MODULATION OF EPIDERMAL CELL CYTOKINE SECRETION BY STAPHYLOCOCCAL ENTEROTOXIN-A

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PREFACE

Langerhans cells (LCs) are the unique antigen-presenting cells of the mammalian epidermis. They are known to migrate from the epidermis into the draining lymph nodes following cutaneous hapten application. Several attempts have been made to uncover the mechanism(s) of Langerhans' cell (LC) migration. The elucidation of the signalling pathway that leads to LC migration is expected to have a profound impact on human pathology and therapy.

Increasing evidence is accumulating for the role of cytokines in mediating inflammatory and immune responses within the skin. Recent findings suggest that cytokines may also be involved in the phenomenon of LC migration following uptake of antigen in the epidermis. The purpose of this study was to examine the secretion of interleukin(IL)-1-alpha and tumor necrosis factor (TNF)-alpha following the stimulation of epidermal cells with staphylococcal enterotoxin-A(SEA), a superantigen which induces LC migration. The investigations in this study involved specific treatments of epidermal cells in suspension, followed by a one-day culture of the cells, after which the cell-culture supernatant fluids were tested by ELISA for the presence of the two cytokines.

The results indicate that although epidermal cells secrete both cytokines constitutively, SEA induces a further increase in the secretion mainly of IL-1 alpha, and to a lesser extent of TNF-alpha.

The increase of IL-1 alpha is apparently mediated via a protein kinase C-mediated signal transduction pathway, while its constitutive production is affected through G-proteins as well. More specifically, there are indications that Langerhans cells are partly responsible for the constitutive but not SEA-induced secretion of IL-1 alpha while other epidermal cells may be the chief source in the latter case. Dendritic epidermal T-cells were implicated to be either a source of or serve as inducers of constitutive TNF-alpha secretion in the epidermis.

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INTRODUCTION

The Langerhans cell (LC) is no doubt one of the most intriguing cells of the immune system within the skin. It was discovered in 1868 by Paul Langerhans, who is much better known to medical science for his other major discovery - the pancreatic "islet" cells (24). He described these intraepidermal cells as "a system of sensory elements" for carrying "extracutaneous signals to the nervous system" (119). For a long time since then the Langerhans cell (LC) was believed to be a component of the nervous system that served an unknown function. More than a century later, with the recognition of vertebrate skin as a complex organ, there was a resurgence of LC studies. This rediscovery came about when a group of researchers, while investigating the effects of topical estrogen application on human skin enzymes, stumbled upon a dendritic cell located in the suprabasal layer of the epidermis that was adenosine triphosphatase (ATPase) positive (25). This finding laid the foundation of morphological and functional studies of the LC.

It is now known that Langerhans cells (LCs), which constitute 1-8% of epidermal cells in mammalian skin (25,50,93), are bone marrow-derived (50) antigen-presenting cells (103). They are located in the basal and suprabasal layers of stratified squamous epithelia (40,49) like that of the buccal mucosa, vagina, cervix, esophagus, and epidermis. LCs form a continuous network with their dendrites and play a key role in the induction of primary T-cell-

dependent immune responses in the skin (28,84,109). LCs are capable of movement and are constantly migrating from the epidermis (53) to the draining lymph nodes under "normal" (unsensitized) conditions. However, it has been shown that this depletion of LCs in the skin is much more marked at sites of topical or intradermal challenge with an antigen or hapten (5,101), or ultraviolet light exposure (5,113). Contact hypersensitivity studies have shown that antigen-bearing dendritic cells accumulate in the lymph nodes following skin sensitization (20,55,56,57,72). In addition flow cytometric analyses have revealed that following the application of a chemical allergen on skin, a considerable proportion of dendritic cells that arrive in the lymph nodes carry the allergen (54,55,72). Thus substantial evidence now supports the idea that LCs trap antigen in the epidermis and carry it to the draining lymph nodes, where they tend to home to T-cell areas (64). This is the site where the antigen is presented to T-cells in context of major histocompatibility complex (MHC) class II molecules (denoted as HLA-DR in humans and Ia in mice), which ultimately results in the effector function of the immune system. Larsen *et al.* (1990) have shown that the phenotypic and functional maturation of LCs begins in the epidermis, but is not completed within it (64). The maturation continues through the migratory process. Hence, the epidermis represents the primary site for the initiation of immune responses within the skin.

The nature of the stimulus that induces LC migration, the intracellular signalling pathway(s), and the mechanism(s) leading to sensitization after hapten application are yet to be elucidated

(21,26,39,49). Investigations in the last decade have revealed that epidermal cells are capable of secreting immunoregulatory cytokines (67). Under appropriate circumstances epidermal cells can secrete the following cytokines: interleukin(IL)-1 (-alpha,-beta), IL-3, IL-6, IL-8, tumor necrosis factor (TNF) (-alpha,-beta), interferon (-alpha,-beta), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), transforming growth factor (-alpha,-beta), basic fibroblast growth factor, platelet-derived growth factor, epidermal cell suppressor factor, and epidermal cell-derived contra-IL-1 (66,67,96) (see Appendix-I). Although keratinocytes are the major source of cytokines within the epidermis, there is ample evidence which indicates that LCs are another source of some of these immunomodulating factors (67). Cytokines play various roles in the activation, differentiation and proliferation of cells (115). Both immune as well as non-immune cells can secrete and react in response to cytokines.

Recently Lundqvist and Back (1990) discovered that an intraperitoneal injection of recombinant IL-1-beta caused depletion of Ia^+ cells in murine epidermis (71). Further Enk *et al.* (1993) suggested that dendritic cell-derived IL-1-beta may play a crucial role in the initiation of primary immune responses within the skin (26). Previously, having demonstrated that an intradermal injection of TNF-alpha induces LCs to accumulate in draining lymph nodes, Cumberbatch and Kimber (1992) had proposed that TNF-alpha may provide one stimulus for LC migration from the skin (21). GM-CSF was found to increase viability and enhance the function of LCs in

culture (58,118), and in humans, to promote keratinocyte growth and wound healing as well (48). IL-1 alone, on the other hand, does not keep LCs alive, but further enhances LC function when combined with GM-CSF (44). TNF-alpha was found to maintain LC viability *in vitro*, but no enhancement of LC function was observed (58).

It is clear that there is a network of interacting cytokines in the epidermis (68) and one or more of these may constitute the stimulus for LC migration from the skin. However, there are various types of cells in the epidermis [LC, Thy-1^+ dendritic epidermal T-cells (DETC), keratinocytes, melanocytes, merkel cells and indeterminate cells] that may be the source(s) of the cytokines. The viability and function of LC may be modulated by cytokines through autocrine and paracrine regulation within the epidermis. It is vital, therefore, to elucidate the migration-inducing cytokine(s) and its source as a primary step in our understanding of the Langerhans' cells migratory phenomenon.

Immunobiology of the Skin

The skin is the largest organ of the mammalian body. Its principal function, that of an impenetrable physicochemical barrier to water, water-soluble compounds, and potentially harmful microorganisms, was the only function associated with it for ages. Other major physiological functions of the skin that were realized several decades ago include maintenance of body temperature, regulation of stable circulation, production of endocrine mediators, and the bearing of peripheral neural receptors and nerve endings

(9), which enable perception of sensory stimuli. Hence the central role of the skin in host defense has been known for a long time. Although the idea that an interaction exists between the skin and host immunity has also been recognised for several decades, extensive studies of immunity within the skin have been conducted only within the last twenty years. Mammalian skin was found to contain all the components required for the elicitation, performance, and regulation of immune responses to foreign antigens.

Streilein introduced the term Skin Associated Lymphoid Tissues (SALT) in 1978 to depict the special relationship that exists between the skin and the immune system (106). The term SALT resembles the notions of gut-associated lymphoid tissues (GALT) and bronchial-associated lymphoid tissues (BALT) which were formulated to signify the major role of the mucosal surfaces of the gastrointestinal and respiratory tracts, respectively, in the immune defense system. The SALT is a complex of cells that can sort out self from non-self (foreign) antigens and allows the skin to react autonomously to immune challenges. Besides Langerhans cells five other skin cell populations are known to constitute the SALT system. These are keratinocytes, T-lymphocytes (T-cells), endothelial cells, dermal dendritic cells, and dermal macrophages (108). Keratinocytes comprise the majority of the epidermis, and are believed to create a unique microenvironment that is favorable to antigen uptake and lymphocyte function. LCs are the antigen-presenting cells of the epidermis while the chief antigen-presenting cells of the dermis are the dermal dendritic cells and macrophages. These cells are responsible for the uptake, processing, and presentation of antigens

to T-cells *in situ* or in the draining lymph nodes. Almost all conventional T-cells (bearing alpha/beta T-cell receptors) are confined to the dermis while the majority of lymphocytes in murine epidermis are Thy-1^+ dendritic epidermal T-cells (DETCs; bearing gamma/delta T-cell receptors). A homologue of the Thy-1^+ DETC has not been conclusively demonstrated in other animals. Nevertheless, there is some evidence of the occurrence in low numbers of a similar subset of gamma/delta T-cells in humans (38). The exact role of DETCs is not completely resolved. There have previously been suggestions of a relationship to natural killer (NK) cells (87) or a role in the induction of antigen-specific tolerance (107). Recent evidence has implicated the involvement of DETCs in cutaneous immunologic tolerance (117). Endothelial cells, through the expression of cell surface adhesion molecules (CAMs), promote LC and lymphocyte migration into dermal lymphatics and from the vasculature into the dermal and epidermal compartments. Taken together, the functional properties of these diverse cells of the SALT and their their cooperative interactions confer upon the skin the ability to recognise and distinguish between self and non-self cutaneous antigens, and then initiate immune responses which ultimately result in the elimination of the foreign antigens.

The Epidermis

The epidermis is the most superficial layer of the skin. It is avascular and consists of various layers of cells. The uppermost layer of human epidermis which is exposed to the external environment,

called the stratum corneum (or "horny" layer), is composed of flat, anucleate, and cornified dead cells. The layer immediately beneath the stratum corneum is the transitional layer or stratum lucidum which is usually a single layer of anucleate cells. Below it is the stratum granulosum which comprises a couple of layers of granular cells. Underlying the granular layer are a few layers of cells comprising the stratum spinosum (or spinous layer) below which is the basal layer or stratum germinativum (120). The basal layer sits upon the basement membrane that separates the dermis from the epidermis. These five layers may or may not be easily distinguishable depending upon the location of the epidermis on the human body. The epidermis of mice is not structurally comparable to that of humans. Murine epidermis is extremely thin and generally consists of between 3-4 viable cell layers only.

The multilayered epidermis encloses an aqueous microenvironment between the stratum corneum and the basement membrane. The cells of the SALT system mentioned earlier are found in the region between the stratum granulosum and the stratum germinativum (108). Langerhans cells are particularly found in the area near the hair follicle root sheaths.

Morphology of Langerhans Cells

Mammalian epidermis consists of three major cell populations: keratinocytes, melanocytes, and Langerhans cells. All three populations appear as "clear cells" (i.e., cells with nuclei surrounded by relatively unstained cytoplasm) in routine paraffin-embedded

hematoxylin-and-eosin stained epidermal sections (10). LCs are not visible by this method (40,104,119). LCs are usually identified by special light microscopic techniques involving immunohistological stains, and more certainly, by electron microscopy, on the basis of specific marker cytoplasmic structures (10,104).

The most obvious of all LC characteristics that can be viewed by light microscopy is their dendritic appearance. The dendrites are long and extend three-dimensionally between other epidermal cells. The number of dendrites per cell varies upon the location (40) and perhaps also upon the pathologic conditions. Two most common strategies for the identification of murine LCs are to stain for the presence of the ATPase marker and for Ia molecules (encoded by class II genes of the major histocompatibility complex in mice) on the cell surface. These two surface markers are absent on other epidermal cells under normal conditions. Epidermal LCs possess high amounts of ATPase on their cell surface (4,88,111). In the past the ATPase marker was utilized most commonly for the detection of LCs. Nevertheless, the loss of this marker under certain pathologic conditions made it necessary to use immunochemical staining of the Ia molecules (25). LCs also have Ia or 'Immune-response-region-associated' antigens on their surface (90,111) which are expressed constitutively. LCs are the only cells in the epidermis that are capable of MHC-class-II expression under normal circumstances (92,109). Staining for Ia molecules involves the use of either monoclonal or polyclonal antibodies. Both these stains can successfully be performed on paraffin-embedded, frozen tissue sections, as well as on cell suspensions (4) prepared from normal

epidermis. However, recent investigations of cutaneous hypersensitivity have revealed that under pathologic conditions other epidermal cells such as keratinocytes may also express Ia antigens (25). Thus this strategy of staining may not accurately reveal epidermal LC density under certain conditions.

While the ATPase and Ia markers are most commonly targeted for staining LC, the most definitive feature of LCs is the presence of the Birbeck or Langerhans' cell-granule. Birbeck or LC-granules are rod-like trilaminar cytoplasmic structures (49) that can be visualised only through electron microscopy (40). The granule is usually located near the nucleus or golgi apparatus and is believed to be composed of limiting membranes that enclose paracrystalline material. The origin of the Birbeck granule is still controversial. There are two theories regarding its origin. First, that the granule is a kind of "endocytic organelle" (49) formed from the cell membrane, and second, that it originates from the golgi membranes (40) since it is usually found in close association with the golgi apparatus. The exact function of the Birbeck granule is still unknown. Since LCs are the unique antigen-presenting cells of the epidermis, it is hypothesized that these granules may be associated with the presentation of antigens to T-lymphocytes or temporary intracellular storage of antigens (49). This view was supported by a study in which preferential occurrence of LC-granules in the LC at LC-lymphocyte contact zones was demonstrated (19).

Other markers unique to LCs but less often used in histological studies of skin are: the T6 (CD1a) and T-200 antigens in humans (31,80), TL and Ly-5 antigens in mice (65,91), the C3 receptor

(12,105), the F_C-IgG receptor (105), and the high and low affinity Fc-IgE receptors in humans (6,7). Despite their limitations, the ATPase-stain and the Ia-stain are the most frequently used techniques for the identification of Langerhans cells in the epidermis.

Langerhans cells generally lack phagocytic vacuoles and large lysosomes. The cytoplasm contains moderate numbers of mitochondria, golgi bodies, rough endoplasmic reticulum, and some small lysosomes (40). They sometimes display networks of microfilaments and a prominent system of microtubules (112), both of which are characteristics normally seen in motile cells.

Ontogeny of Langerhans cells

As mentioned earlier, it was believed for a long time that the LC was a component of the nervous system. However, ever since the time that LCs were recognized as a component of the immune system, it has been thought that they were of bone marrow origin like other immunocytes. Today the ontogeny of the LC is no longer in question. Over a decade ago, it was demonstrated that LCs are derived from and are continuously repopulated by a mobile pool of precursor cells which originate in the bone marrow. Katz *et al.* (1979) established this fact through a two-step experimental process (50). First, they transplanted sex-matched parental skin on to hybrid mice and after various time periods found MHC class I and class II antigens of the recipient on the LCs within the graft. Next, after irradiating chimeric mice (where recepient bone marrow cells are killed followed by reconstitution with allogeneic or semi-allogeneic

bone marrow cells), they found that a low percentage of F_c-IgG bearing cells in the transplanted skin were of bone marrow-donor origin (LC) while the majority of the cells were of the recipient type (other epidermal cells). The first experiment showed that the LC of the transplanted skin were derived from a mobile pool of recipient cells; the latter experiment proved that the LCs were bone marrow-derived. Since this discovery, Langerhans cells are believed to be of the monocyte/macrophage lineage (89). It is interesting, however, that no cells in the bone marrow show the presence of Birbeck granules (112). Perhaps they are formed during the process of differentiation from a macrophage-like or monocyte-like progenitor.

Functions of Langerhans Cells

Antigen presentation:

There is no longer any doubt about the antigen-presentation function of Langerhans cells. An antigen-presenting cell is any cell that can pick up, process, and present antigen in association with MHC-class-II molecules to helper T-lymphocytes (T_H cells). Strong *in vitro* evidence has shown that LCs are important in this process (103) lending support to suggestions that LCs may have a function similar to that of macrophages (37,112). Langerhans cells were demonstrated by Grabbe *et al.* (1991) to be capable of presenting tumor antigens (36). In addition, other *in vitro* experiments have demonstrated that LCs are potent accessory cells in T-helper-cell responses (49). Accessory cells are those which help or cooperate

with T lymphocytes and enable them in the recognition and ultimately in the removal of antigens from the body. Langerhans cells cultured for 2-3 days exhibit markedly enhanced T-cell-stimulatory ability (94) and antigen-presenting capacity (47,99) while endocytosis occurs with significantly lower efficiency (34) when compared with fresh LCs.

The precise antigen-processing pathway is still unknown. In human Langerhans cells, surface MHC-class-II (HLA-DR) molecules were shown by Hanau *et al.* (1987) to cointernalize with T6 (CD1a) antigens through common coated regions of the cell membrane when treated with anti-Ia antibody (41). This cointernalization was demonstrated to be due to receptor-mediated endocytosis, which was characterized by the presence of coated pits, coated vesicles, receptosomes, lysosomes and Birbeck granules (41). Since MHC-class-II molecules are involved in binding processed antigens prior to antigen presentation, it is possible that antigens may be picked up by LCs in a similar manner. The presence of complement receptors (12,105) and F_c receptors (6,7,78,105) on LCs may be involved in enhancing adherence and the subsequent opsonization of bacteria (ingestion of antibody-coated bacteria). A complement receptor is a member of a family of cell surface receptors that specifically recognize a component of the complement system. Complement is a group of serum proteins (C1-C9) that participate in an enzymatic cascade that ultimately generates the cytolytic membrane attack complex (62). The complement cascade is activated by antigen-bound antibodies of the IgG and IgM isotypes (immune complexes). Once complexed with antigen, the F_c portion of the antibody molecule

undergoes a conformational change that allows it to bind to the F_c receptor on a cell or to the C1 component of the complement system. The subsequent reactions of the complement cascade produce an enzyme that converts a proenzyme called C3 into the active form C3b which is bound to the antigen via the antibody molecule. C3b can bind to the C3 receptor on phagocytes such as macrophages, monocytes, and neutrophils. On the other hand, the F_c portion of the antibodies in immune complexes can directly bind Ig-F_c receptors on the phagocytes. In this manner both C3 and antibody F_c portions facilitate the recognition and uptake of antigen by phagocytes.

LCs are very potent antigen-presenting cells, as measured through T-cell proliferation assays. They have been shown to be more potent antigen-presenting cells on a per cell basis than monocytes in responses to antigens frequently found in the skin such as those from mycobacteria, *Candida albicans*, or tetanus toxin (49).

The main aspect of LC function presented above has been well accepted for a long time. However, whether antigen-presentation occurs in the epidermis or LCs migrate to the lymph nodes for this purpose have been topics of debate for several years. It is widely believed that, following hapten sensitization, LCs migrate from the skin as "veiled cells" into the draining lymph nodes through the lymphatics in the dermis (115). The most convincing evidence for this hypothesis came when Kripke *et al.* (1990) showed that sensitization of nude mice at the site of a skin allograft leads to the appearance of antigen-bearing dendritic cells of donor origin in the recepient's lymph nodes (61). On the other hand, few other researchers hold the view that antigen presentation occurs in the

epidermis and that the apparent dissappearance of LCs in tissue sections is due to the loss of surface markers and/or cell death. Mommaas *et al.* (1993) have recently shown that LC depletion following UVB exposure is not due to migration of LCs, but due to cell destruction (76). This is in contrast to the results of Moodycliffe *et al.* (1992) which indicated that LCs migrate following UVB exposure (77).

It seems likely that both of the above phenomena might be taking place in antigen-challenged skin, depending on the type and amount of antigen present.

LC and contact (delayed type) hypersensitivity:

Much of the knowledge of LC function has been elucidated through the studies of the induction or sensitization stage of contact hypersensitivity. Such responses develop 48-72 hours following activation of a subset of helper T-cells (T_H1) by a hapten (or contact sensitizer). A hapten is a low-molecular-weight compound that lacks antigenic determinants but can elicit immune responses when coupled to a carrier such as dinitrophenol (DNP). This results in the secretion of various cytokines such as interleukin-2 (IL-2), interferon(IFN)-gamma, macrophage inhibition factor (MIF) and TNF-beta (63). The ultimate consequence of this type of reaction is localized non-specific enzymatic lysis of infected cells, and usually some uninfected neighboring cells as well, by activated macrophages. A vast amount of evidence indicates that LCs play a dominant role in

the process by which the immune system is exposed to skin-borne antigens and in its responses to them (83).

Situated within the epidermis, LCs provide a continuous network of dendritic processes that can capture antigens subsequently leading to processing and presentation of the antigens. After challenge with a hapten, the following changes in the LC were noted by Silberberg-Sinakin and Thorbecke (1980): the hapten is localized on the LC surface and within membrane-bound organelles in the LC, and, prominent endoplasmic reticulum, golgi bodies, and lysosomes are present indicating increased LC activity (101). Following this phase, LCs gradually start to dissappear from the epidermis (49).

LC and cutaneous immunologic tolerance:

Topical application of haptens at areas lacking or naturally deficient in LCs (such as mouse tail) does not promote induction of contact hypersensitivity (109), but instead leads to antigen-specific unresponsiveness or tolerance. This suggested that LCs do not themselves induce tolerance; it is their absence that leads to it. Later studies have implicated the Thy-1 dendritic epidermal T-cells as responsible for the induction of immunologic tolerance *in vivo* (117).

LC and graft rejection:

LCs are believed to be critical "passenger leukocytes" (64) that trigger graft rejection by sensitizing the recipient's T-cells. It is

assumed that epidermal LCs from skin allografts migrate into the draining lymph nodes and initiate the rejection. LCs migrate out of both allografts and isografts; however, in the case of an allograft, the entry of the donor's LCs into the recepient's lymph nodes itself provides a powerful stimulus for the initiation of rejection (64).

LC and allergy:

Bruynzeel-Koomen *et al.* (1986) first reported the occurrence of IgE on epidermal LCs in patients with atopic dermatitis, implying that LCs may play a key role in the pathophysiology of atopic disease (11). The majority of resident LCs in human epidermis can bind monomeric IgE through the high affinity receptor, F_c-epsilon-RI (116). Although the mechanism of signal transduction through cross-linked LC-surface IgE and its effects are unknown, Wang *et al.* (1992) suggested that it is conceivable that this could stimulate events in LCs similar to those occurring in mast cells and basophils (116). Under appropriate stimulation (cross-linking of surface IgE by allergens) mast cells and basophils are known to synthesize and secrete various inflammatory metabolites ("degranulation") and cytokines (63).

Mudde *et al.* (1990) showed that IgE-bearing LCs are superior in their ability to present air-borne-house-dust allergens to sensitized T-cells *in vitro* when compared with LCs without surface IgE (78). These *in vitro* results correlate with the positive delayed patch reaction to the same allergens indicating that the F_c-epsilon-RI, when bound to monomeric IgE, appears to play a crucial role in the

uptake and processing of cutaneous antigens by Langerhans cells. Targeting this F_C-IgE receptor on LCs may be an important route in the future for topical therapy of atopic allergy.

Epidermal Cytokines

The discovery of the ability of epidermal cells to secrete immunomodulating cytokines further supported the notion that the epidermis is a primary site for the induction of immune responses within the skin. Cytokines are low-molecular-weight, hormone-like proteins which are produced by various cells and can regulate cell growth and differentiation. They can exert their biological effects at extremely low concentrations via specific surface receptors on target cells. The secretion of cytokines mediates immunologic and inflammatory reactions. In the epidermis keratinocytes, Langerhans cells, and to a lesser extent melanocytes, are known to produce cytokines; however, keratinocytes are the major source (66). Through the secretion of cytokines epidermal cells are capable of exerting autocrine, paracrine, and endocrine effects. Most cytokines have multiple effects and have overlapping activities; some are known to induce each other (23,51) and also influence cell function in a synergistic or antagonistic fashion (68). In recent years it has been found that epidermal cells can produce constitutively, or can be induced to produce, a wide variety of cytokines. The repertoire of epidermal-cell-derived cytokines currently known were listed earlier in this chapter (also see Appendix-I). Among these cytokines, those that are most important for LC maintenance and function are

IL-1 (36,44,60,71), GM-CSF (44,48,118), and TNF-alpha (21,58,59). Recent studies have investigated the influence of other epidermal-cell-derived cytokines such as IFN-gamma (84), IL-10 (27), and TGF-beta (29,36) on LC function. The cytokines IL-1 and TNF are described in some detail in the following sections.

Interleukin-1 (IL-1)

The first cytokine demonstrated to be released by murine keratinocytes was epidermal-cell-derived thymocyte-activating factor (ETAF), which was also found to enhance (costimulate) the proliferative response of thymocytes to mitogens (69). ETAF was soon found to be biochemically and biologically identical to interleukin-1 (70). The sources of IL-1 were originally thought to be cells of the monocyte/macrophage lineage. Within the last decade several other cell types were found to produce IL-1 (81). Two forms of interleukin-1, IL-1-alpha and IL-1-beta, which are encoded by two distinct genes, have been identified (81). Although there is only about 26% amino-acid-sequence similarity between these two forms in humans (73), they recognise the same cell receptor and share the same spectrum of biological activities (22,66). IL-1 is produced in response to damaging stimuli such as infection, ultraviolet irradiation, microbial toxins, inflammatory agents, products of activated lymphocytes, complement, and clotting components (22). Interleukin-1 is multifunctional and exerts both immunological and non-immunological effects. Its most important biological effects are that it is an endogenous pyrogen (a fever-inducing agent), induces

the production of hepatic acute phase proteins, causes neutrophilia, activates T-cells, costimulates T-cell mitogenesis, and induces cytokine production such as IL-2, IL-4, IFN-gamma, and CSF by T-cells (22,66). It is also an activator for dendritic cells and seems to enhance their capacity to bind to T-cells (102). IL-1 induces the expression of intercellular adhesion molecules (ICAMs) which enable adherence of cells (67).

IL-1 is present in significant amounts in the stratum corneum (33) and normal skin (43) in humans. Keratinocytes are capable of constitutive IL-1 production, albeit in low amounts (68). IL-1-alpha is the predominant form in the skin (97).

Tumor Necrosis Factor (TNF)

Tumor necrosis factor was originally described as a substance found in the serum of bacillus Calmette-Guerin (BCG)-infected mice treated with endotoxin that mimicked the tumor necrotic action of endotoxin itself (14). TNF exists in two forms, TNF-alpha (cachectin) and TNF-beta (lymphotoxin), which are antigenically distinct polypeptides, but bind a common cell receptor and share common biological properties (68). Like interleukin-1, TNF is an endogenous pyrogen (23), it can activate neutrophils, and induces the production of other cytokines such as GM-CSF, M-CSF, and IL-1 (51). TNF-alpha can be induced by stimulants such as bacterial endotoxins, ultraviolet irradiation, IL-1, and TNF-alpha itself (68). TNF-alpha is known to exert pleitropic biological effects. It is an important mediator of cachexia (characterized by tissue wasting, negative nitrogen balance,

and loss of body weight) and endotoxic shock (commonly characterized by fever, hypotension, and diarrhoea) (16), and causes cytotoxicity and cytostasis of tumor cell lines *in vitro* (110).

Within the epidermis, keratinocytes have been demonstrated to synthesize and release TNF-alpha upon stimulation (59). TNF-alpha increases the expression of MHC class-I and class-II molecules as well as intercellular adhesion molecule-1 (ICAM-1) on several cell types (68).

Cytokine-Mediated Maintenance, Maturation and Migration of Langerhans Cells

Because Langerhans cells are antigen-presenting cells stationed in the outer layer of the skin and upon antigenic challenge are able to migrate to the lymph nodes (to present antigen to T-lymphocytes), it is important that they are maintained at a particular density in the skin under normal conditions. This homeostasis is believed to be regulated by cytokines, which may be produced by LCs (autocrine regulation) and other epidermal cells (paracrine regulation). If cell culture studies are assumed to approximately reflect *in vivo* cell growth, then both granulocyte and macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-alpha are required for maintaining viability of LCs. Schuler and Steinman (1985) suggested that epidermal LCs represent precursors or immature elements of the dendritic cell system (94). However, following 24-72 hours of *in vitro* culture of epidermal cell suspensions LCs undergo marked changes (118). LCs seem to mature immunologically in bulk

epidermal culture, expressing several new features unique to lymphoid dendritic cells. Since LC maturation leads to the development of accessory function (118) for primary immune responses, the regulation of maturation following skin sensitization (or antigenic challenge) becomes very important. Changes in LCs due to maturation *in vitro* include decrease in antigen uptake ability, loss of antigen processing ability, decrease in the number of LC granules (86), and increase in lymphocyte stimulatory ability.

Koch *et al.* (1990) showed that both GM-CSF and TNF-alpha can maintain LC viability in culture, but unlike GM-CSF, TNF-alpha cannot induce maturation of LC function (58). Interleukin-1 (IL-1) on the other hand does not maintain viability, but enhances LC function two-fold when combined with GM-CSF (58). The stimulus that causes LCs to migrate from the skin, however, remains to be elucidated. Some researchers have suggested that IL-1 may be this stimulus (71) while some others believe that TNF-alpha is responsible for LC migration (21).

An unpublished work of Pickard and Burnham (85) had previously shown that the superantigens Staphylococcal enterotoxin-A (SEA), Staphylococcal enterotoxin-B, and Staphylococcal exfoliative toxin are capable of inducing a dose-dependent depletion of Langerhans cells in murine epidermis. Among these SEA proved to stimulate the most drammatic depletion of LCs. Staphylococcal exotoxins are known to deliver activation signals via Ia similar to that produced by the binding of anti-Ia monoclonal antibody (17). Unlike nominal antigens, SEA interacts with MHC-class-II (Ia)

molecules at a site independent of that involved in antigen presentation, and is also capable of binding the T-cell receptor (TCR). Superantigens like SEA can thus mediate the interaction between antigen-presenting cells (APC) and T-cells without being internally processed by APC. This interaction leads to the antigen-non-specific activation of T-cells. Since LCs are the only cell type capable of expressing Ia in normal murine epidermis and since superantigens are known to interact with surface MHC-class-II (Ia) molecules, it is reasonable to assume that the LC depletion induced by SEA is mediated by activation through Ia. Although SEA produces higher quantitative responses by epidermal cells it probably produces events qualitatively similar to those induced by nominal antigens, since nominal antigens also interact with MHC-class-II molecules and play a role in the activation of antigen-specific T-cells.

Since both IL-1 and TNF have been implicated to play a crucial role in the migration of LCs from the epidermis, the present study examines their secretion by epidermal cells and their subpopulations induced by SEA. The intention of this approach was that an investigation of the depletion-inducing stimuli and molecular-signalling pathway(s) involved in SEA-induced LC depletion may later serve as a model for the signalling pathway(s) and mechanism(s) of LC depletion or migration caused by nominal antigens in the skin. Such studies may have a profound impact on human pathology and therapy. Such knowledge may allow us to manipulate LC function to obtain desirable immune responses (such as tolerance) and prevent undesirable effects (such as allergies).

CHAPTER II

MATERIALS AND METHODS

Animals

Young adult (6-to-8 weeks old) female BALB/c strain mice obtained from Charles River (Wilmington, MA) were used throughout this study. The mice were maintained in a licensed facility at Oklahoma State University.

Buffers and Media

Balanced salt solution (BSS, without calcium and magnesium) was used to keep surgically-removed skin hydrated at room temperature. One liter of BSS contains 8.0 g sodium chloride (NaCl, Baker Chemical Corp., Phillipsburg, NJ), 0.35 g sodium bicarbonate ($NaHCO_3$, Mallinckrodt, Paris, KY), 0.4 g potassium chloride (KCl, Fisher, Fairlawn, NJ), 1.0 g glucose (Fisher), and 0.2% phenol red (Difco, Detroit, MI) in deionized water.

Phosphate buffered saline (PBS, 0.15 M) was utilized to prepare solutions of trypsin (type II, from porcine pancreas, Sigma Chemical Company, St.Louis, MO) and deoxyribonuclease I (DNase I, from bovine pancreas, Sigma). One liter of PBS contains 1.15 g anhydrous sodium phosphate (Na_2HPO_4, Sigma), 0.2 g monobasic potassium phosphate (KH_2PO_4, Fisher), 8.0 g NaCl, and 0.2 g KCl in deionized

water. The pH of the solution was adjusted to 7.2 prior to sterilization in an autoclave at 15 psi for 20 minutes.

In all experiments, cells were washed and cultured in Cellgro™ sterile RPMI 1640 (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine (Sigma), 100 U/ml penicillin (Sigma), and 0.1 mg/ml streptomycin (Sigma).

Cytotoxicity medium recommended by Cedarlane Laboratories (Ontario, Canada) was used in complement-mediated epidermal-cell-subpopulation depletion experiments. One liter of this medium contained 10.4 g powdered RPMI 1640 (Sigma), 25 mM HEPES (Sigma), 0.3% bovine serum albumin (BSA, fraction V, Sigma), 1 mM sodium pyruvate (Sigma), 0.22% Sodium bicarbonate, 2 mM L-glutamine, 0.1 mM non-essential amino acids (Sigma), 100 U/ml penicillin, and 0.1 mg/ml streptomycin in deionized water. The medium was filtered through a 0.45 micron filter (Corning Inc., Corning, NY) and stored at 4°C till required.

Toxins and Chemicals

Staphylococcal enterotoxin A (SEA, Toxin Technology, Sarasota, FL) and Lipopolysaccharide (LPS, Sigma) were applied to epidermal cells in some experiments to induce cytokine production.

Inhibitors of signal transduction in this study included 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7, an inhibitor of protein kinase C, Calbiochem Corp., San Diego, CA) (45,52), N-[2-(methylamino) ethyl]-5-isoquinoline sulfonamide (H-8, an inhibitor

of G-protein associated cyclic-nucleotide-dependent protein kinases, Calbiochem Corp.) (45), and Cholera and Pertussis toxins (inhibitors of separate G-proteins, Sigma) (114).

Antibodies

Purified mouse-anti-mouse-Ia^d monoclonal antibody (Pharmingen, San Diego, CA) was used for several purposes in this study. It was applied as a treatment in some experiments, in experiments requiring complement-mediated depletion of Langerhans cells, and in the separation of Langerhans cells from epidermal cell suspensions using the biomagnetic separation technique described later in this chapter. This monoclonal antibody (mAb) is of the IgG isotype and is specific for the mouse MHC-class-II (Ia) cell surface antigen encoded in the I-A region of the $H\text{-}2^d$ haplotype. The hybridoma J1j10 (anti Thy 1.2) [American Type Culture Collection (ATCC), Rockville, MD] was cultured in supplemented RPMI 1640 and the cell-free supernatant was collected and stored at -20 °C. This supernatant contained a rat IgM monoclonal antibody specific for the Thy-1 antigen on the surface of murine T lymphocytes. Another hybridoma, R4-6A2 (anti murine gamma interferon) (ATCC) was also cultured and stored in an identical manner. This hybridoma secretes a monoclonal rat IgG that was used to neutralize IFN-gamma in some experiments.

Preparation of Epidermal Cell Suspensions

Following hair removal, mice were euthanized in an ether chamber. Total body skin was surgically removed, cut into large pieces, and kept hydrated with BSS at room temperature till required. Ears were split with the aid of fine-tipped foreceps and floated, dermal side down, on a freshly prepared solution of 0.5% trypsin for 45 min at 37°C in the presence of 5% carbon dioxide. After scraping away the subcutaneous layer, pieces of skin were also placed dermal side down on 0.5% trypsin and incubated for 35 min under the same conditions. Following incubation, the loosened epidermal sheets were carefully peeled from the ears and skin and laid dermal side up on the surface of a clean plastic Petri dish. The exposed basal layer of the epidermis was immediately covered with a solution of 0.05% DNase I and gently rubbed with the smooth tip of a glass stirring rod to bring epidermal cells into suspension. Approximately four volumes of supplemented RPMI 1640 were added to the suspension and filtered through a Falcon™ 100 um sterile nylon mesh (Becton Dickinson, Franklin Lakes, NJ) to remove clumps. The cells were pelleted by spinning at 300Xg for 5 min at 4°C in a Damon CRU-5000 centrifuge (IEC, Needham Heights, MA) and washed twice with culture medium (supplemented RPMI 1640) to remove all DNase I. After the final wash the cells were resuspended in 10 ml culture medium, counted by trypan blue (Sigma) exclusion with the aid of a hemacytometer, and adjusted to the required

air. Following incubation, the culture medium was collected, spun at 300Xg for 5 min to pellet the cells, and stored in aliquots at -70°C till required.

Purification of Langerhans Cells by Biomagnetic Separation

An epidermal-cell suspension was prepared in supplemented RPMI 1640 (culture medium), washed twice, and counted by trypan-blue exclusion. The cells were sedimented at 300Xg and resuspended at 1 X 10^7 cells/ml with 5 ug anti-Ia mAb/ml of 10% FBS (in PBS). They were incubated at 4°C for 40 min with gentle bidirectional shaking on a tube rotator. Subsequently, the cells were washed three times with cold culture medium to remove all unbound antibody, and adjusted to a concentration of 2.5 X 10^7 cells/ml of culture medium. A pre-calculated number of sheep-anti-mouse IgG-coated magnetic beads (Dynal, Inc., Lake Success, NY) were taken in a separate test tube and washed at least three times in culture medium (this number was calculated assuming that about 3% of the EC suspension consisted of Langerhans cells). Washing the beads was accomplished by placing the tube on a Dynal magnetic particle concentrator (MPC) and, following a 2 min standing period to allow the beads to adhere to the wall of the tube closer to the magnet of the MPC, the medium was collected with a sterile pasteur pipette and discarded. This step was essential to remove all traces of the preservative sodium azide that was included in the commercial magnetic-bead suspension for storage purposes. The magnetic beads were then added to the highly concentrated cell suspension so that there were about 15 beads per

target cell, which is a significantly high bead-to-cell ratio to allow optimal cell/bead interaction. The test tube containing the cell-bead mixture was incubated at 4°C for 40 min on a tube rotator. All the incubations thus far were done at 4°C to minimize non-specific adherence.

The binding of beads to the anti-Ia-bound cells resulted in rosette formation. Following the last incubation, the rosettes (LCs) and free beads were separated from non-rosetted cells (other epidermal cells, OECs) by using the MPC as described above. While rosetted cells were bound to the wall of the tube closer to the magnet of the MPC the non-rosetted cells were removed with a sterile pasteur pipette into a separate test tube. The fraction containing rosettes (LCs) was washed at least three times with cold culture medium to ensure that all non-rosetted cells (OECs) were removed. To ensure that all LCs were removed from the OEC fraction, the MPC was applied to it twice as described above. Finally, the number of cells in each of the two fractions was determined and adjusted to appropriate concentrations as required. SEA was then applied to some fractions, and the cells were incubated for 24 h at 37°C in the presence of 5% carbon dioxide. Cell-free supernatant fluids were collected as described earlier, aliqouted, and stored at -70°C till required.

Cytokine Assays

IL-1-alpha and TNF-alpha were assayed using commercially prepared Intertest-1 alpha X™ ELISA and Factor-Test-X™ ELISA kits

(Genzyme, Cambridge, MA) respectively. The traditional methods used for IL-1 and TNF detection are bioassays. The thymocyte co-stimulator assay is used for IL-1 while the L929 killing assay is used for TNF. Both of these assays require 2-3 days for completion. These assays are also unable to differentiate between the two forms of each of these cytokines. The kits, on the other hand, require only 3-4 hours for cytokine quantitation and are specific for murine IL-1-alpha and TNF-alpha. The assays were done by sandwich ELISA, for which all reagents were included in the kits.

IL-1-alpha ELISA:

The kit contained anti-mouse IL-1-alpha mAb pre-coated wells, which required blocking prior to sample incubation. Blocking with an unrelated protein solution prevents non-specific binding of antigen to the wells. One hundred microliter samples were added to the wells and incubated to allow binding of IL-1-alpha to the anti-IL-1-alpha mAb. Subsequently biotinylated polyclonal anti-mouse IL-1-alpha was added to the wells. Following this incubation, avidin-conjugated peroxidase was added to the wells and incubated once again, to allow it to bind to biotin. Finally, a substrate solution containing tetramethylbenzidine (TMB) and buffered hydrogen peroxide was added. In between all of the above steps, the wells were washed four times with wash buffer. The substrate reaction was allowed to occur at room temperature for a fixed period of time and then halted by adding an equal volume of 1 M sulfuric acid (stop reagent). The ELISA reader (Vmax™ kinetic microplate reader,

Molecular Devices Corp., Palo Alto, CA) was blanked using culture medium (containing no IL-1-alpha) and the absorbance of the samples was read at 450 nm.

TNF-alpha ELISA:

The kit contained hamster-anti-mouse TNF-alpha mAb pre-coated wells. One hundred microliter samples were incubated in the wells to allow binding of TNF-alpha to the antibody. Following four washes with wash buffer, horseradish peroxidase-conjugated goat polyclonal anti-mouse TNF-alpha was added and the incubation was continued. Subsequently, the wells were washed four times and a substrate solution containing TMB and buffered hydrogen peroxide was added. The substrate reaction was allowed to occur at room temperature for a fixed period of time and then stopped by adding an equal volume of stop reagent. As in the IL-1-alpha assay, absorbances were read at 450 nm after blanking the ELISA reader with culture medium (containing no TNF-alpha).

CHAPTER III

RESULTS

The objective of the present study was to examine the secretion of IL-1-alpha and TNF-alpha by epidermal cells (ECs) and epidermal-cell- subpopulations when stimulated by Staphylococcal enterotoxin-A (SEA). Staphylococcal exotoxins such as SEA are known to deliver activation signals via cell-surface Ia similar to that produced by the binding of anti-Ia monoclonal antibody (17). The data obtained from two separate experiments are shown for each investigation and the standard error of the mean (SEM) was calculated in order to interpret the significance of the results.

Effects of SEA and Lipopolysaccharide (LPS) on EC Cytokine Secretion

In order to ascertain the unique stimulatory action of SEA, its ability to induce cytokine secretion by ECs was compared with that of LPS, a bacterial cell wall component and a common endotoxin. SEA and LPS were applied to EC suspensions at concentrations of 5 ug/ml and 100 ug/ml respectively and the cells were incubated for 24 h at 37°C in the presence of 5% carbon dioxide. Subsequently, the cell-free culture supernatant fluids were obtained by pelleting the cells at 300Xg for 5 min and analyzed for IL-1-alpha and TNF-alpha levels by ELISA. Figures 1 and 2 depict the results of two such experiments. As shown in Figure 1, SEA stimulated a significant increase in IL-1-

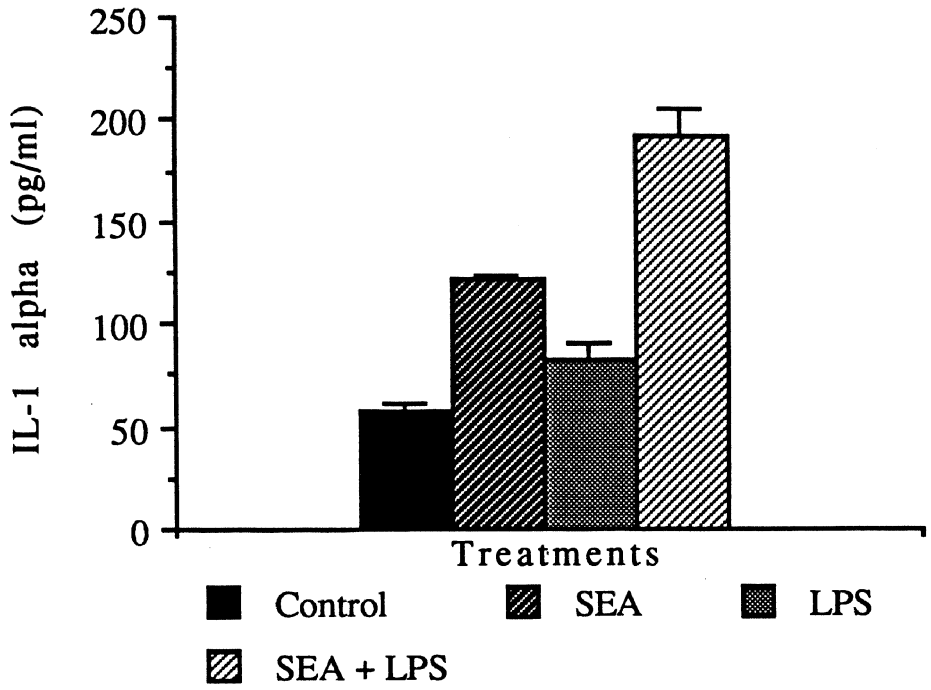

Figure 1. Comparison of the increases in IL-1 -alpha secretion by epidermal cells following stimulation with SEA and LPS. The values expressed are the mean ± SEM (standard error of the mean) generated by pooling the data from two separate experiments. (LPS- lipopolysaccharide; SEA-staphylococcal enterotoxin A)

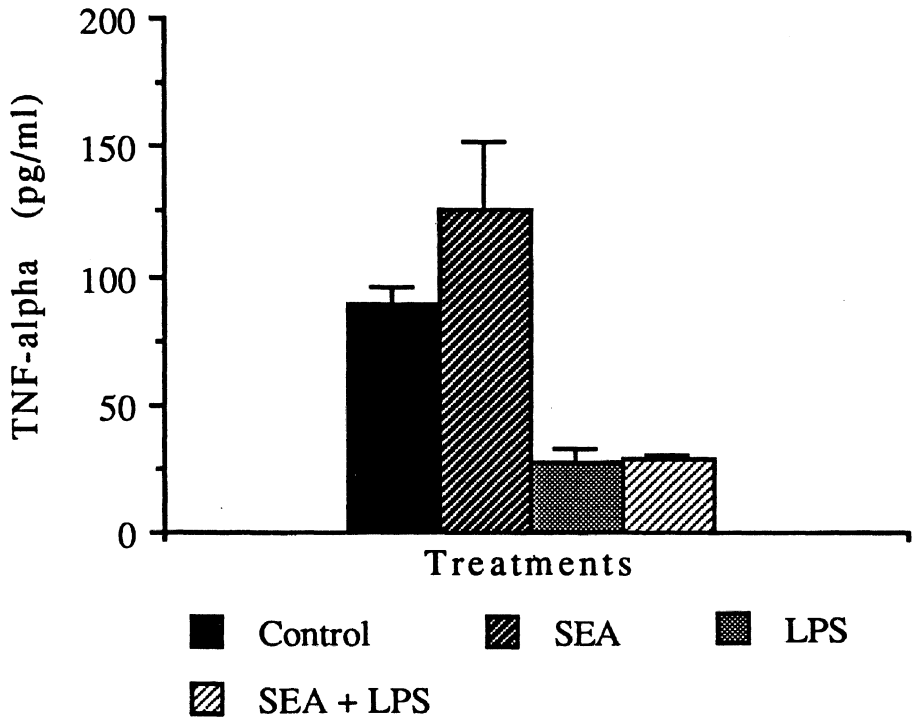

Figure 2. Comparison of the effects of SEA and LPS on TNF-alpha secretion by epidermal cells. The values expressed are the mean ± SEM generated by pooling the data from two separate experiments.

alpha secretion by ECs. LPS was also capable of stimulating an increase in IL-1-alpha secretion, but to a lesser extent. However, it was discovered that when applied simultaneously, SEA and LPS acted in a synergistic fashion. They induced a massive increase in EC-secreted IL-1-alpha which was greater than the sum of the effects of each taken independently. Figure 2 shows the influences of SEA and LPS on the secretion of TNF-alpha by ECs. There was a small increase of TNF-alpha when induced by SEA. Surprisingly, however, LPS by itself caused a considerable decrease in TNF-alpha secretion and also blocked the action of SEA on the EC suspension. These results demonstrate an increase in IL-1-alpha secretion by ECs following stimulation with SEA, which is more marked than that induced by LPS.

Effect of SEA on Langerhans' Cell Morphology

The morphology of LCs in normal epidermis and following SEA application on the extracutaneous surface are shown in Figures 3 and 4. Visualization of LCs was enabled through an Ia stain which stains all cells in the epidermis that express cell-surface MHC-class-II molecules. Under normal conditions, LCs appeared to have dendrites extended out from the cell body. However, the application of SEA followed by a 48 h incubation caused a notable difference in LC morphology in addition to a decrease in LC density observed previously (85). Under stimulation by SEA, LCs appeared round in shape and the dendrites were not visible. This suggests that the LCs either retracted their dendritic processes or that their surface Ia

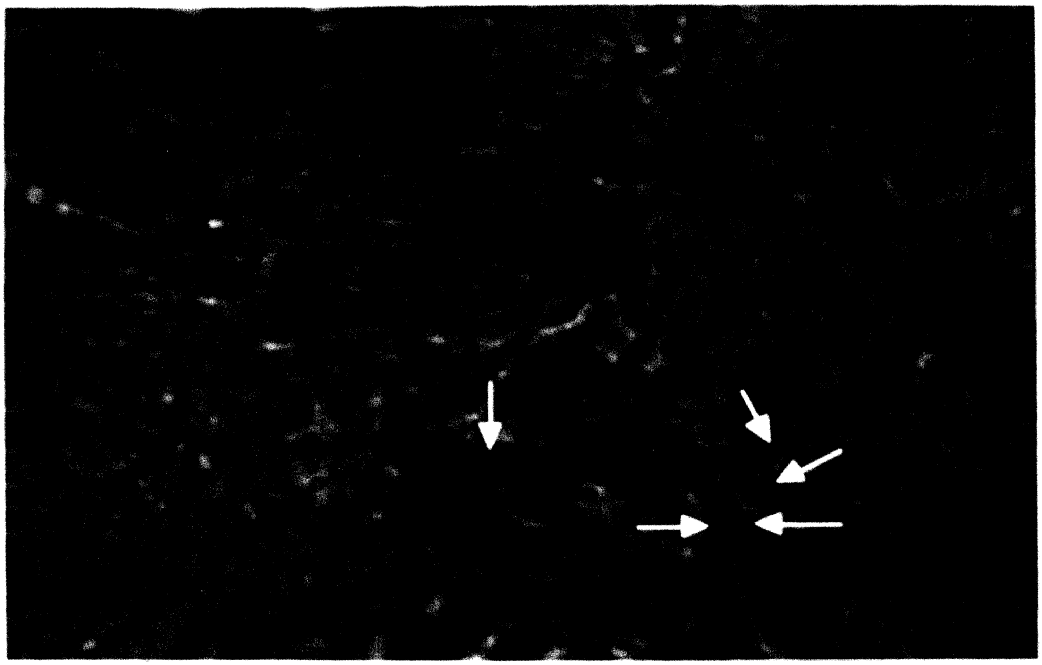

Figure 3. Normal morphology of Langerhans cells in the epidermis. Arrows indicate dendritic processes of one Langerhans cell.

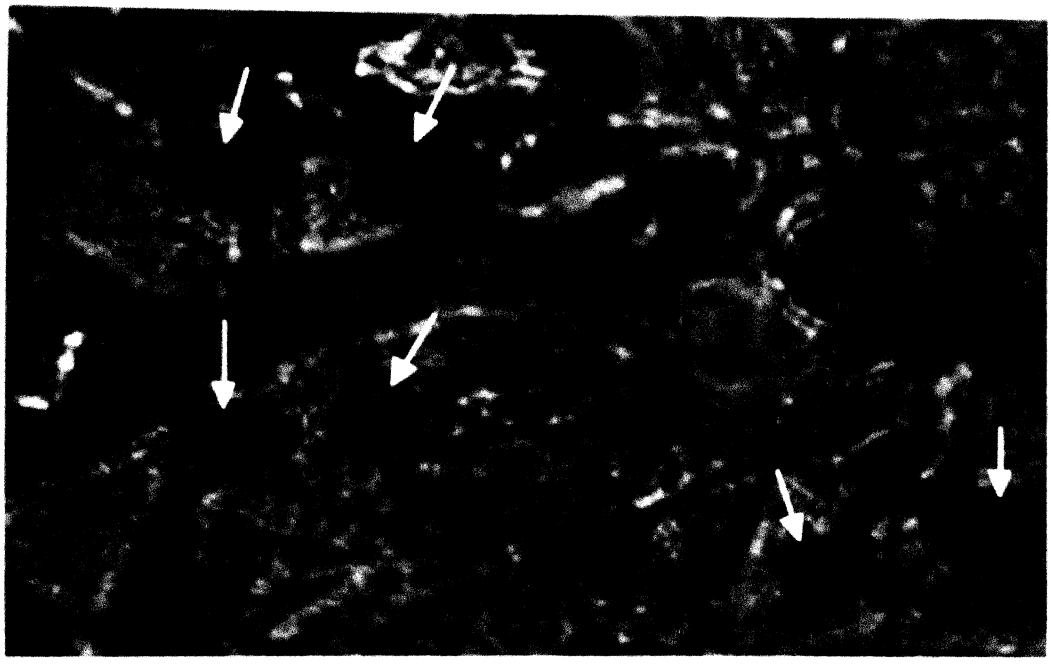

Figure 4. Effect of extracutaneous SEA application on the morphology of Langerhans cells in the epidermis. Arrows indicate stained LCs.

molecules were clustered together over the cell body. Such an aggregate of Ia molecules would produce a rounded appearance after the Ia stain. In either case, it is evident that SEA induces not only a change in cytokine secretion but also in LC morphology.

Role of G-proteins and Second Messengers Involved in the Transduction of the SEA-induced Stimulus

Extracellular stimuli such as the binding of biochemical transmitters, cytokines, or hormones to their corresponding cellular receptors ("signals") are known to be converted into intracellular events by complex, and yet highly regulated, signal transduction pathways. Such pathways are usually composed of signal transducers (GTP-binding proteins; alternatively called G-proteins), enzymes (adenylate cyclase or phosphoinositide-specific phospholipase C), and second messengers (cyclic AMP or calcium-dependent protein kinase C) that interact with each other near the cytoplasmic end of a cell-surface receptor to produce the intracellular event or change in cell behaviour.

In order to investigate the role of G-proteins and second messengers in the secretion of IL-1-alpha and TNF-alpha, inhibitors of signal transduction were examined for their capacity to block secretion of these cytokines. This was accomplished by culturing epidermal-cell (EC) suspensions with H-7, H-8, Cholera toxin, or Pertussis toxin for 1 h at 37°C prior to the addition of SEA. Following the addition of SEA to some groups, the cells were further incubated

for 24 h. 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) was employed because of its property to inhibit the function of protein kinase C (52). N-[2-(methylamino) ethyl]-5-isoquinoline sulfonamide (H-8) is known to specifically inhibit G-protein-associated kinases (45). Purified toxins from *V.cholerae* and *B.pertussis* were employed to further examine the role of G-proteins. These two toxins are inhibitors of distinct but overlapping spectra of G-proteins (114). H-7 and H-8 were applied at a final concentration of 10 uM while cholera and pertussis toxins were applied at a final concentration of 1 ug/ml to EC suspensions. Following the 24 h incubation, cell culture supernatants were tested by ELISA for IL-1-alpha and TNF-alpha levels. Results of this study are illustrated in Figures 5 and 6. While the constitutive IL-1-alpha levels were significantly lowered by all the inhibitors, the application of SEA to EC suspensions containing H-8, cholera toxin (CT), and pertussis toxin (PT) resulted in an approximate 100% increase in IL-1-alpha, identical to that seen when SEA was applied in their absence (Figure 5). Thus all the inhibitors of signal transduction were able to block constitutive IL-1-alpha secretion while only H-7 was efficient in blocking the SEA-induced IL-1-alpha level as well. These data implicate protein kinase C as the second messenger (and hence phospholipase C as the effector enzyme) that mediates increased IL-1-alpha secretion following stimulation with SEA. On the other hand, the results indicate that constitutive IL-1-alpha secretion involves both G-proteins and protein kinase C. There was a small increase in TNF-alpha production induced by SEA, which was not significantly blocked by any of the inhibitors. Pertussis toxin was found to decrease constitutive

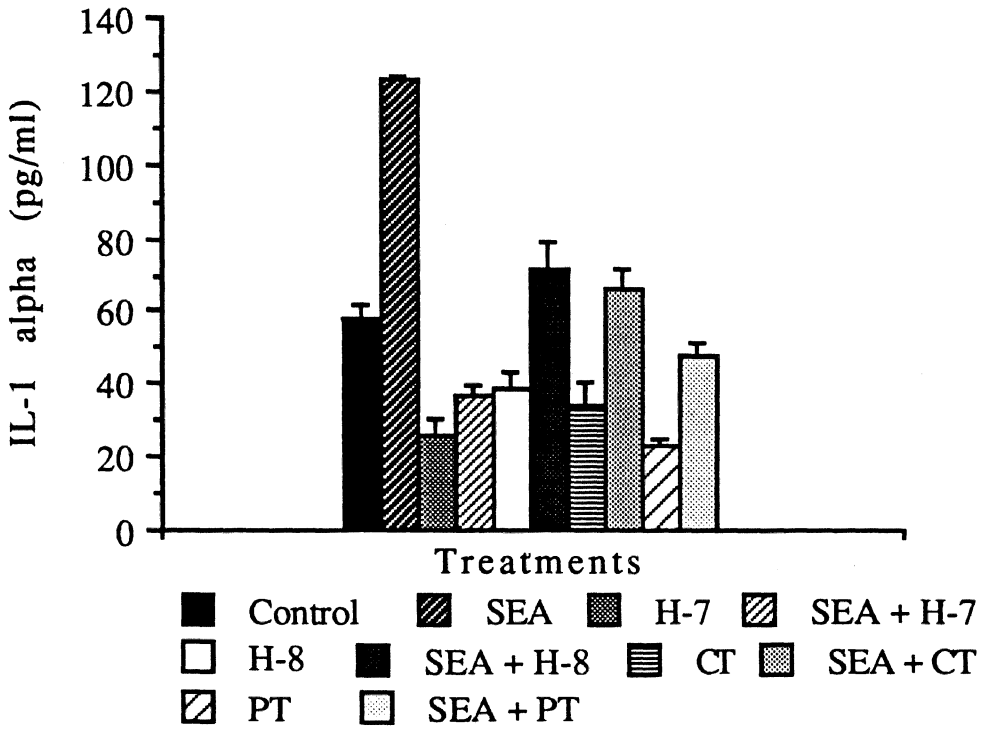

Figure 5. Inhibition of SEA-induced increase in IL-1-alpha secretion by EC with the use of specific inhibitors of signal transduction. The values expressed are the mean ± SEM generated by pooling the data from two separate experiments. (CT-cholera toxin; PT-pertussis toxin)

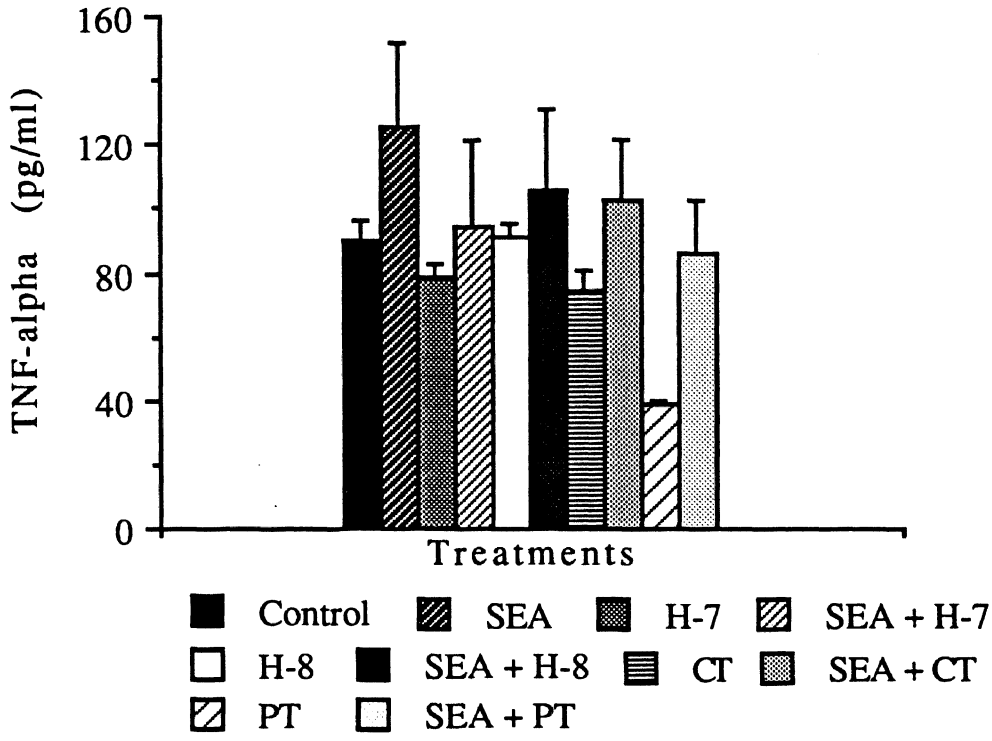

Figure 6. Effect of the application of specific inhibitors of signal transduction on the SEA-induced increase in TNF-alpha response by epidermal cells. The values expressed are the mean ± SEM generated by pooling the data from two separate experiments.

secretion of TNF-alpha considerably. The mechanism leading to increased TNF-alpha secretion due to such stimulation may or may not involve a G-protein and the above mentioned second messengers.

Effect of Binding Cell Surface Ia Molecules with Anti-Ia mAb Prior to Stimulation with SEA

Surface Ia (murine MHC-class-II) molecules were bound by a 1 h incubation at 37°C with anti-Ia monoclonal antibody (mAb) prior to the addition of SEA to EC suspensions. This was done to investigate the route of action of SEA via Ia molecules. After SEA application, the cells were further incubated for 24 h before testing the supernatant fluids for IL-1-alpha and TNF-alpha. As shown in Figure 7, the constitutive IL-1-alpha secretion was significantly lowered but no effect was seen on the SEA-induced response when the cell surface Ia molecules had been blocked. Nevertheless, the overall SEA-induced IL-1-alpha level was markedly reduced. Results of the effect of adding anti-Ia mAb to EC suspensions on the production of TNF-alpha were variable and statistically not meaningful, as depicted in Figure 8. These results indicated that the mAb and SEA were specific for two separate epitopes on the Ia molecules and that binding of the mAb down-regulated IL-1-alpha secretion. This shows that the use of anti-Ia mAb in some investigations within this study did not adversely affect the stimulatory action of SEA. This may also be the case with the induction of TNF-alpha as well, but is not supported by this study.

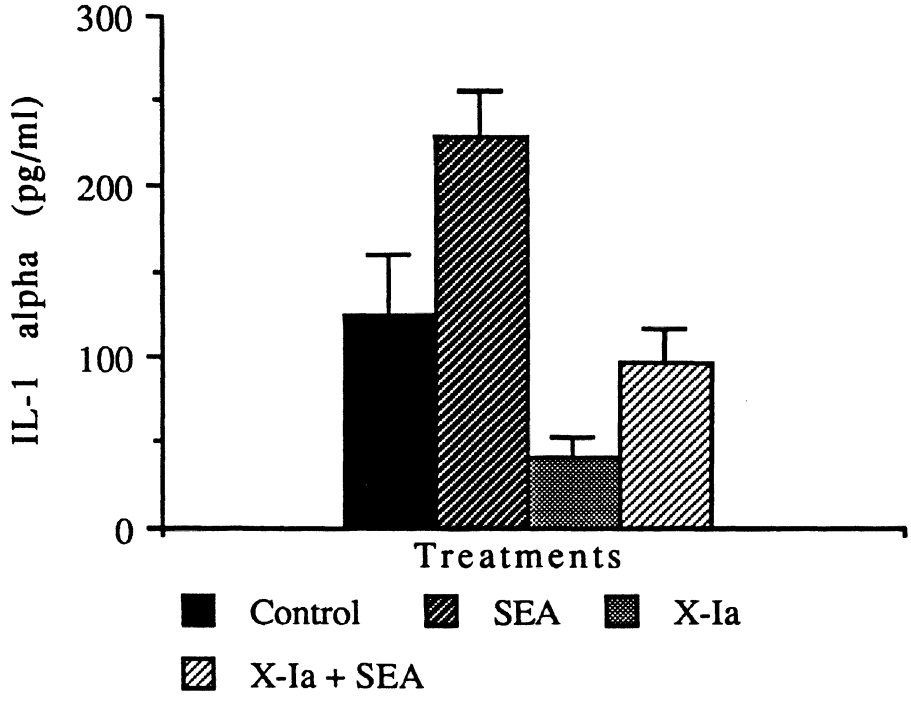

Figure 7. Effect of binding cell-surface Ia molecules with Anti-Ia mAb on SEA-induced IL-1 -alpha secretion. The values expressed are the mean ± SEM generated by pooling the data from two separate experiments. (X-Ia - anti-Ia monoclonal antibody)

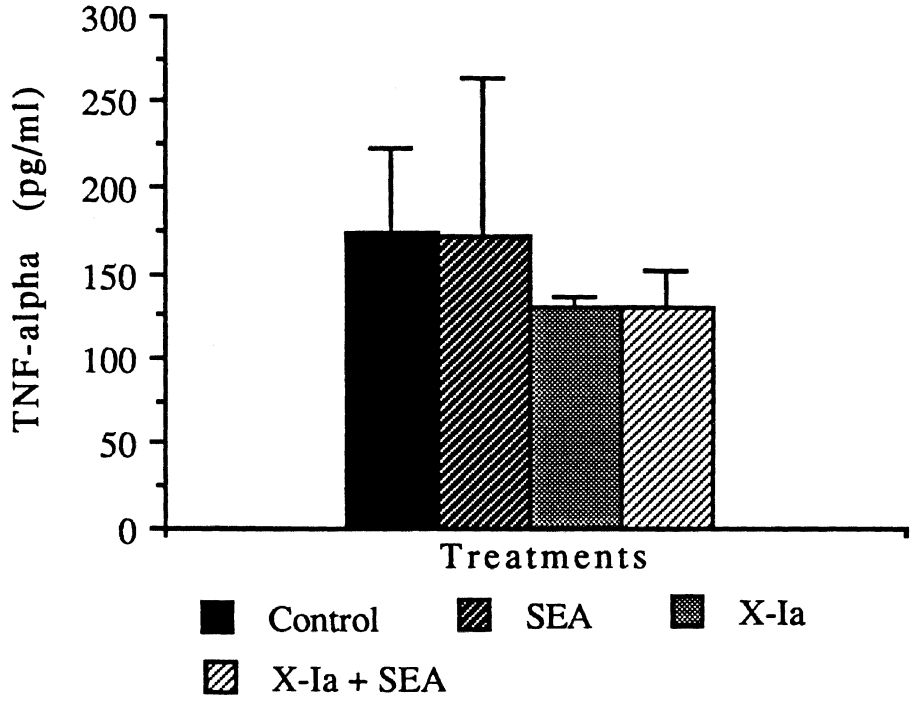

Figure 8. Effect of binding cell-surface Ia molecules with Anti-Ia mAb on SEA-induced TNF-alpha secretion. The values expressed are the mean ± SEM generated by pooling the data from two separate experiments.

Effect of Depleting Specific EC-Subpopulations on Cytokine Production

LCs, DETCs, or both subpopulations were eliminated from EC suspensions by treating ECs with anti-Ia, anti-Thy1, or both. This was followed by incubation with rabbit complement. Since the cells did not show equal amounts of constitutive IL-1-alpha production between the two experiments, these results could not be grouped and are presented separately. As shown in Table I, the LC-depleted fraction of ECs secreted lower quantities of IL-1-alpha constitutively. The results obtained with the DETC-depleted fraction and LC- and DETC-depleted fraction were quite variable.

Consistent with the results of earlier experiments, the SEA-induced increase in TNF secretion by ECs was statistically insignificant. As shown in Figure 9, LC-depleted fractions exhibited no significant change in TNF-alpha secretion. The depletion of DETC from EC suspensions, however, markedly decreased both constitutive and SEA-induced total TNF-alpha secreted by ECs. A similar decrease was seen in the EC fractions depleted of both LCs and DETCs.

These results suggest that LCs are partly responsible for the constitutive but not SEA-induced IL-1-alpha secretion, while the production or regulation of IL-1 secretion by DETCs is inconclusive. There is a strong indication that DETCs may either be a source of or serve as inducers of constitutive TNF-alpha secretion in the epidermis. The presence of DETCs in the LC-depleted fraction correlated with lower constitutive and SEA-induced IL-1-alpha when compared to the EC fractions depleted of both LCs and DETCs. These

Table I

Constitutive and SEA-Induced IL-1 alpha Secretion* by Specific Subpopulation-Depleted Epidermal Cells

	EC	EC + SEA	LC depleted	LC depleted + SEA	DETC depleted
Trial 1	104	135	70	102	56
Trial 2	46	58	38	50	51

	DETC depleted + SEA	LC and DETC depleted	LC and DETC depleted + SEA
Trial 1	73	84	114
Trial 2	51	46	56

* the values given in the table are in pg/ml.

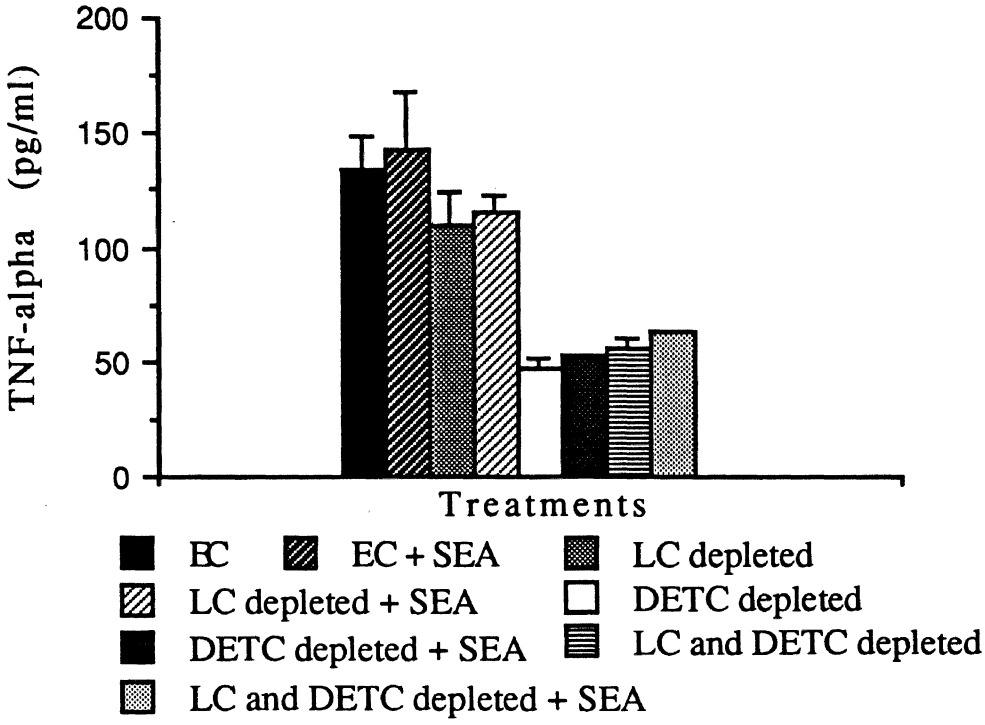

Figure 9. TNF-alpha response of epidermal-cell suspensions depleted of LC, DETC, or both, to stimulation with SEA. The values expressed are the mean ± SEM generated by pooling the data from two separate experiments.

data suggest that in the absence of LCs in the epidermis, a product of the DETCs may be present that partially inhibits the secretion of IL-1-alpha by ECs, in contrast to the apparent stimulatory role of DETCs in the secretion of TNF-alpha or enhancement of TNF-alpha secretion by other cells.

An Investigation of the Potential Involvement of Dendritic Epidermal T-cells (DETC) in EC Cytokine Secretion

A preliminary investigation was conducted to test whether Thy-1$^+$ DETCs are involved in a complex intercellular-signalling network that modulates EC secretion of IL-1-alpha and TNF-alpha. Cell-free supernatant fluid of cultured hybridoma R4-6A2 (anti murine IFN-gamma) cells was utilized at a final concentration of 25% v/v in the culture medium to block any IFN-gamma that might be present. Following 1 h incubation at 37°C with or without the antibody, SEA was added to specific groups and the cells were incubated for 24 h. Subsequently, the culture supernatant fluids were harvested and tested for the two cytokines. No significant effect of IFN-gamma on constitutive or SEA-induced IL-1-alpha secretion was noticeable (Figure 10). Although SEA-induced TNF-alpha secretion remained unaffected, the constitutive TNF-alpha level was significantly lowered by blocking the IFN-gamma (Figure 11).

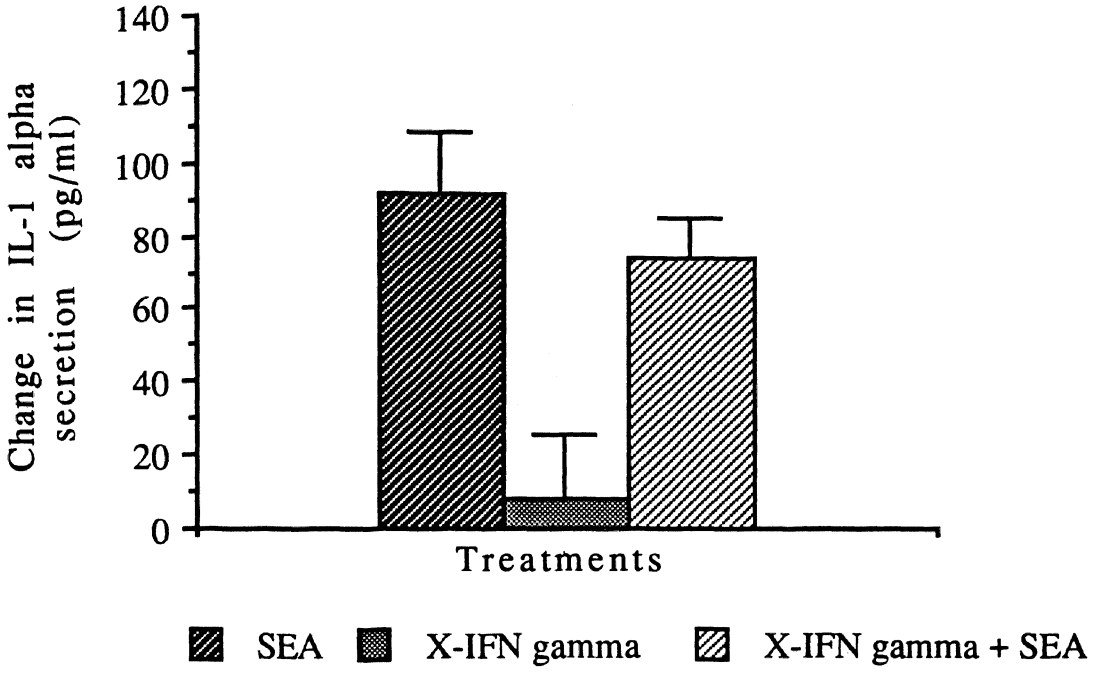

Figure 10. Effect of blocking IFN-gamma in epidermal cell suspensions on SEA-induced secretion of IL-1-alpha. The change in IL-1 -alpha secretion was calculated by subtracting the constitutive IL-1-alpha level from levels detected in the treatment groups.The values expressed are the mean $\pm$ SEM generated by pooling the data from two separate experiments.

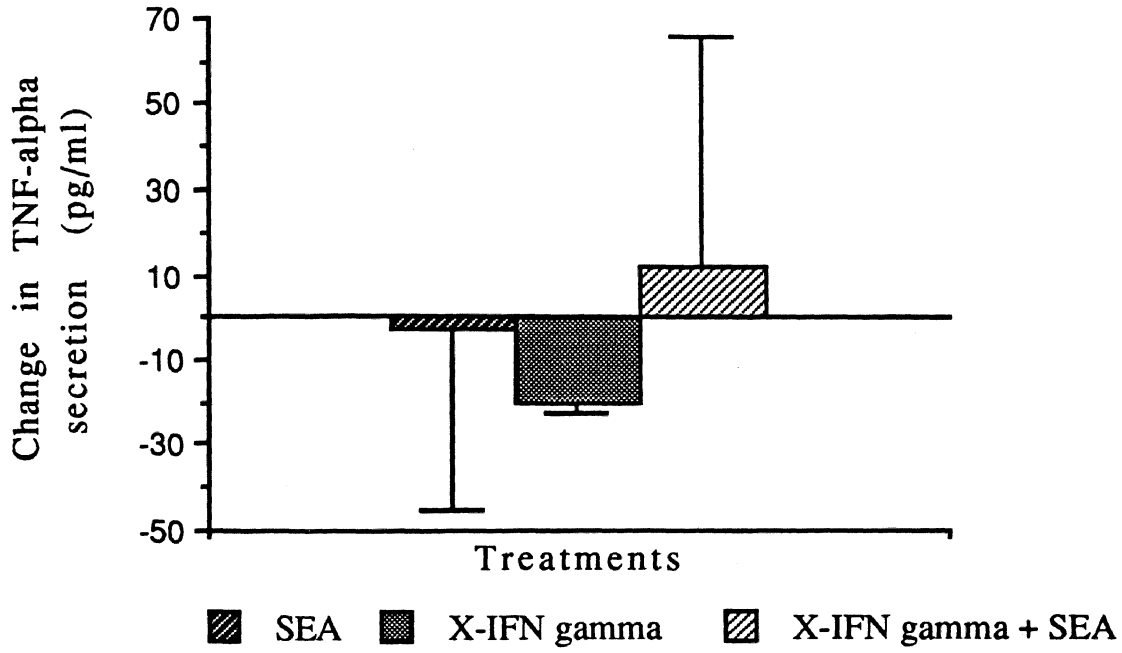

Figure 11. Effect of blocking IFN-gamma in epidermal cell suspensions on SEA-induced TNF-alpha secretion. The change in TNF-alpha secretion was calculated by subtracting the constitutive TNF-alpha level from levels detected in the treatment groups. The values expressed are the mean $\pm$ SEM generated by pooling the data from two separate experiments.

Cytokine Secretion by Purified Langerhans Cells

The final set of experiments in this study involved an examination of IL-1-alpha and TNF-alpha secretion by purified LCs. LCs isolated by biomagnetic separation contained magnetic microspheres (beads) attached on the cell surface via Ia molecules. The fraction of cells that remained after Ia^+ cell removal were labelled "other epidermal cells (OECs)" and were used as another group in this study. Two concentrations of each of these two groups, LC and OEC, were cultured in the presence or absence of SEA. Since all our studies utilized ECs at a concentration of 2 X 10^6 viable cells/ml, one group of purified LCs were cultured at 4 X 10^4 viable cells/ml which is 2% of this cell number and represents the normal density of LCs in the epidermis. Another group of LCs were cultured at a concentration of 5 X 10^5 viable cells/ml, which was previously determined to produce cytokine levels in the lower part of the range detectable by the ELISA kits that were used. To be comparable with the latter group of LCs, one group of OECs was cultured at 5 X 10^5 viable cells/ml while another was cultured at 2 X 10^6 viable cells/ml. Figures 12 and 13 illustrate the results of this study. LCs at 4 X 10^4 cells/ml were found to produce negligible amounts of both cytokines. As shown in Figure 12, the increase of LC-derived IL-1-alpha (at 5 X 10^5 LC/ml) induced by SEA was insignificant while OECs at either concentration exhibited statistically significant increases. The EC group, comprising a mixture of the two epidermal cell subpopulations showed a considerable increase in IL-1-alpha secretion following

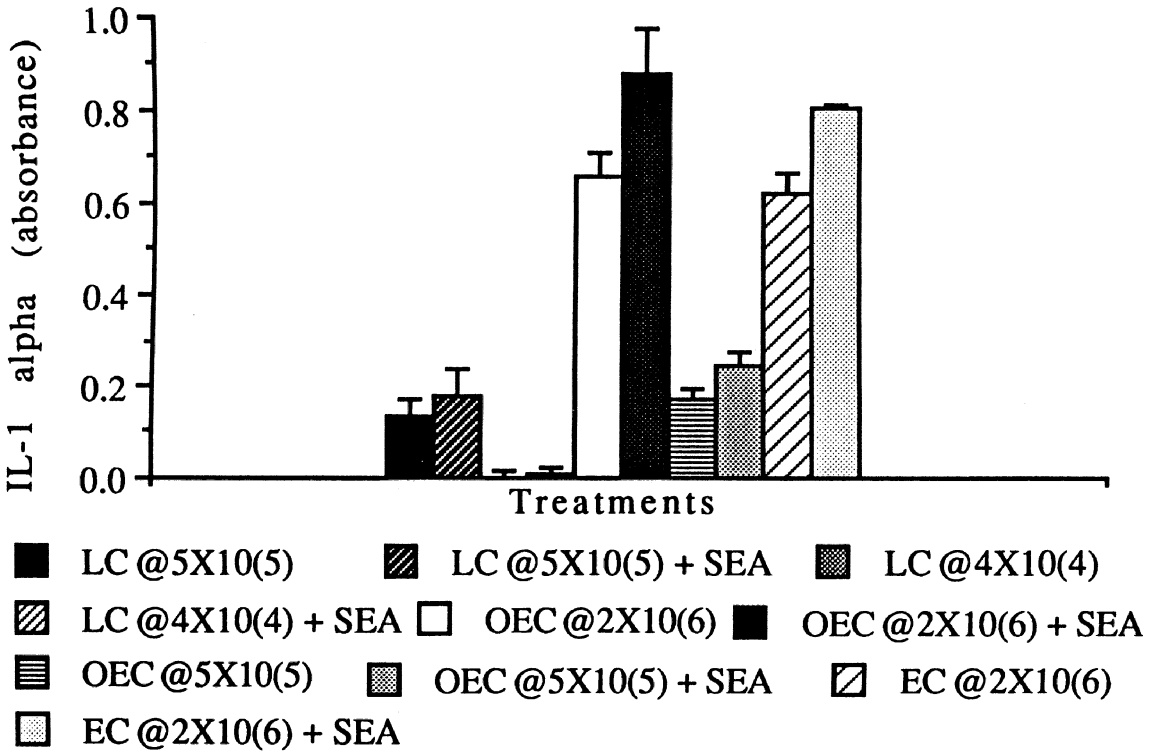

Figure 12. IL-1-alpha response of purified LC and a LC-depleted epidermal-cell population (OEC) to stimulation with SEA. The figures in the legend indicate the number of cells/ml. The values expressed are the mean $\pm$ SEM generated by pooling the data from two separate experiments.

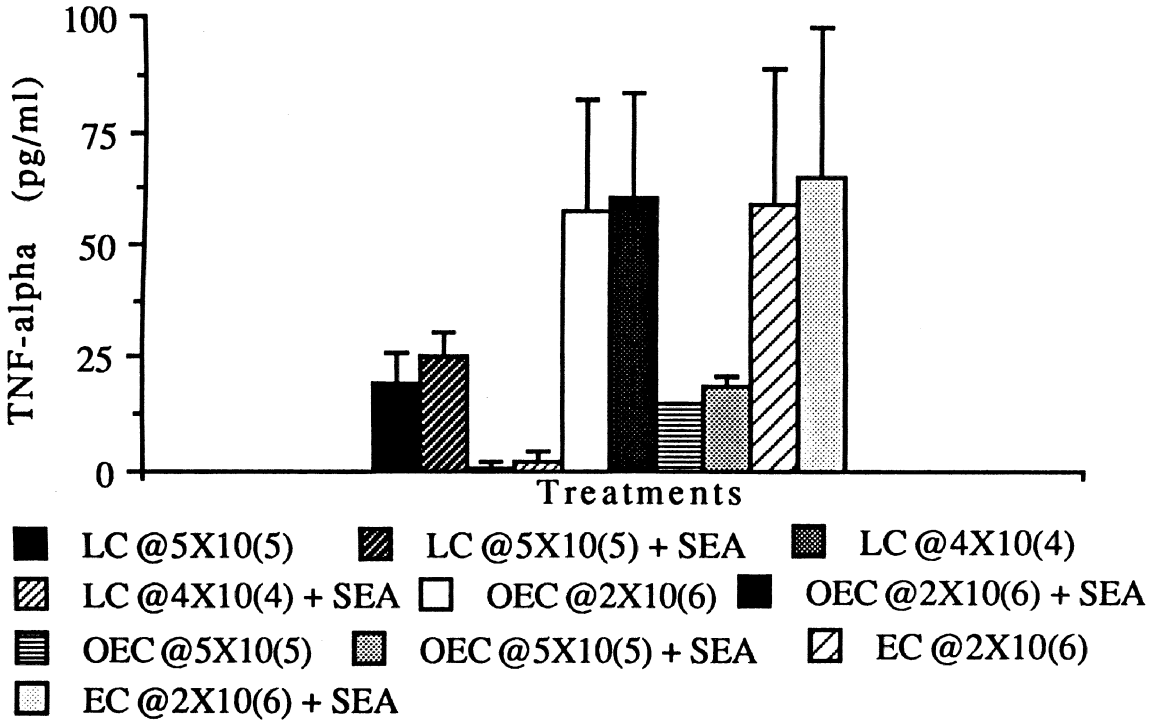

Figure 13. TNF-alpha response of purified LC and a LC-depleted epidermal-cell population (OEC) to stimulation with SEA. The figures in the legend indicate the number of cells/ml. The values expressed are the mean ± SEM generated by pooling the data from two separate experiments.

stimulation with SEA. The analysis of TNF-alpha in these experiments, as depicted in Figure 13, did not provide meaningful information due to considerable variation between experiments.

The results of this study indicate that rosetted LCs are capable of cytokine secretion and, more importantly, that while isolated LCs are not a significant source of the increased IL-1-alpha secretion by ECs, OECs may be chiefly responsible for the increase induced by SEA. Although the SEA-induced increase in IL-1-alpha secretion by purified LCs was statistically insignificant, the possibility still exists that the LCs may be partly responsible for this increase in the presence of OECs, but subsequently cease this response once they are separated from them, such as when LCs migrate out of the epidermal compartment en route to the lymph nodes.

Summary

Altogether the various investigations in this study have revealed the following aspects of the epidermal cell (EC) and Langerhans cell (LC) cytokine modulation. It was found that ECs constitutively produce IL-1-alpha and TNF-alpha. They increase their production of mainly interleukin(IL)-1-alpha, and to a lesser extent of tumor necrosis factor(TNF)-alpha, upon stimulation with bacterial toxins, especially staphylococcal enterotoxin-A (SEA). The application of SEA on the external surface of the skin alters the morphology of LCs within the epidermis from a dendritic shape to a rounded appearance. The increased secretion of IL-1-alpha by ECs stimulated by SEA apparently involves protein kinase C in the

molecular intracellular signalling cascade within ECs while the constitutive IL-1-alpha secretion is probably induced through the G-protein-mediated pathway. More specifically, there are indications that LCs are partly responsible only for the constitutive secretion of IL-1-alpha while other epidermal cells (OECs) are chiefly responsible for the SEA-induced response. On the other hand, the dendritic epidermal T-cells (DETCs) may either be a source of or serve as inducers of constitutive TNF-alpha secretion in the epidermis. Furthermore, some results indicate that in the absence of LCs in the epidermis, a product of the DETCs may be present that partially inhibits the secretion of IL-1-alpha by ECs. Although one investigation within this study indicated the potential role of interferon(IFN)-gamma (possibly produced by DETC in the epidermis) in the induction of constitutive TNF-alpha, but not of SEA-induced TNF-alpha and both constitutive and SEA-induced IL-1-alpha secretion, the possibility cannot be entirely ruled out. Thus the potential role of DETCs as regulators of EC-cytokine secretion still exists. The findings from this study constitute a fundamental step in the ultimate elucidation of cytokine networks within the skin and the signalling mechanism(s) following hapten/antigen application on the skin that results in LC migration.

CHAPTER IV

DISCUSSION

The nature of the stimulus which causes Langerhans cells (LCs) to migrate out of the epidermis following exposure to cutaneous antigens poses an interesting and yet unresolved question. There are several biological mediators that could potentially constitute such a stimulus. Since cytokines are known to regulate several immunologic mechanisms, epidermal-cell-derived cytokines have been under close scrutiny for the last few years. Granulocyte/macrophage colony stimulating factor (GM-CSF) was found to increase viability and enhance the function of LCs in culture (58,118), and in humans, to promote keratinocyte growth and wound healing (48). TNF-alpha has been shown to maintain LC viability *in vitro*, but no enhancement of LC function was noted (58). IL-1 alone, on the other hand, was previously found to enhance LC function but not viability when combined with GM-CSF (44).

In addition to maintaining LC viability and function, the involvement of cytokines in the phenomenon of LC migration has also been recently suggested. Lundqvist and Back (1990) discovered that a systemic or intracutaneous injection of recombinant IL-1-beta caused a decrease in Ia^+ dendritic cells in the epidermis of C3H/HeN mice (71). Subsequently, Enk *et al.* (1993) found that such an injection of IL-1-beta given to BALB/c mice resulted in 5- to 100-fold enhancement of mRNA signals for IL-1-alpha, IL-1-beta, macrophage inflammatory protein (MIP)-2, IL-10, TNF-alpha, and

MHC-class-II (Ia) molecules, mimicking the changes induced by antigens that cause LC depletion (26). Thus they were able to suggest that dendritic cell-derived IL-1-beta may play a critical role in the initiation of primary immune responses in the skin. The synthesis of IL-1-beta mRNA has been demonstrated by other researchers as well (74,95). Enk and Katz (1992) had previously reported that the secretion of IL-1-beta protein was below the level of detection in sensitive bioassays, and by the analysis of mRNA signals showed that LCs mainly produced IL-1-beta while keratinocytes were the major sources of secreted IL-1-alpha, GM-CSF, MIP-2, and TNF-alpha (28). On the other hand, Cumberbatch and Kimber (1992) demonstrated that an injection of recombinant murine TNF-alpha into the dermis of BALB/c mice leads to the concentration- and time-dependent accumulation of dendritic cells in the draining lymph nodes (21), while GM-CSF was shown to be incapable of stimulating dermal dendritic cells in an identical manner.

Previous studies have shown that staphylococcal superantigens are capable of inducing a dose-dependent depletion of LCs from murine epidermis (85). The underlying stimulatory signal(s) and mechanism(s) may be similar to the situation of LC depletion induced by nominal antigens. Since both IL-1-alpha and TNF-alpha are produced by keratinocytes (the major EC subpopulation), their presence in epidermal cultures is easily detectable by ELISA. Thus the purpose of this study was to examine the effect of SEA on the production of IL-1-alpha and TNF-alpha by epidermal cells. An incubation period of 24 h following SEA application was initially

determined to result in the secretion of optimal levels of both cytokines (data not shown).

Lipopolysaccharide (LPS) was originally discovered as the endotoxin that induced TNF secretion in bacillus Calmette-Guerin(BCG)-infected mice during a study of hemorrhagic necrosis of transplanted tumors caused by material from gram-negative bacteria (14). It was found to be toxic for tumors and thus caused their necrosis, a function previously attributed to the endotoxin itself. LPS is known as a potent non-specific activator of macrophages and monocytes (3), inducing them to produce cytokines such as TNF and IL-1 (15). We have found that both LPS and SEA were capable of stimulating increased IL-1-alpha secretion. SEA was more potent than LPS in this stimulatory capacity. When applied simultaneously, SEA and LPS seemed to synergistically induce very high IL-1-alpha levels. LPS has previously been found to act in a synergistic fashion with IL-1-alpha, TNF-alpha, IFN-gamma, and M-CSF in various experimental systems (30,79,98). Two possibilities might explain the tremendous increase of IL-1-alpha under simultaneous stimulation with SEA and LPS. The first possibility is that the two toxins may be directly synergising through co-stimulation, while the second is that the SEA or LPS induced IL-1-alpha synergises with LPS. These possibilities could be further be tested by including purified recombinant murine IL-1-alpha, both alone and with LPS, as additional treatment groups in an identical study.

TNF-alpha was found to be secreted constitutively in high amounts, but much variation was observed between experiments.

Thus most of the following discussion focusses on the significant findings relevant to IL-1-alpha.

The binding of Ia ligands to cell surface receptors has previously been demonstrated to result in elevated intracellular cAMP levels and in the translocation of protein kinase C (PKC) to the nucleus in murine B cells (13), and in the secretion of IL-1 by human monocytes (82). Ia molecules are members of the immunoglobulin supergene family which have been described to commonly have src-type protein tyrosine kinases (PTK) associated with their cytoplasmic domains (17). Activation of receptor-coupled PTK triggers a cascade of tyrosine phosphorylations of which the first event is the phosphorylation of an enzyme (referred to as the effector). This leads to the activation of a second messenger, which is then followed by several other events (2).

Previous studies have revealed that H-8, cholera toxin(CT), and pertussis toxin(PT) are able to significantly block SEA-induced LC depletion from the epidermis (85). This suggests that G-proteins and their associated cyclic nucleotide-dependent protein kinases are involved in the phenomenon of LC migration. GTP-binding proteins (G-proteins) are located near the cytoplasmic end of some cell-surface receptors and function as signal transducers. G-proteins consist of three protein chains - alpha, beta, and gamma, of which the critical role in signal transduction is played by the alpha subunit. When a ligand-bound receptor binds to a G-protein, the alpha subunit is activated. It then dissociates from the beta-gamma dimer and binds to an appropriate enzyme (adenylate cyclase or phosphoinositide-specific phospholipase C). The activated enzyme

then acts as an amplifier and produces a specific second messenger (cAMP or protein kinase C) which in turn affects the production of a third and perhaps even a fourth messenger that ultimately alters cell function or behaviour (1).

The property of G-proteins that is commonly exploited to study their involvement in transmembrane signalling mechanisms is their sensitivity to cholera and pertussis toxins. These toxins were used in this study because of their ability to inhibit the transduction of a signal by ADP-ribosylating specific G-proteins (114). H-8, a specific inhibitor of G-protein-associated cyclic-nucleotide-dependent kinases (45) was used in this study to examine the role of these cyclic nucleotide-dependent protein kinases. In addition, H-7 was utilized in this investigation because of its selective ability to inhibit protein kinase C (52). Moreover, the use of this inhibitor in conjunction with the others mentioned above was useful in implicating protein kinase C as the second messenger (and therefore phospholipase C as the effector enzyme) in the SEA-induced stimulus mediated through surface Ia molecules causes increased IL-1-alpha secretion by ECs.

It was found in this study that constitutive IL-1-alpha secretion involves a G-protein-mediated-transduction pathway while the SEA-induced IL-1-alpha response involves protein kinase C. Both the G-protein-mediated pathway and the PTK-mediated pathway may involve protein kinase C as the second messenger. If the protein kinase C was a part of the G-protein-transduced pathway then H-8, cholera toxin, and pertussis toxin should also have, in addition to H-7, been able to block the IL-1-alpha response to SEA stimulation. Thus it is evident that the SEA-induced signal is transduced via the PTK

pathway. Indeed, Chatila and Geha (1993), having reviewed in a recent article (17) the work of several other groups, presented a scheme for transmembrane signalling in which PTK is shown to be associated with the cytoplasmic end of the Ia molecule. The results of the present investigation are supported by the work of Fuleihan *et al.* (1991) who demonstrated that staphylococcal exotoxins (superantigens) induced a transient accumulation of cAMP in HLA-DR^+ human B cells (32). But this does not account for the effects of bacterial superantigens induced via surface-MHC-class-II molecules, which were shown by the failure of forskolin (a direct activator of adenylate cyclase) to reproduce activation events through Ia molecules similar to those induced by staphylococcal exotoxins. Moreover, the use of HA 1004 (an activator of cAMP-dependent protein kinases) to abolish these activation events also proved to be a failure (32). These results are particularly interesting in the face of a recent finding by Halliday and Lucas (1993) that protein kinase C transduces the signal for Langerhans'-cell migration from the epidermis (39). The fact that G-proteins have been implicated in LC depletion from the epidermis (85) indicates that SEA does not directly induce LC migration but that it induces a primary mediator which may itself, or via secondary mediators, signal the LCs to migrate. Whether IL-1-alpha could, in fact, provide such a signal is intriguing in view of previous reports of the stimulation of cAMP synthesis by IL-1 in a variety of cells (100) and the involvement of a G-protein in the transduction of this stimulus (18). Normal resting keratinocytes have been found to express IL-1 receptors in low numbers in humans (8) while the expression by LC of these receptors

has not yet been reported. The use of H-8, cholera toxin(CT), and pertussis toxin(PT) apparently did not have any effect on the SEA-induced increase in IL-1-alpha secretion by EC but did in fact significantly lower the total IL-1-alpha levels (see Figure 5). It is possible that this overall decrease in IL-1-alpha level was responsible for the inhibition of LC depletion following treatment with H-8, CT, or PT seen in the study by Pickard and Burnham (85). Based on such a possibility the following hypothesis can be considered for the induction of Langerhans'-cell migration from the epidermis following appropriate stimulation. ECs constitutively secrete IL-1-alpha; upon stimulation with SEA, however, the LCs or DETCs are activated which results in the production of an unknown mediator that induces OECs (mainly keratinocytes) via a protein kinase C-dependent signal-transduction pathway to increase the IL-1-alpha secretion. These increased IL-1-alpha levels, upon exceeding a threshold level, provide the migration signal to LCs via a pathway mediated by G-proteins. IL-1 has previously been shown to provide signals to dendritic cells (DC) that enhance responses mediated by them (60).

The study involving the analysis of the IL-1-alpha levels following binding of surface-Ia molecules with anti-Ia monoclonal antibody (mAb) clarified the binding site of SEA on the Ia molecule. This receptor is independent of the mAb binding site. This was evident from the observation that SEA was able to induce an increase in IL-1-alpha secretion comparable to that of the controls despite the EC surface-Ia molecules being bound to the mAb (see Figure 7). This experiment also showed that the mAb alone was able to lower

constitutive IL-1-alpha secretion, which cannot be explained as yet. Although it is likely that the SEA-induced signals are affected by Ia molecules, this mechanism is not confirmed in the present study. It is possible that such a signal is transmitted by other, as yet uncharacterized, superantigen receptors.

An examination of the role of Langerhans cells (LCs) and dendritic epidermal T-cells (DETCs) revealed much variation between experiments of IL-1-alpha levels. One possible reason for this may be the presence of variable numbers of dead cells after complement-mediated lysis of the specific cell types. Ideally, the removal of dead cells prior to treatment application would eliminate such a possibility. The experiments in the present study did not employ techniques to remove dead cells because of several other complications involved in the process. There are no known reports of the negative effects of dead cells on cytokine secretion by cells. Despite the variation in IL-1-alpha levels, one distinct pattern was noted in each experiment. The depletion of LCs from the suspensions markedly lowered the constitutive IL-1-alpha levels (see Table I). The TNF assay for these experiments showed little variation. Even though SEA did not significantly induce increased TNF-alpha secretion, the depletion of DETCs either separately or in conjunction with LCs resulted in a major decrease in both constitutive and SEA-induced total TNF-alpha secretion. Based on this, it can be proposed that the DETCs are either a source of TNF-alpha in the epidermis or that they serve as enhancers of the constitutive secretion of this cytokine by other epidermal cells. It was recently found that the induction of functional responses in Ia^+ cells, such as the secretion of

TNF by monocytes, by staphylococcal exotoxins was potentiated by the presence of V-beta-compatible T-cells (17). In a similar way DETCs, which express the products of the V-gamma-3- and V-delta-1-gene segments, may potentiate TNF-alpha secretion by LCs. This is in contrast to observations of Matsue *et al.* (1992) who reported that the depletion of LCs from EC suspensions caused a marked decrease in TNF and macrophage inflammatory protein(MIP)-2 (74).

To probe the regulatory role of DETCs, a preliminary investigation was performed that examined the effect of adding anti-IFN-gamma to the culture medium prior to SEA application. The purpose of adding anti-IFN-gamma was to block any IFN-gamma that may be produced following culture of ECs. IFN-gamma is known to activate macrophages (75) and therefore may also affect dendritic-antigen-presenting cells such as LCs. It must be stressed that this was only a preliminary investigation since the mAb in the supernatant fluid of the R4-6A2 hybridoma cell culture was not previously titred. Thus the amount of mAb added may not have been sufficient to block all IFN-gamma in the medium. However, if it is assumed that all the IFN-gamma was in fact blocked, the results suggest no apparent regulatory role of DETCs in the secretion of IL-1-alpha or TNF-alpha by the common T-cell cytokine, IFN-gamma. Another possibility that was not tested in this study, is the involvement of other T-cell cytokines in the potential role of DETCs. T-cells belonging to the type-2 class of helper cells (T_H2 type) are known to secrete mainly IL-4, IL-5, and IL-10, while those of the type-1 class (T_H1 type) secrete mainly IL-2 and IFN-gamma. It is not currently known to which class the DETCs belong. Hence there are at

least three cytokines of the T_H2 type and at least one more of the T_H1 type that would need to be further examined through similar studies. There is an indication from this investigation that IFN-gamma is involved in regulating constitutive TNF-alpha secretion. This possibility also needs further clarification.

To extend the study of the role of LCs in EC-cytokine secretion, highly purified (approximately 90-95%) Langerhans'-cell populations were obtained by biomagnetic separation. This procedure results in LCs rosetted by immunomagnetic spheres. Previous studies have demonstrated that the adherence of these immunomagnetic spheres does not effect LC function (42). We have shown that rosetted LCs are capable of cytokine secretion following stimulation with SEA. This is in agreement with the unpublished findings of Hanau *et al.* (1988) that rosetted LCs retained their capacity to secrete cytokines upon stimulation with endotoxin (42). Nevertheless, there is the likelihood that the absolute amount of cytokines secreted may have been altered. Recall that one of the investigations in this study has revealed that binding a mAb to Ia molecules caused a decrease in IL-1-alpha secretion. Since LC-surface-Ia molecules were saturated with anti-Ia mAb prior to biomagnetic separation, the IL-1-alpha levels seen in purified LCs may be assumed to be far less than those attained under normal conditions (that is, in the absence of blocked Ia molecules). However, this assumption cannot be made in the case of the purified LCs if they are indeed, as indicated by another investigation within this study, not a source of SEA-induced IL-1-alpha. This investigation indicates that OECs, of which keratinocytes are the main subpopulation, secrete the majority of IL-1-alpha. This

is supported by previous observations by other laboratories (67). When stimulated with SEA, OECs increased their secretion of IL-1-alpha. This suggests that OECs may have either been induced to express surface Ia molecules through which the stimulus was transduced, or that SEA may act on cells through additional or alternative means which are not yet known.

In the midst of extensive research going on presently on staphylococcal superantigens and their effects on the immune system, this study is the first to demonstrate that staphylococcal enterotoxin-A is capable of stimulating the secretion of two important immunomodulating cytokines by epidermal cells: IL-1-alpha and TNF-alpha. However, the roles of these cytokines in LC depletion by SEA still remain to be elucidated.

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APPENDIX-I

CYTOKINES FOUND IN THE SKIN

CYTOKINE	SOURCE	IMMUNOLOGICAL ACTIVITY
IL-1 (-alpha, -beta)	Several cell types	Co-stimulates activation of T-cells; enhances activity of NK cells; increases expression of adhesion molecules on endothelial cells; chemoattractant for macrophages and neutrophils
IL-3	T_H cells, NK cells, mast cells	Supports growth and differentiation
IL-6	monocytes, macrophages, T_H cells, bone marrow stromal cells	Stimulates antibody secretion; induces secretion of acute phase proteins
IL-8	macrophages, endothelial cells	Chemoattractant for neutrophils
TNF (-alpha, -beta)	macrophages	cytotoxic to tumor cells; Induces cytokine secretion by cells
IFN	leukocytes, fibroblasts	Inhibits viral replication; stimulates activity of accessory cells
G-CSF, M-CSF, GM-CSF	T-cells	Influence inflammatory responses
TGF (-alpha, -beta)	Platelets, macrophages, lymphocytes	Chemoattractant for macrophages and monocytes; induces increased IL-1 production; promotes wound healing
bFGF	macrophages, endothelial cells	Mitogen for fibroblasts and endothelial cells
PDGF	Platelets, endothelial cells, macrophages	Increases MHC-class-II expression on macrophages; chemoattractant for monocytes and neutrophils

CYTOKINE	SOURCE	IMMUNOLOGICAL ACTIVITY
Epidermal cell Suppressor Factor	Epidermal cells	Inhibits contact hypersensitivity responses
Contra-IL-1	Epidermal cells	Inhibitor of IL-1

ABBREVIATIONS: IL- interleukin; TNF- tumor necrosis factor; IFN- interferon; G-CSF- granulocyte colony-stimulating factor; M-CSF- macrophage colony-stimulating factor; GM-CSF- granulocyte/macrophage colony-stimulating factor; TGF- transforming growth factor; bFGF- basic fibroblast growth factor; PDGF- platelet-derived growth factor.

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