penetrate skin grafts within 24 h following engraftment such that by the 5th or 6th day post transplantation there is usually an elaborate growth of new blood vessels and lymphatics within the grafted tissue (Billingham and Medawar, 1953). Furthermore, our laboratory has shown that BPD is taken up somewhat selectively by activated or rapidly dividing cells and endothelial cells of neovasculature and that these activated or rapidly dividing cells may be targets for photoinactivation by BPD (Richter *et al.*, 1994b; Obochi *et al.*, 1995). Therefore, it is conceivable that the neovasculature of engrafting skin tissues may be especially vulnerable to the effects of PDT. Thus, this might explain why transdermal PDT of recipient mice at various days post transplantation failed to prolong the survival of skin allografts.

However, when donor skin tissues were treated with low levels of BPD and light prior to engraftment, there was a significant ($p < 0.0001$ by the regular analysis of variance) increase in the survival of the skin allografts. We found that an increase of BPD dose did not result in longer-surviving skin allografts. Rather, the most beneficial effects were observed at a lower doses of BPD (0.25 - 0.5 μg/mL) and light (10 J/cm^2, 690 $\pm$ 10 nm wavelength) as opposed to 1.0 μg/mL and light. Further analysis by the Bonferroni multiple comparison test revealed that there were intra-group differences within the treated groups. It showed that graft survival on mice given donor skins which were pretreated with 0.5 or 0.25 μg/mL of BPD and light appeared significantly different from both treated and

control groups, although they were not significantly different from each other. The fact that the relatively higher dose of BPD (1.0 μg/mL) and light was not optimal for prolonging skin allograft survival suggested that the modulation of the anti-graft immune responses induced by the low-dose PDT treatment was not likely to be a consequence of a cytotoxic event on the donor tissue by this treatment regimen.

The immunomodulatory mechanisms by which low-dose PDT affected skin allograft immune responses or the specific cell targets of this kind of treatment had not yet been identified. This was the subject of ongoing research (chapters 5 - 7 of this thesis). However, possible candidates were thought to likely include either the bone marrow-derived epidermal Langerhans cells (LC) or the keratinocytes (KC). Based on this assumption, a number of possible explanations could be advanced for the role of PDT-treated LC or KC in the prolonged skin allograft survival that we obtained in our model. First, at the level of the keratinocytes, it might be possible that low-dose PDT induced the release of immunosuppressive cytokines. KC are known reservoirs of a number of pro-inflammatory and immunoregulatory cytokines including tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-10 (Schwartz and Luger, 1992). These cytokines can be released as a result of a number of insults including injury, microbial attack or ultraviolet irradiation (Matsue *et al.*, 1992; Schwartz and Luger, 1989). On that basis, there is the likelihood that the prolonged skin allograft survival may be partly attributed, in part, to the KC-derived immunosuppressive cytokines. Second, at the level of LC, there may two possible outcomes which might explain the prolonged allograft survival that we have observed. On the one hand, the treatment regimen could have attenuated epidermal LC, the known initiators of

skin contact hypersensitivity and allograft rejection (Kripke *et al.*,1990), and thereby render them inefficient in their ability to present antigens to alloreactive T cells. On the other hand, it was possible that the treatment selectively depleted most of the epidermal LC and thus, prevented the initiation of the rejection process. Both possible outcomes could result in a prolonged allograft survival and therefore might account for the prolonged survival that we observed. However, the fact that the lower doses of BPD were most beneficial for engraftment argued against Langerhans cell depletion by selective killing as the major mechanism but did not rule out possible contributions by keratinocytes.

Thus, our observations suggested that the immunomodulatory effects of transdermal photodynamic therapy of tissue grafts associated with extended engraftments might depend upon a selective effect upon immune cell populations within the skin graft tissue. It might not require depleting cells from the donor tissue in order to induce the prolonged allograft survival that we observed. In addition, higher doses of photosensitizer might possibly compromise graft survival by damaging tissue repair mechanisms in light-exposed tissues. The mechanism(s) by which low-dose PDT could affect anti-graft immune responses will be the subject of subsequent chapters.

CHAPTER FIVE

MECHANISMS OF LOW-DOSE PDT-INDUCED ANTI-GRAFT IMMUNE RESPONSE

I. MODIFICATION OF DONOR TISSUE ANTIGENICITY

5.1 Abstract.

In order to identify mechanisms by which pretreatment of donor skin could effect anti-allograft immune responses, we investigated the effect of low-dose PDT on Langerhans cells (LC) within the epidermis. We determined whether treatment of skin grafts with PDT, under conditions that characteristically resulted in prolonged engraftment, might alter skin histological features. Skin samples were treated with low-dose PDT and maintained for an additional 24 h *in vitro*, fixed in formalin and prepared for histological examination. In addition, unfractionated epidermal cells and purified epidermal LC were used to evaluate the effect of low-dose PDT on the surface antigen expression specific for Langerhans cells and the ability of LC to stimulate the proliferation of alloreactive T cells. We found that the low-dose PDT treatment produced certain minimal histological changes in the epidermis. These included nuclear enlargement, perinuclear vacuolation and a decrease in eosinophilia of epithelial cells, a general increase in cytoplasmic volume of keratinocytes and an increase in the intracellular space between keratinocytes on the epithelial surface. There were, however, no associated dermal changes. Flow cytometric analysis of epidermal LC, enriched from treated donor skin (0.25 μg/mL BPD and 10 J/cm^2 light), showed that low-dose PDT down-regulated the LC surface antigen expression of MHC class I ($H2^b$) (62.8% of untreated control), class II (I-A^b) (90.8% of untreated control), B7-1 (CD80) (89.2% of untreated control), and B7-2 (CD86) (80% of untreated control). Low-dose PDT at 1.0 μg/mL BPD and light gave a similar profile. Nevertheless, viabilities of freshly isolated LC ($\geq$ 79 % of untreated control) as well as their surface molecules of ICAM-1 (CD54),

Leukocyte Common Antigen (CD45), and DEC-205R (recognized by the monoclonal antibody, NLDC-145) were unaffected, suggesting that the decreased cell surface molecules are unlikely to be a consequence of a nonspecific, low-dose PDT-induced cytotoxic event. Furthermore, this treatment significantly suppressed the ability of Langerhans cells to stimulate the proliferation of alloreactive T cells (68.7 $\pm$ 7.6 % metabolic activity) in the mixed epidermal cell lymphocyte reaction. The observed down-modulation of the ability of LC to stimulate alloreactive T cells following low-dose PDT is probably a result of inefficient allo-antigen presentation by Langerhans cells. This, in turn, is likely to be a consequence of the decreased expression of MHC and B7 molecules, key determinants of the two signals, required for T cell activation. Thus, our data suggest that low-dose PDT of tissue grafts associated with extended engraftment may depend in part upon a selective immunomodulatory effect of this treatment on epidermal LC. Langerhans cell depletion by selective cell cytotoxicity, therefore, appears not to be a requirement for the prolonged acceptance of skin allograft following low-dose PDT.

5.2 Introduction.

The rejection of MHC disparate allografts is believed to be largely the consequence of recipient T lymphocyte recognition of alloantigens expressed by dendritic cells (DC) present within the grafted tissue (Woodward *et al.*, 1982). The immunostimulatory DC ("passenger leukocytes") migrate from the allograft to regional lymph nodes and sensitize alloreactive T lymphocytes, which ultimately cause the destruction of the graft (Lafferty *et al.*, 1983, Lechler and Batchelor, 1982). A similar sequence of events have been shown to occur both in avascular tissues (e.g. skin) (Larsen *et al.*, 1990a; Rosenberg and Singer, 1992) and vascularized tissues (e.g. pancreas, heart, liver, and kidney) (Lafferty *et al.*, 1983; Faustman *et al.*, 1984). As a result, many anti-rejection strategies which have focused mainly on the elimination of these "passenger leukocytes" from the donor tissue prior to transplantation have resulted in prolonged acceptance across mouse MHC barriers (Faustman *et al.*, 1984; Iwai *et al.*, 1989). However, recent data suggest that the depletion of LC may not be sufficient to sustain skin and epidermal sheet allograft survival (Rouabhia *et al.*, 1993).

Ultraviolet (UV) B radiation is known to suppress induction of cutaneous immune responses in both mice and humans. In the mouse, UVB exposure of the skin has been shown to inhibit the development of contact hypersensitivity reactions when the hapten is applied on the UVB-irradiated site (local immunosuppression) (Toews *et al.*, 1980) and to cause the induction of specific tolerance detected by painting the sensitizing hapten later on unirradiated skin areas (systemic immunosuppression) (Noonan *et al.*, 1981a & b). Both

local and systemic UVB-induced immunosuppression have been related to the deleterious effects of these radiations on epidermal cells. In the murine system, for example, immunosuppression has been shown to result both from direct effects of UVB radiation on LC antigen-presenting function (Simon *et al.*, 1992) and from indirect effects mediated through keratinocytes-derived soluble suppressive factors (Noonan and De Fabo, 1992). Tang and Udey (1991) reported that low doses of UVB radiation inhibited the ability of freshly isolated murine epidermal LC to support anti-CD3 monoclonal antibody-induced T cell mitogenesis and selectively inhibited the up-regulation of ICAM-1 expression by LC without causing appreciable cytotoxicity in short-term ($\leq$ 24 h) incubations. The upregulation of I-A antigen or CD45 antigen expression was not affected by this treatment (Tang and Udey, 1991). However, levels of UV radiations (UVB, UVC or psoralen + UVA radiation) that inhibited LC accessory cell function and selectively modulated ICAM-1 expression in short-term cultures were ultimately cytotoxic for LC (Tang and Udey, 1992).

Similarly, PDT using BPD and light at a wavelength of 690 nm has been shown to have potential as an immunomodulatory technology (Richter *et al.*, 1994a & b; Ratkay *et al.*, 1994b; Chowdhary *et al.*, 1994; Simkin *et al.*, 1995) which effects specific immune responses. In our laboratory, working with BPD, we demonstrated that this photosensitizer could be activated in the blood by whole body illumination with red light, without causing skin photosensitivity (Richter *et al.*, 1994b). This procedure was termed transdermal PDT. Furthermore, using transdermal PDT, we showed that we could modulate immune responses in certain disease models such as adjuvant arthritis in MRL/*lpr* mice (Ratkay *et al.*, 1994b; Chowdhary *et al.*, 1994) as well as inhibit the development of skin contact hypersensitivity

(Simkin *et al.*, 1995). Similarly, in this thesis, we have shown (chapter 4) that transdermal PDT of donor tissues prior to engraftment could prolong the survival of skin allograft survival. We showed that the optimum dose of PDT for the prolongation of allograft survival time (82.9 %, Table 4.3.3) was 0.25 μg/mL of BPD and 10 J/cm^2 light (λ = 690 $\pm$ 10 nm). The fact that high dose of BPD (1.0 μg/mL) + light (10 J/cm^2 light; λ = 690 $\pm$ 10 nm) were not optimal for allograft survival suggested that the observed anti-graft immune response was unlikely to be a consequence of a PDT-induced cytotoxic event such as is associated with PDT of tumors.

In transdermal PDT, it was possible to activate BPD in the blood and in tissues because of the transparency of tissues to red light (Wilson *et al.*, 1984; Lin, 1991) where BPD is activated (λ = 690 $\pm$ 10 nm). However, little is understood about the specific cellular targets or the mechanisms by which transdermal PDT might modulate immune responses. As a result, the possible mechanisms by which transdermal PDT of donor graft tissue could modulate specific immune responses were the subject of this chapter. Since the exposure of donor skin tissue to low-dose PDT prior to engraftment resulted to prolonged allograft acceptance, it is likely that the donor cells, especially epidermal cells (EC) which are known to take part in skin allograft rejection, are the cellular targets for the transdermal PDT of donor skin tissues.

Epidermal cells consist of two major immunologically active cell populations: the keratinocytes which make up to 95 % of the epidermal cell populations (Nickoloff and Turka, 1993) and the bone marrow-derived Langerhans cells (LC) which make up 2 - 8 % of epidermal cells (Rouabhia *et al.*, 1993; Mommaas *et al.*, 1994). Keratinocytes are potent

producers of active pro-inflammatory and immunosuppressive cytokines (Kupper, 1988; Stingl *et al.*, 1989) while LC play an essential role in immune responses initiated in the skin (Schmitt *et al.*, 1986; Hauser, 1990). LC constitute the only accessory and antigen presenting cells of the skin-associated lymphoid tissue. *In vitro*, LC have been shown to induce allogeneic, antigen-specific, and cytotoxic T cell proliferation (Bertraux *et al.*, 1986). *In vivo*, they may be involved in various immunological functions, such as the induction of contact hypersensitivity to haptens, and seem to take part in skin allograft rejection as well as the presentation of non-self-antigens to T cells (Streilein *et al.*, 1982; Aubock *et al.*, 1988; Lerner-Tung and Hull, 1989).

Langerhans cells are capable of inducing such arrays of immunological functions because of their endowment with MHC (Shimada *et al.*, 1987) and costimulatory molecules required for T cell activation (Cumberbatch *et al.*, 1992; Inaba *et al.*, 1994). In 1987, Jenkins and Schwartz showed experimentally that IL-2 and interferon-gamma producing T cells (Th1 cells) require two signals from antigen presenting cells (Jenkins and Schwartz, 1987) in order to produce these cytokines (Jenkins and Schwartz, 1987; Mueller *et al.*, 1989). The antigen specific "signal 1" is provided when T cell receptor (TCR) interacts with antigen presented in the context of MHC expressed on antigen presenting cells (APC). The "signal 2" or costimulatory signal is provided by a set of receptor-ligand interactions distinct from the TCR interactions. Both B7-1 and B7-2 molecules, members of the immunoglobulin super gene family, expressed on the surface of APC can provide costimulatory function (Freeman *et al.*, 1989; Linsley *et al.*, 1991a; Norton *et al.*, 1992; Galvin, 1992; Harding *et al.*, 1992; Hathcock *et al.*, 1993; Azuma *et al.*, 1993; Freeman, 1993 a,b,c). CD28

(Gross *et al.*, 1992; Linsley and Ledbetter, 1993), and CTLA-4 (Mueller *et al.*, 1989; Linsley *et al.*, 1991b) have been identified as molecules expressed on T cells through which costimulatory signals can be delivered. TCR occupancy in the absence of costimulation results in suboptimal activation of T cells, with no IL-2 production and no resultant proliferation (Jenkins and Schwartz, 1987). In addition, such cells are unresponsive (anergic) to subsequent stimulation with normal APC plus antigen (Jenkins and Schwartz, 1987).

Thus, because of the pertinent roles played by LC in the immune responses initiated in the skin, we deemed it necessary to evaluate the effect of low-dose PDT on the surface antigens as well as the accessory and antigen presenting functions of epidermal LC. Initially, we used the routine haematoxylin/eosin staining procedure to evaluate the effect of low-dose PDT on any possible alteration of the histological features of the donor graft tissue. Furthermore, we used crude epidermal cells to determine the effect of this treatment regimen on the capacity of epidermal cell suspension to stimulate the proliferation of alloreactive T cells in the mixed epidermal cell lymphocyte reaction (MECLR). Subsequent experiments evaluated the effect of this treatment regimen on epidermal cells that had been enriched for LC using the density gradient centrifugation. First, we evaluated the ability of enriched LC to stimulate the proliferation of alloreactive T cell in the MECLR. Second, we established whether low-dose PDT affected the viability of Langerhans cells enriched from treated or untreated donor skin. Finally, we characterized the cell surface antigens of enriched LC using monoclonal antibodies and flow cytometry.

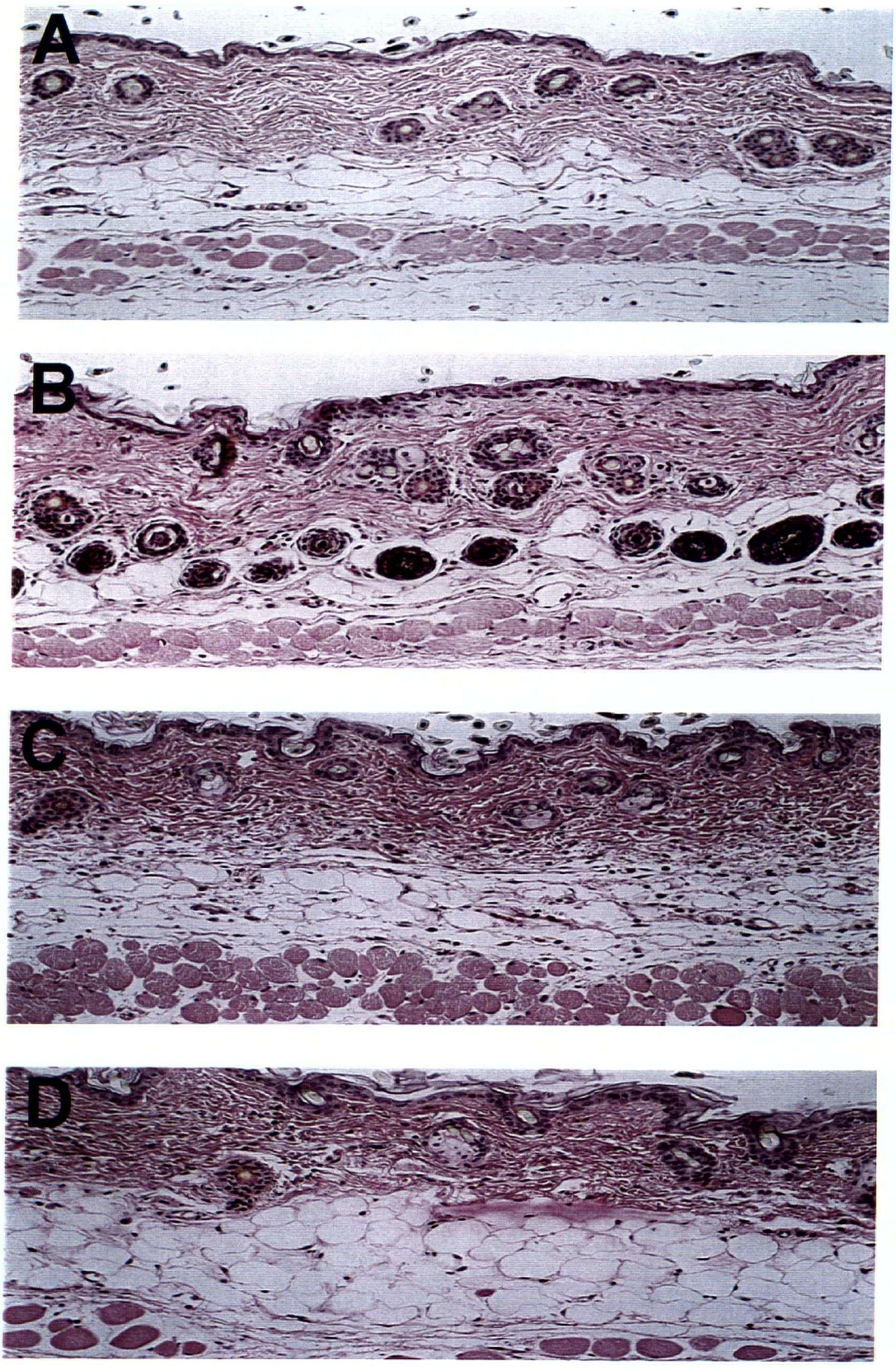
5.3 Results.

a. Effect of low-dose PDT on skin histological features.

In order to determine whether treatment of skin grafts with PDT, under conditions that characteristically resulted in prolonged engraftment, might alter skin histological features, samples were treated with low-dose PDT as described (chapter 4) and maintained for an additional 24 h *in vitro*, following which they were fixed in formalin and prepared for histological examination (materials and methods section 2.17).

Tissues incubated in the electrolyte solution with or without BPD at 1.0 μg/mL appeared unaltered (Fig. 5.3.1a and b). However, certain minimal histological changes were noted in the epidermis of graft tissues treated with BPD at 0.25 or 1.0 μg/mL and light at 10 J/cm^2 (Fig. 5.3.1c and d) These included nuclear enlargement, perinuclear vacuolation and a decrease in eosinophilia of epithelial cells, a general increase in the cytoplasmic volume of keratinocytes and an increase in the intercellular space between keratinocytes on the epithelial surface. There were no associated dermal changes and no evidence of apoptosis or necrosis. These histological findings suggest that low-dose PDT treatment of donor tissues produced a small degree of cellular damage and tissue reorientation rather than noticeable cell death. Figure 5.3.1 shows a). tissue incubated with electrolyte solution; b). tissue incubated with electrolyte solution containing BPD at 1.0 μg/mL; c). tissue treated with BPD at 0.25 μg/mL and light (10 J/cm ^{2}, λ = 690 $\pm$ 10 nm), and d). tissue treated with BPD at 1.0 μg/mL and light (10 J/cm ^{2}, λ = 690 $\pm$ 10 nm).

Fig. 5.3.1 Histology of A, untreated, B, treated with BPD only and C and D, low-dose PDT-treated full thickness donor skins (C57BL/6). Tissues were further maintained in electrolyte solution (Plasmalyte A, Baxter Corp.) for an additional 24 h, stained with haematoxylin and eosin and observed under a light microscope. The samples treated with BPD at C) 0.25 μg/mL and D) 1.0 μg/mL concentrations and light (10 J/cm^2, 690 $\pm$ 10 nm wavelength) showed certain histological changes in the epidermis but no associated dermal changes or evidence of cell death following 24 h of incubation. Tissues incubated with electrolyte solution (A) or incubated with electrolyte solution containing BPD at 1.0 μg/mL appeared unaltered. Stain magnification for all the histology sections (A-D) was 200X.


A
B
C
D

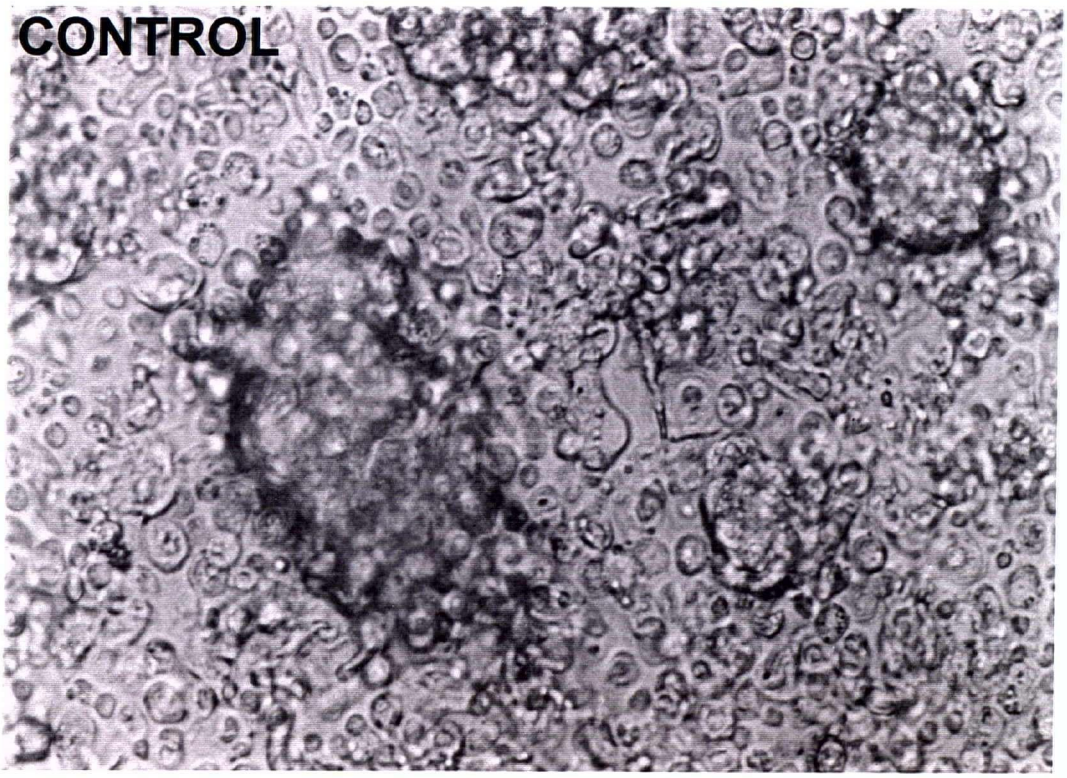
b. Effect of low- dose PDT on the LC-dependent proliferation of alloreactive T cells in mixed epidermal cell-lymphocyte reaction (MECLR).

We evaluated the effect of BPD and light, at levels which did not cause significant tissue damage, on the ability of LC to induce the proliferation of alloreactive T cells. In our preliminary experiments, we used crude epidermal cell suspensions, prepared from control donor skin or donor skin following low-dose PDT (1.0 μg/mL of BPD and light) as the stimulator cell population in a co-culture of epidermal cells and nylon wool-purified allogeneic T cells (materials and methods section 2.16). This co-culture is a modification of the mixed lymphocyte response assay and is referred to as the mixed epidermal cell-lymphocyte reaction (MECLR) assay.

We found that the ability of the treated epidermal cells to stimulate the proliferation of allogeneic T cells was significantly impaired in comparison to the non-treated controls. Micrographs of the cultures, at 48 h, showed typically large clusters of cells in the untreated controls (Fig. 5.3.2a) while there were only limited clusters in the treated samples (Fig. 5.3.2b). The clusters of cells most likely represents clusters of alloreactive T cells around LC stimulators. It has been shown that dendritic cells such as LC are the principal APC which stimulate clustering of alloreactive T cells (Flechner *et al.*, 1988) and are necessary for T cell proliferation in the mixed leukocyte reaction (MLR) (Metlay *et al.*, 1990). The assessment of proliferating cells following 120 h of culture using the MTT assay (Blackman *et al.*, 1990; Chen *et al.*, 1990; Wooley *et al.*, 1993) (materials and methods section 2.10) suggested that epidermal cells from treated donor skin might have an impaired accessory cell activity in the MECLR. We obtained a significant (32.5 ± 2.2 %; $p < 0.01$ by the

Student's *t*-test) suppression of the LC-dependent proliferation of alloreactive T cells relative to epidermal cells from untreated donor skin in our assay system (Fig. 5.3.3).

Fig. 5.3.2. Micrographs of clusters of allogeneic T cells in the mixed epidermal cell lymphocyte reaction. Photographs of cell cultures were taken 48 h following co-culture of untreated or treated (1.0 μg/mL BPD + 10 J/cm^2 light) epidermal cells (C57BL/6) and allogeneic T cells (BALB/c). The micrographs are representative of 4 - 8 independent experiments. Magnification: 400X.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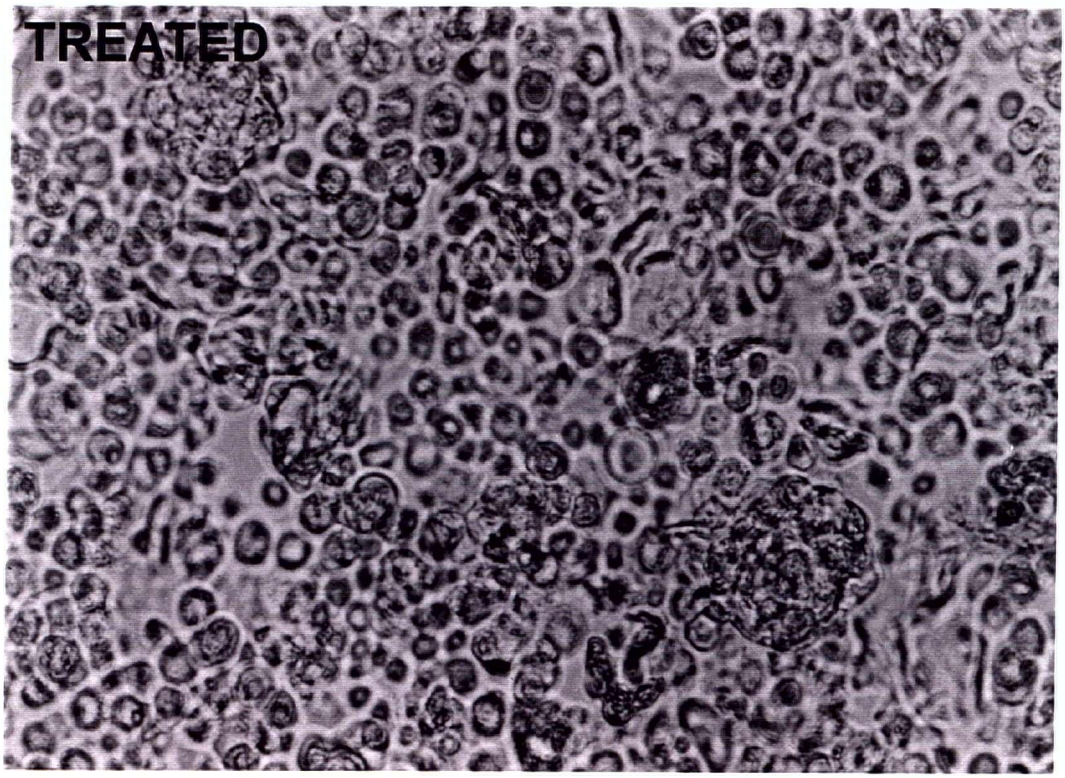

TREATED

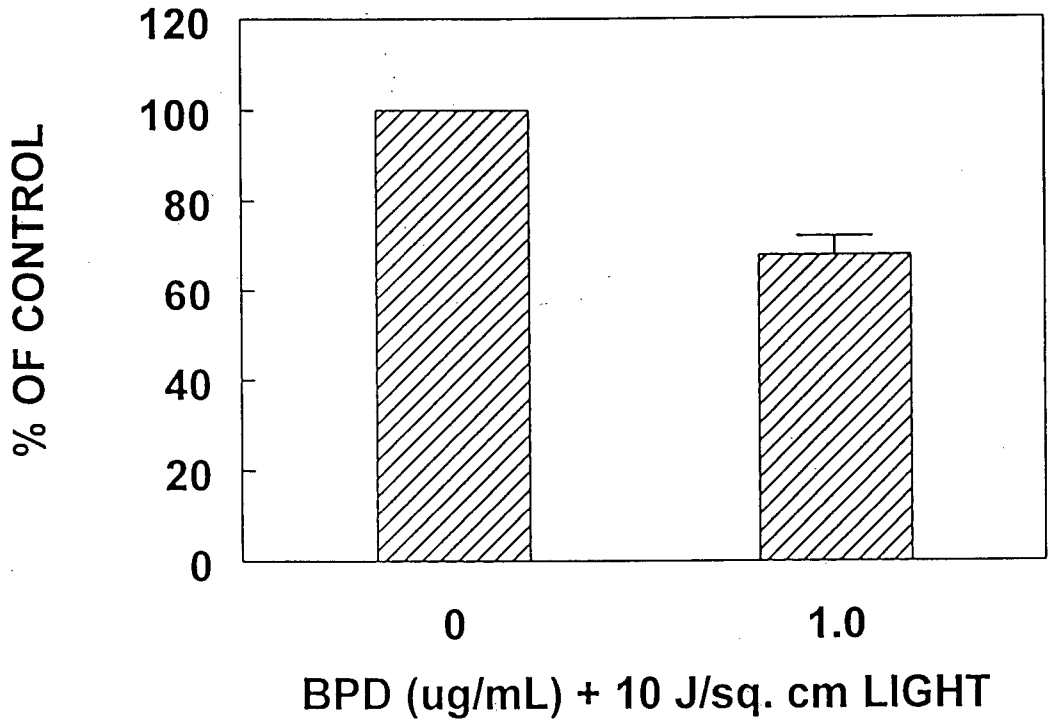


Fig. 5.3.3 Proliferation of allogeneic T cells in the MECLR using treated or untreated epidermal cells. Epidermal cells were isolated from treated (1.0 μg/mL BPD + 10 J/cm^2 light) or untreated donor skin (C57BL/6). Nylon wool-purified T cells from BALB/c mice were co-cultured for 5 days with Mitomycin C-treated (100 μg/mL at 37 °C for 30 min) epidermal cells. The LC-dependent proliferation of allo-T cells were quantified via the MTT assay and presented as a percent of control cells treated with light only and expressed as mean (n = 4) $\pm$ standard deviation (SD).

Since LC are known to be responsible for the induction of the T cell response in the MECLR (Stingl *et al.*, 1978) and epidermal cells are mainly composed of keratinocytes, we clarified the effect of low-dose PDT on the alloreactivity of LC by using epidermal cells that were enriched for LC by step-wise density gradient centrifugation (materials and methods section 2.7F). We used LC, enriched from epidermal cells prepared from donor skin tissues following low-dose PDT (0.125 - 1.0 μg/mL of BPD and light) and enzyme treatment, as the stimulator cell population in the MECLR. Optimal induction of allogeneic T cell proliferation was obtained at LC to T cell ratio of 1:4 i.e. at 1 x 10^5 LC/well and, all proliferation assays were performed at this cell concentration. Data are expressed as percentages of T cell proliferation (metabolic activity) obtained with LC isolated from either untreated skin samples or samples given light in the absence of BPD, taken as 100 % response (Fig. 5.3.4).

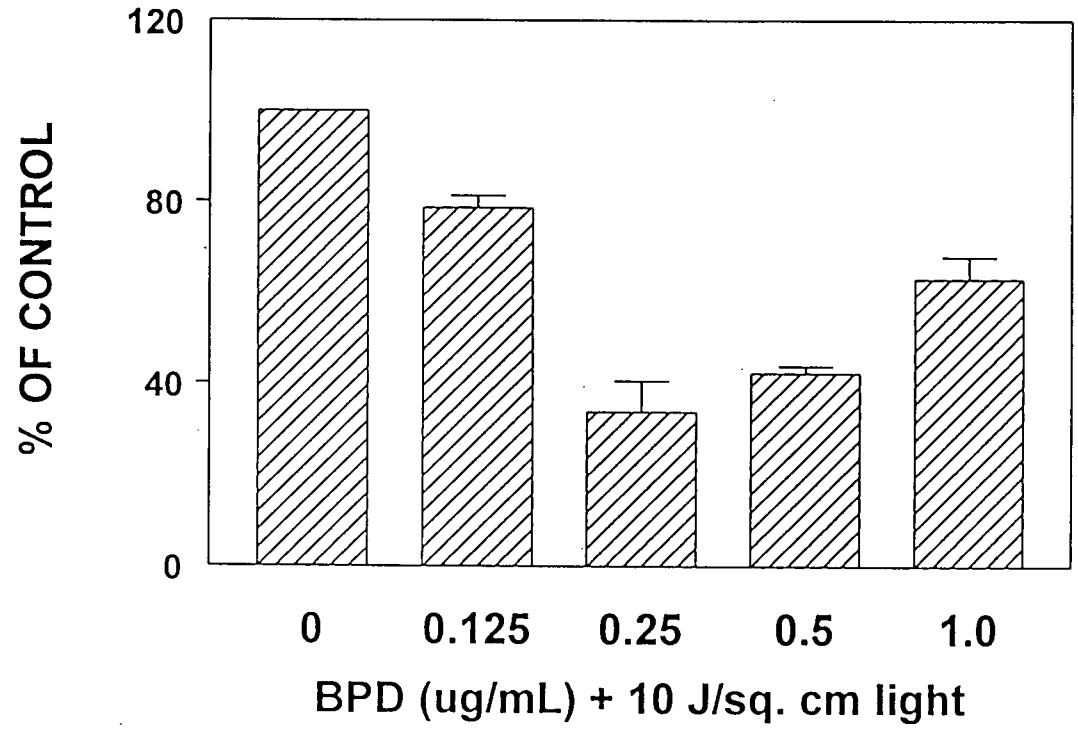


Fig. 5.3.4. LC-dependent proliferation of allogeneic T cells in the MECLR using LC from treated or untreated epidermis as stimulators. LC were enriched from treated (0 - 1.0 μg/mL BPD + 10 J/cm^2 light) or untreated donor skin (C57BL/6) using a two-step density gradient centrifugation. Nylon wool-purified T cells (4 x 10^5/well) from BALB/c mice were co-cultured for 5 days with enriched LC (1 x 10^5/well). The LC-dependent proliferation of allogeneic T cells were quantified via the MTT assay and presented as a percent of control cells treated with light only and expressed as mean (n = 3) $\pm$ SD.

Our results confirmed our initial observation that low-dose PDT significantly impaired the alloreactivity of epidermal LC. We found that Langerhans cells, enriched from donor skin tissues given 1.0, 0.5, 0.25 and 0.125 μg/mL BPD and a constant dose of light (10 J/cm^2, λ = 690 $\pm$ 10 nm), inhibited the proliferation of allo-T cells in MECLR by 39.1, 57.0, 74.1 and 24.5 % respectively relative to the control group, taken as 100% response (Fig. 5.3.4). Optimal suppression of T cell proliferation in MECLR was obtained at 0.25 μg/mL of BPD when constant light was used; thus correlating with the optimal conditions for prolongation of skin allograft acceptance (chapter 4; Table 4.3.3).

Using the regular analysis of variance, we found that the treated groups were significantly different ($p < 0.0001$; term significant at alpha = 0.05) from the untreated control group. We performed the Bonferroni (all-pairwise) multiple comparison test to identify possible intra-group differences within the treated group. Our analysis showed that there were significant intra-group differences among the groups treated with different doses of BPD (0.125 - 1.0 μg/mL) and light (10 J/cm ^{2}, λ = 690 $\pm$ 10 nm) (Table 5.3.1). Specifically, the impaired alloreactivity of LC in MECLR observed by using LC enriched from donor skin pretreated with BPD concentrations of 0.5 or 0.25 μg/mL + light were significantly different from those pretreated with BPD doses of 1.0 or 0.125 μg/mL + light. Whereas data obtained with BPD dose of 1.0 μg/mL + light (λ = 690 $\pm$ 10 nm) were significantly different from those obtained with 0.125 μg/mL of BPD and light, there was no significant differences between those with BPD concentrations of 0.5 and 0.25 μg/mL + light (10 J/cm ^{2}, λ = 690 $\pm$ 10 nm).

Table 5.3.1 Bonferroni (all-pairwise) Multiple Comparison Test on the decreased capacity of pretreated LC to stimulate the proliferation of allogeneic T cells (Fig. 5.3.4).

	Untreated Control	PDT (μg/mL BPD + 10 J/cm^2 LEDs)			
		1.0	0.5	0.25	0.125
GROUP*	**1**	**2**	**3**	**4**	**5**
1		++	++	++	++
2	++		++	++	++
3	++	++			++
4	++	++			++
5	++	++	++	++	

* The untreated and treated groups (one to five) are arranged vertically and horizontally such that vertical group 1 is same as horizontal group 1 and so on. The summary of the Bonferroni multiple comparison analysis is read by considering the cell at which the vertically arranged groups intersect with the horizontally arranged groups. The plus sign (++) within shaded cell (intersecting cell) indicates an intra-group significant difference between the vertical and the intersecting horizontal group.

c. Effect of low-dose PDT on the Langerhans cell surface antigens.

It has been suggested that UVB radiation may inhibit LC antigen presenting cell function by preventing the expression of critical costimulatory molecules (Simon *et al.*, 1991; Tang and Udey, 1991; Young *et al.*, 199; Pequet-Navarro *et al.*, 1993; Romani *et al.*, 1989). Since the responses induced by both UV radiation and PDT may involve a common mechanism, the reactive oxygen species-mediated responses, we studied whether the impaired alloreactive function observed in LC following low-dose PDT could also be

explained by a decrease in the surface antigens on LC. For these experiments, we used both immunobead-purified and density gradient centrifugation-enriched Langerhans cells.

i. Immunobead-purified Langerhans cells.

In our preliminary experiments, we studied the effect of low-dose PDT on MHC class II (I-A^b) and ICAM-1(CD54) antigen expression using immunobead-purified Langerhans cells. 4 - 6 hours post the application of low-dose PDT treatment, we purified epidermal LC from untreated and treated donor skin tissue and analyzed them for changes in their surface antigen molecules of class II (I-A^b) and ICAM-1 using anti-mouse monoclonal antibodies and flow cytometry (materials and methods section 2.12).

Data shown (Table 5.3.2) represent the specific mean fluorescence intensities (intensity with relevant monoclonal antibody minus the intensity with isotype-matched control IgG) expressed as mean (n = 3) $\pm$ standard deviation. We determined that low-dose PDT significantly ($p < 0.0001$ by Student's *t*-test) down-modulated the cell surface expression of MHC class II antigens from the mean intensity of 13.5 $\pm$ 6.8 (untreated group) to 2.7 $\pm$ 1.8 (treated group) (Table 5.3.2). A modest decrease in the level of ICAM-1 was obtained although, this was not significantly different (by the student's *t*-test) from the untreated controls. Low-dose PDT did not alter the percentage of F4/80-positive epidermal cells, presumably mainly Langerhans cells, isolated using the immunobead separation technology. The percent cell yield (obtained by dividing the procured LC by the starting crude epidermal cells and multiplying the quotient by 100) was 2.9 $\pm$ 0.1% (control group) and 2.23 $\pm$ 1.4 % (treated group). 70 -80 % of these cells stained positive for I-A^b

and DEC-205 (Table 5.3.2 footnote 2). Cell viability, assessed by trypan blue exclusion was $> 95\ \%$ both in cells isolated from donor tissues that were either treated with or without low-dose PDT (Table 5.3.2). Therefore, we inferred that it was unlikely that the decrease of class II antigens expression observed was a consequence of direct toxic effects of this treatment regimen.

Table 5.3.2 Mean fluorescence intensity of class II and ICAM-1 surface antigens of the immunobead-purified Langerhans cells.

Group[1]	% Viability[2]	Mean Fluorescence Intensity[3] ± Standard Deviation	
		MHC class II (I-A^b)	ICAM-1
Control	95.8 ± 0.78	13.5 ± 6.8	2.2 ± 0.5
Treated	95.4 ± 1.13	2.7 ± 1.8[4]	1.3 ± 0.6[NS]

1 LC were purified from the epidermis of skin (C57BL/6 mice) which were untreated or treated with BPD (1.0 μg/mL) and light (10 J/cm^2).

2 Cell viability was assessed by trypan blue exclusion immediately following isolation and expressed as mean (n = 3) ± standard deviation. The percent yield of LC (percent procured LC divided by crude epidermal cells) was 2.9 ± 0.1% (control group) and 2.23 ± 1.4 % (treated group) with 70 - 80 % staining positive for I-A^b and DEC-205.

3 Mean fluorescence intensity of the surface antigens were obtained by subtracting the fluorescence intensity obtained with isotype-matched control IgG from the intensity of the relevant monoclonal antibody.

4 $p < 0.0001$, by the student's *t*-test, relative to control group.

NS Not significantly different, by the student's *t*-test, from the control group.

ii. Density gradient-enriched Langerhans cells.

On the basis of the above findings (Table 5.3.2), we performed a series of experiments to assess the effect of low-dose PDT on various cell surface molecules of freshly isolated LC. For these experiments we used epidermal cells, obtained from either

untreated donor skin tissues or skin tissues that received low-dose PDT treatment and enriched for LC by a two step density gradient centrifugation. Langerhans cells were analyzed by monoclonal antibodies and flow cytometry (materials and methods section 2.12) within 6 h post isolation for changes in their surface antigen molecules of MHC class I ($H2^b$), and class II (I-A^b), ICAM-1 (CD54), B7-1 (CD80), B7-2 (CD86), Leukocyte common antigen (LCA) (CD45), and DEC-205 (recognized by the monoclonal antibody, non-lymphoid dendritic cell (NLDC)-145). Data shown (Fig. 5.3.5) represent the specific mean fluorescence intensities (intensity with relevant monoclonal antibody minus the intensity with isotype-matched control IgG) expressed as mean (n = 3) $\pm$ standard deviation.

We found that low-dose PDT, at a BPD dose of 0.25 μg/mL and light, significantly decreased ($p < 0.001$ by ANOVA) the LC surface molecules of MHC class I ($H2^b$) (62.8 %), class II (I-A^b) (90.1 %), B7-1 (89.2 %), and B7-2 (80.0 %) relative to the untreated groups (Fig. 5.3.5). Similar levels of down-modulation were obtained with low-dose PDT at a BPD dose of 1.0 μg/mL and light. However, the surface expression of CD45, ICAM-1 and DEC-205 were unaffected at both doses of BPD (1.0 or 0.25 μg/mL) and light.

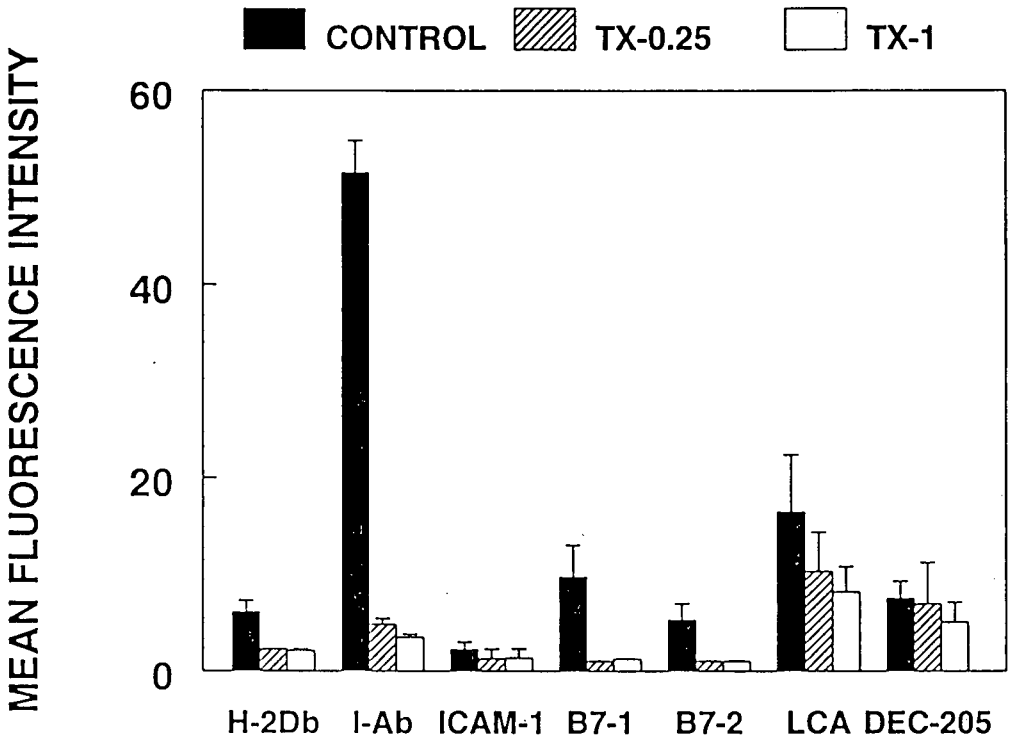
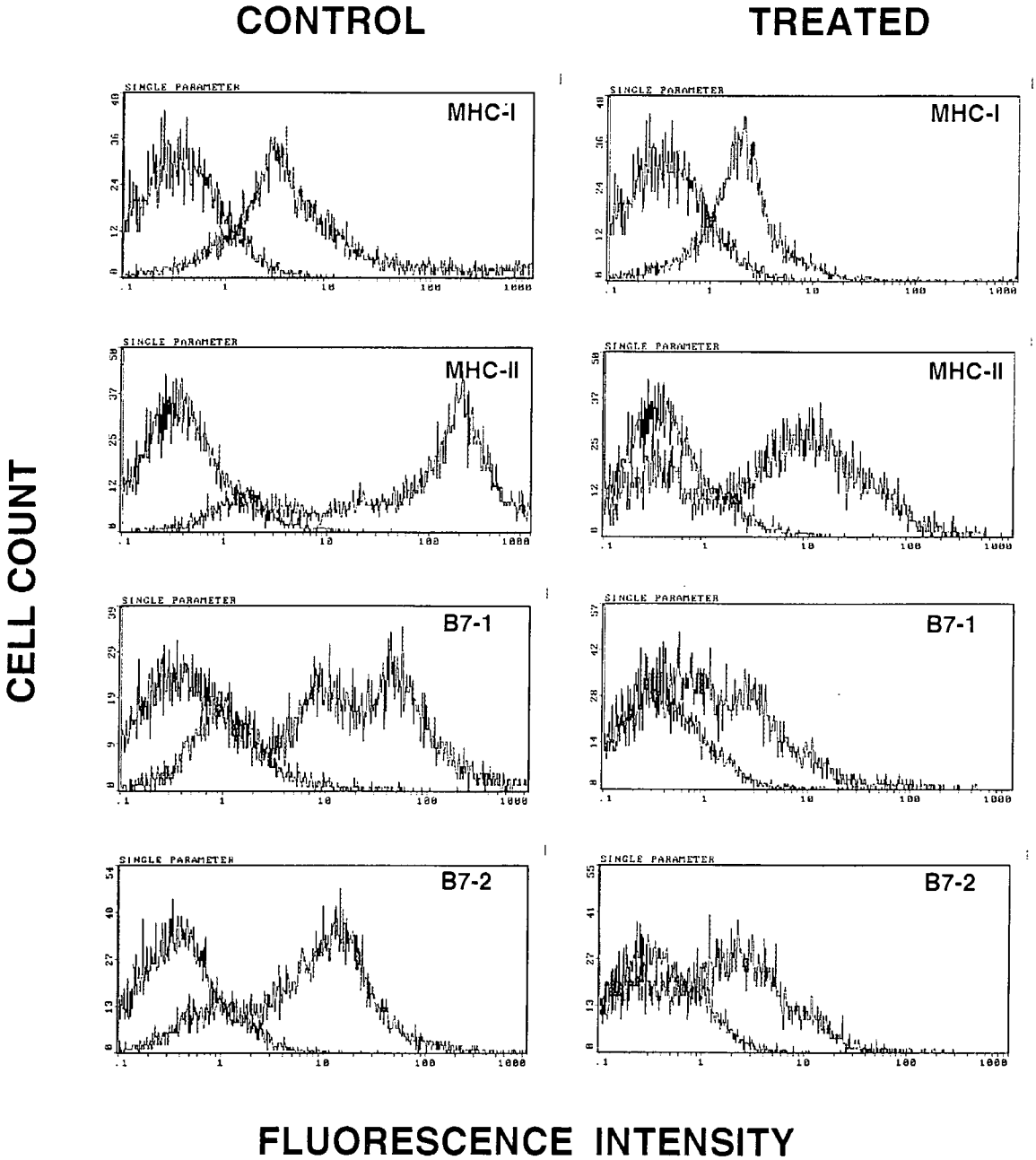


Fig. 5.3.5. Flow cytometric analysis of Langerhans cells surface antigens. LC were enriched from treated (1.0 and 0.25 μg/mL BPD + 10 J/cm^2 light) or untreated donor skin (C57BL/6) using a two-step density gradient centrifugation. Epidermal LC were stained with a panel of monoclonal antibodies (materials and methods section 2.12) and analyzed with an EPICS XL® flow cytometer. Data shown represent the specific mean fluorescence intensities (intensity with the relevant monoclonal antibody minus the intensity with isotype-matched control IgG) expressed as mean (n = 3) ± SD.

Further analysis by the Bonferroni (all-pairwise) multiple comparison test revealed that the down-modulation obtained with the treated groups (1.0 or 0.25 μg/mL BPD + light) were not significantly different ($p > 0.05$) from each other. The enriched LC expressed distinct, albeit relatively low, expression of ICAM-1, B7-1 and B7-2 molecules but medium levels of class I and high levels of class II MHC molecules (Fig 5.3.6). This was in agreement with previous reports that LC freshly procured from mice, were $Ia^{low\ or\ high}/B7\text{-}1^{-\ or\ low}/B7\text{-}2^{-\ or\ low}$, rather than cultured LC, which are known to express phenotype $Ia^{high}/B7\text{-}1^{high}/B7\text{-}2^{high}$ (Inaba *et al*, 1994; Shimada *et al.*, 1987; Xu *et al.*, 1995a; 1995c).

Furthermore, comparable numbers of LC (2 -3 % LC yield, 60 -70 % positive for $I\text{-}A^b$ and DEC-205) were procured from untreated or treated donor skin tissues with viabilities, assessed routinely by trypan blue and propidium iodide exclusion, > 79.0 % both in LC enriched from untreated or treated donor skin tissues (Fig. 5.3.7). The viabilities of LC enriched from treated donor skins were not significantly different ($p > 0.05$ by ANOVA) from those procured from control donor skin tissues.

Fig 5.3.6 Surface expression of MHC molecules (class I, and class II), costimulatory molecules (B7-1, and B7-2), adhesion molecule, ICAM-1, Leukocyte common antigen (LCA) (CD45), and DEC-205 (recognized by the monoclonal antibody, non-lymphoid dendritic cell (NLDC)-145). LC were enriched from treated (1.0 and 0.25 μg/mL BPD + 10 J/cm^2 light) or untreated donor skin (C57BL/6) using a two-step density gradient centrifugation. Epidermal LC were stained with a panel of monoclonal antibodies (materials and methods section 2.12) and analyzed with an EPICS XL® flow cytometer. Representative histograms of treated or untreated LC are shown with staining profiles for the indicated antigens (red lines) or with isotype-matched control IgG (blue lines). Page 147 = profiles for MHC and B7 molecules. Page 148 = profiles for ICAM-1, LCA and DEC-205.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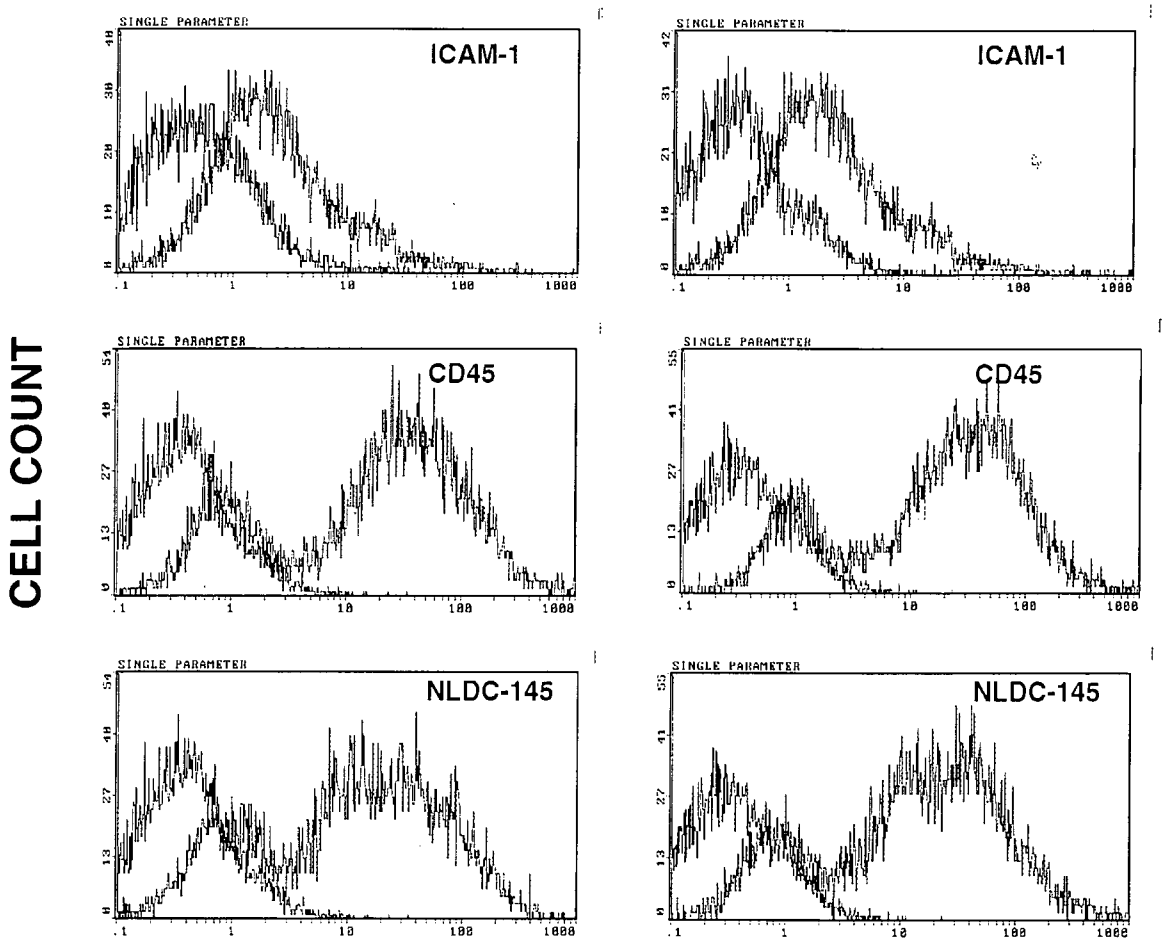
TREATED
CELL COUNT
SINGLE PARAMETER
MHC-I
MHC-II
B7-1
B7-2
FLUORESCENCE INTENSITY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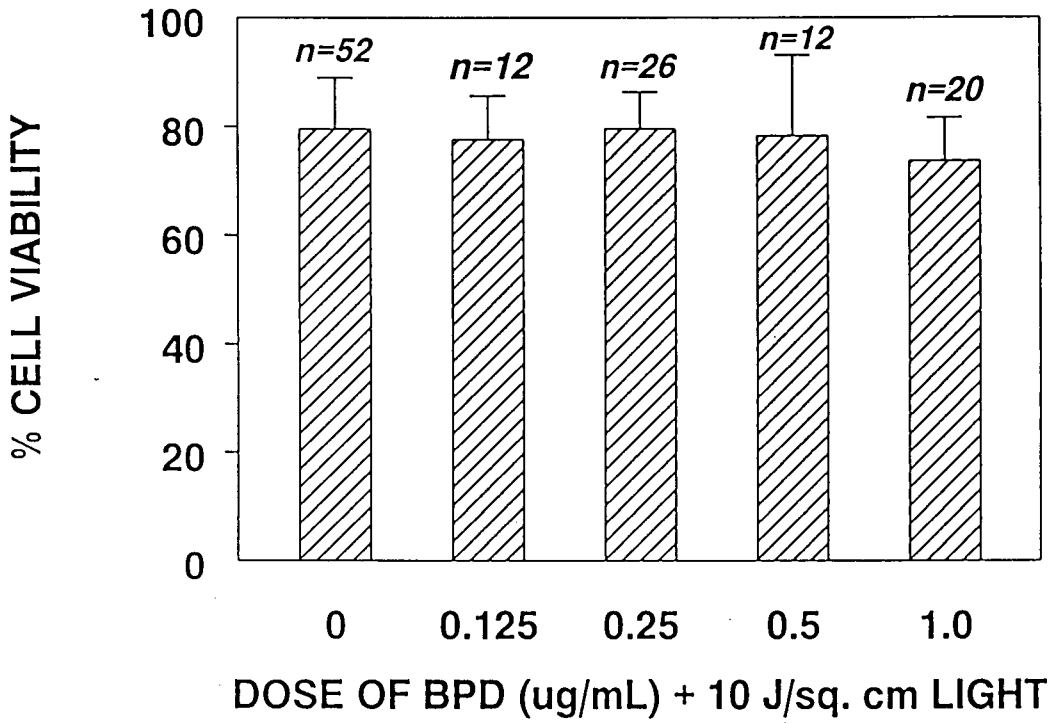


Fig. 5.3.7. Viabilities of enriched Langerhans cells. Epidermal cells were obtained from untreated or treated (0 - 1.0 μg/mL BPD + 10 J/cm^2 light at a wavelength of 690 $\pm$ 10 nm) donor skin following which they were enriched for LC using a two-step density gradient centrifugation (materials and methods section 2.7F). Cell viability was assessed by trypan blue exclusion and by flow cytometry using propidium iodide stain. Data is expressed as mean percent viable cells $\pm$ SD. n = number of experimental animals.

d. Effect of low-dose PDT on enriched LC in short- and long-term cultures.

Several authors have suggested that exposure of LC to ultraviolet (UV) B or psoralen plus UVA radiation (PUVA) causes a loss of surface markers (including ATPase and class II MHC antigens) without causing overt cytotoxicity (Aberer *et al.*, 1981 and 1986; Hanau *et al.*, 1985; Humm and Cole, 1986; Odling *et al.*, 1987; Stingl *et al.*, 1986). However, Tang and Udey (1992) showed that the levels of UV radiation that inhibited LC accessory cell function and selectively modulated ICAM-1 expression in short-term cultures were ultimately cytotoxic for LC. To exclude cytotoxicity of low-dose PDT for LC as a potential explanation for the decrease in cell surface antigens that we observed, we studied the effect of low-dose PDT on the *in vitro* survival of enriched murine LC. LC were enriched from untreated donor skin tissues or tissues treated with low doses of BPD (1.0 or 0.25 μg/mL) and light. Cells were maintained in short-term (24 h) or in long-term ($\geq$ 7 days) cultures in RPMI-10 in the presence of equal concentrations of granulocyte/macrophage-colony stimulating factor (GM-CSF) and macrophage-colony stimulating factor (M-CSF) (20 ng/mL; R & D Systems, Minneapolis, MN). GM-CSF and M-CSF have been shown to be optimal for LC survival *in vitro* (Xu *et al.*, 1995a and b; Kitajima *et al.*, 1995). At the end of the incubation periods, cells were recovered, enumerated, assayed for viability by trypan blue or propidium iodide exclusion. Furthermore, we stained the cells enriched from untreated or treated (0.25 μg/mL BPD and light) for MHC class II (I-A^b), B7-1, and B7-2 antigens following the short-time incubation period.

We found that following an overnight culture *in vitro*, the levels of LC surface antigens of class II MHC, B7-1 and B7-2 antigens remained lower in the treated (0.25

μg/mL of BPD + 10 J/cm^2 light) group than in the control group (Table 5.3.2). The level of reduction of the LC surface antigens obtained after the short-term *in vitro* culture was similar to those obtained when analysis was performed within the first 6 h post pretreatment and isolation.

Table 5.3.2 Comparison of the mean fluorescence intensity of the surface antigens of LC, isolated from untreated or treated (0.25 μg/mL of BPD + 10 J/cm^2 light), after a short-term *in vitro* culture.

	MEAN FLUORESCENCE INTENSITY ± STANDARD DEVIATION[1]					
	Fresh Isolates			Short-term culture[NS]		
	I-A^b	B7-1	B7-2	I-A^b	B7-1	B7-2
Control	51.6 ± 3.2	9.7 ± 3.3	5.2 ± 1.7	44.9 ± 14.8	10.8 ± 3.8	6.2 ± 0.3
Treated	4.8 ± 0.6	1.04 ± 0.1	1.04 ± 0.1	5.8 ± 2.2[2]	2.3 ± 0.03[2]	2.8 ± 0.1[2]

1 Mean fluorescence intensity of the surface antigens were obtained by subtracting the fluorescence intensity obtained with isotype-matched control IgG from the intensity of the relevant monoclonal antibody. Freshly isolated LC or LC that had been maintained in culture medium supplemented with 10 % FBS, 20 ng/mL M-CSF and GM-CSF for 24 h (short-term) were stained with anti-mouse I-A^b, B7-1 and B7-2 monoclonal antibodies and analyzed by flow cytometry.

2 $p < 0.001$ by ANOVA or the Student's *t*-test relative to control group.

NS Not significantly different ($p > 0.05$ by ANOVA or Student's *t*-test) from the mean fluorescence intensity of fresh isolates of LC.

Furthermore, we found that low-dose PDT at BPD dose of 0.25 μg/mL did not affect the viabilities of cells ($\geq$ 80 %) after an overnight culture (Table 5.3.3). The viability of cells recovered from a short-term culture of LC was 80.2 $\pm$ 4.0 % (untreated group) and 82.9 $\pm$ 4.1 % (treated group at a BPD dose of 0.25 μg/mL and light). On the contrary, low-dose PDT at a BPD dose of 1.0 μg/mL significantly decreased (49.7 $\pm$ 2.0 %) ($p <$ 0.001 by Student's *t*-test relative to control group) the viability of LC after an overnight culture *in vitro* (Table 5.3.3). This suggests that low-dose PDT, at a dose of BPD (1.0 μg/mL) and light, that modulated surface antigens of LC was ultimately cytotoxic for LC, whereas the lower dose of BPD (0.25 μg/mL) and light (which was more effective in allograft extension) was not.

Similarly, the viabilities of LC recovered from a long-term ($\geq$ 7 days) culture were significantly decreased ($p <$ 0.0001 Student's *t*-test) from 80.1 $\pm$ 2.4 % (control group) to 28.0 $\pm$ 4.9 % (treated group; 1.0 μg/mL of BPD + light). Low-dose PDT at BPD dose of 0.25 μg/mL, however, did not significantly affect the viability of LC (78.8 $\pm$ 1.7 %) in long-term cultures (Table 5.3.3).

Table 5.3.3. Effect of low-dose PDT on LC, enriched from untreated or treated donor skin tissues, in short-term (24 h) and long-term (7 days) cultures.

	VIABILITY OF ENRICHED LANGERHANS CELLS* ± STANDARD DEVIATION (%)		
GROUP	**Fresh**	**Short-term culture**	**Long-term culture**
Untreated Control	79.6 ± 9.5	80.2 ± 4.0^{NS}	80.1 ± 2.4^{NS}
Low-dose PDT (μg/mL BPD + 10 J/cm^2 light (λ = 690 nm)			
1.0 μg/mL BPD	73.8 ± 8.0	49.7 ± 2.0†	28.0 ± 4.9‡
0.25 μg/mL BPD	79.7 ± 6.8	82.9 ± 4.1^{NS}	78.8 ± 1.7^{NS}

* The viability of LC was determined via trypan blue or propidium iodide exclusion. Viabilities were evaluated with freshly isolated LC or LC that had been maintained in culture medium supplemented with 10 % FBS, 20 ng/mL M-CSF and GM-CSF for 24 h (short-term) or 7 days (long-term) at 37 °C. Data is expressed as mean ± standard deviation of 3 independent experiments using ≥ 10 mice for each experiment.

† $p < 0.001$ by Student's *t*-test relative to the fresh isolates.

‡ $p < 0.0001$ by Student's *t*-test relative to the fresh isolates.

NS Not significantly different ($p > 0.05$ by Student's *t*-test) from the fresh isolates.

5.4 Discussion.

In skin grafts, the main candidates for affecting cutaneous immune responses are the LC or keratinocytes. During the initial phases of alloantigen sensitization, it is thought that the primary initiator is the LC within the donor graft. These immature DC migrate from the graft and relocate, via the lymphatics, in the draining lymph nodes. There, these MHC class I and class II expressing cells present their antigens to both $CD4^+$ and $CD8^+$ T cells. Activation and proliferation of these alloantigen specific T cells and their subsequent migration to the graft mediate the rejection process. Thus, it was reasonable to determine whether LC were modified in any way by low-dose PDT such that they were less effective in antigen presentation or T cell activation.

First, analysis of skin histological features that received low-dose PDT, under conditions which characteristically resulted in prolonged engraftment (chapter 4), showed that low-dose PDT of donor skin tissue induced certain minimal histological changes in the epidermis of graft tissues without associated dermal changes and no evidence of apoptosis or necrosis. This suggested that low-dose PDT treatment of donor tissues produced a small degree of cellular damage and tissue reorientation rather than wide spread cell death. Our viability studies using enriched LC confirmed the apparent non-cytotoxicity of this treatment regimen.

Second, since LC are responsible for the induction of T cell responses in MECLR (Stingl *et al.*, 1978), we therefore compared the influence of low-dose PDT on the allostimulatory capacity of these cells. In the preliminary study, we analyzed the effect of

low-dose PDT of donor skin tissue on the capacity of crude epidermal cell (EC) suspensions to induce a primary allogeneic T cell response. We showed that BPD (1.0 μg/mL) and light (10 J/cm^2, λ = 690 $\pm$ 10 nm) reduced the capacity of EC to induce T cell proliferation. These results are in line with those reported by Czernielewski *et al.* (1984) using UVB irradiated crude EC suspensions. Furthermore, we observed that LC, enriched from donor skins treated with low-dose PDT, were inefficient allostimulators in the MECLR. Similar results were obtained earlier by Gruner *et al.* (1985) which demonstrated that treatment of human peripheral blood mononuclear cells with Photofrin® and light rendered them unable to stimulate allogeneic cells in the MLR. Unlike Gruner's work, however, optimal effects were obtained at a lower dose of BPD (0.25 μg/mL) and light (10 J/cm^2, λ = 690 $\pm$ 10 nm). Our *in vivo* skin graft data (chapter 4) have shown the same optimal dose of BPD and light prolonged skin allograft acceptance best, thus suggesting a correlation between our *in vitro* and *in vivo* data.

Finally, using Langerhans cells that were isolated from full thickness skin grafts which had been treated with low-dose PDT, we analyzed (by flow cytometry) the expression of the MHC (class I and II) and adhesion molecules (ICAM-1), the costimulatory molecules (B7-1 and B7-2), the leukocyte common antigen (CD45), and the endocytic receptor (DEC-205) recognised by the monoclonal antibody, NLDC-145. We found that the levels of MHC and B7 molecules were present in significant lower amounts on freshly isolated LC, obtained from low-dose PDT-treated skin, than in control preparations. On the contrary, the levels of expression of CD45, ICAM-1 and DEC-205 were unchanged. These observations suggested that the decreased levels of LC surface molecules observed in our experiments

were unlikely to result from a random event representing a total collapse of the cell membrane of LC. We obtained similar levels of decreased surface antigens irrespective of treating graft tissues with 1.0 or 0.25 μg/mL of BPD and light (10 J/cm^2, $\lambda = 690 \pm 10$ nm) prior to LC preparation. The data obtained from both doses of BPD at a constant light dose were not significantly different from each other. However, low-dose PDT, at a BPD dose of 1.0 (but not 0.25 μg/mL) and light, that modulated surface antigens of LC was ultimately cytotoxic for LC when the cells were maintained in short- or long-term cultures *in vitro*. This suggests that low-dose PDT, at a relatively higher dose of BPD (1.0 μg/mL) and light, that modulated surface antigens of LC was ultimately cytotoxic for LC, whereas the lower dose of BPD (0.25 μg/mL, which was more effective in allograft extension) was not. This is in agreement with the findings of Rouabhia *et al.* which suggested that the depletion of LC may not be sufficient to sustain skin and epidermal sheet allograft survival (Rouabhia *et al.*, 1993). The lower dose of BPD (0.25 μg/mL) and light which modulated rather than depleted the epidermal LC was not cytotoxic to LC (Table 5.3.2) but was most effective in prolonging skin allograft acceptance (chapter 4). This is an important observation because the control of rejection and subsequent graft acceptance and tolerance has been suggested to depend upon the establishment of mixed, long-term microchimerism in the recipient tissues as well as the transplant (Starzl *et al.*, 1992; Rao *et al.*, 1994). This involves the migration into the recipients after transplantation of the bone-marrow derived "passenger" leukocytes that normally reside in the interstitial tissues of whole organs (Talmor *et al.*, 1995; Thomson *et al.*, 1995; Rao *et al.*, 1994; Starzl *et al.*, 1993b) and the reciprocal migration of circulating recipient leukocytes, which repopulate the interstitium

of whole organ allografts (Rao *et al.*, 1994; Starzl *et al.*, 1992). This bidirectional exchange and interaction of bone marrow-derived cells after organ transplantation is considered a seminal event in the acceptance of allografts and in the induction of donor-specific tolerance (Murase *et al.*, 1995). Therefore, it appears that the depletion of donor-derived DC may be ineffectual and unlikely to be the best approach for achieving the much desired donor-specific tolerance in transplantation. It is interesting to note that attempts which had focused mainly on the depletion of the dendritic cells within the donor tissue have achieved moderate, but not long-term, prolongation of allograft survival in the skin, pancreatic islets, and thyroid allografts (Lafferty *et al.*, 1975; Lafferty *et al.*, 1976; Bowen *et al.*, 1979; Faustman *et al.*, 1984; Iwai *et al.*, 1989; Lafferty *et al.*, 1983; 1986; Gruner *et al.*, 1984; Gruner *et al.*, 1985; Morison *et al.*, 1981). However, the establishment of mixed chimerism has been shown to induce long-term unresponsiveness (tolerance) to allografts across major and minor histocompatibility barriers (Orloff *et al.*, 1995; Colson *et al.*, 1995; Li *et al.*, 1995).

The impairment of allostimulatory capacity of LC in MECLR (treated group) may be a consequence of the decreased surface antigen expression on LC of MHC and B7 molecules. The roles played by the MHC or the costimulatory molecules in the two distinct signals required for T cell activation is well documented (Mueller *et al.*, 1989; Linsley and Ledbetter, 1993; Jenkins and Schwartz, 1987). "Signal 1", the antigen specific, is provided when T cell receptor (TCR) interacts with antigen presented in the context of MHC expressed on APCs. The "signal 2" or costimulatory signal is provided by a set of receptor-ligand interactions distinct from the TCR interactions. It has been shown that the B7-1 and

B7-2 molecules expressed on APCs (Freeman *et al.*, 1989; Hathcock *et al.*, 1993; Azuma *et al.*, 1993; Freeman, 1993a) and the CD28 and CTLA-4 molecules expressed on T cells (Gross *et al.*, 1992; Linsley and Ledbetter, 1993) provide this costimulatory signal (June *et al.*, 1994; Boise *et al.*, 1995; Linsley and Ledbetter, 1993). Importantly, TCR occupancy in the absence of costimulation results in suboptimal activation of T cells, with no IL-2 production and no resultant proliferation (Jenkins and Schwartz, 1987). In addition, such cells are unresponsive (anergic) to subsequent stimulation with normal APC plus antigen (Jenkins and Schwartz, 1987).

Thus, it is possible that inefficient alloreactivity of LC from treated donor skin may explain the suppressed proliferation of allogeneic T cells in MECLR and the prolonged survival of skin allograft survival following low-dose PDT of donor skin grafts.

Chapter Six

MECHANISMS OF LOW-DOSE PDT-INDUCED ANTI-GRAFT IMMUNE RESPONSE

II. FATE AND ACTIVITY OF LANGERHANS CELLS AFTER LOW-DOSE PDT

6.1 Abstract.

It is known that as a response to antigen encounter, injury or skin graft, LC migrate to the draining lymph nodes where they present antigens to T cells. As a result, activated T cells emigrate from the lymph node via the blood into the site of injury or antigen encounter in the skin. Thus, to study the effect of low-dose PDT on the migration of LC, we investigated the effect of low-dose PDT on the LC-specific ATPase activity on the epidermal sheets of donor skins (C57BL/6) and on the migration of LC *in vivo* in response to topical application of a contact sensitizer (Cell Tracker™ Green BODIPY®). Subsequently, we evaluated the ability of T cells from graft recipients to respond to restimulation with mitogen (con A) or with donor-derived alloantigen.

We found that the ATPase activity was not affected by low-dose PDT at the dose of BPD (0.25 μg/mL) that was most beneficial in prolonging skin allograft survival. On the contrary, PDT at higher dose of BPD (1.0 μg/mL) led to more than 90% reduction in ATPase activity. Furthermore, transdermal PDT (0.25 - 1.0 mg/kg) had no significant ($p > 0.05$ by the ANOVA) effect on the migration of LC in response to topical application of the contact allergen. However, the response of T cells from animals that received treated donor graft tissues to restimulation with untreated LC was significantly reduced relative to the response of T cells procured from animals that received untreated donor graft tissues. The optimal effect (69.5 $\pm$ 1.9 % reduction relative to the untreated controls, taken as 100 % response.; $p < 0.001$ by ANOVA) was observed when T cells were derived from graft recipients that previously received donor skins pretreated with low-dose PDT at BPD dose

of 0.25 μg/mL + light (the dose at which optimal prolongation of graft allograft acceptance was observed). Nonetheless, the cell surface antigens of these primed T cells or their response to con A was not jeopardized, suggesting that low-dose PDT, unlike many immunosuppressive agents, might not compromise the general immune response of the graft recipients.

6.2 Introduction.

Epidermal Langerhans cells (LC), a component of the dendritic cell system, are well equipped to provide the principal sensitizing signal for initiation of cutaneous immune responses (Steinman *et al.*, 1995). First, they have the unique ability to take up antigen in the epidermis and then migrate to the draining lymph nodes where they present the antigen to T cells (Macatonia *et al.*, 1987). Second, LC constitutively express MHC class II molecules (Unanue, 1981; Beller, 1984). Concomitant with their migration out of the skin, MHC class II expression is further increased and expression of the costimulatory molecules, B7-1 and B7-2, are strongly up-regulated (Larsen *et al.*, 1994; Schuler and Steinman, 1985). Thus, they develop into highly efficient antigen-presenting cells with the potential to induce the primary stimulation of specific T cells in the paracortex of lymph nodes. As a result, activated T cells emigrate from the lymph node via the blood into the site of injury or antigen encounter in the skin.

Langerhans cells are distinguishable by their dendritic morphology, and by a unique cytoplasmic organelle, the Birbeck granule (Stingl *et al.*, 1980; Streilein and Bergstresser,

1984). Cell surface markers that are used commonly to identify LC as distinct from other epidermal cells are the enzyme ATPase and CD1a (Mommaas *et al.*, 1994). Epidermal LC display intense formalin-resistant adenosine triphosphatase (ATPase) staining on their cell membrane (Mackenzie and Squier, 1975). The enzyme is located on the plasma membrane with its active site facing the exterior, thus its designation as membrane ATPase (mATPase) and an ectoenzyme (Wolff and Winklemann, 1967). ATPase has been used extensively and reliably as a histochemical marker for these cells both *in situ* and in suspension (Mackenzie and Squier, 1975; Cormane and Kalsbeek, 1963; Wolff and Winklemann, 1967).

Extensive studies by Kripke and colleagues (Kripke *et al.*, 1990) on the induction of contact hypersensitivity (CHS) to a fluorescent contact allergen, fluorescein isothiocyanate (FITC) have provided a very useful model for studying the fate and activity of epidermal LC. They showed that after the topical application of the fluorescent contact allergen, the FITC-bearing LC migrated out of the skin and collected in the local draining lymph nodes, where they interacted with T cells to initiate a CHS response (Kripke *et al.*, 1990). Also, several studies have shown that exposure of skin to ultraviolet (UV) radiation affected antigen presenting functions, impairing the ability of LC and other antigen presenting cells to induce CHS (Elmets *et al.*, 1983; Okamoto and Kripke, 1987; Cruz *et al.*, 1989). Similarly, transdermal PDT had been shown to suppress the development of the dinitrofluorobenzene (DNFB)-induced skin contact hypersensitivity (Simkin *et al.*, 1995).

The effects of UV irradiation on LC are thought to be due to decreased expression of MHC, adhesion, and costimulatory molecules (Tang and Udey, 1991; Simon *et al.*, 1991; Young *et al.*, 1992; Shimada *et al.*, 1987; Harding *et al.*, 1992). Similarly, it has been

shown that the irradiation of mouse skin with suberythemal doses of UV-B reduced the population and altered the morphology of ATPase$^+$ epidermal LC at the site of UV irradiation (Bergstresser *et al.*, 1980c; Toews *et al.*, 1980; Aberer *et al.*, 1981; Lynch *et al.*, 1981; Elmets *et al.*, 1983). PUVA (psoralen plus UVA irradiation) was shown to deplete the epidermis of both ATPase-positive (Lynch *et al.*, 1981; Horio and Okamoto, 1982) and Ia-positive cells (Nordlund *et al.*, 1981). Similarly, Photofrin® and visible light have also been used to deplete ATPase$^+$ epidermal LC (Gruner *et al.*, 1985).

In this chapter, we evaluated the fate of LC following transdermal PDT. This was achieved by designing experiments that closely mirrored the studies on the fate of LC following UV irradiation. However, there might be several possible outcomes in relation to the fate of LC after low-dose PDT in our system. One possibility might be that the LC were depleted by low-dose PDT treatment. However, the results we have obtained so far (chapters 4 and 5) seem to indicate otherwise. We showed that similar numbers of LC were procured from treated and untreated donor skins (Table 5.3.2) and that our treatment regimen did not affect the level of expression of LC-specific DEC-205 (recognized by the monoclonal antibody, NLDC-145) (Fig. 5.3.5). To further clarify the effect of low-dose PDT treatment on LC in the donor skin, we studied the effect of this treatment regimen on an LC-specific marker, ATPase, which are found on the surfaces of epidermal Langerhans cells by staining for the enzyme on the epidermal sheets of treated and untreated donor skins.

The second possibility might be that low-dose PDT could have reduced the level of LC migration to the local draining lymph nodes in response to a contact allergen. Our initial

experiments focused on the enumeration by flow cytometry of LC that might have emigrated from the grafted skin to the draining lymph nodes of the recipient mice at varying days post transplantation. However, this attempt to directly detect the emigrating LC from the grafted donor skin to the draining lymph nodes of the graft recipient was not successful. Thus, we adopted an *in vivo* model of transdermal PDT in which the dorsal skin of mice were topically painted with contact allergens immediately following transdermal PDT (1 mg/kg of BPD plus 15 J/cm^2 light at a wavelength of 690 nm) on the assumption that levels of BPD and light delivered to the skin would be roughly equivalent to that used to treat grafts. The draining lymph node cells were analyzed 24 h later for the fluorescence of the allergen-bearing Langerhans cells.

The third possible outcome might be that LC emigrated from the epidermis to the draining lymph nodes following low-dose PDT and transplantation but remained functionally impaired. We studied the fate and activity of LC by using T cells that were isolated from the draining lymph nodes of graft recipients at varying times (4 - 8 days post the engraftment of low-dose PDT-treated donor skins). Specifically, we evaluated the ability of the draining lymph node T cells from graft recipient to respond to restimulation with mitogen (con A) or with donor-specific alloantigen (untreated LC). We adopted this indirect approach because it had been implied that UV-altered LC may be responsible for the induction of T cell anergy, as suggested by *in vitro* experiments using UV-irradiated LC and purified T cells in the mixed leukocyte reaction (Simon *et al.*, 1991). We speculated that low-dose PDT-modulated LC might migrate from the donor graft to the draining lymph nodes and induce T cell anergy or at least, induce a reduced T cell proliferation. This was

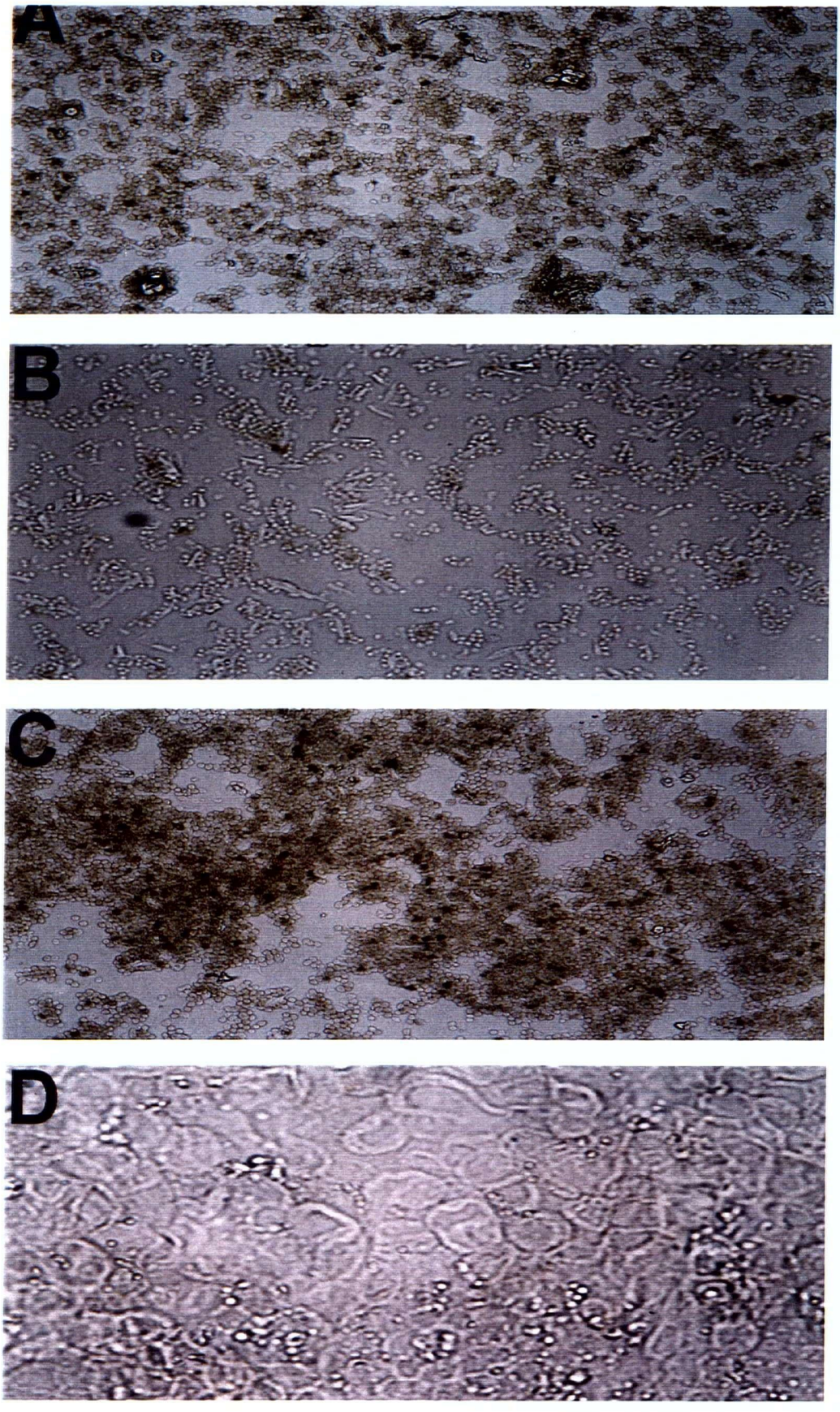
suggested by our earlier findings that low-dose PDT down-modulated the LC surface molecules of MHC and B7 surface molecules (Fig. 5.3.5) and impaired the capacity of low-dose PDT-altered LC to stimulate T cell proliferation (Fig. 5.3.4). To evaluate this possibility, we purified T cells from the draining lymph nodes of graft recipients that received treated or untreated donor skins and rechallenged the T cells with enriched LC (donor-specific antigens) in the mixed epidermal cell lymphocyte reaction. Second, we evaluated the response of the lymphocytes from the graft recipients to con A. Third, we characterized the surface antigen profile of the splenic lymphocytes from these allograft recipients with a panel of monoclonal antibodies and flow cytometry. Lastly, we immunostained the lymph nodes from the recipient mice given untreated or pre-treated donor skin for the intensity of $CD4^+$ and $CD8^+$ T cells within the nodes at various times post transplantation.

6.3 Results.

a. Effect of low-dose PDT on the ATPase activity.

The results of the histochemical staining for the LC-specific ATPase activity on epidermal cells of treated and untreated donor skins are shown in Fig. 6.3.1. We used epidermal cells that were enriched for LC and cytocentrifuged them onto microscopic slides following which they were fixed in formaldehyde, stained with ATP, and examined with a light microscope (materials and methods section 2.17). The keratinocyte (KC) cell line, PAM 212, was used as the negative control (Fig. 6.3.1d).

Fig. 6.3.1. Epidermal cells stained for ectophosphatase activity using extracellular adenosine triphosphate (ATP). LC were enriched from epidermal cells obtained from the truncal skin of a) untreated donor mice (C57BL/6) or donor skin treated with BPD at b) 1.0 μg/mL and c) 0.25 μg/mL concentrations and light (10 J/cm^2, 690 $\pm$ 10 nm wavelength). Specimens were cytocentrifuged onto microscopic slides, fixed with formalin and stained with ATP. (materials and method section 2.19). Slides were observed under a light microscope. Keratinocyte cell line (PAM 212) was used as the negative control (d). Micrographs are representative of 3 independent experiments. Magnification: 100X.


A
B
C
D

We found that enriched LC, but not the KC cell line, stained for the ATPase, confirming that the presence of ATPase was restricted only to the epidermal LC. The intensities of ATPase staining on epidermal cells from the untreated donor skin (Fig. 6.3.1a) were similar to those observed on the epidermal cells from the donor skin treated with low-dose PDT at BPD dose of 0.25 μg/mL plus light (10 J/cm^2; λ = 690 $\pm$ 10 nm). In certain instances, the intensities of ATPase on the epidermal cells from donor skins treated with low-dose PDT at BPD dose of 0.25 μg/mL plus light (10 J/cm^2; λ = 690 $\pm$ 10 nm) appeared to be slightly more than those from control donor skins. On the contrary, low-dose PDT at a higher dose of BPD (1.0 μg/mL of BPD) and light (10 J/cm^2; λ = 690 $\pm$ 10 nm) led to a significant reduction ($\geq$ 90 % reduction) in number of ATPase$^+$ cells in comparison to the untreated controls.

b. Effect of transdermal PDT on LC migration.

In our preliminary experiment, we investigated the fate of LC in the epidermis in response to topical application of FITC immediately following transdermal PDT. Briefly, untreated mice or mice injected with BPD (1.0 mg/kg) intravenously were illuminated with or without direct light exposure (15 J/cm^2 light at a wavelength of 690 $\pm$ 10 nm) 60 minutes later. Immediately following treatment, animals were topically painted on the right lateral thoracic wall with FITC (1.0 mg/mL) in acetone. 24 h post treatment, we obtained lymph node cells by preparing single cell suspensions from the draining lymph nodes of these mice. Cells were stained with a panel of monoclonal antibodies and subsequently analyzed for differences in mean fluorescence intensities of surface antigens characteristic

of antigen presenting cells (MHC class II, ICAM-1, B7.1) or surface antigen found specifically on LC (DEC-205, recognized by the monoclonal antibody, NLDC-145) and an antigen found on LC as well as lymph node B cells (HSA) (materials and methods section 2.12). We compared results obtained from mice given BPD alone or transdermal PDT followed by topical application of FITC to those which received FITC alone (positive control) or PDT alone (PDT control) or none of the treatments (negative or naive control). The result of the initial experiment using FITC as the contact allergen is shown in figure 6.3.2.

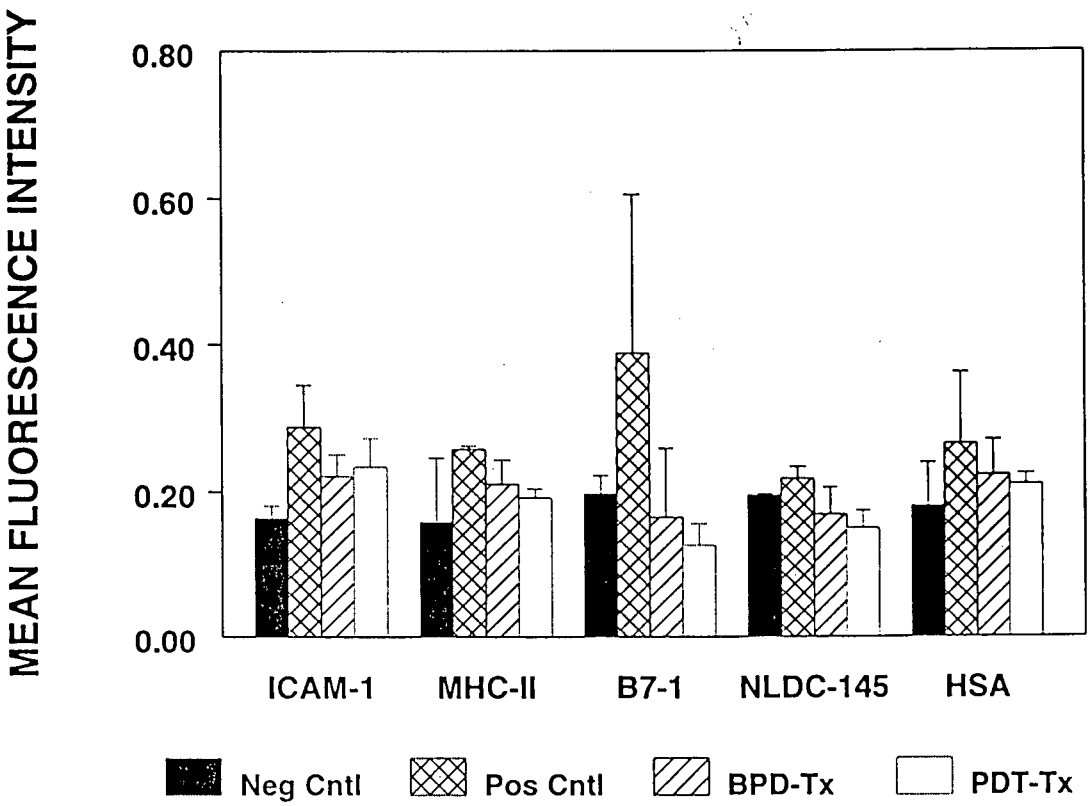


Fig. 6.3.2. Effect of transdermal PDT on the migration of LC in response to topical application of fluorescent contact allergen (FITC). Untreated BALB/c mice or mice injected with BPD (1.0 mg/kg) and illuminated with (15 J/cm^2 light at a wavelength of 690 $\pm$ 10 nm) or without direct light exposure were topically painted on the right lateral thoracic wall with FITC (300 μL, 1.0 mg/mL) in acetone immediately following transdermal PDT. Lymph node cells from the draining lymph nodes of these mice were stained with a panel of monoclonal antibodies (materials and methods section 2.15) at 24 h post treatment. Results obtained from mice given BPD alone or transdermal PDT followed by topical application of FITC were compared to those which received FITC alone (positive control) or PDT alone (PDT control) or none of the treatments (negative or naive control). Data shown represent the specific mean fluorescence intensities (intensity with the relevant monoclonal antibody minus the intensity with isotype-matched control IgG) expressed as mean (n = 3) $\pm$ SD.

We found that mice which were painted topically with the allergen alone (positive control group) exhibited a moderate increases in the levels of the mean fluorescence intensity (MFI) of surface molecules of ICAM-1 (0.16 ± 0.02 (negative control) to 0.29 ± 0.06 (positive control group); MHC-II (0.16 ± 0.09 (negative control) to 0.26 ± 0.01 (positive control group); B7-1 0.20 ± 0.03 (negative control) to 0.39 ± 0.2 (positive control group); and HSA (0.18 ± 0.06 (negative control) to 0.27 ± 0.1 (positive control group); but not NLDC-145 in comparison to the negative (naive) controls (Fig. 6.3.2). The fluorescence intensity observed in the PDT only control group was similar to that observed in the negative (naive) controls. Our findings implied that epidermal cells might have migrated in response to the topical application of FITC. Furthermore, we found that transdermal PDT seem to have no significant effect on the migration of FITC-bearing epidermal LC to the draining lymph nodes. The intensities of the surface antigens observed in the positive control group were not significantly different ($p > 0.05$ by ANOVA) from those observed in the groups given BPD (1.0 mg/kg) with or without light (Fig. 6.3.2). Therefore, it appears unlikely that the mechanism by which transdermal PDT induced the suppression of the development of cutaneous immune response in the skin contact hypersensitivity model (Simkin *et al.*, 1995) was a consequence of targeted depletion of epidermal LC or inhibition of their migration. However, because we observed no significant increase in the mean fluorescence intensity of the LC-specific surface antigen (DEC-205, recognized by the monoclonal antibody, NLDC-145) in response to the contact allergen in the positive control in comparison to the negative control groups (Fig. 6.3.2), this led us to question the sensitivity of this assay. It was not possible to conclude from these data that

the moderate increases observed in the surface antigens of MHC class II, ICAM-1, B7-1 and HSA following transdermal PDT and FITC application was a consequence of the migration of epidermal cells. It was thought that a better alternative to evaluate the fate of LC might be a direct detection of the fluorescent intensity of the contact allergen-bearing epidermal LC in the lymph nodes of mice following *in vivo* transdermal PDT and topical application of a contact allergen.

Consequently, we evaluated the migration of LC in response to topical application of another contact allergen, 8-chloromethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-indacene (Cell Tracker™ Green BODIPY®, Molecular Probe Inc., Eugene OR), following transdermal PDT. 24 h following treatment and the application of the Green BODIPY®, the draining lymph node cells were analyzed for the allergen-bearing cells. Using the Coulter XL® flow cytometer, we assessed the fluorescence intensity of the draining lymph node cells from treated or untreated mice. This approach was deemed to be a more direct approach than the former for evaluating the migration and activity of LC following transdermal PDT and contact sensitization. Alternatively, we enumerated the total viable cells obtained from the superficial inguinal and axillary lymph nodes of these mice. We compared results obtained from mice given transdermal PDT and Green BODIPY® to those that received only Green BODIPY® without transdermal PDT (positive control) or to the unmanipulated litter mates (naive or negative control). Our observation are summarized in figure 6.3.3.

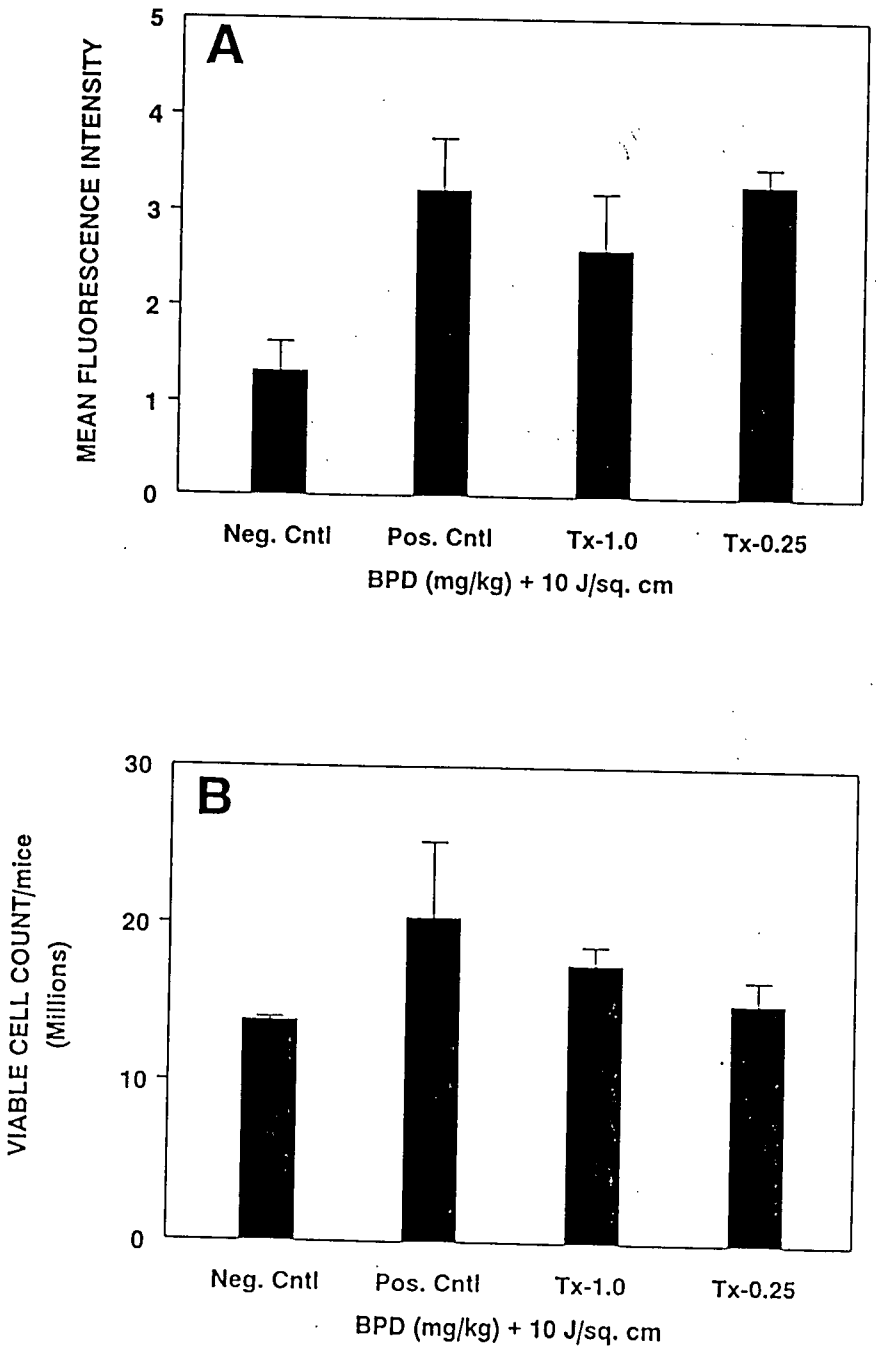


Fig. 6.3.3. Effect of transdermal PDT on the migration of LC in response to topical application of contact sensitizer. Balb/c mice given BPD (1.0 or 0.25 mg/kg) were exposed to light (15 J/cm^2 light at a wavelength of 690 $\pm$ 10 nm) following which they were topically painted on the right lateral thoracic wall with Green BODIPY$^•$ cell tracker (300 μL, 1 mM solution) (materials and methods section 2.15). 24 h later, the draining lymph node cells were counted and analyzed for the fluorescence of the allergen-bearing LC by flow cytometry. Data is expressed as a) mean (n = 4) fluorescence intensity or b) mean (n = 4) cell count $\pm$ SD. Mice that received no treatment or contact allergen were used as negative control while mice given topical application of Green BODIPY$^•$ without transdermal PDT were used as positive control.

Our data suggest that transdermal PDT did not appear to affect the migration of epidermal LC to the local draining lymph nodes (Fig. 6.3.3a). We found that the contact sensitizer induced the migration of BODIPY-bearing epidermal cells (possibly LC) from the epidermis to the local draining lymph nodes. The mean intensities of the allergen-bearing cells from mice painted with the Green BODIPY® alone (positive control) were significantly ($p < 0.001$ by the regular ANOVA) greater than the background fluorescence intensity obtained from naive mice that received no contact allergen (negative control). On the contrary, we found that transdermal PDT at the doses of BPD (0.25 or 1.0 mg/kg) and light (15 J/cm^2; $\lambda = 690 \pm 10$ nm) had no significant effect on the migration of the Green BODIPY®-bearing epidermal cells to the draining lymph nodes.

Furthermore, we found that the contact allergen induced a moderate cellular increase ($p < 0.05$ by the ANOVA) in the lymph nodes from 1.39×10^7 viable cells/mice (negative control group) to 2.04×10^7 viable cells/mice (positive control group). However, the cellularity (viable lymph node cells) of the lymph nodes from the positive control group was not significantly different ($p > 0.05$ by the regular ANOVA and the Bonferroni multiple comparison test) from those of the treated groups (Fig. 6.3.3b) receiving antigen.

c. Effect of low-dose PDT on the immunocompetence of presensitized T cells.

Kripke *et al.* (1990) showed that epidermal LC migrate to the draining lymph nodes in response to topical application of contact allergen. Further, it has been implied that UV-altered LC may be responsible for the induction of anergy, as suggested by *in vitro*

experiments using UV-irradiated LC (Simon *et al.*, 1991). Thus, we evaluated the immunocompetence of the T cells from the draining lymph nodes of graft recipients undergoing graft rejection.

At various days post transplantation, we isolated lymph nodes or spleens from graft recipients which had received donor grafts that were either untreated or pretreated with low-dose PDT (1.0 or 0.25 μg/mL BPD + 10 J/cm^2 light at $\lambda = 690 \pm 10$ nm) prior to transplantation. Subsequently, single cell suspensions were prepared from the lymph nodes or spleens (materials and methods section 2.5a and b) and resuspended in RPMI-10.

We enriched the lymph node cells for T cells using the nylon wool column technique described earlier (materials and method section 2.7c) following which the T cells were re-stimulated with freshly isolated untreated LC by coculturing them in the mixed epidermal cell lymphocyte reaction (MECLR) as described earlier (material and methods section 2.16). For this experiment we used T cells obtained from graft recipients on the 6th day following transplantation. Data were expressed as percentages of results obtained with T cells isolated from graft recipients that received untreated donor skins, taken as 100 % response. We found that the response of the nylon wool-enriched T cells (procured from graft recipients given donor skins treated with low-dose PDT at BPD dose of 0.25 μg/mL) to restimulation with untreated LC was 30.5 $\pm$ 1.9 % in comparison with the untreated control group, taken as 100 % response (Fig. 6.3.4). Similarly, the response of T cells from graft recipients transplanted with donor skins pretreated with low-dose PDT at higher BPD dose (1.0 μg/mL) to restimulation was 79.5 $\pm$ 8.5 % relative to the control group (Fig. 6.3.4).

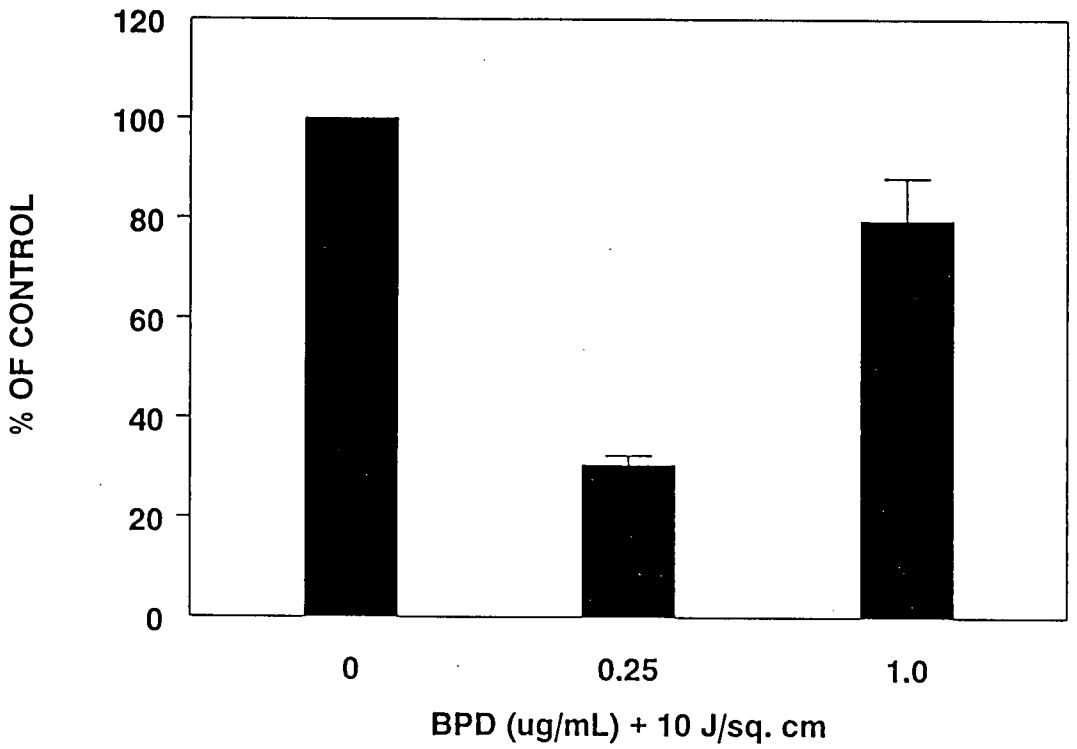


Fig. 6.3.4. The response of primed T cells to restimulation by donor derived-alloantigen in the MECLR. T cells were procured from day 6 graft recipients (Balb/c) by nylon wool enrichment while LC were enriched from untreated donor skin (C57BL/6) using a two-step density gradient centrifugation. Nylon wool-purified T cells (4 x 10^5/well) and enriched LC (1 x 10^5/well) were co-cultured in humified atmosphere for 5 days at 37 °C. The proliferation of the primed T cells were quantified via the MTT assay and presented as a percent of control cells treated with light only and expressed as mean (n = 3) ± SD.

Using the regular analysis of variance, we found that the responses from the treated groups were significantly different ($p < 0.001$; term significant at alpha = 0.05) from the control groups. We performed the Bonferroni (all-pairwise) multiple comparison test to identify possible intra-group differences within the treated groups. Our analysis showed that there was an intra-group difference at the level of restimulation obtained with the two doses of BPD. The data supported our earlier observation that low-dose PDT affected the ability of LC to stimulate alloreactive T cells. Thus, inefficient stimulation of T cells by LC during their initial encounter in the draining lymph nodes might likely explain the reduced proliferation of T cells that was observed upon restimulation with LC.

We also re-stimulated the splenic or lymph node single cell suspensions with con A (materials and methods section 2.11) for 72 h. Cell proliferation was determined at the end of the incubation period using the MTT assay (materials and methods section 2.10) and the data presented as mean (n = 3) metabolic activity (OD_{590nm}) $\pm$ standard deviation. For this experiment we used only the dose of BPD (0.25 μg/mL) and light at which optimal prolongation of skin allograft survival was obtained. We found that the response of the splenic or lymph node lymphocytes to Con A was very similar in both the control and treated graft recipients (Fig. 6.3.5). There were no significant differences ($p > 0.05$ by Student's *t*-test) between the responses of lymphocytes isolated from the control and treated graft recipients for all the times tested (4 - 8 days post engraftment). Our data suggest that low-dose PDT-induced down-regulation of allogeneic T cell response to alloantigen is antigen specific.

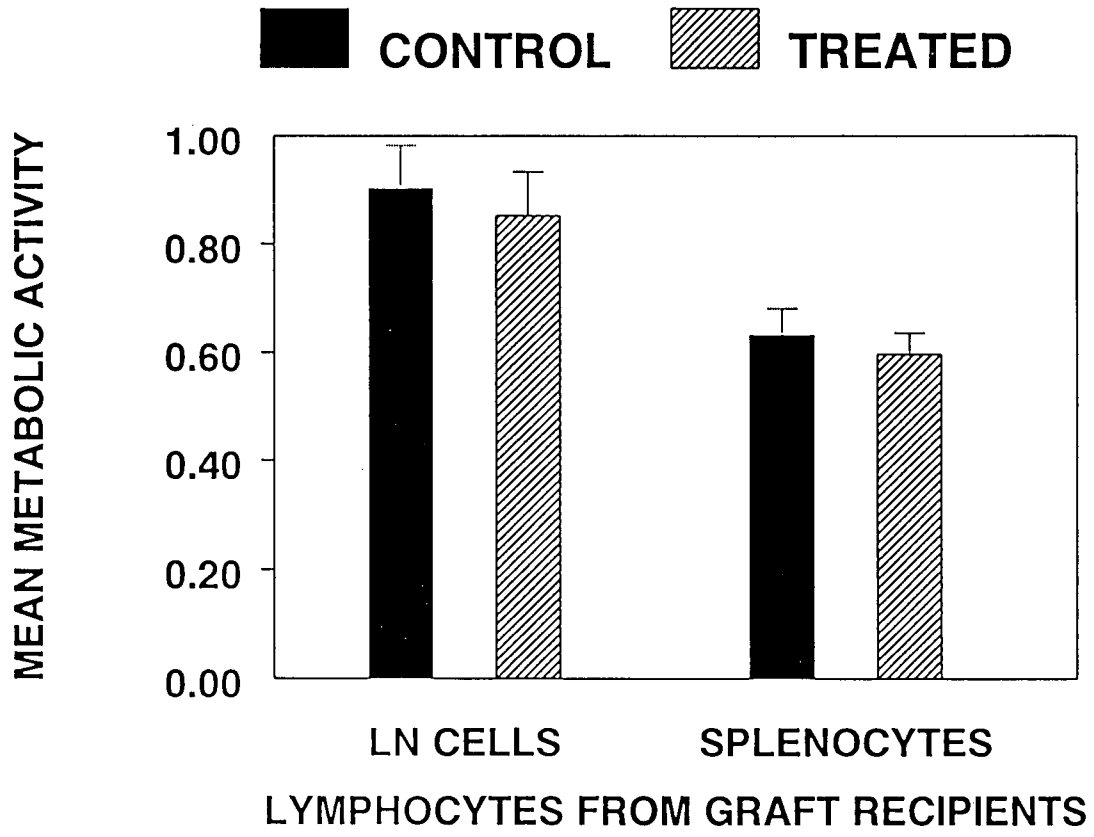
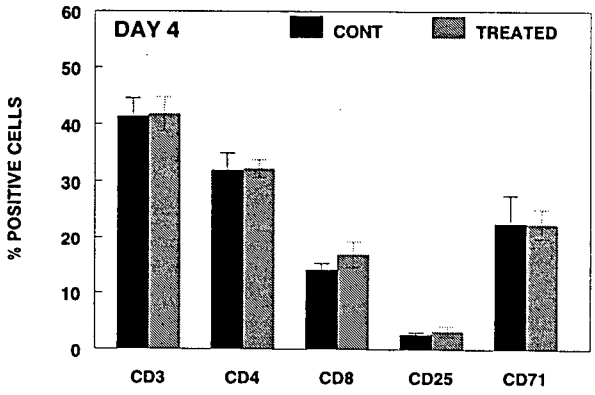
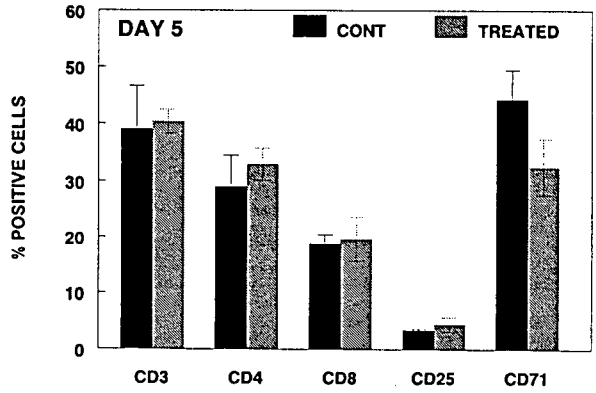


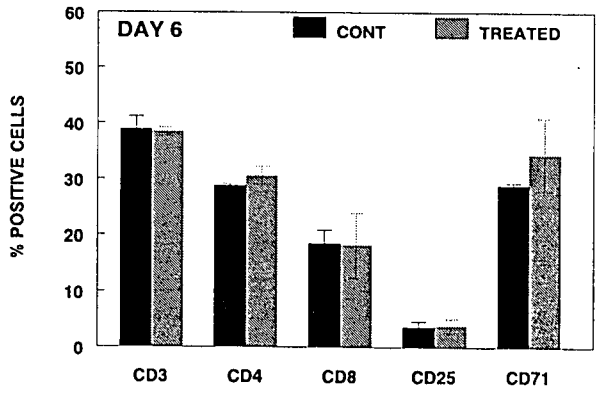
Fig. 6.3.5. Response of lymphocytes to concanavalin A. Splenic and lymph node cells were obtained on day 6 post engraftment from mice given untreated or treated (0.25 μg/mL BPD + 10 J/cm^2 light at a wavelength of 690 $\pm$ 10 nm) donor skin. Cells (2 x 10^6/mL) were cultured in a humidified atmosphere at 37 °C for 72 h in RPMI-1640 medium containing 10 % FBS and Con A (2.5 μg/mL). Cell proliferation was determined by the MTT assay and data expressed as mean (n = 3) metabolic activity (OD 590 nm) $\pm$ SD. Comparable levels of OD readings were obtained at various times (4 - 8 days) post engraftment.

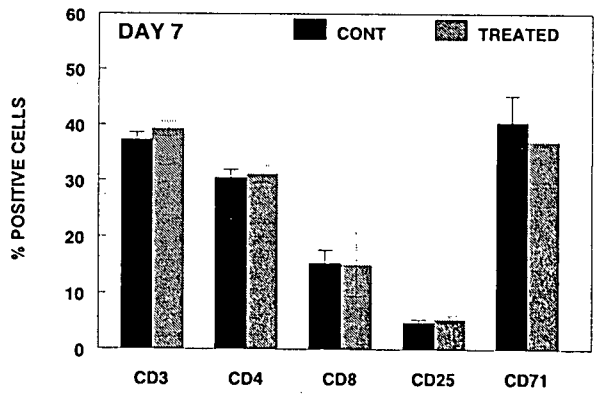
Subsequently, using monoclonal antibodies and flow cytometry (materials and methods section 2.10a) we analyzed the lymphocytes from the spleens of graft recipients for differences in the expression of the surface antigens characteristic of T cells (CD3, CD4 and CD8), and activated cells [interleukin (IL)-2 receptor (CD25) and transferrin receptor (CD71)]. The results are shown in figure 6.3.6 and represent the average of four independent experiments. Percent of cells positive for each surface antigen were expressed as mean (n = 4) $\pm$ standard deviation. We found that the pretreatment of donor graft tissues with low-dose PDT, at the dose of BPD (0.25 μg/mL) and light (10 J/cm^2; λ = 690 $\pm$ 10 nm) that was optimal for graft acceptance, had no significant effect ($p > 0.05$ by Student's *t*-test) on the cell surface antigens (CD3, CD4 and CD8, CD25, and CD71) of the splenic lymphocytes from graft recipient at various times post transplantation in comparison to untreated controls (Fig. 6.3.6). Similar levels of the surface antigens were observed in both the control and treated groups and at all time points tested (4 - 8 days post transplantation) (Fig. 6.3.6). Our data suggest that grafting low-dose PDT-treated donor skin may not compromise the immune cells of the graft recipient. It appears that low-dose PDT treatment of donor tissues was unlikely to compromise the general immune system of the graft recipients.

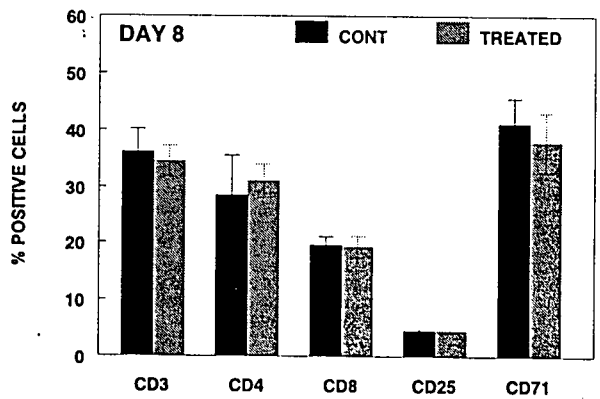
Fig. 6.3.6. Flow cytometric analysis of lymphocyte cell surface antigens from graft recipients. Splenic lymphocytes (1 x 10^6/mL) from graft recipients at various times (4 - 8 days post engraftment) were stained with a panel of monoclonal antibodies and analyzed via flow cytometry (materials and methods section 2.12). Non-specific binding was eliminated by subtracting the % of cells positive for the isotype-matched IgG from those positive for the specific monoclonal antibody. Data represent percent of cells positive for each surface antigen expressed as mean (n = 4) $\pm$ SD. Solid bars represent lymphocytes from mice given untreated donor skin (control) while hatched bars represent lymphocytes derived from mice given donor skin treated with BPD (0.25 μg/mL) and light (10 J/cm^2) at a wavelength of 690 $\pm$ 10 nm (treated).


DAY 4
CONT
TREATED
% POSITIVE CELLS
60
50
40
30
20
10
0
CD3
CD4
CD8
CD25
CD71


DAY 5
CONT
TREATED
% POSITIVE CELLS
60
50
40
30
20
10
0
CD3
CD4
CD8
CD25
CD71


DAY 6
CONT
TREATED
% POSITIVE CELLS
60
50
40
30
20
10
0
CD3
CD4
CD8
CD25
CD71

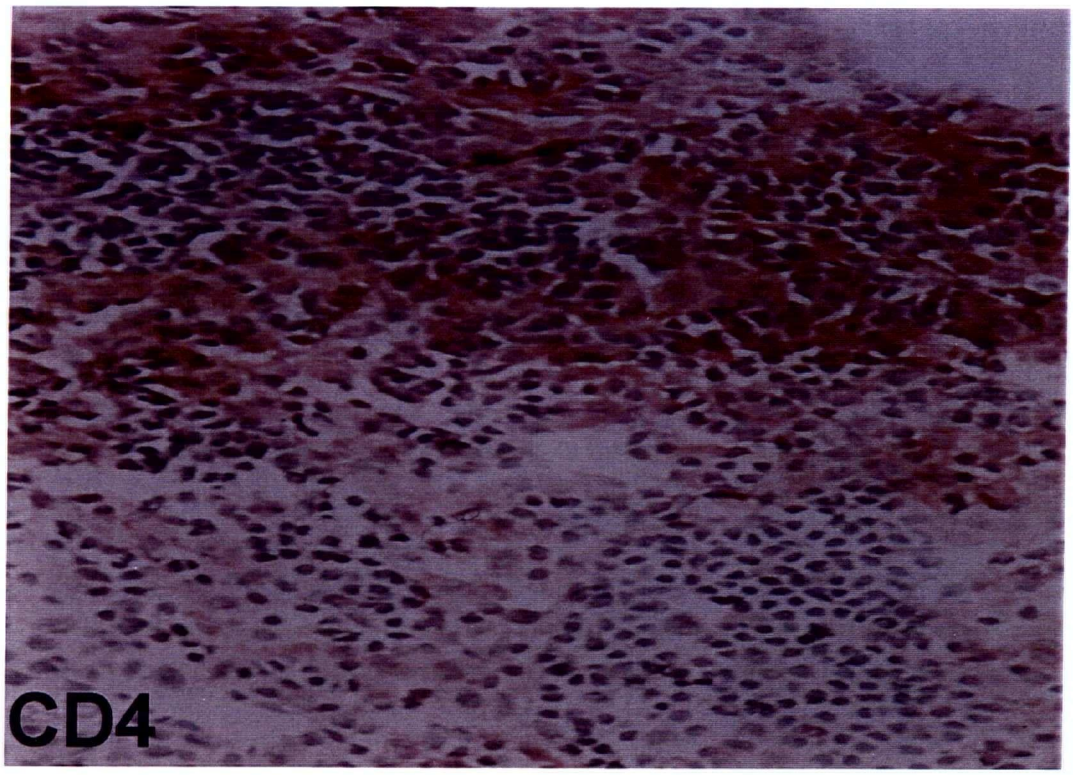

DAY 7
CONT
TREATED
% POSITIVE CELLS
60
50
40
30
20
10
0
CD3
CD4
CD8
CD25
CD71

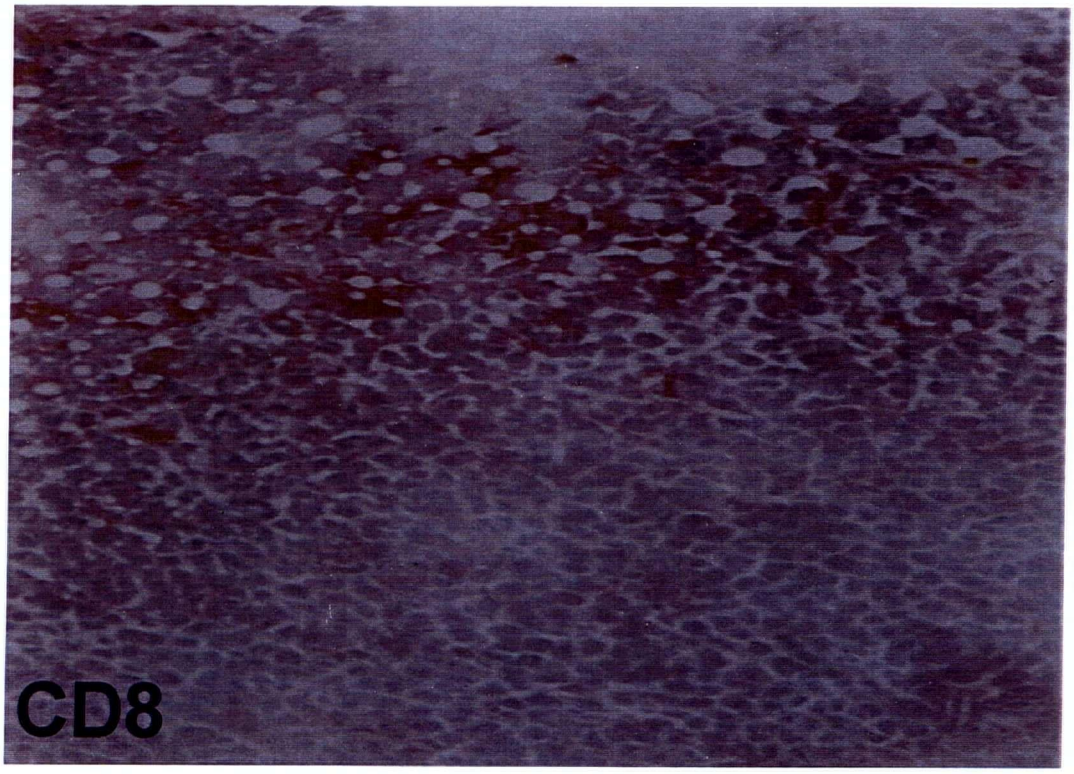

DAY 8
CONT
TREATED
% POSITIVE CELLS
60
50
40
30
20
10
0
CD3
CD4
CD8
CD25
CD71

Lastly, we excised lymph nodes from graft recipients at various times (4 - 8 days) post transplantation. Sections of the lymph nodes were immunostained with the alkaline-phosphatase streptavidin-biotin method described earlier (materials and methods section 2.18) applying biotinylated anti-mouse CD4 and CD8 monoclonal antibodies. We compared the lymph nodes from graft recipients given pretreated skin tissues (0.25 μg/mL of BPD and light (10 J/cm^2) at a wavelength of 690 nm) to that from the untreated controls at different times (4 - 8 days) post transplantation. We found that low-dose PDT at the dose of BPD (0.25 μg/mL) and light (10 J/cm^2 at a wavelength of 690 nm) where the optimal prolongation of skin allograft was observed did not affect the intensities of staining for both CD4 and CD8 in the lymph nodes of recipients at the time points studied. Figure 6.3.7 shows the representative micrographs of the staining intensities for CD4 and CD8 from the lymph nodes graft recipients given untreated or treated donor skins at various days post transplantation. At each time point, we observed that there were no significant differences in the intensity of T cells in the lymph nodes from the treated group in comparison to the untreated group.

Our results suggested that the transplantation of low-dose PDT- treated donor skin might not in any way compromise the graft recipients' T cell response to mitogens or alter their surface antigen profiles. However, the transplantation of low-dose PDT-treated donor skins might decrease the ability of the recipients' T cells to respond to restimulation by the donor-specific alloantigen-bearing LC.

Fig. 6.3.7. Immunohistology of lymph node sections from graft recipients on day 6 post transplantation. Draining lymph nodes (superficial inguinal or axillary) were obtained from graft recipients given untreated or treated (0.25 μg/mL BPD + 10 J/cm^2 light at a wavelength of 690 $\pm$ 10 nm). Frozen sections of lymph nodes from BALB/c recipients were stained with the alkaline-phosphatase avidin-biotin method (materials and methods section 2.18) for CD4 and CD8. At each time point (4 - 8 days post transplantation), there were no significant differences in the intensity of T cells in the lymph nodes from the treated group in comparison to the untreated group. The staining intensity from day 6 post engraftment was very similar to those of other days. Magnification: 400X.


CD4


CD8

6.4 Discussion.

We had shown in previous chapters(4 and 5) that low-dose PDT of donor skin may inhibit LC antigen presenting function (Fig. 5.3.4) by down-modulating the expression of MHC and critical costimulatory molecules (Fig. 5.3.5). Similarly, our laboratory has shown that transdermal PDT might suppress the development of the skin contact hypersensitivity in mice (Simkin *et al.*, 1995). However, the fate of LC following low-dose PDT of donor skin was not known. Kripke *et al.* showed that following topical application of FITC, the FITC-bearing LC migrate out of the skin and collect in the local draining lymph nodes, where they interact with T cells to initiate a cutaneous immune response (Kripke *et al.*, 1990). Thus, these studies provided a very useful model for studying the fate and activity of epidermal LC. We attempted to answer definitively the question of the fate of Langerhans cells after transplanting low-dose PDT-treated donor skin. We considered several possible outcomes beginning with their fate in the epidermis to their fate and activity in the draining lymph nodes.

The initial outcome we considered was the possibility of LC depletion by low-dose PDT. We used the enzyme ATPase activity which has proved to be a reliable histochemical marker for LC *in situ* and in suspension (Cormane and Kalsbeek, 1963; Wolff and Winklemann, 1967; Mackenzie and Squier, 1975). Our data showed that low-dose PDT at the optimal BPD dose of 0.25 μg/mL (Table 4.3.4), did not affect the staining intensity of ATPase on the epidermal cell suspension. However, low-dose PDT at a BPD dose of 1.0 μg/mL led to a significant reduction ($\geq$ 90 %) in the staining intensity of the enzyme. This

observation suggested that the doses of BPD that we used in the low-dose PDT treatment regimen might have different mechanisms through which they prolonged the survival of the skin allografts. We had shown in the preceding chapter that low-dose PDT at a BPD dose of 1.0 and light {the dose at which ATPase activity on LC was depleted (Fig. 6.3.1)} was cytotoxic for LC when the cells were maintained in short- or long-term cultures *in vitro* (Table 5.3.3). Thus, it appears that the mechanism of the low-dose PDT-induced prolongation of skin allograft survival depended on the dose of BPD. Cytotoxicity of LC likely contributed a significant part to the effects observed at the relatively higher dose of BPD (1.0 μg/mL) while the modulation of LC surface antigens may be the major mechanism at the lower (optimal) dose of BPD (0.25 μg/mL). However, it seemed unlikely that the reduction in ATPase was as a result of LC depletion since we had earlier shown that comparable numbers of LC with similar viabilities were procured from both treated (1.0 - 0.125 μg/mL BPD + 10 J/cm^2 light at a wavelength of 690 nm) and untreated donor skins (Fig. 5.3.7). Furthermore, it has been suggested that the reduction in the number of LC following UV irradiation of mouse skin might be due to a loss of the enzymatic marker of LC and not necessarily to a physical absence of LC (Aberer *et al.*, 1981). Aberer and colleagues showed that at the UV dose of 600 J/cm^2, a dose at which sunburn cells and edema were easily detected on the skin of outbred Swiss ha/ha mice, LC cells were still present in the epidermis but had simply lost their ATPase and Ia surface markers (Aberer *et al.*, 1981).

The second outcome of LC following low-dose PDT that we considered was the possibility that the treatment regimen affected LC migration by reducing the number of

migrant cells collecting at the draining lymph nodes. An ideal approach to evaluate this possibility was to enumerate in the graft recipients' draining lymph nodes, the number of LC that might have emigrated following transplantation. However, this approach was unsuccessful. One reason for the failure might be that the numbers of LC migrating from the 1 cm^2-donor skin graft to the draining lymph nodes following transplantation was too low for detection with the flow cytometer. As a result, we used an *in vivo* model of transdermal PDT on the assumption that levels of BPD and light delivered to the skin would be roughly equivalent to that used to treat grafts and evaluated the fate of LC following transdermal PDT in response to topical application of contact allergens. We acknowledge that this model was different from our skin graft model. However, our laboratory has shown that transdermal PDT suppressed the development of cutaneous immune response in the skin contact hypersensitivity model (Simkin *et al.*, 1995) and modulated immune responses in certain disease models such as adjuvant arthritis in MRL/*lpr* mice (Ratkay *et al.*, 1994b; Chowdhary *et al.*, 1994). Based on our earlier findings *in vitro* (chapter 5), it could be implied that epidermal cells might be targets for transdermal PDT. Therefore, we contend that results which we might obtain from this *in vivo* transdermal PDT study would most likely provide relevant information regarding the probable fate and activity of epidermal LC following low-dose PDT of donor skin.

Initially, we used topical application of FITC to induce the migration of LC (Kripke *et. al.*, 1990) following which we characterized the surface antigens of the draining lymph node cells with monoclonal antibodies and flow cytometry. Our data suggested that transdermal PDT affected the immunologic profile in the local lymph nodes in response to

topical application of FITC but did not elucidate the fate of LC following transdermal PDT. Subsequently, we adopted a different approach whereby we used the topical application of another allergen, Green BODIPY®, to induce the migration of LC following which we obtained the draining lymph node cells and measured the fluorescence intensity of the BODIPY®-bearing cells by flow cytometry. Alternatively, we counted the number of viable cells obtained from the draining lymph nodes. The use of Cell Tracker™ Green BODIPY® was deemed to be a more direct approach to evaluating the migration and activity of LC following transdermal PDT and contact sensitization because unlike FITC, the Green BODIPY® contains a mildly thiol reactive chloromethyl reactive group. Once inside the cell, the chloromethyl group reacts with intracellular thiols, transforming the probe into a cell-impermeant fluorescent dye-thioether adduct (Haugland, 1992a). Interestingly, the Cell Tracker™ probes have been used extensively as a long-term living cell tracer and cell fusion monitor (Haugland, 1992a; Cumberledge and Krasnow, 1993; Burghardt *et al.*, 1992). Our data showed that epidermal LC migrated in response to the topical application of the contact allergen. However, transdermal PDT had no effect on the migration of the Green BODIPY®-bearing epidermal cells. Thus, on the basis of our findings using the *in vivo* transdermal PDT model, we can infer that low-dose PDT of donor skin might not affect the migration of donor-derived LC in our model.

The last outcome of LC following low-dose PDT that we considered was the possibility that the treatment regimen affected the antigen presenting functions of LC in a way that they remained functionally impaired in the draining lymph nodes. We adopted an indirect approach to probe this possibility by evaluating the response of the graft recipient's

lymphocytes to restimulation with donor-specific alloantigen and mitogen. It had been implied that UV-altered LC may account for the T cell anergy observed in the *in vitro* experiments using UV-irradiated LC (Simon *et al.*, 1991). We observed that T cells from graft recipients transplanted with low-dose PDT-treated donor skin maintained their cell surface profiles and responded normally to con A. Furthermore, the intensities of T cells within the lymph nodes of graft recipients were not affected by low-dose PDT. On the contrary, their response to restimulation with donor-specific alloantigen was significantly reduced. This is in agreement with our earlier observations *in vitro* which suggested that low-dose PDT of donor skin may inhibit LC antigen presenting function (Fig. 5.3.4). Just as was suggested from the MECLR data (Fig. 5.3.4), the present observation may be due to down-modulation of the expression of MHC and critical costimulatory molecules (Fig. 5.3.5).

In summary, our results suggested that the inefficient stimulation of the recipients' T cells by the "low-dose PDT-altered donor-derived LC during their initial encounter in the lymph nodes may explain their decreased proliferation upon *in vitro* restimulation with donor-specific alloantigens. Furthermore, our results while implying that the transplantation of low-dose PDT-treated donor tissues selectively down-modulates the recipient's anti-graft immune responses, suggests that low-dose PDT of donor skin is unlikely to compromise the general immune system of the graft recipient.

Chapter Seven

MECHANISMS OF LOW-DOSE PDT-INDUCED ANTI-GRAFT IMMUNE RESPONSE

III. EFFECT OF LOW-DOSE PDT ON THE INFILTRATION OF CELLS INTO GRAFTS DURING SKIN GRAFT REJECTION

7.1 Abstract.

Several types of host cells infiltrate an allograft during rejection. Most of the infiltrating cells (mainly mononuclear leukocytes) are equipped with receptors specific for the alloantigens on the graft. These cellular infiltrates are responsible for mediating the rejection process. In order to establish the effect of low-dose PDT on the development of histologic and immunohistologic changes consistent with graft rejection, we obtained graft tissues at various times (4 - 8 days post grafting). Our immunohistological and histological results showed that low-dose PDT kept the level of the cellular infiltration into the graft low compared to the control grafts. This was evident histologically in the infiltration of the inflammatory cells into the grafts and immunohistologically in the intensities of staining for CD8, MHC class II and ICAM-1 surface antigens. In skin allografts, Langerhans cells (LC) are known to initiate graft rejection in the draining lymph nodes of graft recipients by presenting their antigens to both $CD4^+$ and $CD8^+$ T cells. These alloantigen-specific T cells subsequently migrate to the graft to mediate the rejection process. On the basis of our earlier findings which suggested that low-dose PDT might down-modulate the antigen presenting functions of LC, it appeared that the lower levels of inflammatory cellular infiltrates into the graft might be a consequence of the inefficient allostimulatory functions of LC.

7.2 Introduction.

The "passenger leukocyte" hypothesis suggested that allograft rejection might be initiated by the immunostimulatory cells within the donor graft (usually the dendritic cells) (Lafferty *et al.*, 1983; Woodward *et al.*, 1982). The immunostimulatory cells migrate from the allograft to the regional lymph nodes of the graft recipient and sensitize alloreactive T lymphocytes, which ultimately cause the destruction of the graft (Lafferty *et al.*, 1983, Lechler and Batchelor, 1982). The epidermal dendritic cells are known as Langerhans cells (LC), and their density correlates with the immunogenicity of skin allografts (Mathieson *et al.*, 1975; Sena *et al.*, 1976; Chen and Silvers, 1983). In skin allografts, LC are known to initiate graft rejection in the draining lymph nodes of graft recipients by presenting their alloantigens to both $CD4^+$ and $CD8^+$ T cells (Rosenberg and Singer, 1992; Lerner-Tung and Hull, 1989; Aubock *et al.*, 1988). These alloantigen-specific T cells subsequently migrate to the graft to mediate the rejection process (Kobayashi and Fujiwara, 1992).

Following LC migration and subsequent allospecific-stimulation, several types of host cells infiltrate the allograft. Most of the infiltrating cells are mononuclear leukocytes (blast, lymphocytes, monocytes and macrophages) although the more necrotic areas of the graft also display the infiltration of granulocytes (Roberts and Hayry 1977). Approximately 60-80% of the allograft-infiltrating cells are equipped with receptors directed to the antigens of the graft (Roberts and Hayry, 1976). Skin allograft rejection, however, is effectively a T cell response involving different proportions of the $CD4^+$ and $CD8^+$ sub-populations depending on the nature of the antigen (Rosenberg and Singer, 1992).

Many studies have indicated that $CD4^+$ and $CD8^+$ T cells share overlapping functional capacities in skin allograft rejection (Widmer and Bach, 1981; Roopenian *et al.*, 1983; Rosenberg and Singer, 1992). Nevertheless, it remains possible that there may be a number of different mechanisms which may contribute to the rejection of fully-incompatible skin grafts and may contribute to differing degrees, depending upon the circumstances. For example, depletion of $CD4^+$ or $CD8^+$ T cells with monoclonal antibodies was not successful in prolonging the survival of grafts with class I plus class II incompatibilities or with *H2* plus non-*H2* incompatibilities (Wheelahan and McKenzie, 1987). But, nude mice rejected grafts expressing all types of antigens if reconstituted with $CD4^+$ or $CD8^+$ cells alone as well as with both cell types together (Rosenberg *et al.*, 1987). Similarly, $CD4^+$ or $CD8^+$ cells adoptively transferred into severe combined immunodeficiency (scid) mice mediated rejection of allografts with full mismatch (incompatibilities in all types of antigens) (Shelton *et al.*, 1992). Investigations into the cellular basis of graft rejection revealed that both CD4 and CD8 T cells can migrate into the graft and mediate graft rejection alone or in concert (Auchincloss and Sachs, 1989).

This chapter describes the results of experiments that evaluated the effect of low-dose PDT on the development of histologic and immunohistologic changes consistent with skin allograft rejection. BALB/c mice were engrafted with untreated donor skins (C57BL/6) or with donor skins pretreated with low-dose PDT as described earlier (materials and method sections 2.11 and 2.12). After sacrificing the animals, graft tissues were dissected at various times (4 - 8 days) post grafting. For these experiments we used low-dose PDT at the dose of BPD (0.25 μg/mL) and light (10 J/cm^2; $\lambda = 690 \pm 10$ nm) where optimal skin allograft

survival was observed. The pre-treated skin tissues were compared to the untreated controls samples at matched time point post transplantation.

Skin tissues were snap frozen in liquid nitrogen-chilled isopentane. The frozen tissue sections were immunostained with an alkaline-phosphatase streptavidin-biotin method as described earlier (materials and method section 2.16) applying commercially available biotinylated monoclonal antibodies directed against mouse cell surface antigens: MHC class II ($I\text{-}A^d$) and ICAM-1 antigens (LC, macrophages), CD4 (T helper) and CD8 (T cytotoxic). The immunostained slides were later evaluated "blind" using a light microscope. Scores were assigned reflecting the intensity of each stain using set scoring criteria such that 0 = absent of stain, 1 = minimal stain at some areas, 2 = mild stain, 3 = mild to moderate areas of stain, 4 = moderate areas of stain, moderate to marked areas of stain, and 6 = areas of marked staining intensity.

In addition, untreated or pretreated graft tissues were fixed in 10 % formalin and sections stained with haematoxylin and eosin following which microscopic assessment of the H/E stained skin tissues were performed "double blind" (materials and methods section 2.17). Scores were assigned to various anatomic/pathologic changes that reflected the degree of inflammation and vascular change using a set criteria in which 0 = absent of inflammatory infiltrates, 1 = minimal infiltrating cells, 2 = mild infiltration, 3 = mild to moderate infiltration, 4 = moderate infiltration, 5 = moderate to marked infiltration, and 6 = marked infiltration.

7.3 Results.

a. Effect of low-dose PDT on cellular infiltration into grafts.

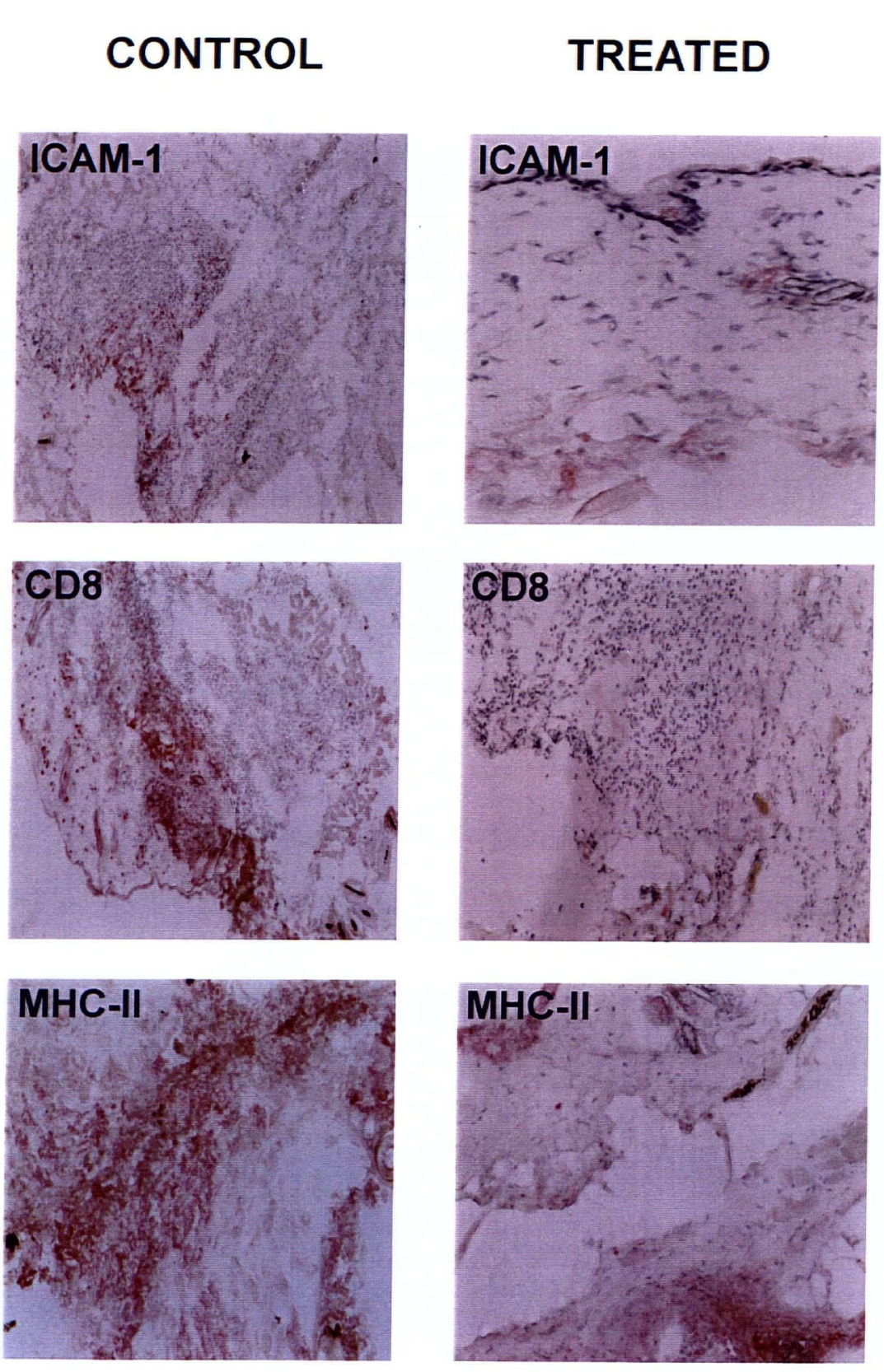
We used histological and immunohistological techniques to evaluate the effect of low-dose PDT on the level of cellular infiltration in the grafted tissue at various times (4 - 8 days) following transplantation.

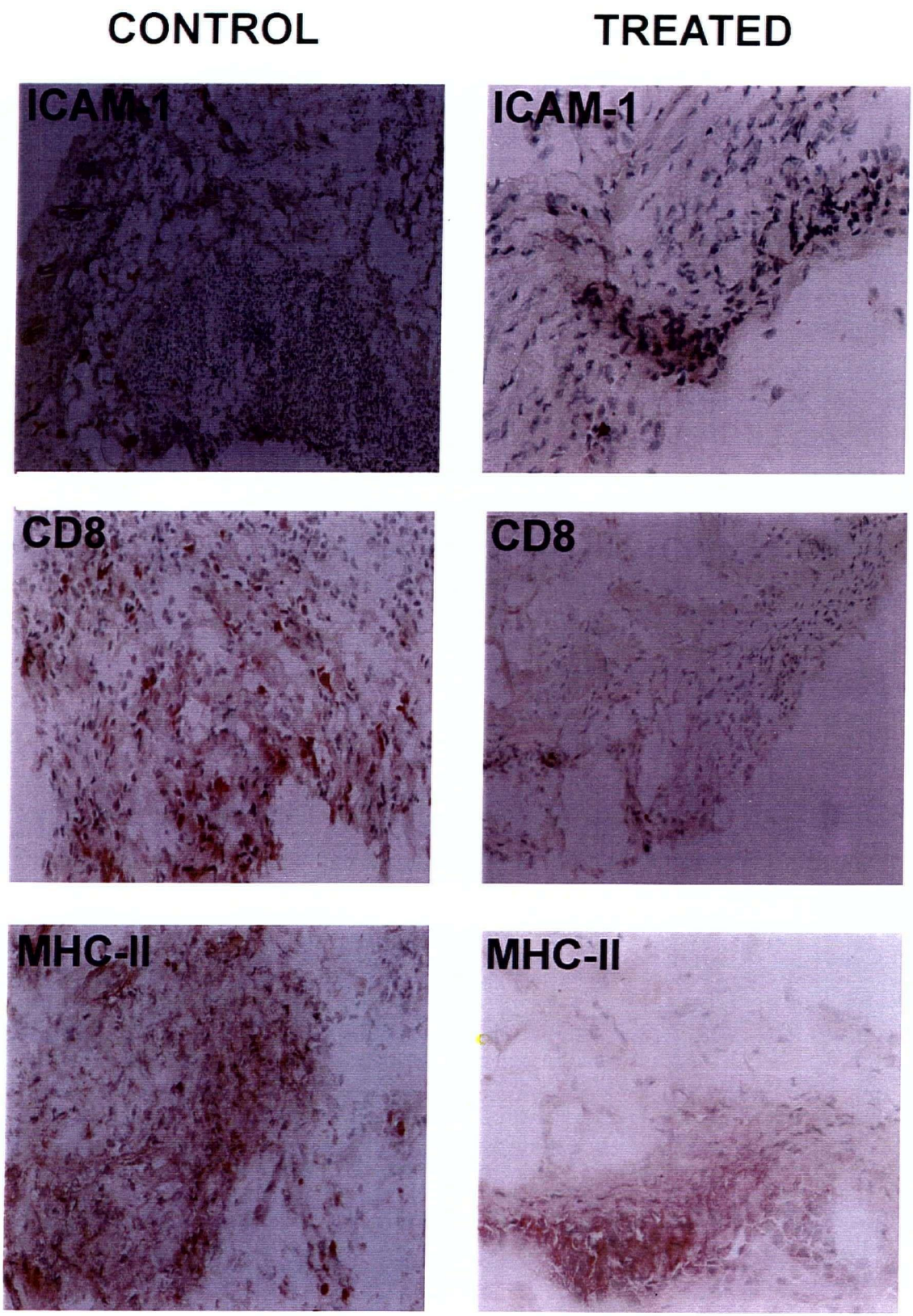
i. Immunohistology.

i.1 Control grafts. We observed three distinct stages of the rejection process. Stage I was characterized by a marked focal infiltration at the graft's dermal base (Fig. 7.3.3b). This progressed into stage II which was typified by strips or layers of cellular infiltrates at the areas of vascular anastomosis (Fig. 7.3.3c). This stage normally progressed to the third stage (stage III) when the infiltrates had peaked as diffuse dermal infiltrates (Fig. 7.3.3d). In general, by day 4 post transplantation, the untreated control grafts showed marked focal infiltration at the grafts dermal base (stage I). ICAM-1 positive cells seemed to surround the focal infiltrates at the base of the dermis (Fig. 7.3.1a). The focal infiltrates were rich in CD8 staining cells (Fig. 7.3.1b), characteristic of an ongoing graft rejection. The area had low staining for CD4 but there were significant MHC class II-positive infiltrates (Fig.7.3.1c). By days 6 - 8 the infiltrates had covered areas of vascular anastomosis in a progressively thickening layer (stage II). The infiltrates were positive for ICAM-1, MHC class II and CD8 as was observed on day 4 (Fig. 7.3.1). However, by days 7 - 8, they had climaxed as diffuse dermal infiltrates (stage III).

Characteristic of the histopathology of skin allograft rejection, the ICAM-1 and MHC class II-expressing cells are normally the mononuclear infiltrates and the dendritic cells. Similarly, the vascular endothelial cells express ICAM-1 surface adhesion molecules. The $CD4^+$ staining cells are characteristically the helper T cells while the $CD8^+$ cell infiltrates are the killer T cells.

Fig. 7.3.1 Immunohistochemical characterization of cellular infiltration into grafts obtained from graft recipient mice 4 (page 198) and 6 (page 199) days following implantation. Skin tissues were obtained from graft recipients given untreated or treated (0.25 μg/mL BPD + 10 J/cm^2 light at a wavelength of 690 $\pm$ 10 nm). Frozen sections of skin grafts from BALB/c recipients were stained with the alkaline-phosphatase avidin-biotin method (materials and methods section 2.18). In the control grafts, we observed a marked focal infiltration at the basal dermis of the graft: a) surrounded by ICAM-1 positive cells, b) rich in CD8 staining cells, characteristic of an on-going graft rejection and c), a significant MHC class II-positive infiltrates. However, in the low-dose PDT-treated grafts, we observed a smaller focal infiltrates that is low in a) ICAM-1, b) CD8 and c) MHC class II. 10 - 20 animals were used per group. Magnification: a) day 4: 100X (ICAM-1 control; CD8); 200X (MHC-II control); 400X (ICAM-1 treated; MHC-II treated). b) day 6: all sections are 400X except for ICAM-1 control which is 100X.

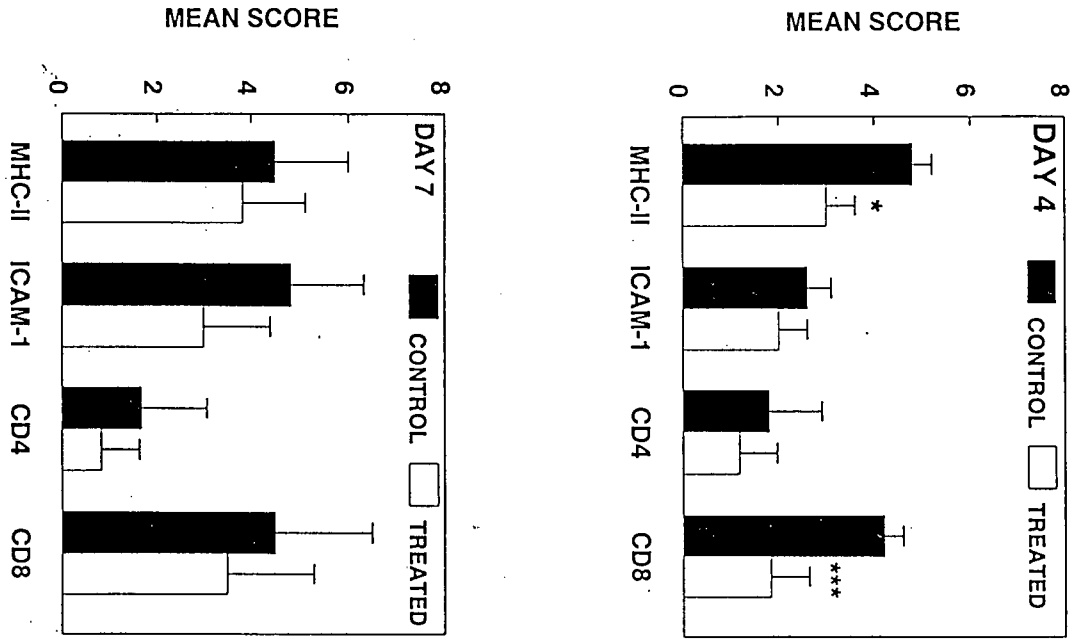

CONTROL
TREATED
ICAM-1
ICAM-1
CD8
CD8
MHC-II
MHC-II


CONTROL
TREATED
ICAM-1
ICAM-1
CD8
CD8
MHC-II
MHC-II

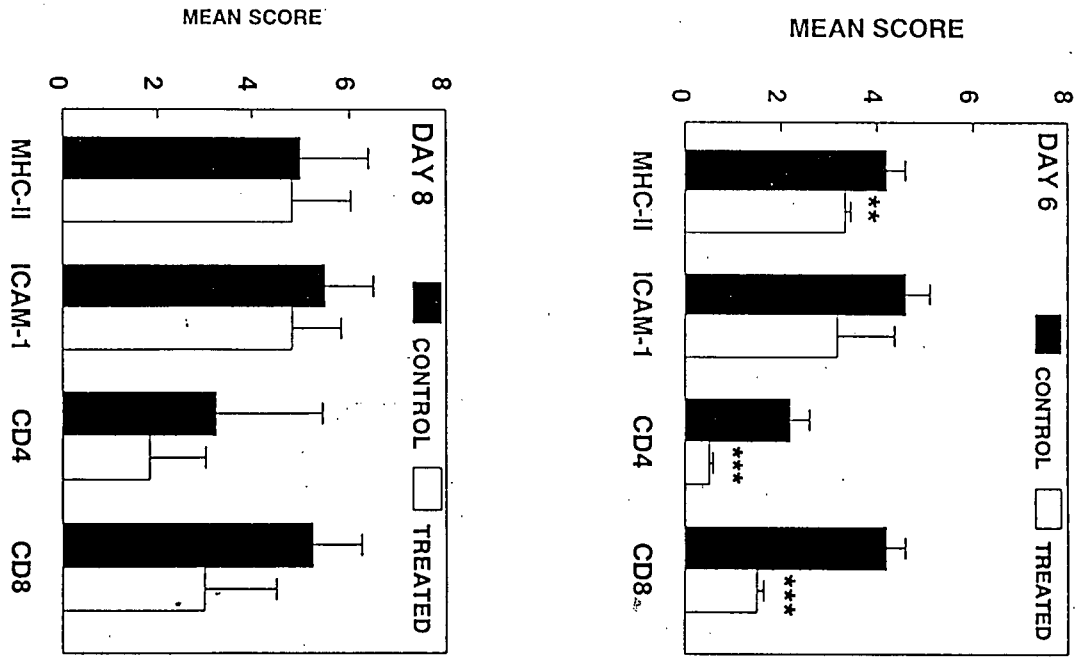
i.1 Treated grafts. In contrast to the control group, the speed of the cellular infiltration which is a prelude to graft rejection was less intense in the low-dose PDT-treated grafts. A general overview of all the skin samples assessed showed that the treated samples usually had smaller focal infiltrates staining for ICAM-1(Fig. 7.3.1a) and MHC class II (Fig. 7.3.1c) at the basal dermis of the graft than the control samples. The infiltrates were significantly lower in CD8-staining cells (Fig. 7.3.1b) and low or negative for CD4-staining cells in the treated groups in comparison to the control skin sections.

A summary of the semiquantitative scores is shown in figure 7.3.2. Overall, we found that low-dose PDT significantly reduced the number of cells staining positive for MHC class II, CD8, CD4, and ICAM-1 in the treated group in comparison with the control group. Our data suggested that the cellular infiltrates in the treated groups were significantly (days 4 - 6 by the Kolmogorov-Smirnov test) different from those of the control samples. By days 7 and 8, comparable staining intensities were observed in both groups.

Fig. 7.3.2. Semiquantitative immunohistological scores of graft tissues from day 4 - 8 skin allograft. Skin tissues were obtained from graft recipients given untreated or treated (0.25 μg/mL BPD + 10 J/cm^2 light at a wavelength of 690 $\pm$ 10 nm). Tissue sections were immunostained with alkaline-phosphatase streptavidin-biotin method applying biotinylated monoclonal antibodies (materials and methods section 2.18). The slides were observed "blind" using a light microscope. Scores were assigned reflecting the intensity of each stain using set scoring criteria such that 0 = absent of stain, 1 = minimal stain at some areas, 2 = mild stain, 3 = mild to moderate areas of stain, 4 = moderate areas of stain, moderate to marked areas of stain, and 6 = areas of marked staining intensity. Data were expressed as mean score of results obtained from untreated and treated graft tissues using 10 - 20 mice for each group. * = $p < 0.03$ by the Kolmogorov-Smirnov test. ** = $p < 0.05$ by the Kolmogorov-Smirnov test. *** = $p < 0.005$ by the Kolmogorov-Smirnov test.


MEAN SCORE
DAY 4
CONTROL
TREATED
0
2
4
6
8
*
***
MHC-II
ICAM-1
CD4
CD8

MEAN SCORE
DAY 7
CONTROL
TREATED
0
2
4
6
8
MHC-II
ICAM-1
CD4
CD8

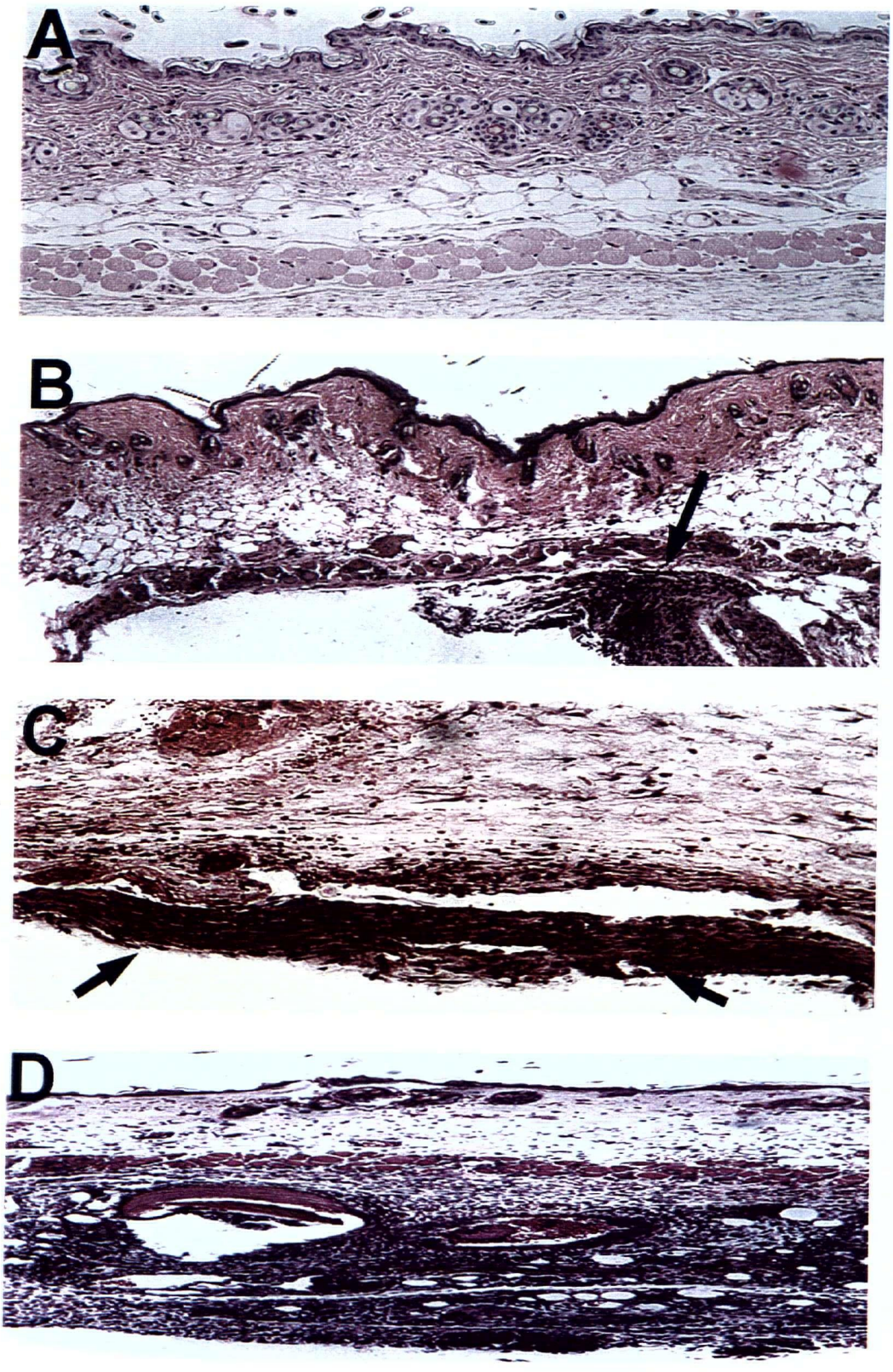

MEAN SCORE
DAY 6
CONTROL
TREATED
0
2
4
6
8
**
***
***
MHC-II
ICAM-1
CD4
CD8

MEAN SCORE
DAY 8
CONTROL
TREATED
0
2
4
6
8
MHC-II
ICAM-1
CD4
CD8

ii. Histology.

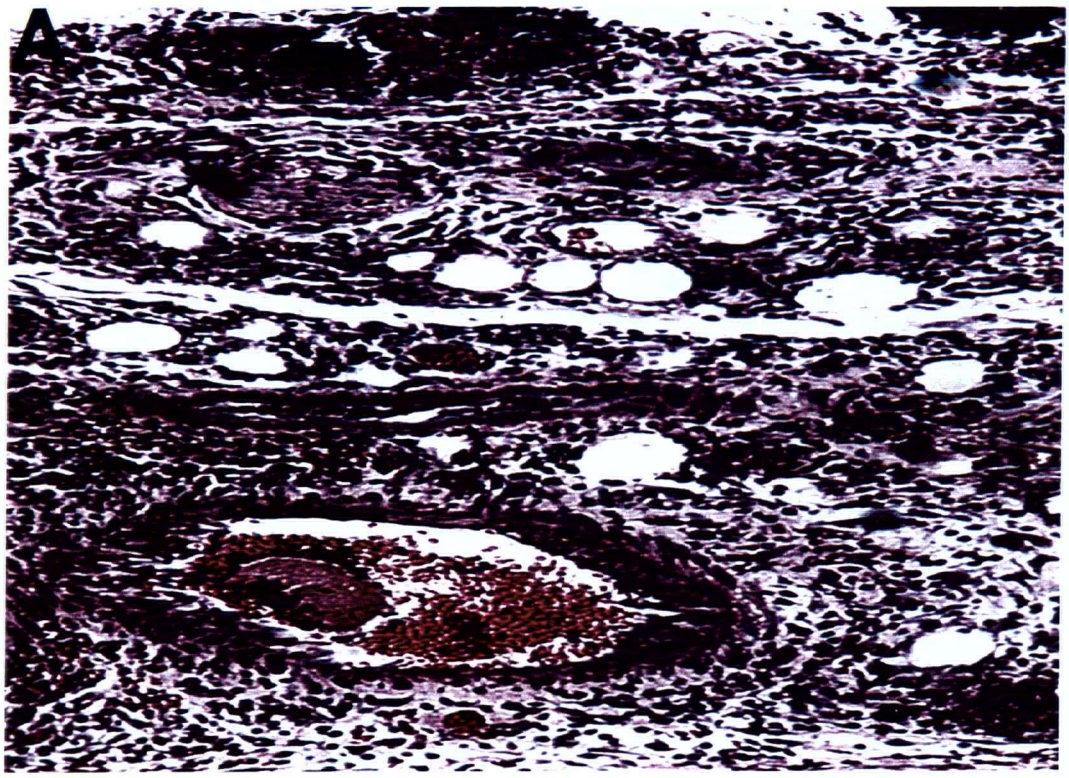
ii.1 Control grafts. There was a progressive intensification of the inflammatory response as the days post transplantation went by in control grafts as in the immunohistology experiment where we observed the three stages of the inflammatory process. The first stage was observed on day four post transplantation and was portrayed by a marked focal infiltration at the graft's basal dermis (stage I) (Fig. 7.3.3b) at which about 40 % of the graft samples had significant inflammatory changes. This was followed by the second stage (days 4 - 6) in which the infiltrates had covered areas of vascular anastomosis in a progressively thickening strip (stage II) (Fig. 7.3.3c). Ultimately, by days 7 - 8, the infiltrates had peaked as diffuse dermal infiltrates (stage III) (Fig. 7.3.3d) at which > 80 % of the grafts had significant inflammatory changes. Another important indicator of skin rejection that we evaluated was hemorrhage. We observed that as the severity of the rejection process progressed in the control animals, so too did the amount of hemorrhage present at all levels of the skin sections. Likewise, edema increased in severity to become a fairly constant feature. Furthermore, there were continuous increases in the epidermal reaction and/or degenerative changes. Similarly, the cutaneous musculature showed degeneration of myofibres with reaction of the sarcolemmal nuclei which was typical of the rejection process in skin allografts.

Fig. 7.3.3. Progression of the inflammatory response in skin allografts at 4 - 8 days post transplantation. Skin tissues were obtained from graft recipients given untreated or treated (0.25 μg/mL BPD + 10 J/cm^2 light at a wavelength of 690 $\pm$ 10 nm). Untreated or pretreated graft tissues were fixed in 10 % formalin and sections stained with haematoxylin and eosin following which microscopic assessment of the H/E stained skin tissues were performed "double blind" (materials and methods section 2.17). a) Normal donor graft tissue (magnification: 200X). b) marked focal infiltration at the graft's basal dermis (arrow) (stage I) (magnification: 100X). c) infiltrates covering areas of vascular anastomosis in a progressively thickening strip (arrows) (stage II) (magnification: 100X). d) diffuse dermal infiltrates (stage III) (magnification: 100X).


A
B
C
D

ii.1 Treated grafts. We observed that low-dose PDT induced a delay in the onset and a decrease in intensity of the inflammatory cell infiltrate (7.3.4). In general, the treated samples seemed to be one inflammatory stage behind the untreated control samples at the duration of this experiments. Semiquantitative comparisons of the treated samples with the time matched untreated control samples showed that between days 4 - 6 the number of inflammatory cells in the treated graft tissue was significantly ($p < 0.01$ by the Kolmogorov-Smirnov test) less than that seen in the control samples (Fig. 7.3.5). Furthermore, we found that there was a significant decrease in the extent of hemorrhage in the grafts of the treated mice, especially at four to six days post transplantation. Edema was slightly less or similar at all days in both the treated and control groups. Furthermore, the onset of the cutaneous muscle degeneration was delayed; the degree of degeneration was also less in the treated than in the control samples. Mineralization of the muscle fibres was occasionally present in some of the animals that received low-dose PDT-treated donor skins.

Fig. 7.3.4. Comparison of the inflammatory cellular infiltrates on day 6 untreated (a) and treated (b) skin grafts. Skin tissues were obtained from graft recipients given untreated or treated (0.25 μg/mL BPD + 10 J/cm^2 light at a wavelength of 690 $\pm$ 10 nm). Untreated or pretreated graft tissues were fixed in 10 % formalin and sections stained with haematoxylin and eosin following which microscopic assessment of the H/E stained skin tissues were performed "double blind" (materials and methods section 2.17). Day 4 micrographs were similar to day 6 and was therefore not shown. Magnification: 100X.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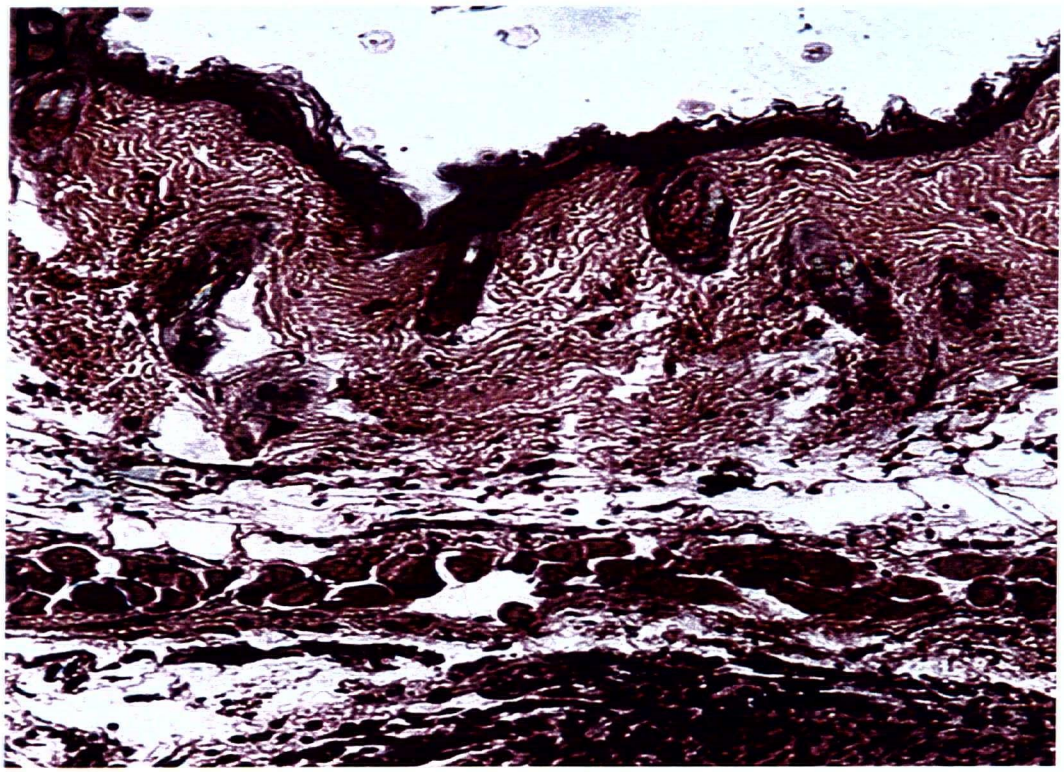
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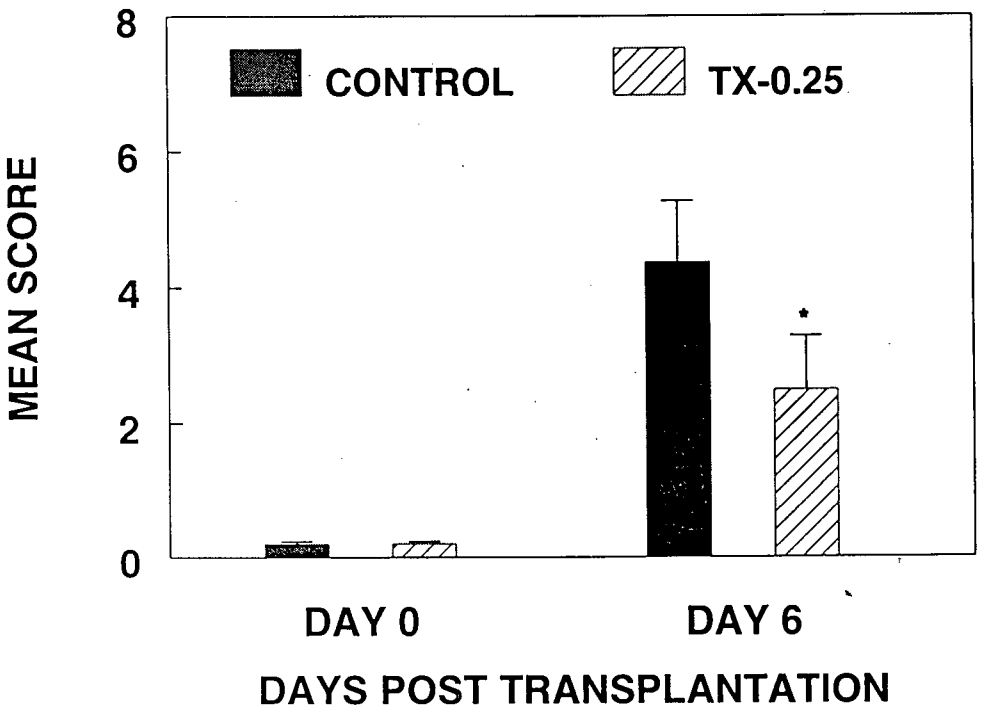

Fig. 7.3.5. Semiquantitative histological scores of graft tissues from day 6 skin allograft. Skin tissues were obtained from graft recipients given untreated or treated (0.25 μg/mL BPD + 10 J/cm^2 light at a wavelength of 690 $\pm$ 10 nm). Untreated or pretreated graft tissues were fixed in 10 % formalin and sections stained with haematoxylin and eosin following which microscopic assessment of the H/E stained skin tissues were performed "double blind" (materials and methods section 2.17). Scores were assigned to various anatomic/pathologic changes that reflected the degree of inflammation and vascular change using a set criteria in which 0 = absent of inflammatory infiltrates, 1 = minimal infiltrating cells, 2 = mild infiltration, 3 = mild to moderate infiltration, 4 = moderate infiltration, 5 = moderate to marked infiltration, and 6 = marked infiltration. Data were expressed as mean score of results obtained from untreated or treated graft tissues using 10 - 20 mice for each group. * = $p < 0.02$ by the Kolmogorov-Smirnov test.

7.4 Discussion.

LC are known to initiate skin allograft rejection by migrating to the draining lymph nodes of graft recipients and presenting their MHC antigens to both $CD4^+$ and $CD8^+$ T cells which are specific for these antigens (Rosenberg and Singer, 1992; Lerner-Tung and Hull, 1989; Aubock *et al.*, 1988). These alloantigen-specific T cells subsequently migrate to the graft to mediate the rejection process (Kobayashi and Fujiwara, 1992).

The principal histological measures of graft rejection over the time course of these experiments were hemorrhage and cellular infiltrates into the graft area. We found that hemorrhage was less severe in the treated group on days four to six than in the untreated controls. Likewise, inflammatory changes generally appeared less severe in the dermis and the subcutaneous tissues of the treated group than in controls, particularly between day four and day six post transplantation. There was a lesser degree of degeneration of the cutaneous muscle layer in the treatment groups than in the control group. Taken together, these observations suggested that there was an effect of low-dose PDT in the reduction of the severity of inflammatory response at least in the early stages of graft rejection. Similarly, our immunohistological data showed that low-dose PDT kept the level of the cellular infiltration into the graft low compared to the control grafts. This was evident in the progression of infiltration and the intensities of staining for CD8, MHC class II and ICAM-1 surface antigens.

In conclusion, data from both studies (histology and immunohistology) suggested that low-dose PDT kept the levels of cellular infiltrates into the graft low. This in turn resulted

in the lesser severity of the inflammatory response observed in the treated samples in comparison to the controls. Inflammatory responses observed during the skin allograft rejection are known to be a consequence of the LC-induced proliferation of the effector T cells in the recipients' draining lymph nodes and subsequent effector functions of these alloreactive T cells. On the basis of our earlier findings which suggested that low-dose PDT might down-modulate the antigen presenting functions of LC, it appeared that our recent findings could be the consequence of inefficient allostimulatory functions of PDT-treated LC. Our data seemed, therefore, to support our central concept that suggested that low-dose PDT treatment of donor skin prior to transplantation may have significant immunomodulatory effects on the epidermal LC, and that these effects might result in a series of down-modulatory events, including decreased MHC and costimulatory molecule expression resulting in impaired allostimulatory capacity, which ultimately culminated in the prolongation of skin allograft survival.

CHAPTER EIGHT

GENERAL DISCUSSION AND OVERVIEW

Therapeutic interventions in the rejection of mismatched cells, tissue or organ transplants may involve two main approaches - the immunosuppression of the recipient after transplantation with a variety of immunosuppressive agents including cyclosporin A, FK506, corticosteroids, antilymphocyte antibody, and ultraviolet radiation; and the diminution of the donor tissue antigenicity before grafting. The former has been studied extensively and requires severe suppression of the recipients' immune system to maintain graft survival. This leaves transplant recipients at high risk of developing unusual neoplasms such as B-cell lymphomas and potentially deadly infections (Trucco and Stassi, 1996), the leading cause of death in human transplant patients (Jarowenko *et al.*, 1986). The alternative strategy is currently under investigation in this study. The catalyst was the clarification of the requirements for donor strain dendritic cells (DC), the "passenger leukocytes", in allograft rejection (Lechler and Bachelor, 1982).

Since this elegant work by Lechler and Bachelor, several attempts have been made to remove donor-strain DC prior to transplantation. Examples include organ culture (Lafferty *et al.*, 1975; Lafferty *et al.*, 1976; Bowen *et al.*, 1979), pretreatment of donor tissue with monoclonal antibodies (Faustman *et al.*, 1984; Iwai *et al.*, 1989; Lafferty *et al.*, 1983; 1986), or a combination of UVA (320 - 400 nm) and 8 methoxy-psoralen (PUVA) (Gruner *et al.*, 1984, Morison *et al.*, 1981) and a combination of Photofrin® and light (Gruner *et al.*, 1985). Such attempts have focused mainly on the depletion of the dendritic cells within the donor tissue and have achieved moderate prolongation of allograft survival in the skin, pancreatic islets, and thyroid allografts. Recent evidence has suggested that the control of rejection and subsequent graft acceptance and tolerance might depend on the

establishment of mixed, long-term microchimerism in the recipient and graft tissues (Starzl *et al.*, 1992; Rao *et al.*, 1994). Bone-marrow derived "passenger" leukocytes within the interstitial tissues of organs migrate into the recipient after transplantation (Talmor *et al.*, 1995; Thomson *et al.*, 1995; Rao *et al.*, 1994; Starzl *et al.*, 1993b). Similarly, there is a reciprocal migration of circulating recipient leukocytes which repopulate the interstitium of whole organ allografts (Rao *et al.*, 1994; Starzl *et al.*, 1992). This bidirectional exchange and interaction of bone marrow-derived cells after organ transplantation is considered, though with lots of controversy, a seminal event in the acceptance of allografts and in the induction of donor-specific tolerance (Murase *et al.*, 1995). It is interesting to note that attempts which had focused mainly on the depletion of the dendritic cells within the donor tissue have achieved moderate prolongation of allograft survival in the skin, pancreatic islets, and thyroid allografts (Lafferty *et al.*, 1975; Lafferty *et al.*, 1976; Bowen *et al.*, 1979; Faustman *et al.*, 1984; Iwai *et al.*, 1989; Lafferty *et al.*, 1983; 1986; Gruner *et al.*, 1984; Gruner *et al.*, 1985; Morison *et al.*, 1981). None of these attempts produced long-term donor-specific tolerance in the recipient animal. However, mixed chimerism offered long-term unresponsiveness (tolerance) to allografts across major and minor histocompatibility barriers (Orloff *et al.*, 1995; Colson *et al.*, 1995; Li *et al.*, 1995). As a result, one could infer that the total depletion of donor-derived DC may be ineffectual and unlikely the best approach for achieving the much desired donor-specific tolerance in transplantation. Thus, it seemed logical to speculate that an anti-rejection strategy focused on the attenuation or modulation, rather than depletion of these MHC-bearing "passenger leukocytes", may be a more selective and less toxic approach for the prevention of allograft

rejection. In this thesis we evaluated whether transdermal PDT could modulate epidermal Langerhans cells and as a result prolong the survival of skin allografts.

We found that there was a significant increase in the survival of the skin allografts in animals given pretreated donor skin tissues in comparison to the untreated controls (Table 4.3.1). Higher doses of BPD did not necessarily translate to the optimal prolongation of the skin allograft survival. Rather, the most beneficial effects of the treatment were observed at lower doses of BPD (0.25 - 0.5 μg/mL) as opposed to 1.0 μg/mL and light (10 J/cm^2, 690 $\pm$ 10 nm wavelength). This was contrary to the findings of Gruner *et al.* (1985) who pretreated donor mouse tail skin grafts with Photofrin® and light prior to transplantation but found higher doses of Photofrin® and light to be most beneficial. Our strategy was, therefore, termed low-dose PDT to reflect the fact that we used low doses of BPD and visible light, doses where, even at the highest level, the histological features of pretreated donor skins revealed no obvious tissue damage (Fig. 5.3.7).

The next series of experiments attempted to evaluate the mechanisms by which low-dose PDT affected skin allograft immune responses or the specific cell targets for this kind of treatment. In the skin graft, the main candidates for affecting cutaneous immune response are likely the epidermal Langerhans cells (LC) and keratinocytes (KC). However, during the initial phase of alloantigen sensitization, it is thought that the primary initiator is the donor-derived epidermal LC (Rosenberg and Singer, 1992). The LC migrate from the graft and relocate, via the lymphatics, in the draining lymph nodes (Larsen *et al.*, 1990b) where they present their antigens to both $CD4^+$ and $CD8^+$ T cells (Streilein and Bergstresser, 1984). The subsequent activation and proliferation of these alloantigen specific T cells and

their migration to the graft mediates the rejection process (Kobayashi and Fujiwara, 1992; Auchincloss and Sachs, 1989). Thus, it seemed reasonable to determine whether LC were modified by low-dose PDT such that they were less efficient antigen presenting or accessory cells.

On the basis that LC are responsible for the induction of T cell responses in the mixed epidermal cell lymphocyte reaction (MECLR) (Stingl *et al.*, 1978a & b; Sontheimer, 1985), we compared the influence of low-dose PDT on the capacity of epidermal LC to induce T cell responses in the MECLR. We observed that LC enriched from donor skins treated with low-dose PDT were inefficient allostimulators, as suggested by their reduced capacity to induce allogeneic T cell proliferation in MECLR (Fig. 5.3.4). Similar results were obtained earlier by Gruner *et al.* (1985) who demonstrated that treatment of human peripheral blood mononuclear cells with Photofrin® and light rendered them unable to stimulate allogeneic cells in mixed leukocyte reaction (MLR). Unlike Gruner's work, however, optimal effects were obtained at a lower dose of BPD (0.25 μg/mL) and light (10 J/cm^2, $\lambda = 690 \pm 10$ nm), the same dose where we obtained optimal skin allograft survival *in vivo* (Table 4.3.3), thus suggesting a possible correlation between our *in vitro* and *in vivo* data.

Furthermore, we evaluated the possible explanation for the decreased allostimulatory potential of the treated donor skin tissue-derived epidermal LC. Using flow cytometry, we found that the levels of expression of the MHC (class I and II) and the costimulatory (B7-1 and B7-2) molecules was substantially reduced on LC isolated from treated skin in comparison to the control preparations (Fig. 5.3.5). On the contrary, the levels of the

leukocyte common antigen (CD45), the adhesion molecule (ICAM-1) and the endocytic receptor (DEC-205) recognised by the monoclonal antibody, NLDC-145 were unchanged. Similar results have been reported elsewhere (Tang and Udey, 1991) using ultraviolet B (UVB) which showed that low doses of UVB inhibited the up-regulation of some (ICAM-1) but not all (class II and CD45) LC surface antigen expression. Thus, it was unlikely that the decreased levels of LC surface molecules observed after low-dose PDT resulted from a random event representing a total collapse of the cell membrane or cytoskeletal structure of LC. Furthermore, based on the important roles played by the MHC and costimulatory molecules (B7-1 and B7-2) in signalling for T cell activation (Mueller *et al.*, 1989; Linsley and Ledbetter, 1993; Jenkins and Schwartz, 1987), it is likely that the impaired allostimulatory capacity of the treated donor skin-derived LC results from the decreased LC surface antigen expression of MHC and B7 molecules.

Other factors such as cellular toxicity by apoptosis might be another mechanism through which low-dose PDT was likely to elicit its down-modulatory properties. Low-dose PDT, at a BPD dose of 1.0 (but not 0.25 μg/mL) and light, depleted the ATPase activity on LC (Fig. 6.3.1) and was ultimately cytotoxic for LC when the cells were maintained in short- or long-term cultures *in vitro* (Table 5.3.3). Our finding was similar to those reported earlier in studies involving ultraviolet (UV) radiations of LC *in vitro* (Tang and Udey, 1992) which suggested that the levels of UV radiation that inhibited LC accessory cell function and selectively modulated ICAM-1 expression in short-term cultures were ultimately cytotoxic for LC. We did not pursue the mechanisms by which low-dose PDT at the BPD dose of 1.0 μg/mL and light induced cytotoxicity (apoptosis or necrosis) in this thesis. However, it is

likely that the cytotoxic event that we observed could be as a result of PDT-induced apoptosis. Both rapid and delayed apoptosis after PDT has been demonstrated both *in vitro* (Agarwal *et al.*, 1991; He *et al.*, 1994; Luo *et al.*, 1996) and *in vivo* (Zaidi *et al.*, 1993). It is possible that the stripping of the ecto-adenosine triphosphatase (ATPase) enzyme on LC could signal the cells to commit suicide, since it was suggested that the enzyme ATPase on LC provides protection against extracelluar adenosine triphosphate (ATP)-induced permeabilization (Girolomoni *et al.*, 1993) and the subsequent induction of apoptosis upon exposure to extracelluar ATP (Zanovello *et al.*, 1990; Zheng *et al.*, 1991). Taken together, it appeared that the mechanism of the low-dose PDT-induced prolongation of skin allograft survival depends on the dose of BPD. Cytotoxicity of LC possibly contributes significantly to the effects observed at the relatively higher dose of BPD (1.0 μg/mL), while the modulation of LC surface antigens is likely to be the major mechanism at the lower (optimal) dose of BPD (0.25 μg/mL).

LC migrate out of the skin following transplantation and collect in the local draining lymph nodes (Larsen *et al.*, 1990b), where they interact with T cells to initiate graft rejection (Streilein and Bergstresser, 1984). Our attempt to evaluate the migration of Langerhans cells after transplanting low-dose PDT-treated donor skin was inconclusive. This approach was difficult because the number of LC migrating from the 1 cm^2-donor skin graft to the draining lymph nodes following transplantation was too low for detection with the flow cytometer. Thus, we used an *in vivo* model of transdermal PDT on the assumption that the lower levels of BPD and light delivered to the skin would be roughly equivalent to that used to treat grafts (Richter *et al.* 1990a; 1991). We observed that transdermal PDT had no

effect on the migration of the epidermal Langerhans cells (Fig. 6.3.3) in response to topical application of allergen. Even though this model was different from our skin graft model, we anticipated similar results with low-dose PDT-treated donor skins. However, for a conclusive definition of the quantitative fate of LC in our model, it would be important to evaluate the migration of donor skin derived LC following transplantation. An ideal approach still remains the enumeration of the donor-derived LC in the draining lymph nodes of graft recipients. This might be achieved by the use of sensitive assay systems such as cell sorting by flow cytometer (Kripke *et al.*, 1990) or the reverse-transcriptase polymerase chain reaction (RT-PCR) technique (Kawasaki, 1991).

We used the measurement of the immunocompetence of lymphocytes from the draining lymph nodes and spleens of graft recipients to evaluate the activity of donor-derived LC post transplantation. We found that low-dose PDT significantly impaired the response of graft recipient-derived T cells to restimulation by normal donor-derived LC; the optimal dose of BPD (0.25 μg/mL) and light being most effective (Fig. 6.3.2) Similarly, low-dose PDT decreased the anti-graft inflammatory response, as suggested by the reduced levels of cellular infiltrates in the graft in comparison to the controls (chapter 7). Both down-modulatory effects observed at the level of the recipients lymphocytes could be the result of the low-dose PDT-induced down-modulation of the LC surface molecule expression of MHC and costimulatory molecules (Fig. 5.3.5). It has been implied that UV-altered LC may account for the T cell anergy observed in *in vitro* experiments using UV-irradiated LC (Simon *et al.*, 1991). Furthermore, it was shown that T cell receptor (TCR) occupancy in the absence of costimulation resulted in suboptimal activation of T cells and to

unresponsiveness (anergy) upon restimulation with untreated antigen presenting cell (APC) plus antigen (Jenkins and Schwartz, 1987). Thus, the inefficient stimulation of the recipients' T cells by the low-dose PDT-altered donor-derived LC during their initial encounter in the lymph nodes may account for their decreased proliferation upon *in vitro* restimulation and the reduced cellular infiltration into the graft. Unlike other immunosuppressive agents, however, low-dose PDT was unlikely to compromise the general immune system of the graft recipients. This is consistent with the observation that low-dose PDT affected neither the response of the recipients' T cells to mitogen (Fig. 6.3.5) nor their surface antigen profiles (Fig. 6.3.6) following the transplantation of pretreated donor skin tissues.

It is notable that the prolonged allograft may not necessarily be the result of direct effect on the LC allostimulatory capacity *per se*, but rather the generation of a response that does not destroy the graft due to the pattern of cytokine production (Dallman, 1992). It is possible that low-dose PDT induced a switch from a T helper 1-like cytokine profile to a T helper 2 cytokine pattern. The variable cytokine-producing potential of activated T cells (both $CD4^+$ and $CD8^+$), as demonstrated by T helper 1 (type 1 or Th1) versus T helper 2 (type 2 or Th2), heavily influences the quality of the immune responses (inflammatory versus humoral) (Mosmann *et al.*, 1991; Salgame *et al.*, 1991; Romagnani *et al.*, 1993; Subash *et al.*, 1995). Cytokines produced by each subset of T cells can regulate the function and activity of the other set (Fiorentino *et al.*, 1989; Tripp *et al.*, 1993; Manetti *et al.*, 1993; Hsieh *et al.*, 1993; McKnight *et al.*, 1994; Hsieh *et al.*, 1992). Interestingly, the nature of allograft immunity is greatly influenced by cytokines present during the response

(Dallman, 1992). For example, the rejection of islet allografts by $CD8^+$ T cells has been suggested to be a cytokine-dependent process *in vivo* (Hodgkin *et al.*, 1985), probably due in part to production of interferon-gamma (IFN-γ) by the responsive T cell (Hao *et al.*, 1990). This is also true for skin allograft rejection across a MHC class II barrier (Rosenberg *et al.*, 1990). It is intriguing that the antigen presenting cell may play an active role in dictating the cytokine profile of activated T cells (Coffman *et al.*, 1991; Fiorentino *et al.*, 1991; Fox, 1992). In this regard, it has been shown that IL-10 inhibited production of IFN-γ by Th1 clones, not by direct effect on the responsive T cell, but rather indirectly by affecting the function of the antigen presenting cells in the reaction (Fiorentino *et al.*, 1991). Ozawa and colleagues showed that IFN-γ and IL-10 inhibited antigen presentation by LC for Th1 cells by suppressing their B7-1 expression (Ozawa *et al.*, 1996). In general, the prevention of Th1 response and the expansion of Th2 immune response are critical in the induction of tolerance in skin allografts (Chen *et al.*, 1996). Therefore, it is possible that low-dose PDT altered LC antigen presenting function so that a tolerance-inducing signal is delivered to Th1 cells, whereas an activation signal is delivered to Th2 cells. Although normal LC present antigen to both Th1 and Th2 cells, low-dose PDT-treated LC may be unable to present antigen to Th1 cells but might retain their capacity to present antigen to Th2 cells (Simon *et al.*, 1990). In that case, the low-dose PDT-induced prolonged skin allograft acceptance could be associated with a form of immune regulation whereby the nature of cytokine production in the response was altered (Dallman, 1992; Takeuchi *et al.*, 1992). However, the extent to which low-dose PDT influences the cytokine pattern of the effectors cells in skin allograft remains to be determined. The pattern and levels of cytokines

secreted by LC and activated T cells can be quantified *in vitro* (MECLR) by the enzyme-linked immunosorbent assay (ELISA). Ultimately, *in vivo* (skin allograft) and *in vitro* (MECLR) studies using culture supernatant from mixed cultures of LC and allogeneic T cells would be required to test the extent to which low-dose PDT influences the cytokine pattern of effector T cells during skin allograft rejection.

In this thesis we have focused on the contributions of LC in the initiation of skin allograft rejection. However, it may be possible that other cells (keratinocytes (KC), $\gamma\delta$-T cells, melanocytes, and the recipient's antigen presenting cells) or factors (cytokines, and apoptosis) which we did not consider might have played a significant role in the modulation of the anti-graft immune response by low-dose PDT. Such factors or cells require delineation before the mechanisms through which low-dose PDT induced the prolongation of skin allograft survival can be fully comprehended. A few of these cells or factors are briefly discussed below. First are the epidermal keratinocytes. The KC-derived pro-inflammatory and immunoregulatory cytokines can have both positive (Grabbe *et al.*, 1991; Grabbe *et al.*, 1992; Beissert *et al.*, 1995) and negative (Cumberbatch and Kimber, 1992; Cumberbatch *et al.*, 1994) effects on cutaneous immune responses. It is possible that low-dose PDT, like a number of other insults including injury, microbial attack or ultraviolet irradiation, induces the release of KC-derived cytokines (Matsue *et al.*, 1989). Such low-dose PDT-induced KC-derived cytokines, for example the immunosuppressive IL-10 and prostaglandins (PGE_2), might be working in concert *in vivo* to suppress Th1 function and augment Th2-like reactions. In this regard, it is interesting to note that IL-10 has been shown to block the synthesis of many proinflammatory cytokines (Moore *et al.*, 1993;

Fiorentino *et al.*, 1991) while PGE_2 was shown to favour the activation of Th2 cells (Araneo *et al.*, 1989; Fiorentino *et al.*, 1989; Betz and Fox, 1991). Second, other cells within the skin associated lymphoid tissues such as melanocytes and dendritic epidermal gamma delta ($\gamma\delta$)-T cells (DETC) might also play a significant role in the skin allograft immune responses. Melanocytes can synthesize and secrete cytokines, and thereby play a role in the skin immune system (Swope *et al.*, 1994). The $\gamma\delta$-T cells are thought to recognize antigens (heat shock proteins) expressed on stressed epidermal cells (Tigelaar and Lewis, 1995). This reasoning led to the hypothesis that DETC function in cutaneous immune surveillance and/or immunoregulation by recognizing of a common self-antigen expressed by transformed, damaged, or altered cells in the epidermal microenvironment (Asarnow *et al.*, 1988; Janeway *et al.*, 1988). Third, another potential contributor to the altered cutaneous immune responses are the recipient-derived inflammatory macrophages. It is possible that following low-dose PDT exposure of the skin and transplantation, infiltration of inflammatory macrophages appear in the epidermis (Cooper *et al.*, 1993) and play a critical role in the down-modulation of the cutaneous immune response by *in vivo* low-dose PDT-exposed epidermal cells (Hammerberg *et al.*, 1994), perhaps via IL-10 production (Kang *et al.*, 1994; Rivas and Ullrich, 1992; Enk *et al.*, 1993). Thus, before a full understanding of the mechanism of the down-modulatory activities of low-dose PDT in the cutaneous immune system can be achieved, it will be important to characterize various cells (by flow cytometry) or factors/cytokines involved in skin allograft immune responses. The low-dose PDT-inducible cytokines could be quantified by assessing the cytokine protein production in culture supernatant by enzyme-linked immunosorbent assay (ELISA) or the increase in

intracellular transcription levels of the cytokine-specific messenger ribonucleic acid (mRNA) by RT-PCR (O'Connel *et al.*, 1993). The involvement of low-dose PDT-inducible cytokines in down-modulation might be delineated by evaluating the effects of cytokines on the alloreactivity of LC *in vivo* (skin allograft) and *in vitro* (the MECLR) employing both the cytokines and the neutralizing anti-cytokine antibody treatments. Ultimately, *in vivo* studies involving *in situ* techniques detecting low-dose PDT-inducible cytokine expression in mouse skin would be required to prove the involvement of PDT-induced cytokines in the prolongation of skin allograft survival.

The molecular mechanisms by which low-dose PDT down-modulated the LC surface antigens is not known and was not the subject of this thesis. In order to understand the mechanisms of low-dose PDT-induced down-modulation, however, the molecular targets and intracellular pathways affected by the treatment regimen would have to be defined. This will be the subject of future research in our laboratory. Nevertheless, a number of hypotheses could be advanced which might expedite the characterization of these molecular targets and pathways necessary for understanding the mechanisms by which low-dose PDT down-regulated LC surface antigens.

First, at the molecular level, PDT with BPD has been shown to stimulate the stress-activated c-Jun protein (SAP) kinase and p38 HOG1 mitogen-activate protein (MAP) kinases in murine keratinocytes (Tao *et al.*, 1996) and to induce stress protein expressions in both tumor cells and tissues (Curry and Levy, 1993). Similarly, PDT with Photofrin® was shown to enhance the translation of stress genes (heat shock proteins, glucose regulated proteins and heme oxygenase) (Gomer *et al.*, 1988; 1989; 1991) as well as early response genes such

as c-fos, c-jun, c-myc and erg-1 that encode for proteins that regulate gene expression at the level of transcription (Luna *et al.*, 1994). It is possible that low-dose PDT could induce early response genes with negative regulatory functions on the surface antigen expression on LC. Alternatively, these early response genes might lead to the upregulation of such cytokines as the KC-derived interleukin (IL)-10 and PGE_2 (Henderson and Donovan, 1989) whose immunosuppressive properties on antigen presenting cells have been well established (Enk *et al.*, 1993; Ullrich, 1994; Strassmann *et al.*, 1994). So far, no such early response gene with negative regulatory functions or genes that can induce the formation of immunosuppressive cytokines has been associated with PDT. Currently in our laboratory, there is an active search for signals, transcription factors and response genes with negative regulatory functions which may be associated with low-dose PDT-induced down-regulation of surface antigens. Delineation of these possibilities may come from characterization of signals in LC derived from mice with germline deletions of the MHC genes (Grusby and Glimcher, 1995; Zijlstra, *et al.*, 1990; Koller, *et al.*, 1990) , or in the B7 gene (Freeman, *et al.*, 1993), or in both (Sun *et al.* 1996) following low-dose PDT treatment.

An alternative mechanism for low-dose PDT-induced down-modulation of surface molecules might be through direct extracelluar cleavage of the molecules from the cell membrane (Krutman *et al.*, 1989). However, this appears to be unlikely following low-dose PDT because studies by others in our laboratory (Hunt *et al.*, 1996, unpublished data) have shown that the secretory form of ICAM-1 was not detected in cultures of splenic dendritic cells (DC) whose ICAM-1 expression was down-modulated by the *in vitro* exposure to low doses of BPD and light. Furthermore, direct extracelluar cleavage suggests that the reduced

surface antigens (down-modulatory event) would be transient. However, the reduction in the levels of LC surface antigens of MHC and B7 molecules even after 24 h in culture (Table 5.3.2) were similar to those obtained when analysis was performed within the first 6 h post treatment and isolation. Similarly, using splenic DC that were treated *in vitro* with low doses of BPD and light, the levels MHC molecules remained down-modulated even after 48 h in culture (Hunt *et al.*,1996, unpublished data).

Further, it is possible that low-dose PDT does not cause a loss of MHC and B7 molecules from the cell surface but is capable of inducing a structural alteration of the surface molecules (Krutman *et al.*, 1989) which in turn prevents recognition of selected epitopes and the ligand binding sites. It is conceivable that low-dose PDT induced folding of the receptor molecule which altered the accessibility of epitopes on MHC or B7 molecules for the monoclonal antibodies tested. However, this might be unlikely since this treatment did not prevent the expression of a variety of other surface receptors, such as the leukocyte common antigen (CD45), the adhesion molecule (ICAM-1) and the endocytic receptor (DEC-205) recognised by the monoclonal antibody, NLDC-145 (Fig. 5.3.5). Nonetheless, this could be evaluated by comparing the molecular weights of cell surface markers purified from low-dose PDT-treated cells from those obtained from control cells following immunoprecipitation on SDS-polyacrylamide gels. If the decreased expression of the surface antigens that we observed was the result of low-dose PDT-induced folding of the surface molecules as opposed to intracellular or molecular modulation, then the molecular weight of the receptors purified from treated cells would be indistinguishable from those obtained from the control cells.

Another mechanism by which low-dose PDT could selectively down-regulate LC surface antigens of MHC and B7 molecules might be by attenuating the intracellular vesicles (endosome and lysosome) through which antigenic peptides and antigens must pass enroute to the cell surface (Cresswell *et al.*, 1987). However, this was unlikely since a random attenuation of the intracellular vesicles will suggest that many cell surface proteins might be altered. Many proteins have a common biosynthetic pathway that involves transport from the endoplasmic reticulum (ER) through the golgi complex (Bakke and Dobberstein, 1990). Nonetheless, it has been reported that PDT acts primarily to alter cell membranes and membrane associated structures by oxygen-dependent mechanisms (Athar *et al.*, 1988; Girotti, 1983). Many intracellular targets including lysosomes (Zdolsek *et al.*, 1990), endoplasmic reticulum (Candide *et al.*, 1989), and mitochondria (Varnes *et al.*, 1990) have been identified as targets for PDT. It is possible that low-dose PDT treatment of LC could induce perioxidative injury to the membrane-associated intracellular vesicles such as ER and endocytic compartment. An injury to the ER will affect the biosynthetic pathway of proteins while an injury to the endocytic compartment will neutralize them thereby rendering these acidic compartments unfit for proteolysis. It is possible that the neutralization of the endosome by low-dose PDT induced the degradation of the trafficking antigenic peptides and prevented their procession for proper association with MHC molecules (Benaroch *et al.*, 1995), thus resulting in the down-regulation of the LC surface antigen expression that we observed. It is interesting to note that treatment of cells with agents that inhibited their acidification of the low pH vesicles (endosome and lysosomes) involved in proteolysis rendered them incapable of presenting antigen (Unanue, 1984). Using laser scanning

confocal microscopy and florescent probes, the intracellular localization of BPD could be traced. In addition, the hypothesis that low-dose PDT may induce alterations in pH of the acidic vesicles in epidermal LC can be evaluated with the endosome- and lysosome-localizing pH-sensitive fluorescent probes (Haugland, 1994b) which changes color in response to changes in the pH of the acidic vesicles. The vesicular pH can be determined using flow cytometry, confocal microscopy, and emission ratio imaging (Haugland, 1994b). Alternatively, using SDS stability studies, it is possible to fractionate cell extracts and evaluate the possibility that low-dose PDT neutralized the endosome and prevented the breakdown of the invariant chain, thus blocking the conversion of MHC class II molecules to peptide-loaded, SDS-stable alpha beta dimers (Benaroch *et al.*, 1995).

Besides affecting the intracellular vesicles, low-dose PDT treatment is capable of attenuating the actin- and microtubule-based systems (Margaron *et al.*, 1996) which provide the tracks for movement of membraneous organelles in eukaryotic cells (Kuznetsove *et al.*, 1992; Fath *et al.*, 1994). It has been reported that microtubules and microtubule-based motor proteins are important in intracellular trafficking of proteins (secretion, endocytosis, and sorting of subcellular constituents) (Vallee and Sheetz, 1996). Thus, it is possible that low-dose PDT down-modulates LC surface antigen expression by depolymerising the actin- and microtubule-based systems. The mechanism by which low-dose PDT depolymerises the microtubule-based systems is not understood. However, it is possible that low-dose PDT-induced membrane depolarization (Specht and Rodgers, 1990) leads to a rapid increase in intracellular calcium (Ben-Hur *et al.*,1991) which induces microtubule depolymerization (Schliwa *et al.*, 1981; Boekelheide *et al.*, 1987; Berg *et al.*, 1990; Sporn and Foster; 1992).

It has been shown that microtubules are unstable at increased calcium concentration (> 1 μM) (Schliwa *et al.*, 1981). As a result of the disruption of the microtubular systems by low-dose PDT (Margaron *et al.*, 1996), the intracellular trafficking of proteins (such as the internalization and recycling of surface antigens) may be affected. These events possibly resulted in the down-regulation of the LC surface antigen expression that we have observed. In this regard, it is interesting that the removal of microtubule motors from cell lysates have been found to inhibit fusion between early and late endosomes (Bomsel *et al.*, 1990; Aniento *et al.*, 1993). The fate of microtubules and actin filaments following low-dose PDT of LC can be evaluated by immunofluorescence techniques (Margaron *et al.*, 1996). In addition, intracellular trafficking of proteins can be assessed by tracing the intracellular fate of internalized surface antigens. The LC surface antigen can be tagged with fluorescent-labelled peptides or probes. Following a short incubation period, the fluorescent probe can be traced using laser scanning confocal microscopy. Alternatively, low-dose PDT-treated donor skin-derived LC surface antigens may be labelled with iodinated (I^{125})-monoclonal antibodies. The intracellular localization of the iodinated antibody may be detected by radioactive counts (I^{125}) of various subcellular fractions.

In summary, we have presented data which support the possibility that low-dose PDT of donor tissues induces a series of down-modulatory events in the graft recipient. Langerhans cells, following low-dose PDT treatment of donor skin, lose the capacity to stimulate efficient proliferation of allogeneic T cells. Although not completely delineated, this impaired alloreactivity of LC appear to be the result of decreased expression of LC surface expression of MHC and costimulatory molecules such that T cells are suboptimally

activated. Such suboptimal activation of T cells accounted for their reduced proliferation upon subsequent restimulation with normal donor-derived alloantigens and the decreased anti-graft inflammatory cell infiltration into the graft. The low-dose PDT-induced decreased antigen presentation and costimulation as well as the suboptimal activation of alloreactive T cells climaxed in the *in vivo* prolonged survival of skin allografts. However, it is possible that other cells (e.g. KC, $\gamma\delta$-T cells, melanocytes and recipient's antigen presenting cells) or factors (e.g. cytokines and cytotoxicity by apoptosis) may also have contributed to the prolonged allograft survival. It is anticipated that low-dose PDT-induced down-modulatory events would be relevant to vascularized internal organ transplants such as pancreas, liver, lung or cardiac allografts and in preparing arterial allografts for the development of vascular bioprostheses for use in humans (LaMuraglia *et al.*, 1995). The application of low-dose PDT in internal organ transplants will be the subject of future research.

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