PHYSIOLOGIC MODULATION OF DENDRITIC EPIDERMAL T CELLS BY STAPHYLOCOCCAL ENTEROTOXIN A

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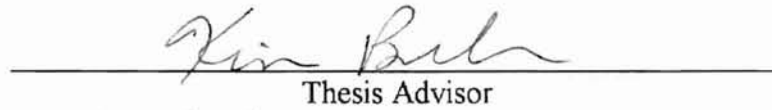

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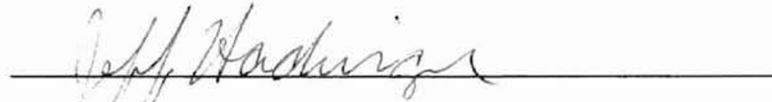

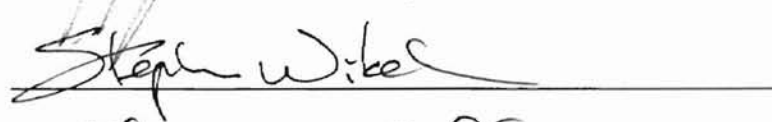

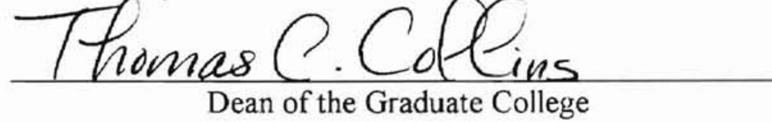

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NOMENCLATURE

BSA	bovine serum albumin
CD	cluster designation
CH	contact hypersensitivity
DETC	dendritic epidermal T cell
DMSO	dimethyl sulfoxide
ELISA	enzyme linked immunoadsorbent assay
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte/macrophage colongy stimulating factor
ICAM-1	intercellular adhesion molecule-1
IFNγ	interferon-gamma
LC	Langerhans cell
LFA-1	lymphocyte function-associated antigen-1
LPS	lipopolysaccharide
MHC	major histocompatability complex
Mls	minor lymphocyte stimulating antigen
MoAb	monoclonal antibody
PBS	phosphate-buffered saline
PE	phycoerythrin
SALT	skin-associated lymphoid tissue
SEA	Staphylococcal enterotoxin A
SEB	Staphylococcal enterotoxin B
TCR	T cell receptor
TNF	tumor necrosis factor
TSST	toxic shock syndrome toxin

CHAPTER 1

INTRODUCTION

It has long been recognized that there exists a close relationship between the skin and the immune system. The skin, acting as the barrier between the body's external and internal environment, is bombarded daily with numerous insults, both physical and biological. Although it has long been recognized as a physiochemical barrier and an organ of temperature control, only in the past 20 years have the immunocompetent attributes of the skin come to be appreciated. First, the true role of epidermal Langerhans cells (LC) as antigen presenting cells began to be realized by 1978 (98), after originally having been thought to be a part of the nervous system. Another finding that gave credence to the idea of the skin having immunologic function was the finding of certain T cell malignancies with an inclination toward infiltrating the skin (100). Streilein was the first to give the idea of this relationship between the skin and immune system a name -- Skin-Associated Lymphoid Tissue or SALT (104) -- and he included within this system keratinocytes, the growing layer of the epidermis; the LC, the major antigen-presenting cell of the epidermis; skin-seeking T lymphocytes; endothelial cells, that direct homing T cells; and draining lymph nodes (104, 101).

However, in 1983, a discovery was made that would force researchers to slightly alter their concept of the SALT system. Groups in both Dallas and Vienna, Austria reported a cell population in mouse epidermis that bore a dendritic morphology and Thy-1 cell surface glycoprotein, but no IA molecules (10, 111). These cells did not resemble any other cell present in the epidermis and it was impossible at the time to place them in any type of classification scheme, so these cells were simply referred to as Thy-1+ dendritic epidermal cells (10, 111).

Within a few years, however, much was learned about these cells, the most significant being that they possessed a T cell receptor (TCR), upon where they were renamed dendritic epidermal T cells (DETC). This TCR was found to be of the CD3-associated (97) γ/δ type, rather than the α/β heterodimer, as is found on most circulating peripheral T cells (53), and it lacked both the mature T cell surface markers CD4 and CD8 (8). Despite their lack of these markers, DETC were found to proliferate in the presence of the T cell mitogen Concanavalin A and also Interleukin-2 (IL-2) (72, 106), as well as anti-CD3 monoclonal antibody (MoAb), phorbol ester and calcium ionophore (72, 41, 110). Also unlike most T cells, DETC exist as non-circulating cells in the suprabasal layer of the epidermis, where they constitute only 0.8-2% of cells there (8, 108). Radiation chimera studies have shown that, along with LC, DETC are of bone marrow origin (9, 17). Evidence has also shown that they are dependent on the thymus for complete development (71, 58, 42) which lends additional support to the DETC being of the T cell lineage.

Much has been learned about DETC since their discovery in 1983 (8, 108, 107), but many questions still persist and at the present time, their physiologic role has not been elucidated. Also interesting is the fact that, to date, no resident γ/δ+ cells with a dendritic morphology have been found in the epidermis of any mammalian species, including humans. One of the keys to understanding the true role of these cells lies in the determination of the antigen DETC recognize, as their TCR is characterized by very limited diversity (40, 5), even to the point of being virtually monomorphic (107). This suggests that the number of ligands that the DETC can recognize is extremely limited and some investigators have suggested that there is only a single physiologic ligand for the DETC (4). The γ/δ TCR is thought to be more primitive than its α/β counterpart, as evidenced by the fact that γ and δ chains are rearranged and expressed earlier than the α/β heterodimer (46), and because of this ontogenic difference, were termed TCR1 and TCR2,

respectively. Since most $\gamma/\delta+$ T cells reside in various epithelial compartments, it has been proposed that γ/δ T cells form a first line of defense against invading pathogens and may play a crucial role in immune surveillance (46).

To maintain and coordinate this complex system of cutaneous immune responders is an interwoven network of cytokine production. To illicit an immune response in the skin and to eliminate the offending antigen, cells of the SALT system must work in conjunction with one another and coordinate their actions. Keratinocytes bear the largest burden of supplying soluble protein signals as nonspecific activators for other cells in a cutaneous immune response. This is certainly their primary immune function, but it could be argued that their role in capturing antigen is rather significant, although this point remains a topic of debate. Keratinocyte-derived Interleukin-1 (IL-1), and to a lesser extent tumor necrosis factor, have a significant effect in mediating inflammatory and contact hypersensitivity reactions in the skin. Both cytokines have been implicated in upregulation of endothelial cell adhesion molecules (88) and may be necessary primary signals in LC migration from the epidermis. IL-1 has proved important as an autocrine stimulatory factor for neighboring keratinocytes and a chemoattractant of T cells (100). Little is known about what signaling effects epidermal keratinocytes have on DETC. It is known that IL-7 from keratinocytes serves as a growth factor for DETC (62) and DETC will migrate toward PAM 212 keratinocyte culture supernatants, but not toward keratinocyte cytokines administered individually (24).

Immunobiology of the Skin

The role of the skin as a protective organ has long been known, but in recent years, much has been learned about the skin's relationship with the immune system. Although the skin is effective acting as a physiochemical barrier to environmental insults and

invading organisms, it cannot keep all pathogens from getting through its layers. Therefore, the skin, along with its draining lymph nodes, must act as an active immune organ in fighting against external infections and cutaneous neoplasms (88), althewhile maintaining its physiologic functions.

The concept of cutaneous immunobiology was first reported by Alexandre Besredka in 1925 (11) when she pointed out the existence of organ-specific immunity, where she wrote two books on the subject (15). In 1970, a group published an article (31) in which they stated that the skin was a first-level lymphoid organ, comparable to the primary lymphoid status of the thymus (15). Much of what is known about cell-mediated immunity came about through delayed type hypersensitivity skin reactions in experimental assays (100). The idea of organ or tissue-specific immunity was first documented for the respiratory tract as BALT (or bronchial-associated lymphoid tissue) (12) and the gastrointestinal tract as GALT (gut-associated lymphoid tissue) (39), but Streilein's definition of skin-associated lymphoid tissue or SALT (104), was the first documentation suggesting resident keratinocytes, Langerhans cells, epidermotropic T cells, dermal endothelial cells and draining lymph nodes working together to provide immune protection to the skin.

The functional components of the SALT system work together to perform four basic functions, which are: (1) induction of primary immune responses to new cutaneous antigens, (2) expression of immunity in the skin to previously encountered antigens, (3) avoidance of deleterious responses to nonpathologic cutaneous antigens and (4) performing immune functions without compromising the skin's physiologic integrity (100). To do this, the SALT system employs a number of cells within both the epidermis and the dermis in the elicitation of contact hypersensitivity reactions. The most common type of antigens that induce this type of contact hypersensitivity reaction are haptens such as pentadecacatechol from poison ivy and poison oak, nickel, chromate, and chemicals used to make

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rubber (83). As haptens, these substances are too small to illicit an immune response on their own, but once they penetrate the epidermis, they probably bind to body proteins, where they become active targets for cells of the immune system. First, keratinocytes comprise the growing portion of the epidermis and constitute approximately 90% of the cells found there. The outer layers of the skin are comprised of dead, densely packed keratinocytes, but the basilar layers are made up of keratinocytes which produce a number of immunologically-relevent cytokines and which are believed to create an appropriate environment for antigen uptake and recognition. Epidermal Langerhans cells (LC) are the major antigen-presenting cell of the epidermis (while dermal macrophages and dendritic cells play this role in the dermal layer) and are the only cells within normal, unperturbed epidermis to constitutively express MHC class II molecules. The job of LC is to induce primary T cell responses by binding to foreign antigens and migrating to draining lymph nodes, where they present these antigens to T helper cells. The T lymphocytes to which LC present their bound antigen may be found in regional somatic lymph nodes or in the skin itself. These T lymphocytes find their way to the skin and ultimately, to the site of the antigenic stimulation, by "sticking" to dermal and blood vessel endothelial cells which can become activated to upregulate cell surface adhesion molecules that the skin-directed T cells can recognize and bind. In the midst of all of these cellular interactions is a network of cytokine release that coordinates immunological events within the skin that help drive the immune response from initiation to resolution.

Layers of the Skin

The cutaneous membrane constitutes one of the four major types of organs of the integumentary system, but is most-commonly referred to as the skin (43). As previously stated, the skin acts as a physiochemical barrier that provides protection against harmful

substances such as microorganisms, ultraviolet rays from the sun, toxins and harmful chemicals. It also prevents water loss from deeper tissues, allows for sensory reception through heavy innervation, excretes wastes, regulates body temperature (43) and, as just described, mediates cutaneous immune responses.

The skin itself is divided into two major tissue layers, the epidermis and dermis, which are separated by the dermal-epidermal junction. These tissues are held in place by a layer of loose connective and fatty tissue termed the subcutaneous layer. The epidermis comprises the outermost, avascular layer of the skin which is made up of stratified squamous epithelium and is comprised of a number of layers -- layers that vary from region to region in the body. The deepest layer of the epidermis has access to nutrients provided by blood vessels from the underlying dermis, and so, possesses cells that are capable of growth and division. This layer is termed *stratum germinativum* or *stratum basale* and the cells here take on a columnar-cuboidal morphology. As keratinocytes -- the growing cell population of the epidermis -- from this layer grow, they are pushed vertically, where they flatten and acquire desmosomal connective junctions that help hold them to one another to form a consistent epidermal covering. This thick layer is termed the *stratum spinosum*. Two more distal layers may or may not be present, depending on the thickness of the skin in a particular region. They are, a granular layer called the *stratum granulosum* and the *stratum lucidum*, which is found in palmar--plantar regions. As keratinocytes are pressed even further away from the basal and spinous layers, their internal organelles and nuclei disappear and they undergo a hardening process called keratinization, where a tough, fibrous protein called keratin develops within the cell. This outermost layer of densely-packed keratinized cells is called the *stratum corneum* and this is the final fate of keratinocytes before they are sloughed off (95, 43). Binding the epidermis to the underlying tissues is the dermis. The membrane separating these two skin regions is made up of

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both epidermal and dermal components in a series of folds, where both layers extend into one another through small finger-like projections. This basement membrane is referred to as the dermal-epidermal junction and this structure binds the epidermis to the dermis and acts to prevent the movement of materials and cells across the junction (95). The dermis itself is made up of collagen, elastic tissue and gel-like ground substance and can be divided into two layers, the upper *papillary layer* and the lower *reticular layer*. Within these layers can be found sensory and motor nerves, hair follicles, sweat and sebaceaous glands, smooth muscle, and blood and lymphatic vessels. The dermis is essential for the viability of the epidermis, as it cannot be maintained in culture for long periods of time without dermal tissues present (95). The blood vessels of the dermis provide thermal regulation to the skin and are also crucial for cell trafficking in cutaneous immune responses. Connective tissues of the dermis come from fibroblasts and it is the networking of these tissues that give the skin its elasticity and toughness (95, 43). Underneath the dermis lies the subcutaneous layer. This layer, composed mainly of loose connective and adipose tissues, supplies blood to the skin through major blood vessels and also functions as a heat insulator (43).

Langerhans Cells

It was in 1868 that a medical student by the name of Paul Langerhans inoculated human skin with gold and discovered a population of dendritic epidermal cells, which he originally ascribed as a cell of the nervous system (55). It took more than a century for researchers to begin to understand the function of the cell that bears Langerhans' name. Proof came about that Langerhans cells were not a part of the nervous system in the late 1960s (116) and eventually it was learned that these cells are of bone marrow origin (8) and are believed to be of the monocyte/macrophage lineage (8). In the mid-1970s, it was

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realized that these cells possessed the ability to act as antigen presenting cells (98) and much research has been done to corroborate this idea, as Langerhans cells (LC) have been shown to possess a great ability to stimulate T cell responses within the skin and with much greater efficiency than macrophages, splenic adherent cells and peripheral blood monocytes(116, 49). This would be consistent with the fact the LC are the only cell in normal epidermis to express surface IA antigens, the human corollary of which is HLA-DR (85). The ability of LC to induce these T cell responses comes from their ability to migrate out of the epidermis to draining lymph nodes upon encountering antigen as shown by depletion of these cells from the skin after hapten painting and that hapten-bearing MHC class II+ cells appear in draining lymph nodes 24 to 48 hours after skin painting (8). Also, it is believed that LC only reach their full potential as antigen presenting cells after having left the epidermis (15). Studies have implicated LC as being involved in contact hypersensitivity reactions within the skin, showing that application of a chemical allergen on the skin results in antigen-bearing dendritic cells in draining lymph nodes (26). Although much of the antigen presented by LC is believed to take place in draining somatic lymph nodes, if the appropriate T lymphocytes are already in or near the skin, antigen presentation may take place within the skin itself (96). LC are also critical in graft rejection, as they represent the "passenger" leukocytes critical in allosensitization (103). Important to note, though, is the fact that application of haptens in LC-deficient skin leads to suppresser T cell-mediated suppression (102), suggesting that it is the absence of LC, not the LC themselves, that induce tolerance.

Langerhans cells constitute approximately 2-8% of the cells of the epidermis and reside in its suprabasal region (88). LC can also be found in other epithelial tissue such as the oral cavity, vagina, cervix and esophagus, as well as the dermis, and draining lymphatic vessels and nodes (116). These cells are regularly distributed within the skin, although the

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density varies from region to region, and their dendrites intercalate between keratinocytes to form a sort of immunological net, so that each LC covers an area greater than adjacent epidermal cells (116). As previously stated, LC constitutively express MHC class II markers, but they also have receptors for the Fc portion of IgG, the complement component C3b (100) and are also ATPase positive (115) . Unique to these cells is an organelle originally described by Birbeck (14). The exact function of this organelle remains undiscovered at the present time, but it is the only reliable morphologic marker that the LC can be identified by through electron microscopy (100).

Keratinocytes

Keratinocytes comprise the growing cell population of the epidermis and consist of approximately 90% of the cells found there. These cells begin life in the basal layer of the epidermis and as they proliferate, become pushed distally from that region, differentiating into keratinized non-proliferative cells that form the outer layer of skin in direct contact with the outside environment. Once they reach this layer, they are sloughed off and replaced by cells underneath, as this process repeats itself again and again. Over the past few years, though, it has been realized that keratinocytes are not the immunologically-silent cells scientists once thought they were, but possess properties that give them a vital role in maintaining the immunologic integrity of the skin.

First and foremost, keratinocytes are able to produce a number of cytokines with a wide range of immunologic influences. Under the appropriate circumstances, keratinocytes can produce IL-1α and β, IL-3, IL-6, IL-7, IL-8, IL-10, IL-12, tumor necrosis factors alpha and beta (TNFα and β), granulocyte/macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), monocyte colony stimulating factor (M-CSF), interferon alpha and beta (IFNα and β), platelet-derived growth factor,

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fibroblast growth factor and transforming growth factors alpha and beta (TGFα and β) (65, 15, 100, 62). Few of these factors are expressed constitutively in vivo, but many can be induced by a wide variety immunological insults as non-specific, pro-inflammatory substances, the most significant being IL-1. This cytokine, along with TNFα, has been shown to increase MHC class II and intercellular adhesion molecule (ICAM)-1 expression on LC (7) and may well be one of the primary signals in inducing their migration from the epidermis (80). IL-1 can cross the semi-permeable membrane of the dermal-epidermal junction, as is demonstrated by its ability to be a strong chemoattractant to T cells (89) as well as to upregulate surface adhesion molecules on the endothelial cells of post capillary venules of the papillary and reticular dermis (57, 15). Keratinocytes also secrete cytokines that can downregulate inflammatory responses. TGFβ and prostaglandin E2 can inhibit inflammation by blocking the effects of both IL-1 and IL-2 and IL-10 downregulates MHC class II expression and immune responses mediated by Th1 cells (23, 83).

Keratinocytes are also able to influence immune responses within the skin by upregulating certain surface markers, namely MHC class II and ICAM-1. In non-inflamed skin, keratinocytes only express MHC I antigens (EE), but in perturbed skin, IFNγ secreted by T lymphocytes can upregulate class II MHC expression (112). There is some speculation, however, as to the significance of class II MHC expression on these cells. No studies have shown IA+ keratinocytes as possessing the ability to act as accessory cells in antigen presentation (23). This may be due to the lack of the ability to process antigen as shown in studies where IA+ keratinocytes could present viral peptides which required no processing, but not intact viruses in which processing was necessary (23). Interestingly enough, there is evidence suggesting that T cell recognition of antigen-derivatized keratinocytes does occur, but that this recognition leads to clonal anergy, suggesting a role for IA+ keratinocytes in the downregulation of immune responses (36). IFNγ and TNFα

induce the expression of ICAM-1 on keratinocytes, which may play a role in recruiting or trapping immune cells to the site of antigenic challenge, as this molecule binds to lymphocyte function-associated antigen (LFA-1) expressed on activated T cells (88).

A phagocytic ability is demonstrated by keratinocytes, even much more so than LC. It is theorized that antigen phagocytized by keratinocytes may be modified and reintroduced to the epidermal microenvironment, where they are picked up by the poorly-phagocytizing LC in an essential first step in LC antigen presentation. However, internalization of antigen by keratinocytes may prevent access of those antigens to other immune cells and may actually prevent proper antigen presentation (100).

Epidermotropic T Lymphocytes

It has been well-known that T lymphocytes play a major role in inflammatory, allergic and autoimmune responses within the skin, but the discovery of certain T cell malignancies with a propensity for infiltrating the skin, led researchers to believe that there existed a population of actual skin-seeking lymphocytes (101). Research has demonstrated that lymphoid cells recovered from mesenteric and peripheral lymph nodes display different homing patterns, with mesenteric lymph node cells infiltrating the gut and peripheral lymph node cells infiltrating the skin (38, 84). In 1990, Picker *et al* found a subset of peripheral T cells with cutaneous lymphocyte-associated antigen (CLA) by using the monoclonal antibody HECA-452, which binds to a 200 kD surface glycoprotein found on 16% of peripheral blood T cells, but on 85% of T cells derived from various dermatological diseases (81). CLA is expressed on approximately 43% of T lymphocytes in normal skin (15). Along with the LFA-1-ICAM-1 interactions of T cells with endothelial cells and keratinocytes, this surface marker may well interact with E-selectin (formerly known as

endothelial cell leukocyte adhesion molecule-1), which may act as an adhesion molecule for cutaneous homing of T cells.

There exists a vast difference in dispersion patterns of T cells in the skin, depending on the region. Of the total number of T lymphocytes in normal skin, 90% are located in dermal perivascular regions. These numbers in the epidermis are 2% and most of these cells are of the CD8+ phenotype (88). In the dermal regions, most of the cells are in the activated state, as demonstrated by expression of HLA-DR antigens and IL-2 receptors. Also, there are nearly equal numbers of CD4+ inducer and CD8+ cytotoxic/suppressor cells, which are of the α/β variety (16), clustered around post capillary venules in the dermis, but only CD8+ T lymphocytes are commonly found in "free" dermis, away from these structures, occasionally venturing as far as the epidermis itself (100).

During initiation of an immune response in the skin, and subsequent migration of antigen-derivatized LC, if the relevant memory T cell is already located in the post capillary region of the dermis, antigen presentation will most likely occur there. These induced cells will then secrete IFNγ, which causes upregulation of class II MHC and ICAM-1 molecules on keratinocytes and activates them to release pro-inflammatory cytokines such as TNFα, GM-CSF, IL-3, IL-6 and most importantly, IL-1. This activation of keratinocytes can then induce the migration of non-antigen specific T cells from peripheral lymph nodes, which home back to the site of the original challenge. If, however, the antigen in question has not been previously encountered, LC will migrate to the regional lymph nodes themselves, and there, present the antigen to naive T cells. Then, binding their lymphocyte homing receptors to the expressed adhesion molecules on endothelial cells, these newly activated T cells will migrate to the skin to mediate an immune response. However, Chin, Sackstein and colleagues have proposed, through studies involving lymphocyte trafficking in psoriatic lesions, that upregulation of endothelial adhesion molecules may

capture T lymphocytes with the appropriate receptors that are normally circulating through the blood, implying that they may not even be T cells of the skin-seeking variety (21, 22). It has been hypothesized that there is an "imprinting " of T cells based on the unique tissue environment where the antigen is encountered, leading to tissue tropism (31).

Ontogeny of DETC

When DETC were first discovered, scientists were unable to place them in any particular classification scheme. The only indication of the potential lineage of these cells came from the presence of the murine T cell differentiation marker Thy-1 on their surface, hence the somewhat generic name, Thy-1+ dendritic epidermal cell. The first key to unraveling the mystery of this particular cell population lie in discovering their origin. Bone marrow chimera studies in AKR mice unequivocally showed that Thy-1+ cells of bone marrow origin could infiltrate the epidermis of recipients, resulting in repopulation of allogeneic DETC, although reconstitution of significant numbers took much longer than similar studies employing LC (9, 17). Once this was established, the next issue to be dealt with involved asking if these cells were derived from the thymus as precursor cells which migrate into the epidermis during the neonatal period or whether they developed independently of the thymus. Fetal thymocytes do bear the characteristic Vγ3-Vδ1 TCR of DETC (108) and the disappearance of Vγ3-Vδ1 cells in the fetal thymus precedes the initial appearance of DETC in the epidermis (40, 42). A thymic dependence on DETC maturation was observed by the fact that grafting of thymic lobes or injection of thymic precursor cells resulted in the gradual appearance of CD3+, TCR-Vγ3-Vδ1 DETC in the epidermis of athymic or Thy-1 disparate mice, expanding in clusters and slowly becoming more dendritic. This, along with the fact that CD3- or Vγ3/Vδ1- thymocytes failed to

repopulate the epidermis as CD3+ or Vγ3/Vδ1+ DETC, supported the idea that DETC arrange and express their TCR before entering the epidermis (108, 8, 58, 28). Interestingly, very few Thy-1+ cells are present in the epidermis of newborn mice and these cells are CD45+, but CD3-. Only after a month are there cell densities in the skin comparable to those of adults (107, 108). Elbe *et al*, demonstrated this extra-thymic development from precursor to mature CD45+/Thy-1+/CD3+ cells by grafting day 16 fetal skin onto athymic or Thy-1-disparate adult mice, whereupon fetal skin originally devoid of CD3/TCR-bearing cells showed Thy-1+/CD3+/TCR Vγ3+ cells in increasing numbers over time of donor grafted, but not host, skin (78). This showed that although the precursor cells of DETC originate in the thymus, their maturation is thymus-independent, as it takes place within the skin.

Functional and Structural Attributes of DETC

Surface Markers and T Cell Receptor

When first discovered in 1983, DETC were named Thy-1+ dendritic epidermal cells because of the presence of the murine T cell differentiation marker, Thy-1, expressed by this cell population. This marker is found on all murine T lymphocytes, but usually coinciding with either of the two distinguishing mature T cell alloantigens, CD4 or CD8, of which DETC display neither (107). DETC constitutively express, along with Thy-1, CD45, asialo-GM1, a γ/δ T cell receptor complexed with CD3, but no IA molecules or surface IgG (107, 71).

The vast majority of T cells in the thymus and periphery display a polymorphic heterodimeric T cell receptor consisting of an α chain and a β chain. This receptor is linked to a CD3 complex, which is composed of 3-5 transmembrane subunits which transmit

activation signals from the TCR to the cell (108). A T cell of the α/β variety can be further classified by the presence of either surface CD4 or CD8 molecules, each with its own unique MHC requirements for activation: CD4+ cells require antigen in context with MHC class II molecules and CD8+ cells need antigen and MHC class I (83). However, there exists a minor subset of T lymphocytes that are CD4- and CD8- which bear a CD3-associated heterodimeric glycoprotein composed of a γ and a δ chain (46). The antigen-MHC binding portion of the TCR, of both T cell classes, is composed of four sets of genes: V (variable), D (diversity), J (joining) and C (constant) segments. Diversity among T cells comes about through somatic rearrangement of these different germline-encoded gene segments, giving the entire T cell repertoire the ability to recognize a vast array of foreign antigens (107, 108). The overall ability of γ/δ T cells to generate combinatorial diversity is greater than that of α/β T cells and this is demonstrated by TCR analysis of circulating peripheral γ/δ T cells, but this is not the case with resident γ/δ T cell populations in various epithelial compartments (29). And, indeed, circulating γ/δ T cells show marked variability in their TCR, but those found preferentially in these various epithelium seem to be biased toward certain conserved TCR types that are site-specific. In murine lung, γ/δ T cells demonstrate a TCR bearing predominately $V\gamma2$ and $V\delta1$ or $V\delta6$ components (6). In gut epithelium, there is no δ chain preference, but a strong γ chain preference toward $V\gamma7$ (54) and in the tongue and mouse female reproductive tract, receptor chain preference is toward $V\gamma6$ and $V\delta1$ (44). T cells of the murine dermis show $V\gamma5$ preferences (54). Although the precise ligands of these γ/δ T cells have not been elucidated, researchers believe that each cell type has a conserved TCR, specific for that site, to recognize antigens that are most likely confronted in that particular anatomic site. It has been speculated that the importance of γ/δ T cells, in general, be as a first line of

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defense in response to infectious agents and altered or transformed self cells, initiation of autoimmunity and downregulation of α/β T cell responses (107). This speculation is primarily based on the fact that increased numbers of γ/δ T cells have been found to infiltrate certain disease sites involving granulomatous skin lesions (66), cutaneous melanomas, discoid lupus erythematosus and infection sites involving *Toxoplasma gondii, Listeria monocytogenes* (107, 108) and *Mycobacterium tuberculosis* (73).

Consistent with other γ/δ populations, DETC also display extremely limited $\gamma\delta$ pairing. Bergstresser et al have produced a number of DETC cell lines, all of which bear identical TCR, composed of $V\gamma3/J\gamma1/C\gamma1$ and $V\delta1/D\delta2/J\delta2/C\delta$ gene segments (108, 5, 4), while others have shown similar results with cultured DETC lines being $V\gamma3/V\delta1+$ (42, 5, 108). However, there has been some disparity, with some groups finding DETC with considerable TCR heterogeneity. Possible explanations stem from different strains of mice, variations in protocols, or differences in culture techniques. Monclonal antibody studies were carried out by independent groups in Berkeley and Dallas in order to determine the TCR variability of DETC in epidermal sheets and in culture. Researchers in Berkeley developed a MoAb (named F536) which was specific for all γ/δ TCR-bearing $V\gamma3$ segments, irrespective of the δ chain. Using disaggregated EC and flow cytometric analysis, this group demonstrated that over 90% of CD3+ epidermal cells were positive for the F536 MoAb (40). The MoAb developed by the Dallas group (17D1) was also specific for $V\gamma3+$ cells, but only when those cells were associated with a $V\delta1/D\delta2/J\delta2$-containing δ chain. Using whole epidermal sheets, this group determined that approximately 98% of all CD3+ epidermal cells expressed TCR that were recognized by the 17D1 MoAb (58). Interestingly, the $V\gamma3/V\delta1$ TCR characteristic of DETC is found in no other epithelial compartment, peripheral blood or secondary lymphoid tissue. They have only been found in the

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fetal thymus and are the earliest fetal thymocytes to express a CD3/TCR complex. These $V\gamma3/V\delta1$ thymocytes are predominant until day 17 of gestation, at which time their numbers decline while the numbers of α/β+ cells increase (107, 108, 8).

Growth and Proliferation

Along with the presence of a T cell receptor, one attribute of the DETC that led researchers toward classifying this cell as being of a T cell lineage was its proliferative response to T cell mitogens. It has been shown that DETC proliferate significantly in the presence of Concanavalin A and IL-2 and IL-2 alone after this initial activation (72, 106), although evidence shows that activation signaling for DETC may be different than for other types of T lymphocytes. For example, lymph node or spleen-derived T cells show strong mitotic responses to phorbol myristate acetate (PMA) plus ionomycin, but DETC do not and PMA plus ionomycin and IL-2 showed only marginal proliferative effects on DETC. Also, IL-2 seems to be the primary T cell-derived growth factor for DETC while other T cell classes respond to either IL-2 or IL-4 (110). The ease of establishing and maintaining DETC lines as opposed to the difficulty in culturing γ/δ cells from other sites also contribute to the idea that DETC may have unusual activation requirements (107). DETC have also been known to proliferate in response to anti-CD3 MoAb (110) and application of certain preservatives or antioxidants in topical medications (82). It was also found that keratinocyte-derived IL-7 could serve to maintain growth of DETC in culture and cause proliferation in Con A stimulated cells (62). This cytokine is believed to be a major relevant growth factor since DETC are the only cells in the epidermis to secrete IL-2, and only after cell activation. So, it seems unlikely that IL-2 is the lone DETC growth factor.

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Cytotoxicity

A well-known feature of epithelial γ/δ T cells is their ability to demonstrate non-MHC restricted cytotoxicity toward certain cellular targets and the γ/δ+ DETC possesses this ability, as well. Nixon-Fulton *et al* have shown DETC-directed non-MHC-restricted cytotoxicity toward the YAC-1 lymphoma, a natural killer cell-sensitive target. This ability was seen only in Con A and IL-2 stimulated cells and was not seen in cells that were freshly isolated (70). This stimulation and expansion necessity for cytotoxic activity is reminiscent of lymphokine activated killer (LAK) activity. Researchers in the same laboratory also found that 3 long-term DETC lines, but not their supernatants, were able to lyse the transformed keratinocyte line PAM212 and 2 transformed endothelial cell lines in addition to a number of mouse melanoma and fibrosarcoma tumors (48). Others have found that DETC can also lyse an ultraviolet light (UVL)-induced fibrosarcoma (74). DETC cytotoxic activity only after stimulation, coupled with evidence that intestinal γ/δ T cells show cytotoxicity in normal, but not germ-free, animals (56) have led researchers to believe that DETC in unperturbed epidermis must be induced to become mature lymphocyte killer cells. Also in support of this is the fact that DETC have mRNA for perforin, a protein capable of forming transmembrane pores in the cell membranes of target cells (52). Overall, the demonstration of these cytotoxic abilities toward transformed cells of the epithelia support the notion that DETC play a role in immunosurveillance of the skin. That is, DETC can recognize self cells that have been altered by a variety of means (transformed, physically damaged, infected) and eliminate them before they threaten the skin's physiologic and functional integrity.

Tolerance

Despite their role in cytotoxic immune reactions which are not MHC-restricted, DETC also seem to play a role in normal immune responses. Contrary to the essential role

Langerhans cells play in induction of contact hypersensitivity (CH) and delayed type hypersensitivity, DETC downregulate these α/β T cell-mediated responses since the ability to mount a CH response has been shown, by one investigator, to be inversely proportional to the ratio of LC to DETC in the skin (13) . Hapten-conjugated DETC injected intravenously or into the footpad resulted in an inability to mount an antigen-specific contact hypersensitivity response, while injecting hapten-derivatized normal lymphocytes or unconjugated DETC, by these same routes, caused no impairment in mounting such a response (107, 108). The mechanism for this tolerance may involve cytotoxic activity toward normal lymphocytes (108). Furthermore, cutaneous hapten painting after UVB radiation, a standard protocol for inducing immunologic tolerance, results in hapten-derivatized Thy-1+ cells with downregulatory abilities in draining lymph nodes (75). These data seem to support the idea that, in addition to being effector cells in the epidermis, DETC may also be involved in the downregulation of cutaneous immune responses.

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Antigen Recognition

To elucidate the true role of the DETC, it is first necessary to recognize the physiologic ligand or ligands of this cell population. As stated previously, the TCR of DETC are extremely restricted, with nearly all established clones being monomorphic. This TCR homogeneity would suggest that the number of ligands that DETC can recognize is also very restricted. To reiterate their cytotoxic ability, DETC show non-MHC-restricted lysis toward a certain transformed keratinocyte and endothelial cell lines, as well as a number of lymphoma, melanoma and fibrosarcoma tumors (70, 48, 74). It is believed that the DETC expression of this invariant TCR, along with the fact that these cells are not motile, indicate that DETC may not encounter a wide array of antigens, except on rare occasions. However, they are in intimate contact with surrounding keratinocytes and may very well

recognize a common antigenic marker induced on damaged or transformed cells in their microenvironment (8, 107, 108). Asarnow *et al* have suggested that this ligand might be mammalian heat shock proteins or stress proteins, a highly conserved family of proteins induced in many cell types by a variety of cellular insults, including heat, starvation, malignant transformation and infection (4). Reports have also cited that certain γ/δ T cells, distinct from DETC, are able to respond to various species of Mycobacteria, whose antigens bear striking similarity to heat shock proteins (66, 73). The current popular theory has DETC playing their major role in cutaneous immune surveillance, where they recognize heat shock proteins or other stress-induced markers on damaged epidermal cells, responding to these antigens and eliminating the stressed cells before they compromise the physiologic integrity of the skin. This recognition of stress ligands seems not to be restricted by normal polymorphic class I or II MHC molecules, but instead by more non-polymorphic β_2-microglobulin-associated MHC-like molecules such as tissue-restricted CD1, Qa, or Tla molecules (117).

Epidermal Cytokines

Cytokines are low molecular weight protein substances secreted by their producer cells in response to certain stimuli. They have much the same function as classical hormones, but are classified differently, as hormones are secreted by particular endocrine organs and cytokines are produced by a number of different cell types in various tissue regions. Like hormones, though, cytokines act on their target cells after initial release from their producer cell, where they bind by specialized surface receptors specific for that particular cytokine. The target cell is then induced to perform a particular function, based on the cytokine acting on it. Cytokines play a great role in coordinating numerous types of immune responses, wound healing, cell regeneration and differentiation and they can

also act as paracrine and autocrine growth factors. Rarely are these molecules produced at a constant rate, but most are induced by a stimulus and they are usually present for only a short period of time.

The discovery of the ability of epidermal cells to secrete various cytokines reinforced the notion that the skin was fully capable of carrying out immune functions. As previously described, Langerhans cells, keratinocytes, as even melanocytes, are capable of secreting cytokines. It has also been shown that DETC possess this ability, too. The largest source of epidermal cytokine production has been attributed to keratinocytes, where IL-1 and TNFα may have the greatest role as keratinocyte-derived immune response modifiers.

Studies using reverse transcriptase polymerase chain reaction have shown that DETC possess mRNA for a number of cytokines, including: IL-1α, IL-2, IL-3, IL-6, IL-7, TNFα, TNFβ, GM-CSF, and IFNγ (64, 105). They also secret keratinocyte growth factor (KGF) and so, may play a role in promoting epidermal cell growth after injury (107). The cytokine produced in largest quantities by DETC is probably IL-2, which would be consistent with the large amounts produced by normal α/β T cells. However, while mRNA for another Th1-type cytokine, INFγ, is expressed constitutively in DETC, IL-2 is not and is only expressed after activation, especially by Con A (64). The cytokine repertoire of DETC seem to be of the Th1 classification. The only Th2 cytokine observed is IL-4 and only upon secondary stimulation by both Con A and rIL-2 in short-term culture (264). Stimulation of long-term DETC lines by anti-TCR MoAb showed increased levels of IL-2 and IFNγ, but no IL-4. These results are consistent with DETC producing a Th1 cytokine profile and would correlate with cytokine production by T cells infiltrating the skin in a normal delayed-type hypersensitivity reaction, in which no humoral response is present. As previously mentioned, IFNγ is capable of upregulating MHC class II and

ICAM-1 on keratinocytes, but this cytokine can also inhibit the growth of these cells (63). T lymphocyte-derived IFNγ can stimulate keratinocytes to secrete IL-7 (3), which in turn, acts as a growth factor for DETC. This along with the fact that DETC secrete KGF, suggest an intimate interaction between keratinocytes and DETC.

Interferon-gamma (IFNγ) was first recognized over 30 years ago for its anti-viral activity (114). Research concerning IFNγ over the past decade, however, has determined a much broader immunologic role for this cytokine, with its role an as anti-viral agent actually being quite minor. IFNγ belongs to a protein family which is divided into three classes -- interferon-α, -β and -γ -- all of which are related by their ability to give protection against viral infection (30). However, IFNγ is unrelated to its interferon counterparts at both the genetic and protein levels and its antiviral activity is much lower than the other two interferons while its ability to function as an immunomodulator far exheeds that of its interferon counterparts (77). Although the major source of IFNγ is T lymphocytes, all cells, with the exception of erythrocytes, possess a recptor for it (30). Possibly the most important function of this cytokine is its antiproliferative effect on Th2 murine CD4+ cells, which makes it key in determining the type of T-helper immune function that develops during an immune response, steering it away from the Th2 humoral response and toward the Th1 cell-mediated branch of immune effector function (30). In contrast, IL-10 inhibits IFNγ production by Th1 CD4+ T cells, inhibiting cell-mediated immune responses (68). In effect, IFNγ and IL-10 regulate the development of immune effector functions between the Th1 cell-mediated branch and the Th2 humoral branch. Another well-known attribute of this cytokine is its ability to upregulate MHC class I and II molecules on the surface of immunologically-important cells that do not normally express them, such as mononuclear phagocytes, endothelial cells and keratinocytes (30, 109, 51), which is an important step in antigen presentation. IFNγ is also capable of helping immature myeloid precursor cells

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differentiate into mature monocytes (30). It promotes antigen presenting abilities of macrophages, not only by upregulating MHC class II, but also by increasing the levels of some intracellular enzymes that may be vital to this task (47, 2) and its ability to induce nitric oxide production suggests that IFNγ plays an indirect role in the killing of intracellular pathogens (30). In addition, IFNγ may enhance the interaction between macrophages and T cells by inducing adhesion molecule expression on macrophages (35) and it plays a role in lipopolysaccharide (LPS)-induced tumor necrosis factor alpha (TNFα) by various murine macrophage populations (25, 19).

The most important cytokine involved in T cell proliferation is Interleukin-2 (IL-2). The activity of IL-2 was first observed in cell culture supernatants of human peripheral blood lymphocytes which had been stimulated with certain T cell mitogens (93). It was soon discovered that the activities of T cell growth factor (TCGF), thymocyte stimulation factor (TSF), thymocyte mitogenesis factor (TMF), T cell replacing factor (TRF) and killer helper factor (KHF) could be attributed to the same cytokine (93). The activity of IL-2 is derived from a single protein of 15.4 kD, which provides all of its signaling for T cell DNA duplication and subsequent mitosis and is produced by Th1 CD4+ T cells following mitogen or MHC-restricted antigen activation (93). Following this finding, it was determined that IL-2 bound to activated T cells by way of a unique receptor. This receptor is a heterodimer consisting of α and β subunits (37). The β subunit is constitutively expressed on resting T cells, but the α subunit is only found on those T cells whose antigen receptor has already been triggered (92). With the fully-assembled receptor confined to activated cells only, T helper cells can ensure conservation of clonality of a T cell-mediated immune response through the secretion a non-clone specific cytokine. So, in effect, responsiveness to IL-2 becomes an antigen dependent autocrine or paracrine proliferative factor for those T cell-bearing functional IL-2 receptors. In addition to this

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important activity, IL-2 also plays a role in growth of B cells, natural killer (NK) and lymphokine-activated killer (LAK) cells (93, 20), augmenting IFNγ expression (76); and, inducing IL-6 production by human monocytes (69).

Superantigens

The concept of superantigens was first brought to light in 1973, when antigens from unmapped genetic loci able to generate intense mixed leukocyte reactions between MHC-identical mice were found (1). These loci were mapped outside the MHC and were termed minor lymphocyte stimulating (Mls) antigens. However, their functions were not discovered for some time when in the late 1980s, two groups found the deletion of certain T cell subsets in mice expressing Mls antigens and it was found that the recognition by the TCR of the Mls antigens was determined by the TCR Vβ domain (1). Roughly at the same time, other groups discovered that certain bacterial toxins from various strains of *Staphylococcus aureus*, responsible for causing food poisoning, possessed properties similar to those of Mls antigens. These bacterial proteins were the staphylococcal entertoxins. They are recognized by a high frequency of T cells determined by the particular type of TCR Vβ domain (45, 1), much like that of Mls antigens. Scientists were somewhat perplexed as to any significance in the similarities between bacterial proteins and endogenous Mls antigens in mice until the true source of the Mls antigens was determined. A type B retrovirus transmitted through the mammary glands of mice was found to encode a superantigen within the 3' long terminal repeat of its genome (1) and was called mouse mammary tumor virus (MMTV). This discovery demonstrated the first correlation between bacterial enterotoxins and Mls antigens: both came from infectious sources.

Bacterial superantigens produced by the organism *Staphylococcus aureus* cause a number of disease states in humans, including food poisoning and shock, caused by

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staphylococcal enterotoxins A, B, C1, C2, C3, D and E; toxic shock syndrome, caused by toxic shock syndrome toxin; and scalded skin syndrome, caused by exfoliating toxins A and B (61). Of these, the enterotoxins have been best described and characterized of all exogenous superantigens. These are highly related proteins of intermediate size that share similar functions, but have been divided into two groups: SEA, E, and D in one and SEB, C1, and C3 in the other.

As previously stated, these staphylococcal superantigens activate their respective T cell subset by intimate interaction with the TCR Vβ domain of those T cells. Recent work has shown that these superantigens interact with an exposed region of the Vβ domain that appears to be a β-pleated sheet and also, the toxins engage both the TCR and MHC class II surface antigens that are found on a number of immune cells within the body. However, their initial target ligand is the MHC class II molecule itself (61) and its presence is essential for T cell activation by these superantigens. TCR affinity for the toxins is relatively low and is only increased after interaction with MHC class II (33). Work with the Raji B cell line has demonstrated a binding of SEA to certain portions of its MHC class II markers (64), but not to MHC class II negative mutant Raji B cells (67). Also, fibroblasts, which are not normally able to bind SEA, are capable of doing so after transfection with HLA-DR genes (67). It is believed that this interaction with class II MHC is not to bind the MHC molecule to the TCR, but rather to induce a conformational change in the toxin to increase its affinity toward the TCR Vβ region (33).

The ability of the staphylococcal superantigens to activate such a vast heterogeneity of T cell specificities lies in the way in which these toxins are presented to the body's immune system. When a nominal antigen is encountered by an antigen presenting cell, it is internalized and broken down into peptide fragments by proteolytic enzymes. The relevant antigenic determinant is then complexed with newly-synthesized MHC class II

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molecules with the entire complex finally expressed on the cell surface for presentation to the appropriate T helper cell (49). The MHC class II molecule is made up of a membrane-bound alpha and beta chain, each comprised of two extracellular domains (60). The most significant structure of this molecule bears two near-parallel α-helical structures that form a cleft which is supported by an extended β-pleated sheet (60). This forms the peptide binding groove where an antigenic peptide fragment is displayed for presentation. Encountering the antigenic epitope, along with the self MHC markers, provide the first activating signals for the T cell. In the case of superantigens, however, this entire process is much different. First, these proteins do not need to be processed by the antigen presenting cell and proteolytic fragments of these toxins will not activate T cells at all (34). Also, they are able to associate with various MHC class II alleles and isotypes from a number of different mammalian species (33). Another unique feature of superantigen presentation is the fact that they do not occupy the peptide binding groove of the MHC class II molecule when recognition by the T cell takes place. This has been seen by studies showing that SEB association with class II MHC molecules does not inhibit subsequent antigen presentation of hen egg lysozyme peptides to T cell clones (27) and further supported when it was found that the binding of TSST-1 did not prevent the binding of a HLA-DR-restricted peptide (50).

Although the mechanisms by which α/β CD4+ T cells are activated by staphylococcal superantigens has been well documented, the means by which these toxins interact with γ/δ T cells is less well understood. Recent work has shown, however, that γ/δ T cells are capable of proliferating in response to staphylococcal enterotoxins and also show cytotoxic activity, possibly by more than one pathway. Studies using human γ/δ T cells have demonstrated an ability for these cells to show proliferation (32, 94) in the presence of, and cytotoxicity toward, SEA-coated targets, restricted to those cells bearing the Vγ9

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receptor and only in the presence of MHC class II positive accessory cells. This type of response usually responds to only SEA, unlike α/β T cells which are known to respond to a wide variety of staphylococcal enterotoxins (87). Other findings have also shown that peripheral blood $V\gamma9+$ T cells were capable of lysing SEA-coated target cells. Interestingly, some of these cells possessed the IgG FcRIII and were capable of mediating antibody-dependent cellular cytotoxicity (ADCC) against SEA-coated target cells, but only in the presence of certain human sera. When this sera was depleted of anti-SEA antibodies, cytotoxicity of these T cells was inhibited (86). These works and others (79, 59) seem to indicate that responses of γ/δ T cells to SEA are restricted to those cells bearing the $V\gamma9$ chain, but others have indicated that this may not necessarily be the case (32, 94). A certain $V\gamma9$ subset was shown to expand by in vitro culture with a 1 to 3 kD protease-resistant fraction from a mycobacterial lysate at a degree of 1 out of every 6 cells. This response was dependent upon MHC class II+ presenting cells and overall, demonstrates a superantigenic capacity of certain mycobacterial ligands (79). This, along with the fact that $V\gamma9+$ cells are the most prevalent γ/δ T cell in the periphery suggest that these cells may be significant in fighting off bacterial infections.

In these processes, the response to SEA-coated target cells requires accessory or presenting cells positive for MHC class II as evidenced by inhibition of cytotoxicity or proliferation by addition of anti-MHC class II antibodies or removing accessory cells (86, 87, 79, 94, 59). However, γ/δ T cells obtained from peripheral blood and cerebrospinal fluid of patients with multiple sclerosis were able to proliferate in response to, and show cytotoxic activity toward, B cells pulsed with either SEA, SEB, or TSST, all in a non-MHC-restricted fashion (99). Most of what is known about the activity of γ/δ T cells toward staphylococcal enterotoxins has been derived from the human system. Little is

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known about this relationship in mice and nothing at the present time is known about superantigen response to DETC.

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CHAPTER 2

MATERIALS AND METHODS

Animals

All animals used in this study were 6-to-8 week old Balb/c female mice obtained from Charles River (Wilmingtion, MA). These mice were maintained in a licensed facility at Oklahoma State University.

Toxins

Staphylococcal Enterotoxin A (SEA, Toxin Technologies, Sarasota, FL) was applied to dorsal trunk skin and epidermal skin sections of Balb/c mice in DETC depletion studies. SEA was also added to whole epidermal cell cultures to induce production of cytokines. Biotinylated SEA (Toxin Technologies) was used in the immunofluorescent SEA binding studies of epidermal cell suspensions.

Media and Solutions

Phosphate-buffered saline (PBS, 0.15 M) was used to make up solutions of trypsin (type II from porcine pancreas, Sigma Chemical Company, St. Louis, MO), deoxyribonuclease I (DNase I, from bovine pancreas, Sigma), antibody dilutions, resuspension of lyophilized SEA and hydration of murine dorsal and ventral skin sheets. One liter of 0.15 M PBS contains 1.15 g. dibasic anhydrous sodium phosphate (Na_2HPO_4, Fisher), 8.0 g. reagent grade sodium chloride (NaCl), 0.2 g. potassium chloride (KCl, Fisher) in deionized water and pH adjusted to 7.2 before autoclave sterilization for 20 minutes at 15 psi. PBS

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with Tween 20 (Sigma) was used in washing steps in immunohistochemical staining of epidermal skin sections. PBS with 1% BSA (Fisher) and 0.05% NaN_3 (Sigma) was used in the immunofluorescent staining of epidermal cells.

Dimethyl sulfoxide (DMSO, Sigma) was used to resuspend SEA for use in DETC depletion studies or as an SEA control in the same experiments.

In immunohistochemical staining of DETC, skin sections were floated on 2 M sodium bromide (NaBr, Fisher Chemical) to separate the epidermis from dermis.

Cell washings in the generation of epidermal cell suspensions were performed in Cellgro RPMI 1640 tissue culture media (Fisher) supplemented with 5% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA), 100 U/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma), 0.1 mM MEM non-essential amino acids (Sigma), and 0.1 mM sodium pyruvate (Sigma). Epidermal cells were cultured in the same media with the addition of 2 mM L-glutamine (Sigma), 5mg/L gentamycin (Sigma) and 15% FBS.

Antibodies and Conjugates

Purified monoclonal anti-mouse CD90 (Thy-1.2, Pharmingen, San Diego, CA) and monoclonal anti-mouse γ/δ TCR (Pharmingen) were used as primary antibody in the immunohistochemical staining of DETC in fresh epidermal skin sections. Both of these monoclonal antibodies (MAb) are of the IgG isotype. Phycoerythrin (PE)-labelled anti-mouse CD90.2 (Pharmingen) was used in immunofluorescent binding studies of epidermal cell suspensions. Fluorescein isothiocyanate (FITC)-conjugated avidin (Accurate Antibodies, Westbury, NY) was used to verify biotinylated SEA binding to epidermal cells in the same studies.

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Epidermal Cell Suspensions

Body trunk hair was removed from mice by clippers and a depilatory before being sacrificed in an ether chamber. Dorsal and ventral body trunk skin was surgically removed and placed in a clean 100 mm x 15 mm Petri dish (Fisher), keeping skin rolled up to prevent drying out. Ears were removed, split with forceps and placed epidermis-side up on a fresh solution of 0.25% trypsin. The tail was cut off, split down one side by a scalpel and skin removed and also placed epidermis-side up on the trypsin solution. Dorsal and ventral trunk skin were then laid epidermis-side down in the clean Petri dish and the subcutaneous layer scraped away by a scalpel. After cutting the dorsal skin into two equal parts, the sections were placed epidemis-side up on the trypsin. Body skin, ears, and tail were then incubated for one hour at 37° C in the presence of 5% CO_2. Following incubation, skin and ear sections were transferred, epidermis-side down, to a 100 mm x 20 mm deep well Petri dish (Fisher). Immediately after removing the dermal layer, all sections were immersed in a 0.05% DNase I solution and rubbed with a blunt-tip glass rod to bring cells into suspension. The epidermal layer of the tail was separated from its dermal layer and rubbed with the glass rod. Three to four equal volumes of RPMI 1640 with 5% FBS were added to the suspension and the suspension aspirated approximately 20 times with a 60cc syringe (Fisher), then filtered through a Falcon 70 μm nylon mesh cell strainer (Becton Dickinson, Lincoln Park, NJ) into a Sarstedt 50 ml test tube (Sarstedt, Princeton, NJ). The cells were then pelleted in a Damon CRU-5000 centrifuge (IEC, Needham Heights, MA) for 8 minutes at 4° C and washed three times to remove all traces of DNase I. Cells were resuspended in 10 ml of tissue culture media and quantitated by trypan blue (Sigma) exclusion on a hemacytometer and adjusted to the desired concentration.

Immunohistochemical Staining of Epidermal Skin Sections

Hair was removed from the dorsal side of a female Balb/c mouse and the mouse then sacrificed in an ether chamber. Dorsal trunk skin was surgically removed and placed epidermal-side down in a clean Petri dish and kept hydrated with culture media or PBS. Skin was then cut into a desired number of 0.7 cm^2 sections and each placed in separate wells of a Falcon 24-well flat-bottom tissue culture plate (Becton Dickinson, Lincoln Park, NJ) containing three 1.3 cm antibiotic filter discs and 600 μl culture media. Subsequently, 25 μl of SEA or DMSO were applied to the skin sections. Sections were incubated for 48 hours at 37° C in the presence of 5% CO_2. After incubation, skin was floated on 2 M sodium bromide and incubated for 1 hour at 37° C with 5% CO_2. In a Petri dish of PBS, epidermis was removed from the dermis by scraping with a scalpel and then transferred to a new 24-well plate. Epidermal sections were rinsed with PBS and fixed in cacodylate-buffered formaldehyde for 15 minutes at 4° C. Hydrogen peroxide was added and skin sections incubated for 30 minutes on a plate shaker, washed three times with PBS-Tween and incubated for another 20 minutes with fresh hydrogen peroxide. Sections were washed again and blocked with 10% FBS for 20 minutes on a plate shaker at room temperature. After washing, epidermal sections were incubated overnight at 4° C with anti-mouse Thy-1.2 or anti-mouse γ/δ TCR. After incubation with the 1° Ab, sections were washed and incubated overnight at 4° C with biotinylated anti-mouse IgG from a Vectastain Elite Mouse IgG ABC kit (Vector Laboratories, Burlingame, CA). After washing, skin was incubated with Vectastain ABC reagent at room temperature for 15 minutes on a plate shaker. During this incubation, substrate solution was prepared from a Vector AEC peroxidase substrate kit or Vector VIP peroxidase kit (Vector), depending on the color desired. After the final washing, substrate solution was added and sections incubated for 5-15 minutes at room temperature until the desired color was achieved. Sections were

then mounted in glycerol on microscope slides and stained cells quantified with the aid of a epifluorescent microscope.

Double Immunofluorescent Staining of Epidermal Cells

An epidermal cell suspension was prepared from 1 Balb/c female mouse and resuspended in PBS-BSA-NaN_3 at a concentration of 1×10^7 cells per ml. One ml of the cell suspension was placed in a 15 ml test tube as the control and 1 ml placed in another test tube as the experimental group. Biotinylated-SEA (Toxin Technologies) was added to the experimental group at a final concentration of 10 μg/ml, while the control group received an equal amount of PBS. The tubes were placed on a tube rotor and rotated slowly for 2 hours at 4° C. Cells were subsequently washed twice with PBS and then fixed in cacodylate-buffered formaldehyde for 15 minutes at room temperature on the tube rotor. The cells were washed again and transferred to clean microcentrifuge tubes at a concentration of 1×10^7 cells per ml, spun at low speed (8500 rpm) in a table top BHG Z230 M high speed centrifuge (BHG & Co, Germany) for 15 seconds and the supernatant discarded. Cells were suspended in both 50 μl of avidin-FITC at 1:50 dilution in PBS-BSA-NaN_3 and 50 μl of PE-labelled anti-CD90.2 at 1:20 dilution in PBS-BSA-NaN_3 and incubated on a tube rotor for 30 minutes at 37° C. Cells were washed twice and each pellet resuspended in 50 μl of glycerol, mounted on microscope slides and percentages of labeled cells enumerated.

Cytokine Production by Epidermal Cells After SEA Application

An epidermal cell suspension was obtained, as previously described, from 1 Balb/c female mouse and cells resuspended at a concentration of 1×10^6 cells/ml. After the

correct cell concentration was obtained, 1 ml of the cell suspension was then plated out in a Falcon 24-well tissue culture plate (Fisher), in duplicate, for each of the time points for 12 hours, 24 hrs, and 48 hrs. SEA was then added to each experimental well at a final concentration of 5 μg/ml (50 μl) and 50 μl of PBS added to each control group. Supernatants for each group were taken at their respective time points and stored at -80° C until all points were taken. Once all supernatants were collected, 1.6 ml of each group was loaded into a 4 kD NanoSpin Plus microconcentrator (Gelman Sciences, Ann Arbor, MI) previously blocked overnight with a 1% BSA solution. Supernatants were then spun in a Sorvall RC 5B Plus centrifuge (Du Pont, Newtown, CT) at 6800 g for 2 1/2 hours at 4° C to obtain 4x concentrated samples. Concentrated supernatants were then analyzed for levels of IFNγ and IL-2 production (Genzyme, Cambridge, MA). Supernatants were also tested for IL-10 levels by ELISA (Genzyme), but were assayed directly from culture and were not concentrated.

Interferon-γ ELISA

IFNγ from epidermal cell culture supernatants was assayed by Intertest-γ Mouse Interferon-γ ELISA kit (Genzyme). This assay employed the multiple antibody sandwich principle, where a 96-well microtiter plate was precoated with monoclonal anti-mouse IFNγ. Tissue culture samples with the suspected IFNγ were added to the necessary number of wells in 100 μl volumes and incubated overnight at 4° C to allow IFNγ to bind to anti-mouse IFNγ mAb. Wells were washed four times with the provided wash buffer and 100 μl of biotinylated polyclonal anti-mouse IFNγ was added to each well and again, incubated overnight at 4° C. Washing was performed as before and horseradish peroxidase-conjugated streptavidin was added and incubated for two hours at room temperature to allow binding to the biotin. Wells were washed one final time and substrate reagent of

tetramethylbenzidine and buffered hydrogen peroxide added, then the subsequent enzyme-substrate reaction allowed to proceed for 15 minutes at room temperature. This reaction was stopped by the addition of 100 μl of 1 M sulfuric acid stop solution and the plate read in a Vmax kinetic microplate reader (Molecular Devices Corp., Palo Alto, CA) at 450 nm.

Interleukin-2 ELISA

Epidermal cell culture supernatants were assayed for IL-2 by the Intertest-2X Mouse Interleukin-2 ELISA kit (Genzyme). Tissue culture supernatants and IL-2 standards were added to wells precoated with monoclonal anti-mouse IL-2 and incubated overnight at 4° C. Addition of biotinylated polyclonal anti-mouse IL-2 followed and after overnight incubation at 4° C, streptavidin-peroxidase for 2 hours at room temperature. Finally, tetramethylbenzidine in buffered hydrogen peroxide substrate reagent was added for 10 minutes, the reaction stopped by 1 M sulfuric acid stop solution and read by ELISA plate reader at 450 nm. All steps involved 100 μl volumes and wells were washed 4 times with the provided wash solution between each incubation period.

Interleukin-10 ELISA

Epidermal cell culture supernatants were assayed for IL-10 by the Intertest-10X Mouse Interleukin-10 ELISA kit (Genzyme). Tissue culture supernatants and IL-10 standards were added to wells precoated with monoclonal anti-mouse IL-10 and incubated overnight at 4° C. Addition of biotinylated polyclonal anti-mouse IL-10 followed and after overnight incubation at 4° C, streptavidin-horseradish peroxidase was added for 2 hours at room temperature. Finally, tetramethylbenzidine in buffered hydrogen peroxide substrate

reagent was added for 15 minutes, the reaction stopped by 1 M sulfuric acid stop solution and read by ELISA plate reader at 450 nm. All steps involved 50 μl volumes and wells were washed 3 times with the provided wash solution between each incubation period.

Summary

The purpose of the present study was to first determine if Staphylococcal enterotoxin A (SEA) could induce dendritic epidermal T cell (DETC) depletion from murine epidermis as has been previously shown with Langerhans cells (80). The second objective was to examine secretion of IFNγ and IL-2 from murine epidermal cells after stimulation with SEA. It has been shown that SEA is able to induce elevated production of IL-1α and to a lesser extent, TNFα in epidermal cell suspensions (91). Also of interest was to determine if SEA was actually bound by the DETC itself. The results of these studies sought out a better understanding of the role of the DETC and how it may interact with other cells in the cutaneous immune response.

CHAPTER 3

RESULTS

The purpose of this study was to determine the physiological effects, if any, of Staphylococcal enterotoxin A (SEA) on dendritic epidermal T cells (DETC) found in murine epidermis and in doing so, help to elucidate the function of DETC within the epidermis. It has already been known that SEA can cause significant depletion of Langerhans cells (LC) from the epidermis (80) and is also capable of inducing non-T cell cytokine secretion from murine epidermal cell suspensions (91). The objective of this study was to determine if SEA could have similar effects on DETC. All data obtained in each investigation was pooled from three separate experiments and expressed as the mean $\pm$ the standard error of the mean for each set of data. The student t-test was also performed to determine statistical significance between control and experimental groups.

Effects of SEA on DETC Depletion

As previously stated, SEA is very effective at causing Langerhans cell depletion from the epidermis (80). Other evidence from the same lab has suggested that these cells do, in fact, migrate out of the epidermis and through the underlying dermis and subcutaneous layers (Kuschell and Burnham, unpublished results), as IA+ cells were detected in these regions, in a time-dependent manner, following SEA treating of the skin. To determine whether or not DETC possess this same ability upon SEA stimulation, dorsal skin sections were treated with 100μg/ml of SEA, incubated for 48 hours at 37° C in the presence of 5% CO_2 and then stained for the γ/δ TCR surface antigen by peroxidase and Vector VIP substrate. As Figure 1 shows, SEA application on murine epidermis does not

deplete DETC. The original procedure with Langerhans cells was repeated to verify the stimulatory properties of the SEA and the results shown side-by-side with the DETC studies. Statistics performed, by way of the student t-test, verified that the differences in the results from the control group and SEA group were not statistically significant. The γ/δ TCR monoclonal antibody used in the immunohistochemical staining of the epidermal tissue sections was chosen due to the fact that although, on extremely rare occasions, an α/β+ T cell will migrate up into the epidermis, the DETC is the only cell in that compartment possessing a T cell receptor of the γ/δ variety (16). To corroborate the results given in this first experiment, the study was repeated, but a mouse anti-Thy-1.2 monoclonal antibody was used this time to visualize the DETC. Langerhans cells were not stained for in this experiment, since the SEA was proved to have been effective against it in the first depletion experiment utilizing mouse anti-γ/δ TCR as the DETC marker. The results from this follow-up experiment are displayed in Figure 2. As the figure shows, there is no depletion of Thy-1+ cells from the epidermis after SEA application to the skin. Again, the student t-test was utilized and it was determined that the numerical differences between the control and experimental groups was not statistically significant. The overall average numbers of cells per mm^2 were slightly lower in this instance than in the results from Figure 1 and may be caused by a difference in receptor density between the γ/δ TCR surface antigen and Thy-1, but was not verified. The cell number differences between the two experiments was not however, statistically significant as evidenced by the student t-test.

Although it was shown that DETC were not depleted from sections of murine skin *in vitro*, an experiment had not been attempted to ascertain the possibility that these cells would respond differently to SEA *in vivo*, and so, see reduced numbers. On the other hand, certain preservatives used in topical medications and foods possessed the ability to

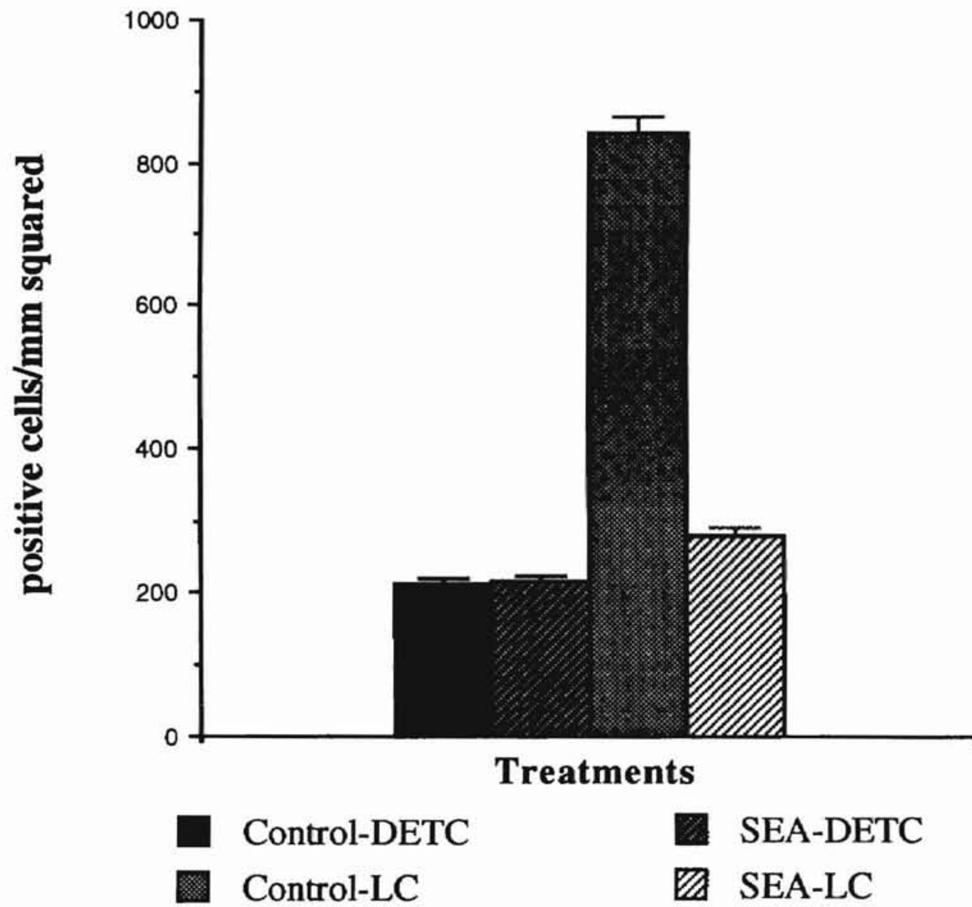

Figure 1. Effects of SEA on the depletion of Langerhans cells and DETC from murine epidermis. The values shown represent the mean ± SEM determined by pooling the data from three separate experiments.

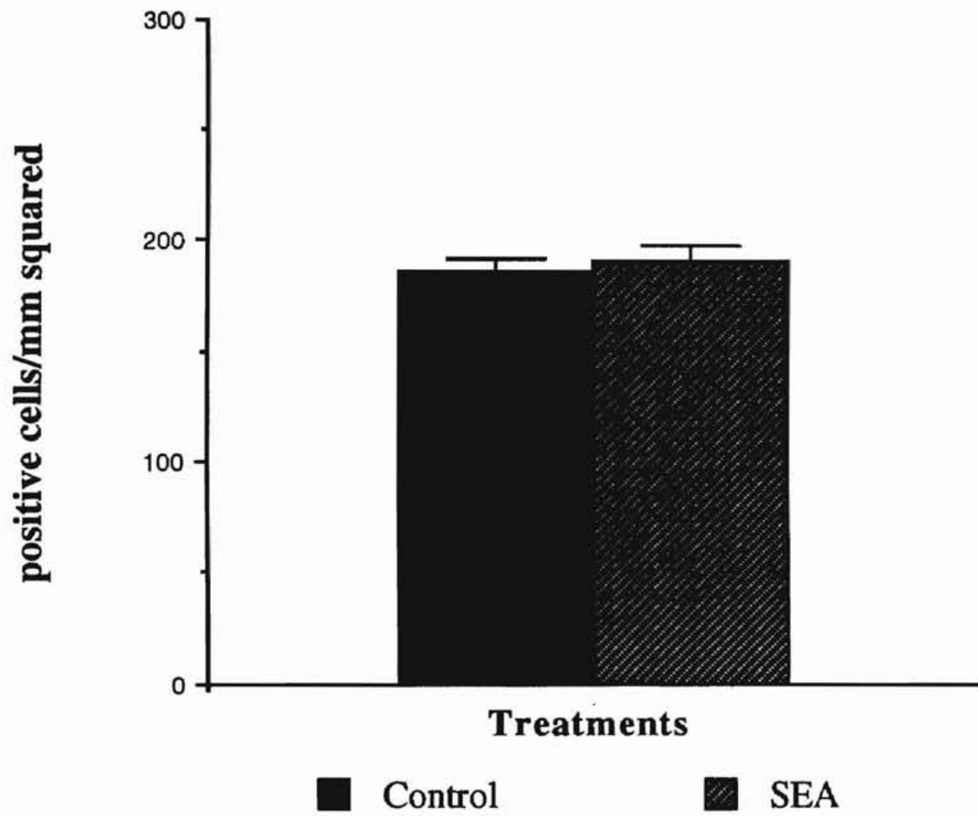

Figure 2. Effects of Thy-1.2 positive DETC depletion from murine epidermis following SEA application. The values shown represent the mean ± SEM determined by pooling the data from three separate experiments.

increase the number of resident DETC in vivo (82), and although SEA is not structurally similar to any of those chemical compounds, the possibility that SEA may actually increase the number of DETC had not been explored. To establish the possibility of a significantly increased or decreased number of DETC upon *in vivo* application of SEA, 25 μl of SEA at a concentration of 100 μg/ml was painted onto bare dorsal trunk skin of a female Balb/c mouse once per day over a period of four days. The mouse was sacrificed, the treated skin cut into sections and stained with mouse anti-Thy-1.2 monoclonal antibody, as previously described. As the results show in Figure 3, the number of DETC present in SEA and control groups was comparable. However, there was a slightly more noticeable decline in the SEA group's numbers, but in comparing the two groups by the student t-test, differences were determined to not be statistically significant.

Therefore, unlike Langerhans cells, DETC do not appear to migrate, or are not depleted, from murine epidermis following stimulation with SEA. These results may not be surprising, however, since the role played by the LC is that of premier antigen presenting cell of the epidermis. Currently, the functional role of the DETC is not known and their ability to interact with or respond to superantigen has not been previously explored. These data demonstrate that SEA shows no significant ability to affect the number of Thy-1.2+ or γ/δ TCR+ cells in murine epidermis.

Binding of SEA to Thy-1 Positive Epidermal Cells

One of the significant questions to be asked in determining the ability of DETC to play a role in SEA-induced immune responses addresses whether or not DETC have receptors for the toxin itself. It has been shown that in Langerhans cell migration caused by SEA stimulation, IL-1α and to a lesser extent, TNFα may be the primary signals that initiate this migratory response (90). Although the application of SEA to mouse skin

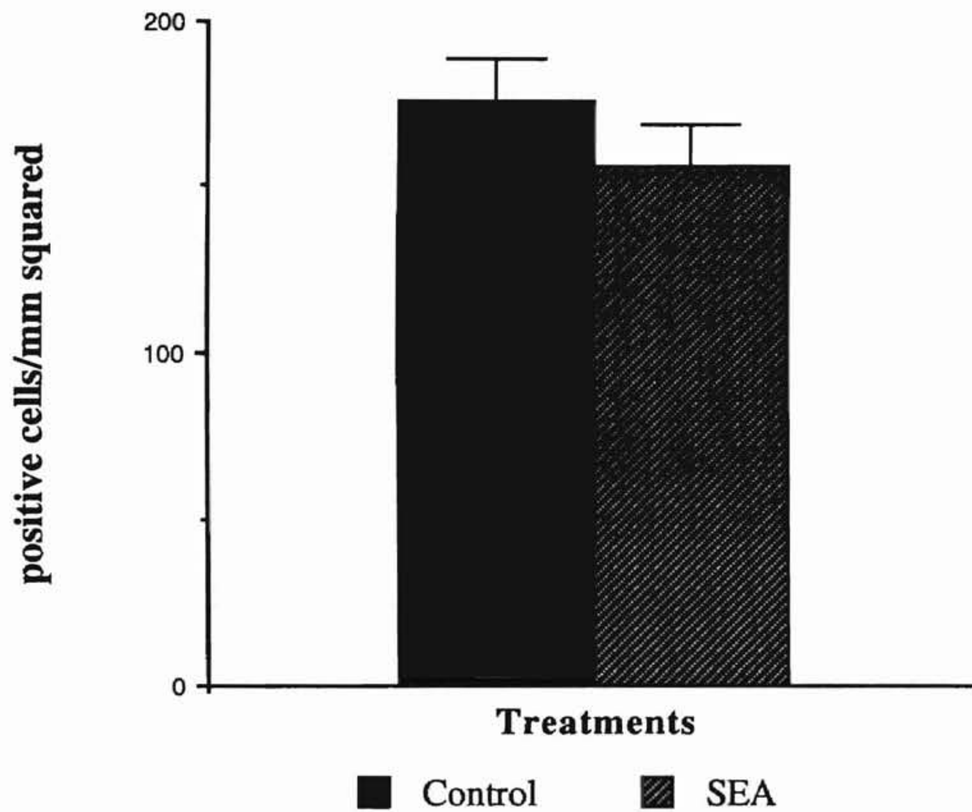

Figure 3. Effects of DETC depletion from murine epidermis after in vivo application of SEA. The values shown represent the mean ± SEM determined by pooling the data from three separate experiments.

induces LC migration from the epidermis, it is still not known whether or not the toxin actually does bind to the cell.

To determine the binding affinity of DETC toward SEA, whole epidermal cell suspensions were incubated with biotinylated SEA, fixed in cacodylate-buffered formaldehyde and incubated with avidin-FITC and phycoerythrin-conjugated mouse anti-Thy-1.2 monoclonal antibody. Cells were then mounted on microscope slides and fluorescent cells enumerated at 400x total magnification on a fluorescent microscope. The percentages of both single- and double-labelled cells are shown in Table 1. Those cells which bound SEA were determined to be those that displayed a bright optic yellow color under the appropriate filter. Numerous cells portrayed a dull, faint yellow color, but only those cells giving off a color many magnitudes higher were counted as positive. PE-labelled cells excited under the appropriate filter as a brilliant red. Again, a number of cells gave off a faint reddish color, but only those few of a great intensity were recorded as being positive. Also, when both FITC- and PE-positive cells were counted for a field of view, the number of double-positive cells was determined. As Table 1 shows, no cells were ever found to be both FITC- and PE-positive. Interestingly, out of the many cells that gave a somewhat background or low level of yellow color, no cell found to be Thy-1.2+ displayed any level of this color yellow color, whatsoever. Hence, there were no cells that were determined to bind SEA that were also positive for the DETC surface marker, Thy-1.2. In both groups, that which was incubated with SEA and that which was incubated without, the percentage of total epidermal cells expressing the Thy-1 surface marker was approximately 1%, which was consistent with the average number of DETC found in the epidermis (10, 111). Those cells binding SEA were not stained for any other surface markers, so whether they were Langerhans cells or keratinocytes remained undetermined.

Table 1

Binding of SEA to Thy-1+ epidermal cells as shown by immunoflourescence microscopy

	Average number of cells per trial	Percentage of FITC-positive cells	Percentage of PE-positive cells	Percentage of FITC and PE positive cells
Control Group (No SEA)	1,184.67	$0.06 \pm .04$	$1.12 \pm .18$	0
Experimental Group (SEA added)	1,738.33	$0.56 \pm .09$	$0.94 \pm .14$	0

* Biotinylated SEA used and captured with avidin-FITC (floursceinisothiocyanate) added

* PE (phycoerythrin)-labeled anti-mouse Thy-1.2 monoclonal antibody

IFNγ Secretion by Whole Epidermal Cell Suspensions

Previous studies have shown that epidermal cell subpopulations are capable of cytokine secretion upon stimulation with SEA (91). To determine if epidermal cells could secrete levels of IFNγ, whole epidermal cell suspensions were obtained at a concentration of 1×10^6 cells/ml and plated out in a 24-well tissue culture plate at 1 ml per well. The experiment was carried out over 48 hours, with 12, 24, and 48 hour time points. SEA was given to each well at a final concentration of 5 μg/ml for the experimental group, and PBS only for the control, and tissue culture supernatants harvested at the appropriate time point. After supernatants had been collected at each time point, IFNγ levels were analyzed for both groups by ELISA. The experiment was repeated three times and the results averaged. These results are displayed in Figure 4. As the figure shows, levels of IFNγ, in pg/ml, increased over the course of the 48 hour period, but the control groups increased, as well. This was consistent throughout repeated experiments. With the fear of possible lipopolysaccharide (LPS) levels in the media, due to bacterial contamination, stimulating the epidermal cells, the experiment was repeated with a certified pyrogen-free media, but levels of IFNγ in control groups were not diminished (results not shown). Again, the experiment was repeated, but after the normal RPMI media, used for cell culture, was run over an endotoxin-depleting column in another attempt to remove possible pyrogen contamination. Again, IFNγ control levels remained high (results not shown).

Although both SEA and control groups were increased in their levels of IFNγ production over the course of the 48 hours of the experiment, the levels in the control group did tail off somewhat at 24 hours and did remain lower than the levels recorded from the SEA group. However, results from analysis of the data by the student t-test showed that the differences between the SEA and the control groups were not statistically significant.

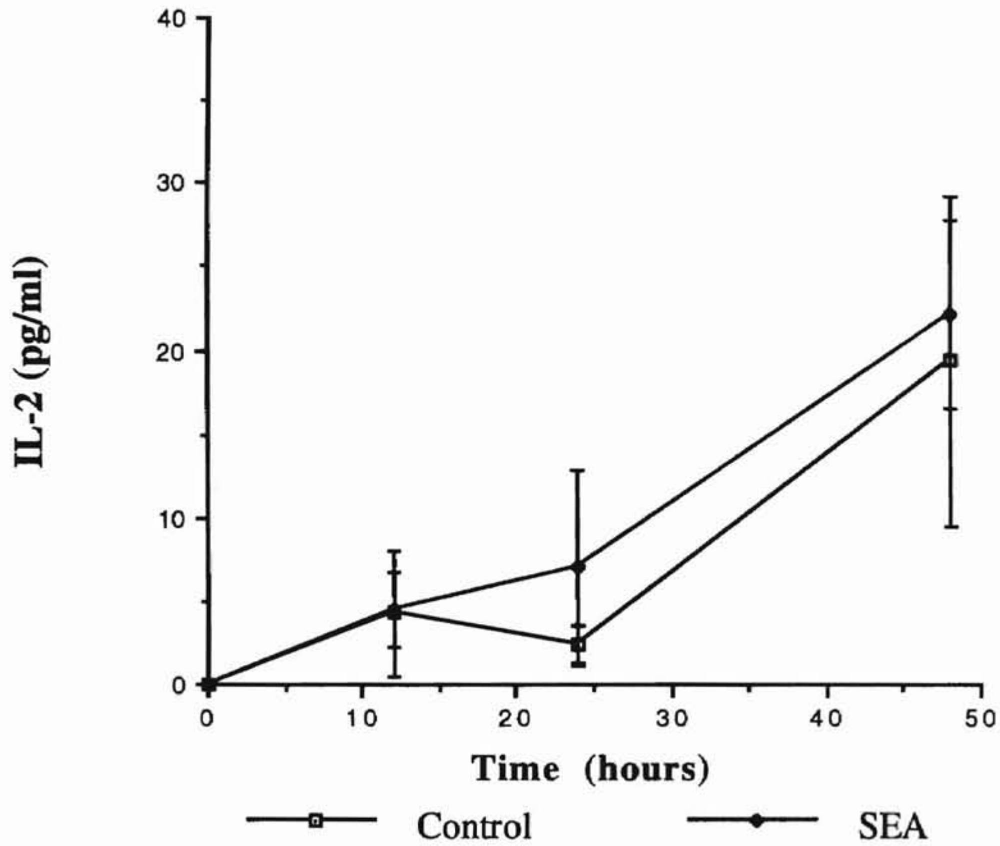

Figure 4. SEA-induced IFNγ production by murine epidermal cells. The values determined represent the mean ± SEM for each time point by pooling the data from three separate experiments.

Since DETC are the only cell in the epidermis capable of secreting IFNγ (64, 105), it was determined that in the culture conditions of the experiment, DETC are capable of secreting increasing levels of IFNγ over a period of time, at least for 48 hours. However, it was not determined what the nature of the stimulating factor was since IFNγ levels consistently rose in the control group, which was not treated with SEA, as well as the SEA group itself.

IL-2 Secretion by Whole Epidermal Cell Suspensions

The second T cell cytokine examined for its ability to be produced by epidermal cells after SEA stimulation was IL-2. As with the experiments set up to look for levels of IFNγ production, epidermal cell suspensions were generated, plated out in a 24-well tissue culture plate, SEA added to one group, and tissue culture supernatants collected at three time points over a 48 hour period. Finally, supernatants were examined for levels of IL-2 by ELISA and the results displayed in Figure 5. The results show that small amounts of IL-2 are produced in increasing fashion over time, although those levels did not quite reach the quantity of IFNγ produced in the prior experiment. These experiments were also performed three times and the results from each time point averaged. The figure shows, much like the ELISA studies on IFNγ, that IL-2 levels increase over time in both the SEA and the control groups. Experiments using certified commercial pyrogen-free media and endotoxin-depleted media were performed, but the control levels observed remained similar to those previously observed (results not shown).

Although IL-2 levels in the control group increased with the SEA levels, they too, remained lower than the pg/ml levels expressed by the SEA group after 24 hours onward. The student t-test was performed, but the differences between the two groups was not shown to be statistically significant.

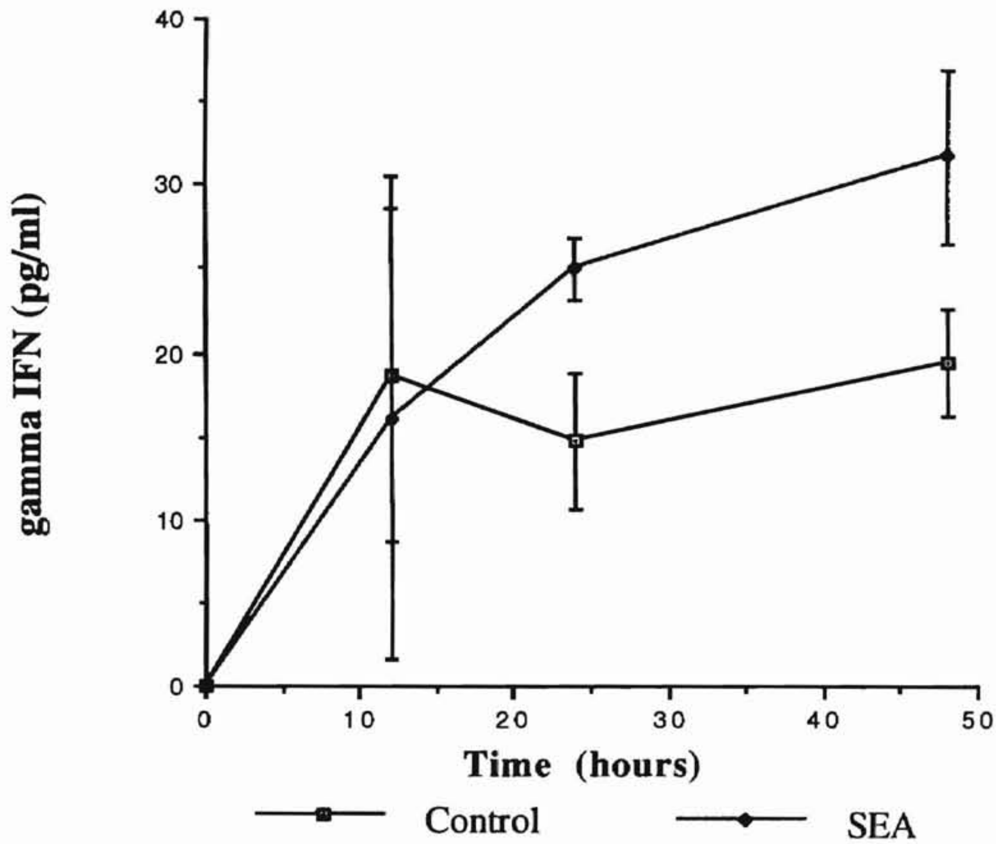

Figure 5. SEA-induced IL-2 production by murine epidermal cells. The values determined represent the mean ± SEM for each time point by pooling the data from three separate experiments.

Like IFNγ, IL-2 can only be produced by a single epidermal cell type -- the DETC -- and the results show that IL-2 can be produced by DETC over a 48 hour period in culture, but the source of stimulation remains to be elucidated.

IL-10 Secretion by Whole Epidermal Cell Suspensions

In addition to IFNγ and IL-2, whole epidermal cell suspensions were exposed to 5 μg/ml levels of SEA over a 48 hour period and assayed for IL-10 by ELISA. In three separate experiments, it was determined that there were negligible levels of IL-10 produced in whole epidermal cell suspensions throughout the duration of the 48 hours of the experiment. These results were consistent in both experimental and control groups. As Figures 6 and 7 demonstrate, there is a propensity for the Th1 cytokine profile in epidermal cell suspensions in these studies, as shown by the accumulation of IFNγ and IL-2, while a shift away from the Th2 profile was demonstrated by a lack of IL-10 production.

Summary

The results from the investigations undertaken in this study have determined a number of things about the interaction between dendritic epidermal T cells (DETC) and the bacterial superantigen, Staphylococcal enterotoxin A (SEA). First, unlike Langerhans cells (LC), DETC are not depleted from the epidermis after exposure to SEA. When SEA was painted on mouse skin and stained for DETC cell surface markers Thy-1.2 and γ/δ T cell receptor, there was not any DETC depletion. Even *In vivo* experiments where daily painting of SEA on a live mouse was performed for four consecutive days, there was no

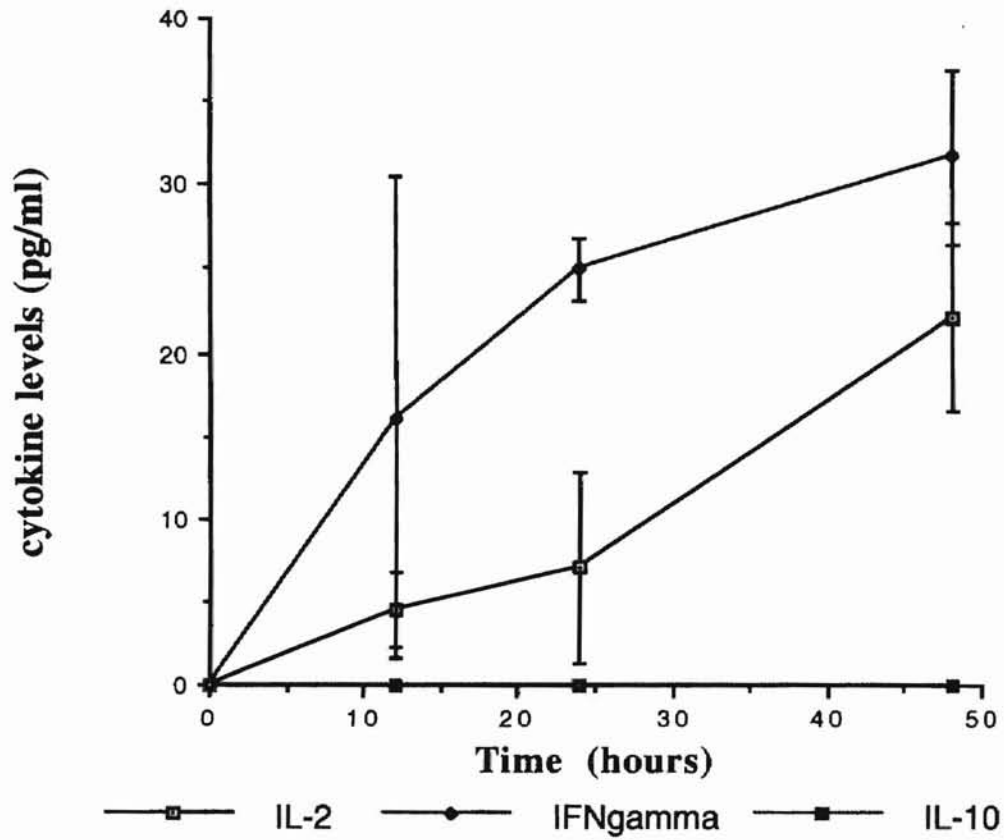

Figure 6. Overall cytokine secretion by murine epidermal cells following SEA stimulation. The values determined represent the mean ± SEM for each time point by pooling the data from three separate experiments.

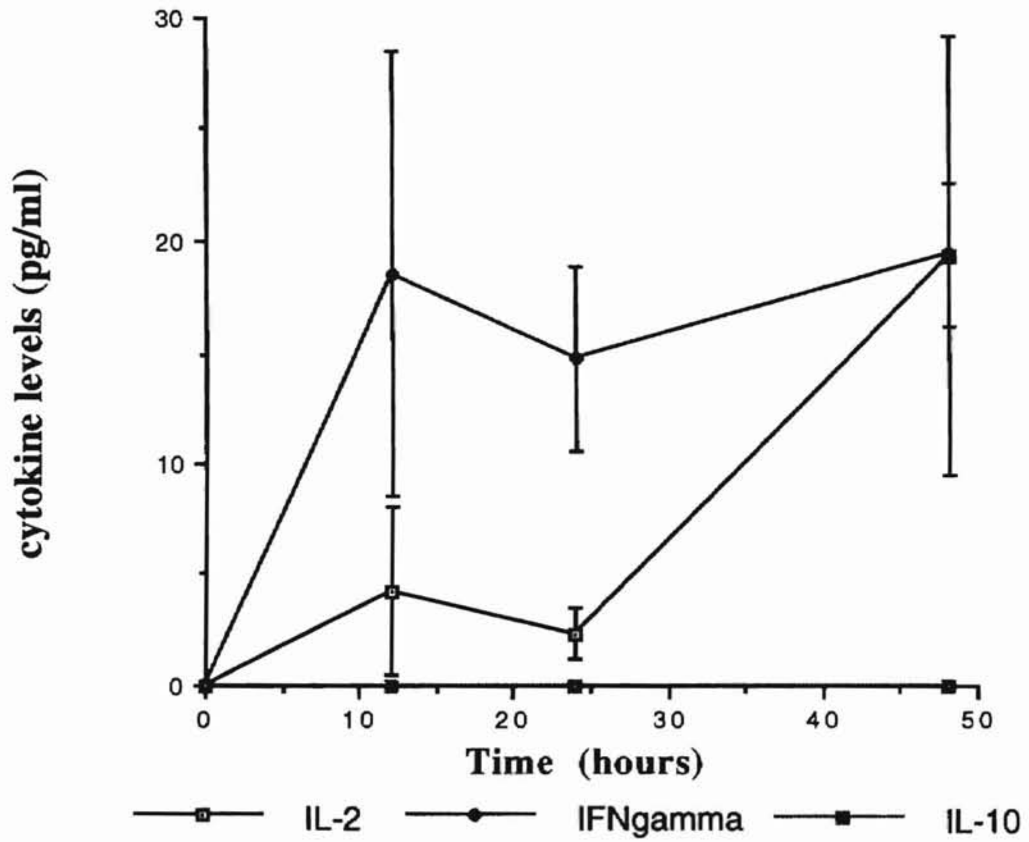

Figure 7. Overall cytokine secretion by murine epidermal cells in culture without SEA application. The values determined represent the mean ± SEM for each time point by pooling the data from three separate experiments.

statistically significant reduction in the number of Thy-1.2 positive cells from the epidermis. Double fluorescent staining experiments demonstrated that no DETC, detected by phycoerythrin-labelled mouse anti-Thy-1.2 monoclonal antibody, were able to bind biotinylated SEA, which was detected by conjugating with avidin-florosceinisothiocyanate (FITC). No cell was detected for simultaneous binding by both florescent markers. In whole epidermal cell suspensions, DETC were able to produce IFNγ and IL-2, but no IL-10, over a 48 hour period after initial stimulation by SEA. Although pg/ml levels of IFNγ and IL-2 were relatively low, both increased over time. However, both SEA and control groups increased with the progression of the experiment. The cytokine levels from the control groups of both IFNγ and IL-2 remained lower than its corresponding SEA group, but they were still increased and the differences determined to be statistically insignificant. Experiments were performed to determine the possibility of cell stimulation by bacterial lipopolysaccharide contamination in the tissue culture media, but results proved that this was probably not the cause of the cytokine production in the control groups of both experiments. The true cause of this stimulation remains unknown.

CHAPTER 4

DISCUSSION

The dendritic epidermal T cell (DETC) exists as a resident dendritic cell in murine epidermis which constitutes a part of the cutaneous immune response system in mice. It has been a curious phenomenon that this cell population should exist as a resident epidermal cell bearing a γ/δ T cell receptor which is found in no other mammal than mice. First reported in 1983 (10, 111), it was not until the discovery of the T cell receptor (53) on the DETC that researchers began to ask what role, if any, this cell played in the cutaneous immune response. A number of characteristics have been attributed to DETC, both in vivo and in vitro, since their discovery, but their true physiologic role remains unknown. Since this cell has only been found in the murine system, the true immunologic role of DETC, and especially how DETC interact with the other cellular components of the skin immune system, is of great interest.

Previous studies using bacterial superantigens -- specifically, Staphylococcal enterotoxins -- have been effective in elucidating epidermal Langerhans cell cytokine production, migration and possible mechanisms for how this migration is initiated (80, 90, 91). It is known that these superantigens have a great capacity to stimulate peripheral α/β T lymphocytes by their novel interaction with only the Vβ domain of the α/β TCR. Thus, much of the molecular mechanisms behind this interaction are now understood. However, superantigen interaction with γ/δ T cells is not well understood and studies in this area have been fewer in number than those of their α/β counterparts. The work that has been done in the area of γ/δ T cell-superantigen interaction has been done in the human system and no studies to date have attempted to determine what effects Staphylococcal superantigens have on DETC.

The purpose of this study was to use Staphylococcal enterotoxin A (SEA) to alter the physiology of DETC in an attempt to better understand their physiologic function in the skin. Moreover, we wanted to use superantigens as a model for looking at how DETC may interact with the other immune cells within the epidermis, namely Langerhans cells and keratinocytes, and what their possible role may be in a cutaneous immune response.

Being the premier antigen-presenting cell in the skin, the Langerhans cell (LC) must pick up foreign antigen that has compromised the skin's outer layers and present it to the proper T cell clone to induce a contact hypersensitivity response. To accomplish this, they must travel out of their resident environment and migrate to draining lymph nodes where antigen presentation can take place. Previous studies have shown that staphylococcal enterotoxins are capable of inducing a migratory response in LC after toxin painting on dorsal trunk skin of Balb/c mice (80), which was similar to results obtained after UVB exposure of murine epidermis (18). In these studies, staphylococcal enterotoxin A exhibited the greatest effect on LC depletion. Although UVB-induced depletion of LC shows similar effects as that of SEA-induced LC depletion, DETC are not depleted from the skin after UVB exposure, but hapten painting on mouse skin, followed by UV exposure resulted in a number of hapten-conjugated cells in draining lymph nodes that were Ia- but Thy-1+ (75). The same toxin was used in this study to determine if DETC could be depleted from murine epidermis in a manner similar to LC. After SEA painting on murine skin sections and immunohistochemical staining for γ/δ TCR surface antigen, it was demonstrated that, contrary to LC, DETC were not depleted in any way from the epidermis by this toxin. These results were confirmed by repeating the experiment with Thy-1 as the DETC staining marker. In this second experiment, there were no significant differences between the number of DETC in the group that received SEA and the control group that received only DMSO, but there was a higher number of stained cells, overall, in the groups that were

stained after capture by the anti-γ/δ TCR antibody. This could be the result of a slight difference in the number of T cell receptor antigens on the surface of DETC as opposed to that of the Thy-1 marker. It is also possible that the antibody for the γ/δ T cell receptor had a slightly higher affinity for its epitope and so, was able to bind more quickly, or to a higher degree within the time frame of the antibody incubation step. Regardless, both of these markers are highly selective for DETC in the epidermal environment and the DETC is the only cell in this region to be found with a γ/δ TCR. There are occasional α/β+ CD8+ cells that make their way into the epidermal layers that might be counted along with DETC in these experiments, as they are also Thy-1+. However, this would be a highly unlikely source contributing to the difference in DETC numbers between the two experiments, since it was the group stained for γ/δ TCR that displayed the higher numbers, not those stained for Thy-1. In the in vivo study, our goal was to determine if SEA had the capacity to reduce the number of DETC in the living system or conversely, to determine if SEA could cause a proliferation of DETC. It has been suggested that certain topical substances applied to mouse skin could cause an increase in the population density of resident DETC (82). Although these substances are structurally unrelated to SEA, they do behave somewhat like pertussis toxin, which possesses the capacity for polyclonal T cell activation. All of the results from this previous study were evident within five days, which was the basis for the five day time period of our in vivo DETC depletion study. The results from this experiment show that the density of Thy-1+ DETC in murine epidermis after SEA application in vivo remains consistent with those results obtained from the same experiments conducted in vitro. However, Figure 3 shows that the number of DETC per mm^2 in the in vivo SEA group was slightly lower than that of the control group. The student t-test was performed on these data and determined that the differences between the two groups were not statistically significant. It is possible though, that the continual

application over the four day period was able to cause an affect in the cells that a single dose was not able to induce. The numerical differences between the groups might be reduced if the number of trials was increased, as standard deviation usually decreases with the increase in the number of data sets.

Overall, the results have shown that unlike LC, DETC numbers are not reduced in the skin by SEA application, but this may be consistent with the role of this cell. Although, the true role of the DETC has not been elucidated, one popular theory is that DETC are highly involved in the process of immune surveillence within the epidermis (8, 107, 108) as evidenced by their ability to show non-MHC restricted cytotoxicity toward a number of tumor targets (70, 48, 74). Cells in the epidermis that were transformed would display altered self antigens on their surface and damaged cells may produce certain stress signals that the DETC would react to so as to rid the body of these cells before they compromised the immunologic integrity of the animal. This would be further supported by the limited diversity of the T cell receptor of DETC, implying that this cell recognizes a single antigen or highly conserved set of antigens. Since this cell is sessile, this antigen would have to be found in a much higher degree in the skin than in the periphery for DETC to be effective in playing out its physiologic role. In light of this, immune surveillence would be a role consistent with the properties of DETC. It is possible that DETC are not stimulated by SEA and even if this were, in fact, the case, migration out of the skin may not be how DETC would react to such a stimulus. With the highly conserved TCR of DETC, it may be very likely that SEA is not an antigen that it recognizes.

While LC modulation by SEA has demonstrated a migratory ability in tissue sections, it has also proven effective in cytokine induction of epidermal cells. Previous studies have indicated increased levels of IL-1α and TNFα in epidermal cell suspensions that have been stimulated by SEA (90). These cytokines were, themselves, implicated in the

induction of LC depletion from the epidermis since depletion could be inhibited by antibodies specific for these cytokines (90). One of the focuses of this study was to examine T cell cytokine secretion in epidermal cell suspensions in the presence of SEA. It is known that DETC have the capacitiy to produce IFNγ and IL-2 (41, 106, 64, 105), although it is unclear as to whether or not they produce IL-10, as well. Our studies focused on the SEA-induced secretion of these three cytokines by epidermal cells, but results suggest that the cytokine production observed in these epidermal cell cultures did not require stimulation by SEA. Over a 48 hour period, levels of INFγ and IL-2 accumulated in increasing amounts, both SEA-treated and control groups. The student t-test was performed and it was determined that the differences between the SEA and control groups were not statistically significant. These results were consistently seen in three separate experiments and it was first thought that bacterial pyrogen contamination may have contributed to the elevated cytokine levels in the controls. However, a repeat of these experiments first using certified pyrogen-free tissue culture media and second, using media which had been passed over an endotoxin-depleting column proved ineffective at bringing control group levels down. It is possible that the conditions for obtaining the cell suspensions could be contributing to the production of activating factors responsible for the level of cytokine production observed in these studies. During epidermal cell suspension preparation, tissue is floated on a trypsin solution and the epidermis mechanically disrupted by glass rod scraping and syringe aspiration. It is very likely that during this preparation, a number of cells are damaged or destroyed. These cells could be producing substances such as heat shock proteins in response to this stress since it has been hypothesized that DETC play an active role in immune surveillance by recognizing heat shock proteins expressed by damaged or transformed cells (4). Also, it has been shown that DETC are able to respond to certain Mycobacterial antigens that are considerably similar to heat shock

proteins (66, 73). It may be possible that breakdown products of collagen, elastin or other cellular debris from damaged epidermal cells released into the culture may be activating either DETC directly or indirectly through the induction of cytokine release from LC or keratinocytes. It has been shown that cell separation techniques involving the passing of cells through glass wool and/or nylon columns could cause lymphocyte activation (113). Therefore , the culture techniques used here may result in the activation signals to DETC for cytokine secretion. Another possibility is that these cytokine levels are constitutive, but this is probably unlikely, especially with IL-2 since it is usually only found after cell stimulation of normal circulating T lymphocytes (93) and DETC (64, 72). Although SEA was not the likely source of the activation of these cells, the cytokine pattern expressed is consistent with a cell-mediated immune response which is expressed in the skin (100). Both IFNγ and IL-2 are of the Th1 cytokine profile, which promote a T cell-mediated immune response (30, 93). It is known that within the dermis and epidermis, only CD4+ and CD8+ T lymphocytes have been found -- as well as DETC in mice. There is a complete lack of B cells within this region (100). Also, contact hypersensitivity falls under the class of Type IV (delayed) hypersensitivity, which is the only type of hypersensitivity which is cell mediated (83). The IL-10 studies included in these experiments further support this cell-mediated theory, since IL-10 is a Th2 type cytokine and its activities include the downregulation of Th1 type immune responses (83, 68).

The investigations previously described in this study have demonstrated an inability of SEA to affect particular functions of DETC. One of the basic questions that must be addressed in examining a role of DETC modulation by SEA is whether or not SEA binds directly to the cell itself. In a further attempt to determine whether or not any interaction exists between SEA and DETC, binding studies were performed using immunofluorescent antibody staining. The results determined that no epidermal cells were simultaneously

positive for Thy-1.2 surface antigen and SEA binding. In other words, cells either bound SEA or anti-Thy-1.2 antibody, but not both. This would suggest that DETC do not possess a receptor specific for SEA. If this is true, the results from the depletion studies and cytokine production studies would make perfect sense since SEA was determined in these experiments to be unable to bind to DETC. The interactions between staphylococcal superantigens and normal circulating α/β T lymphocytes has been well-studied and documented. However, many areas in γ/δ T cell-superantigen interactions remain unclear. Furthermore, most superantigen studies performed with γ/δ T cells have been in the human system and not in mice. Those studies have demonstrated, with some consistency, a capacity for staphylococcal enterotoxins to activate certain γ/δ T cell clones. Most work in this area has shown that clones bearing the $V\gamma9$ designation are the most reactive toward these toxins, SEA in particular (86, 87, 79, 59). They also appear to act independent of the J and C region of the γ chain or the δ chain altogether (59). There is some evidence, however, that T cells with γ chain designations other than $V\gamma9$ can be activated by SEA and that SEA is not necessarily the only staphylococcal superantigen that $\gamma\delta$ reactive T cells respond to (86, 99). If this is the case, DETC might be expected to exhibit reactivity toward SEA. The TCR on DETC are highly conserved, to the point of being virtually monomorphic and nearly all of these DETC bear γ chains of the $V\gamma3$ designation (42, 5, 4, 108). In light of this, it would make sense that these studies would provide no positive results in the modulation of DETC by SEA when only $V\gamma9$+ T cells seem to be activated by SEA. The interpretation of these results would only hold true, however, if they could be carried across the human-mouse species barrier since all of the data implicating $V\gamma9$ as the lone target for SEA on $\gamma\delta$ T cells has been demonstrated in humans. There is certainly structural variability between homologous γ chain designations in mice and humans and so, the assumption carried over from the human studies that DETC should not become

activated in any way by SEA may not be valid. Conversely, it is known that staphylococcal superantigens can stimulate T cells across species barriers (61), so SEA reactivity with DETC may still be feasable. If direct interaction of DETC with SEA does not occur, there is still a possibility for DETC to be activated by signals from other cells within its environment when they have been activated by the toxin. It is known that IL-7 and TNFα promote the growth of DETC (63) and IL-1 has been shown to have a proliferative effect (110) on these cells, as well. All of these cytokines are produced by cells that share the epidermal microenvironment with DETC (65) and it has been shown that at least IL-1α and TNFα can be induced in epidermal cells by SEA stimulation (91). The possible indirect effect of DETC stimulation or activation by SEA is unlikely in the present studies, though, since results in each independent study were negative. Without observable effects on DETC in these studies, no source for modulation need be explored. However, no dose response curves were persued in the migration and cytokine studies and it is possible that DETC may be modulated by SEA, but only in appropriate concentrations, which were not employed in these studies. In the depletion studies, SEA was applied to skin sections at a concentration of 100 μg/ml, although it is difficult to say how much of the toxin is penetrating the outer epidermis to reach the suprabasilar compartment occupied by DETC. This concentration applied to skin was sufficient, though, to induce significant depletion of LC from the epidermis (80). Also, SEA-induced cytokine secretion by LC was most effective at a final concentration in culture of 5 μg/ml (91). This same concentration of SEA was employed in the present cytokine studies based on the prior success in using it with LC. This, too, may be an improper concentration for inducing cytokine secretion by DETC, though. One experiment was performed using 10 μg/ml SEA for DETC cytokine induction, but showed results no different than those described here (data not shown). Concentrations of SEA lower than 5 μg/ml were not used, but it may be possible that only

significantly lower concentrations of SEA would yield positive results. Studies with human γδ T cells determined SEA ranges of 0.01-1 μg/ml to be most effective (86, 87, 99) and similar concentrations of SEA may be needed to induce a response in DETC.

In summary, DETC numbers were not reduced in the epidermis, either in vitro or in vivo, by SEA application to the skin, although identical applications of SEA does significantly reduce the number of Langerhans cells. It is entirely possible that even if SEA were found to bind to DETC that it would not cause them to deplete or migrate from the epidermis, as their main function may not require such action. DETC are the only epidermal cells capable of producing IL-2 and IFNγ and our ELISA studies showed that they are capable of secreting both of these cytokines, over time, in culture. Also, these results support that fact that T cell responses within the skin are of the Th1 variety and IL-2 and IFNγ fall under the Th1 cytokine profile. This is also supported by the absence in production of IL-10, a Th2-type cytokine and would be consistent since the expression of either cytokine class is normally mutually exclusive in relation to the other (83). However, the cytokine production observed was probably not due to SEA, but some other stimulatory factor in culture since experimental and control groups of both cytokine studies exhibited increased cytokine production over the course of the 48 hour study. Contaminating bacterial endotoxins were ruled out as a stimulus of cytokine release through experimentation, but cells stressed or mechanically damaged through the protocol could have contributed, in some fashion, the stimulus necessary for DETC activation in culture. In performing double immunoflourescent staining of epidermal cells for SEA and Thy-1 surface antigen, it was determined that there were no cells that were positive for both fluorescent markers used. We therefore concluded that DETC did not bind SEA. This would be consistent with literature which states that only Vγ9+ γδ T cells respond to SEA since virtually all DETC are Vγ3+. However, these data were obtained using human, not murine, γδ T cells

and these results do not prove the findings of the previous experiments, but they do support them. No literature was found to determine if DETC respond in any way to SEA, or to superantigens, in general. All of these results would indicate that SEA does not modulate, or interact with, DETC. These studies are the first to address DETC-superantigen interaction and provide preliminary evidence in determining as to whether SEA can modulate DETC function.

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