SKIN LANGERHANS (DENDRITIC) CELLS IN VIRUS INFECTIONS AND AIDS

DEVELOPMENTS IN MEDICAL VIROLOGY

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Preface

Over the generations the skin has been the site for immunization against smallpox. This method of immunization was described in a letter written by Lady Mary Montagu on April 1, 1717 in Adrianopole, Turkey:

"The small-pox, so fatal, and so general amongst us, is here entirely harmless by the invention of ingrafting, which is the term they give it...The old woman comes with a nut-shell full of the matter of the best sort of small-pox...She immediately rips open (the skin) with a large needle...and puts into the vein as much venom as can lie upon the head of her needle, and after binds up the wound. There is no example of anyone that died of it; and you may believe that I am satisfied of the safety of this experiment since I intend to try it on my dear little son" (Letters from the right Honourable Lady Mary Montagu 1709-1762. Published by J.M. Dent and Co. London, 2nd edition, September, 1906, p. 124.)

The "variolation" method was, 80 years later, markedly improved by the use of cowpox virus, as reported by Edward Jenner in 1796.

The successful method of intradermal immunization against smallpox and later against other virus diseases is in fact based on the presence of anitigen-presenting dendritic cells in the skin. Although skin dendritic cells were described by Paul Langerhans in 1868, the first study on the involvement of skin dendritic (Langerhans) cells in infection of the skin with vaccinia virus was published by Nagao and Inaba in 1976 (Arch. Dermatol. Res. 256:23-31)

The importance of skin dendritic (Langerhans) cells in virus infections is becoming more apparent with the rapidly accumulating evidence of the bone marrow origin of these cells and their ability to function as antigen-presenting cells. This book on viruses and skin dendritic cells at the present state of

research has been prepared to provide virologists and immunologists with the current knowledge on the subject. It is hoped that this knowledge will enhance the research on dendritic cells in virus diseases. I have taken the liberty of presenting the first chapter which reviews the subject, with full references in order to assist the reader.

The recent findings that human immunodeficiency virus (HIV-1) infects skin dendritic (Langerhans) cells and develops latently in these cells provides another aspect of AIDS as described in four chapters in the book.

Dendritic cells are also involved in the ability of the skin to succumb to wart virus infections that may lead to the development of skin tumors as well as to responses that cause "spontaneous regression" of a tumor. Due to their involvement in processes in the skin such as aging and responses to environmental agents like chemicals and ultraviolet light, dendritic cells may be studied to determine not only the effects of various chemical agents, but also to find ways of protecting these cells from damage (e.g. by retinoic acid treatment).

I wish to thank all those who contributed the chapters of this book and Dr. Julia Hadar for excellent editorial assistance. I hope that the present book will be of use for developing ideas on a new generation of virus vaccines as well as treatments to improve the ability of man to overcome environmental-induced diseases.

Yechiel Becker
Jerusalem

SKIN LANGERHANS (DENDRITIC) CELLS IN VIRUS INFECTIONS AND AIDS

GENERAL

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DENDRITIC CELLS IN THE EPIDERMIS AND THE LYMPH NODES - A REVIEW

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ABSTRACT

The present article reviews several aspects of Langerhans cell biology that have received much attention during recent years. Much has become known in the past decade about the structure and function of Langerhans cells, particularly regarding the functional relationship between Langerhans cell and other dendritic cell subsets in the lymph nodes and the epidermis. The role of Langerhans cells may not only be restricted to that of regular antigen-presenting cells. Indeed, recent findings indicate that Langerhans cells may control important intraepidermal processes of differentiation and may be involved in the pathogenesis of various disorders.

BRIEF HISTORICAL SURVEY

Paul Langerhans' extraordinary sense of observation and his outstanding capacity to recognize the novelty and peculiarity of biological structures allowed him to introduce many new concepts in the various fields of biology (reviewed in ref. 1). Not only did he describe for the first time Langerhans cells (LC) in the skin (2) and a novel cellular structure in the pancreas (3) [renamed after him, by the French histologist Laguesse, " îlots de Langerhans " (4)], but he also left a monumental contribution to the science of invertebrates (1). These latter studies were performed in Madeira where he had settled to recover from tuberculosis and where he died on July 20, 1888, at the age of 41, one hundred years ago (1).

Paul Langerhans was still a medical student in Berlin when he discovered a new nonpigmentary dendritic cell (DC) type by staining

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human skin with the gold-staining method that had been developed by his teacher, Conheim (2). Since the gold chloride stain has a specific affinity for nerve tissues, LC were considered for about a century to be part of the nervous system (2,5). This theory was first challenged in the late 1950's when Masson (6) and later on, Breathnach (7) and Zelickson (8) advanced the hypothesis that LC may be related to melanocytes, or may be worn-out products of these cells or their precursors. In 1968, Breathnach et al. (9) performed a decisive series of experiments which definitely ruled out this possibility. Indeed, after transplantation of limb buds of 10-day-old mouse embryos deprived of their neural crest elements, into the spleen of histocompatible animals, LC (but not Schwann cells or melanocytes) could be readily observed in the developing skin (9).

It was not until the late 1960s, one century after their discovery, that the real nature of LC began to be more clearly understood. Reports were then published which indicated that LC could be found in various tissues of mesenchymal origin (10-11). Later, electron microscopic studies showed that during sensitization to allergenic compounds, LC migrate from the skin through the lymph vessels and come into close contact with lymphocytes in the draining lymph nodes (reviewed in ref. 12). These findings suggested that LC might play an important role in the development of the immune response to skin antigens and triggered various groups to investigate the possibility that LC may function as the most peripheral arm of the immune system. Throughout the 1970s and early 1980s, it was established that the cell discovered by Paul Langerhans in 1868 represented an essential element of the immune system playing a central role in the mechanisms of defense of the body in a wide range of pathological processes.

DENDRITIC CELLS IN THE EPIDERMIS: MORPHOLOGY AND PHENOTYPE

Many different cell types are known to populate the epidermis: these include keratinocytes, that constitute the bulk of the skin cell population, melanocytes that synthesize the skin pigment, melanin and Merkel cells that are neural crest-derived and are known to synthesize and secrete various neuropeptides (13).

In 1983, three groups of investigators simultaneously described a new type of DC, termed Thy-1$^+$ dendritic epidermal cell (Thy-1$^+$DEC) in the murine epidermis (14-16). Thy-1$^+$DEC have been shown to be derived from a cell progenitor originating in the bone marrow (17). They were shown to be Thy-1$^+$, Ly-5$^+$, Ia$^-$, asialo GM1$^+$, Lyt-1$^-$, Lyt-2$^-$, L3T4$^-$, sIg$^-$ and weakly Fc receptor$^+$ (18-19) and to proliferate in response to concanavalin A (ConA) and interleukin 2 (IL-2) (20). The function and lineage of these cells is still a matter of controversy. When injected by the intravenous (IV) route they were shown, in contrast to LC, to induce immune suppression (21). It has also been suggested that Thy-1$^+$DEC belong to the natural killer (NK) cell lineage on the basis of both phenotypical (19) and functional (22) studies. However, unlike NK cells, Thy-1$^+$DEC express the $\gamma\delta$ T cell receptor (TCR)/CD3 complex (23). They are known to express IL-2 receptor and to release IL-2 and IL-3 when stimulated with ConA (24). In addition, mice homozygous for the mutation "severe combined immunodeficiency" (SCID), that are known to be deficient in both T and B cells (25) lack Thy-1$^+$DEC (26).

These data support the hypothesis that Thy-1$^+$DEC belong to the T-cell rather than the NK lineage. Since their phenotypical and functional features strikingly resemble those of a recently described T cell early precursor cell population, they might represent an immature T-cell related population in the murine epidermis (27-28). Studies on the structure of Thy-1$^+$DEC TCR indicated that they might represent the earliest emigrants from the pool of maturing T cells within the fetal thymus (29). Thy-1$^+$DEC are thought to play a role in the immune surveillance of the epidermis since they respond to activation in a manner similar to $\alpha\beta$ TCR$^+$ T cells (24). A human analog of the murine Thy-1$^+$DEC has been recently described (30-31).

The second DC type belonging to the immune system present in the epidermis is the LC (32). LC represent about 1-3% of the total epidermal cell (EC) population (32) and can be easily recognized at the ultrastructural level by the presence in their cytoplasm of a characteristic organelle named the Birbeck granule (33). This granule is rod-shape with a hemispherical blep at one end which gives it its characteristic "tennis-racket" shape (34). The origin and the function of this unique structure have still not yet been entirely elucidated. Recent findings by Hanau et

al. (35) indicate that the granule probably originates from the plasma membrane. The Birbeck granules might be related to the antigen-presenting function of LC as their number increases with sensitization of the skin to allergens (36). Other ultrastructural features characterizing LC, include a clear cytoplasm, the lack of tonofilaments, melanosomes or desmosomes; a lobulated nucleus and numerous cytoplasmic projections (32-34).

A large number of histochemical and immunochemical techniques have been devised that today allow the unequivocal identification of LC in the epidermis. In addition to gold chloride (2), LC can be stained with other heavy metals such as silver, cobalt, mercury or chrome (37-38). However these methods lack specificity. In contrast, the activity of various hydrolytic enzymes can be specifically demonstrated on their membranes (38-41). Adenosine triphosphatase (ATPase) (39-40) and adenosine diphosphatase (ADPase) activities (41) serve as the most widely used markers of LC in the epidermis.

LC express several markers on their membrane that are not expressed under normal conditions by other epidermal cells. Fc-IgG and complement receptors can be demonstrated using rosetting with IgG-coated ox red blood cells and IgM- or complement-coated bovine red blood cells respectively (42) or with appropriate monoclonal antibodies (43). LC have been shown to bear HLA-D/DR, T-200, CD1 and CD4 antigens in humans (44-47) and Ia, Ly-5, TL and F4/80 antigens in rodents (18,48-50). The expression of these markers is under the control of various humoral factors (reviewed in ref. 51). LC express IL-2 receptors when cultured in vitro (52). Their cytoplasm also stains positive with an anti-S-100 (a protein found in Schwann cells and melanocytes) (53) and an antiserum against vimentin (a protein also found in endothelial cells and fibroblasts) (54). Several subsets of LC have been identified by selective binding to various lectins (55). LC were shown to lack membranal class I antigens (56) although recent findings suggested that this might not be true (57).

Although a large number of markers are today available for identifying LC, most of these are not specific to LC, depending on the site they are studied and the pathological state of the the tissue being examined. For instance, keratinocytes can also express class II antigens in

various pathological conditions (58-59). Recently a new monoclonal antibody, named NLDC-145 has been shown to specifically stain murine LC and related cells in the dermal lymphatics, the peripheral lymph nodes and the thymus (60). Also, a monoclonal antibody has been described which specifically reacts with Birbeck granules in human LC (61).

THE LANGERHANS CELL FAMILY: ORIGIN AND DISTRIBUTION

LC belong to a large family of DC, distributed all over the body (reviewed in ref. 62). Using a number of markers, it was possible to identify LC and related cells in many tissues in the body including the skin (2), the gastrointestinal tract (62), the genital tract (63), the lymphatics where they are termed veiled cells (62,64-66), the thymus (67-71), the lymph nodes (62,66,72) and the spleen (73) where they are named interdigitating cells (IDC). All these cells share most of the properties that are known to characterize LC, including in the skin, thymus and lymph nodes, the presence of intracytoplasmic Birbeck granules (74-75).

The availability of membranal markers for LC allowed Katz and his colleagues (76) to elucidate the origin of LC. Using bone marrow chimerization techniques, they demonstrated that three months after bone marrow transplantation, 85% of LC originated from the bone marrow of the donor whereas keratinocytes displayed markers of the host animal. Similarly, using cytogenetic marking, donor-derived LC were detected in the skin of human recipients of allogeneic bone marrow (77). Several groups have reported the identification of a progenitor cell for LC in the bone marrow (78-79) and in the spleen (80). In addition, it has been suggested that the rare $T6^+$ peripheral blood cells (81), which increase in numbers in burned patients (82) and are readily found in cord blood (83), are the circulating precursors of LC (82,84). Phenotypic transformation of dermal macrophages into epidermal LC was observed in bone marrow transplant patients, suggesting that a certain subset of bone marrow-derived circulating monocytes may transform into LC precursor cell population in the dermis (85). In addition, cells termed indeterminate cells that are identical to LC, except for the absence of Birbeck granules in their cytoplasm, can be found in the epidermis and are thought to represent the epidermal precursors of LC (86).

In spite of the fact that LC seem to originate from the bone marrow, it is clear from a number of studies that a small fraction replicates in situ (87-88). In addition, a number of factors have been identified which stimulate LC replication in the epidermis (89-90).

LANGERHANS CELL FUNCTION IN THE SKIN AND THE LYMPH NODES

As mentioned above, early studies suggested that LC subserve immunological functions as they could be seen during contact dermatitis to bear the sensitizing antigens, to migrate in the lymph vessels and to be in close apposition with lymphocytes in the draining lymph nodes (12). Their role as potent antigen-presenting cells was definitively demonstrated in vitro by Stingl et al. in mice (91) and Braathen et al. in humans (92). Both groups succeeded in showing that purified LC were able to present antigens to T cells at least as efficiently as macrophages (91-92). These initial observations were later confirmed in a large number of studies (reviewed in refs. 93-94).

It was also shown that the capacity of epidermal cell suspensions to stimulate the proliferation of allogeneic T cells was entirely dependent on LC as it could be abolished by pretreatment of the cell suspensions with anti-Ia antibody and complement (92,95). Since this reaction represents the in vitro correlate of allogeneic graft rejection reaction in vivo, the role of LC as inducer of this reaction was investigated. A correlation was found between decrease in their numbers and enhanced survival of allogeneic grafts (96-97). However, recent experiments in humans have partly failed to reproduce these previous results (98). Finally LC were shown to be able to induce cytotoxic T cell responses in vitro (99).

In vivo, LC and Thy-1$^+$DEC have been shown to function as an integrated and autoregulated local immune system. LC appear to function as sensitizing cells whereas Thy-1$^+$DEC function as suppressor cells (21). The ratio between the two cell populations was shown to determine the susceptibility of different mouse strains to sensitization (100).

LC are thought to function in vivo by taking up antigens in the skin (37) and then migrating through the lymphatics as veiled cells (64-

66) to the lymph nodes, where they are called IDC and where they trigger the immune response (62). Following skin antigenic stimulation, IDC are found in the lymph nodes at the paracortical zone where they specifically interact with T cells (62,72). Birbeck granules and additional LC markers can be found in these cells (75,101). IDC were shown to carry sensitizing antigens in the lymph nodes (12,101-103) where they form specific clusters with T cells (104). The numbers of veiled cells (64,66) and of IDC (72,101,103) have been shown to increase upon sensitization with soluble antigens.

Despite extensive functional and phenotypical similarities between skin LC and DC in different lymphoid organs, several differences exist between the two cell subsets such as variations in the expression of specific markers or the poor stimulatory activity of fresh LC as compared with DC in various functional assays (105-106). Recent studies indicate that short-term in vitro incubation of LC (94-95,105) or in vivo migration of LC from the skin to the draining lymph nodes (101) lead to the transformation of LC into phenotypically and functionally mature DC. Immature LC function as highly potent antigen-presenting cells (94). In contrast, they are poor stimulators of T-cell proliferation. Only after transformation into mature DC are LC able to induce clonal T-cell expansion (94).

Several reports have shown that LC might be able to release important mediators of the immune function including IL-1, TNF-α, thymosin β4, prostaglandin(PG)D_2 and IFN-γ (107-111). Not much is presently known concerning the physiological importance of the release of these mediators.

It has been hypothesized that LC might also be involved in T-cell extrathymic differentiation. It is known that several subsets of T cells are able to differentiate in the absence of a functional thymus (112-113). The skin could represent the principal site of T-cell extrathymic maturation (114-116). It was shown that epidermal cells as well as thymic epithelial cells but not fibroblasts or endothelial cells are able to cause the maturation of T cells (117). It was also shown that both the skin and the thymus produce several proteins like keratin, thymopoietin, thymic serum factor and thymosin β4 (108,115,118,119-120). Moreover, experiments done with the nude mouse mutant that lacks both hair and

a normal thymus, suggested that a single gene may regulate the development of the mouse fur and thymus as it was not possible to achieve segregation of the two traits despite intensive breeding experiments (115). In addition, immature T-cell precursors have been shown to reside in the murine and human epidermis which strikingly resembles very early precursors of T cells in the thymus (14-31). Thus, striking phenotypical and functional similarities exist between the thymus and the skin. In addition, thymic dendritic cells are known to be part of the LC lineage in the thymus (62,67-68,71) and are known to control T-cell intrathymic differentiation (121-122).

Similarly, LC in the epidermis are thought to play a central role in T-cell intraepidermal differentiation. Indeed, LC were found to contain thymosin β4 (108) which induces T-cell differentiation in the thymus (123). In addition, several epidermotropic T-cell clones were recently described (124). These clones were shown upon intradermal injection to migrate into the epidermis. LC depletion prevented T-cell clone infiltration into the epidermis, suggesting that LC may direct specific T cell subset circulation into the epidermis (124).

MODULATION OF LANGERHANS CELL DENSITY AND ACTIVITY IN THE SKIN

Various physicochemical and biological factors and agents are known to augment or decrease LC density and/or function in the epidermis. Exposure to ultraviolet (UV)-light or X-irradiation, administration of a low protein diet, administration of immunosuppressive drugs or steroids, and abrasion of the skin were shown to cause a decrease in LC numbers or function in vivo and/or in vitro (96,125-130). This decrease might in certain cases be due to the loss of expression of membranal markers rather than to the actual disappearance of LC (128).

In contrast, bacterial antigens, interferon-γ (IFN–γ), monobenzyl ether or hydroquinone and retinoids are known to augment LC numbers and/or activity in the skin and other epithelia (131-135).

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LANGERHANS CELLS IN DISEASE STATES

A major breakthrough in research on LC biology was achieved when it was recognized that they might play a cardinal role in the mechanisms of defense of the body against various diseases. Many studies as well as a number of chapters in the present book indicate that LC are involved in the pathogenesis of viral infection (reviewed in ref. 136). In addition, they were shown to play an important role in the immune response against bacterial (137-138), parasitic [(139-140); although this issue is still controversial (141)] and fungal infections (142).

A large number of very recent reports indicate that LC might control the development of cancer in humans. Indeed, it is known that LC disappear or their function is impaired following treatments that are known to promote carcinogenesis, including X-irradiation, UV-light irradiation, infection with oncogenic viruses and exposure to tumor promoters or carcinogens (97,127-128,143-147). In addition, the numbers of LC are known to correlate with the activity of benign tumors or the degree of malignancy of the tumors (145-148). The number of LC (but not of other cell types) found in the tumors was shown to correlate with the prognosis of patients affected by different kinds of cancer (149-152). Recently, it has been shown that intratumoral injection of a killed streptococcal preparation was accompanied by a local increase in LC numbers in gastric carcinoma (stage III) patients and by a concomitant increase in the survival rate (post-resection) of the same patients from 36 to 75% (134).

These findings suggest that LC may represent a key element in the control and the pathogenesis of cancer. Although at this stage nothing is known about the mechanims underlying the putative antitumor activity of LC, it seems likely that these may include release of various anti-cancer cytokines, known to be secreted by cells that can be activated by LC (153-154). In addition, LC has been shown to release various cytokines including IL-1, TNF-α, IFN-γ and PGD_2 (107,109-11) that can function as potent antineoplastic agents (153-157). Thus, new anti-cancer immunotherapeutic strategies might include potentiation of LC function in the skin or elsewhere. Supporting this idea are recent results by Halliday et al. who showed that retinoic acid which is known to suppress

tumor promotion in mice, also protected LC from the deleterious effects of the tumor promoter 12-O-tetradecanoyphorbol 13-acetate (143).

Finally, since LC play an important role in the immune system, it is not surprising that they are also involved in the pathogenesis of immunological disorders. It is well-known that they function as the key mediators of contact hypersensitivity states (reviewed in ref. 125). Recently, they were shown to be involved in the pathogenesis of various autoimmune disorders (158-160). LC density was shown to decrease at the onset of diabetes mellitus type I (158). It is possible that LC mediate the autoimmune reaction leading to the pathology seen in bullous pemphigoid (159). Cutaneous lupus erythematosus was shown to affect the LC population in the skin of patients (160).

CONCLUSION

The data reviewed above show that LC might be involved in the development of different types of pathological processes. Further understanding of their role in the pathogenesis of disease states might open the way for novel diagnostic and therapeutic approaches based on adequate evaluation and modulation of LC function in the skin.

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MONOCLONAL ANTIBODIES TO LANGERHANS CELLS IN MAN AND EXPERIMENTAL ANIMALS

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INTRODUCTION

Langerhans cells belong to the family of bone marrow-derived dendritic cells which play a key role as antigen-presenting cells in various T-cell-dependent immune responses. Langerhans cells can leave the skin and will enter draining lymph nodes as veiled cells whereafter they can be found as interdigitating cells in the T-dependent areas of the lymph node and also of other lymphoid organs. Although these cells, which are the *in vivo* equivalent of the *in vitro* isolated dendritic cells, are generally included within the mononuclear phagocyte system it is not clear whether they share a common monocyte precursor with macrophages or whether an independent precursor line exists. Nor is it clear what the differentiation pathways are within the population of Langerhans-dendritic cells. Does the continuous influx of Langerhans cells into lymph nodes via the afferent lymphatics mean that all interdigitating cells are derived from Langerhans cells or do separate pathways exist? In addition to questions on the origin and relationships of these cells their importance in antigen presentation is well recognized and many questions on how these cells can act as such potent accessory cells are now being addressed.

The presence of surface markers on Langerhans cells has been an extremely valuable tool to study their function, and ongoing research into the relationship of various markers within the CD notations, as well as the development of new monoclonal antibodies, will help to unravel the differentiation pathways of the Langerhans cells.

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In this chapter an overview of the markers known to be present on Langerhans cells of man and experimental animals will be given and where possible the implications for their function and relationship is discussed.

SURFACE MARKERS ON LANGERHANS CELLS

MHC class II antigens

The first indications for an immunological role of the Langerhans cells came from the demonstration of MHC class II antigens on their surface. Using allo-antisera against Ia- encoded antigens, Hämmerling and coworkers (1) were the first to demonstrate the presence of these antigens within the murine epidermis. That the cells that reacted with anti Ia reagents were indeed Langerhans cells was simultaneously demonstrated in human skin by Rowden et al. using allo-antisera (2) and by the group of Klareskog with heterologous antisera (3). This was later confirmed in mouse and guinea pig (4,5) and in several experimental approaches it was demonstrated that isolated Langerhans cells were capable of presenting antigen (6,7).

The human MHC class II molecules are the gene products of the HLA-D locus on the 6th chromosome, which contains at least three subloci, HLA-DP, HLA-DQ, and HLA-DR, coding for a heterodimer consisting of non-covalently associated glycoprotein chains (8). All three of these gene products have now unequivocally been identified on Langerhans cells (9). Although many cell types express MHC class II antigens, or can be induced to expression, Langerhans cells are the only cells in the skin that constitutively express Ia and will continue to do so *in vitro* (10). The mode of expression of MHC class II antigens however can be modulated by interferon-γ (11,12). In the constitutive presence of Ia on the cell's surface Langerhans cells resemble the dendritic cells from lymphoid organs, but are quite different from macrophages which express little or no Ia under normal conditions but can be induced to Ia expression e.g. after infection (13).

MHC Class I antigens

MHC class I antigens (HLA-A, B, and C in man, H-2K, H-2D in mouse) are classical serologically detectable glycoproteins expres-

sed on virtually all nucleated cells. They are involved in the recognition by T cells of hapten-modified, virus-modified of tumor-antigen bearing cells (14). Although Langerhans cells express MHC Class I antigens the mode of expression is low in comparison with other intradermal or non-dermal cells. This is observed in both man and mice (15,16) but it is unclear whether this has any functional significance.

CD1 antigens

CD1 antigens were originally described on human thymocytes by Reinherz and Schlossman (17) in their studies on differentiation antigens of T-cell development. Within the group of CD1 antigens three forms can be recognized on T cells, CD1a, CD1b, and CD1c, whereas on Langerhans cells CD1a and CD1c are expressed, but not CD1b (18). Before their CD annotation (19) these antigens were designated by the monoclonal antibodies OKT6, and NA1/34 for CD1a, and M241 for CD1c respectively (20-22). Recently a fourth CD1 molecule (CD1d) has been identified by cDNA coding of a thymocyte library (23). It remains to be seen whether this molecule is also expressed on Langerhans cells.

Immunoprecipitation studies with the OKT6 and M241 antibodies have shown that biochemically the antigens on T cells and Langerhans cells are identical and closely resemble MHC-class I antigens in size as well as in their association with β-2 microglobulin (24,25). Further structural analysis of the CD1 antigens has revealed that there is a high sequence identity between the β-2 microglobulin binding region of the MHC I molecules and CD1a and an overall similarity in the predicted secondary structure between CD1a and HLA-A2 (26). The antigens however are encoded for by different chromosomes, the CD1 antigens are encoded by genes on chromosome 1, unlike MHC class I genes which are present on chromosome 6 (27). All these data have been derived from man, only in sheep the expression of a CD1 equivalent has been reported (28).

That the CD1 epitopes on the cortical thymocytes can differ from those on Langerhans cells was demonstrated with a larger panel of anti CD1 monoclonal antibodies which could clearly be divided in groups based on the differential expression on the two cell types (29). Whether this reflects functional differences of the molecules

or isoforms based on cell-specific glycosylation is not clear.

Although the CD1 antigens on T cells are clearly associated with discrete differentiation stages in the thymus, no function has been attributed to these antigens on both T cells or Langerhans cells. The expression of the CD1 marker on Langerhans cells may reflect a differentiation stage of these cells in the skin environment (25,30), in view of the relationship between T cell differentiation and the ectodermal epithelium of the thymus, or, alternatively, it could be that Langerhans cells play a role in extra-thymic T cell differentiation. In this respect it is striking that CD1 expression is modulated by IL-1 (31), which has an important function in the interaction of accessory cells and T cells (12).

Considering the inverse relationship of MHC class I and CD1 on both cortical thymocytes and Langerhans cells it is likely that CD1, as MHC class I, is a receptor molecule or linked to a receptor. Evidence for this comes from the demonstration of receptor mediated endocytosis of CD1 molecules on Langerhans cells, whereby the molecules are internalized via coated pits and recycled on the external membrane (32,33). Such mode of internalization has sofar only been described for molecules with a clear receptor function (34).

Adhesion molecules; Integrins

The integrin receptors belong to a superfamily of transmembrane proteins comprising non-covalently linked heterodimers. These cell surface adhesive receptors can be divided into three families based on their common β subunit. The first family of antigens, (CD11), which is predominantly expressed on leucocytes, consists of LFA-1 (CD11a), CR3 (Mac-1, CD11b), and p150,95 (CD11c). The second family comprises the VLA (Very Late Antigens) group in which at least 5 heterodimers can be found, and the third family includes the fibronectin receptor, the vitronectin receptor, and the platelet membrane glycoprotein IIb-IIIa complex (35). Antigens from the first two families, CD11a (36), CD11b and CD11c (37) as well as the common β subunit of the VLA family (38), have been demonstrated on Langerhans cells by the use of monoclonal antibodies. The precise role of any of these antigens on Langerhans cells is speculative but

since these surface receptors are involved in several aspects of cell-cell interactions it is assumed that they play a role in either the interaction of Langerhans cells with lymphocytes or keratinocytes by conjugate formation, or that they are involved in the migration and localization of these cells.

Leukocyte common antigen

The presence of CD45 on Langerhans cells has been demonstrated in both man and mouse (39,40). CD45 has originally been defined as the leukocyte common antigen and is found on all hematopoietic cells, except erythrocytes and their precursors (41). It therefore confirms the hematopoietic origin of the Langerhans cell. CD45 is a family of membrane glycoproteins, with several isoforms in the range from 180-240 kDa, due to differences in the O- and N-glycosidically linked, carbohydrate chains (42). Designated as a 'differentiation' antigen it has now become clear that CD45 is a protein tyrosine phosphatase, which may act as a regulator of signal transduction in lymphocytes and antigen presenting cells by influencing the mobility of receptors, e.g. the CD3 complex on T cells (43). It is furthermore assumed that the isoforms as found on various celltypes will have different roles in the binding to receptors. CD45 is an important molecule in cell-cell interactions and it remains to be investigated in what isoform it is present on Langerhans cells to establish its precise role.

Lineage specific markers: macrophage-related antigens

The Langerhans cell originates from a precursor in the bone marrow as has been documented in reconstitution experiments (44) and also the presence of the CD45 molecules on its surface are, as mentioned above, a clear indication of its hemopoietic origin.

It is likely that the direct precursor for the Langerhans cell is the monocyte but formal proof for the incorporation of the Langerhans cell lineage within the mononuclear phagocyte system is hard to give. There are however many indications that the Langerhans cell is closely related to the macrophage, such as the demonstration of transitional forms between macrophages and Langerhans cells in patients undergoing bone marrow transplantation (45) and the presence of macrophage specific markers on Langerhans cells. The

expression on Langerhans cells of Fc-IgG receptors (39,46,47) and complement receptors (see integrins, CD11b), which are typically found on macrophages has been well documented. In addition it has recently been found that low affinity Fc receptors for IgE (FcεR2/CD23) are expressed on Langerhans cells from patients with atopic dermatitis (48). The CD23 antigen could also be induced on isolated Langerhans cells from nonatopic individuals after incubation with IL-4 and interferon-γ (49).

Other markers that are even more restricted for macrophages such as F4/80 and BM-8 in the mouse and Leu M3 (CD14) in man are also expressed on Langerhans cells (50,51,52,53). Although these three antigens are not found outside the macrophage lineage they are probably different entities considering the differences in molecular mass of the antigens (52,54).

It is striking that the interdigitating cell in lymph nodes which is so closely related to the Langerhans cells does not bear F4/80 and BM-8 antigens, but also lacks both Fc and complement receptors (55,56). This may indicate that the Langerhans cells are an intermediate stage between the macrophage and the dendritic, interdigitating cell with many characteristics still of the macrophage, or, that the skin environment is important for the expression of these types of membrane determinants. In contrast the 55 kDA human CD14 molecule has been reported to be also present on interdigitating cells (56).

Also in other species evidence for a relationship of the Langerhans cell with the monocyte-macrophage lineage has been found based on the expression of surface markers. In the rat two reports on new panels of monoclonal antibodies with macrophage specificity have recently been published with a clear reactivity of some of the antibodies for Langerhans cells (57,58). In the first description no function or molecular analysis of the markers has been given, in the second a relationship with CD11/CD18 integrin molecules is suggested based on the distribution of the molecules and their molecular make up (58).

Lineage specific markers: lymphocyte antigens

In addition to the CD1 molecule which has been demonstrated on Langerhans cells and cortical thymocytes (see CD1 antigen), other

lymphocyte specific markers have been found on the Langerhans cell as well. These includes several B cell specific markers such as CD39, CD40, and CD9 (59). Of these the CD39 and CD40 antigens have also been demonstrated on dendritic cells isolated from tonsils (57). The functions of these antigens are now under study.

The expression of CD4 on Langerhans cells as reported by several groups (60,61) is intriguing, although the function of CD4 on Langerhans cells is not clear. On T cells its expression is directly correlated with the recognition of antigenic determinants together with MHC class II antigens on antigen presenting cells. The expression of such MHC class II receptors on the very cells that constitutively express high levels of MHC class II antigens may therefore be related to a regulatory function, especially since the expression of CD4 is clearly under influence of local inflammatory activity. In normal control skin very weak staining was observed whereas in skin of patients with cutaneous lymphoma or inflammatory conditions the expression of CD4 was very prominent (61). Using gingival organ cultures Walsh and coworkers were able to demonstrate that the CD4 expression of Langerhans cells could be positively modulated by interferon-γ, whereas prostaglandin E2 could down-regulate the influence of interferon-γ (62).

The presence of the receptor for IL-2 on the surface of Langerhans cells has also been described (63,64). Although the expression of this receptor is weak on cells in tissue sections, it is increased considerably after culturing isolated Langerhans cells (65,66).

In experimental animals additional markers that are shared by lymphocyte populations and Langerhans cells have been described. In mouse the J11D monoclonal antibody, which has originally been found on B cells has recently been demonstrated on Langerhans cells and dendritic cells (63). In the guinea pig the MSgp2 antibody has been described to react with the majority of lymphocytes and Langerhans cells (67), whereas the MSgp9 antibody was found to react with B cells and Langerhans cells (68). For J11D and the two anti-guinea pig antibodies no functions or CD notations are yet available.

The CD44 antigen

Another antigen that has recently attracted much interest is

the CD44, Pgp-1 molecule, present on most leukocytes but absent from immature thymocytes (69). It has now been established that CD44 is directly related to the lymphocyte homing receptor gp90 (HERMES) in man (70,71) and that the CD44 antigens can be considered as a family of adhesion molecules. Its presence has been demonstrated on interdigitating cells in man (72), and on Langerhans cells in the mouse (63). Although the function of CD44 on lymphocytes is clearly related to the interaction with high endothelial venules in lymphoid organs (70) its function on cell types outside the lymphocyte lineage is less specific and may involve the interaction with extracellular matrix proteins (73).

The Lag antigen

The Lag antibody is an intriguing antibody since it reacts with Birbeck granules in man and is therefore one of the most specific markers of Langerhans cells and cells directly originating from them (74). The function of the 40 kDa molecule precipitated by the antibody is as unclear as the cell organelle from which it is isolated (see also relevant chapter in this volume).

The S100 antigen

The S100 antigen was orignally found in the brain on glia cells and Schwann cells (75) but subsequent immunohistological analysis showed that S100 was also present on Langerhans cells (76,77). These observations were performed with heterologous antisera against the bovine S100 molecule which cross-reacts with the human antigen. Recently also monoclonal antibodies against S100 have been described (78). Using these antibodies it was shown that the S100 antigen has a very broad distribution and can be found in an extensive variety of tissues and cells. Also a differential expression of the antibodies was found in different tissues suggesting that S100 consists of a group of antigens which expression is related to the functional activity of the cell. The function of this antigen is sofar unknown.

Other membrane antigens

In the mouse the NLDC-145 antibody recognizes a 145 kDa trypsin-sensitive glycoprotein on the surface of Langerhans cells

Table 1

Monoclonal antibodies that react with Langerhans cells

Antigen*	CD	kD	typical Moab	species	References
MHC class I	-	40	various	man, mouse	15,16
MHC class II	-	33,25	various	man, mouse	1-4
				rat, cattle	85
				guinea pig	5
CD1	CD1a	49,12	OKT6,NA1/34	man	20-22
	CD1c	43,12	M241	man	20-22
Helper T cell	CD4	55	Leu-3, OKT4	man	60,61
B cells	CD9	24	Ba2	man	59
LFA-1	CD11a	180	CIMT	man	36
Mac-1, CR	CD11b	165	MO1, OKM1	man	37
p150,95	CD11c	150,95	Leu-M5	man	37
macrophages	CD14	55	Leu-M3	man	56
Fc ε RII	CD23	45-50	M-L25, 3-5	man	48.49
VLA	CD29	105,145	K20, 4B4	man	38
Fc IgG R	CD32	40	C1KM5, 2.4G2	man, mouse	39,46,47
B cells	CD39	80	G28/8	man	59
B cells	CD40	50	G28/5	man	59
Pgp-1	CD44	90	HERMES	man, mouse	63
Leukocyte common antigen	CD45	180-240	T29/33, H201	man, mouse	39,40
				guinea pig	28
				sheep	84
IL-2 receptor		52,104	3C7, 7D4	man, mouse	63,64,65
macrophages		160	F4/80	mouse	50
macrophages		125	BM-8	mouse	51
macrophages		-	RM-1	rat	57
macrophages		-	TRPM-1	rat	57
macrophages		160,95	ED8	rat	58
macrophages		-	ED9	rat	58
B cells		-	J11D	mouse	63
lymphocytes		-	MSgp2	guinea pig	67
macrophages		-	MSgp9	guinea pig	68
Birbeck granules		40	Lag	man	74
brain S100		100	S1-61-64,S2-20	man	78
dendritic cells		145	NLDC-145	mouse	79
macrophages		15	MI-8	mouse	83
brain endothelium		30,25,23	EBA	rat	86

References listed in this table are the references in which the expression of the antigen on Langerhans cells has been described and can therefore be different from the original description of the monoclonal antibody. * Antigen refers to original specificity described for particular antibody.

(79). This determinant is shared by lymph-borne veiled cells and dendritic cells in thymus and lymph nodes and, to a lesser extent, by spleen dendritic cells (62,80). Interestingly, this determinant is also expressed on cortical epithelial cells of the thymus. Again, as is the case with CD1, the relationship with thymus and skin is striking. Unfortunately very little information on the function of the NLDC-145 molecule has sofar been obtained (81). In an attempt to produce new monoclonal antibodies against the 145 kDa molecule which may recognize more functional epitopes we have come up with a series of monoclonal antibodies that reacted with dendritic cells, but differently with thymus epithelial cells (82). One of these antibodies, DC-9B5, also reacts with Langerhans cells, but, in contrast with the original NLDC-145, in the thymus it recognizes both medullary and cortical epithelium cells.

Recently an additional monoclonal antibody against Langerhans cells in the mouse has been described (83). This antibody, M1-8, has a somewhat broader distribution than NLDC-145 because it also reacts with monocytes. It recognizes a small membrane glycoprotein of 15 kDa. No function has been attributed to this antigen sofar.

CONCLUDING REMARKS

A variety of surface markers for Langerhans cells of man and experimental animals has been described in the literature (see Table 1). The vast number of monoclonal antibodies that react with these cells is a reflection of the expanding hybridoma technology but also of the growing interest for the Langerhans cell. It is remarkable that to many of the antigenic determinants no function can be attributed and that they are merely used as markers to classify Langerhans cells in tissue or culture. This is of course a consequence of the way many of these antibodies have been produced.

Another remarkable aspect is that, with perhaps the exception of the Lag antigen which seems to be restricted to the Birbeck granules, none of these monoclonal antibodies is specific for Langerhans cells only. In fact, Langerhans cells share many determinants with both macrophages and lymphocytes, a confusing characteristic when one likes to use these parameters for lineage analysis. However, it is obvious that the more we learn about the cellular membrane constituents, the more it will become clear that

the specific function or phenotype of a given cell is usually not limited to the presence or absence of single membrane glycoproteins. The specificity of a cell will be determined by the combination of cell surface structures and their possible isoforms. Furthermore, such a complex arrey of cell surface enzymes, adhesion molecules, and various receptors must be changing constantly as the cell differentiates or is modulated by local stimulatory or suppressive influences. It is this interplay that one has to keep in mind when studying the surface structures of Langerhans cells, or any other cell type, which are continuously influenced by the dynamic state of the cell and the demands of the microenvironment.

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3

DETECTION OF LANGERHANS CELLS WITH MONOCLONAL ANTIBODIES

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ABSTRACT

We prepared a mouse monoclonal antibody that reacts specifically to human Langerhans cells (LC). The protein recognized by this antibody was mainly in the membranes of Birbeck granules and related structures. Using this antibody (Lag), we could identify LC in normal various tissues; these cells were in the skin, stratified squamous mucosal epithelia, lymph nodes, and the thymus. Lag did not react with monocytes, tissue macrophages, lymphoid dendritic cells, follicular dendritic cells, or interdigitating cells. The antigen purified with this antibody was a heterogenously glycosylated protein of Mr 40,000 without interchain disulfide bonds. In Letterer-Siwe disease, both lymph nodes and lesional skin contained abundant Lag-positive cells. By two-dimensional gel electrophoresis, antigenic subtances in the lymph nodes of patients with Letterer-Siwe disease were found to have the same molecular weight of 40,000 dalton and isoelectric points extending from 4.7 to 6.5 as those in normal human skin and lymph nodes. Our results support the contention that Letterer-Siwe disease is a proliferative disorder of Langerhans cells. This antibody (Lag) may be useful for identifying normal or abnormal LC in various human tissues , and for studying the origin and fate of Birbeck granules of LC.

IINTRODUCTION

Langerhans cells (LC) in the epidermis were first identified by their dendritic features (1) and later by the presence of Birbeck granules in their cytoplasm (2). The only reliable morphologic way to identify LC is by the detection of their Birbeck granules, which can be done only under the electron microscope.

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Membrane-bound ATPase is often used as a marker for Langerhans cells (3). S-100 antigen has been shown to exist in LC (4). Also, several immunohistochemical cell surface markers such as Fc and C3 receptors (5), and human lymphocyte antigen (HLA)-DR (6), have been used.

LC can be detected in skin using monoclonal antibodies such as CD1a, OKT6 (monoclonal antibody to T6) (7,8), NA1-34 (9), and V1-CY-1 (10). However, these monoclonal antibodies are not quite specific; OKT6 also reacts with thymocytes, as does NA1-34, and V1-CY1 also reacts with human B cells as well as monocytes. So, on detection of LC using these monoclonal antibodies, we have usually used it together with membrane-bound ATPase or antibdy to (HLA)-DR.

The human epidermis is a heterogenous epithelium composed of five cell types : keratinocytes, melanocytes, Merkel cells, LC and indeterminate cells (IC). LC are now considered as the most peripheral outpost of the immune system. In the human epidermis, LC and indeterminate dendritic cells are the only epidermal cells that express the specific surface antigen T6. Indeterminate dendritic cells, without Birbeck granules, have been reported to play similar functions as LC (11). It is suspected that LC and IC are developmentally and functionally related and can be differentiated only by the presence of Birbeck granules. In normal human epidermis, monoclonal antibody to T6 is a useful and simple way to detect LC and indeterminate dendritic cells (8, 11). However, it is still important to detect LC with Birbeck granules easily in different tissues, including lymphatic tissues in various conditions such as lymphoproliferative disorders.

We established a mouse monoclonal antibody that reacts specifically with human LC (12). In an immunoelectron microscopic study, we showed that this antibody bound to Birbeck granules and related structures in LC. Using this antibody, we can find LC in various organs. This antibody can be used successfully to identify Birbeck granules containing LC in normal and pathologic human tissues (13). We have also isolated and characterized the specific antigen reactive to this monoclonal antibody in normal human epidermis and in the lesional skin and lymph nodes from the patients with Histiocytosis X. Since Histiocytosis X appears to be a proliferative disorder of LC, it has been proposed to be renamed "Langerhans cell granulomatosis" (3, 14).

MATERIALS AND METHODS

Preparation of Monoclonal Antibodies

Skin fragments were taken from specimens of resected breast. The epidermis was stripped after incubation with Dispase (1000 U/ml; Godoshusei, Tokyo) solution (15). Single cells from the epidermal sheet were obtained by treatment with 0.02 % EDTA and 0.25 % trypsin in PBS. The cells were washed and used for the immunization.

Seven-week-old BALB/c mice were immunized i.p. 3 times with 2 X 10^7 cells in 1 ml of PBS every 2 weeks. Three days after the last immunization, spleen cells from the mice were fused with mouse myeloma cells (X63Ag8.653) in a 50 % solution of polyethylene glycol 1500. The fusants were incubated in flatbottomed 96-well microculture plates (Coster, Cambridge, Massachusetts) with irradiated thymocytes of BALB/c mice, and the fused cells were selected in HAT medium. After 14 days of culture, 0.1 ml of medium obtained from each well containing colonies was assayed. Hybrid cells secreting antibodies of interest were cloned by the limiting-dilution method. To determine the immunoglobulin subclass of the antibodies, we used the Ouchterlony double-immunodiffusion method with rabbit monospecific antisera to mouse immunoglobulins (Miles, Elkhart, Indiana) (16). To obtain a large quantity of antibody, hybrid cells were injected into the peritoneal cavity of BALB/c mice given pristane (2,6,10,14-tetramethylpentadecane, Aldrich Chemical Co., Milwaukee, Wisconsin). Ig G was purified from ascites by ammonium sulfate precipitation and chromatography on protein A-Sepharose CL-4B columns (Pharmacia Fine Chemicals, Uppsala, Sweden) (17). Purified immunoglobulins were stored in PBS containing 10 mM sodium azide.

Staining and Observations

Materials. For immunofluorescence or immunoperoxidase staining, we used normal human skin and other organs from autopsies done at Kyoto University Hospital. Bone marrow cells were obtained from a patient with skin amyloidosis without any systemic involvement. Peripheral blood cells were obtained from healthy donors.

To detect Langerhans cells in the pathognomonic tissues, three cases of Letterer-Siwe disease were selected. Letterer-Siwe disease is one form of Histiocytosis X. From these patients, both lymph nodes and lesional skin were biopsied and used for immuno-staining.

Indirect Immunofluorescence with Monoclonal Antibodies. Normal and pathognomonic tissues were frozen in acetone at -70°C and stored at -80°C until use. Cryostat sections 4 um thick were prepared, overlaid with culture supernatant from hybrid cells, incubated in a moist chamber for 45 min at room temperature, and washed twice with PBS. These sections were treated for 45 min at room temperature with fluorescein-labeled rabbit antibodies against mouse immunoglobulins (Cappel Laboratories Malvern, Pennsylvania). After being washed with PBS, the sections were examined under a Nikon epifluorescence microscope (Tokyo, Japan).

Indirect Immunoperoxidase Staining. Frech specimens of the tissue were cut into small pieces using a razor blade, immediately fixed with 5 % paraformaldehyde in water for 2 h at 4°C, immersed overnight in PBS containing 10-20 % sucrose and 10 % glycerine at 4°C, and embedded in Tissue-Teck II O.C.T.compound (Miles Laboratories, Naperville, Illinois). Serial sections 10 μm thick were mounted on albumin-coated glass slides, and air-dried. They were stained with mouse monoclonal antibody (2 μg/ml) as the first antibody, and with peroxidase-conjugated goat antibodies against mouse immunoglobulins (25 μg/ml; Cappel Laboratories) as the second antibody. After being fixed with 1 % glutaraldehyde in PBS, the sections were treated with a solution of diaminobenzidine in PBS for 30 min and then with a mixture of diaminobenzidine and hydrogen peroxide for 2 min.

For immunoelectron microscopic studies, these stained sections were washed with PBS, postfixed with 1 % osmium tetroxide in water for 1 h, dehydrated in increasing concentrations of ethanol, and embedded in polyethylene capsules containing epoxy resin.

Ultrathin sections were stained with uranyl acetate and observed under an H-300 electron microscope (Hitachi, Japan). We chose one monoclonal antibody for the study described here. We named this antibody Lag for Langerhans cell granules, for reasons given later.

Double staining. Purified Lag and anti-T6 monoclonal antibody were conjugated with fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (RITC), respectively. Cryostat sections were incubated with a mixture of FITC-conjugated Lag (20 μg antibody/ml) and RITC-conjugated anti-T6 monoclonal antibody (10 μg antibody/ml) for 1 hour at room temperature. After being washed with PBS, the sections were examined under a Nikon epifluorescence microscope.

Immunoadsorbent, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), and Immunoblotting

Immunoadsorbent Isolation of Antigen Recognized by Lag. Purified Lag (IgG_1) or affinity-purified mouse anti-trinitrophenyl (anti-TNP) monoclonal antibody (IgG_1) (18) was immobilized with cyanogen bromide. The epidermal sheets and biopsy specimens of the lymph nodes that had been stored frozen in liquid nitrogen were homogenized at 0°C in the lysis buffer containing 50 mM phosphate buffer (PB), pH7.3, 150mM NaCl, 5mM EDTA, 0.5 % Nonidet P-40 (NP-40), and 2mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 3000 rpm for 5 min at 4°C, and the supernatant was applied to the anti-TNP antibody Sepharose CL-4B column. The fraction that passed through was incubated at 4°C for 2 h with 0.2 ml of the Lag-Sepharose CL-4B with constant shaking. The Lag-Sepharose CL-4B was washed by centrifugation with 50mM PB (pH 7.3) containing 150 mM NaCl, 5 mM EDTA, and 0.1 % NP-40, and then with 10 mM PB (pH7.3) containing 50 mM NaCl and 0.1 % NP-40. The antigen was eluted with 50 mM citric acid containing 0.1 % NP-40. The eluate was neutralized with 1.5 M Tris-HCl buffer, pH 8.6 , unless otherwise noted.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Immunoblotting. The prepared sample was run, after addition of 1 % SDS and 0.1 M dithiothreitol, in 12 % polyacrylamide gels containing 0.1 % SDS (13 cm X 14.5 cm, 1 mm thick) with 3 % stacking gels (19, 20). Proteins were stained after SDS-PAGE by the silver-staining method (Daiichi Chem., Osaka, Japan) (21). In some cases, the proteins in the gel were transferred to nitrocellulose paper (Schleicher and Shuell, Inc., Keene, New Hampshire) using a Trans-Blot Cell (Bio-Rad Laboratories, Richmond, Carifornia) with 25 mM Tris, 192 mM glycine, and 20% methanol overnight at 7 V/cm (22). These blots were incubated with PBS containing bovine serum albumin (10 mg/ml), Lag in culture medium, and then with peroxidase-conjugated goat IgG against mouse immunoglobulins diluted 1:200 in PBS containing bovine serum albumin. The reaction was visualized with 4-chloro-1-naphtol (Wako Pure Chemical Industries, Osaka, Japan).

Two-Dimensional Gel Electrophoresis. The antigen purified by affinity chromatography, in 0.15 ml of citric acid, was treated with 0.3 ml of a solution of neuuraminidase (EC3.2.1.8, 1 U/ml, Nakarai Chemicals, Ltd., Kyoto, Japan) in 0.1 M citrate buffer, pH 5.5, at 37°C for 1h. The solution was neutralized with

1.5 M Tris-HCl, pH 8.6, and 2 ml of the buffer for lysis was added. It was applied to Lag-Sepharose CL-4B column, and the antigen was eluted with 0.5 M NH_4OH containing 0.1 % NP-40. After the sample was freeze-dried, it was subjected to 2-dimentional electrophoresis with pI markers (Pharmacia) according to the method of O'Farrell et al (23). Ampholine (pH 3.5-10, LKB, Sweden) was used as the pH carrier. The proteins in the gel after SDS-PAGE were visualized by silver staining.

RESULTS

Monoclonal Antibody Against LC

Of the 283 culture supernatants assayed, 95 reacted to normal human epidermis. One specifically bound to cells with dendrites scattered in the suprabasal epidermis and the hair follicles (Fig. 1). Some cells were also

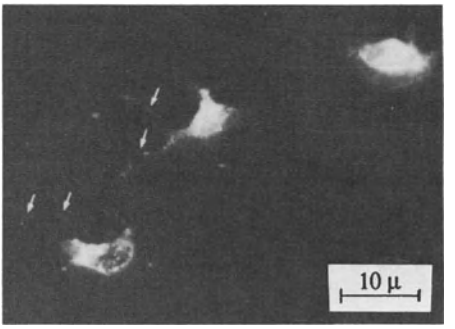

Fig. 1. Indirect immunofluorescent staining of normal human skin. Frozen human skin was sectioned perpendicular to its surface. These sections were reacted with culture supernatant from hybrid cells and then with fluorescein-labeled rabbit antibodies against mouse immuno-globulins. Suprabasal cells with many dendrites (*arrows*) were stained.

stained in the upper dermis. Indirect immunoperoxidase assay showed that this monoclonal antibody reacted with some cytoplasmic components of suprabasal epidermal cells with dendrites (Fig. 2). Neither keratinocytes nor melanocytes

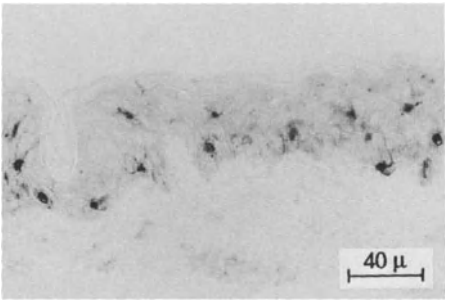

Fig. 2. Indirect immuno-peroxidase staining of normal human skin. Some cells with dendrites were stained with culture supernatant from the hybrid cells, followed by peroxidase-conjugated goat antibodies against mouse immunoglobulins. The peroxidase reaction was observed mainly in the inside of the cell body, not on its cell surface.

were stained. Tissue sections treated with anti-TNP monoclonal antibody as the first antibody were unstained. Immunoelectron microscopic observation showed that the reaction products of peroxidase were generally very close to the rod-shaped and racket-shaped structures, or to vacuoles of the nonkeratinocytic cells of the epidermis (Fig. 3). Some of these rod-shaped structures had striated lamellae, and we identified them as Birbeck granules.

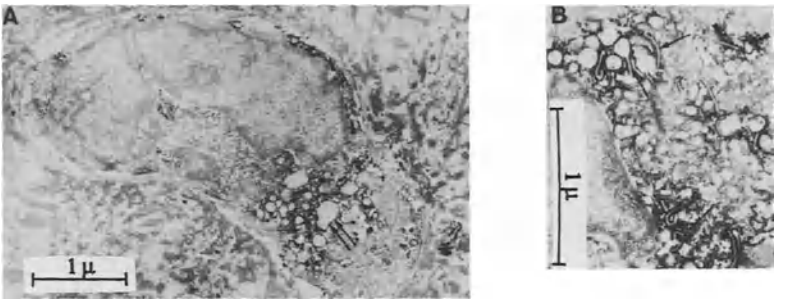

Fig. 3. Immunoelectron micrograph of a cell reacted to Lag. The cell determined by Lag shows characteristic feature of LC with the cytoplasmic process (*asterisk, A*) in normal human skin. The reaction products are mainly very close to the membrane of Birbeck granules (*arrows, B*) in the cell body, and of the irregular or polygonal vacuoles (*double arrows, A*) that are morphologically distinguishable from the round portion of the granules. The reaction products are also present diffusely near Birbeck granules (*arrowheads, B*).

The reaction products were clearly seen on the cytoplasmic side of the rod and the bulb portions of these granules, but not inside the granules. Some vacuoles containing reaction products were polygonal or irregularly shaped and were morphologically distinguished from the round portion of Birbeck granules, judged from their size. We also found small vacuoles and Birbeck granules with reaction products in cross-sections of dendrites of the cell. There were no reaction products on or near the surface membrane, mitochondria, or lysosomes. The reaction products were also present diffusely in the cytoplasmic space near the Birbeck granules. These results indicate that the monoclonal antibody reacts with Birbeck granules and the related structures of epidermal LC, for which reasons we have named this antibody Lag.

The reactivity of the antibody to the skin of other animals was examined by indirect immunofluorescence and immunoperoxidase staining. The pattern of staining of skin from the African green monkey was similar to that from human

skin. The antibody did not react at all with skin from mice, gunea pigs, rabbits, or pigs.

Detection of Lag-Positive Cells in Tissues Other Than the Skin

We examined the reactivity of the monoclonal antibody (Lag) with cells in various other normal human organs. Lag-positive cells were detected in the stratified squamous epithelia of the lip, tongue, buccal mucosa, esophagus, vagina, and uterine cervix. The immunoelectron microscopic observation ascertained that those cells reacting with Lag in these organs were LC having Birbeck granules, and that most of the reaction products of peroxidase were distributed on Birbeck granules. Reactive cells were not found in the transitional epithelia of the bladder and the columnar epithelia of the intestines.

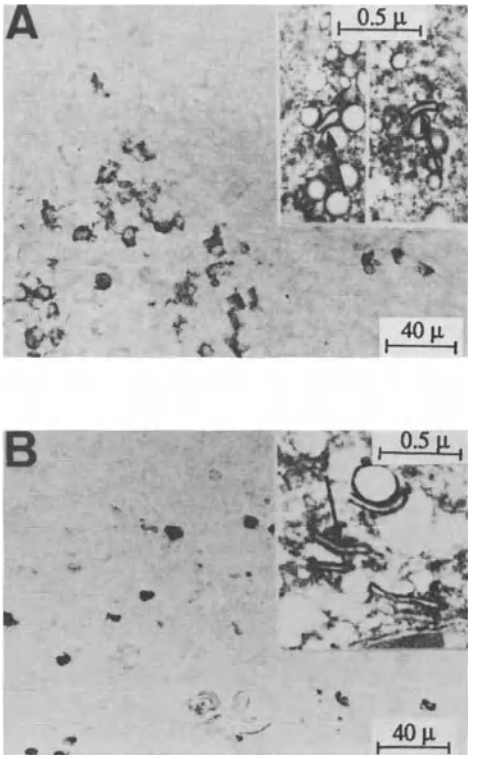

Fig. 4. Indirect immunoperoxidase staining of Lag-positive cells in various human organs.

A. In the lymph nodes, reactive cells were seen on the paracortical area and in the marginal sinuses.

B. In the thymus, they were near Hassal's corpuscles and within the interstitial connective tissue.

These reactive cells in the lymph node and thymus are not clearly branched, but they have the Birbeck granules around which the products of the peroxidase reaction were found (arrows).

In the lymph nodes, most cells that reacted with Lag were in the paracortical area and some were in the marginal sinuses (Fig. 4A). In the thymus, such cells were found both in the medulla and the interstitial connective tissue (Fig. 4B). In other areas of the lymph nodes and thymus, there were no reactive cells. The cells reactive with Lag in the lymph nodes and thymus were not so clearly branched as were LC in the epidermis. Under the electron microscope, however, these cells had Birbeck granules containing reaction products of peroxidase, and also some vacuoles containing reaction products, as in the skin. Lag did not react with some nonlymphoid cells lacking Birbeck granules but having vacuoles of irregular or polygonal shapes, which lay scattered in the lymph node or thymus.

In the spleen, kidney, pancreas, or bone marrow, we did not find any cells that reacted with Lag. In the lung, liver, or heart, in which tissue macrophages have been reported, reactive cells were absent. Lag did not react to peripheral blood cells.

Antigen Recognized by Lag

The epidermal sheets were lysed, and the antigen was purified by affinity chromatography with Lag-Sepharose. The isolated antigen displayed a broad band centered at Mr 40,000 after SDS-PAGE (Fig. 5,A). The electrophoretic

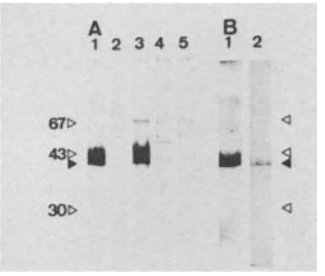

Fig. 5. A, SDS-PAGE profile of the isolated antigen. The antigen isolated from human epidermal lysate with Lag-Sepharose has a broad band at the Mr 40,000 and a minor band at Mr 64,000 under reducing conditions (*lane 3*). Under nonreducing conditions, the antigen has a major band at 40,000 (*lane 1*). Eluate of the lysate of human liver from Lag-Sephalose does not have any distinct protein band (*lane 5*); neither does the antigen-free lysis buffer eluated from Lag-Sepharose (*lane 2*), or the human epidermal lysate eluated from anti-TNP antibody Sepharose (*lane 4*). SDS-PAGE was performed in 12% acrylamide gel under reducing conditions in lanes 2-5, and nonreducing conditions in lane 1. Proteins were visualized by silver staining.
B, Immunoblot profile of the purified antigen. The antigen isolated from a lysate of human epidermal cells was electrophoresed. The separated proteins were transferred to nitrocellulose paper, and stained with Lag as the first antibody and the peroxidase-labeled second antibody. Lag reacted to isolated macromolecules electrophoresed at Mr 40,000 (*lane 2*). The antigen isolated in this part of the experiment has one broad band at Mr 40,000 after SDS-PAGE (*lane1*). It was visualized by silver staining.

patterns were similar when SDS-PAGE was carried under reducing and nonreducing conditions, which indicates that interchain disulfide bonds were absent. There was inconsistently a minor band at Mr 64,000. The eluate of liver lysate from Lag-Sepharose did not display any distinct protein band after SDS-PAGE. Similarly, no distinct band was seen with the eluate of epidermal lysate from anti-TNP antibody-Sepharose nor with the lysis buffer without antigen that was eluted from Lag-Sepharose.

Various amounts of lysate of the epidermal sheets were electrophoresed, and the proteins were transferred to nitrocellulose paper. Staining on this paper by Lag and the peroxidase-labeled second antibody was negligible (data not shown). When the antigen isolated as above was transferred after SDS-PAGE, an indirect immunoperoxidase assay was perfomed (Fig. 5,B). Lag was found to react with the isolated molecule. This result confirms that the macromolecule isolated was the antigen recognized by Lag. It also indicates that the antigenic determinant is intact after SDS-PAGE, and that the seeming lack of antigen in the blotted proteins of the whole lysate was caused by the ratio of antigen to that of proteins from whole epidermal sheets being too small.

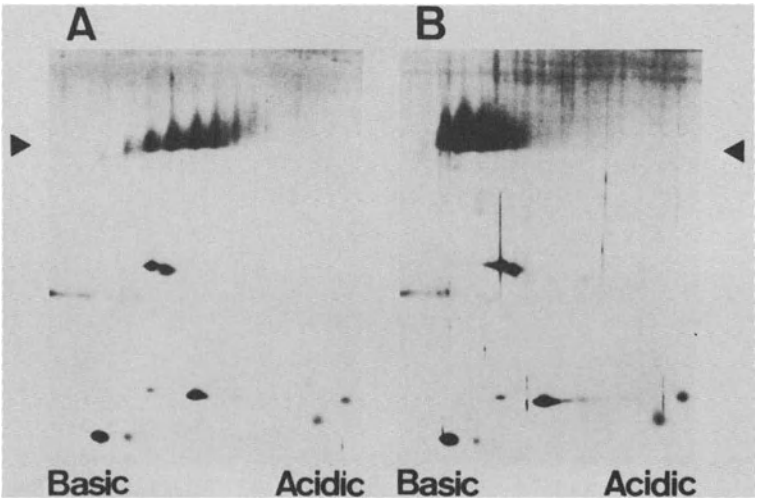

Fig. 6. Analysis of the antigen by 2-dimentional PAGE. The antigen, purified from a lysate of human epidermal sheets with Lag-Sepharose, was subjected to 2-dimentional electrophoresis and visualized by silver staining. A, The isolated antigen without further treatment. B, Materials digested with neuraminidase (0.7 U/ml at 37^{o}C for 1h). Note that the neuraminidase treatment results in a shift in the protein spots to the basic side of the gel and a decrease in their number. The spots at lower molecular weights are pI marker proteins.

The broadness of the band in the SDS-PAGE profile suggested that the antigen recognized by Lag was glycosylated. To test this hypothesis, the antigen was further characterized by 2-dimentional PAGE, in which the first dimention was performed under nonequilibrium conditions to retain basic as well as acidic proteins. The purified antigen had several components similar in molecular weight but different in isoelectric point from pI 4.7-6.5 (Fig. 6,A). Digestion of the antigen with neuraminidase resulted in a shift of the components to more basic points (pI 5.9-6.7) and in the decrease of the number of components (Fig. 6,B). These results indicate that the antigen purified with Lag was heterogenously glycosylated, and the heterogeneity of the isoelectric point of the antigen was mainly due to the sialic acid in the sugar chains.

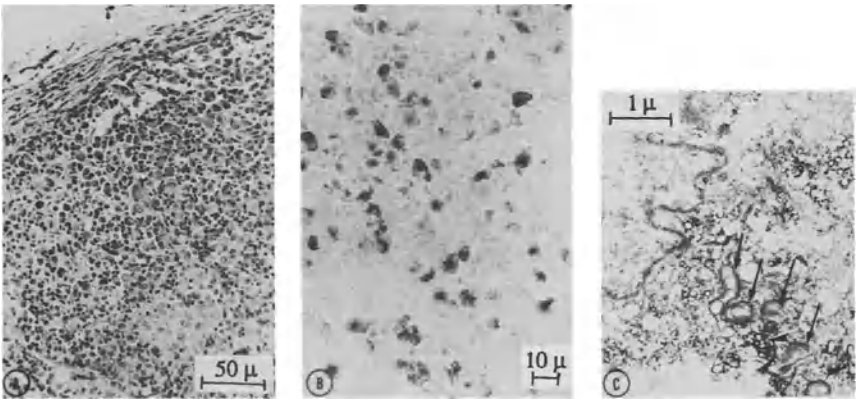

Fig. 7A-C. A, Histologic findings of a cervical lymph node of Letterer-Siwe disease. The follicular structures are destroyed by the cellular infiltrate composed mainly of large numbers of histiocytic cells. Note distinctive histiocytic cells with folded nuclear membranes, inconspicuous nucleoli, and clear, abundant cytoplasm. B, Immunoperoxidase staining of the lymph node with Lag shows intracytoplasmic staining of the histiocytic cells. C, Immunoelectron micrograph of a Lag-reactive cell in the lymph node. Nuclear invagination, many vacuoles, and characteristic myelinated body (*arrows*) are seen. The reaction products are close to the membrane of small vacuoles and Birbeck granules (*arrowheads*).

Lag-Positive Cells In Lymph Nodes of Letterer-Siwe Disease

The cortex of lymph nodes of Letterer-Siwe disease was expanded by the massive infiltration, consisting mainly of large, formy histiocytic cells that possessed abundant clear cytoplasm and folded nuclear features (Fig. 7,A). The majority (70 %) of large, formy cells were Lag-positive in the cytoplasm, as determined by indirect immunofluorescence (data not shown) and immuno-peroxidase methods (Fig. 7,B). Tissue sections treated with the control monoclonal anti-trinitrophenyl antibody as the first antibody were unstained. The immunoelectron microscopic observation of Lag-positive histiocytic cells showed that the reaction products were generally located very closely to the small vacuoles and the typical Birbeck granules (Fig. 7,C). Reaction products were not observed on the vacuoles near the surface membrane. Lag-positive histiocytic cells showed nuclear invagination and possessed characteristic myelinated bodies. These results indicated that Lag reacted to histiocytosis X cells containing Birbeck granules and the related structures. The double staining method with Lag and anti-T6 monoclonal antibody showed that almost all large, foamy histiocytic cells were reactive to anti-T6 monoclonal antibody and that about 70 % of T6-positive cells were reactive to Lag (Fig. 8), indicating that only about 70 % of of T6-positive proliferating cells in the lymph nodes of patients with Letterer-Siwe disease have Birbeck granules and the related structures.

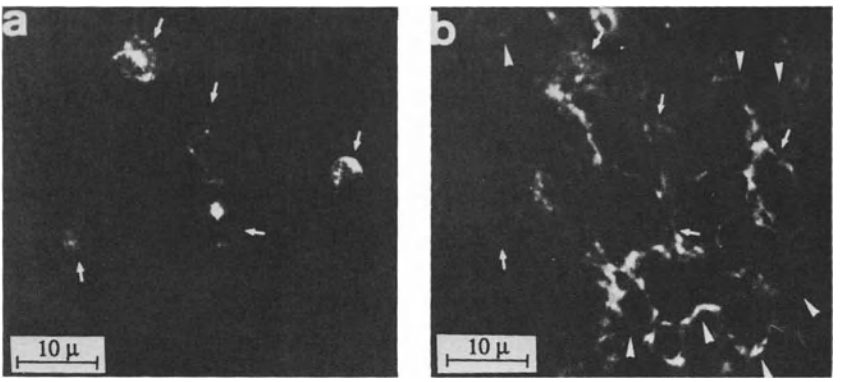

Fig. 8. Immunohistologic micrographs of a cryostat section of a lymph node of Letterer-Siwe disease. FITC-conjugated Lag reacts to the cytoplasm of some infiltrating cells (a) (*arrows*). Lag-positive cells also express T6 antigens on the cell surface and dendrites as visualized by RITC-conjugated anti-T6 monoclonal antibody (b) (*arrows*). Some cells react only to RITC-conjugated anti-T6 antibody (b) (*arrowheads*).

Lag-Positive Cells in the Skin of Letterer-Siwe Disease

Histiocytic cells that had the folded nuclear configulation were shown to infiltrate in the upper dermis of papular lesions. Electron microscopic observation revealed that each large, foamy, mononuclear cell with folded nuclei in the upper dermis contained numerous cytoplasmic vacuoles and typical Birbeck granules. The heterochromatin was more abundant than in normal epidermal Langerhans cells (Fig. 9A), By immunoperoxidase staining the majority of these large, foamy cells were Lag positive (Fig. 9B).

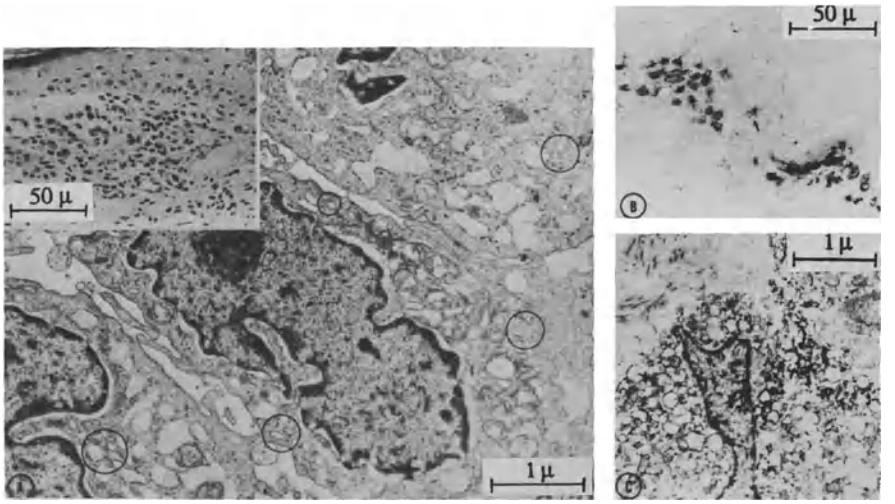

Fig. 9A-C. A, Histologic findings of a papule on the skin of Letterer-Siwe disease. Distinctive histiocytic cells with irregular nuclear shapes are seen in characteristic folded nuclei, abundant cytoplasm, and Birbeck granules (*circle*) and vacuoles in the cytoplasm. B, Immunoperoxidase staining of the papular lesions. In the upper dermis, infiltrating cells show intracytoplasmic staining with Lag. In the epidermis a Langerhans cell with dendrites is stained (*arrow*). C, An immunoelectron micrograph of the Lag-reactive cells in the skin. Reaction products are present mainly very close to the Birbeck granules (*arrows*) and the small vacuoles.

Ultrastructually the reaction products were very close to the small cytoplasmic vacuoles and Birbeck granules (Fig. 9C). The double-staining method with Lag and anti-T6 monoclonal antibody revealed that all T6-positive cells were reactive to Lag and vice versa (Fig. 10).

Antigens Recognized by Lag in Lymph Nodes of Letterer-Siwe Disease

The Lag antigen was purified by the affinity chromatography with Lag-Sepharose from the lysates of the normal human epidermal sheets, the normal lymph nodes, and lymph nodes of Letterer-Siwe disease. By two-dimensional gel-electrophoresis, each isolated antigen was found to consist of several components with molecular weight of 40,000 daltons that differed in isoelectric points from pI 4.7 to 6.5 (Fig. 11). The antigen purified from the lymph node of Letterer-Siwe disease showed the same pattern as that from the normal human epidermis and the normal human lymph nodes.

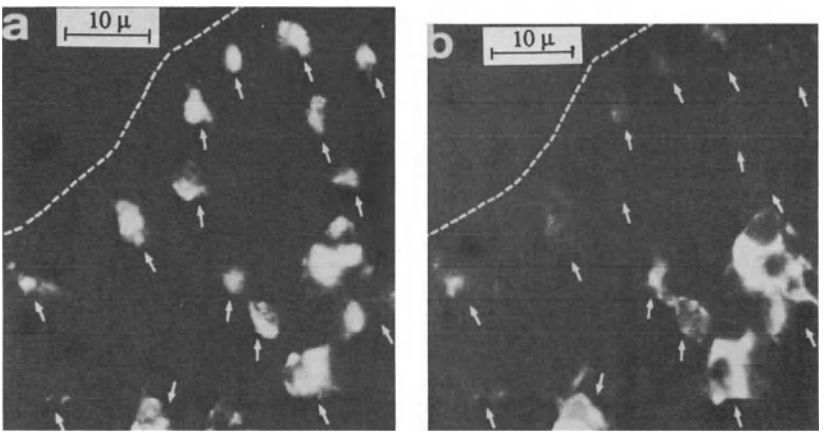

Fig. 10. Immunohistologic micrographs of cryostat section of the papular skin lesions on Letterer-Siwe disease. By direct immunofluorescence, the proliferating cells in the upper dermis coexpress Lag (a) (*arrows*) and T6 (b) (*arrows*) antigens. The dotted lines mark the dermo-epidermal junction.

DISCUSSION

Langerhans cells are considered to play an important role in the immunologic reactions in the skin, and are present in the epidermis, skin appendages, and certain stratified squamous epithelia (24). Silberberg et al. (25) reported that after contact sensitization such cells from the epidermis seemed to migrate to the dermis, and then to the paracortical area of regional lymph nodes. Langerhans cells have also been observed in the medulla of the thymus (3). Knowledge of the distribution of these cells would help us to understand how they participate in the immunologic defenses of organisms.

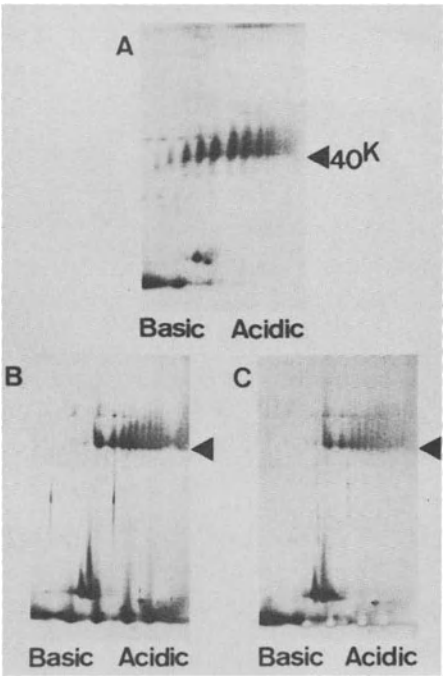

Fig. 11. Two-dimensional gel electrophoresis of the Lag antigen.

The antigen was purified with Lag-Sepharose from normal human epidermal sheets (A), normal human lymph nodes (B), and lymph nodes obtained from a patient of Letterer-Siwe disease (C), respectively.

The several protein spots are present at 40,000 daltons in each gel profile. The spots at lower molecular weights are pI marker proteins.

Birbeck granules are the only morphologic marker for LC (2) and considerable effort is required to identify LC by detection of Birbeck granules using electron microscopy. Here, we describe a monoclonal antibody, Lag, that reacts specifically with these granules and related structures in normal human skin. Using Lag, LC in various organs can be identified easily using light microscopy.

We found these cells in several noncutaneous tissues: the stratified squamous mucosal epithelia, lymph nodes (paracortical areas and marginal sinus), and thymus (medulla and interstitial connective tissue). Lag did not react with cells in the liver, heart, lung, spleen red pulp, or peripheral blood in which tissue macrophages or monocytes are thought to be present. No Lag-positive cells were found in the white pulp of the spleen or in peripheral blood where there were lymphoid dendritic cells. Lag did not react with the cells in lymphoid follicles in which follicular dendritic cells were present. These results show that Lag does not react with monocytes, tissue macrophages, lymphoid dendritic cells (26, 27), or follicular dendritic cells (28). Interdigitating cells have been reported in the medulla of the thymus and in the thymus-dependent paracortical area of the spleen and lymph nodes. Typical interdigitating cells are similar in morphology and distribution to LC, but they lack Birbeck granules (29). We conclude that Lag does not react to typical interdigitating cells. Birbeck granules are observed frequently in cells that resemble interdigitating cells in the thymus (30). It is not clear whether LC and interdigitating cells represent different cell populations. Probably LC can be classified precisely and specifically with the monoclonal antibody Lag.

The antigen recognized by Lag is a glycoprotein with Mr 40,000, without interchain disulfide bonds. The sugar chain of membrane glycoproteins is generally outside of the surface membrane or inside the intracellular organelle membrane (31). We found that the products of the peroxidase reaction were predominantly on the cytoplasmic side of the membrane of Birbeck granules and polygonal vacuoles. These results indicate that this glycoprotein is an integrated membrane protein penetrating the membranes of Birbeck granules and polygonal vacuoles. The reaction products were distributed not only in close approximation to these distinctive structures but also diffusely in the cytoplasmic space near Birbeck granules. Why these products are not always confined to the membranes is unknown. Perhaps molecules with antigenic properties similar to the Lag-antigen were present near these structures, or perhaps a part of the antigen was dissociated from the Birbeck granules during our preparation procedure.

Also, Lag reacts to the proliferating histiocytic cells in the lymph nodes and skin of patiets with Letterer-Siwe disease. Immunoelectron microscopy revealed that the antigen was present on the membranes of Birbeck granules

and the small vacuoles of histiocytosis X cells containing typical myelinated bodies. We have also shown that the Lag antigen in the proliferating cells had the same biochemical properties such as molecular weight and pI as that in normal human epidermal Langerhans cells. These results indicate that histiocytosis X cells share the common antigenicity (Lag antigen) with Langerhans cells in normal human tissues.

Using this monoclonal antibody Lag, we found that Birbeck granules had the specific membrane glycoprotein, Lag-antigen, which was not found in the surface membranes or lysosomes. As the sialic acid residue was added to the sugar chains of glycoproteins in the Golgi apparatus (32), it is obvious that this marker protein of Birbeck granules, Lag-antigen, originates from the Golgi apparatus. Irregular or polygonal vacuoles did have this antigen. These vacuoles, which can be morphologically distinguished from the bulb part of Birbeck granules, may be the related structures of these granules during formation or degradation.

The origin of Birbeck granules is still being disputed. For long time, the function and the origin of Birbeck granules have been the subject of confrontation between two theories based on static electron microscope images. It has been proposed by supporters of the "secretion theory", such as Wolff (33) and Niebauer et al (34), that Birbeck granules derived from the Golgi apparatus adhere to the cell membrane and expel their contents outside the cell. In contrast, some advocates of the "endocytosis theory", such as Hashimoto and Takahashi (35-38), suggested that Birbeck granules are formed by the invagination of the surface membrane of LC and move to the interior of the cell enable LC to take up substances from the extracellular compartment. But recently Birbeck granules have been suggested to be involved in receptor-mediated endocytosis or adsorptive endocytosis (39-42). Hanau et al (43) have observed internalization of T6 in human epidermal LC, suggesting that this is due to receptor-mediated endocytosis involving coated pits, coated vesicles, endosomes, the smooth endoplasmic reticulum, and lysosomes. They suggest that Birbeck granules could represent T6 intracellular transport organelles carrying T6 from the central part of the cell to an unknown destination (43,44). We propose that elements of the endocytosis theory and secretion theories may both be correct : invaginated endocytotic vesicles containing T6 or other antigens acquire Lag antigen centrally in LC during the

transport process from the surface. Acquisition of the Lag protein may determine subsequent functional roles of the Birbeck granule containing T6 and other antigens.

Histiocytosis X is a disease of unknown cause, characterized by a proliferation of a distinct histiocytic cell type (containing Birbeck granules in the cytoplasm). Histiocytosis X includes three clinical forms: Letterer-Siwe disease, Hand-Schuller-Christian disease, and eosinophilic granuloma. Letterer-Siwe disease is recognized as the acute disseminated form of histiocytosis X. For the early diagnosis of histiocytosis X, it is important to detect histiocytosis X cells in various tissues. Lag reacts specifically to Birbeck granules and the related structures in histiocytosis X cells, as well as in Langerhans cells on normal human tissues. The lack of cross-reactivity of Lag to other known organelles suggests the superiority of Lag to so-far reported tools for the detection of histiocytosis X cells. With Lag, Birbeck granule-positive histiocytosis X cells can be identified easily by light microscopy without the need for time-consuming and technically difficult electron microscopy.

Our results with Lag and anti-T6 monoclonal antibody revealed that almost all the proliferating cells in the lymph nodes of patients with Letterer-Siwe disease expressed T6 antigen and that about 70% of these cells were positive for Lag antigen. This indicates that some of proliferating cells in the lymph nodes lack Birbeck granules and the related structures. However, in the lesional skin the proliferating cells expressed both T6 and Lag antigens. We do not know the nature of these T6-positive, Lag-negative cells in the lymph nodes. However, it might be possible that T6-positive, Birbeck granule-negative cells represent subpopulations different from Birbeck granule-positive cells on the Langerhans cell lineage and that such cells are so-called indeterminate cells (11) or immature Langerhans cells that proliferate in the special situation such as "Langerhans cell granulomatosis".

We express our sincere appreciation to Dr. David A. Norris at Department of Dermatology, University of Colorado for critically reading this chapter and his appropriate advice.

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4

EPIDERMAL DENDRITIC CELLS IN IMMUNODEFICIENT MICE

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ABSTRACT

The expression of Ia$^+$ Langerhans cells (LC) and Thy-1$^+$ dendritic epidermal cells (Thy-1$^+$DEC) was investigated in the skin of C57BL/6J mice homozygous for the viable motheaten, beige, rhino or nude mutations. These mutations cause various types of immunodeficiencies associated with abnormalities in skin structure. At one month of age viable motheaten (me^v) mice possess normal numbers of LC compared with control heterozygotes. However by five weeks, LC density decreases in me^v/me^v homozygotes reaching 404 ± 119 cells/mm^2 compared with 1198 ± 215 in control heterozygotes ($p < 0.002$). This decrease in LC numbers correlates with a decrease in the number of interdigitating cells (IDC) isolated from the draining lymph nodes of motheaten mice. However, the ability of remaining viable motheaten mice-derived LC to transport antigens from the skin to the lymph nodes is retained. Nude mice possess significantly decreased numbers of Thy-1$^+$DEC compared with heterozygote controls from the age of four months. Rhino mice possess significantly increased numbers of Thy-1$^+$DEC at all ages tested. Thus these studies define autosomal recessive mutations in mice that are accompanied by striking changes in the numbers and function of epidermal dendritic cells.

INTRODUCTION

Two distinct dendritic cell (DC) populations have been described in the murine epidermis. These are the epidermal LC and the Thy-1$^+$DEC (1-3) Both cell populations are derived from bone marrow (4-5). LC

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display membranal ATPase activity (6) and strongly express Ia antigens (7). Recently a new monoclonal antibody, NLDC-145, has been shown to react specifically with LC and related non-lymphoid DC in dermal lymphatics and in peripheral lymph nodes (8).

LC function in the skin as potent antigen-presenting cells by taking up antigens in the epidermis and migrating through the lymphatics, as "veiled" cells, to the peripheral lymph nodes where they are called interdigitating cells (IDC) and where they were shown to trigger the lymphocyte response (9-10).

Thy-1$^+$DEC have been shown to be strongly Thy-1$^+$, asialo GM1$^+$ (asGM1$^+$), Ia$^-$, Lyt-1$^-$, Lyt-2$^-$, L3T4$^-$, sIg$^-$ and weakly Fc receptor$^+$ (11). They proliferate in response to Con A and interleukin-2 (12) and might represent an immature T-cell population in the murine epidermis (13-15).

Immunodeficient mouse mutants are useful models to study the development and function of the immune system (reviewed in ref. 16). As recently surveyed in (17), several defined mutations affecting the immune system also affect the structure and function of the skin in the mouse. The effects of four autosomal recessive immunological mutations on epidermal DC populations in C57BL/6J mice was determined. The viable motheaten (me^v), nude (nu), beige (bg), and rhino (hr^{rh}) mutations were chosen for this investigation because of their known pleiotropic effects on the immune system and on the skin.

Mice homozygous for the mutation "motheaten" (me) are characterized by a mean lifespan of only three weeks (18), even under germ-free conditions, indicating that autoimmunity might be the cause of early death in this mouse mutant (19). More recently, a new mutation at the motheaten locus termed "viable motheaten" (me^v) occurred (20). Homozygosity for this mutation resulted in a threefold increase in lifespan compared with me/me mice. This increased lifespan appears to be due to a delay in the development of autoimmunity and hemorrhagic pneumonitis which is the immediate cause of death in these animals (21). Although onset of autoimmunity is delayed in me^v/me^v mice, they show similar immunological impairments as me/me mice (16). Such impairments include decreased proliferative responses to B and T cell mitogens (22-23), reduced T cytotoxic (24) and NK (25) activities and development of early, severe autoimmune disease (18-21).

Autoimmunity in these mice is accompanied by abnormal proliferation of macrophages (25) and high levels of autoantibodies (22) due to polyclonal B-cell activation by soluble factors (26).

B cells of me^v/me^v mice are abnormal in that they display Russel bodies (indicative of impaired secretory potential) (27) and are characterized by an unusual phenotype (28). The thymus of me^v/me^v mice is histologically normal until 4 weeks of age. Thereafter, it diminishes in size until atrophy due to the inability of prothymocytes to home into the thymus (29-30). Recent data indicate that hemopoiesis is markedly impaired in me^v/me^v mice (31). Focal accumulation of neutrophils and macrophages is detectable in the skin by five days of age and gives the skin of me^v/me^v mice its characteristic "motheaten" appearance (16).

"Nude" (nu) mice are characterized by the absence of hair and thymic aplasia at birth as well as by a number of well-characterized T cell-dependent immune defects (reviewed in refs. 16-17). The "beige" (bg) mutation is considered as a homologue for the Chediak-Higashi syndrome in man since anomalous lysosomal granules are found in neutrophils and other cell types of this mutant (16). Moreover NK function (32), cytotoxic T-cell function (33), humoral responses (34) and macrophage tumoricidal capacity (35) have been reported to be impaired in this mutant. The fur of bg/bg shows reduced pigmentation compared with that of normal bg/+ littermates due to abnormal melanosome development (16). "Rhino" (hr^{rh}) mice are characterized by hairlessness and wrinkled skin from the age of five weeks (36) as well as morphological abnormalities in lymphoid organs, including early thymic involution, and systemic lesions in connective tissue (37). Impaired T helper cell function, response to alloantigens and development of autoimmunity, have also been reported in this mutant (38-39). The aim of the present study was to investigate the state of epidermal DC populations in four murine models of congenital immunodeficiency.

MATERIALS AND METHODS

Mice

C57BL/6J +/+, me^v/me^v, nu/nu, bg/bg, and hr^{rh}/hr^{rh} mice as well as their littermates were obtained from research colonies at the Jackson Laboratory. C57BL/6J-me^v/me^v mice were propagated by +/me^v sib

matings. Littermate controls (referred to as +/-) consisted of approximately two thirds +/me^v and one third +/+, the two genotypes being phenotypically indistinguishable. Generation of +/me^v breeders was accomplished by transplanting ovaries from me^v/me^v females to histocompatible female hosts and mating to C57BL/6J +/+ males. The other mutants (nude, beige and rhino) were maintained by mating homozygous males with heterozygous females. These crosses provided equal numbers of affected homozygotes and phenotypically normal heterozygote controls. Each mutant mouse was compared with a sex- and age-matched littermate control. All mice were raised under SPF conditions. They were kept at three to five per cage on sterilized pine bedding, fed a standard diet of pasteurized Wayne Lab Blox, and received chlorinated tap water ad libitum.

Epidermis separation

Mice were killed by $C0_2$ asphyxiation immediately prior to excision of skin from the rear footpads. Skin samples were incubated for two hours at 37°C in 5% CO_2/95% air in a 8.2 g/liter solution of $Na_4EDTA \cdot 2H_20$ buffered with 116 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4 and 1.4 mM KH_2PO_4 (pH 7.3, adjusted with 0.1M HCl). The epidermis was then mechanically separated from the dermis and washed in PBS prior to subsequent treatments (40).

Monoclonal antibodies and fluorescein-conjugated antisera

The following antibodies were used to identify LC: monoclonal anti-Ia^b (mouse IgM, clone 25.516) followed by fluorescein-isothiocyanate (FITC)-conjugated Fab'2 goat anti-mouse IgM obtained from Southern Biotechnology Associates Inc. (SBA); NLDC-145 (rat IgG2a, obtained from Dr. G. Kraal, Free University of Amsterdam, The Netherlands) followed by FITC-conjugated goat anti-rat Ig (SBA). The following antibodies were used to identify Thy-1^+ DEC: monoclonal anti-Thy 1.2 (mouse IgM, clone HO-13-4) followed by FITC-conjugated rabbit anti-mouse Ig (SBA); rabbit anti-asGM1 (Wako, Pharmaceutical Co.) followed by FITC-conjugated goat anti-rabbit Ig (SBA). All primary monoclonal antibodies were hybridoma cell culture supernatants. All FITC-conjugated secondary antibodies were affinity purified. Optimal concentrations for each antibody were determined on epidermal sheets from C57BL/6J +/+ mice.

Immunofluorescence microscopy

Epidermal sheets were fixed for 20 min at 23°C in acetone. Specimens were washed three times in PBS containing 5% FCS for 30 min at 4°C before incubation with the appropriate unlabeled monoclonal antibody in a total volume of 0.2 ml. The specimens were then incubated at 4°C for 16 hr and washed three times at 23°C for 2 hr. The sheets were then incubated for 100 min in the appropriate FITC-conjugated antiserum. The specimens were finally washed three times in PBS on a rocker platform for 2 hr at 23°C, mounted in a 9:1 solution of glycerol/PBS and examined with an Orthoplan fluorescence microscope equipped for epi-illumination (E. Leitz, Inc., FRG). Labeled cells were counted with the aid of an eyepiece grid (for Ia and NLDC-145 staining, 0.046 mm^2 = one field; for asGM1 and Thy-1 staining, 0.75 mm^2 = one field; 10 to 20 fields chosen at random were counted for each specimen) (41). Controls consisted of specimens incubated with an irrelevant primary antibody or medium alone followed by incubation with the appropriate fluorescein-conjugated antiserum.

ATPase staining

Epidermal sheets were stained for ATPase as described (6). Briefly, epidermal sheets were washed three times in Tris maleate buffer (containing 6.85% sucrose) at 4°C for 20 min and then fixed for 20 min at 4°C in 4% cacodylate-buffered formaldehyde. The sheets were then washed three times in Tris maleate buffer (6.85% sucrose) for 30 min at 4°C and stained with a solution containing 10 mg of ATP, 3 ml of 2% $PbNO_4$, 5 ml of 5% $MgSO_4$ and 92 ml of Tris maleate buffer (8.53% sucrose) at 37°C for 20 min. The preparations were then washed again three times in Tris maleate buffer at 23°C and put into a 1% solution of ammonium sulfide for 5 min. They were finally washed twice with distilled water, mounted in a 9:1 solution of glycerol-PBS and counted with the aid of an eyepiece grid (0.046 mm^2 = one field; 20 fields chosen at random were counted for each specimen)

Sensitization procedures

In order to sensitize the mice to rhodamine B isothiocyanate (RITC) or challenge them with fluorescein isothiocyanate (FITC) or RITC, they were painted on their shaved abdomen or thorax (sensitization) or ears (challenge) with 5 mg of FITC or RITC (Sigma) dissolved prior to experiment in 0.4 ml of acetone:dibutyl phthalate (50/50, v/v) (42).

Increase in ear thickness was quantitated with a micrometer 24 hr after challenge.

Cell suspensions

For obtaining lymph node cell (LNC) suspensions, mice were killed by cervical dislocation, their lymph nodes (axillary, subscapular and inguinal) were removed aseptically and pressed through sterile nylon mesh. The cell suspensions were filtered through nylon mesh and washed twice. Cell viability was assessed by trypan blue exclusion and shown to be always greater than 95%. LNC were enriched for DC populations as described by Knight et al. (43). Cells were resuspended at a concentration of 5 X 10^6 per ml in RPMI containing 10% fetal calf serum (FCS), 10^{-5}M 2-mercaptoethanol and 100 μg/ml of gentamycin. Six to 8 ml of each cell suspension was layered onto 2 ml of 14.5% metrizamide (Nygaard, analytical grade) in medium and centrifuged for 10 min at 600g. Cells at the interface were recovered and washed twice before subsequent use. Cell suspensions were incubated for 30 min on ice with anti-NLDC-145 antibody in PBS supplemented with 5% FCS and 0.1% sodium azide at 100 ml/10^6 cells. The cells were washed twice and resuspended at the same concentration in FITC-conjugated goat anti-rat Ig (SBA) for 30 min on ice. The cells were finally washed and counted by immunofluorescence microscopy as noted above.

Statistics

Experimental group means and standard errors were calculated for each experiment. Comparisons of group means were performed by using the Student's *t* test. Differences between values were considered statistically significant at $p < 0.05$.

RESULTS

Langerhans cell density in the epidermis of immunodeficient mice

LC density in the epidermis of immunodeficient mice was determined by immunochemistry with the NLDC-145 and an anti-Ia^b monoclonal antibodies.

We analysed LC density in the epidermis derived from four immunodeficient mouse mutants, maintained on a C57BL/6 strain background that are also known to be characterized by skin abnormalities. At one month of age, there were no significant differences in numbers of LC in me^v/me^v, hr^{rh}/hr^{rh}, nu/nu and bg/bg mice compared with their

normal littermates as assessed by reactivity with both anti-Ia^b and NLDC-145 antibodies. In contrast, me^v/me^v mice were found to possess significantly fewer Ia^+-cells than their normal littermates at the age of 5 weeks (491 ±10 in comparison with 1254 ±143 in controls, $p < 0.03$). LC density was also analysed by staining with the NLDC-145 antibody the skin of 1, 3, 6 and 8 week-old me^v/me^v mice. Decrease in LC density was shown to progress with age after four weeks. Epidermal sheets derived from two-month-old me^v/me^v mice were shown to contain 404 Ia^+-cells/mm^2 and 456 NLDC-145^+ cells in comparison with 1198 Ia^+ cells/mm^2 and 1425 NLDC-145^+ cells/mm^2 respectively, in control littermates ($p < 0.002$). These results were later confirmed using ATPase staining (43a).

Thy-$1^\pm$ dendritic epidermal cell density in the epidermis of immunodeficient mice

Density of Thy-1^+DEC in the skin of immunodeficient mice was assessed by staining epidermal sheets from the footpads with a monoclonal anti-Thy-1.2 antibody. Thy-1^+DEC density was lower than density of LC in all specimens examined as has been reported for other anatomical sites (1-2).

A marked and early decrease in Thy-1^+DEC density occured in the epidermis of nu/nu mice. At one month of age, nu/nu mice possessed normal numbers of Thy-1^+DEC (77 ± 4 cells/mm^2 vs 80 ± 3 cells/mm^2 in nu/+ mice) whereas at 4 months of age, they possessed significantly lower numbers of Thy-1^+DEC than young age-matched normal littermates (44 ± 7 cells/mm^2 vs 101 ± 16 cells/mm^2 in nu/+ mice, $p < 0.002$). At 8 months, nu/nu mice showed a two fold-decrease in the density of Thy-1^+-cells in comparison with normal heterozygotes (17 ± 6 cells/mm^2 vs 46 ± 8 cells/mm^2 in nu/+ mice, $p < 0.01$). In contrast with nude mice, another hairless mutant, the rhino hr^{rh}/hr^{rh} mutant showed a 3-5-fold increase in density of Thy-1^+-cells as compared with control animals at all ages tested. At one month 330 cells/mm^2 were found in the mutant while there were only 85 cells/mm^2 in the controls ($p < 0.003$). At eight months of age the numbers were 341 and 61 cells for mutants and controls, respectively ($p < 0.003$). Changes in Thy-1^+DEC density were confirmed by staining with anti-AsGM1 (43a). No $L3T4^+$ cells were found in the epidermis of nude or rhino mice. Beige mice did not show any significant

changes in Thy.1$^+$-DEC density in the epidermis as compared with normal littermates.

NLDC-145$^+$ cell isolation from the lymph nodes of motheaten mice

It is known that LC function by migrating along the lymphatics as veiled cells and presenting antigen to T cells in the paracortical area of peripheral lymph nodes as IDC (9). Veiled cells, IDC as well as LC stained positively with the NLDC-145 antibody (8).

Six week-old me^v/me^v mice and their control littermates were sensitized with RITC as previously described by Macatonia et al. (42) by painting the abdominal and truncal skin with RITC dissolved in acetone and dibutylphthalate. Twenty four hours later, their lymph nodes (axillary, subscapular, inguinal) were removed and DC were isolated at the interface of metrizamide gradients as the low density cell fraction (43). Subsequently, the cells were stained with the NLDC-145 or anti-Ia^b antibody which were revealed with a goat anti rat or goat antimouse Ig FITC-conjugated antisera respectively (Fig. 1). Using double immunofluorescence, antigen-containing cells were therefore visualized as cells emitting red fluorescence whereas the NLDC-145$^+$ marker was visualized by green fluorescence.

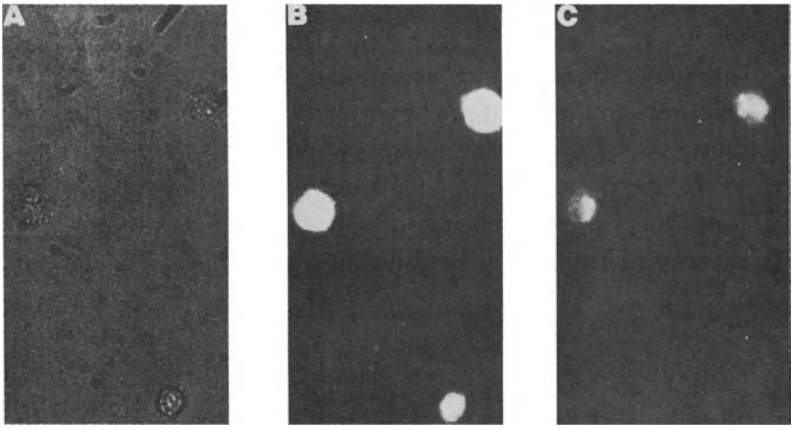

Fig. 1. Low density LNC from normal C57BL/6J mice. Mice were sensitized to RITC 24 hr prior to LNC removal. LNC were stained with NLDC-145 antibody followed by FITC-conjugated antiserum. The cells were examined by (A) phase contrast microscopy and for (B) red or (C) green fluorescence (X400).

The cell suspensions isolated on metrizamide gradients from normal C57BL/6J mouse LNC consisted of 60-80% NLDC-145$^+$ cells. Morphologically, the contaminating cells were 10-20% lymphocytes and about 5% of the isolated cells phagocytosed latex beads.

Preliminary experiments indicated that cell suspensions in sensitized normal C57BL/6 +/+ mice before centrifugation on the metrizamide gradient contained 2-5% of RITC$^+$ cells. After centrifugation on metrizamide gradient, the cell suspension contained approximately 70-80% RITC$^+$-cells, 50-70% NLDC-145$^+$-cells and 70-80% Ia$^+$-cells. Virtually, all (95-99%) NLDC-145$^+$-cells were also RITC$^+$ (Fig. 1, Table 1). The low density LNC represented approximately 0.75-1.25% of the total number of cells isolated from the lymph nodes.

Table 1 shows that the percentage of RITC- and NLDC-145$^+$ cells in the low density LNC population derived from me^v/me^v mice was not

Table 1. Isolation and staining with the NLDC-145 antibody of low density lymph node cells in me^v/me^v mice.

Exp#	Genotype	Total LNC	Low density LNC (%)			
		Cells x 10^6	Cells x 10^3	RITC$^+$	NLDC$^+$	NLDC$^+$/RITC$^+$
1	me^v/me^v	37	47	77	58	95
	+/-	33	250	80	67	96
2	me^v/me^v	33	55	55	39	97
	+/-	20	110	76	48	99
3	me^v/me^v	44	44	81	56	99
	+/-	33	175	81	66	99

Total LNC were obtained from RITC-sensitized 5 to 8-week-old me^v/me^v and control littermates. Low density LNC were recovered as described in Materials and Methods. Cells = total number of cells obtained per mouse (from each mouse two inguinal, axillary and subscapular lymph nodes were removed, cells were pooled from three or four animals), RITC$^+$ = % of fluorescent cells per 200 cells examined for red fluorescence, NLDC$^+$ = % of fluorescent cells per 200 cells examined for green fluorescence, NLDC$^+$/RITC$^+$ = % of cells staining positive for NLDC-145 (revealed with FITC-conjugated anti-rat antiserum) that were simultaneously rhodamine-positive.

significantly different than that found in littermate controls. Moreover, as found for normal littermate controls, almost all NLDC-145$^+$ cells were also RITC$^+$, suggesting that transfer of antigen from the skin to the lymph nodes is normal in me^v/me^v mice. However, it was consistently observed that fewer low density LNC could be recovered from the lymph nodes of me^v/me^v mice in comparison with normal littermates (Table 1). Consequently, a lower number of NLDC-145$^+$ cells was found to be present in the total (unfractionated) LNC population of me^v/me^v mice in comparison with normal littermates.

In order to determine whether LNC from me^v/me^v mice could be sensitized to RITC and could transfer responsiveness to normal recipients, cells were obtained from the lymph nodes of RITC-sensitized me^v/me^v and normal littermates (Fig. 2). It has been shown that the ability of LNC to transfer sensitivity to RITC entirely resides within the IDC fraction (44). According to the method described by Thomas et al. (45), 2 x 10^6 LNC from me^v/me^v and normal littermates were injected into

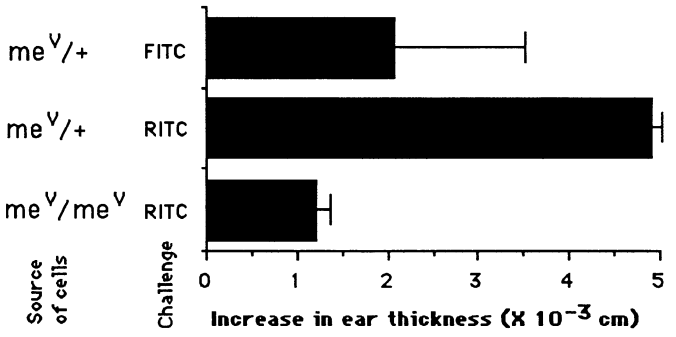

Fig. 2. Delayed-type hypersensitivity reaction in recipients of LC derived from me^v/me^v mice. Me^v/me^v or $me^v/+$ control littermate were painted with RITC 24 hr before harvesting of LNC. 10^6 LNC derived from each group were injected into the footpad of 6 week-old +/+ C57BL/6J mice. Recipients were challenged 7 days later with RITC or FITC and ear swelling was measured as the mean increase in ear thickness 24 hours after challenge in each group of 2-4 mice. Results are expressed in 10^{-3} cm $\pm$ SEM.

the footpad of 6-week-old +/+ C57BL/6J recipients (Fig. 2). One week later, the recipient mice were challenged with RITC or FITC as a control in the ear, and 24 hours later increase in ear thickness was measured. A significantly lower response was measured in mice sensitized with LNC derived from me^{v}/me^{v} mice as compared with recipients of LNC derived from normal littermates (Fig. 2).

DISCUSSION

In the present study, LC were examined in various mutants maintained on one single genetic background (C57BL/6J). This allowed us to compare the effect of various recessive mutations on epidermal DC. In addition, the mice were raised under highly controlled conditions. This point is particularly relevant since different reports have indicated that infection and/or exposure to several physicochemical agents have the ability to modify LC density in the epidermis (46-53).

We showed that LC numbers are normal in nude, beige and rhino mice compared with age-matched normal control littermates. However a threefold reduction in density of LC was found in the epidermis of viable motheaten mice as compared with their normal control littermates by 8 weeks of age.

Since LC density was found to be decreased in the skin of me^{v}/me^{v} mice, it was of interest to examine LC activity in the skin of those mice. In order to examine the function of LC in taking up antigens at the periphery and transferring them to the peripheral lymph nodes as previously shown (3,9), we used the method described by Macatonia et al. (42) who reported the isolation of antigen-carrying LC in the lymph nodes of mice having been sensitized to RITC or FITC. Thomas et al. (45) demonstrated that transfer of sensitization to FITC, in contrast with oxalozone, was conferred on virgin recipients by non-T cells. The different nature of the immunogenic complexes formed by the two sensitizers could be due to the fact that FITC is an hydrophilic molecule, whereas oxalozone is known to be highly lipophilic (45). It was shown that the cells responsible for the transfer were non B, non phagocytic antigen-presenting cells (42) and more recently, these cells were found to be identical to LC (44).

It is known that LC function in vivo by carrying antigens from the skin to the draining lymph nodes where they sensitize T cells (9). As the NLDC-145 antibody is known to react with LC as well as with IDC (8), we assessed the function of LC as antigen-presenting cells by monitoring the

efficiency of the transfer of RITC to the lymph nodes by low-density NLDC-145$^+$ cells. Virtually all NLDC-145$^+$ cells stained positively for RITC in me^v/me^v mice as well as in controls. Thus, the function of LC in me^v/me^v mice in taking up the antigen seems to remain intact. However, RITC$^+$ NLDC-145$^+$ cells were (in three successive experiments) recovered in lower numbers from metrizamide gradients as compared to the number of NLDC-145$^+$ cells recovered from the lymph nodes of normal control littermates. This suggests that less LC are able to migrate from the skin of me^v/me^v mice to the peripheral lymph nodes as compared to LC derived from the skin of normal littermates.

Our results also indicate that when injected in vivo into the footpads of normal recipients, LNC derived from me^v/me^v mice elicited a significantly weaker sensitization to RITC than LNC derived from normal littermate controls. This correlates with the results showing decreased numbers of IDC in LNC from me^v/me^v mice since transfer of sensitization in this system is known to be dependant on IDC exclusively (44). Therefore, me^v/me^v mice are characterized by a decrease in the numbers of functional LC in the skin and the lymph nodes and an associated decreased capacity of LNC to transfer sensitization to RITC to normal recipients. However, our results suggest that the remaining LC function normally.

The decrease in LC density as revealed by immunochemistry could be due to three different causes. It is unlikely that the decrease in Ia$^+$ cell density in the skin of me^v/me^v mice was due to reduced Ia expression on LC membrane since the paucity of LC in motheaten mice was confirmed by using two other independant staining methods. Secondly, decrease in LC could be secondary to the chronic skin inflammation evident in me^v/me^v mice by 1 week of age. Indeed it has been shown that inflammation in the skin resulted in the transient loss of LC because of increased keratinocyte proliferation and subsequent shedding of the suprabasal cell layers, including LC (54-55). However, the ontogeny of the decrease in LC density does not coincide with time of appearance or severity of inflammatory lesion in the skin of me^v/me^v mice.

The decrease observed in LC density in the skin of me^v/me^v mice could reflect a more generalized quantitative deficiency in other accessory cell populations. Regarding this possibility, it is interesting to note that the multifaceted immunodeficiency syndrome that affects me^v/me^v

mice includes the inability of prothymocytes to home into the thymus (29-30). The reason for the impaired ability of normal prothymocytes to home to the thymus of me^v/me^v mice has been partly elucidated in a recent report by Komschlies et al. (30). These authors showed that when C57BL/6 (Ly.1.2.) me^v/me^v-derived bone marrow cells were injected IV to lethally irradiated normal congenic C57BL/6 +/+ (Ly.1.1.) recipients, no donor-derived thymocytes were detected in the thymus of the recipients. In contrast, the number of me^v/me^v donor-derived Ly.1.2.$^+$-thymocytes was found to reach normal levels in congenic (Ly.1.1.) C57BL/6 recipients if the bone marrow cells were injected intrathymically. This demonstrated that prothymocytes are present in normal numbers in bone marrow derived from me^v/me^v mice but are unable to home into the thymus via the blood.

Addition of normal bone marrow or bone marrow derived from scid/scid mice (lacking in functional prothymocyte activity) to bone marrow derived from me^v/me^v mice restored the ability of the me^v/me^v bone marrow prothymocytes to home to the thymus following IV injection. Therefore, it was concluded that the inability of me^v/me^v prothymocytes to home to the thymus is due to the absence in me^v/me^v mice of a particular bone-marrow-derived accessory cell subset (16,30). Recent findings (56) suggest that this deficiency might be related to impaired capacity of bone marrow derived from me^v/me^v mice to respond to a thymus-derived soluble factor, that was shown to direct prothymocyte homing into the thymus in vitro. Alternatively, the specific lack of expression of a molecule present on bone marrow-derived accessory cells and directing the homing or proliferation of immature T cells to the thymus as previously reported (57), might provide an interesting explanation for these puzzling data.

Impaired function of an accessory cell subset of the marrow stroma has also been shown to be responsible for abnormal lymphopoiesis in me^v/me^v mice (58-59). Thus, particular subsets of accessory cells in various lymphoid organs including the bone marrow, the thymus, the skin and the lymph nodes are affected by the me mutation. All of these have been implicated in the control of T cell differentiation (59-61). It is tempting to understand these findings as pointing to the existence of a general deficiency in LC lineage primarily due to the deleterious effects of the motheaten mutation.

Two hairless mutants were found to possess abnormal density of Thy-1$^+$ DEC. Nude mice were found to possess relatively normal number of Thy-1$^+$ DEC at one month of age but their density fell down rapidly with age. At the age of 8 months, only 20 cells/mm^2 could be found in the plantar epidermis of nu/nu mice. Abnormal Ia expression on thymic epithelial cells has been proposed to be responsible for the inability of precursor T cells to populate the thymus of nude mice (62). Our finding that Ia antigens are expressed normally on nu/nu mouse-derived LC supports the hypothesis (17) that epidermal Ia antigen expression in the skin of nude mice might promote extrathymic maturation of Lyt-2$^+$ and L3T4$^+$ T cells that appear in the lymphoid tissues of 6 month-old nude mice (63). Consistent with this hypothesis, English et al. recently showed that T-cell competence can be induced in young nude mice by grafting of normal skin (64). Thy-1$^+$ DEC might represent an immature T cell population (13-15,65-67). The decrease in Thy-1$^+$ DEC density in the skin of nu/nu mice with age could therefore reflect the mobilization of this pool of immature T cells which after subsequent intraepidermal maturation could populate the nude mouse lymphoid organs (63). In agreement with this hypothesis are the findings of Yoshikai et al. who showed that functional T cells in nude mice mostly express the $\gamma\delta$ TCR (68) (although it has been suggested that Thy-1$^+$ DEC might also give rise to $\alpha\beta$ TCR$^+$ T cells (15) and small numbers of such cells are also found in nude mice).

A recent report indicated that Thy-1$^+$DEC of nude mice differ from Thy-1$^+$DEC derived from normal littermate controls in their response to mitogenic stimulus (13). In addition nude mice-derived Thy-1$^+$DEC failed to produce functional CD3/$\gamma\delta$ TCR complex, were found at a lower density than in normal littermates (13) and were present mostly around the hair follicles whereas normal mice-derived Thy-1$^+$DEC were distributed interfollicularly (13). In this study, nude mice derived-Thy-1$^+$DEC were found by phenotypical and functional criteria to belong to a more immature T cell population than Thy-1$^+$DEC from normal controls (13). This suggested that although Thy-1$^+$DEC seems to be a thymus-independant cell population, part of their intraepidermal differentiation is not possible in nude mice, possibly due to the microenvironmental defect known to characterize the skin of this mutant mouse strain (13). Alternatively, it is likely that Thy-1$^+$DEC differentiation is dependent on

the presence of a functional thymus or on the availability of some of its secretory products. Indeed, it was found that Thy-1$^+$DEC are found in normal numbers in the skin of lethally irradiated thymectomized animals, reconstituted with normal bone marrow. However, CD3/TCR complex expression in Thy-1$^+$DEC was shown to be deficient in these animals unless mice were also injected with fetal thymocytes (69).

In contrast to our observations in nude mice, increased numbers of Thy-1$^+$ DEC were found in the skin of rhino mice. This could reflect the accumulation of the T cell precursor Thy-1$^+$DEC due to the severe structural abnormalities known to characterize the connective tissues of these mice (37) and could be related to the specific T helper cell functional deficiency (38) which characterizes rhino mice. Similarly, abnormalities were demonstrated in the architecture of the thymus of young rhino mice (38,39).

In summary, we have described abnormalities in the density of LC and Thy-1$^+$DEC in the skin of several immunodeficient mice known to have pleiotropic defects in the skin and the immune system. The ability of these individual mutations to selectively affect either LC or Thy-1$^+$DEC suggests that numbers of these epidermal DC are independently regulated. Each of these models should prove as a valuable tool for investigating the biological properties and the ontogeny of the epidermal DC function.

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CANCER AND AGING

5

LANGERHANS CELLS IN TUMOUR DEVELOPMENT AND REGRESSION

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ABSTRACT

This Chapter reviews the role of Langerhans cells in the development and regression of cutaneous tumours. Experimental evidence both with chemical carcinogens and ultraviolet light-induced cutaneous tumours suggest that alteration in Langerhans cell number and function is an important feature of the early development of experimental skin tumours. This appears associated with activation of suppressor cells via I-J restricted epidermal antigen-presenting cells. Such events would favour the generation of immunosuppressive mechanisms allowing developing tumour cells to bypass local specific immune reactions. However, while Langerhans cells are present in cutaneous tumours, their role in tumour regression of developed lesions remains unclear. Ultimate answers to these questions will depend on future studies examining the ability of Langerhans cells to process and present tumour antigens and on their capacity to generate immune responses. Such investigations in part will need to be performed on Langerhans cells extracted from neoplastic lesions.

INTRODUCTION

The skin immune system contains unique populations of antigen-presenting cells, of which the best characterized are Langerhans cells (LC). LC take up antigen and then migrate via lymphatics to local lymph nodes where they present antigen to specific lymphocytes resulting in the initiation of an immune response. Other dendritic cells found in murine skin are Thy-1+ and I-J+ cells, the functions of which are still debated (1). Since LC have a key role initiating cutaneous immunological defence reactions,

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it is likely they may take part in any immunological protection against skin tumours. This Chapter reviews changes in LC in skin tumours and then proceeds to examine the effects of carcinogens on LC number and function; comparisons are made with the early stages of ultraviolet light-induced carcinogenesis. However, a difficulty with these studies is that at present little is known on the tumour-associated antigens in cutaneous tumours and hence precise functional investigations on LC in skin tumours are still awaited.

LANGERHANS CELL INFILTRATION OF SKIN TUMOURS

Dendritic antigen-presenting cells (APC) have been observed in human and murine skin tumours (1) although the function and phenotype of these cells have not been adequately characterized. Some investigators have found these cells present at increased densities while others have reported decreased levels; however, it is difficult to know with what to compare the density of these cells infiltrating tumours. Hence the major worthwhile observation is that these cells have infiltrated tumours at all. Due to the lack of functional data, it is entirely unclear whether these cells have any influence on the development or growth characteristics of the tumours.

Our group studied LC infiltration of a variety of human tumours using anti-S100 antibodies and immunohistochemistry to identify these cells. We found large numbers of LC infiltrating the lesions and in epidermis adjacent to 11 cases of Bowen's disease, 13 cases of keratoacanthoma, 18 cases of squamous cell carcinoma (SCC), and 18 cases of basal cell carcinoma (BCC). In 16 examples of actinic keratosis there were high numbers of cells in the lesion but not in the epidermis adjacent to the lesion. In keratoacanthoma, SCC, and BCC, the LC density was highest at the interface between normal and neoplastic tissue, but were more evenly distributed in Bowen's disease and actinic keratosis (2). LC have previously been reported infiltrating basal cell papillomas, BCC, SCC and keratoacanthomas, although very few cases were examined (3). In a small series of patients LC have also been studied in the epidermis above SCC; three patients were found to have normal LC density and morphology, one had normal density but altered morphology, and in six patients a decrease in LC density was observed (4). LC in these lesions were mainly

round or oval rather than dendritic. Large numbers of Ia+ dendritic cells have also been identified in chemically induced murine tumours (5).

Elevated numbers of LC have likewise been observed in cervical intraepithelial neoplasia (CIN; 6). Papillomavirus infection has been implicated as an aetiological agent in human cervical cancer (7) and thus it has been of interest to study LC density in cervical wart virus lesions (cervical condyloma) as these could be pre-cancerous. The number of LC in these lesions was found to be decreased compared to normal epidermis, suggesting that the decreased LC density induced by wart virus infection may impair immunosurveillance of the cervix, and as the tumours develop, LC migrate into the lesions (6).

It is now known that there are at least 36 different types of human papillomavirus. Recently, Hawthorn et al found that CIN lesions infected with moderate and high numbers of papillomavirus type 16 had low numbers of LC infiltrating the lesions (8). CIN infected with even low copy numbers of human papillomavirus type 18 also had low LC densities, whereas CIN lesions with no detectable human papillomavirus were infiltrated with high numbers of LC. Hawthorn and co-workers concluded that in part, the carcinogenecity of human papillomavirus types 16 and 18 may be due to the ability of these viruses to affect the afferent limb of the immune response (8).

LC have also been identified with anti-S100 antibodies in 48 SCC of the tongue (9), within bronchiolo-alveolar tumour nodules (10) and within 7 out of 37 bronchiolo-alveolar cell carcinomas (11). LC have also been identified within an intracranial histiocytic lymphoma (12). The significance of the presence of LC in non-squamous epithelium related tumours is unclear and warrants study.

LC have also been examined in the epidermis above melanomas. Stene et al (1988) found that, compared to normal epidermis adjacent to the melanomas, LC were depleted from the epidermis of deeply invasive melanomas but remained at normal density above nevi, early invasive melanomas and cutaneous metastatic melanoma nodules (13). In contrast, dermal LC were increased around in situ and early invasive melanomas but not around deeply invasive melanomas of cutaneous metastatic nodules. Stene and co-workers suggested dermal LC might present neoantigens to T lymphocytes in situ or in the draining lymph

node.

To examine in more detail the effect of tumour growth on LC density Bergfelt, Bucana and Kripke (1988) examined the density of LC in epidermis overlying subcutaneous transplantable tumours at various times during progressive tumour growth compared to normal epidermis (14). The LC densities in the epidermis above a UVB-induced line (UV-2237), a tumour induced by PUVA treatment (PUVA-2210), and a melanoma line (K-1735) were all increased. The number of LC did not change during growth of the tumours. The epidermis above UV-2237 injected into athymic nude mice also contained increased numbers of LC, as did the epidermis above tumours which were regressing when they were injected into mice which had been pre-immunised with the tumour line. There was no clear relationship between the immune status of the mice and LC density above the tumour, nor between whether the tumours were regressing or growing and LC density. Bergfelt et al concluded that non-immunological factors regulate the number of LC in the skin associated with skin tumours (14).

Thus whereas there has been considerable documentation of the density of LC in different types of skin and non-skin neoplastic lesions at various stages of development, at present no clear pattern has emerged to indicate whether these infiltrating cells are playing a role in the development or regression of these lesions. This is largely because it is still unclear what role the immune system plays in the biology of these lesions. To answer this question it will be necessary to correlate the antigenicity of individual tumours with LC density and the function of these infiltrating cells. This is a difficult objective which we are far from reaching.

CHANGES IN THE SKIN IMMUNE SYSTEM DURING THE EARLY STAGES OF CHEMICAL CARCINOGENESIS

It is during the early stages of tumour development when the interactions between any developing neoplastic cells and the immune response of the host are likely to be important for the development of immunity against the tumour. Once regulatory lymphocyte populations specific for the tumour have been activated, it would be difficult to override the effects of these cells by subsequent events unless the antigens recognised by these lymphocytes are lost and

others are gained. As the initial cells of the immune system to interact with developing tumour cells are the local cells resident in that organ, it is this interaction which is likely to be of critical importance for the development of anti-tumour immunity. For skin tumours, the important local cells of the immune system are the dentritic antigen-presenting cells, of which the best characterized are the LC which express class II major histocompatibility complex encoded glycoproteins (Ia in mice, HLA-DR in humans) on their plasma membrane.

For these reasons, it has been of great interest to study LC during the early stages of carcinogenesis, prior to the appearance of macroscopically visible tumours. As this is not possible in humans, this work has been restricted to animal models of cancer. Two different procedures have been used to produce skin tumours in animals - ultraviolet light irradiation and topical application of chemical carcinogens, both of which have profound effects on LC and the function of the skin immune system. Chemical carcinogens will be discussed in this section, and ultraviolet light in the next.

Initial experiments showed that the chemical carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) applied weekly to the skin of mice reduces the density of adenosine triphosphatase (ATPase)-positive LC (15). The LC were depleted very early during the process of carcinogenesis, within 3 days of a single application of DMBA. Further treatments continued to reduce the LC to about 2/3 of their original density. Electron microscopic examination of skin from DMBA-treated epidermis revealed that the DMBA treatment had depleted these cells from the epidermis rather than modulating ATPase from the surface of the LC. No cells containing Birbeck granules, a cytoplasmic organelle only found in LC, were present in epidermis from DMBA-treated mice (16). Additionally, upon cessation of DMBA treatment, the LC did not return to their normal density for 6 - 8 weeks (15). If carcinogen treatment had altered the markers used to identify the LC, then the marker should have been re-synthesised within a much shorter time period.

Chemical carcinogenesis is a multi-step process. The initial genetic event in the pre-neoplastic cells is not sufficient for it to develop into a cancer. The subsequent steps involved in cancer

development are poorly understood, but the recognition that some chemicals, which are not able to induce tumour formation on their own, are carcinogenic in combination with other chemicals has greatly facilitated dissection of the steps involved in carcinogenesis. These chemicals can be broadly divided into two groups, initiators and promotors, where the initiator is the mutagen which induces the genetic changes in the cell which leads to carcinogenesis, and the promotors provide the further signals necessary for the cell to develop into a tumour (17). Tumour formation requires a single application of an initiator, followed by multiple applications of a promotor, indicating that the promotor may alter the environment of the initiated cell, thus enabling it to develop into a tumour.

In order to determine whether the carcinogen-induced alteration in LC density is associated with the initiation or promotion of tumours, we examined the effects of a range of tumour initiators and promotors on LC density. None of the initiators assessed - urethane, chrysene or benz(a)anthracene - effected the density of ATPase-positive LC over a three week period. In contrast, all promotors examined - croton oil, 12-0-tetradecanoylphorbol 13-acetate (TPA), and teleocidin - depleted LC from murine epidermis within one week of a single application, and they remained low, with subsequent treatments for up to three weeks. Further, time course studies indicated that LC required more than six weeks to return to control values, after cessation of croton oil treatment, indicating that the promotors had depleted the cells from the epidermis, rather than modulating ATPase from their surface (18).

This depletion of LC by tumour promotors but not initiators was confirmed by immunohistochemical identification of LC, using monoclonal anti-Ia antibodies, indicating that the disruption in LC density is associated with steps involved in tumour development, rather than initiation (19). Thus, this disruption in epidermal antigen-presenting cells may alter the environment of the initiated cell in such a way that it is able to grow into a tumour. It is possible that the promotor-induced disruption in local antigen-presenting cells enables pre-neoplastic cells to grow into a tumour without inducing an immune response capable of destroying the tumour.

This effect of tumour promotors on LC has now been shown to

also occur in humans. Croton oil applied to human skin depletes LC density and reduces their allo-antigen-presenting function (20). Baxter et al (1988) have also found TPA to alter LC morphology but not density when applied topically to SENCAR mice, which are particularly sensitive to chemical carcinogenesis (21).

To investigate whether these carcinogen-induced disruptions in local antigen-presenting cells alters the immunological capabilities of the skin, we used a contact sensitivity model. In these experiments BALB/c mice were treated with the carcinogen by topical application to the dorsal trunk and then the contact sensitizer was applied to the treated skin. The mice were then examined for the development of sensitization by challenging their ear 5 days later and then measuring the increase in ear thickness after a further 24 h as a measure of the cellular immune response. The contact sensitizer 2,4-dinitrofluorobenzene (DNFB) applied to DMBA-treated skin did not induce as large a contact sensitivity response as when applied to solvent-treated skin. The time course for the reduced contact sensitivity response paralleled the reduction in LC density (22).

However, when the contact sensitizer was applied to abdominal skin distal to the DMBA-treated dorsal trunk, then a response was initiated which did not differ from that induced in solvent-treated control mice, indicating that the effect of the carcinogen on the immune system was restricted to disruption in the local immune response of the treated skin. Adoptive transfer of spleen cells from mice sensitized with DNFB through DMBA-treated skin into immune hosts demonstrated that effector suppressor lymphocytes had been activated which inhibited the development of contact sensitivity upon challenge of the immune hosts. This suppressor cell activation required contact with antigen as spleen cells from DMBA-treated but unsensitized mice did not contain suppressor cells (22).

DNFB applied to DMBA-treated skin also activated suppressor cells which inhibited the development of contact sensitivity when transferred into naive mice. These suppressor cells were specific as they would not inhibit the development of an immune response against a very similar antigen, 2,4,6-trinitrochlorobenzene (TNCB); they were long lived, remaining active for the longest time examined, 6 months. These suppressor cells retained their specificity over this time

period (23). Additionally, DNFB applied to the skin of mice treated with tumour promotors but not initiators likewise activated suppressor cells (19).

Besides the activation of suppressor cells for cellular immunity, DNFB applied to DMBA-treated skin activates suppressor cells for humoral immunity; these cells are also specific and long lived. This was shown by measurement of specific serum antibody by an enzyme-linked immunosorbent assay. However, they show a different dose course for induction; a 10-fold increase in the dose of contact sensitizer still activates suppressor cells for cellular but not humoral immunity. This indicates that either the suppressor cells for cellular and humoral immunity are separate populations of cells, or that whereas cellular immunity is only induced by LC presentation of antigen, humoral immunity may be induced by two distinct mechanisms, one involving LC (23).

To examine how the suppressor cells were activated in the absence of antigen presentation by LC, we induced immunity in naive mice by subcutaneous or intravenous inoculation of trinitrophenyl (TNP)-conjugated antigen-presenting cells from the epidermis (EC) and lymph nodes (LN) draining the skin. In these experiments, to examine presentation of the antigen by the inoculated cells rather than reprocessing of injected cellular material by host antigen-presenting cells, it was necessary to inject no more than 10^4 TNP-conjugated cells. Reprocessing of inoculated antigen-presenting cells and presentation by host cells was controlled by examining whether the antigen-presenting cells were able to induce a response if they were killed by slow freezing and thawing prior to inoculation. These experiments demonstrated that, whereas LN antigen-presenting cells from DMBA-treated mice, unlike control LN APC, were unable to induce a contact sensitivity response, they were also unable to activate suppressor cells, and hence this DMBA-induced alteration in lymph node APC was not responsible for the tolerance induction. In contrast, EC from DMBA-treated mice, were unable to present antigen in a way which induced a contact sensitivity response, but were able to activate transferrable suppressor cells when injected subcutaneously or intravenously; EC from solvent-treated mice induced contact sensitivity. Thus the suppressor cells are activated by an

antigen-presenting cell present in the epidermis, not the local lymph nodes of DMBA-treated mice (Fig.1; 24).

Contact sensitivity (CS) and suppressor cells (S) induced by 10^4 TNP-epidermal cells or TNP-lymph node cells

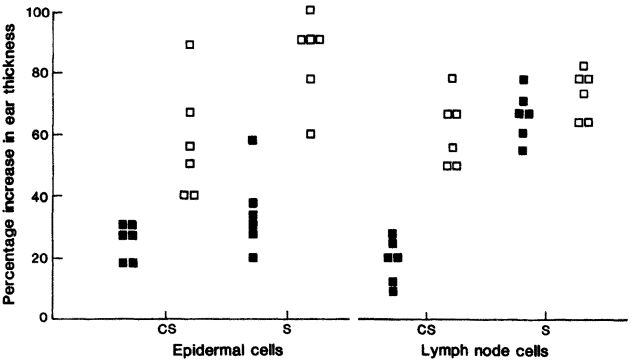

Fig. 1. Epidermal or lymph node cells removed from DMBA (■) or solvent (□) treated mice were conjugated to TNP and injected subcutaneously into syngeneic recipient mice. Mice were challenged with the antigen on their ears 5 days later, and contact sensitivity induced by the inoculated cells assessed after a further 24h by measurement of the increased ear thickness. Suppressor cell activation by the inoculated cells was determined by adoptive transfer of spleen cells from the recipients into host mice who were then sensitized with the antigen, and challenged by applying the antigen to their ears 5 days later, and the ear thickness measured after a further 24h (see reference 24 for details).

The suppressor cells are activated in the local lymph nodes within 3 - 6 h following application of antigen. This was shown by removing the local lymph nodes or spleens from mice at various times ranging from 0.5 - 12 h following application of antigen to DMBA or solvent treated skin, and transferring these organs separately as cell suspensions into naive hosts. These hosts were allowed 2 weeks for any signal present to activate suppressor cells and then they were treated with the contact sensitizer. Lymph nodes but not spleens contained the suppressor cell activating signal indicating that the suppressor cells are activated by an epidermal antigen-presenting cell which migrates to local lymph nodes within 3 - 6 h of antigen exposure, where they present the antigen to suppressor cells (Fig.2; 24).

Contact sensitivity response of mice injected with spleen or lymph node cells prepared 12 hrs following sensitization through DMBA or solvent treated skin

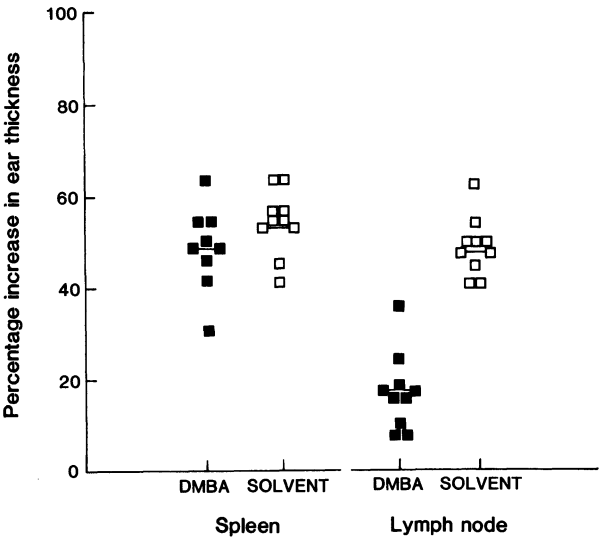

Fig. 2. Mice treated with DMBA or solvent were sensitized by topical application of antigen applied to the treated skin; 12h following sensitization the draining lymph nodes or spleens were removed, cell suspensions prepared and the lymph node or spleen cells injected intravenously into separate syngeneic host mice. After 2 weeks the hosts were sensitized by topical application of the antigen and the ability of the hosts to respond to this sensitization was assessed by ear challenge 5 days later with measurement of ear thickness following 24h after challenge (see reference 24 for details).

Recently, an Ia+ I-J restricted cell which presents antigen to suppressor cells has been identified in murine epidermis by Granstein et al (25). The demonstration of this cell indicates that suppressor cell activation is not simply due to the absence of LC, but requires antigen presentation by a distinct epidermal antigen-presenting cell. It has been confirmed that LC and these I-J restricted APC are distinct cell populations by showing that I-J depleted EC can activate a delayed-type hypersensitivity response (26). These Ia+ I-J restricted cells are also of high density, are Thy-1-negative, and adhere to glass (27).

The EC which presents antigen to suppressor cells in the local lymph node in our experiments were found to be Ia+ dendritic cells which remain in the epidermis following DMBA treatment. These EC did not express the Thy-1 glycoprotein and thus were not Thy-1+ cells induced to express Ia. By using congenic mouse strains which differ only at the I-J genetic loci (Bl0 A[3R] and Bl0 A[5R]), we found that, unlike LC, the Ia+ EC which activates suppressor cells are genetically restricted by the I-J locus, thus resembling the I-J restricted epidermal APC described by Granstein et al (28). This suggests that murine epidermis contains at least two different populations of epidermal antigen-presenting cells, LC - which are Ia+, I-A restricted dendritic cells - and Ia+, I-J restricted APC. Both of these cell types take up antigen in the epidermis and migrate to the local lymph nodes within 3-6 h following contact with antigen.

In the lymph nodes, the LC present antigen in a way that activates effector lymphocytes whereas the I-J restricted cell presents antigen by a procedure which activates suppressor cells. If both of these APC arrive at the lymph node at appropriate densities, then a co-ordinated, controlled immune response will be induced which can be switched off by the suppressor cells. However, in the absence of LC arriving at the lymph node, only the I-J restricted cells will present antigen and thus the suppressor cells will predominate, with tolerance resulting (28). Hence, DMBA may disrupt the local skin immune system by altering the distribution of different populations of local antigen-presenting cells.

We have also demonstrated using skin grafting experiments that chemical carcinogens alter epidermal antigen-presenting cell function. Depletion of local LC by treatment with DMBA or tumour promotors but not initiators enabled the treated skin to survive longer than solvent-treated skin when grafted onto histo-incompatible host mice (16,19). This enhanced survival of carcinogen-treated skin was due to disruptions in local Ia+ antigen-presenting cells and was demonstrated by using congenic mouse strains which differ only at the class I or II MHC loci. Skin from Bl0 A(2R) mice treated with DMBA survived for the same length of time as skin from solvent-treated Bl0.A(2R) mice when grafted onto class I MHC disparate Bl0.A mice, but survived indefinitely when grafted onto class II MHC disparate

B10.A(4R) mice (16). Hence, the enhanced skin graft survival was due to the carcinogens reducing Class II MHC (Ia) expression in the epidermis. As this is limited to the local dendritic APC, these experiments provide further evidence that carcinogens alter the local APC.

Examination of the effects of other carcinogens on local antigen-presenting cells showed that in contrast to DMBA and tumour promotors, the tobacco-derived carcinogens benzo(a)pyrene (BP) and catechol both increased the density of local Ia-positive dendritic cells (5). However, the morphology of these cells was abnormal; they were smaller with blunted dendrites. At this stage it is not known whether the increased Ia+ cells are I-A-restricted LC or I-J-restricted APC which activate suppressor cells. However, DNFB applied to BP-treated skin induced a lower contact sensitivity response than that which occurred in solvent-treated mice (5). Thus, it appears that in addition to LC depletion alterations in the number and morphology of local antigen-presenting cells can also suppress the induction of skin immunity. The simplest explanation for this is again the presence of several functionally distinct populations of local Ia+ APC, with the appropriate ratio of these cells being required for the induction of an effective immune response.

An effective means of protecting against skin cancer may be to protect these local antigen-presenting cells during the early stages of carcinogenesis, as this may allow the induction of effective skin immune responses. We have investigated the effects of two different therapeutic reagents on local epidermal APC during carcinogenesis, retinoic acid and indomethacin. All trans retinoic acid (RA) applied topically to murine skin prior to TPA treatment prevented TPA from depleting the Ia+ dendritic cells from the epidermis. Additionally, in the presence of RA, TPA was unable to alter the skin immune system, so that a normal contact sensitivity response was induced when DNFB was applied to skin treated with both TPA and RA (29). Retinoids reduce tumour growth (30) and incidence in some instances, (31,32) and appear to be useful for inhibition of the promotor phase of carcinogenesis (33,34). This anti-carcinogenic activity of RA may be due to a protection of LC during the promotor phase of carcinogenesis. This could enable the induction of an immune

response, capable of destroying the tumour or at least inhibiting its development, rather than the generation of tolerance against developing tumours (29).

Indomethacin, a prostaglandin (PG) synthetase inhibitor was found to inhibit BP from increasing the density of Ia+ epidermal dendritic cells but did not curtail DMBA from reducing the number of these cells in the epidermis. In these experiments BP, but not DMBA, increased the level of PGE_2 compared with untreated skin. Indomethacin reduced this BP-induced PGE_2 increase indicating that increased levels of PG may be responsible for the elevated number of Ia+ dendritic cells in BP treated epidermis, but not the decreased level in DMBA treated epidermis. Thus, it appears that reagents which protect Ia+ dendritic cells during carcinogenesis may not be effective in all forms of carcinogenesis (35).

CHANGES IN THE SKIN IMMUNE SYSTEM DURING THE EARLY STAGES OF ULTRAVIOLET LIGHT INDUCED CARCINOGENESIS

Short wave length ultraviolet light (UVB; 290-320 nm) found in sunlight readily induces tumours in mice, and is thought to be the prime aetiological agent responsible for non-melanocytic skin cancer in humans (36,37). In mice, UVB-induced skin cancers have been demonstrated to be very antigenic as they are immunologically rejected when transplanted into normal syngeneic animals, but grow when transplanted into UVB-irradiated or immunocompromised hosts, due to the activation of T-suppressor lymphocytes (38,39,40). Thus, UVB, like chemical carcinogens, not only initiates tumour induction, but also immuno-compromises the host in such a way that immunological destruction of the tumour is prevented, allowing tumour development. The effects of UVB on the skin immune system have recently been reviewed by Cruz and Bergstresser (41) and will only be dealt with briefly here.

Irradiation of mice with UVB has been shown to reduce the level of detectable LC in local irradiated skin (42). Low dose UVB radiation may modulate cell surface antigens so that they are no longer detectable within the epidermis, although this may also alter their function (43,44,45). Higher doses of UVB have been reported to deplete LC from the epidermis (46). However, it remains

controversial whether different doses of UVB deplete LC from the epidermis or alters their cell surface properties.

Low dose UVB irradiation, as well as depleting the density of LC, inhibited mice from becoming sensitized to the contact sensitizer DNFB applied to irradiated skin, but both LC and the ability to induce contact sensitivity had recovered by two weeks following irradiation (42,47). Activation of transferrable suppressor cells for the induction phase of contact hypersensitivity was demonstrated in this model (48).

More direct evidence that the inability to induce contact sensitivity through UVB-irradiated skin was due to an effect of UVB on LC has come from studies using hapten-conjugated epidermal cells injected subcutaneously into syngeneic mice. Sauder et al demonstrated that whereas hapten-conjugated epidermal cells presented antigen in such a way that contact sensitivity was induced, UVB-irradiated hapten-conjugated EC induced unresponsiveness (49). Using a fluorescence-activated cell sorter to fractionate epidermal cell populations, Cruz and co-workers purified Ia+ dendritic epidermal cells which were then UVB irradiated, conjugated with hapten and injected intravenously into syngeneic mice. These cells induced unresponsiveness, as the mice not only failed to develop a contact sensitivity response upon ear challenge, but also did not respond to a subsequent sensitization on their abdomen (50).

Unlike LC, the I-J restricted Ia+ epidermal APC is resistant to the effects of ultraviolet light (25). This suggests that both chemical carcinogens and UVB have similar effects on local epidermal APC; LC are sensitive to both, whereas the I-J restricted APC are resistant to carcinogens and UVB. This similarity between these two methods of skin cancer induction suggests that depletion of LC, and hence the ability to induce an effector immune response, with retention of the I-J restricted APC and thus the ability to activate suppressor cells may be an important factor in the early stages of carcinogenesis.

Low dose UVB alteration of the distribution of Ia+ dendritic epidermal cells has been convincingly demonstrated to be responsible for the subsequent induction of tolerance, rather than sensitivity to contact sensitizers. While it is likely that this is related to

suppressor cell activation during the development of UVB-induced cancers, this has not yet been proven. Recently, Alcalay, et al have shown that Ia+ dendritic cells are depleted from murine epidermis exposed to carcinogenic doses of UVB, but they reappear late in the latency period prior to tumour appearance (51). These LC which appeared in the epidermis prior to tumour formation were of altered morphology, and their functional activity is unknown; however, this could further indicate that any role of LC in tumour development might be very early in the latency period before tumours appear.

When mice exposed to higher doses of UVB are painted with contact sensitizer on unirradiated skin contra-lateral to the irradiated site, specific suppressor cells are also activated (52). It has been proposed that this systemic immunosuppression is mediated by a photoreceptor in the outer layers of the skin, trans urocanic acid, which is converted to the immunosuppressive cis isomer upon irradiation (53). While both chemical carcinogens and UVB irradiation induce local unresponsiveness upon antigen exposure, chemical carcinogens do not induce systemic unresponsiveness (22). Like chemical carcinogens UVB irradiation only induces tumour formation at the irradiated, not the unirradiated site. What role UVB induced systemic immunosuppression has on carcinogenesis at other sites requires investigation.

PERSPECTIVE

While there is considerable evidence that LC are altered during the early development of cutaneous tumours, their role in tumour regression is still unclear. Our own experimental studies on DMBA-induced murine tumours suggested that tumour regression was accompanied by the repopulation of the skin by LC (15). The experiments reviewed in this Chapter indicate that during the early stages of carcinogenesis both induced by chemical carcinogens and UVB irradiation, there is alteration in LC leading to the induction of immunosuppressor mechanisms which would favour the emergence of malignant cells.

There is now ample evidence that tumours possess tumour-associated antigens (1) and hence should generate specific anti-tumour immune mechanisms. However, at present there is

little precise data on these antigens in cutaneous tumours. Whether LC are able to present tumour-associated antigens to the immune system requires study. LC are able to present complex cellular antigens such as red cells to the immune system to induce an immune response (54) so it is likely they will also present tumour associated antigens. Critical investigations are needed in this area linked to the functional ability of LC to generate immune responses to particular tumours.

While it is evident that LC are present in cutaneous tumours, what their precise role in the biology and regression of these lesions is unclear. Future developments in this field will come from precise characterisation of the antigens associated with cutaneous skin tumours and by experiments on the functional capacity of LC recovered from neoplastic lesions.

The fact that chemical carcinogens and ultraviolet light both induce skin tumours and alter the local immune system so that there is impairment of LC but not I-J restricted cells leading to suppressor cell activation, suggest that these changes are important in the ultimate biology of tumours. However, to date these detailed analyses have been confined to murine systems. Whether they apply to the induction of tumours in human skin is unknown. Likewise studies of similar changes in local immunity at other body sites of tumour development is another area requiring investigation.

The central theme of this volume is viruses. Our own investigations have shown that papillomavirus will decrease LC in the cervix (6). Studies are now required on the functional effect of LC depletion from the cervix linked to changes in local immunosurveillance mechanisms.

Finally, while it is evident that LC are present in cutaneous tumours, the mechanism whereby they migrate into these sites is unknown. Further understanding of the factors controlling LC migration into the skin will partly clarify this question.

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6

EPIDERMAL DENDRITIC CELLS IN AGED C57BL/6 J MICE

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ABSTRACT

Aging is known to affect the immune function and structural integrity of the skin. The density and function of epidermal dendritic cell (DC) populations was investigated in old mice. Langerhans cell (LC) and Thy-1^+ dendritic epidermal cell (Thy-1^+DEC) density was found to decrease with age. This decrease correlated with an impairment in epidermal cell function as assessed in vitro by the skin-lymphocyte reaction assay and by measuring the ability of epidermal cell suspensions to stimulate the proliferation of sensitized T cells in the presence of the sensitizing antigen. However, the ability of LC to transport antigens from the skin to the draining lymph nodes was found in vivo to be normal in old mice as compared with young mice. Normal numbers of interdigitating dendritic cells (IDC) were recovered from the lymph nodes of old mice. Bone marrow from old and young donors was transplanted into young irradiated recipients. Results from these experiments indicated that low LC density in the skin of old mice probably results from impaired capacity of LC progenitor cells in the bone marrow of old mice to home into and repopulate the epidermis. Studies with herpes simplex virus type 1 (HSV-1) showed that old mice were markedly more susceptible to HSV-1 infection by the intraperitoneal or footpad routes of inoculation as compared with young mice. Systemic administration of thymosin $\alpha 1$ to old mice partially protected them from a lethal challenge with the virus. The higher suceptibility of old mice to HSV-1 infection was correlated with an impaired capacity to mobilize immune cells in the draining lymph nodes and with the absence, in old mice, of an increase in LC numbers following infection, which is usually seen in young mice,

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infected with HSV-1. In contrast, a normal increase in the numbers of IDC in the draining lymph nodes was observed in old mice. Systemic administration of thymosin α1 to old mice partially restores their ability to mobilize immune cells in the draining lymph nodes.

INTRODUCTION

Senescence is known to lead to severe changes in the structure of the dermis and epidermis structure (1). Such changes include thinning of the epidermis, cytoheterogeneity, decrease in the barrier function of the stratum corneum, flattening of the dermoepidermal junction, decrease in proliferative capacity of keratinocytes and their response to growth factors, decrease in numbers and impairment in function of other cellular components of the epidermis, including melanocytes and LC (1-4).

As briefly mentioned above, aging is also associated with changes in various immunological parameters (reviewed in refs. 5,6) including delayed allograft rejection, reduced proliferative response of lymphocytes to mitogens, depressed humoral immunity, diminished responsiveness in the mixed-lymphocyte reaction, lowered interleukin-2 (IL-2) and IL-2 receptor synthesis and reduced production and response to IL-1 (7-14). In contrast to specific immune responses, non-specific immune responses (natural immunity) are generally normal or elevated in old individuals as compared with young ones. Natural killer cell activity augments with age (15). Contradictory results (reviewed in ref. 16) on the effects of aging on antigen-presenting cell (APC) function have been reported including impaired function of adherent spleen cells in supporting IL-2 production, impaired ability of macrophages to process antigens, increased phagocytic activity of macrophages, increased activity of macrophage lysozomal enzymes and intact function of spleen DC (12,16-20).

The etiology of the various changes observed in the activity of the immune system with age is not well-defined. Decline in immunological functions with age might be related to intrinsic degenerative changes in bone marrow stem cells (21) since old recipients are immunologically rejuvenated when given young marrow and an infant thymus (22). However when young and old mice are parabiosed, the immune responses of the young mice decrease, whereas the immune function of old mice is not affected (23-24). These experiments indicate the presence in the blood of old mice of suppressive bone marrow-derived factors that

would account for the immunological deficiencies known to occur in aged mice (24-25). In the same studies, old marrow stem cells were shown to function equally well as young marrow stem cells in reconstituting hemopoietic functions in lethally irradiated recipients (24).

Different authors demonstrated the importance of microenvironmental factors in the thymus and bone marow in determining the capacity of progenitors to develop into potent immunocytes (26-27). In addition to changes at the level of bone marrow progenitors and their microenvironment, other factors that contribute to immunological changes with age include: impaired cytokine and thymic hormone production (reviewed in ref. 28) and abnormalities in lymphocyte functions (29), like alterations in the recognition repertoire expressed by suppressor cells (30) and production of autoantibodies (31).

Since aging affects both the skin and the immune system, we investigated the effect of aging on the epidermal local immune system which, as reviewed in Chapter 1, mainly consists of LC and Thy-1$^+$ DEC (32-34). In addition we examined the effect of aging on the immune response to skin infection with herpes simplex virus type 1 (HSV-1).

MATERIALS AND METHODS

Animals

Old (>10 months) C57BL/6J and (CBA X B6)F1 mice were obtained from the aging colonies maintained by Dr. D. Harrison at the Jackson Laboratory. C3H/He and C57BL/6 mice were obtained from the Jackson Laboratory or from the Hebrew University.

Virus

The HSV-1 Justin strain was obtained from Dr. H. Locker, Dept. of Molecular Genetics, Hebrew University. Virus was propagated in BSC-1 cell cultures grown in Dulbecco Modified Eagle's Medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 10% calf serum (CS) (Beith Haemek) and antibiotics [400 units/ml stretomycin (GIBCO), 160 μg/ml penicillin (GIBCO)].

Cytokines, antisera, monoclonal antibodies and hybridomas

The following monoclonal antibodies were used: anti-Ia^b (IgM, dilution:1/200, clone 25.516); anti-Ia^k (IgM, dilution 1/2, clone MCA 179); anti-Thy.1.2. (IgM, dilution:1/50, clone HO-13-4); NLDC-145 (IgG2a,

dilution 1/20, obtained from Dr. G. Kraal, Free University of Amsterdam, The Netherlands).

The following antisera were used: affinity-purified (AP) fluorescein isothiocyanate (FITC)-conjugated Fab'2 goat anti-mouse IgM (dilution 1/50, Southern Biotechnology Associates Inc. (SBA); AP FITC-conjugated goat anti-rat Ig (dilution 1/100, SBA); AP FITC-conjugated goat anti-mouse Ig (dilution 1/50, Sigma).

The NLDC-145 hybridoma (34a) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Beit Haemek, Israel), 2 mM glutamine and antibiotics.

Thymosin α1 (Tα1) was obtained from Dr. A.L. Goldstein (George Washington University, Washington DC) and dissolved in saline prior to use.

Animal infection

Mice were anesthesized with ether and then injected intradermally (ID) in the abdominal and upper chest skin or through the footpad (FP) with 0.05 ml of virus using a 25 gauge hypodermic needle. Mice were infected intraperitoneally (IP) by injecting them with 0.05-0.1 ml of virus using a 25 gauge needle.

Animal sensitization to rhodamine or fluorescein

In order to sensitize the mice to rhodamine B isothiocyanate (RITC) or challenge them with RITC, they were painted on their shaved abdomen or thorax (sensitization) or ears (challenge) with 5 mg of RITC (Sigma) dissolved prior to experiment in 0.4 ml of acetone:dibutyl phthalate (50/50, v/v) (35). Increase in ear thickness was quantitated with a micrometer (Ozaki Engineering) 24 hours after challenge.

Epidermis separation

Skin samples were obtained from the footpad of the mice. Skin was excised and incubated for two hours at 37°C and 5% CO_2 in an incubator in a 8.2 g/L solution of $Na_4EDTA{\cdot}2H_20$ in a buffer containing 116 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4 and 1.4 mM KH_2PO_4 (pH 7.3, adjusted prior to experiment with HCl 0.1 M). The epidermis was then mechanically separated from the dermis (36).

ATPase staining and immunochemistry

Epidermal sheets were washed three times in Tris maleate buffer (containing 6.85% sucrose) at 4°C for 20 min and then fixed for 20 min at 4°C in 4% cacodylate-buffered formaldehyde. The sheets were then washed

three times in Tris maleate buffer (6.85% sucrose) for 30 min at 4°C and stained with a solution containing 10 mg of ATP, 3 ml of 2% $PbNO_4$, 5 ml of 5% $MgSO_4$ and 92 ml of Tris maleate buffer (8.53% sucrose) at 37°C for 20 min. The preparations were then washed again three times in Tris maleate buffer at 23°C and put into a 1% solution of ammonium sulfide for 5 min. They were finally washed twice with distilled water, mounted in a 9:1 solution of glycerol/PBS and counted with the aid of an eyegrid piece (0.046 mm^2 = one field, 20 fields chosen at random counted for each specimens) (37).

For immunochemistry, epidermal sheets were fixed for 20 min at 23° in acetone. Specimens were washed three times in PBS containing 5% fetal calf serum (FCS) for 30 min at 4°C before addition of the appropriate monoclonal antibody in a total volume of 0.2 ml. The specimens were then incubated at 4°C for 16 hours and washed three times at 23°C for two hours. The sheets were then incubated for 100 min in the appropriate secondary FITC-conjugated antiserum. Controls consisted of specimens incubated with an irrelevant primary antibody or medium followed by incubation with the secondary labeled antiserum. The specimens were finally washed three times on a rocker platform for two hours at 23°C, mounted in a 9:1 solution of glycerol/PBS and examined with an Orthoplan fluorescence microscope equipped for epi-illumination (E. Leitz, Inc., FRG). Labeled cells were counted with the aid of an eyegrid piece (for Ia and NLDC-145 staining, 0.046 mm^2 = one field, for Thy.1 staining, 0.75 mm^2 = one field, 10-20 fields chosen at random counted for each specimen) (38).

Preparation of cells

Lymph node cells. For obtaining lymph node cell suspensions, mice were killed by cervical dislocation, their lymph nodes removed aseptically and forced through a sterile nylon mesh. The cell suspensions were filtered and washed twice. Cell viability was assessed by trypan blue exclusion and shown to be always greater than 95%.

Lymph node interdigitating cells. In order to increase the IDC in lymph node cell populations, the method described by Knight et al. (39) was used. Cells were resuspended in RPMI containing 10% FCS, 10^{-5}M 2-β-mercaptoethanol (Sigma) and 100 μg/ml of gentamycin at a concentration of 5 X 10^6 cells/ml. Eight ml of the cell suspension were layered onto 2 ml of a solution of 14.5% metrizamide (Nygaard, analytical grade) in RPMI

1640 medium and centrifuged for 10 min at 600 g. Cells at the interface were recovered and washed twice before subsequent use.

Bone marrow cells. Mice were sacrificed by cervical dislocation and each femur was disarticulated and stripped of muscle. Small needle punctures were made at each end and the marrow cavity was flushed with medium. The marrow was forced through a 19 gauge syringe needle and the cell suspension was filtrated. Bone marrow cells were washed, counted and resuspended in medium at a concentration of 25 X 10^6 cells/ml.

Bone marrow transplants

Recipients were administered total body irradiation from a ^{137}Cs source (Shepard Mark I) at a rate of 240 rads per min to a total dose of 1000 rads per mouse. Following irradiation, they were injected with 5 X 10^6 bone marrow cells in the lateral tail vein.

Immunofluorescence on cell suspensions

Cell suspensions were incubated for 30 min on ice with the appropriate dilution of monoclonal antibody or antiserum in PBS supplemented with 5% FCS and 0.01% sodium azide at 10^6 cells/100 µl. The cells were washed twice and resuspended at the same concentration in the appropriate dilution of the secondary labeled immunoreactant for 30 min on ice. The cells were finally washed and counted by using an Orthoplan fluorescence microscope equipped for epi-illumination (E. Leitz, Inc., FRG).

Statistics

Experimental group mean and standard error from the mean (SEM) were calculated for each experiment. Statistical significance was evaluated using the Student's *T*- test or the χ^2 test. Differences between values were considered statistically significant for $p < 0.05$.

RESULTS

Dendritic cell density in the epidermis of old mice

LC density in the epidermis of old mice was determined by immunofluorescence microscopy with the NLDC-145 and anti-Ia^b monoclonal antibodies. In addition ATPase staining was used to confirm the results obtained by immunochemistry. Mice were considered old at the age of 20-22 months according to the accepted definition of aging in mice (40).

A progressive decrease with age in LC density in C57BL/6J mice as assessed by the three staining procedures was observed. LC density in one

month-old mice was approximately 1300 cells/mm^2 whereas 20 month-old mice were found to possess less than 500 cells/mm^2 as assessed by staining with the anti-Ia antibody ($p<0.001$) (41).

Decrease in NLDC-145$^+$ cell density was found to be less marked but nevertheless significant: epidermis from one month-old mice was found to contain 1113 cells/mm^2 whereas epidermis from 22 month-old mice contained 733 cells/mm^2 ($p<0.008$) (41).

Staining of epidermal sheets for ATPase activity yielded similar results: at the age of 1 month, C57BL/6J possessed 1190 ± 59 cells/mm^2, at the age of 4 months, this number dropped to 765 ± 65 cells/mm^2. By the age of 20 months, only 406 ± 48 cells/mm^2 were found in the epidermis of C57BL/6J mice ($p < 0.001$).

The Thy-1$^+$DEC density in the skin of old mice was assessed by staining epidermal sheets with a monoclonal anti-Thy-1.2 antibody. A progressive decrease in Thy-1$^+$DEC density was found to develop with age in the epidermis of C57BL/6J mice. At the age of one month, epidermis from C57BL/6J mice contained approximately 100 Thy-1$^+$ DEC/mm^2 whereas at the age of 11 months, this density dropped to half this number. At the age of 22 months, a three-fold decrease in Thy-1$^+$ DEC density was found in comparison with one month-old young mice (41).

The low density of LC found in the epidermis of old mice was associated with a decrease in the capacity of epidermal cells to function as accessory cells in the skin lymphocyte reaction and in an antigen-presentation assay (41).

NLDC-145$^+$ cell isolation from the lymph nodes of old mice

Four week- and 20 month-old C57BL/6 mice were sensitized with RITC as previously described by Macatonia et al. (35). Twenty-four hours later, their draining lymph nodes were removed and DC were isolated on metrizamide gradients. Subsequently, the cells were stained with the NLDC-145 monoclonal antibody which was revealed with FITC-conjugated goat anti-rat antiserum (41).

Old mice-derived LC did not differ from young mice-derived LC in their ability to transport the antigen from the periphery to the local draining lymph nodes as most of NLDC-145$^+$ cells recovered from the metrizamide gradient were observed to contain rhodamine when examined by immunofluorescence microscopy (41). In addition, a similar

number of low density and NLDC-145$^+$ cells was recovered from the lymph nodes of old mice as compared with young mice (41).

In order to assess their functional capacity, lymph node cells were obtained from the lymph nodes of RITC-sensitized 20-month-old and 6-week-old C57BL/6 mice. It has been previously shown that the ability of these lymph node cells to transfer sensitivity to RITC entirely resides within the IDC fraction (35,42). According to the method previously described (43), 2 X 10^6 lymph node cells from old and young sensitized mice were injected into the footpads of 6 week-old +/+ C57BL/6 recipients. A group of non-injected mice served as a control. One week later, the recipient mice were challenged with RITC in the ear, and 24 hours later increase in ear thickness was measured. Similar responses were obtained from recipients injected with lymph node cells derived from old and young mice (Fig. 1).

Effect of bone marrow transfer from old mice to young recipients on Langerhans cell density in the epidermis

The decrease in LC and Thy-1$^+$ DEC densities in the epidermis of old C57BL/6J mice could be related to changes in bone marrow-derived progenitors or, alternatively, could be due to extrinsic (microenvironmental) causes. In order to distinguish between these two possibilites, the following experiments were performed.

Two groups of two-month-old C57BL/6J mice were lethally irradiated and immediately following irradiation injected with bone marrow derived from two-month- or 20-month-old C57BL/6J mice. Twelve and 16 weeks following irradiation, the recipients of young bone marrow had significantly elevated LC numbers (as assessed by staining the epidermal sheets with an anti-Ia^b antibody) compared with recipients of old bone marrow (41).

This decrease could have resulted from a lower rate of disappearance of host LC or a more rapid rate of homing of donor LC in the epidermis of recipients of young bone marrow. In order to distinguish between these two possibilities, one month-old C57BL/6J were lethally irradiated and immediately following irradiation injected with bone marrow derived from two-month- or 24-month-old (CBA X C57BL/6J)F1 mice. Six and 10 weeks after irradiation, there was no significant difference in the Ia^{b+} cell density between recipients of old and young bone marrow. However at 10

weeks post-transplantation donor-derived Ia^{k+}-cell density was observed to be lower in the recipients of old marrow (41). At 14 weeks after irradiation, recipients of young marrow showed significantly higher numbers of Ia^{b+} cells and donor-derived Ia^{k+} cells as compared with recipients of old marrow (41).

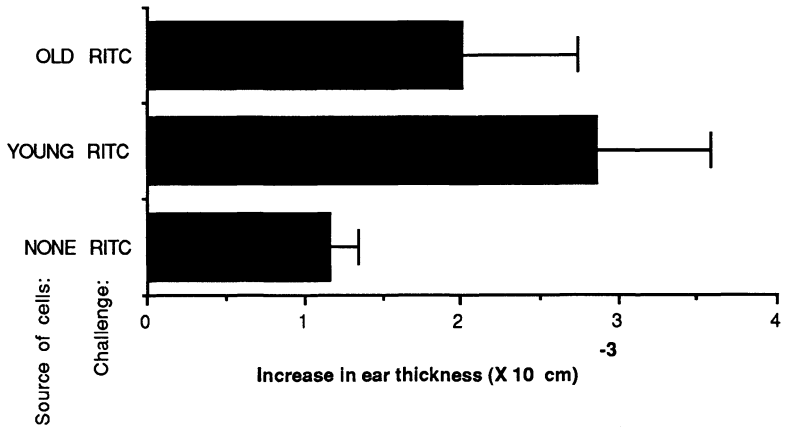

Fig. 1. Delayed-type hypersensitivity reaction in recipients of lymph node cells derived from old mice. Six-week-old (YOUNG) and 20- month-old (OLD) C57BL/6 mice were painted with RITC. Twenty four hours later, lymph node cells were harvested and 10^6 cells were injected into the footpads of 6-week-old +/+ C57BL/6J mice. Control mice did not receive cells. All mice were challenged 6 days later with RITC and ear swelling was measured as the mean increase in ear thickness 24 hours after challenge in each group of 2-4 mice. Results are expressed in 10^{-3} cm $\pm$ SEM.

Role of Langerhans cells in HSV-1 infection of the skin in old mice

Mice at different ages were infected IP with the Justin strain of HSV-1 at 10^6 pfu/mouse and survival was recorded. Fig. shows that at one week of age, C57BL/6 mice were highly suceptible to infection with the Justin strain of HSV-1 (survival: 0%). They became fully resistant to the virus by four weeks of age and were still totally resistant (survival: 100%) to a challenge with the virus at 12 and 15 months of age. By 22 months of age, the mice were found to be more susceptible to IP infection as compared

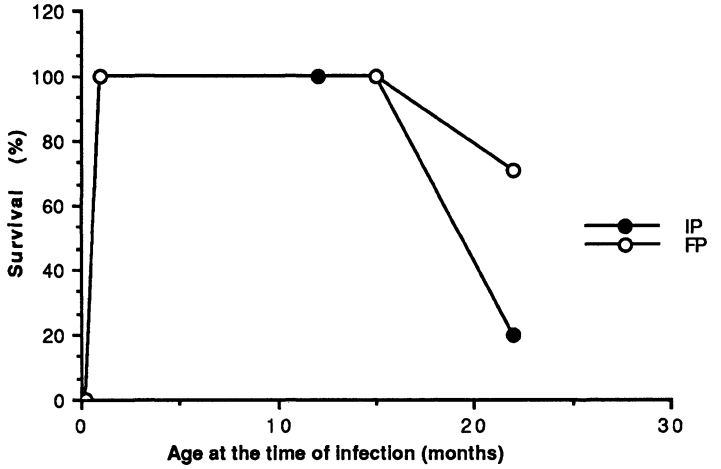

Fig. 2. Survival of C57BL/6 mice as a function of age at the time of infection. C57BL/6 mice were infected IP or into the FP at different ages with 5 X 10^5 pfu/mouse of the Justin strain of HSV-1 and survival was recorded for three weeks. Results are expressed as the percentage of surviving mice from the total number of infected mice.

with four-week-old mice (survival: 20%) as previously reported in an intravaginal model of HSV-1 infection (44).

Mice were injected into the FP with 5 X 10^5 pfu/mouse of the HSV-1 Justin strain (Fig. 2). One week-old mice were highly susceptible (survival: 0%) while four-week-old mice were fully resistant to the virus (survival: 100%). Old mice were less susceptible to HSV-1 FP infection (survival: 71%) as compared to the IP route.

LC density was shown to significantly increase following HSV-1 FP infection in young normal mice (45). Here we compared the increase in LC density found in young mice to that recorded in old mice following HSV-1 FP infection. Five-month-old and 23-month-old male C57BL/6 mice were infected via the FP with 5 X 10^5 pfu/ml of the Justin strain of HSV-1. One week following inoculation, footpad skin specimens were processed for ATPase. LC response to HSV-1 infection in old mice was deficient as compared with LC response in young adult controls (Table 1).

Table 1. Langerhans cell response to HSV-1 infection in old mice

Age of mice[a]	Infection	ATPase$^+$ cells/mm^2 $\pm$ SEM
5 months	Mock	658 $\pm$ 27
	HSV-1	840 $\pm$ 73 ($p<0.05$)
22 months	Mock	399 $\pm$ 23
	HSV-1	462 $\pm$ 163

[a] C57BL/6 mice were infected into the FP with 5 X 10^5 pfu/mouse of the Justin strain of HSV-1 or an equivalent amount of uninfected BSC-1 cells (mock) and one week following infection, the footpads were excised and stained for ATPase activity. Results are expressed in cells/mm^2 $\pm$ SEM

In an attempt to find some correlation between the decrease in resistance of old mice to infection with HSV-1 in C57BL/6 mice and changes in LC, the following experiment was conducted. Twenty-two-month-old and four-week-old C57BL/6 mice were injected ID with 10^5 pfu/mouse of the Justin strain of HSV-1. The animals were sacrificed at various time intervals post-infection (p.i.) and their axillary, subscapular and inguinal lymph nodes were removed. Cells were obtained and counted. Some of the cells were passed through a metrizamide gradient (39), thus enriching the cell suspension for IDC.

Results shown in Fig. 3A indicate that untreated old mice possess as many lymph node cells as young mice. However, in contrast with young mice, they were unable to mobilize cells into the draining lymph nodes following HSV-1 challenge. Indeed whereas the lymph node cell number in young mice increased more than four times following HSV-1 infection (Fig. 3A), at the same time, there was no statistically significant increase in the number of lymph node cells in old mice infected with HSV-1. A similar increase in IDC in the draining lymph nodes was noticed in young and old mice challenged with HSV-1 (Fig. 3B). All cell suspensions contained between 73 and 81% NLDC-145$^+$ IDC.

Effect of thymosin α1 systemic administration on HSV-1 infection in old mice.

Tα1, a synthetic peptide consisting of 28 amino acid residues, has been shown to enhance production of lymphokines, to modulate IL-2 receptor expression and to control T-cell development (46). In addition, its level

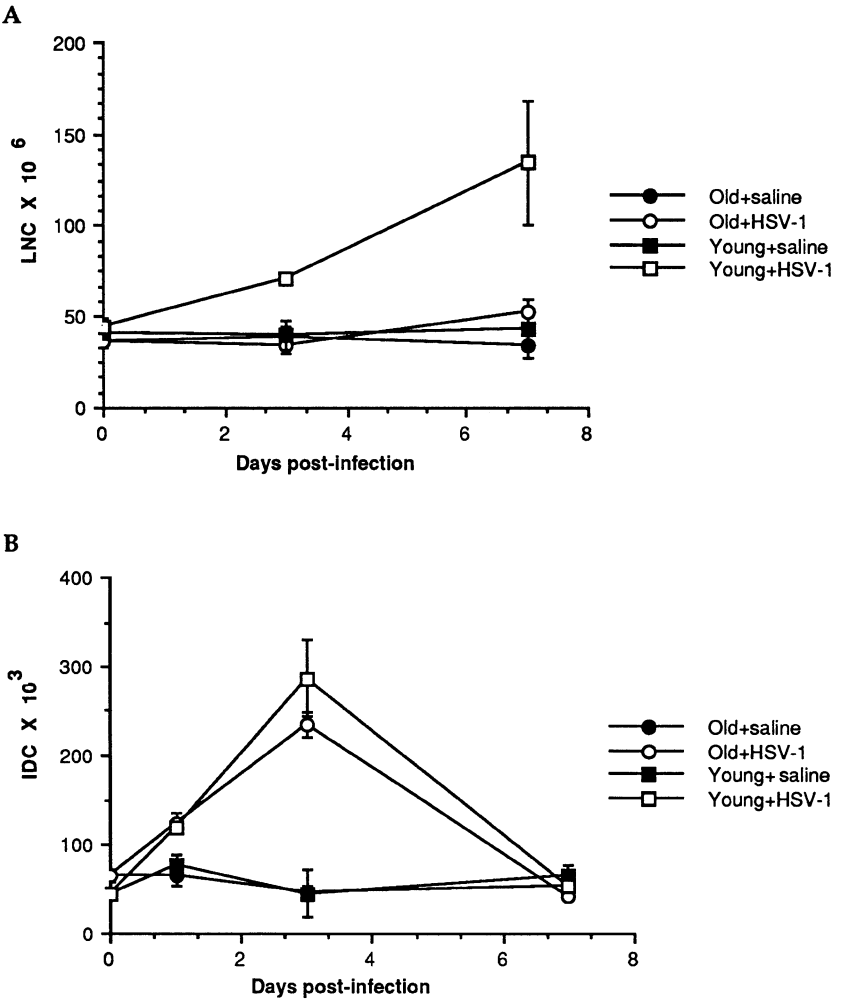

Fig. 3. Lymph node cell and IDC response to HSV-1 infection in old mice. Twenty-two-month-old (old) and 4 week-old (young) C57BL/6 mice were injected ID with 10^5 pfu/mouse of the Justin strain of HSV-1 (HSV-1) or with an equivalent volume of saline. At various times p.i., the animals were sacrificed and the draining lymph nodes were removed. The numbers of lymph node cells (A) and IDC (B) recovered per mouse were determined. From each mouse two inguinal, axillary and subscapular lymph nodes were removed, cells were pooled from three or four animals. Results are expressed in number of cells/mouse $\pm$ SEM as a function of time p.i.

was shown to decrease with age (47) whereas it was able to restore T cell function in aged mice (48) as well as to boost the suppressed antibody titers in elderly volunteers, immunized with tetanus or influenza vaccines, to the normal levels seen in young people (47). Tα1 was shown to protect mice against infection with various pathogens (49).

Tα1 dissolved in saline or saline alone as control was administered at various doses to different groups of mice by intraperitoneal injection. Old mice that were pretreated with Tα1 (Fig. 4) demonstrated significantly increased numbers of lymph node cells following HSV-1 infection as compared to old mice pretreated with saline only.

As shown in Table 2, mice pretreated for five days with saline were more susceptible to IP HSV-1 infection than mice that were pretreated for five days with Tα1. In one experiment, 5 out of 5 22-month-old mice pretreated for five days with Tα1 survived a challenge with 10^6 pfu/mouse of the Justin strain of HSV-1 given into the FP. Four out of 5 mice that were treated with saline and FP infected with HSV-1 Justin strain as a control, survived. The difference was not significant.

DISCUSSION

Relationship between age-associated decrease in Langerhans cell density and the biology of aging

The present results as well as results from other laboratories have shown that LC density decreases with age in man and mice (4,50-53). Since LC are thought to play an important role in the control of infectious and proliferative disorders, a decrease in LC density could contribute to the higher incidence of skin infections and neoplasms that occurs with aging (54,55). In addition, as reviewed in Chapter 1, the epidermis is considered today to play an essential role in the control and initiation of immune-mediated reactions as well as to serve as an important extrathymic site of T-cell differentiation (56,57). It thus seems logical to assume that LC

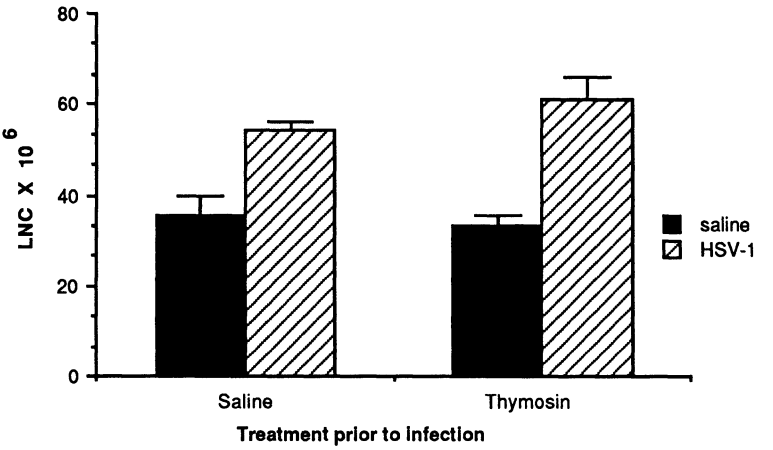

Fig. 4. Thymosin α1 systemic administration effect on lymph node cell response to HSV-1 infection. Twenty-two-month-old C57BL/6 mice were injected IP with 200 μg/kg/day of Tα1 or saline for five days prior to infection and then injected ID with 10^5 pfu/mouse of the Justin strain of HSV-1 or with an equivalent volume of saline. The animals were sacrificed on day 7 p.i. and their axillary, subscapular and inguinal lymph nodes were removed. Lymph node cells were obtained and counted. Results are expressed as the number of cells/mouse ± SEM.

Table 2. Effect of thymosin α1 systemic administration on resistance of C57BL/6 mice to HSV-1 infection

Age[a]	Route	Treatment[a]	Survival[b]
22 months	IP	Tα1 200 μg/kg/day	12/16 (75) §
22 months	IP	Saline	2/18 (11)
22 months	IP	None	2/10 (20)
22 months	FP	Tα1 200 μg/kg/day	5/5 (100)
22 months	FP	Saline	4/5 (80)
4 months	IP	None	20/20 (100)
4 months	FP	None	8/8 (100)

[a] Male 22-month-old and 4-month-old C57BL/6 mice were injected IP or into the FP with 10^6 pfu/mouse of the Justin strain of HSV-1 with or without treatment with Tα1 administered at 200 μg/kg/day for five days prior to infection.

[b] Number of surviving mice three weeks after infection/total number of mice infected (in brackets, % of surviving mice). Mortality of Tα1-treated mice was compared with mortality in untreated or saline treated mice using the χ^2 test. Differences were considered as significant for $p < 0.05$ (§)

deficiency in the skin of old individuals could contribute to abnormalities in T-cell function known to characterize aged animals and humans (5-6).

Modulation of LC activity in the epidermis of old individuals might lead to the correction of impairment in various local and systemic age-associated deficiencies in the immune system. Further studies are needed in order to disclose whether LC activation by a number of immunomodulators, which are known to stimulate LC function and replication (58-62), will lead to a decrease in the susceptibility of old individuals to infection and neoplasia and perhaps to the restoration of certain T lymphocyte functions.

Various reasons could account for the finding that in spite of low LC density in the epidermis of old mice, LC appear to function normally in the transfer of antigen to the draining lymph nodes and normal numbers of LC are recovered from the lymph nodes of old mice. Firstly, it is possible that a compensatory mechanism exists that cause sthe mobilization of a greater number of LC at the site of sensitization than in young mice, thus helping the fewer resident LC to perform the function of the high number of resident LC found in the young mouse epidermis. Such compensatory mechanisms are thought to account for the enhanced activity of macrophages observed in old mice (18,63). Secondly, it is possible that only a few LC are actually transporting the antigen from the skin to the peripheral lymph nodes where they could transfer it to resident NLDC-145$^+$ DC in the lymph nodes. Age might not affect the number and/or function of these resident cells. Such a discrepancy between the effect of age on cells of the same lineage depending on their location has been reported for humoral responses and T cell responses to mitogens and alloantigens (64-65). However it is difficult to reconcile this possibility with the actual timing of antigen transfer to the draining lymph nodes (42).

Finally, it is possible that the observed decrease in LC density and impaired epidermal cell function are due to reduced expression of LC membranal markers. It is likely that the lack of Ia expression does not interfere with the uptake of antigens (66). LC may acquire cell surface Ia and other functional markers as they migrate to the lymph nodes as has been demonstrated in vitro (67) and is thought to occur in vivo (42). Such decreased Ia expression in aged BALB/c mice is thought to be due to impaired cytokine regulation (52).

Investigation into the cause of decreased Langerhans cell density in the skin of old mice

In order to investigate the cause(s) for LC low density in the epidermis of old mice, we examined LC density in the epidermis of young recipients injected with old and young bone marrow. We choose this system, since the results obtained by injecting old recipients with young marrow cells might be influenced by microenvironmental defects in the skin (1) and bone marrow (27) of old animals. Our results show that the deficiency in LC in the skin of old mice was not due to microenvironmental lesions. This deficiency could have resulted from a lower rate of disappearance of host LC or a more rapid rate of homing of donor LC in the epidermis of recipients of young bone marrow. In order to distinguish between these two possibilities, the experiment was repeated using (CBA X C57BL/6J)F1 mice as bone marrow donors. This method allowed us to distinguish between host-derived and donor-derived LC. Results indicated that most of the change in LC density due to aging resulted in fact from a decreased capacity of old marrow-derived LC progenitors to home into the epidermis.

This decreased capacity could reflect two different deficiencies, associated with aging. The decreased capacity of stem cells to populate the epidermis of the recipient mice was shown to result from an intrinsic impairment in stem cell function (reviewed in ref. 68). However, more recent results suggest that the poor function of old stem cells is due to the deleterious influence of old microenvironment rather than being intrinsic to the stem cells (26-27). Indeed, in striking contrast with previous results (68), Astle and Harrison demonstrated that normal function of stem cells is retained upon sequential transfer for at least 15-50 life spans of a normal mouse, i.e. for more than a century (24). In addition, old bone marrow stem cells were found to have normal ability to generate red blood cells and lymphocytes in young recipients (69). These contradictory results could be explained by the fact that earlier studies used mice that were affected by diseases or other stressful conditions (24,69-70).

The decreased function of old marrow might have been due to the presence of high numbers of suppressor cells in the peripheral blood of old mice (25,29) since parabiosis of young and old mice induces a decrease in the immune functions of young mice (23-24). However, recent results (41) indicate that when young and old mice are parabiosed for a period of 4

months, the old partners show numbers of LC typical of young skin. Thus, loss of LC with age seems to be associated with a precursor defect and not with the presence of suppressor functions affecting old marrow ability to generate normal density of LC in the epidermis. Consistent with this possibility, new data (71) indicate that suppressor cells in old mice affect differentially the various components of the immune system.

In conclusion, whether the decrease in LC density is indeed due to impaired function of committed stem cells (as suggested by the preliminary results with parabiosed mice) or to circulating factors influencing the function of these stem cells, it is clear that the low density of LC in the epidermis of old individuals results from deleterious effects of aging at the level of the bone marrow.

Langerhans cell response to HSV-1 infection in old mice

LC in the skin of old mice showed a significantly lower response to HSV infection than LC in the epidermis of young mice (Table 1). This result corroborates recent observations by Gu et al. who showed decreased response of LC in old mice to contact allergens (53). Similarly, lymph node cells appeared in lower numbers in the draining lymph nodes of old mice infected with HSV than in young mice (Fig. 3). In contrast, IDC numbers increased to a similar level in infected old mice as IDC in young infected mice (Fig. 3). Tα1, which is known to lack activity on accessory cells (49) corrected the deficient response of old mice to HSV-1 infection.

Thus, in spite of a deficient epidermal LC response to HSV-1 infection in old mice, it seems that impaired function of accessory cells is not responsible for the higher susceptibility of old mice to the viral infection. These observations are in line with our observations on normal antigen transfer by LC in old mice as compared with young mice (41) and with previous findings which showed that in experimental systems where resistance to infection is solely dependent on accessory cell functions, mortality and morbidity in old infected animals is even reduced when compared with mortality and morbidity in young mice (63).

Taken together, these results suggest that the effector and not LC response is responsible for the higher susceptibility of old mice to infection with HSV-1 in the skin.

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HIV INFECTION OF LANGERHANS CELLS IN AIDS

7

IMMUNOHISTOLOGY OF SKIN FROM AIDS PATIENTS

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ABSTRACT

Autoimmune phenomena and Langerhans cells (LC) have been studied in clinically normal skin from patients with the acquired immunodeficiency syndrome (AIDS) by several investigators using immunohistological (IH) techniques. In addition, IH techniques have been used to compare cytokine expression by skin overlying AIDS—associated Kaposi's sarcoma to adjacent normal skin.

At present there is no evidence that autoantibody- or immune-complex depositions occur in normal skin of AIDS patients. However, epidermal LC are reduced in number and located more superficially in uninvolved skin of AIDS patients compared to healthy controls. The altered distribution and reduced density of LC may impair the functional capacity of these immunological active dendritic cells, and thus have a role in several of the skin manifestations seen in AIDS patients. The finding of

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increased epidermal expression of tumor necrosis factor α (TNFα) and interleukin-6 in AIDS-associated Kaposi's sarcoma indicates that immunoinflammatory mechanisms are involved in this characteristic AIDS manifestation.

INTRODUCTION

The rapidly increasing number and variety of specific mono- and polyclonal antibodies has extended the usefulness of IH techniques in medical science. IH techniques can provide not only structural information, but also insight into physiological mechanisms and pathological conditions. The fact that the skin is easily accessible for biopsy and it is possible to obtain specimens simultaneously from pathological sites and clinically normal areas, makes IH examination of skin a useful instrument in clinical studies. Thus, IH skin examination has been of great clinical value both in the study of dermatological disorders (1), as well as of the auto-immune inflammatory connective diseases (2).

Along with the increasing recognition of dermatological involvement in AIDS (3) several IH studies of the skin in the past 5 years have been carried out on patients with AIDS. The relevance of such studies is further accentuated by the possibility of epidermal LC becoming infected with HIV (4), and with the potential for secondary immune manifestations in the skin of these patients (5).

This chapter will summarize and discuss our experience with IH skin investigations of immune deposits (6), LC (6) and epidermal cytokine expression in HIV-infected patients (7).

MATERIALS AND METHODS

Examination for in vivo deposits of plasma proteins and distribution of Langerhans cells (LC)(6)

Twenty-one male patients with AIDS, 6 male patients with AIDS-related complex (ARC) and 23 healthy age-matched males had a biopsy taken from clinically unaffected skin sites not exposed to the sun. We examined cryosections of each specimen by direct immunofluorescence with labeled antibodies against IgG, IgM, IgA, IgD, IgE, C1q, C3, fibrinogen and albumin, and by indirect immunofluorescence with anti-CD1 (T6) antibody.

Examination for epidermal cytokine expression (7)

We examined skin specimens from 6 male patients with AIDS-associated Kaposi's sarcoma (nodular tumor stage) and 6 age-matched healthy males. In the patients, biopsies were obtained from both tumor and unaffected adjacent skin.

Tumor necrosis factor α (TNFα) was visualized using polyclonal antiserum against human recombinant TNFα. Interleukin-6 (IL-6) was indirectly demonstrated using a polyclonal antiserum against partially purified supernatants of activated human blood monocytes before and after absorption with recombinant human IL-6. Bound antibodies were identified by a biotin-avidin-peroxidase complex technique.

RESULTS

Cutaneous immune deposits (6)

Deposits of immunoglobulins, complement, fibrinogen or albumin in dermal vessel walls, dermo-epidermal junction zone (DEJ) or in epidermis could not be demonstrated in any of the lesion or control specimens. Granular IgM DEJ deposits were found in one patient with ARC.

Cutaneous Langerhans cells (LC)(6)

A semiquantitative evaluation of number and distribution of LC revealed the ratio of the mean number of CD1-positive epidermal LC in patients compared to controls to be 0.77, and that the epidermal LC were more superficially located in the patients. No differences in epidermal LC morphology could be observed. CD1 (T6) positive LC were only shown in the dermis of a few patients and controls.

Epidermal cytokine (IL-6 and TNFα) expression (7)

A granular staining pattern in the intercellular spaces of the upper cellular epidermis was similar with the two anti-cytokine antisera in all specimens from normal skin. Staining was localized either to the membranes of single cells or to groups of cells in the epidermis. In titration experiments, maximum dilutions of anti-cytokine antibodies, giving epidermal cell-membrane staining, were almost equal when unaffected skin from the patients was compared to the healthy controls.

Biopsies from Kaposi's sarcoma consistently revealed a much more intense intercellular staining and a wider epidermal distribution compared to normal skin. The epidermal staining now included the basal cell layers and in all cases further titration (x)(2-3) of anti-cytokine antibody were still capable of staining the tissue. Neither IL-6 or TNFα were identified in or around the endothelial cells of the tumor.

DISCUSSION

Cutaneous immune deposits

Autoimmune phenomena have been suggested to play a role in the pathogenesis of AIDS (5). Our search for cutaneous immune deposits was stimulated by a previously report of in vivo IgM deposits in

epidermal sheets from AIDS patients (8). This, together with evidence for polyclonal B-cell activation in these patients (5), suggested that deposits of immune complexes or antibodies directed against epidermal antigens might be found in the skin. However, failure to demonstrate immunoglobulins or complement in the vertical skin sections indicates that B-cell mediated autoimmune phenomena do not take place in uninvolved skin of AIDS patients. This is in agreement with a recent serologic study (5) which concluded that the immunological dysregulation in AIDS does not result in serological or clinical autoimmunity.

Cutaneous Langerhans cells

Examination of LC in AIDS has been a subject of major interest during recent years, encouraged by the recognition that LC have phenotypic and functional properties in common with mononuclear phagocytes. Furthermore the expression of CD4 molecules on LC have drawn attention to the possibility of LC as targets for HIV infection. Conflicting results have, however, been obtained when monoclonal antibodies to HIV antigens have been used in IH techniques in order to examine the presence of HIV within epidermal LC (9, 10)(discussed in this volume).

LC are important antigen-presenting dendritic cells of bone marrow origin (11). They migrate to the skin and mucosal surfaces where they interact with lymphocytes. Epidermal LC normally comprise 2-3% of the cells within the epidermis, and are mainly located in the suprabasal layers. After trapping external antigens, LC may migrate to regional lymph nodes, or the lymphocyte-LC contact may take place in the skin (12).

Reduced numbers of epidermal LC have been found in some systemic autoimmune diseases (2), and during

contact allergic reactions the number of LC have been found to vary in a time-dependent manner (4). In a comprehensive IH study of 68 biopsy specimens of viral skin and mucosa lesions (warts, condylomas, mollusca contagiosa and herpes simplex) it was found that epidermal LC were reduced in number or absent in almost all viral lesions (13).

Using different staining and counting techniques we (6) and others (8, 14, 15) have found the density of epidermal LC to be reduced in uninvolved skin of patients with AIDS. However, one study (16) comprising only a small number of patients reported a normal number of epidermal LC in AIDS patients. In addition to a reduced density we found the epidermal LC of the AIDS patients located more superficially compared with normal controls (6). It is not known if the depletion of epidermal LC in the various virus diseases, including AIDS, is the result of a direct cytotoxic effect of the virus, an indirect effect via mediators or a migration of antigen-primed LC from the epithelium to the regional lymph nodes. It is also unknown whether the reduced number, and the altered location of epidermal LC, is associated with an impaired functional capacity. It has, however, been hypothesized that the alterations in epidermal LC might explain some of the skin manifestations seen in AIDS patients, e.g. cutaneous anergy to multiple skin test antigens (17) and the increased frequency of dermatitis (3) and malignancies (3). In this context it is of interest that LC have been shown to be absent or greatly reduced in lesions of the AIDS-associated oral hairy leukoplakia (18).

The evidence for suggesting that enumeration of epidermal LC has any prognostic value is conflicting. Thus, Dreno et al. (15) reported a correlation between the number of epidermal LC, CD4-positive

blood lymphocytes and clinical disease stage, while Kanitakis et al. (10) failed to do so. Future IH studies of LC in the various skin lesions of AIDS patients will probably bring insight into the pathogenetic role of LC in this disease.

Epidermal cytokine (IL-6 and TNFα) expression

The last decade has witnessed a remarkable expansion in the understanding of the keratinocyte as a cell which may be actively involved in immunologic reactions. The discovery that not only LC, but also keratinocytes can produce and secrete immunoregulatory glycoproteins (cytokines) is a prime example of this. The cytokines IL-1/epidermal thymocyte activating factor (ETAF), IL-3, IL-6, TNFα and granulocyte-macrophage colony stimulating factor (GM-CSF) can be produced by keratinocytes (19). LC have also been shown to secrete IL-1 (20). By use of an IH technique we have shown that IL-6 and TNFα can be visualized in the epidermis of healthy persons (21) and patients with psoriasis (22). The cytokines are located solely to keratinocyte membranes, and double-labelling experiments have confirmed that the epidermal LC fail to express these mediators (Oxholm A. et al, unpublished data). Subsequently, we addressed the question whether the expression of cell membrane-bound epidermal cytokines are altered in AIDS-associated Kaposi's sarcoma (7).

TNFα has been shown to possess potent anti-tumor activity both in vivo and in vitro (23, 24). It may also reduce cellular susceptibility to infection with HIV (25), and it also appears to enhance antibody-dependent cytotoxity and phagocytosis (26). IL-6 has a broad range of biological activities in common with TNFα (27), and TNFα is moreover a potent inducer of IL-6 (27). The keratinocyte-derived cytokines also appear to be of importance for LC as both IL-1 and

GM-CSF have potent stimulatory effects on LC functions (28). Whether the two keratinocyte-cytokines in our study also affect LC remains to be examined. It is, however, reasonable to suggest that our finding of an increased amount and extension of IL-6 and TNFα in the epidermis overlying Kaposi's sarcoma could implicate that both cytokines are regulators of the HIV infection and tumor growth in AIDS related Kaposi's sarcoma. However, Kaposi's sarcoma consists mainly of endothelial cells, and we were not able to detect TNFα or IL-6 in or around the vascular cells of the tumor. This is interesting because normal umbilical cord endothelial cells produce IL-6 after stimulation with IL-1 or TNFα (29).

The continued use of IH techniques for the demonstration of in situ variations in the expression of epidermal cytokines may enable us to understand more of epidermal cell involvement during the HIV infection.

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8

HIV INFECTION OF SKIN LANGERHANS CELLS

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ABSTRACT

Human Langerhans cells in the skin and mucous membranes may play an important role in the HIV infection. They carry CD4 molecules which serve as a binding site for HIV, and HIV-infected Langerhans cells have been demonstrated in the skin of ARC/AIDS patients.

Using epidermal cell suspensions obtained with a suction blister technique, we could, in a series of studies demonstrate HIV infection of Langerhans cells in vitro. Furthermore we could demonstrate that HIV produced and released by Langerhans cells was able to infect allogeneic and autologous peripheral blood mononuclear cells and Langerhans cells. Examination of Langerhans cells from symptomfree HIV sero-positive individuals demonstrated infection after few days in culture, indicating that they were latently infected.

We raise the hypothesis that the Langerhans cells may be the primary target cell for the sexually transmitted HIV infection.

INTRODUCTION

Cutaneous and mucous membrane manifestations associated with the HIV infection are frequent. It seems that acute HIV infection is associated with a roseola-like

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exanthema together with malaise, fever and enlargened lymphnodes, the rash usually lasting about two weeks (1,2). Later in the course of the infection numerous skin and mucous membrane symptoms may develop, many of which are caused by infectious agents, and which are usually more severe, widespread and longlasting than the same infections in otherwise healthy individuals (3-7). It therefore seems that the skin and the mucous membrane immune systems are also deficient in the later stages of the HIV infection. Intracutaneous tests of the delayed type hypersensitivity reactions with microbial antigens demonstrate reduced or abolished reactions.

LANGERHANS CELL FUNCTIONS

A crucial limb of the immune system of the skin and mucous membranes is the Langerhans cells.

First described by Paul Langerhans in 1868 (8), the Langerhans cells derive from the bone marrow, comprise about 2 - 4% of the epidermal cells and are situated over the basal layer. They have intracytoplasmic granules (9). In various skin diseases both the number and the location within the epidermis may vary.

Their presence have also been observed in normal dermis (10,11), in dermal lymphatic vessels and draining lymphnodes (12) as well as in mucous membranes, including oral mucous membranes (13-16), esophagus and gastric mucosa (17,18) and vaginal and cervical epithelium (19-21).

They carry receptors for FcIgG and C3 and the MHC class II antigens characteristic for classical macrophages (22-26). Furthermore they carry the T-cell markers CD1 and CD4 (27-29).

THE MAKING OF EPIDERMAL CELL SUSPENSION

In order to separate epidermal cells from the skin we use a suction blister technique to form blisters, usually on the lower abdominal wall (30). The roofs of the bli

sters are epidermal sheets which are removed and dissociated with enzyme to a single cell suspension (31). Viability is usually over 90% and 2-4% of the cells are Langerhans cells (31). In some experiments we used Langerhans cells enriched by Hypaque-Ficoll gradient centrifugation.

SEPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

PBMC were separated by the Isopaque-Ficoll method from defibrinated blood using Lymphoprep (Nyco, Oslo, Norway) (32).

CELL CULTURES

The cells were cultured in medium RPMI-1640 with L-glutamine (BIO-Cult, Gibco, Glasgow, Scotland) supplemented with penicillin, streptomycin and 20% autologous or pooled human serum.

LANGERHANS CELL ANTIGEN PRESENTATION

In a series of previously published studies we were able to demonstrate the alloactivating and antigen presenting capacity of the Langerhans cells (31). They present microbial antigens like PPD (31), herpes simplex type 1 live virus or virus antigen (33), Candida antigen (34), trichophytin (35) and antigens from common bacterials like Staphylococcus aureus and Escherichia coli, as well as the contact allergen nickel sulphate (36) to T lymphocytes. The result is an antigen specific T-cell proliferation in previously sensitized individuals. We were also able to demonstrate that the Langerhans cell-dependent antigen activation is restricted by HLA-D/DR molecules (37,38), and that they express more HLA-DR determinants than blood-derived monocytes and dendritic cells (39). For certain antigens they seem to be more potent in inducing T-cell responses than peripheral blood macrophages (40).

IN VITRO INFECTION OF LANGERHANS CELLS WITH HIV

Since the Langerhans cells carry CD4 and are capable of taking up various microbial antigens, and HIV seems to have a special affinity for CD4-positive helper/inducer T-cells (41), mononuclear phagocytes (42,43) and follicular dendritic cells (44); and it is believed that the CD4 molecule serves as a binding site for HIV (45-47), we investigated if human epidermal Langerhans cells can be infected with HIV.

We used human epidermal cell suspensions produced as described above and incubated them with supernatants from the HIV-1 infected cell line H9 for one hour at 37°C. After three washes the cells were cultured in the wells of Linbro plates (Costar) in a humid 5% CO_2 atmosphere at 37°C. Aliquots of cells were then removed from the cultures daily, cytocentrifuged onto slides, and stained with a cocktail of monoclonal antibodies (Du Pont) directed against the HIV-specific proteins p15, p24 and gp120 in the APAAP technique (48).

More than 20 experiments have been performed and they consistently demonstrate HIV-positive Langerhans cells in the epidermal cell suspensions after 4-5 days in culture (49,50). Mock-infected epidermal cells, using heat inactivated virus (56°C for 30 min) and noninfected controls were all negative as was staining with OKT8.

LANGERHANS CELL-PRODUCED HIV INFECT ALLOGENEIC PBMC AND LANGERHANS CELLS

From some of the in vitro-infected epidermal cell cultures we obtained supernatants which were able to infect interleukin-2-treated phytohemagglutinin (PHA)-prestimulated allogeneic peripheral blood mononuclear cells (49,50), as well as allogeneic and autologous epidermal Langerhans cells. The peripheral blood mononuclear cells appeared positive after 11 days in culture, after adding more PHA-prestimulated PBMC and interleukin-2 on day se

ven. Both the allogeneic and the autologous epidermal cells demonstrated positive Langerhans cells after 24 hours in culture, indicating adaption of the virus to the Langerhans cells (51).

LANGERHANS CELLS FROM SYMPTOMFREE HIV ANTIBODY POSITIVES ARE HIV INFECTED

ARC/AIDS patients have decreased numbers of epidermal Langerhans cells (52, 53) and electron microscopical examination have demonstrated HIV-producing Langerhans cells (54,55). In order to investigate if HIV antibody-positive symptomfree individuals have HIV-infected Langerhans cells, we produced epidermal cell suspensions from three such individuals, and cultured them. Langerhans cells from all three turned positive, two after three days in culture and the third after four days (56), and the supernatant from one of these cultures infected interleukin-2-treated PHA-prestimulated allogeneic PBMC. Since the cells were negative the first days in culture it appears that they must have been latently infected, i.e. present as provirus in the cells, and that the culture conditions induced the HIV production.

DISCUSSION

Based on the data outlined above we must conclude that Langerhans cells can be easily infected with HIV in vitro. Few days after the in vitro infection they also produce and release HIV into the medium which is then capable of infecting allogeneic or autologous PBMC and Langerhans cells. Taking into account the distribution of Langerhans cells in epithelia we must assume that they, under natural conditions, are the primary target cells for the HIV infection. Microlesions occurring during sexual acts may be necessary for the infection to occur. Penetration of the virus through an intact stratum cor-

neum of the epidermis is probably unlikely to happen. There are however hairfollicle and sweat gland openings at the surface. Mucous membranes do not have the protecting stratum corneum. Seroconversion after skin contact with HIV-positive blood has been reported to the Surgeon General in the U.S.A., and HIV infection after unprotected orogenital sex was recently reported (57, 58).

The hypothesis that Langerhans cells are the primary target cells for HIV has implications for our unterstanding of the first events of the infection. The previous assumption that wounds, rifts or blood must be present for the infection to occur, and that the passage of the virus occurs through wounds into the recipients blood, can no longer be considered valid. Langerhans cells normally migrate from the epidermis through lymphatics to regional lymphnodes, and there is no reason to believe that a HIV-infected Langerhans cell behaves differently. Our hypothesis therefore implicates that the virus is actively transported from the epithelia to regional lymphnodes, and since the Langerhans cells seem to be relatively resistant to the cytopathic effect of the virus, they may remain a longlasting virus reservoir.

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9

HIV INFECTION OF PERIPHERAL BLOOD DENDRITIC CELLS

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Dendritic cells (DC) are important antigen-presenting cells, particularly in the initiation of immune responses in resting T cells and our studies are providing increasing evidence for their involvement in the immunosuppression seen in AIDS. Peripheral blood DC expressed low levels of the CD4 HIV receptor which was up-regulated by exposure *in vitro* to gamma interferon. Infection of DC with HIV, following *in vitro* exposure to virus, was demonstrated by electron microscopy (EM) and by a combined *in situ* hybridization and immunolabelling technique which facilitated identification of infected DC at the light microscope level. Results of *in situ* hybridization to detect viral RNA and DNA suggest that DC may become latently infected or express very low levels of virus. Thus they could serve as reservoir of potential infection which may escape immune surveillance. DC taken from HIV-infected individuals also contained HIV genome, as identified by the combined *in situ* hybridization and immunolabelling method. In addition, a fall in the number of DC was observed in infected individuals which preceded the decrease in the number of lymphocytes. Functional studies, on both DC infected *in vitro* and on DC from infected individuals, revealed a deficiency in antigen presentation. Since depletion and dysfunction of DC precedes the appearance of T-cell defects, this may be a functional lesion in the development of disease.

Introduction

Dendritic cells (DC) of the peripheral blood belong to a family of

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cells that have their origin in the bone marrow (1,2) and includes the Langerhans' cells of the skin, the veiled cells of the afferent lymphatics and the interdigitating cells of the T-dependent areas of the lymph node and spleen (3). Particularly after acquiring antigen in the periphery, Langerhans' cells of the skin enter the lymphatics as veiled cells and then home to the T-dependent region of a lymph node and differentiate into interdigitating dendritic cells (4,5,6). Similarly, DC in the peripheral blood migrate to the T-dependent areas of the spleen and become interdigitating dendritic cells (7,8). *In vitro* studies have shown that lymphocytes form clusters with DC (9,10,11). This is believed to facilitate highly efficient presentation of antigen to helper T lymphocytes in the context of MHC class II glycoproteins which are constituitively expressed at high levels by DC. Other cells that express MHC class II molecules, such as B cells and macrophages, can present antigens in secondary immune responses (12) but DC alone are able to stimulate resting or memory T-helper cells (13,14,15).

Early studies on HIV showed that the CD4 positive helper T lymphocyte was susceptible to lytic infection by the virus (16,17). Initially this observation seemed to explain the loss of this population of cells in AIDS patients and the subsequent onset of immunodeficiency. However, it is now clear that the pathogenesis of the disease is not so simple. Doubt was cast on this original hypothesis when, by *in situ* hybridization, it was found that only 1 in 10,000 lymphocytes in the peripheral blood of infected patients contained viral RNA (18). Additionally, immunological abnormalities were observed before a marked reduction in CD4-positive cells was apparent (19), whilst other studies showed that impaired immune responses could not be simply accounted for by reduced T-helper cell numbers (20). The possibility of HIV-mediated suppression at other levels in the immune system gained credence when it was found that macrophages (21,22,23,24), EBV transformed B cells (25) and follicular dendritic cells of the B-dependent areas of the lymph node (26) were also susceptible to HIV infection. The first evidence that members of the DC family may be affected in AIDS came from the studies of Belsito *et al* (27) who noted a reduction in the

number of MHC class II-bearing Langerhans' cells in the skin of HIV-infected patients. Subsequently, more direct evidence for the involvement of these cells was obtained from both *in vivo* and *in vitro* studies. Examination of AIDS patients' skin biopsies showed HIV-like particles associated with Langerhans' cells (28) whilst the susceptibility of peripheral blood DC to *in vitro* infection with HIV was demonstrated (29).

The *in vitro* proliferative response to antigens by lymphocytes from HIV-infected individuals is defective even when the number of CD4 cells is in the normal range or only marginally depressed (30). This was interpreted as reflecting a selective depletion of a subset of T helper cells or a functional defect in the responding T cell population or both (31). However, a defect in antigen presentation could also explain these findings and for this reason we have chosen to study DC and their possible role in the pathogenesis of AIDS.

Preparation and morphology of peripheral blood DC. DC of the peripheral blood represent a readily available source of cells of the DC antigen-presenting cell family. They are prepared by incubating peripheral blood mononuclear cells on plastic overnight to remove the adherent cells. The non-adherent cells are then centrifuged over a metrizamide gradient to separate the denser lymphocytes from the low density cells which include the DC (32). Such preparations usually comprise 20-40% DC with most of the remaining cells being macrophages and monocytes; fewer than 3% of the cells are lymphocytes. Ultrastructural examination of these preparations reveals typical DC (2,33) which exhibit an irregular surface with long occasionally branched projections (Fig.1). The cytoplasm contains scattered elements of endoplasmic reticulum, a few mitochondria, and a golgi apparatus. Most of the nucleus is comprised of euchromatin whilst at the margins of the nuclear envelope is a thin rim of heterochromatin. By immunogold labelling these cells are negative after labelling with monoclonal antibodies specific for monocytes/macrophages T, B and NK cells but are labelled with antibodies directed against MHC class II antigens. A second cell type is present that expresses MHC class II antigens and lacks markers for macrophages T, B and NK cells (Fig.2). It is

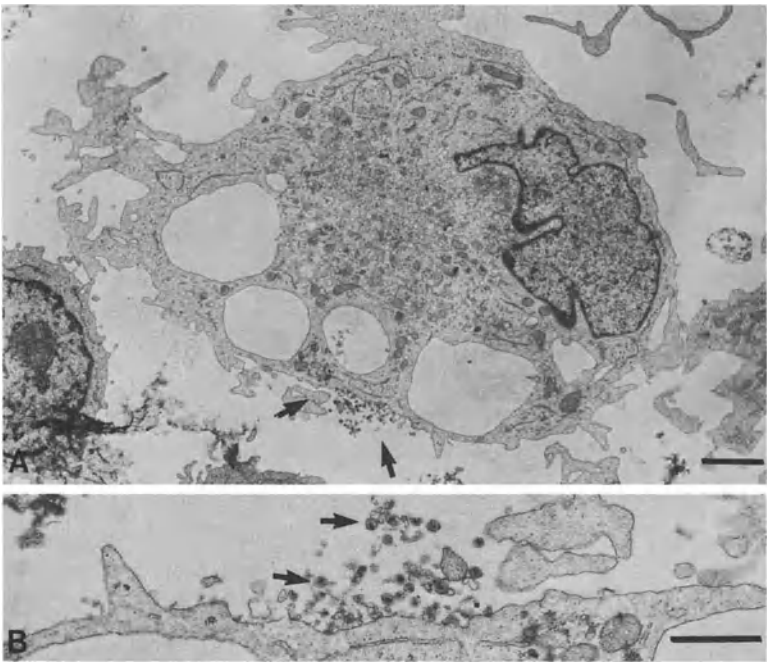

Fig. 1 A) Electron micrograph of a type I DC 5 days after infection *in vitro* with HIV. Bar represents 2 μm. B) Higher magnification of the area indicated by the arrows in (A). Mature HIV particles (arrows) may be observed on the cell surface. Bar represents 1 μm.

characterised by expanses of cytoplasm relatively devoid of organelles, a smooth cell boundary with few processes and overall appears to be in a less "active" state than the typical DC. Cells with a morphology intermediate between these two types are also present, suggesting that they may represent different stages of the same cell lineage. For this reason and because of the similarity in phenotypic profile of the two cells, this second cell was regarded as belonging to the DC family and was termed a type II DC whilst the former was termed a type I DC. Similarly, rat DC from the afferent lymphatics are heterogenous in their morphology (2), whilst DC from the draining lymph nodes of skin painted mice also show two distinct populations of antigen bearing DC (34). Recent evidence (35) suggests this may reflect different stages of cytokine mediated maturation.

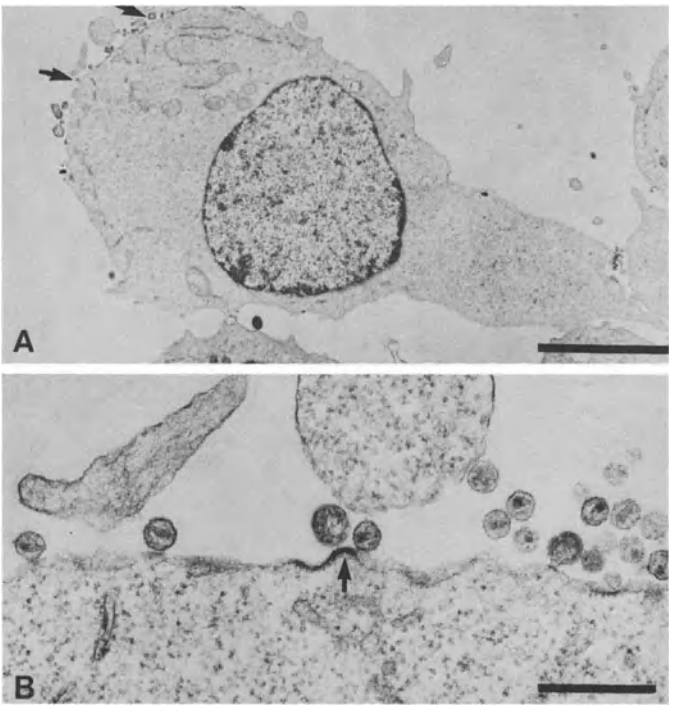

Fig. 2. A) Electron micrograph of type II DC 5 days after infection *in vitro* with HIV. Bar represents 5 μm. B) Higher magnification of area indicated by the arrows in (A). Mature virus particles are on the cell surface and a budding virus (arrow) may be observed. Bar represents 0.5 μm. (Reprinted with permission from ref. 29).

The CD4 receptor on peripheral blood DC. CD4 has been identified as a major receptor for HIV (36, 37) although there is now growing evidence that some CD4-negative cells can be infected (38). By immunogold labelling EM (Patterson, Gross, Bedford and Knight, in preparation) it was demonstrated that DC express low levels of CD4 on their surface (Fig.3). *In vitro* culture of Langerhans' cells with gamma interferon increases the expression of CD4 (39). Similarly, by immunogold labelling, peripheral blood DC can be shown to up-regulate the amount of the membrane CD4 after overnight culture with this lymphokine (Fig.4).

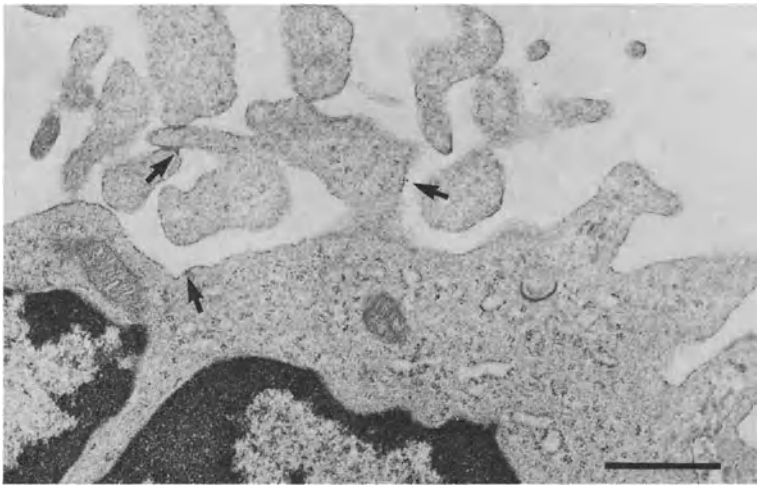

Fig. 3. A type I DC immunogold labelled (arrows) to detect CD4. Bar represents 1 μm. Type II DC show similar low levels of labelling.

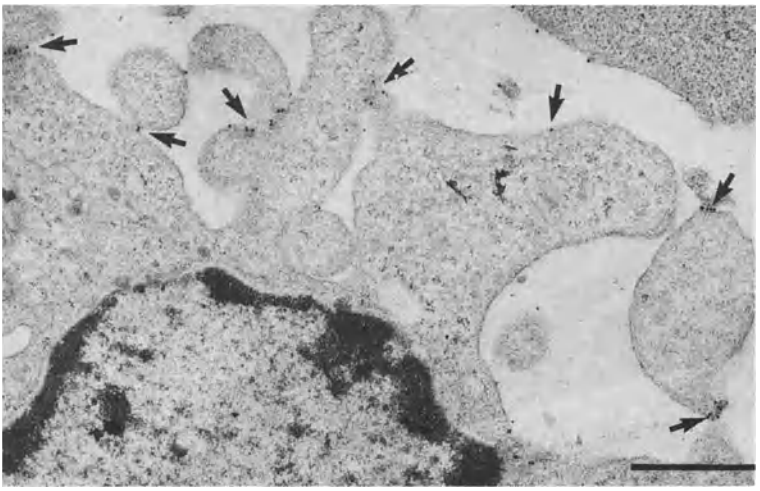

Fig. 4 A type I DC immunogold labelled (arrows) to detect CD4 after overnight (20h) incubation with gama interferon. Bar represents 1 μm.

Interestingly this increase was only observed in type I DC. A low number of untreated type I DC were also detected with high levels of CD4 suggesting that *in vivo* there may be differential expression of this antigen. T cells activated by antigen-presenting cells secrete gamma interferon. Thus increased virus receptor expression and intimate contact with a productively infected cell may promote infection of the antigen-presenting cell.

Susceptibility of peripheral blood DC to *in vitro* infection with HIV

DC enriched cell preparations were infected *in vitro* with the IIIB strain of HIV and then examined by electron microscopy after 5 days in culture (29). Up to 17% of the DC had mature virus particles on their surface and virions were frequently observed budding through the plasma membrane (Figs.1,2). Multinucleate giant cells, which are a feature of lymphocyte infected cultures (16,17), were undetected in most experiments. This may be explained by the observation that a reduced propensity of infected cells to form syncytia correlates with a lower level of membrane CD4 expression (24,40, 41). Virus replication was seen in both morphological types of DC but in 9 separate experiments a greater percentage of type II DC, usually between two and three-fold more, were infected (Patterson, Gross, Bedford and Knight, in preparation). This finding is in contrast to studies on lymphocytes which have shown that activated rather than resting cells support productive HIV infection (42,43). Similarly for cells of the monocyte lineage, visna, a lentivirus of sheep, grows in differentiated mature macrophages rather than in the monocyte precursor (44). For lymphocytes (45) and probably also for cells of the monocyte lineage (46) these differences reflect the presence of NF kappa B-like transcription factor molecules that bind to the enhancer region in the viral LTR and activate virus replication. The presence of similar molecules in DC may explain the present findings but as yet these have not been looked for.

Although the majority of cells in the DC preparations are macrophages and monocytes, there was no evidence of virus growth in these cells. This is probably because the IIIB strain of virus used in these experiments is highly lymphotropic and grows very poorly in macrophages (23).

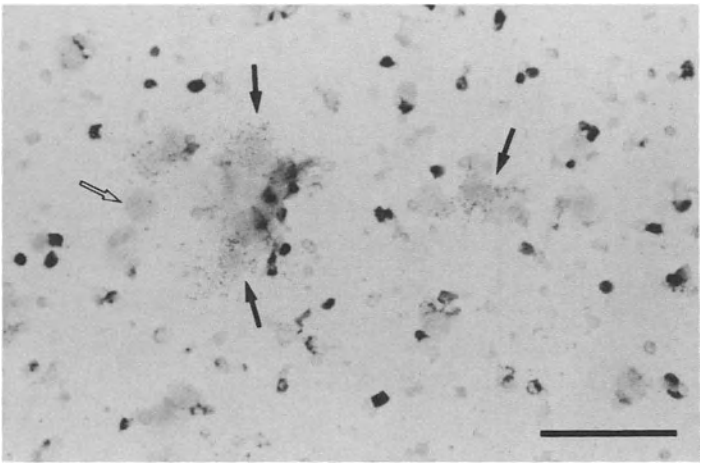

Fig. 5. DC preparation 5 days after *in vitro* infection with HIV processed by the combined immunolabelling (alkaline phosphatase-anti-alkaline phosphatase APAAP) *in situ* hybridization technique. DC are identified by the absence of red alkaline phosphatase staining. Infected cells (arrows) detected by labelling with a ^{35}S labelled DNA probe followed by autoradiography. Bar represents 50μm.

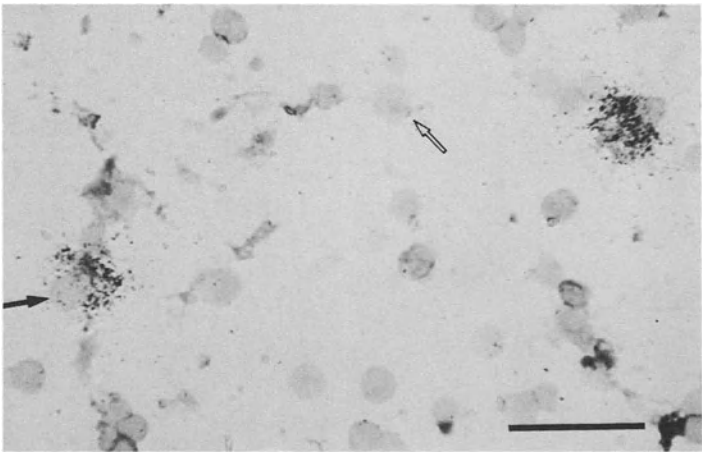

Fig. 6. DC preparation from HIV seropositive individual processed by the combined immunolabelling *in situ* hybridization technique as described for fig. 5. Filled arrow indicates an infected DC, an APAAP positive cell is lying closely apposed to the infected DC. Open arrow indicates an uninfected DC. The other infected cells in the micrograph has been stained by the APAAP procedure and is therefore not a DC. Bar represents 50 μm

The infection of DC by HIV was also monitored by *in situ* hybridization. This technique may be expected to give a more accurate picture than EM of the number of DC infected since it should label cells producing very little virus and may detect latently infected cells. A technical difficulty encountered in this work was that of identifying DC at the light microscope level. This problem was overcome by using a combined immunolabelling *in situ* hybridization technique. Currently there are no monoclonal antibodies available that identify human peripheral blood DC specifically and so DC preparations were labelled with a mixture of antibodies directed against macrophage/monocyte, T, B and NK cell specific determinants. DC were thus identified by the absence of immunolabelling (Fig.5). Employing this technique up to 60% of the DC in an *in vitro* infection were found to be infected after 5 days. When preparations were hybridized to detect either RNA or DNA up to 25% more cells were positive for DNA than for RNA. This may indicate that some DC were latently infected or chronically infected but synthesising very low levels of virus. *In vivo* this would represent a site where the virus could persist and escape immune surveillance. Similar numbers of cells were positive for both RNA and DNA in cultures of H9 cells that were persistently infected with HIV.

HIV in DC of HIV-infected individuals. Having established that peripheral blood DC were susceptible to HIV infection *in vitro* it was important to determine whether the DC of infected patients harboured the virus. DC from seropositive patients were examined for the presence of HIV genome employing the combined immunolabelling *in situ* hybridization technique (Fig.6). HIV infected DC were detected in more than 50% of patients in all clinical categories of the disease indicating that this cell is a major target for the virus *in vivo* (Macatonia, Lau, Patterson, Pinching & Knight, submitted for publication). In contrast to the lymphocyte population, in which the positive cells accounted for less than 0.1%, of the total, up to 24% of the DC were infected with HIV. DC may act as reservoir of virus, infecting T cells as they

form intimate contact during antigen presentation. Total lymphocyte number in the healthy seropositive asymptomatic patients was within normal limits whilst the DC number in this group was significantly reduced. Patients in the persistent generalized lymphadenopathy category showed normal levels of DC but as they progressed to AIDS there was severe depletion of their DC. Thus reduction of DC number precedes changes in other cell types in HIV infection and may prove to be an early indicator of exposure to virus. It could also be a useful parameter for measuring the effectiveness of therapeutic treatment. The data presented here is in agreement with the findings of Eales *et al* (47) who also found a reduction in the number of low density peripheral blood cells expressing high levels of MHC class II antigens in HIV-infected patients.

Functional studies on DC infected *in vitro* with HIV. DC infected with virus may be impaired in their ability to present antigen to helper T lymphocytes and consequently this could contribute to the development of immunosuppression. To test this hypothesis DC from normal healthy controls were infected for 2 days with the IIIB strain of HIV. The DC were then cultured with autologous lymphocytes and concanavalin A for 3 days in 20μl hanging drops (48) and proliferative responses were measured by the uptake of ^{3}H thymidine. A marked suppression in the proliferating response of the lymphocyte population was observed (49). However, examination of these cultures by *in situ* hybridization revealed a small number of infected lymphocytes. Secondary infection of these cells is probably facilitated by the intimate contacts they form with the antigen presenting DC. From these experiments it was not clear whether the suppression of the proliferative response was caused by secondary infection of the responding lymphocyte cell population, to virus impairing the ability of DC to present antigen or to both events. To answer this question, 2',3' dideoxyadenosine was added to infected DC preparations immediately prior to culturing with the lymphocytes. At the appropriate dose this drug blocks virus infectivity by inhibiting the reverse transcriptase enzyme without affecting the lymphocyte proliferative response (50). *In situ* hybridization confirmed that secondary infection of lymphocytes was

prevented and in uninfected preparations there was a normal mitogen induced proliferation response in the presence of the drug. In infected cultures the proliferative response was only marginally less suppressed than in the absence of the antiviral drug (Fig.7).

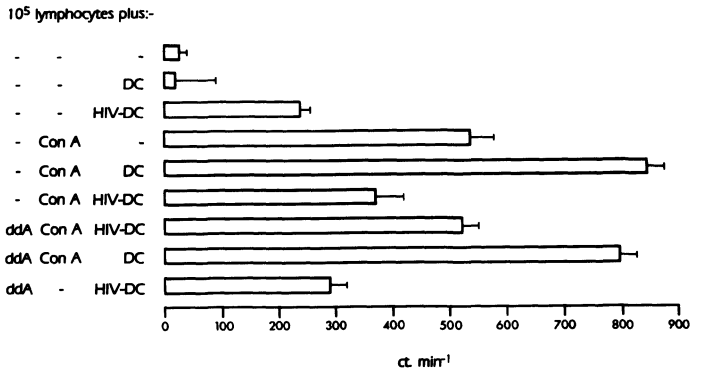

Fig. 7. Capacity of HIV-infected DC to enhance lymphocyte proliferation with Con A in the presence of 2', 3' dideoxyadenosine (ddA) [Reprinted with permission from ref. 49].

This provided the first clear evidence that HIV interfered with the presentation of antigen by DC. It is also apparent that secondary infection of lymphocytes contributes to the suppression of proliferation. Although macrophages are present in these cultures, it is unlikely that the observed effects are due to suppression of antigen presentation by these cells for two reasons. One, depletion of macrophages and monocytes in this system does not significantly alter the proliferative response (15,51,52) and second, the IIIB strain of virus used in these experiments replicates very poorly in macrophages (24,29). Other investigators have shown that antigen presentation by HIV-infected macrophages is impaired (53), however, there is evidence that these cells only present in secondary immune responses and cannot stimulate primary immune responses or memory T cells.

Functional studies on DC from HIV infected individuals.

Investigations of immune function in HIV-infected individuals have shown that their T-cell proliferation and cytotoxic lymphocyte

function is depressed (30,31,54). The conclusion reached in most of these studies is that there is a defect in the responding T lymphocyte population. The question of a possible defect in antigen presentation by DC from HIV-infected individuals has now been addressed. DC and lymphocytes were separated from patients in different disease categories and their ability in MLC reactions to serve as stimulators, a property specifically associated with DC (13), or responding cells was assessed. Lymphocytes from infected healthy asymptomatic individuals proliferated normally to allogeneic DC from uninfected controls but in contrast the DC of this group stimulated normal allogeneic lymphocytes poorly (Macatonia, Lau, Patterson, Pinching & Knight, submitted for publication). In the PGL group the ability of the lymphocytes to proliferate was reduced whilst the stimulation capacity of the DC was severely depleted. Cells from AIDS patients were both poor responders and stimulators. DC from all groups also failed to present concanavalin A to autologous lymphocytes. These findings indicate that a defect in the DC population is an early event in the AIDS syndrome that precedes functional alterations in the CD4 positive lymphocyte population. Other studies have found normal MLC responses in the asymptomatic group of patients whilst observing a defect in specific antigen responses (54). However, the patient cells in these studies were not fractionated into responding and stimulating cells. Studies by Hoffmann *et al* (55) showed that addition of irradiated HLA-DR identical peripheral blood mononuclear cells (PBMC) from normal controls to PBMC from HIV serpositive individuals resulted in an increase in the mitogen responses from the AIDS PBMC. They also reported that AIDS patients' cells were poor stimulators of MLC. As with the present findings, these results may be explained by a defect in the DC population.

Mechanism of HIV mediated impaired DC function. It is not clear how suppression of antigen presentation is mediated. Recent evidence suggests that free gp120 may interfere with CD4 MHC class II interactions and thereby block presentation (56,57,58,59). This was thus examined as a possible suppressive mechanism in the *in vitro* DC-lymphocyte proliferation system. In order to mop up free gp120

soluble recombinant CD4 was added to infected DC immediately prior to pulsing with mitogen and culturing with lymphocytes. Soluble CD4 has been shown to effectively protect T cells *in vitro* against virus infection without affecting lymphocyte proliferation (38,60, 61,62,63). Soluble CD4 did not alleviate the suppression mediated by HIV and thus it seems unlikely that the antigen presentation defect is caused by free gp120 binding to CD4 on the T helper cell (Macatonia, Patterson, Knight, in preparation). This finding is compatible with the MLC data obtained with the asymptomatic infected patients since an effect mediated through free gp120 would be expected to operate at the responder cell level.

Another possible mechanism of suppression of antigen presentation is the down-regulation of MHC class II antigens. Down-regulation of MHC class I antigens has been demonstrated in cells infected with adenovirus (64,65) and HIV has been associated with the down-regulation MHC class II in an infected macrophage cell line (66). In studies on HIV-infected patients, a loss of the MHC class II low density cell population was observed in peripheral blood but it was not clear whether this represented a loss of DC or a down-regulation of MHC class II antigens from their surface (47). However, in preliminary experiments in which DC from patients were immunogold labelled to detect MHC class II antigens, there was no evidence of any marked reduction in the expression of this molecule. Other possible mechanisms include disruption of DC-lymphocyte interactions by down-regulation of cell adhesion molecules (67) as noted in somed EBV-infected cells (68) and impairment of antigen processing. These potential mechanisms are now under investigation.

Conclusions. The mechanism of immunosuppression in HIV-infected patients may not operate solely at the T-helper cell level. The data presented here showing (i) that DC express CD4, (ii) that DC are susceptible to HIV infection *in vitro*, (iii) that the DC of HIV seropositive individuals are infected with virus, (iv) that DC of patients are reduced in number prior to changes in the lymphocyte population and (v) that there are functional defects in DC infected *in vitro* or *in vivo*, supports this notion. We therefore postulate

that HIV infection of DC contributes to the immunosuppression seen in AIDS. Monitoring the number and function of DC in infected individuals could be a useful parameter in plotting and understanding the course of the disease. Furthermore, measurement of DC function may be a good indicator of recovery of immunocompetence in individuals receiving new therapies. Future advances in the elucidation of the role of DC in the pathogenesis of AIDS should lead to new approaches in therapy and the management of infected individuals.

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10

HUMAN IMMUNODEFICIENCY VIRUS (HIV) AND ITS INTERACTIONS WITH EPIDERMAL LANGERHANS CELLS (LC)

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ABSTRACT

Immunofluorescent and immuno-gold-silver staining methods were employed to detect HIV-1-associated p24 core antigen in skin biopsies from proven AIDS patients. Biopsies were taken from non-sun exposed, non-treated sites, showing typical maculo-papular lesions. Co-localization of known Langerhans cells (CD1a) or dermal dendrocyte (Factor XIIIa) markers demonstrated the presence of p24 antigen in 1/16 cases. Although, epidermal LC localization was noted, this was very sparse and the bulk of the staining was associated with dermal histiocytic cells. Quantitation of the numbers of LCs in sheets by means of ATPase, HLA-DR or CD1a methods demonstrated a significant decrease in numbers in all samples.

INTRODUCTION

Since the first reported cases of the acquired immune deficiency syndrome (AIDS) appeared in 1981, an enormous research effort has been made. These studies have led not only to the identification of the etiologic agent responsible, i.e. HIV, but have also clarified the details of the molecular genetics of the viruses in this group. Furthermore, the mechanisms of infection and the pathogenesis of the immune derangement typical of AIDS have been the subjects of intense scrutiny. The involvement of important cells of the immune system in the initial infection and subsequent spread of the disease, explains the susceptibility to opportunistic infections that are ultimately responsible for the demise of the infected individuals. So many excellent reviews of the

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background to the discovery of the viral agents responsible for AIDS have appeared that only a few landmark citations will be given herein (see 1-11).

The selective tropism of HIV for T lymphocytes is now so well known that recapitulation of the details would be superfluous (12-15). In essence, the retroviruses within the HIV classification are lentiviruses that have a specific affinity for cell surface molecules expressed in large amounts on T lymphocytes of the helper/inducer subset, i.e. CD4-positive cells. It is the ability of the virus to utilize this molecule as a ligand for its envelope glycoprotein gp 120, that leads to parasitism of the cells expressing the CD4. Subsequent destruction of the infected cells by various possible mechanisms leads to an eventual depression of immune capabilities and a breakdown in immune recognition of foreign microbial antigens. CD4 is expressed on other cell types besides T lymphocytes. The various members of the mononuclear phagocyte system (MPS) may be especially important in ensuring the persistence and spread of the virus (16-20) but it is probably the deletion of the CD4+ve T lymphocytes that is responsible for the ultimate failure of immune reactivity in the later stages of AIDS.

The role of HIV colonization of the cells of the MPS has been the subject of several excellent reviews(21,22,23,24). In particular, the concept that the virus may set up a persistent infectious focus within such cells that may be protected from detection and destruction by the immune system, i.e. so-called Trojan horse concept associated with visna viruses (25) could account for some of the clinical features of AIDS. The involvement of HIV with brain pathology has been largely explained because of its localization in microglial elements (26-28) which are currently placed in the MPS (29-30).

While it is probable that dissemination of the virus can occur not only from circulating T cells but also from infected monocytes (31), it must be noted that certain elements of the immune system that regularly show viral infection need not necessarily express the CD4 molecule. Follicular dendritic cells

and interdigitating reticulum cells found in B and T cell zones of lymph nodes and spleen do not appear to express CD4, yet both may show heavy viral loads (32). Clearly there may be other receptors involved in the uptake and integration of the virus into cells (see discussion).

It has been noted that AIDS patients may show a variety of skin lesions at different stages of the disease. Apart from the development of an otherwise rare malignancy in a significant percentage of patients, i.e. Kaposi's sarcoma, a variety of macular or maculo-papular rashes or xanthema typify both the early prodromal post-infective period and the late stages of the disease (33-37). In understanding the pathogenesis of these lesions there are clearly difficulties in knowing the exact time periods elapsing between exposure to the virus and development of the characteristic histological changes. The only suitable animal model appears to be infection with simian immunodeficiency virus (SIV) in rhesus monkeys. The virus is closely related to HIV and is tropic for CD4-bearing cells. In addition, it produces disease symptoms which are very similar to AIDS in man. Studies have been carried out that show localization of the virus in macrophages (38). The cutaneous xanthema has been analyzed (39,40). While virus was not actually detected in the skin, the histology suggested a T lymphocyte-mediated cytotoxic destruction of epidermal Langerhans cells. The features of this process were compared to the immune destruction of cells in allograft rejection and in graft-versus host reactions. Other workers have used the model to demonstrate that infection may be achieved across mucosal surfaces of the vaginal vault or the urethera. Despite this evidence for a possible route of viral transmission in heterosexual contact, the virus dosages required to achieve success were much higher than those noted for intravenous infectivity (41).

Although a significant proportion of AIDS victims become infected by transfer of blood products or through a vascular route dependent on their drug habits, this is not presumably the natural portal of viral entry. Infection normally occurs across epithelial barriers which are breached or damaged. The stratified

epithelia that line the vagina, rectum and oral cavity as well as the skin normally combat intrusion from the environment by simple barrier function and by specific immune mechanisms. While infection through the first three epithelia might be achieved as a result of sexual activity, penetration through intact skin seems unlikely. Reports have, however, appeared of health care workers having accidental exposure to contaminated blood products via their skin(42,43). It is likely that the normal integrity of the skin was impaired in these cases either by small cuts and abrasions, or by dermatatic erosions. Transmission of virus has also been noted after skin grafting and it was presumed that the infective agent was either in blood cells transferred in situ or actually within elements of the skin itself (44).

The fact that cells within stratified squamous epithelia may harbour HIV and thereby act as not only a normal route of entry, as well as facilitating spread to adjacent lymph nodes via afferent lymphatic channels, has now been demonstrated by a number of mucosa (45-50).

LCs are intraepithelial dendritic cells associated with areas of stratified squamous differentiation. They are specialized members of a widely distributed dendritic cell family (51-53). The relationship between LCs, mobile veiled cells in the afferent lymph and various dendritic cells in lymphoid organs such as nodes and the spleen has been reviewed (54-57). The major function of LCs appears to be in initiation of T-dependent immune responses to antigens/haptens appearing in the epidermal environment either from the exterior or arising de novo from malignant transformation of epithelial elements or symbionts such as melanocytes. By their position at the interface with the external environment LCs are important sentinels of immune surveillance (58). The characteristic structural enzymatic and immunophenotypic markers of LCs are now well known both in man and in some laboratory rodents (59). Furthermore, the functional capacities of LCs in antigen presentation have been demonstrated in vitro and in vivo (60-62). Recent reviews on cutaneous immunity have integrated the details of LCs and their antigen-presenting role into a broader concept including

the accessory roles of keratinocytes and the cytokines they produce (63). The skin-associated lymphoid system (SALT)(64) or the skin immune system (SIS)(65) are now the models that illustrate the complex inter-relationships of immunocytes and soluble cytokines existing in the skin.

LCs might be predicted to play an important role in the body's defenses against viruses, because of their position in an organ so frequently exposed to the pathogens. Despite the reports in the literature of viruses associated with LCs, the details of the *in vivo* role of the cells in viral immunity are still far behind those studies already published for responses to contact allergens, etc. Morphological studies have shown vaccinia virus (66,67), CMV (59), papilloma virus (68,69,70), HTLV-1 (71) and herpes simplex virus (72-75), to be associated with LCs. The latter virus in particular has been extensively studied in murine models and a relationship between the absence of LCs in the skin (natural or induced) and the recrudescent lesions arising from latent virus activation is well known. LCs appear to have important functional roles in this case in developing anti-viral immunity. A number of *in vitro* studies have demonstrated the ability of LCs to present various viral antigen to appropriate T-cell subsets (76,77) but other studies have not been so positive about the role of LCs in viral immunity (78). Fresh or cultured LCs were not able to present Friend leukemia gp70 to T cells when compared to splenic dendritic cells. The assumption that LCs are potent antigen-presenting cells for all viruses may not be valid. A review of the various aspects of LCs and their interactions with viruses has recently been published (79).

The reports of reductions in numbers of LCs in the skin and oral mucosa of AIDS patients (45-50) were based on staining for ATPase, HLA-DR or CD1a and employed either sheets or frozen sections. There has, however, been some controversy about whether the reduction in LCs occurs as a regular phenomenon and, furthermore whether the identification of HIV viruses or viral antigens in LCs is a reliable observation. A recent study failed to demonstrate any HIV antigens in a large series of cases and this has

cast doubt on the original findings (80). The hypothesis that depletion of LCs in AIDS patients' skin might account for the anergy to skin test antigens seen in such cases as well as serving as an initial resevoir for infection and dissemination to the draining nodes is clearly unconfirmed in the absence of more data on AIDS patients' skin specimens. The previous studies on LCs and HIV will be reviewed more extensively in the discussion section. Since the number of cases studied so far has been rather small, an attempt was made to detect viral antigen in a further series of typical AIDS cases using immunofluorescence and immuno-gold-silver staining with monoclonal antibodies to p24 core protein of HIV-1. In addition, a quantitative study was carried out on epithelial sheets to determine the numbers of LCs present as detected by ATPase, HLA-DR and CD1a markers.

MATERIALS AND METHODS

Punch biopsies were obtained from non-sun exposed areas showing various papular or maculo-papular rashes from 8 patients with proven AIDS. A further 8 biopsies fixed in formalin and paraffin-embedded were studied for presence of viral antigens. Epidermal sheets were obtained by standard ammonium thiocyanate incubation. After fixation in acetone or formalin staining for LCs by means of ATPase, HLA-DR or CD1a markers was carried out (81). Visualization of reactivity for the two antigens was achieved by ABC-peroxidase methods (81). Quantitation of LC/mm^2 was achieved by counting positive cell bodies in 5 separate areas of each sheet at 400 x magnification using a calibrate graticule. Appropriate immunocytochemical and cytochemical controls were carried out to ensure the specificity of the reactions. Frozen sections were stained with a monoclonal antibody against HIV p24 antigen (Dupont) followed by either a biotinylated anti-mouse link reagent and a streptavidin-FITC indicator, or goat anti-mouse IgG gold (Auroprobe 1). Silver intensification was achieved in the latter case by means of the Intense M kit (Janssen Ltd.). This amplification method has been shown to be capable of detecting HTLV-1 antigens in retrospective studies on formalin-fixed specimens (82).

Appropriate controls for reagents and tissues were employed including an HIV-1-infected lymphoblastoid cell line HUT-9. Omission of primary reagent, substitution of inappropriate but isotype matched primary were carried out. Staining for LCs was also achieved on frozen sections using monoclonal anti-CD1a reagents with Texas red-labeled indicator reagents. Dermal dendrocytes were stained by application of rabbit anti-Factor XIIIa antibody with appropriately matched indicator reagents. LCs were isolated from skin biopsies by means of paramagnetic beads (Dynal) coated with anti-CD1a antibodies. Magnetic separation of the rosetted cells was followed by fixation of cells and preparation for conventional transmission electron microscopy (83).

RESULTS

(a) LC quantitation in sheets.

All three markers showed dramatic decreases in numbers in the sheets obtained from the 8 AIDS patients when compared to control skins from breast reduction or residual skin from surgical specimens (Figs. 1 and 2).

ATPase Control - 738 ± 29/mm^2
AIDS - 310 ± 53/mm^2

HLA-DR Control - 714 ± 36/mm^2
AIDS - 282 ± 43/mm^2

CD1a Control - 724 ± 48/mm^2
AIDS - 268 ± 52/mm^2

The numbers present in the AIDS skin were significantly below the normal value for all three markers ($p < 0.0001$). Because of the noted difficulties in quantitating LCs in vertical sections (84,85), no attempt was made to verify the sheet findings on the frozen sections. In addition to the observed reductions in stainable LCs it was noted that the overall distribution was somewhat variable and that the cells showed a marked reduction in their dendritic appearance (Fig. 2).

(b) p24 Antigen staining.

1/16 of the total AIDS specimens showed any staining for p24 when assessed with the fluorescent marker. Occasional strongly

stained cells were present in the epidermis (Fig. 3). These cells stained to show co-localization of CD1a and were considered, therefore, to be LCs. In the same sample, positive cells were seen in the superficial dermis (Fig. 4). These cells co-expressed HLA-DR but not CD1a and were interpreted as histiocytes. One of the retrospective blocks showed the presence of staining in occasional macrophages and giant cells in the dermal infiltrate (Fig. 5). An increase in Factor XIIIa positive dermal dendritic cells was also noted in this case (Fig. 6) but co-localization studies were unsatisfactory. No epidermal staining was seen in the formalin-fixed blocks. All controls were satisfactory demonstrating the specificity of the observed staining (Fig. 7).

(c) Cell suspensions.

Epidermal Langerhans cells were easily obtained from trypsinized epidermal sheets using the CD1a-coated magnetic beads (Fig. 8). The LCs showed an unusually high affinity for the beads and avid phagocytosis (Fig. 9). Birbeck granules were evident in the isolated cells (Fig. 10). Occasional virus-like particules were noted in cytoplasmic vacuoles in some of the isolated LCs (Fig. 11). The particles were not seen budding from the surface and did not have the typical cone-shaped internal nucleoid of mature HIV particles as seen in infected T-cell lines (Fig. 12). No positive staining was noted when isolated LCs were stained for p24 antigen.

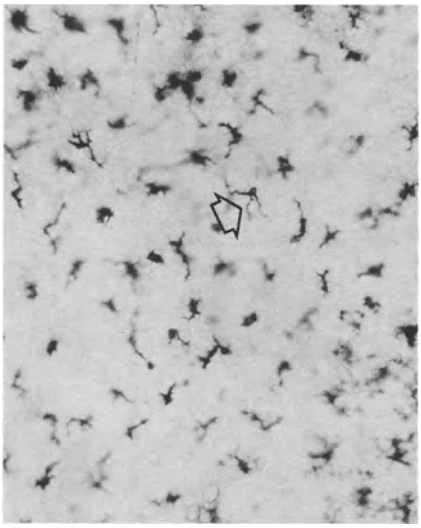

Fig. 1. Normal human epidermal sheet stained for CDla by immuno-peroxidase method. Dendritic LCs showing an even distribution pattern. x 200

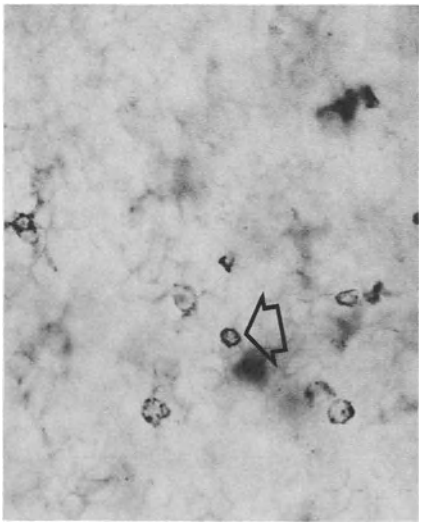

Fig. 2. AIDS patient epidermal sheet stained for CDla. Reduced numbers of LCs showing stubby dendrites and round Profiles. x 850

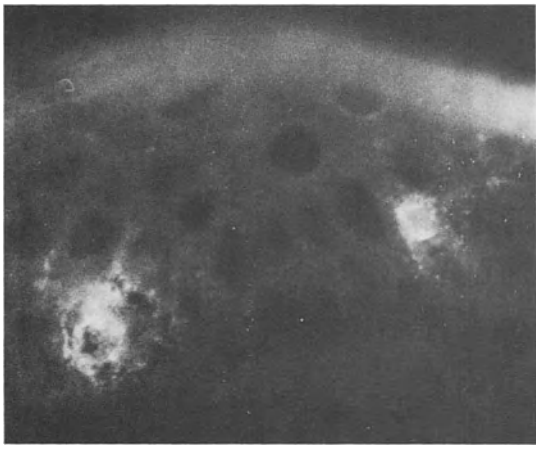

Fig. 3. Indirect immunofluorescent staining for p24 antigen in AIDS patient skin section. Prominent staining of two LCs. x 1500

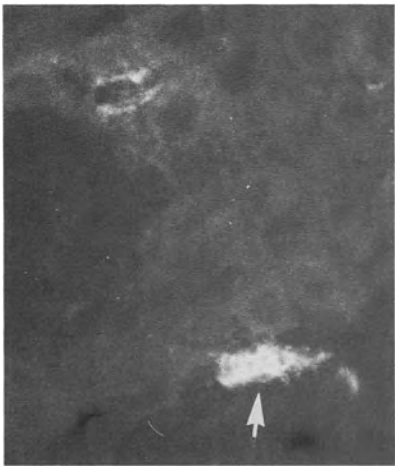

Fig. 4. Details as for Fig. 3. Staining of a dermal histiocyte plus weak reactivity over an epidermal LC. x 1500

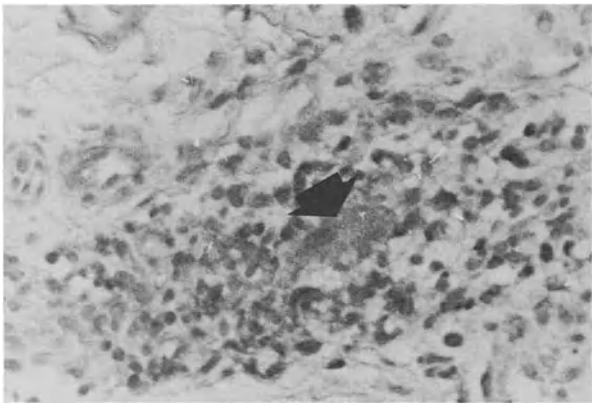

Fig. 5. AIDS patient skin biopsy stained for p24 by immuno-gold-silver method. Multinucleated giant cell in the superficial dermal lympho-histiocytic infiltrate showing weak cytoplasmic staining. x 2000

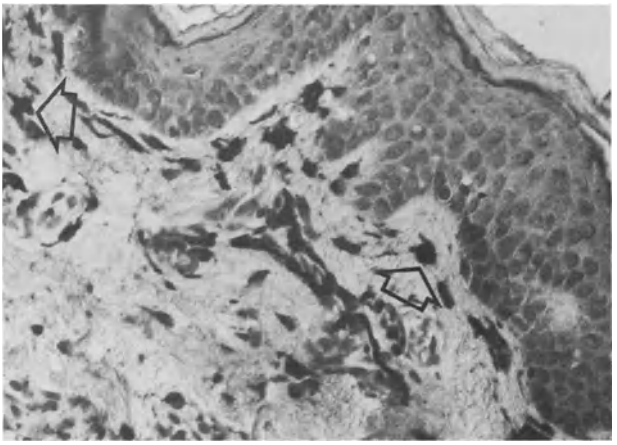

Fig. 6. AIDS patient's skin biopsy stained for Factor XIIIa by indirect immunoperoxidase method. Prominent dermal dendrocytes immediately adjacent to the basal lamina and around peripheral vessels. x 1000

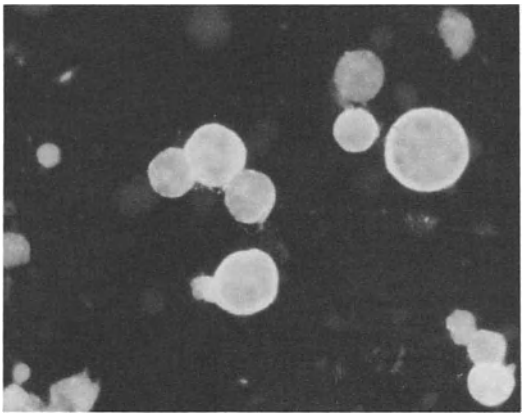

Fig. 7. HUT-9 lymphoblastoid cells infected with HIV-1. Indirect immunofluorescent staining for p24. x 2000

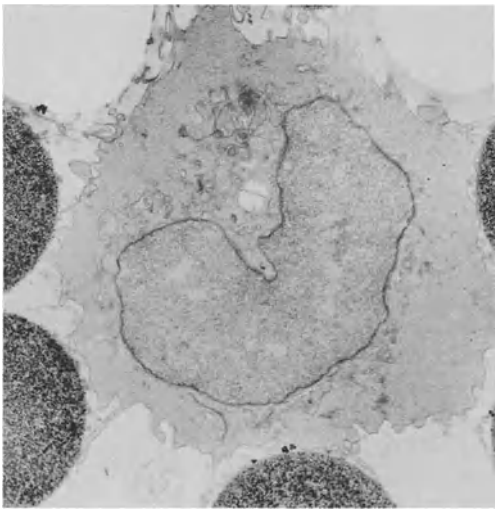

Fig. 8. Langerhans cell rosetted with CD1a-coated paramagnetic beads. x 12,500

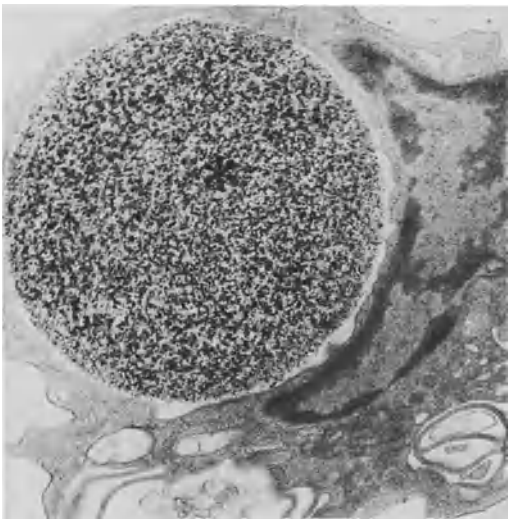

Fig. 9. Rosetted LCs showing phagocytosis of the paramagnetic bead. x 19,500

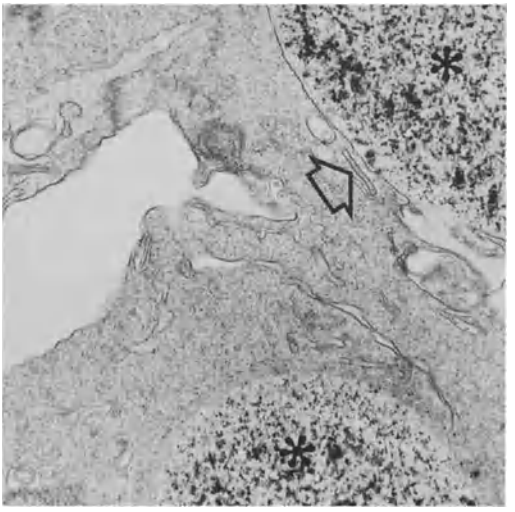

Fig. 10. Details of engulfed paramagnetic bead within a LC. Birbeck granules (arrow). x 30,600

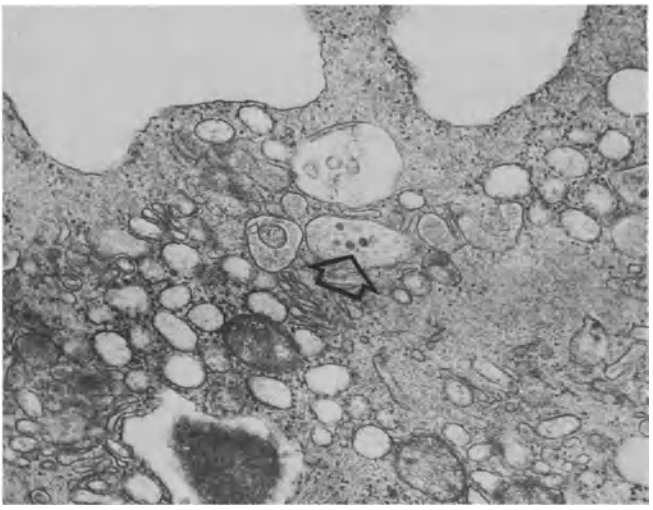

Fig. 11. LC isolated from AIDS patient's skin. "Virus-like" bodies present in a cytoplasmic vacuole (arrows). x 48,700

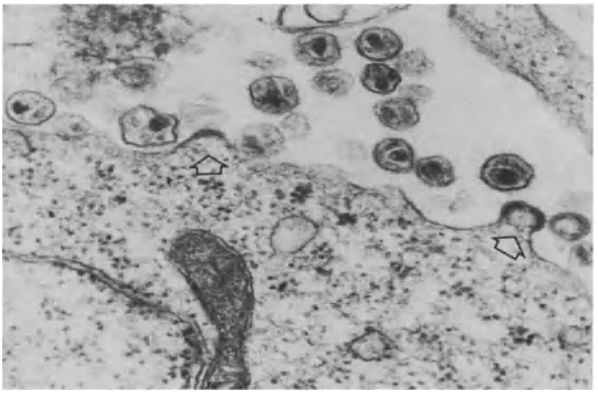

Fig. 12. HIV-1 virus production in infected HUT-9 cells. Free virions near the cell surface and budding particles also shown (arrow). x 48,700

DISCUSSION

The observation of reduced numbers of LCs in the skin of AIDS patients published in 1984 by Belsito and colleagues (45), was the stimulus that led to several groups attempting to clarify this issue. Apart from the fact that the decrease in numbers might result from profound changes in the immune system secondary to the development of opportunistic infections, several workers suggested that the LC might be a primary target for the virus and thus be important in the initial stages of the pathogenesis of AIDS (48,86, 87). Since LC have been shown to express low levels of CD4 (88,89, 90), it was logical to suppose that such cells might become infected by the virus. The question of whether this occurs on a regular basis, however, remains somewhat at issue.

While a number of workers were able to confirm the fall in LC numbers with the stage of the disease (46,49,91,92,93,94), there were other reports that were less confirmatory. Groh and colleagues, although demonstrating reduction in HLA-DR expression failed to demonstrate any linked fall in CDla expression (95). Other reports, although showing alterations in LC expression of CD4, failed to note changes in HLA-DR (50,80,96). Similarly, the correlation between CD4-positive peripheral blood cells, LC numbers and stage of disease noted by Dreno et al. (91), was not confirmed by subsequent studies (80).

The cases examined herein were all Stage III patients showing evidence of opportunistic infections but no evidence of overt Kaposi's sarcoma. The significant decrease in LCs indicated by all three markers confirms the earlier reports. The biopsies were taken from non-sun exposed sites to ensure that UV exposure did not depress normal LC numbers (98,99). No topical corticosteroids were being employed that might similarly be responsible for decreased numbers. It is, however, difficult with this patient sample to come to any firm conclusions about the primary involvement of HIV infection of LCs with the observed decrease in numbers. Anergy is almost a universal observation among AIDS patients but it is not specific for seriologically documented HIV infections (100).

The demonstration of viral antigens in LCs originally

published by Tschachler et al. (47,93) depended on immunostaining with monoclonals against different core or envelope antigens. Seven of 40 patients in different stages of the disease showed positive LC staining for p17 antigen with only one of these also staining for p24. Viral particles were seen by electron microscopy in the intercellular space near LCs and signs of LC damage was noted. Subsequent in vitro studies have demonstrated budding of typical virions from LCs (101-103). There is no doubt that isolated LCs may be infected with HIV-1 and that they support proliferation of the virus, since infective supernatants have been recovered from such experiments. At least two groups have, however, failed to detect any viral antigens within AIDS skin specimens (80,92).

The present series demonstrates that detection of HIV-1 antigens in skin biopsies is not by any means a regular occurrence. In addition, in the single case showing positive results most of the staining was noted in dermal histiocytic cells rather than in LCs. This, perhaps, raises the question of how the virus might gain access to LCs. Although it is possible that the virus might occasionally enter through the skin, it seems now probable that the present positive sightings result from other routes of entry. Since LCs are bone marrow-derived cells and even though they are a slowly cycling population (104-105), it appears that the bulk of their replacement must occur from blood-borne precursors. Clearly a more generalized systemic infection involving bone marrow progenitor elements or even infection of the precursor in the blood would account for eventual seeding of the LC population in the skin.

Infectability and the ability to produce virus has been clearly shown to be related to the maturation stage of cells of the MPS (see 24). Various cytokines and growth factors, as for example, IL-1, TNF, γIF and GM-CSF have all been shown to be important in modulating the expression of HIV-1 in cells of the MPS (106-109). Since there is now good evidence that many of these cytokines may be produced by various cells that make up the SALT system (see 63), it is not difficult to imagine how infected precursor cells might be modified to become viral-producing cells once they differentiate in the microenvironment of the skin. Whether infection with HIV-1

actually alters the normal function of LCs as it has been shown to do for other monocyte/macrophages (110-114), is not known at present. Defective accessory cell functioning of monocytes has, however, been well documented (115-117) and it seems quite probable that HIV-1-infected LCs may also show decreased activities in this sphere.

Another possible mechanism by means of which LCs might become infected through cells in the blood relates to the close association of APCs and T-helper cells during MHC-II class restricted antigen recognition. Since T cells appear to need some activation signal to favour the entry of HIV-1, it has been suggested that this occurs during the presentation of antigens by APCs. If the APC, which in this case is the LC, is infected it is likely that the close proximity of cells and activation signals occurring during the process would ensure efficient transfer of virus (117-121). Conversely, although early reports suggested that very few of the circulating T cells were infected (0.01-0.0001%) (122), it now appears that the actual numbers with integrated (latent) virus is at least 10 x higher (123). The reservoir of HIV-1 in the blood appears to be a T cell that maintains expression of CD4 (124). Whether antigen presentation by LCs occurs in the periphery or after migration to the local lymph nodes is still a debatable point, but clearly if APC functions were to occur in the skin when lymphocytes enter the epidermis, the conditions for exchange of virus from T cell to LC might be as favorable as in the reverse situation.

How the virus actually enters cells is still a matter of dispute (125). Although receptor-mediated endocytosis (RME) has been claimed to be the major mechanism (126), recent studies seem to favor envelope fusion with the plasma membrane, since it is a pH-independent mechanism and does not need endocytosis of the CD4 receptor (127,128). Some recent studies on LCs are pertinent to this subject. Using colloidal gold labelled gp 120 and gp 160 preparations the influence of tryptic removal of the CD4 epitopes responsible for HIV-1 binding has been studied (129,130). Complete removal of the classic binding sites for HIV-1 (12,13) did not prevent the internalization of the labeled glycoprotein probes.

Similarly, uptake was not blocked by any of the various anti-CD4 antibodies employed. The uptake was achieved by the process of RME as has previously been shown to be important in LCs for CD1a and MHC-II antigens. The authors conclude that this is evidence for the existence of other molecules involved in the uptake of HIV-1. Earlier studies on CD4-transfected murine cells also demonstrate that expression of this receptor was in itself insufficient for uptake (17).

The final issue that warrants some comment concerns the presence of demonstrable HIV-1 antigen in dermal histiocytic cells. Recent studies by Nickoloff (131,132) have suggested that the malignant cells in Kaposi's sarcoma are derived from dermal histiocytics that are found normally in the superficial dermis, especially around vessels. These dermal dendrocytes (133) have been shown to be a major component of certain skin tumors (134). At present the proposed histogenesis of Kaposi's sarcoma from such cells is speculative and has not been totally accepted (133,135,136). Other workers favour the development from endothelial elements in both the classic and AIDS related Kaposi's sarcoma cases (136,137,138, 139). A complex system of autocrine and paracrine growth factors released from infected Kaposi's cells in culture has been implicated as controlling the development of this malignancy (140-142). The increase in numbers and size of the Factor XIIIa dermal dendrocytes seen in the infiltrates in the present series of cases may be related to the development of Kaposi's sarcoma or may represent some important immune function of these cells. At present it is not known whether they can perform antigen-presenting or accessory cell functions that might make them relatives of the nearby epidermal LCs. The fact that it was not possible to clearly demostrate whether the dermal cells stained for p24 antigen also expressed Factor XIIIa makes it impossible to confirm that infection of such cells might be important in the pathogenesis of the skin malignancy common to AIDS patients. Further studies are clearly indicated in this area.

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HERPES SIMPLEX VIRUS-1 INFECTION OF THE SKIN AND LANGERHANS CELLS

11

LANGERHANS CELLS AND RECURRENT INFECTION WITH HERPES SIMPLEX VIRUS

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ABSTRACT

Langerhans cells are thought to be the antigen-presenting cells of the epidermis. As such they are likely to be involved in the immune response to epidermally encountered antigens such as herpes simplex virus. Evidence is reviewed which suggests that the involvement of Langerhans cells in the primary immune response to the virus may be severely limited. In contrast it appears that Langerhans cells, by rapidly stimulating a local reaction, provide a potent barrier to the establishment of recrudescent disease. The effects of treating mice with a number of agents known to cause viral reactivation in the sensory ganglia, and in some cases recrudescence, were examined. All the agents tested reduced the numbers of ATPase-bearing Langerhans cells in the epidermis, but DMSO had the most marked effect. Treatment of the pinna with DMSO two days after inoculation of the neck with HSV-1 increased the severity of zosteriform infection of the pinna, and enhanced replication of virus in the cervical ganglia. However, the extent to which agents reduced the numbers of ATPase-staining cells did not correlate with their ability to reduce the antigen-presenting capacity of the cells. The functional assays suggest that effective triggers

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of recrudescent disease may act at least in part by reducing the activity of local Langerhans cells.

INTRODUCTION

It has recently been realised that the epidermis constitutes a highly active, complex tissue within which many components of the immune system are resident. Indeed, keratinocytes, the structural cells of the epidermis, are phagocytic (1), and can be induced to express class II MHC products (2). In addition they secrete a number of soluble factors, for example, interleukin 1 (IL-1) (3), IL-3 (4), beta-interferon (5), granulocyte macrophage-colony stimulating factor (GM-CSF) (6), and thymopoietin (7). In addition to the keratinocytes there are low numbers of other cell types, including T cells. However, it is the Langerhans cell (LC) which appears to be central to the inductive immune response in the epidermis. Investigation of the role of LCs in the immune response is particularly interesting in the study of infections of the skin such as those caused by herpes simplex virus (HSV).

Herpes simplex virus belongs to a large family of double-stranded DNA viruses, the Herpesviridae. The most notable feature of these viruses is their ability to establish a latent infection, which remains indefinitely following the clearance of acute disease. In the case of HSV initial infection occurs at an epithelial site. From such a site virus spreads to the sensory ganglia (8) where latency is established in the neuron cell bodies (9). A feature of particular interest is that in some individuals the latent virus is periodically reactivated in the ganglion, and spreads down the nerves to the epidermis where recrudescent disease may occur. Such individuals do not appear to be overtly immunosuppressed, since problems with other pathogens are rarely apparent (10). Further, the recrudescent HSV infection is usually cleared quickly, and occurs in the face of demonstrable cellular and humoral immune reactivity (11). In addition it is clear that in many cases reactivated virus

enters the periphery without establishing symptomatic infection, a situation designated as recurrence as opposed to recrudescence (11). These observations highlight the need to study local factors involved in the induction of the immune response to HSV, and therefore prompt an investigation into the role of LCs in the disease.

MATERIALS AND METHODS

Animals.

The mice used were 4-week-old female NIH mice bred in the departmental animal facility from stock originally purchased from Olac 1976 Ltd.

Treatment of mice with agents which affect latency.

Fifty µl of dimethyl sulphoxide (DMSO) (100%), xylene (50% in ethanol), or retinoic acid (0.01% in acetone) were applied to the surfaces of the pinna (12).

Virus inoculation.

In order to study aspects of the primary infection with HSV mice were inoculated with 10^5 pfu of HSV-1 strain SC16 (13) by scarification of the right side of the neck (14).

Assessment of viral titres.

Skin from the neck and pinna, and pooled second and third cervical ganglia were removed from the right side of mice and tested for infectious virus using a plaque assay (12). The mean of duplicate wells were calculated and the log taken to produce the $\log_{10}$ titre of virus per tissue sample.

Demonstration of viral antigen by the peroxidase anti-peroxidase (PAP) method.

Whole epidermal sheets were prepared from the pinna of mice by loosening the epidermal-dermal barrier with EDTA (15). Subsequent fixation and staining was according to the method of Shimeld et al (16).

Assessment of disease severity.

The severity of erythema on the pinna was scored from 0 to 4 (17). Neurological involvement was assessed by observations of behaviour, inability to use limbs, and a lack of co-ordination. The numbers of lesions on the pinna was

examined under light anaesthesia using a binocular dissecting microscope.

RESULTS AND DISCUSSION

It is clear that priming alters the response to T-dependent antigens, as evidenced by an increase in the speed and size of the secondary reaction. Studies have also shown that there is a shift in the phenotypes of both the T and B cells available to respond (18,19). Moreover, recent work has suggested that the requirements of primed and unprimed T cells for antigen presentation differ markedly. For example while splenic dendritic cells and macrophages are competent, resting B cells fail to induce a primary response in naive animals (20). On the other hand all three cell types are capable of reactivating primed T cells *in vitro* (20,21). Therefore, to understand completely the role of LCs in infection with HSV-1 it is necessary to consider both the induction of the primary, and the restimulation of the secondary immune responses.

The primary immune response to HSV

One of the major difficulties in studying the reaction of epidermal LCs *in vivo* to HSV is that direct inoculation techniques tend either to cause overt damage, or to by-pass the epidermis. However in the zosteriform spread model (14) study of the site reached by zosteriform spread offers the advantage that virus enters the epithelium in the absence of extraneous pathology. Analysis of epithelial sheets from such sites has revealed that LCs react to viral infection. In the eye a marked infiltration of LCs has been observed (22), and in the epidermis of the pinna clustering of LCs is associated with a small overall decrease in their density (23). Extrapolation to the work of Silberberg-Sinakin (24), who studied the effects of contact sensitisers on the skin, suggests that the changes observed in the pinna may be associated with migration of LCs through the dermis to the lymph nodes. However, other work on HSV has demonstrated that infiltration of the infected epidermis by mononuclear cells does not occur until viral replication spreads as far as the

dermis (25). This suggests that local activation of the immune response does not occur in the infected epidermis.

Data from in vitro studies supports the suggestion that LCs in the epidermis do not possess the capacity to prime T cells. Unless they are cultured for several days in the presence of foetal calf serum and keratinocytes or granulocyte macrophage-colony stimulating factor (GM-CSF) LCs are poor stimulators of non-immune T cells in a primary mixed lymphocyte reaction (MLR) and in antigen-specific plaque-forming cell assays (26). In addition we have shown that suspensions of epidermal cells (containing LCs) induce a very poor primary antigen-specific T helper cell reaction to bovine serum albumin (27), and appear incapable of stimulating any response to HSV (28). In contrast cells from the spleen induce strong primary responses to both antigens (27,28). The observation that prolonged culture, as outlined above, activates LCs so that they are able to induce a primary MLR and antibody production, has led to the proposal that LCs only become mature upon challenge with antigen (6). However, such maturation did not occur in our system (employing only autologous serum) despite the presence of keratinocytes, antigen and T cells (27,28). Nevertheless, it is possible that maturation of LCs may occur under the influence of factors encountered outside the environment of the epidermis, for example during migration to, or in, the lymph nodes. It is noteworthy that when cultured in heterologous serum LCs undergo phenotypic transformations similar to those observed during migration in vivo (29).

In summary it appears that although LCs may be able to initiate an immune response following appropriate activation, the epidermis does not normally possess the ability to mount a primary reaction locally. One can envisage that the advantage of this would be that the immune response to an epidermally encountered antigen could be centralised, allowing a more multi-faceted reaction. In terms of HSV this means that the virus may have longer to establish infection in the skin. However, a more diverse, centralised response may provide

greater protection from viral spread within the nervous system and at other peripheral sites.

The immune response to HSV upon secondary exposure

In contrast to their apparent inability to prime the immune response, epidermal cell suspensions are capable of acting *in vitro* as accessory cells in the restimulation of primed T cells to a variety of immunogens. These include, nickel sulphate (30), keyhole limpet haemocyanin (26), DNP-ovalbumin (31), picryl chloride (32), fluorescein isothyocyanate (33), and HSV (28,34-36). Furthermore, depletion of I-A-bearing cells from the epidermal cells has been shown to abrogate this accessory cell function (28,34,36). In the case of HSV the responding cells are primarily of the T-helper phenotype (28), which can be stimulated by LCs from the cornea as well as from the skin.

The *in vitro* data are supported strongly by observations made *in vivo* on secondary contact with contact sensitisers. Following such a challenge, a number of sensitisers are taken up by LCs (37,38). As following primary challenge, LCs are found in increased numbers in the dermis, lymphatics, and draining lymph nodes which suggests they migrate from the epidermis (24). However, following secondary challenge lymphocytes are found in close apposition to LCs (peripolesis) in the epidermis (39). Peripolesis was observed in guinea pigs as early as 3 to 5 hours after secondary challenge (40), but was not observed in primary irritant reactions or normal skin. The apposition of lymphocytes was associated with LCs showing signs of damage (shrinkage of the plasma membrane, vacuole formation, and even lysis). These observations, along with the *in vitro* data, strongly suggest that LCs may present antigen locally in the delayed type hypersensitivity (DTH) reaction. Unfortunately the difficulties in predicting recurrence in man together with the lack of a satisfactory model of recrudescence in animal models means that analogous experiments have not been performed with HSV. Nevertheless, since the secondary immune response to HSV is also primarily a DTH reaction it seems reasonable to assume that similar

mechanisms may operate. If this is the case then the extreme speed of these changes suggests that a protective T-cell response may be active in the epidermis soon after HSV has entered the site (usually from the peripheral nerves) and before one full round of virus replication has occurred. Therefore, the local reaction mediated by LCs is liable to present a potent barrier to the "secondary" infection which gives rise to recrudescent disease.

Why therefore is recrudescent infection with HSV-1 so common? Previous work has shown that in mice effective "triggers" of recrudescence must act not only to reactivate HSV in the ganglion, but also to create an environment in the skin favourable for viral replication (12). It is noteworthy that two such "triggers", UV light (41) and stripping with cellophane tape (42), have also been shown to decrease LC numbers *in vivo* (43,44). Moreover, treatment of LCs both *in vivo* and *in vitro* with UV light has been shown to reduce their ability to stimulate T-cell responses to HSV (44). However, some caution is necessary before concluding that the LC is the key which determines whether asymptomatic recurrence or symptomatic recrudescence follows reactivation. Both cellophane tape stripping and exposure to UV light, even at sub-erythemal doses, cause pathological changes in the epidermis. In addition, the use of different wavelengths of UV light allowed Noonan *et al* (45) to show that immunological unresponsiveness to contact sensitisers was maximally induced at a wavelength distinct from that which caused an apparent depletion of LCs and gross epidermal damage. Instead, the unresponsiveness appeared to be closely linked to the cis-trans conformational shift in urocanic acid (see chapter by Howie *et al* in this book). This highlights the problem that *in vivo* stimuli have multiple effects all of which need to be considered before any one conclusion can be reached. The work outlined below illustrates this point.

The effects of agents, that trigger reactivation of HSV, on Langerhans cells, and zosteriform viral infection

The studies of Harbour et al (12) demonstrated that in addition to cellophane tape stripping and exposure to UV light, topical application of xylene, retinoic acid, or dimethyl sulphoxide (DMSO) induced reactivation of latent HSV infection in sensory ganglia of the mouse. In some cases reactivation led to symptomatic recrudescence in the skin of the pinna. We therefore studied the effects of topical application of these three chemicals on numbers of LCs in the pinna of uninfected mice (46). Treatment of the pinna with DMSO reduced the numbers of LCs, as assessed by ATPase staining, by up to 99% within 24 hours. The numbers gradually recovered over the next 6 days. In contrast, treatment with xylene, or retinoic acid induced a smaller and more transient reduction in LC numbers. Interestingly a number of observations demonstrate that besides dramatically affecting numbers of ATPase-staining cells in the epidermis, DMSO also alters the severity of primary infection with HSV, as assessed in the zosteriform spread model. In these experiments DMSO was applied to the right pinna (the site reached by zosteriform spread) 2 days after inoculation of the right side of the neck with HSV. Firstly, titres of virus were assessed and it was found that titres in the pinna of treated mice were consistantly higher than those of the untreated controls (Fig. 1), though the difference was significant only on day 5 ($p < 0.01$). Secondly, whole epidermal sheets from the pinna were examined for foci of viral antigens by the peroxidase anti-peroxidase (PAP) method (Fig 2). On every day tested the numbers of PAP-staining foci in treated animals was higher than those in untreated. However, the differences were never significant due to the large variation between mice. Finally, clinical signs of disease (severity of erythema, numbers of lesions on the pinna, and neurological illness) were more severe in mice which had been treated with DMSO (Fig 3). In contrast, treatment of the pinna contralateral to the inoculation site had no effect on disease signs. Hence the

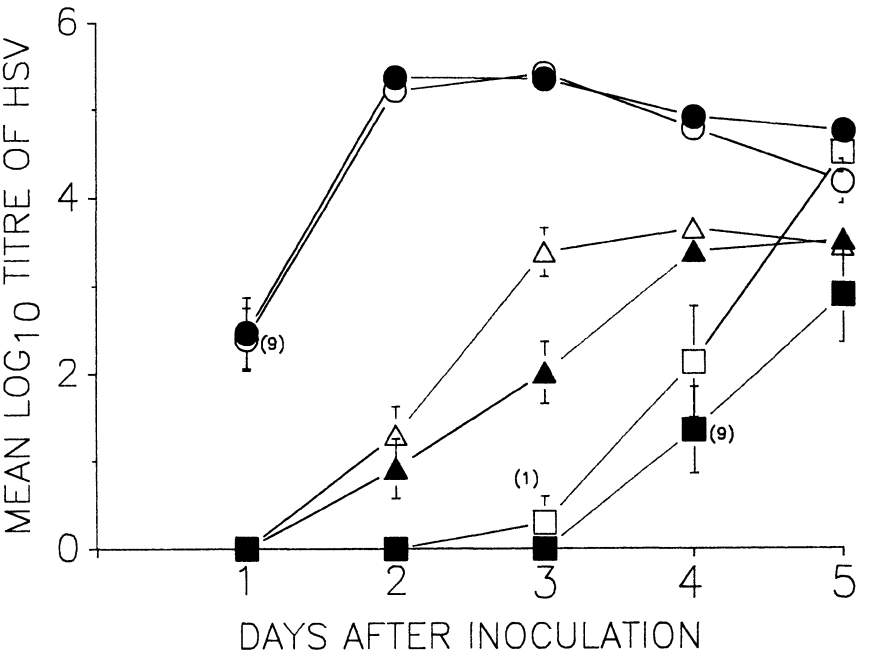

Fig. 1. Mean log titres of virus in the right neck (circles), right cervical ganglia (triangles), and right pinna (squares) of mice inoculated with HSV on the right neck. Groups of animals (n = 10 per day per group) were either untreated (closed symbols), or treated with DMSO (open symbols) on the right pinna on day 2. Figures in brackets indicate the numbers of animals in which virus was detected where not all were positive.

effects of DMSO did not appear to be induced systemically.

Some caution is necessary in interpreting these results in terms of the effects of DMSO on LCs. Thus the levels of virus in the ganglia supplying sensory nerves to the pinna were higher in treated animals than those in untreated controls. Moreover, the enhanced replication in the nervous system occurred before the increased viral titres in the pinna of the treated mice were detectable (Fig 1). It is difficult to imagine how such a change could be the result of alterations to LCs. An effect of the drug on the nerve endings in the skin, and thereby the neurons in the ganglia,

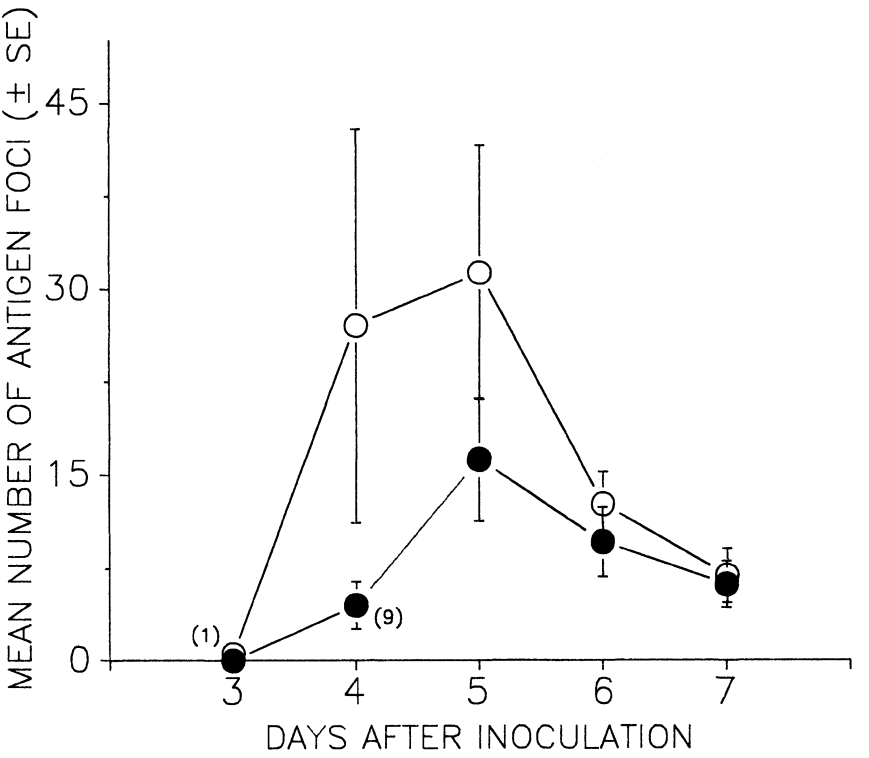

Fig. 2. Mean numbers of HSV antigen foci on whole epidermal sheets from the right pinna (n = 10 per day per group) of mice following inoculation of the right neck with virus. Animals were either untreated (●), or treated with DMSO on the right pinna 2 days after inoculation (○). Figures in brackets represent the number of epidermal sheets with staining foci if not all were positive.

seems a more likely explanation. An alteration in virus/neuron interaction may also explain the ability of DMSO to reactivate the latent infection (12).

Therefore the consequences of treatment with DMSO on zosteriform infection with HSV cannot be entirely attributed to alterations of LCs. Indeed the action of DMSO in the epidermis and on sensory neurons may be complex and indirect since the inflammatory response this chemical induces locally involves the release of serotonin, 5-HT, and a neurogenic

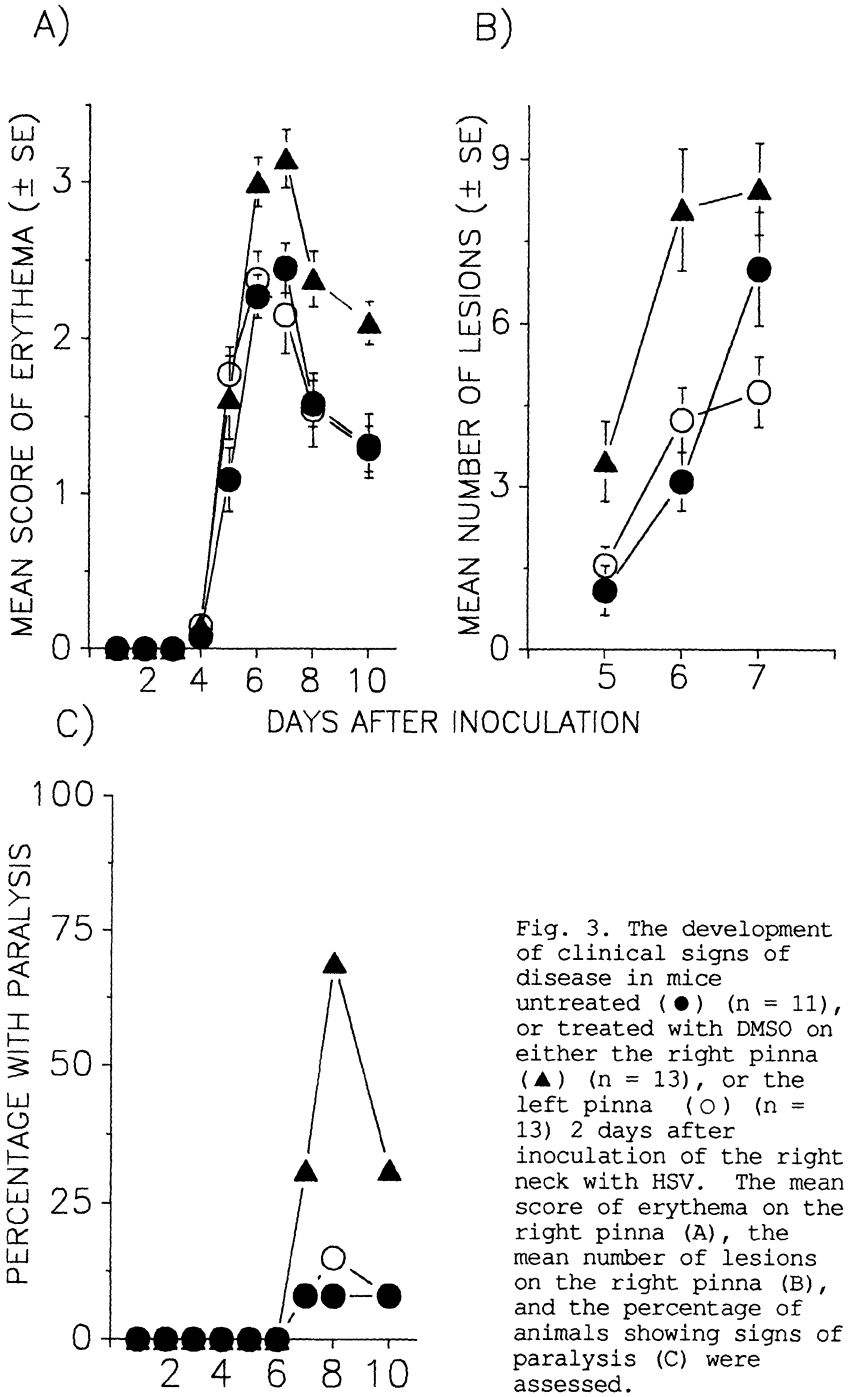

Fig. 3. The development of clinical signs of disease in mice untreated (●) (n = 11), or treated with DMSO on either the right pinna (▲) (n = 13), or the left pinna (○) (n = 13) 2 days after inoculation of the right neck with HSV. The mean score of erythema on the right pinna (A), the mean number of lesions on the right pinna (B), and the percentage of animals showing signs of paralysis (C) were assessed.

agent (probably substance P) (12). However, such problems are not exclusive to the action of DMSO. Other agents employed specifically to deplete LCs from the epidermis also cause the release of inflammatory mediators. For example, subcutaneously injected hypertonic saline in conjunction with abrasion of the skin with sandpaper (47), the injection of prednisolone (36), and even sub-erythemal doses of UV light (48).

Table 1. Effects of drugs on latent infection with HSV and on Langerhans cells in the skin.

	Treatment[a]		
	DMSO	Xylene in ethanol (50%)	Retinoic acid in acetone (0.01%)
Mice with reactivated HSV in the sensory ganglia (%)[b.]	29	12	17
Mice with recurrent HSV in the pinna (%)[b.]	28	44	13
Mice with signs of recrudescent disease (%) [b.]	13	37	30
Number of ATPase-bearing epidermal cells (% of untreated controls) [c.]	6	84	86
T cell proliferation induced by LCs (% of control) [d.]	98	0	43

[a] to skin of right pinna.
[b] Harbour et al (12).
[c] Williams et al (46).
[d] control value (100%) produced by LCs taken from the pinna of untreated animals.

A further note of caution arises from electron microscopical studies of epidermis from the pinna of mice treated with DMSO (N. A. Williams and K. M. S. Townsend unpublished data). At times when ATPase-staining cells were severely depleted, LCs, as determined ultrastructurally, were still present, although many appeared damaged. Similar observations have been made following exposure to UV light (49). From such observations it is clear that many of the treatments used experimentally may not cause a true depletion of LCs from the epidermis. For this reason it is important to support *in vivo* observations with *in vitro* analysis of LC function. Hence, epidermal cell suspensions from the pinna of mice treated 1 day previously with known chemical "triggers" of viral reactivation were used to present HSV to primed T cells. DMSO, which had produced the most dramatic effect on ATPase staining, did not alter the functional activity of the LCs *in vitro* (46) (Table 1). In contrast, application of xylene, or retinoic acid, each of which had only a small effect on LC numbers, reduced the ability of epidermal cell suspensions to stimulate a proliferative response to HSV by 100%, and 57% respectively. Therefore the functional data appears to conflict entirely with that from histological analysis.

In seeking to reconcile these conflicting observations it is interesting to compare the effects of xylene, retinoic acid, and DMSO on LC function *in vitro* with their effects on latent infection using the mouse ear model. Harbour *et al* (12) assessed the ability of these chemicals to induce reactivation, recurrence, and recrudescence (summarised in Table 1). Following the application of xylene to the skin of the pinna (the original site of inoculation), reactivated HSV was detected in the ganglia of only 12% of the mice, yet 44% had virus in the pinna, and 37% showed signs of recrudescent disease. Treatment with retinoic acid elicited reactivation in the ganglia of 17% of the mice, recurrence of virus in the pinna of 13%, and recrudescent lesions in 30%. In contrast, DMSO induced reactivation in the ganglia of 29% of animals,

recurrence in the ears of 28%, yet only 13% exhibited signs of recrudescent disease. Therefore xylene, which appeared to be a relatively poor "ganglion trigger", induced the highest incidence of recrudescence, whereas DMSO, which was the most effective "ganglion trigger" induced the lowest recrudescence. The ability of these agents to induce reactivation in sensory neurons may result from their capacity to damage, and change the membrane permeability, of the neurite endings in the epidermis. However, it is clear that the induction of recrudescent disease does not correlate with the ability to induce reactivation, nor even recurrence. It appears that besides getting virus to the epidermis, effective "triggers" of recrudescence must create a favourable environment for viral replication in the periphery. It is therefore interesting to note that the comparative abilities of xylene, retinoic acid, and DMSO to induce recrudescent disease correlates directly with their relative capacity to inhibit presentation of HSV to T cells by LCs. However, once again some caution is necessary to exclude the possibility that other effects of the chemicals do not correlate in the same manner. It is likely that the effect of any putative "skin trigger" must persist for at least 2 days as virus is not detectable in the skin until this time. It is noteworthy that inflammatory mediators such as histamine, 5-HT, and substance P which are released following application of the chemicals appear to be cleared before virus enters the epidermis (12). However, altered levels of prostaglandins E (PGE) and F were detected over a period of several days. The fact that PGE levels were depressed following application of DMSO, but were raised after treatment with retinoic acid or xylene led Harbour et al to propose such raised levels of prostaglandins as a probable "skin trigger" (12). PGE has a number of effects, including enhancement of viral replication in culture (50). However, it has also been shown that PGE depresses accessory cell activity in peritoneal macrophages (51). If PGE acts similarly on LCs this may be one of the biochemical mechanisms by which the stimuli of recrudescence depress the

immune response in the epidermis thereby allowing the establishment of symptomatic disease.

In conclusion it has been shown that the role of LCs in primary immune responses may differ from that in secondary immune responses to epidermally encountered antigens such as HSV. In the primary reaction their activity may be restrained to allow a more centralised, multifaceted reaction. However, upon secondary challenge evidence suggests that LCs are capable of initiating a rapid local T-cell reaction. Although the precise mechanisms require much further investigation, it appears that the ability of agents to promote symptomatic recrudescence, as opposed to asymptomatic recurrence, may be linked to depression of the local immune response due in part to inhibition of LC activity.

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12

UV TREATMENT OF SKIN: EFFECT ON LANGERHANS CELLS AND HSV-1 INFECTION

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ABSTRACT

The effects of ultraviolet (UV) irradiation of the skin on herpes simplex virus (HSV) infection were investigated. Exposure of the skin to UV-B resulted in a transient dose-dependent reduction of the number of Ia-positive epidermal cells (EC). This decrease of Ia antigen expression of EC was accompanied by a parallel loss of antigen-presenting capacity of EC, which was represented by HSV-induced T-cell proliferative response stimulated by EC. Immune T cells antigen-stimulated in the presence of normal EC effectively cleared HSV from intradermally infected nude mice, while those antigen-stimulated in the presence of UV-irradiated EC did not. In addition UV irradiation of mice at the inoculation site of HSV increased the severity of acute infection and the incidence of latency. These findings indicate that in vivo UV irradiation of the skin abrogates the immune function of EC both in vitro and in vivo, and affects HSV pathogenesis.

INTRODUCTION

Herpes simplex virus (HSV) infection is characterized by its unique course. The virus produces a primary lesion in the skin and travels up the sensory neurons to the ganglion where it remains latent. In some individuals, the virus is reactivated and may cause a recrudescent lesion at intervals.

Animal models that closely approximate human HSV infection have been difficult to develop. If there were, they would provide much information about the pathogenesis of this disease. Thus immune mechanisms of HSV infection have frequently been

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examined with cells (1, 2, 3) from peripheral blood of human patients or with subcutaneous (4) or intraperitoneal (5, 6) animal-inoculation systems except with a few intracutaneous animal-inoculation systems (7, 8, 9, 10). But HSV is an epidermal pathogen and, according to the "skin trigger" theory (11), the recurrence is supposed to arise from a depression of local defense mechanisms. Besides as indicated in the mouse footpad (12, 13), there is a possibility that HSV may exist in a latent state in the skin of man. Accordingly local immune system in the skin will play an important role in controlling primary and latent HSV infection.

Mouse epidermis contains immunologically active elements that include Langerhans cells (LC), keratinocytes and Thy-1-positive dentritic cells. Among these LC are the only cells that express Ia antigen within normal epidermis and, like other Ia-bearing cells, they are able to stimulate the proliferation of allogeneic T cells (14) and to present various antigens to T cells (15). Hence they have attracted attention in the fields of contact hypersensitivity (16, 17, 18), graft rejection (19, 20, 21) and viral infection (22, 23) as well. Furthermore several investigators have demonstrated that Ia-positive epidermal cells (EC), i.e., LC., have HSV-antigen-presenting capacity in vitro (24, 25, 26) and play an important role in the control of cutaneous HSV infection in vivo (24, 27, 28).

On the other hand, in natural infection, sunburn is known to lead sometimes to HSV spread in the manner of photodistribution (29) and to precipitate recurrent HSV infection (30). Experimentally, several animal (10, 30, 31) and human (32, 33, 34, 35) models of recurrent HSV infection induced by UV irradiation have been established. In the past few years, different experimental approaches have been undertaken to dissect the mechanisms underlying these effects of UV irradiation on HSV infection: 1) some noticed the defect of antigen-presenting function of LC (25, 36); 2) others noticed the increaased production of prostagrandins (PGs) (31,37); and 3) still others noticed the induction of suppressor T cells (38, 39, 40) and/or the production of suppressor factors (38, 39, 41). Although the relative roles of each facet remain unclear, we

recently analysed the effects of UV from the aspect of EC-mediated immunity (42).

In this review, we will describe the methodology used for the preparation of murine EC and the effects of UV light on the character of EC, on the function of EC in HSV immunity and on the pathogenesis of HSV infection.

EXPERIMENTAL

Preparation of epidermal cells and UV irradiation

We prepared EC suspensions from non-irradiated or irradiated skin of mice to examine roles of EC in HSV infection and to analyse the mechanisms by which UV influence HSV infection through the function of EC. Although we intended to investigate especially the function of antigen presenting cells, i.e., LC, physical separation of LC from other EC was a difficult task in the mouse system and was, in our hands, not reproducibly successful. Thus our techniques

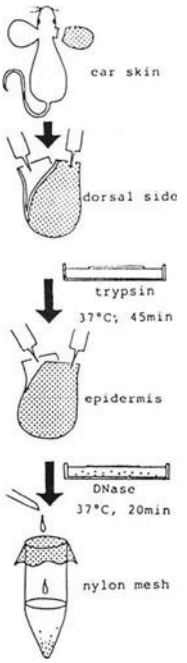

Fig. 1. Preparation of epidermal cells from mouse ear.

that have been used for preparation of EC are essentially the same as described by Stingl et al. (Fig. 1) (43). Cells were obtained from UV-B irradiated or non-irradiated nonimmune mouse ear skin. The ears were rinsed with ethanol, split with forceps and floated (60 min, 37 C) dermal side down on a solution of 1% trypsin in phosphate-buffered saline (PBS) at pH 7.2. The epidermis was separated from dermis and incubated for 20 min at 37 C on 0.025% DNase solution and teased to produce a single cell suspension. Then the viability was assesed by trypan blue exclusion test. In control non-irradiated mice, more than 80% of freshly prepared EC, which contained 5-10% Ia-positive cells as determined by indirect immunofluorescence technique, were viable. However, the EC suspension culture represented a "dying system" in that the viability of EC decreased rather sharply and progressively (per cent viabilities were 89, 62, 24 and 17 on days 0, 1, 3 and 7, respectively).

For UV irradiation, we chose an in vivo rather than in vitro approach, because an in vivo irradiation provided more natural conditions. Mice were anesthesized by intraperitoneal injection of sodium pentobarbitone and their ears were held immobile with adhesive tape. Immediately after these procedures, their dorsal surface was irradiated with various doses of UV-B at a constant angle of 90°. Other portions of the body were protected by a black cloth that did not allow any penetration of UV-B rays (Fig. 2).

The effects of UV irradiation on the skin

The effects of UV irradiation of the skin on the phenotypical

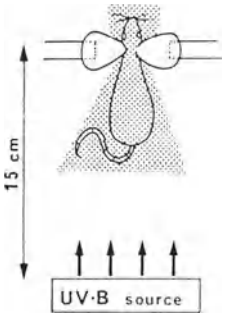

Fig. 2. UV irradiation of mouse ears.

character of EC were examined with mouse ears. Since mouse ear is scanty of hair, we could irradiate it without depilation and could easily observe the skin and, in addition, could assess the effect of UV without considering the influence of depilation. UV-B dose over 200mJ/cm^2 led to erythema of the irradiated ear. The UV-induced alteration in the number of Ia-positive EC in epidermal sheets was examined using immunoperoxidase technique. The epidermal sheets were separated from dermis with mechanical and chemical (25mM Na_4-EDTA) procedures. Ethanol-fixed sheets were incubated with anti-Ia antibody (dilution 1:100) and then with peroxidase-conjugated goat anti-mouse IgG (dilution 1:20). Peroxidase activity was detected by the 3-3'-diaminobenzidine tetrahydrochloride (DAB) reaction.

Fig. 3 shows the time course changes in the number of Ia positive EC after irradiation with suberythemal (120 mJ/cm^2) or erythemal (360mJ/cm^2) dose of UV-B. Ia-positive cells quickly

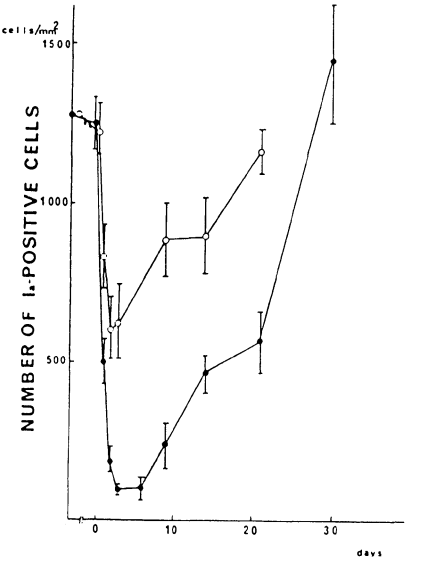

Fig. 3. Time course changes in the number of Ia-positive EC. Mice were irradiated on day 0 with 120 (○) or 360 (●) mJ of UV-B per cm^2 and at various times after irradiation, the density of Ia-positive EC in epidermal sheet preparations was determined. Date represent the mean ± SD. (Reprinted with permission from ref. 42).

decreased in number, reaching a nadir between 2 and 3 days, and then gradually recovered. After 20 to 30 days, their density was virtually the same as that encountered in control mice. Besides, as noted in Fig. 4, the decrease of Ia-positive cells was according to the administered UV dose. Remaining Ia-positive cells were frequently bigger and displayed more elongated dendritic processes than normal counterparts.

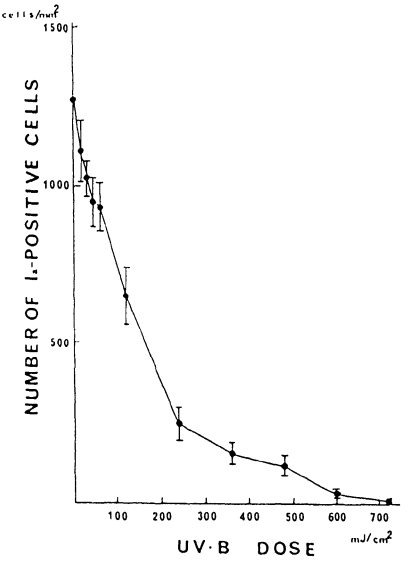

Fig. 4. Dose response of UV-induced depletion of the number of Ia-positive EC. Mice were irradiated with various doses of UV-B 3 days before preparation of the epidermal sheets and the density of Ia-positive EC was determined. Data represent the mean ±SD. (Reprinted with permission from ref. 42).

The effects of UV irradiation on HSV infection mediated through EC

1) In vitro studies: Several studies have shown that HSV-antigen-presenting capacity of EC is impaired by UV-B irradiation (25, 36). To evaluate their HSV-antigen-presenting capacity, EC were assayed for HSV-induced T cell proliferation stimulated by EC (non-irradiated or irradiated in vitro) (25), as well as for HSV-induced antibody producation stimulated by EC (obtained from skin non-irradiated or irradiated in vivo) (36). Alternatively, we examined the HSV-induced EC-mediated T-cell

proliferative response by using EC obtained from skin non-irradiated or irradiated in vivo and studied its time course changes after UV irradiation or its UV-dose response.

Antigen-primed T cells ($4x10^5$/well, immune splenocytes depleted of Ia-positive adherent cells) and mitomycin C-treated EC ($2x10^4$/well, obtained from either irradiated or non-irradiated mouse ear skin), were incubated with HSV antigen ($4x10^6$ PFU/well, calculated before inactivation) at 37C for 5 days. The amount of ^{3}H-thymidine (^{3}H-TdR) uptake during the final 24 hr of incubation was assayed.

Fig. 5 shows that the EC-mediated proliferative response, which reflects the ability of EC to present HSV antigens, was transiently suppressed by suberythemal or erythemal dose of UV irradiation of the skin and the suppression was maximum between 1 and 3 days after

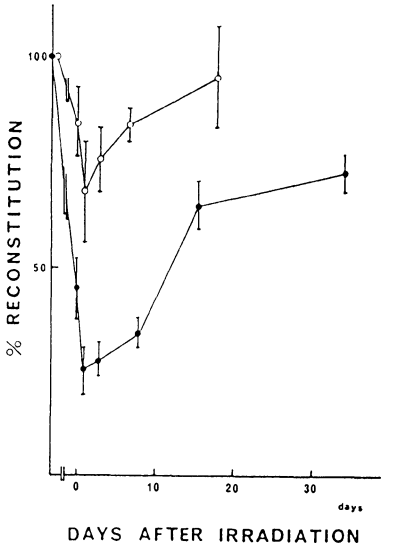

Fig. 5. Time course of UV-induced reduction of the EC-mediated lymphocyte proliferative response. Nonadherent spleen cells ($4x10^5$/well) were cultured with EC ($2x10^4$/well), which were obtained from mice irradiated on day 0 with 120 (○) or 360 (●) mJ/cm^2 UVB at various times after irradiation, and UV-inactivated HSV antigen ($4x10^6$ PFU/well) for 5 days and stimulation was assessed by incorporation of ^{3}H-TdR. The percentage reconstitution is the ^{3}H-TdR incorporation expressed as the percentage of the incorporation detected in control cultures (EC were obtained from nonirradiated mice). Data represent the mean ±SD. (Reprinted with permission from ref. 42).

irradiation. Also the time-course change of the function of EC correlated with that of the number of Ia-positive cells (Fig. 3 and 5). Dose-response effect of UV-B on the accessory cell function of EC (Fig. 6) was examined using EC taken 3 days after UV-B irradiation, when the ability of the skin to handle HSV antigen is markedly reduced. The impairment of antigen-presenting function was dose-dependent and was closely associated with the reduction in Ia-positive cell number (Fig. 4), excepting that the former appears to reach plateau with UV dose higher than 360mJ/cm^2.

Although our data indicate that the UV-induced functional deficit of EC is paralleled by the disappearance of surface Ia antigens, we must be careful to reach the conclusion that UV-induced inhibition of HSV-antigen-presenting capacity of EC is caused only by the reduction of Ia antigen. Considering that it has recently

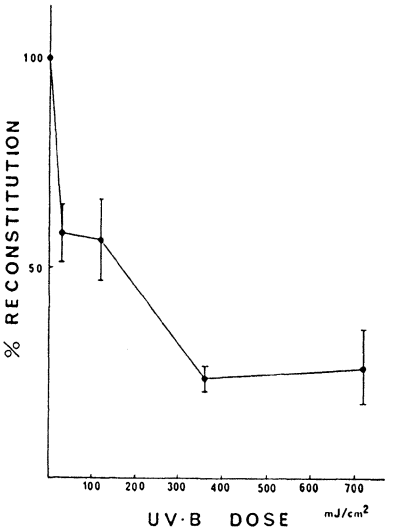

Fig. 6. Dose response of the UV-induced reduction of the EC-mediated lymphocyte proliferative response. Nonadherent spleen cells ($4x10^5$/well) were cultured with EC ($2x10^4$/well), which were obtained from mice irradiated with various doses of UV-B 3 days before preparation of EC, and UV-inactivated HSV antigen ($4x10^6$ PFU/well) for 5 days and stimulation was assessed by incorporation of ^{3}H-TdR. The percentage reconstitution is the ^{3}H-TdR incorporation expressed as the percentage of the incorporation detected in control cultures (EC were obtained from nonirradiated mice). Data represent the mean ±SD. (Reprinted with permission from ref. 42).

been shown in the field of contact hypersensitivity that the suppression of antigen-presenting function is not always associated with the reduction of Ia antigen expression (18, 45), we must also consider the effects of either EC other than LC, i.e., keratinocytes and/or Thy-1-positive cells, or soluble factors.

2) In vivo studies: Nude mice challenged intracutaneously with HSV-1 virulent strain and given no cells develop local skin lesions, followed by the appearance of zosteriform lesions and die within 3 weeks due to encephalitis. Yasumoto et al. (24) has indicated that the transfer of immune T cells stimulated by in vitro culture with HSV antigen in the presence of EC into intracutaneously infected nude mice markedly reduce the virus titer in the skin and inhibit the following formation of zosteriform skin lesions. We therefore investigated the protective activity of immune T cells stimulated with UV-irradiated EC using the same adoptive transfer system.

Immune T cells stimulated with either non-irradiated or UV-irradiated EC (obtained from the skin exposed to UV-B 2 days before preparation of EC) were injected i.v. into infected nude mice and the mice were compared with regard to the development of zosteriform skin lesions and mortality. As noted in Table 1, in the mice given T cells stimulated with normal EC and HSV antigen, development of skin lesions was completely inhibited and all of these mice recovered. On the other hand, immune T cells stimulated

Table 1. Protective activity of EC-stimulated T cells and its abrogation by UV irradiation of EC

Immune T cells cultured with	HSV Ag in culture	No. of mice with lesions / total	No. mice dead/total
Control		10/10 (100%)	10/10 (100%)
None	-	2/8 (25%)	1/8 (13%)
EC	+	0/8 (0%)	0/8 (0%)
UV-EC (120 mJ/cm^2)	+	2/6 (33%)	0/6 (0%)
UV-EC (360 mJ/cm^2)	+	5/8 (83%)	1/8 (13%)

Cultured immune T cells ($5x10^5$) were transferred to nude mice 7 hours after intracutaneous inoculation of the mice with $5x10^4$ PFU of HSV-1 Hayashida strain. (Reprinted with permission from ref. 42).

with irradiated EC could not effectively clear HSV and allowed a considerable number of mice the development of zosteriform eruption, although they protected most mice from death. Furthermore, contrary to our expectation, the incidence of the appearance of zosteriform lesions was higher in mice given the immune T cells stimulated with UV-irradiated EC rather than in those given the unstimulated cells.

Our results indicate that the stimulation of immune T cells with UV-irradiated EC may induce some suppressive immune response, thereby allowing virus to multiply sufficiently to produce lesions. In other words, UV-induced suppression of EC function in the secondary phase of immunity affects the pathogenesis of infection.

The effect of UV irradiation of the skin on the primary HSV infection

In order to examine the effect of UV-B irradiation on the pathogenesis of primary HSV infection, we inoculated mice with HSV under the conditions that markedly reduce the ability of EC to handle HSV antigen in vitro. The right midflank area of 7-weekold female BALB/c mice was depilated and exposed to UV-B (120 and 360mJ/cm^2) 2 days before challenge (Fig. 7).

The development and severity of skin lesions were scored each day and given the following numerical designations: 0, no lesion; 2, local lesion (vesicle or erosion); 6, mild zosteriform lesion; 8, moderate zosteriform lesion; 10, severe zosteriform lesion; and death. Table 2 indicates that mice irradiated with UV-B develop zosteriform skin lesions more frequently and show increased severity

Table 2. Effect of UV-B irradiation on the course of acute infection and the incidence of latency*

UV-B dose mJ/cm^2	No. mice with lesions/total	No. mice dead/total	Mean score ± SD	Incidence of latency No./survivor**
0	9/19 (47%)	3/19 (16%)	5.1±3.4	6/16 (38%)
120	12/18 (67%)	6/18 (33%)	6.7±3.5	5/12 (42%)
360	18/19 (95%)	13/19 (68%)	9.4±2.0	6/6 (100%)

* Mice were inoculated with 2.5x10^4 PFU of HSV-1 intracutaneously 2 days after UV-B irradiation.
**Number of mice yielding virus from ganglia by cocultivation with Vero cells/number of mice surving longer than 50 days after infection. (Reprinted with permission from ref. 42)

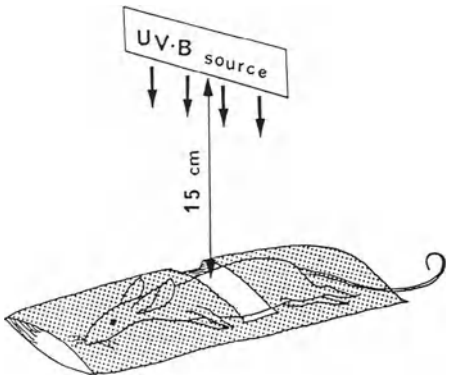

Fig. 7. UV irradiation of midflank of mouse.

of infection compared with non-irradiated control mice. In addition, in UV-irradiated mice the skin lesions developed more rapidly. Second, the amount of virus in tissue homogenates from control and UV-irradiated mice was examined at various intervals after infection. Fig. 8 shows that UV irradiation of the skin increases the severity of infection as the result of the delayed viral clearance from the skin and enhanced viral dissemination to the central nervous system. Finally, mice surviving longer than 50 days after infection were sacrificed and were examined for the existence of latency in the dorsal root ganglia. UV-irradiated mice showed higher incidence of latency than nonirradiated control mice (Table 2).

Similar results concerning the effects of UV irradiation of the skin on the pathogenesis of primary HSV infection were demonstrated by Harbour et al. (46) and Yasumoto et al. (38). The former attributed the effects of UV-B to the increased production of PGs and the latter to the generation of suppressor T cells. However, it is unreasonable to attribute the effect of UV to a single cause. We also cannot limit the cause of UV-induced increase in the severity of primary HSV infection indicated in our experiments only to the UV-induced suppression of EC function in the primary phase of immunity. In fact it is appropriate to consider that several factors that follow irradiation would contribute the production of

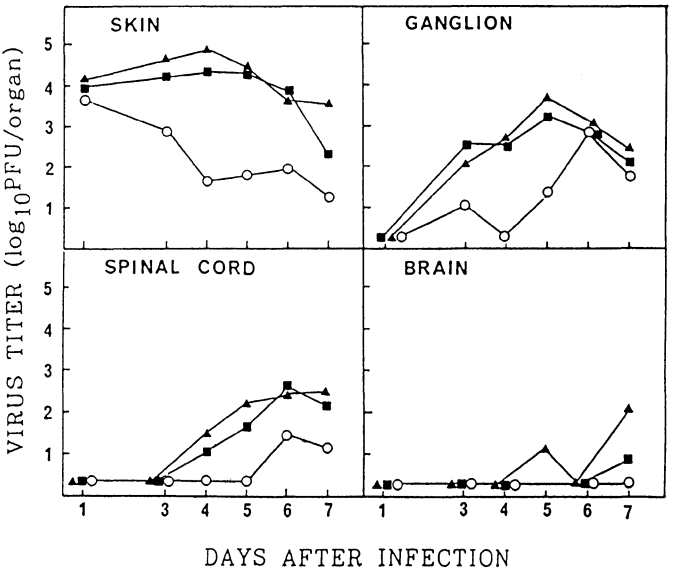

Fig. 8. Effect of UV-B irradiation on viral growth in mouse tissues after intracutaneous inoculation with HSV-1. The midflank area of mice was irradiated with 120 (■) or 360 (▲) mJ of UV-B per cm^2. Control mice (○) were not exposed to UV-B. Two days after irradiation the mice were inoculated with 2.5×10^4 PFU of HSV-1 intracutaneously at the irradiated site. Each point represents the geometric mean titer of virus from 3 mice. (Reprinted with permission from ref. 42).

the conditions favorable for virus growth and would affect the pathogenesis of primary HSV infection.

DISCUSSION

UV light sometimes lead the herpetic lesion to spread over the irradiated area (photodistribution). It also can act as a stimulus to a recurrent herpes simmplex in experimentally infected mice (10, 31) as well as in humans (32, 33, 34, 35). The mechanisms for these phenomena are not clearly understood but there are several possible explanations for the UV-induced enhancement of HSV virulence: 1) UV-induced impairment of the function of EC (25, 36), in particular the antigen-presenting function of LC, would suppress the defense mechanisms; 2) increased production of PGs (31) of LC, would raise the susceptibility of tissue or suppress the immune mechanisms; and

3) the generation of suppressor T cells or soluble immunosuppressive factors would suppress the specific or non-specific defense mechanisms (40, 41). Probably, each of these causes would be relevant to the mechanisms for the UV-induced alteration in HSV pathogenesis.

We investigated how in vivo exposure of the skin to UV would influence HSV infection through the impairment of the accessory cell function of EC. As indicated by transfer experiments, UVinduced suppression of EC function in the secondary phase of immunity render immune mechanisms ineffective and consequently allow the viral multiplication. According to the "skin trigger" theory, subclinical foci of infection frequently occur in the skin. If virus is supplied to the skin by reactivation when EC function is suppressed by UV irradiation, the situation would be similar to that in our experiments and, as a result, clinically apparent lesions might be produced. Accordingly UV induced impairment of EC function and the breakdown in HSV immunity in the skin may be an underlying mechanism for recurrence. In addition, mice primary infected with HSV under conditions that impair EC function in the primary phase of immunity show increased severity of primary infection and a higher incidence of latency compared with control mice. Furthermore, Norval et al. (10) has recently shown that if mice are primary infected with HSV at the time when the antigenpresenting function of EC has been impaired by UV irradiation, the incidence of recrudescence increases after further stimuli. Collectively, UV-induced suppression of antigen-presenting function would affect the pathogenesis of recurrent infection if it occurred at the time of reactivation and would affect the severity of the primary infection, the establishment of latency and the frequency of the following recrudescences if it occurred at the time of primary infection. Since the reasons why the frequency of recrudescence is in great variety among seropositive persons are not clear as yet, the notion that the initial immune response mediated through EC function somehow influences the outcome of the following course of HSV infection is particularly attractive.

On the other hand, UV light may alter several immunological

functions of EC other than antigen-presenting function of LC which we have demonstrated. In herpetic skin lesions keratinocytes are considered to express HLA class II antigen (47) and to act as antigen-presenting cells (47) and are demonstrated to produce β-interferon (48). These functions might be influenced by UV irradiation. Besides keratinocytes produce interleukin-1-like cytokine ETAF (epidermal cell-derived thymocyte-activating factor) whose production/secretion is reported to be adversely influenced (50) by UV-B except a few opposite reports (51, 52). Therefore the recently discovered Thy-1-positive dendritic cells would be related to the immune system, although their function is not clear yet. Some consider from phenotypical and functional studies that they are related to natural killer cells (53, 54) and others consider that they may be operative in the activation of suppressor mechanisms (55, 56). In any case, UV-B irradiation greatly reduces them (57) and may affect epidermal immune functions mediated by them.

Furthermore PGs (in particular E_2), which increase after UV irradiation (58), have been shown to suppress Ia expression (59), inhibit interleukin-2 production (60), reduce antibody-dependent cell cytotoxicity against HSV infected cells (61) and enhance the spread of HSV (62). Hence PGs would suppress the local immune mechanisms and increase the local susceptibility to HSV.

Finally, UV irradiation at the site of HSV infection has recently been shown to induce the generation of antigen-specific suppressor T cells (38, 39) and antigen-specific or -nonspecific suppressor factors (38, 39). It has also been shown that transfer of UV-irradiated EC with live HSV-1 leads to the generation of suppressor T cells rather than effector T cells for delayed hypersensitivity specific for HSV (63). This result indicates that the UV-induced T cell-mediated suppression of delayed hypersensitivity (64) is linked to the alteration in accessory function of EC. UV-induced depletion of the activity and density of LC may induce preferentially the formation of suppressor T cells to HSV antigen. Or else, the recently described I-J-positive, UV-resistant EC (65), which is indicated in contact hypersensitivity, may activate the suppressor cells in HSV infection

as well.

As has been noted, for the last few years, the effects of UV on HSV infection have considerably been investigated in various experimental systems. UV light would produce transient immunosuppression and/or increased susceptibility of EC to infection with HSV. These changes would be mediated through UV-induced alteration in the function of EC (in particular LC) and lymphocytes and in their production of biologically active substances. However, recrudescence of HSV has been shown to be triggered by a variety of stimuli as well as UV light, including colds, fever, menstruation, physical trauma and stress. Nonetheless there have been very few investigations on the effects of stimuli other than UV on HSV infection.

Our task is now to make clear the relative roles of the various immunological factors that follow UV irradiation in the control of HSV infection and, if minor impairments in immune system are involved in the cause of recurrent herpes simplex, to identify the mechanisms common to various stimuli. Such efforts may lead in the future to the development of new strategies which can inhibit HSV recrudescence or even eradicate latent infection.

ACKNOWLEDGMENTS

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13

UV TREATMENT OF THE SKIN AND HSV-1 INFECTION

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ABSTRACT.

Sudden exposure to ultraviolet-B radiation is a well known trigger for recrudescent herpes simplex virus infection in some individuals. In this chapter the known effects of ultraviolet-B radiation on the biology and particularly the immune system of the skin are briefly reviewed. The effects of ultraviolet-B light on experimental herpes simplex virus infections are described and discussed in the context of the induced alterations in the immune response to the virus. The implications of this for virus infections of the skin are considered.

INTRODUCTION.

Exposure to ultraviolet radiation.

Ultraviolet (UV) radiation is divided into three bands according to wavelength, namely UV-A (320-400nm), UV-B (280-320nm) and UV-C (200-280nm). The major source of environmental exposure to UV radiation is sunlight. The relative intensities and wavelengths of solar radiation

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reaching the earth's surface vary depending on location and are affected by a number of parameters including atmospheric absorption, scattering and reflection. UV-C is effectively eliminated by the ozone layer, and is normally only encountered at extreme altitude.Various compilations of measured and calculated exposures of individuals to UV-A and UV-B have been made (1,2). In addition to natural, environmental exposure to UV radiation, individuals may also be exposed to UV-A and UV-B from artificial sources employed for cosmetic or therapeutic reasons (3).

UV radiation and skin carcinogenesis.

Case reports and epidemiological studies have implicated UV radiation in the aetiology of squamous and basal cell carcinomas and malignant melanoma of the skin in humans. Biological data relevant to the evaluation of the risks of UV exposure to humans began to accumulate with the first animal experiments, performed by Findlay in 1928 (4). This work demonstrated that daily exposure of albino mice to UV radiation from a mercury arc lamp induced skin cancer. Since then various studies of UV-radiation-induced carcinogenesis in animal models have been described (5,6). Single exposures to high doses of UV-B have also resulted in experimental skin tumours (7). Other studies have demonstrated mutagenesis in bacteria (8), yeasts and mammalian cells (9). Chromosomal damage to (10) and morphological transformation of (11) mammalian cells exposed to UV-B *in vitro* have also been reported. UV radiation remains implicated as a major causative factor in human skin carcinogenesis.

Effects of UV-B radiation on the skin immune system.

The skin has a complex immune system *in situ* (12). The major responses normally generated by epidermal antigen exposure are the T lymphocyte - dependent hypersensitivity reactions (cell mediated immunity) and antibody production (humoral immunity). Thus exposure to antigen at the level of the skin gives rise to both local and generalized immunity which will clear antigen/pathogen both at the site of infection and systemically.

Numerous studies have documented the effects of UV radiation on the immune system of the skin (12,13). *In vivo* exposure of mice has been shown to induce systemic immunological alterations which are important in the pathogenesis of skin cancer, independent from its carcinogenic effect *per se* (14). Similar systemic disturbances in immune function have been reported to be induced by UV radiation in the context of cell - mediated immune responses to contact sensitizers in mice (15-17) and man (18), photosensitisers (19), parasitic protozoa (20), yeasts (21), histocompatibility antigens (22,23) and herpes simplex virus (HSV) (24). UV - radiation - induced reduction in antibody levels has been reported for contact sensitization (25), but we were unable to confirm this in the HSV system (26). It remains unclear whether epidermal exposure to UV-B can affect the level of antibody responses generated

against antigens encountered by the skin.

The cellular interactions within the immune system involved in UV - mediated induction of unresponsiveness have been investigated in a number of systems. In the experimental models which have been studied the UV - induced lesion in the immune response has resulted in the generation of antigen - specific suppressor T lymphocytes (Ts cells). The cause of this aberrant response to antigens which under normal conditions give rise to positive immunity appears to be due to an alteration in the antigen presenting capacity of the epidermis (15,17,27-29).

Antigen presentation is normally the function of specialized bone marrow - derived cells, predominantly those of the monocyte/macrophage lineage. Antigen - presenting cells (APCs) are characterized by their constitutive expression of cell surface determinants coded for by the species Major Histocompatibility Complex (MHC), the class II MHC antigens. APCs internalize antigen, process it and re-express it at the cell surface in association with MHC II determinants. It is in this form that specific receptors on the surface of T helper lymphocytes (Th cells) interact with antigen and initiate humoral and cell mediated immune responses. Other skin cell types, including dermal fibroblasts (30), keratinocytes (31) and melanocytes (32) can be induced to express MHC II antigens under the control of cytokine (interferon gamma) released

as a consequence of immune stimulation by effector T lymphocytes. Under such circumstances these cells may also act as local APCs and amplify immune responses locally. Interferon gamma is also implicated in regulating lymphocyte traffic through the skin to sites of antigenic stimulation (reviewed in 33) and is thus of especial importance in epidermal immune reactions.

The major APCs of the epidermis are MHC II+ve epidermal Langerhans cells (LCs), reviewed in 12. Exposure of mice to UV radiation has been reported to induce migration of LCs out of the skin, to alter their morphology in situ (34,35), to diminish their ability to act as APCs (15,28), and to deplete LCs of their surface markers (36). Any or all of these effects may result in alterations of the immune response to antigen encountered by the epidermis.

In 1984 it was reported that the alterations in epidermal density and morphology of LCs after UV irradiation occur at a different wavelength from that which induces systemic suppression of contact hypersensitivity (34,35). De Fabo and Noonan (37) demonstrated that the UV wavelength at which suppression of immunity was generated coincided partially with that at which DNA damage occurs and wholly with the wavelength at which a major skin component, urocanic acid (UCA), undergoes isomerization from the trans- to the cis- form. The photochemical properties of UCA have been reviewed elsewhere (38). De Fabo and Noonan proposed that the interaction of cis-UCA with epidermal APCs could alter their

function such that Ts rather than Th lymphocytes were induced when antigen was subsequently encountered (37). Recently it has been reported that the pyrimidine dimers formed when DNA is damaged by UV-radiation can also induce suppression of epidermal immune responses (39).

In 1984, Granstein et al (40) reported that UV-B irradiation induced the function of a novel epidermal APC which caused T lymphocyte suppression of contact hypersensitivity. This cell was not a LC, and expressed I-J antigens, thought to be important in the generation of Ts cells.

The actual molecular and cellular events surrounding the UV - induced alterations in epidermal APC function remain unclear to date. However, the biological consequences of such alterations may have profound effects upon individuals, particularly those encountering or harbouring persistent epidermal pathogens such as HSV. Indeed one of the factors reported to trigger recrudescence of human HSV infection is sudden exposure to UV-B light. UV-B light has also been reported to induce recurrence of experimental murine infection with HSV (41). For these reasons we undertook a series of investigations into the nature of UV-B induced alterations of immune responses to HSV type 1 (HSV-1) in a murine model.

UV RADIATION AND HSV INFECTION.

UV - radiation and natural HSV infections.

HSV-1 is a common pathogen of humans with up to 75% of adults being latently infected depending upon the population studied (42,43). Infection normally manifests itself as groups of sores or blisters on the skin, commonly involving the lips, but occasionally occurring around the eyes, nose or other body parts. Primary infection usually occurs early in life, at one to four years of age, and is followed by the establishment of viral latency in the CNS neurones innervating the dermatome of the original infection. In a proportion of infected individuals the virus may reactivate and recrudesce at the original site on one or more occasions throughout life despite the presence of an apparently strong anti-viral immmune response (43). The course of events leading to recrudescence is not clear, but it may be triggered by internal hormonal factors such as menstruation and stress and by external environmental factors including sudden exposure to UV-B irradiation (44,45).

The role of UV-B irradiation in human primary infection is unknown although it has been postulated (vide infra) that it may alter the initial interaction of the immune system with the virus and thus affect the nature and efficacy of the long term immunity generated against the persistent infection. Similarly UV-B irradiation may trigger the secondary recrudescent

form of infection by transiently suppressing the immune system within the epidermis and allowing reactivated virus to replicate sufficiently to form "cold sores".

Effects of UV-B radiation on experimental HSV infections.

Following from the observations that UV exposure may induce recrudescence in human HSV infections, Blyth et al (41) demonstrated that a similar phenomenon happened in experimental murine infection. Outbred mice, infected by painting virus onto scratched ear skin, developed primary lesions which were demonstrated to contain replicating virus. After resting such animals for varying periods of time they exposed them to UV-B light and found that a small proportion of animals (approximately 15% of those treated) showed reddening of the ear skin from which virus could once again be isolated.

In 1986, we demonstrated in genetically defined, inbred mice of the C3Hf Bu/Kam strain that sub-erythemal UV-B exposure (96mJ per sq cm) 3 days before subcutaneous inoculation with live HSV-1 induced suppression of the delayed hypersensitivity (DH) response on subsequent challenge with viral antigen (24). This suppression was antigen specific in that it did not cross react with immunity against the contact sensitiser picryl chloride.

At this time we also demonstrated that there was a fairly narrow time window after which

antigen exposure would result in suppression of DH. If virus was inoculated 14 days after UV-B exposure, no suppression was seen; at 8 days after slight suppression was seen; if virus was given immediately before UV-B exposure or 3 days before, no suppression of DH was found. Thus if virus infection occurred between 7 and 2 days after a single dose of UV-B, suppression of HSV-1 specific DH was generated, otherwise a positive DH response was seen on subsequent challenge with viral antigen. In contrast to the transient nature of the UV-B irradiation effect on the induction of DH suppression, animals in which this effect had been generated remained suppressed for HSV-1 DH for as long as tested (up to 3 months).

In order to investigate further the effects of UV-B exposure on the immune response to HSV-1 and in particular its effects upon epidermal APC function, we developed an *in vitro* assay for antibody production (28). The experimental system used APCs from normal and UV-B exposed C3H mice, HSV-1 specific Th cells from virus infected syngeneic animals and B lymphocytes from C3H mice immunized *in vivo* with trinitrophenylated calf erythrocytes. These cells were cultured *in vitro* with trinitrophenylated-HSV-1 as antigen. This allowed us to examine the interactions between APCs and virus - specific Th cells by measuring the ability of the Th cells to induce antibody to the hapten trinitrophenol in a classical hapten-carrier system.

APCs derived from epidermal cell sheets and

the peritoneal cavity of normal mice and from animals exposed to UV-B 3 days previously were compared. Epidermal APCs were taken from both the site of irradiation and a distal site which had been protected from UV-B exposure.

These experiments showed that (i) skin cells from normal mice can present HSV-1 antigens in a MHC II - dependent *in vitro* antibody induction system; (ii) that pre-exposure to UV-B *in vivo* 3 days before use *in vitro* substantially reduces the ability of epidermal cells to present HSV antigens; (iii) that the same exposure does not affect the APC capacity of peritoneal cells from the same animals; and (iv) that the UV-B induced reduction in APC function of the epidermis is not local to the site of irradiation.

Having shown that UV radiation suppressed DH responses to HSV-1 and that the same exposure altered the APC capacity of epidermal cells there were several questions to be followed up. We first examined the nature of the DH suppression to determine whether no response at all had been generated or whether a cell mediated suppression had been induced *in vivo* (46). These experiments involved a series of cell transfers of spleen cells from mice infected with HSV-1 3 days after exposure to UV-B. These cells were treated in various ways to isolate lymphocyte subpopulations and then inoculated intravenously into syngeneic animals which had been infected with HSV in the absence of UV-B exposure, 24 hours before challenge with HSV-1 antigens was

administered to provoke a DH reaction; i.e. cells with putative HSV-1 specific Ts activity were transferred to animals which would normally give positive DH responses on challenge with viral antigens.

These experiments showed that the lack of DH reaction seen after UV-B treatment was indeed due to the presence of antigen specific Ts cells. Further, there appeared to be two disinct populations of Ts cells involved in the suppression. One Ts population had the classical CD8+ve, CD5-ve phenotype, whilst the other was CD8-ve, CD5+ve and was subsequently shown to be CD4+ve.

We then examined the role of epidermal APCs in the generation of the Ts cells in more detail (47). These experiments again involved cell transfers from UV-B irradiated animals to syngeneic untreated recipients. However, instead of transferring spleen cells, epidermal cells enriched for LCs were used. Mice were exposed to the standard dose of UV-B (96mJ per sq cm). Three days later epidermal cell suspensions were made from the ears of these animals. These epidermal cells were transferred to naive syngeneic recipients subcutaneously at the same time as, and in the same site as, live HSV-1. Under these conditions suppression of DH was generated in the recipient animals.

Transfer of epidermal cells from non-irradiated animals did not have the same effect, nor did transfer of epidermal cells from UV-B treated mice given at a distal site to live HSV-1

virus. In both these cases DH to HSV-1 was generated rather than suppression.

Thus the status of the APCs which originally came into contact with the virus in the intact animal determined the functional nature of the immune response generated. A single dose of UV-B irradiation appeared to be sufficient to alter the way in which epidermal APCs processed antigen in vivo 3 days later. These experiments showed that this was not restricted to HSV antigens as the same phenomenon was demonstrated with Semliki Forest virus. In contrast to the experiments described above (46) the antigen-specific Ts cells generated in these experiments were of a single phenotype, namely CD4+ve, CD8-ve.

The results of these experiments with HSV-1 indicate that there are at least two aspects to the effect of whole body UV-B irradiation in terms of immunity to antigens encountered by the skin. First the APC function of the epidermis is altered in such a way as to generate antigen specific Ts cells of the CD4+,CD8-ve phenotype in vivo, and also such that the APC capacity to induce antibody responses in vitro is substantially reduced. Second there is a non-epidermal, systemic effect which must be responsible for the generation of the CD8+ve,CD4-ve Ts cells which are found after whole body exposure to UV-B, but are not seen in those animals which received only epidermal cells from UV-B irradiated donors.

The implications of such alterations in

immunity to epidermal pathogens, particularly persistent infections such as HSV-1, may be profound. The ability of a single dose of UV-B irradiation to alter APC function 2-7 days later may be of importance in determining the host-persistent pathogen balance when virus is first encountered at this time. This may predispose such an individual to be more susceptible to recurrent and recrudescent episodes of infection. To test this hypothesis animals were exposed to UV-B or sham-irradiated 3 days before epidermal infection with live HSV-1 (48). Those animals which subsequently recovered from skin lesions containing virus were rested for several weeks. The groups were then divided and half the animals in each group were exposed to UV-B irradiation and minor skin trauma (4x tape stripping) and monitored for the appearance of viral lesions. The group which had received no UV-B at all showed no evidence of recrudescent infection. The groups which received UV-B only on one occasion showed a small number of recrudescences, comparable to the numbers reported by Blyth et el (41). However, the group which received UV-B before primary infection and again before the skin trauma treatment showed recrudescent lesions in 75% of animals. Thus infection with virus at a time when the skin immune function was altered by UV-B exposure did indeed predispose animals towards a greater tendency for recrudescence on subsequent exposure to the same stimulus. The morbidity associated with the infection is also increased with prior UV-B

exposure (26). Similar findings were reported by other workers (49).

DISCUSSION.

The mechanisms by which UV-B exerts its effects on the immune system remain unclear to date. Genetic factors related to the species MHC and unrelated to the pigmentation of the skin have been reported in the UV-B - induced suppression of contact sensitivity (50). The erythematous effects of UV-B exposure are also unrelated to its effects upon the immune system as shown by our own work using sub-erythemal doses of UV-B to study responses to HSV-1, that of others in models of contact hypersensitivity (27,34,35,37), and also recently from the work of Fisher et al (51) who showed that protecting the skin against erythema by the use of sunscreens did not affect the induction of suppression of contact sensitivity.

Many effects of UV-B on the skin have been described which are implicated in altered cellular functions of APCs in the skin. These include alterations in LC integrity, trafficking (34,35) or function (15,28), the generation of oxygen free radicals (52), the release of prostaglandins (53,54), reduction in the expression of MHC II antigens (36,55), reduction in interleukin 1 production (56), altered antigen processing (57) and effects on intercellular adhesion molecules (58).

Investigations of the initial event following

UV-B irradiation of the skin which may provide the UV-B induced signal for alteration of APC function have concentrated on two main areas : DNA damage due to the generation of pyrimidine dimers (59,60) and the generation of the cis-isomer of urocanic acid (UCA) (37). Evidence supporting both these photoformats as the means of altering antigen presentation has been advanced (37,39,61-65). In the context of epidermal virus infections no studies have been reported to study the influence of DNA damage on the host immune response.

We have reported a series of studies to investigate the effect of cis-UCA on the immune response to HSV in the murine model descibed above (61-64). Epidermal painting of mice with UCA of known cis : trans proportions before infection with HSV generates subsequent suppression of the DH response in mice (61). This is due to the generation of antigen specific Ts cells of two distinct phenotypes, one CD4+CD8- and one CD4-CD8+, (62). If, however, epidermal cells from cis-UCA treated mice are transferred to naive syngeneic animals, at the same time as and in the same site as live HSV-1, Ts cells of the CD4+CD8- phenotype only are generated (64). These results are analogous to those discussed above for UV-B irradiation <u>per se</u> (24,28,46,47). Systemic administration of cis-UCA (63), before epidermal HSV infection again results in suppression of the DH response to HSV with the induction of two distinct Ts subsets. Intriguingly the two theories regarding the

initial interaction of UV with the immune system and the subsequent cascade of events resulting in antigen - specific suppression may not be mutually exclusive. There is strong evidence that the cis-isomer of UCA has the ability to bind to DNA in a UV-dependent manner and a capacity to form photodimers with mammalian DNA (reviewed in 38).

In the context of epidermal HSV-1 infections the complex interactions of a persistent viral pathogen with a host may be further complicated by the effects of environmental UV-B exposure upon the immune system of the skin. In particular the alterations induced within the APC component of the skin (presumably Langerhans cells) may alter the state of immunological competence of the host at the time of primary infection. This may affect the establishment of the host - virus balance and predispose such individuals to recurrent infection on subsequent exposure to various stimuli including sudden UV-B exposure.

The molecular and cellular mechanisms underlying the avenue of response taken by the immune system of the UV-B exposed host in dealing with epidermal pathogens are not yet clear. However, it is hoped that an understanding of these areas may in the future provide a rational basis for better therapeutic approaches to many such problems.

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14

INVOLVEMENT OF EPIDERMAL AND LYMPH NODE DENDRITIC CELLS IN SKIN HSV-1 INFECTION

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ABSTRACT

The present article analyzes the role of Langerhans cells (LC) in the defense of the murine skin against herpes simplex virus type 1 (HSV-1) infection. Intradermal infection with HSV-1 resulted in an increase in LC numbers. Activation and increase in LC density by a streptococcal preparation, OK-432, was accompanied by a striking decrease in HSV-1-induced mortality in infected mice. Decrease in LC density led to an increase in the virulence of the virus and synthesis of viral DNA in the brain and the mouse footpad skin. LC were found to induce proliferation of HSV-1-sensitized T cells in vitro in the presence of the virus and were able to transfer viral material from the skin to the draining lymph nodes. HSV-1-sensitized T cells could be recovered from the draining lymph nodes following immunization in the footpad skin with live virus, but not when the immunizing virus was injected into the skin after depletion of LC.

INTRODUCTION

Two different types of dendritic cells (DC) are found in the murine epidermis: a) Langerhans cell (LC)-bearing Ia antigens (Ia^+ cells; 1) that display strong membranal ATPase activity (2) and function as potent antigen-presenting cells in the epidermis (3), and b) Thy-1^+ dendritic epidermal cells (Thy-1^+DEC) which are Thy-1^+, asialo GM1^+ (asGM1^+), Ia^-, Lyt-1^-, Lyt-2^-, L3T4^-, sIg^- and weakly Fc receptor$^+$ (4). These cells proliferate in response to concanavalin A (Con A) and interleukin-2 (5) and belong to an immature T-cell population (6-8).

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LC can be found in the skin and lymphatics (designated veiled cells), and in the thymus and lymph nodes (designated interdigitating cells: IDC) (9). LC function by engulfing the sensitizing agents in the epidermis and migrating to the lymph nodes as IDC where they trigger the immune response through specific interactions (clustering) with T lymphocytes in the paracortical zone of the lymph nodes in vivo (9). These clusters were recently isolated and characterized in vitro (10). IDC numbers have been shown to increase following antigenic stimulation and after sensitization through the skin, the sensitizing antigen was found exclusively in IDC (11,12).

In recent years, it has become clear that LC are involved in various infectious diseases (13-18). LC were shown to play an important role in the defense against viral diseases (reviewed in ref. 13). In the present article recent developments in our research on the role of LC in herpes simplex virus type 1 (HSV-1) infection are reviewed.

MATERIALS AND METHODS

Animals

C57BL/6 and A strain mice were obtained from the Hebrew University animal facilities. They were kept at ten per cage, fed water and standard diet (Ambar, Hefer, Israel).

Virus

The HSV-1 Justin strain was obtained from Dr. H. Locker, Dept. of Molecular Genetics, The Hebrew University; the LP strain was plaque-purified and selected on the basis of plaque size from NIH strain 11124 of HSV-1 (19).

Viruses were propagated in BSC-1 cell cultures grown in Dulbecco Modified Eagle's Medium (DMEM)(GIBCO) supplemented with 10% calf serum (CS) (Beit Ha'Emek) and antibiotics [400 units/ml stretomycin (GIBCO), 160 mg/ml penicillin (GIBCO)].

For titrating the virus, 10-fold dilutions of the tested sample were inoculated to BSC-1 cell monolayers that were after one hour of absorption, covered with a layer of 1% agar in DMEM supplemented with 2% calf serum (CS), 5% $NaHCO_3$ and antibiotics. The cells were incubated for three days at 37°C and 5% CO_2. The cells were then fixed with 2.5% formalin and stained with Gentian violet. The plaques were counted and

the number of plaque-forming units of virus (pfu) per ml or per cells was determined.

Animal infection

Mice were anesthesized with ether and then injected into the hind footpad (FP) or intradermally (ID) in the abdominal and upper chest skin with 0.05 ml of virus using a 25 gauge hypodermic needle. Mice were infected intraperitoneally (IP) by injecting them with 0.05-0.1 ml of virus using a 25 gauge needle.

Depletion and activation of Langerhans cells in the epidermis

To deplete the footpad skin in LC, mice were injected in the footpad skin with 0.625 mg of a sterile prednisolone acetate suspension (Dell Laboratories, Inc., Teaneck, N.J.).

Preparation of cells

For obtaining lymph node cell (LNC) suspensions, mice were killed by cervical dislocation, lymph nodes (axillary, subscapular and inguinal) were removed aseptically and forced through a sterile nylon mesh. The cell suspensions were filtered and washed twice. Cell viability was assessed by trypan blue exclusion and shown to be always greater than 95%.

In order to enrich LNC populations in IDC, the method described by Knight et al. (20) was used. Cells were resuspended in RPMI containing 10% fetal calf serum (FCS), 10^{-5} M 2-β-mercaptoethanol (Sigma) and 100 μg/ml of gentamycin at a concentration of 5 X 10^6 cells/ml. Eight ml of the cell suspension were layered onto 2 ml of a solution of 14.5% metrizamide (Nygaard, analytical grade) in medium and centrifuged for 10 min at 600 g. Cells at the interface were recovered and washed twice before subsequent use.

Peritoneal exudate cells were harvested from mice by washing the peritoneal cavity with 5 ml/mouse of Hank's balanced salt solution supplemented with 5 U/ml heparin (Sigma) and 25 mM HEPES buffer at 4°C.

Bacteria and plasmids

E. coli HB101 was used for cloning of DNA fragments from different plasmids. These were: a) EcoRI murine IL-1β cDNA fragment cloned in pBR322 (obtained from Dr. P. Gray, Department of Developmental Biology, Genentech, Inc., San Francisco, CA) (21); b) PvuII-BamHI IL-1α cDNA fragment cloned in pBR322 (obtained from Dr. P. Lomedico,

Department of Molecular Genetics, Hoffman-La Roche, Inc., Roche Research Center, Nuttley, NJ) (22); c) EcoRI rat β-actin gene DNA fragment cloned in the plasmid pAC 18.1 (obtained from Dr. H. Cedar, Department of Cell Biology, Hebrew University, Jerusalem) (23); d) EcoRI rat fibronectin cDNA fragment cloned into the EcoRI site of pGEM-2 (obtained from Dr. R.O. Hynes, MIT Center for Cancer Research) (plasmid prlf-1) (24); e) BamHI viral DNA fragment containing the BamHI J fragment of HSV-1 genome (including the glycoprotein D (gD) gene) cloned in pBR322 (plasmid PBY7op); f) HpaI viral DNA fragment containing the HpaI P fragment of the HSV genome cloned in pBR322 (plasmid PBY RI 4[3]).

Extraction of nucleic acids from tissues and cells

For DNA extraction, animals were sacrificed by cervical dislocation, and tissues were removed and Dounce-homogenized in 0.5 mM EDTA pH 8.0, 10 mM Tris HCl pH 8.0. Skin from the footpads was frozen and broken in liquid nitrogen prior to homogenization. Sodium dodecyl sulphate (SDS) (1% w/v) and 100 mg/ml of Proteinase K were added to the tissue homogenates and incubated at room temperature overnight. The homogenates were twice extracted in water-saturated phenol and once in chloroform and dialyzed overnight against TNE buffer (Tris HCl 50 mM, EDTA pH 8.0 10 mM and NaCl 10 mM). The preparations were then treated with DNAse-free RNAse (100 mg/ml), extracted twice in phenol-chloroform, concentrated with butanol and dialyzed overnight against TE buffer (25).

Total RNA was extracted by mixing cells at 10^7 cells/ml of 4.5 M guanidinium thiocyanate (Fluka), 0.5% sodium lauryl sarcosine (Sigma), 25 mM sodium citrate pH 7.0, (Sigma), 47 mM 2-β-mercaptoethanol and 0.1% antifoam A emulsion (Sigma). The lysate was then centrifuged at 36000 rpm for 14 hours through a CsCl gradient as described by Chirgwin et al. (26). The pelleted RNA was resuspended in sodium acetate 0.3 M and precipitated in ethanol.

Nucleic acid concentrations in the tissue and cell extracts was determined by measuring their optical density at 260 nm with a Gilford 2400-S spectrophotometer.

Dot-blot and vacuum blot hybridization

The DNA fragments were radiolabeled with [α-^{32}P]dCTP (Amersham, U.K.) by nick-translation (BRL kit) to specific activities of no less than 5 X

10^8 cpm/mg DNA. The dot-blot hybridization procedure was performed according to the instructions supplied with the mini-fold (Schleicher and Schüll). Autoradiograms were developed and read with the aid of an ELISA reader (Microplate reader, MR 700, Dynatech). Results are expressed in arbitrary units of optical density (27).

Hybridization with a cellular probe ruled out the possibility of non-specific hybridization due to excessive DNA loading on the filters since the absolute concentration of cellular DNA per cell does not vary during the course of infection. The results obtained by hybridization with the viral DNA probe were normalized by dividing the densitometry reading obtained from hybridization with the viral probe by the densitometry reading obtained from hybridization with the cellular probe. No major correction had to be introduced on the basis of the results from hybridization with the rat fibronectin gene. This indicates that approximately equal quantities of total DNA were indeed loaded on each well and that hybridization signal did not result from excess loading of total DNA.

The values for the optical density of each sample are expressed as the corrected mean of the duplicates $\pm$ SEM in arbitrary units of optical density.

Vacuum-blot hybridizations were performed as follows: DNA samples were digested with appropriate restriction enzymes according to manufacturer instructions, electrophoresed in 0.7% agarose gel for 14 hours at 30 V. The gels were washed for 20 min in 250 mM HCl, for 30 min in 0.5 M NaOH,1.5 M NaCl and for one hr in 1.5 M NaCl, 1 M Tris HCl. Vacuum blotting was performed according to the instructions of the manufacturer (Pharmacia) at a low vacuum pressure of 50 mm Hg for 35-40 min. The filters were baked under vacuum at 80-85°C and DNA-DNA hybridization was performed as described for dot-blot filters.

Northern blot hybridization

Prior to electrophoresis, the RNA was denatured in a solution containing 50% formamide and 7% formaldehyde and was subjected to electrophoresis for five hours at 75 V on 1.5% agarose-formaldehyde gels containing 18% formaldehyde in MOPS (3-[N-morpholino] propanesulfonic acid) buffer (20 mM MOPS, 5 mM sodium acetate, 0.01 mM EDTA) as previously described (27). The RNA was then transferred to nitrocellulose filters (Schleicher and Schuell) by the capillary blot

procedure for 14 hours (27). The filters were prehybridized at 42°C, hybridized with the nick-translated probes and subjected to autoradiography at -70°C in the presence of an intensifying screen. Quantification of relative RNA levels in autoradiograms was performed by densitometry.

RESULTS

Effect of HSV-1 infection on Langerhans cells in the skin and the lymph nodes

The role of the LC during HSV-1 infection in the skin was examined using murine models of HSV-1 infection (28,29). A marked increase in LC density at the site of virus infection was observed following infection with a pathogenic strain of HSV-1. Such an increase was not seen when the skin was inoculated with an apathogenic strain. (30). These results have been confirmed by other groups using different routes of infection of animals with HSV-1 (31,32). Similar results were obtained in mouse models of infection with vaccinia virus, foot and mouth disease virus (FMDV), P. aeruginosa and K. pneumoniae (13-14,32a,33).

In analogy with what has been shown for some antigens (11), infection with HSV-1 induced a marked increase in the number of IDC in the lymph nodes draining the area of infection (34).

The interaction of HSV-l strain Justin with LC immediately after infection of mice was studied in epidermal whole mounts prepared from Sabra mice sacrificed at 2 hr p.i. Footpad skin was stained both for ATPase activity and membranal expression of viral gD by indirect immunofluorescence. Numerous cells were ATPase$^+$ and also stained with the HSV-l gD monoclonal antibodies (Fig. 1). This result demonstrates active uptake of virus by LC. However some ATPase- cells other than LC also stained positive for gD.

Variations in Langerhans cell density influences the course of HSV-1 infection in the skin

As LC were shown to play a critical role in the triggering of the immune response to antigens presented to the skin (9), we hypothesized that LC might play a role in the immune defense against HSV-1 infection in the skin. We assume that if our hypothesis is correct, a decrease or

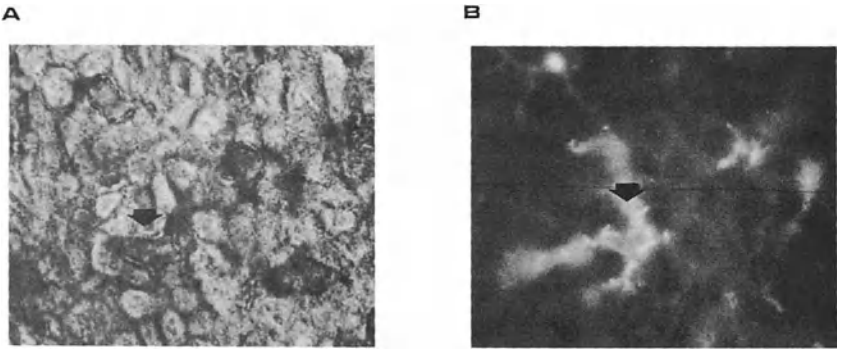

Fig. 1. Interaction between HSV-l and LC after infection of Sabra mice. Epidermal whole mounts were prepared from Sabra mice infected into the FP with 5 X 10^5 pfu of the Justin strain and sacrificed at 2 hr p.i. Footpad skin was incubated for 2.5 hr in a buffered 20 mM EDTA solution and fixed for 20 min in cacodylate-buffered formaldehyde. LC were stained both for ATPase activity and membranal expression of viral glycoprotein D (gD) by indirect immunofluorescence using a monoclonal antibody against HSV-1 gD followed by FITC-conjugated goat anti-mouse IgG antibody. Numerous $ATPase^+$ cells (A, arrow) also stained positive with the HSV-l gD monoclonal antibodies (B) (magnification X 400).

increase in LC density and/or activity should be accompanied by a concomitant increase or decrease in HSV-1 virulence, respectively.

Depletion of LC in the skin by abrasion or local injection of steroids was followed by a marked increase in HSV-1 virulence (30,35). In contrast, progressive recovery of LC density in the mouse footpad skin was accompanied by a parallel decrease in HSV-1 pathogenicity (30). Similar results were reported using exposure of skin to UV light (36,37) or application of DMSO (38) as additional methods of depleting the skin of LC.

HSV-1 infection was also studied in the ear skin since this anatomical site contains half the number of LC present in mouse footpad skin. The virus caused a severe infection in the mouse when inoculated into the ear skin (35). On the other hand, stimulation and local increase in the density of LC in the footpad skin due to injection of the immunomodulator, OK-432, almost completely abolished HSV-1 virulence by this route (34).

To further correlate virulence with a decrease in LC density, the effect of LC elimination on viral DNA synthesis was studied in skin keratinocytes of five week-old female A and C57BL/6 mice. Two control groups of A mice were injected in the hind footpad with 0.05 ml of saline (group 1) and 0.31 mg of prednisolone in 0.05 ml saline (group 2), respectively.

Six additional experimental groups were injected as follows: A mice were injected with prednisolone and nine days later were infected in the footpad with 2.5 X 10^5 pfu/mouse of the HSV-1 LP (group 3) or HSV-1 HFEM (group 7) strains respectively. Strain A mice were injected with 0.05 ml of saline and nine days later were infected with 2.5 X 10^5 pfu/mouse of the LP (group 4) or HFEM (group 8) strains of HSV-1 respectively and served as untreated infected controls. Two groups of C57BL/6 mice were used: one was treated with prednisolone prior to infection with the LP strain of HSV-1 (group 5) and the second served as a control of untreated infected mice (group 6). Three to 5 mice were sacrificed for each time point on day 0, 1, 4 and 8 postinfection. DNA was extracted from various tissues and viral DNA content was determined. DNA was prepared for dot-blotting on duplicate filters. Hybridization of the extracted tissue DNA was performed using as probes: a) the BamHI-J fragment of the HSV-1 genome containing the gD gene of HSV-1 on the first filter and b) an EcoRI fragment containing the rat fibronectin gene on the second filter (24). The same filters were hybridized with the fibronectin gene probe. Fig. 2 (A-C) shows the results of dot-blot hybridization of DNA extracted from the footpads, brains and adrenals of the different groups of mice with the viral probes. No hybridization with HSV-1 occurred with DNA extracted from the tissues of mice of the control groups 1 and 2, that were not infected with HSV-1.

DNA synthesis progressed in the footpads of mice infected with the LP strain of HSV-1 when treated with prednisolone as shown in Fig. 2A with groups 3 (A mice) and 5 (C57BL/6 mice). In contrast, viral DNA gradually disappeared from the footpads of mice that were not given prednisolone (groups 4, 6 and 8) and in mice that received prednisolone but were infected with the nonpathogenic HFEM strain of HSV-1 (group 7).

Similar results were obtained in the brain (Fig. 2B), except that viral DNA synthesis in the brain of A and C57BL/6 mice that were treated with prednisolone (groups 3 and 5) was maximal on day 4 and not on day 7. No

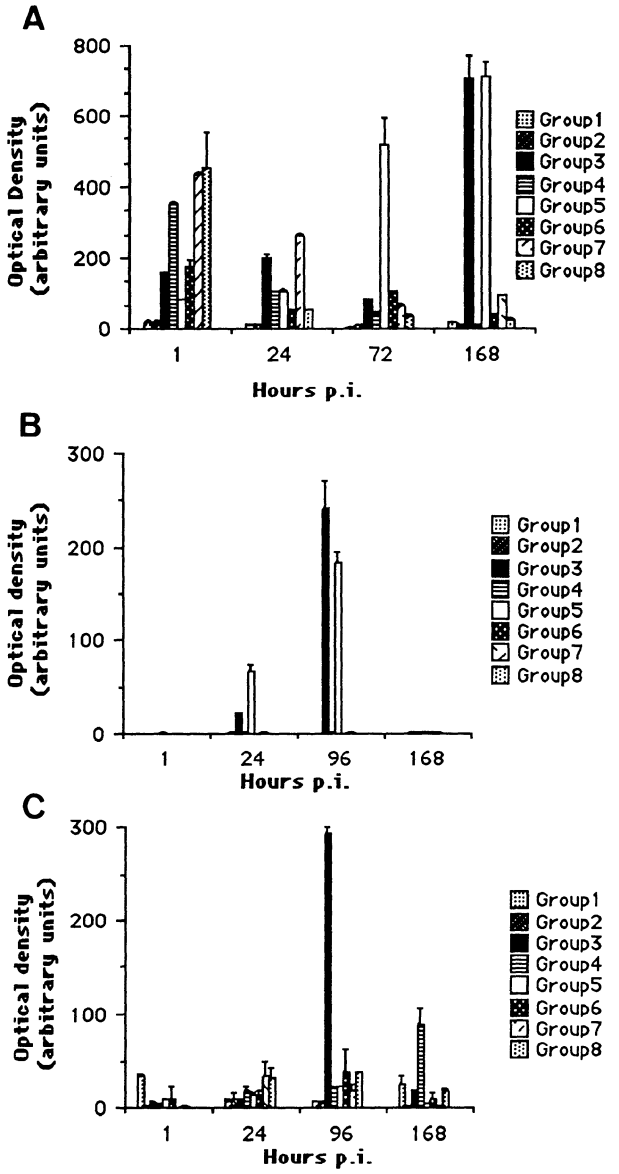

Fig. 2. Detection of viral DNA in various organs following HSV-1 infection. DNA extracted from footpads (A), brains (B) and adrenals (C) of mice in groups 1-8 was hybridized with the BamHI-J probe by the dot-blot technique as described in the text. Histograms represent densitometry results from the corresponding filters corrected according to hybridization with the fibronectin probe as described in the text.

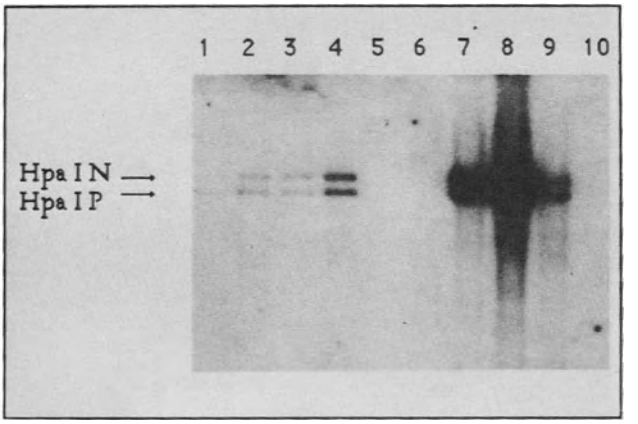

Fig. 3. Detection of viral DNA in footpads and brains following HSV-1 infection. DNA extracted from footpads (lanes 1-4) or brain (lanes 6-9) of A mice injected locally with prednisolone prior to infection with the LP strain of HSV-1 as described in the text (group 3) was hybridized to the viral HpaI-P probe by the vacuum blot technique. Tissues were sampled on days 0 (lanes 1 and 6), 1 (lanes 2 and 7), 4 (lanes 3 and 8) and 8 (lanes 4 and 9) p.i.

viral DNA was detected in the brains of mice that were infected with the HFEM strain (groups 7 and 8) or the LP strain of HSV-1 and not injected with prednisolone (groups 4 and 6).

No viral DNA was found in the liver of infected mice (not shown). In the adrenal glands (Fig. 2C) a peak of viral DNA synthesis was observed in group 3 on day 4 p.i. and in group 4 on day 7 p.i.

DNA from the brains and footpads of group 3 mice was analysed by the vacuum-blot technique. The filters was hybridized to the HpaI-P fragment of HSV-1, detecting two allelic sequences in the genome of HSV-1 (39). Fig. 3 indicates that every positive result by the dot-blot procedure was also positive by the vacuum-blot hybridization technique. Plots of the relative intensity of hybridization obtained by each technique as determined by densitometry were also comparable (not shown).

HSV-1 presentation by LC to sensitized T lymphocytes

The immune response in the skin is mainly cell-mediated (40-43), and thus dependent on functional LC that are potent antigen-presenting cells (9). We found an inverse correlation between LC density and virulence of HSV-1. In an attempt to provide an explanation for these findings, we investigated the capacity of LC to present HSV-1 to T lymphocytes. HSV-1 induced a strong proliferative response of spleen T lymphocytes derived from immunized animals in the presence of epidermal cells only (35). Such a proliferation did not occur when non-primed T cells were cultured with epidermal cells and HSV-1. Pretreatment of the epidermal cells suspension with an anti-Ia monoclonal antibody and complement, in contrast to treatment with complement alone, abolished the response of the T lymphocytes (35).

In addition, we found that when virus was injected into the epidermis, sensitized T cells could be recovered from the draining lymph nodes, except when virus was injected into skin that had been depleted of epidermal LC (34).

Interaction of HSV-1 with lymph node interdigitating cells

It was shown that HSV-1 infection in the skin induces a marked increase in IDC numbers in the lymph nodes (34). In addition, it has been postulated that LC play a central role in the immune response to HSV-1 infection as potent antigen-presenting cells (30). In order to test this hypothesis in vivo, the possibility that infectious virus is carried over to the draining lymph nodes was investigated.

Mice were infected through the skin and IDC and total LNC were recovered at various time intervals p.i. IDC but no other LNC contained infectious virus at day 1 p.i. It seems reasonable to consider that this low quantity of infectious virus reflects the uptake and transport of viral antigen to the lymph nodes. Cook and Stevens (28) previously reported the absence of infectious virus in the peripheral lymph nodes draining the area of infection after HSV-1 infection of the footpad skin. However, they infected the skin after abrasion, a method which has been shown to enhance virulence of HSV-1 (44,45). We showed that abrasion leads to the disappearance of LC from the epidermis (30). Therefore, as no LC are present in the skin infected with the method of Cook and Stevens (28), it seems logical to assume that no virus should arrive to the draining

lymph nodes as no antigen-presenting cells are present in the epidermis to carry it to the lymph nodes.We do not know at this time in what state the virus occurs in the draining lymph nodes. In situ hybridization studies might help determine whether the virus exhibits transcriptional activity in LNC and whether its presence is confined to specific subsets of DC only.

LC and IDC are thought to function as the first line of defense against HSV-1 infection in analogy with resident macrophages in the peritoneum (13). A correlation can be found between the virulence of a given strain of HSV-1 and its ability to induce a cytopathic effect in macrophages (46). In order to investigate more closely the interaction of the virus with IDC, isolated IDC derived from C57BL/6 and A mice were infected in vitro with the Justin strain of HSV-1. The cultures were infected at a MOI of 2 or 10. After two hours of absorption, the medium was washed out and new medium was added. The cell cultures were harvested at various times p.i. and the cells were frozen at -70°C. The cells were then thawed, sonicated and the lysate was seeded on BSC-1 cells for titration.

The virus titer steadily decreased after infection (Fig. 4) whereas the viability of the cells by the trypan blue exclusion test did not vary significantly as compared with uninfected cell cultures. The titer of the virus decreased to the same extent in A and C57BL/6-derived IDC. In contrast, virus replicated in BSC-1 cells although the rise in titer was seen only 24 hour p.i., due to the high MOI chosen (Fig. 4). In addition no cytopathic effect was observed in infected IDC. Virus was nevertheless recovered from infected IDC cultures harvested after the absorption period, indicating that the virus is taken up by the cells but is not able to replicate in them (Fig. 4). The experiment was repeated three times with identical results.

Effect of HSV-1 infection on IL-1 RNA synthesis in lymph node interdigitating cells

Results from this (13,46) and other laboratories (47-49) have demonstrated in various experimental models of HSV-1 infection that resident antigen-presenting cells play an essential role in the defense of the host against the viral infection.

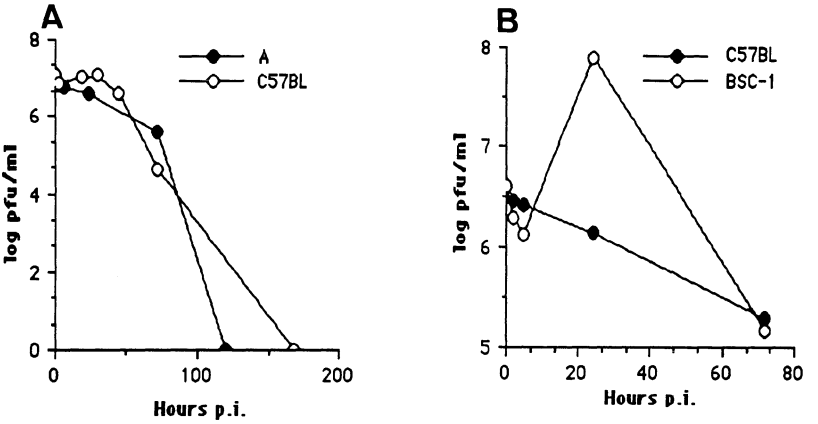

Fig. 4. In vitro infection of IDC with HSV-1. IDC were obtained from C57BL/6 or A mice. Isolated IDC (10^5 cells/well) were infected at a MOI of 10 (A) or 2 (B) with the Justin strain of HSV-1. Virus absorption lasted for 2 hr. The cells were then washed and incubated for various time intervals, after which they were kept frozen until titration on BSC-1 monolayers. As a control, BSC-1 cells were infected the same way and virus titer was determined at various time intervals p.i. Results are expressed in log pfu/ml.

In the skin model of HSV-1 infection, LC have been shown to function as critical elements in the immune response to HSV-1 infection (reviewed in ref. 13). In the intraperitoneal model of HSV-1 infection, peritoneal macrophages have been shown to have a determining role in clearance of virus from the peritoneal cavity, in the prevention of virus systemic spread (47,48,50,51) and in the restriction of virus replication depending on their state of activation and differentiation (46,52,53).

In both systems, antigen-presenting cells are thought to induce an antiviral immune response by interacting with T cells, activating them, and therefore triggering both the cellular and humoral immune response (40-43). In the course of this interaction, antigen presentation is thought to occur in conjunction with IL-1 gene activation in antigen-presenting cells (54).

Two different genes, named IL-1α and IL-1β, are known to code for IL-1 activity in the mouse (21,22). Both species of IL-1 share the same range of biological activity (reviewed in ref. 55). IL-1α and β gene cDNAs have been

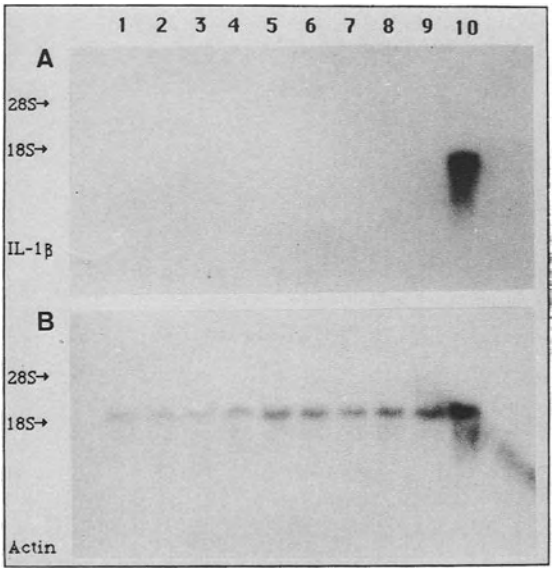

Fig. 5. IL-1β RNA synthesis in IDC. Total RNA derived from IDC (lanes 3, 6, 9), high density lymph node cells (lanes 2, 5, 8) and spleen cells (lanes 1, 4, 7) (2 μg RNA/lane) was hybridized with ^{32}P-labeled IL-1β (A) and β-actin (B) probes. The cells were obtained from mice infected with the Justin strain at 0 (lanes 1-3), 24 (lanes 4-6) and 72 (lanes 7-9) hours p.i. The 28S and 18S markers correspond to the position of the ribosomal RNA bands on the ethidium bromide stain of the corresponding gel after blotting. In addition RNA derived from activated peritoneal exudate cells was run in lane 10 as a positive control.

cloned and shown to detect 2.1 and 1.4 kb RNA species respectively by the Northern blot technique (21,22).

Studies on IL-1 gene activation in vitro are hampered by the fact that the purification and culture of antigen-presenting cells involves adherence to plastic which by itself can induce IL-1 gene expression (56). Therefore, the effect of HSV-1 infection on IL-1 gene expression was studied in vivo, under conditions known to prevent spontaneous induction of the gene (56). Recent in situ hybridization studies, confirming previous reports, have shown that IL-1 gene transcription in peripheral leukocytes is confined to cells of the monocytic lineage (57).

In the present study, we investigated the possibility that HSV-1 induces IL-1 gene expression in IDC isolated from the lymph nodes

draining the skin of infected mice. We also compared the effect of HSV-1 infection on IL-1 RNA synthesis in IDC with IL-1 RNA synthesis in peritoneal exudate cells obtained from mice infected IP. When mice were infected IP, a marked induction of IL-1 genes in peritoneal exudate cells was observed 6 hr p.i. (58).

C57BL/6 mice were injected intradermally in the abdomen and chest skin with 5 X 10^5 pfu/mouse of the Justin strain of HSV-1. Mice were sacrificed at 0, 24 and 72 hours p.i. and their spleen, inguinal, subscapular and axillary lymph nodes were removed. LNC suspensions were prepared and fractionated on a metrizamide gradient into a high and a low density (IDC-enriched) cell populations. RNA was extracted from the spleen and from each fraction and blotted on nitrocellulose filters. Fig. 5 shows the autoradiograms of Northern blot hybridization with the probe for IL-1β (Fig. 5A) or for the β-actin gene (Fig. 5B).

Results show that no induction of IL-1β gene expression could be found at any time in the cell populations tested. As a control, an equal amount of RNA derived from peritoneal exudate cells of mice infected IP with the Justin strain and harvested 6 hours p.i. was run in parallel and found to yield a strong signal (Fig. 5A, lane 10). Hybridization of the Northern blots with the probe for the β-actin gene (Fig. 5B) revealed that approximately identical amounts of RNA had been loaded in each lane. The experiment was repeated three times with identical results. In one additional experiment, similar results were obtained with an IL-1α probe (not shown).

DISCUSSION

This study reveals that LC in the mouse skin respond to a virulent HSV-1 infection by an increase in cell numbers. Yet, in spite of the increased LC density the virus replicates in the skin keratinocytes and penetrates into the nervous system. The present study provides additional information on the interaction of LC with HSV-1. We have shown that: a) the viral antigen can be found in LC as early as 2 hrs p.i. and b) infectious virus can be isolated from LC that migrate to the lymph nodes after infection of the skin. However in-vitro incubation of IDC with HSV-1 results in the inactivation of the virus, a property probably connected with the degradation of virus particles by LC for antigen presentation.

The absence of LC eliminates an important defending cell type from the skin. The study of HSV-1 DNA replication in the murine footpad skin clearly revealed that HSV-1 replication in the infected mice can be correlated with a decrease in LC. The molecular mechanisms utilized by LC in the defenses against HSV-1 replication are not known. The Northern blot hybridization using IL-1 cDNA probe did not reveal IL-1 mRNA in the dendritic cells. However it was reported (59) that activated skin LC contain tumor necrosis factor (TNF) mRNA.

Activation of LC by the injection of the immunomodulator OK-432 revealed an increase in the local number of LC and a marked protection against HSV-1 infection (34). It is possible that activation of LC by OK-432 stimulates the expression of cellular genes which are involved in the interaction of LC with HSV-1 and its degradation for antigen presentation.

Taken together the data presented here indicate that LC play an essential role in the defense of the skin against HSV-1 infection by taking up the virus in the skin, migrating to the draining lymph nodes and there, triggering the immune response by presenting the viral antigens to T cells. The observation that LC play an active, and very often critical, role in the pathogenesis of HSV-1 infection and other viral diseases clearly proves the skin to be much more than a passive receiver of antigenic stimuli as it was thought to be several years ago.

Factors influencing the activity and density of LC in the epidermis have been under study for more than a decade (60-64). Studies on the effect of these immunomodulators on LC activity in viral infections may lead in the future to the development of new strategies aimed at inhibiting primary virus infections or even eradicating latent infection. In this regard, our observations on the effect of OK-432 on the course of HSV-1 infection suggest that it might be possible to interfere with the course of viral infections by modulating LC function in the skin. Retinoic acid which is known to activate LC (62) has been shown to be effective in the treatment of recurrent HSV-1 infections (65). Further understanding of LC role in the pathogenesis of viral diseases might open the way for novel diagnostic and therapeutic approaches, based on adequate evaluation and modulation of LC activity in the skin.

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HUMAN WARTS AND LANGERHANS CELLS

15

WARTS, CARCINOMA AND LANGERHANS CELLS

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ABSTRACT

Langerhans cells (LC) are dendritic cells present in all squamous epithelia; they are required for antigen presentation and for stimulation of antigen-specific T lymphocyte activation. These cells could play a major role in the local host immune response to human papillomavirus (HPV) infections and in the surveillance against malignant conversion.

The distribution and markers of LC and mononucleated cells of the infiltrate were studied with indirect immunofluorescence method, in a series of benign and malignant papillomas excised from various skin and mucosa sites. They were correlated with the presence of virus group-specific antigen detected by indirect immunofluorescence and typing of viral DNA by DNA-DNA hybridization.

Most of the lesions were characterized by the presence of a mild or a moderate infiltrate of CD4, CD8 and HLA-DR positive cells. The number of LC was reduced in the epithelium of all specimens as compared to the normal epithelia. There was no correlation between the LC density and infecting HPV type. Morphology of LC was also altered with a loss of dendrites.

Epidermal lesions showed a mild inflammatory infiltrate without predominance of either CD4 or CD8-

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positive cell subsets. On the contrary, mucosal lesions (laryngeal papillomas and CIN) contained a dense infiltrate, with predominance of CD8-positive cells in the epithelium and presence of LC at the junction of epithelium and connective tissue. In some of these lesions HLA-DR antigen was expressed by epithelial cells.

In conclusion, the reduced LC density in HPV-induced lesions is not related to the infecting virus type; it is directly related to the amount of viral infected cells. The exact mechanism of this modification remains unknown; it may be that virus-induced changes in the epithelial microenvironment influence the LC population thereby causing a decrease in the immunological surveillance. Local host tolerance may be induced and thus favor the persistence of the lesions and their conversion towards the malignant state. In this conversion, although LC depletion may be a favoring factor, the association of potentially oncogenic HPV types with other factors remains essential.

INTRODUCTION

More than 50 different human papillomavirus (HPV) types are known to induce epithelial proliferations of the skin and mucosa (1). Most of these lesions are benign. However, some of them such as dysplasia of the cervix uteri, macules of patients with epidermodysplasia verruciformis and laryngeal papillomas in adults may progress towards squamous cell carcinoma (2,3).

Several observations indicate that the cell-mediated immune responses play an important role in the outcome of HPV infections : high incidence of warts in patients receiving immunosuppressive treatment (4), a weak delayed type hypersensitivity to tuberculin, a decreased number of peripheral blood T lymphocytes or reduced in vitro blastogenic response of lymphocytes to mitogens (5-7).

Langerhans cells (LC) are dendritic cells which are

sentinels of the epithelia. They are found in all squamous epithelia (8-9); they are considered as a population of specialized antigen-presenting cells of bone marrow origin. They migrate to and localize in the epithelia; they have a role in the recruiting of specific T lymphocytes for the local cellular immune response in epithelial lesions (See review 10).

The purpose of this study was to determine the distribution of LC and their phenotype expression in various cutaneous and mucosal papillomas. We examined simultaneously the relationship between the presence of LC and the presence of virus antigen and/or viral DNA within the infected epithelia in a attempt to ascertain whether changes in LC are involved in the pathogenesis of HPV lesions.

MATERIAL AND METHODS

Tissue specimens

About 150 non-regressing papillomas or carcinomas including benign, premalignant or malignant lesions from various sites were taken from patients aged 11-70 years without any sign of immunodeficiency (Table 1). They were removed by surgery, immediately frozen in liquid nitrogen and stored at -20°C. They were usually excised 2-6 months after onset. A few patients had 2-4 biopsies. Several tissues were used as controls : normal human tissues from similar locations were included (normal skin, oral and cervical mucosa) as well as unrelated HPV lesions such as molluscum contagiosum.

Antibodies

Monoclonal antibodies directed against specific antigens were used : Leu 2a, Leu 3a (Becton-Dickinson, USA) BL6 and BL2 (Immunotech, France) respectively specific for CD8, CD4, CD1a and HLA-DR antigens.

Viral antigen was detected with an HPV genus-specific antiserum raised in rabbit against SDS-dissociated highly

purified human papillomavirus (11).

Morphological studies

Hematoxylin and eosin stained sections were examined.

Indirect immunofluorescence technique

Four µm serial cryostat sections of papillomas and normal tissues were prepared, air dried and fixed in cold acetone for 10 min. Adjacent sections were used for comparative analysis.

Indirect immunofluorescence assays were performed as described elsewhere (12). The specificity of the immunofluorescent test was shown by the absence of staining in sections incubated with PBS instead of primary antibody.

Detection of viral DNA

It was done by Southern technique and/or by in situ hybridization. Cloned HPV DNA type 1a, 2a, 6a, 11a, 16 and 18 (provided by G. Orth, Paris and H. Zur Hausen, Heidelberg), were purified through cesium chloride gradients.

Total DNA was extracted and digested for 2 h at 37°C with 1 or 2 appropriate restriction enzymzes (BamHl, EcoRl, Pst, HindII + HindIII, Boehringer, Mannheim, Germany) under conditions described by Orth (13). Electrophoresis of the DNA fragments were performed in 1% agarose gels. HPV types were determined after Genescreen Plus (NEN) transfer and Southern blot hybridization with ^{32}P-labelled probes under stringent conditions.

In situ hybridization was performed according to a technique previously described (14), with HPV DNA biotinylated probes on 4 µm adjacent frozen sections prepared on slides coated with aminopropyl-triethoxy-silane, air dried and fixed 3 min in methanol : acetic acid (3:1). Plasmid DNAs were biotinylated by nick translation using biotinylated lld-UTP (BRL). Stringent conditions were used for typing HPV at Tm-12°C. The DNA-DNA hybrids were detected after successive incubations in

an antibiotin rabbit antiserum, a biotinylated goat antiserum, a streptavidin alkaline phosphatase complex and a mixture containing nitroblue tetrazolium (NBT) and 5-bromo-4 chloro-3 indolyl phosphate (BCIP) from a Blue Gene kit (BRL). The slides were mounted with a glycerin-gelatin solution. Positive nuclei showed a purple precipitate under a light microscope.

Several controls were performed to assess the specificity and the sensitivity of the reaction. Serial tissue sections were negative after incubation in the hybridization mixture where the DNA probe was omitted or replaced by pBR322 probe. Other tissues with lesions unrelated to HPV infection were negative. Positive controls with HPV 16 and 18 probes consisted of cell lines containing different copy numbers of HPV DNA. Nuclei of CaSki cells (600 copies of HPV DNA type 16), SiHa cells (1-5 copies of HPV DNA type 16) were labeled only with HPV DNA type 16 whereas HeLa cells (10-50 copies of HPV DNA type 18) were positive only with HPV DNA type 18.

RESULTS

1. Immunological studies

Examination of hematoxylin and eosin stained sections of each tissue sample allowed classification of the lesions according to their histological features. The epithelium was disorganized as compared to normal tissues.

Normal tissues : Normal epidermis was characterized by a high number of LC with their typical dendritic shape (Fig 1) and CD1a positive cells outnumbered HLA-DR positive cells. This was consistent with histological features in the absence of epidermal or dermal infiltrate.

In the epithelium of normal oral or tracheal mucosa, high numbers of CD1a and HLA-DR positive cells, with their usual dendritic shape were detected; some CD4 and CD8 positive T cells were also seen. In the subepithelial connective tissue numerous T lymphocytes were present;

a few CDla positive cells were observed. Many of the dermal cells were HLA-DR positive.

A large number of CDla positive cells were present in the epithelium of normal cervical mucosa; they displayed their typical dendritic processes and most of them expressed HLA-DR antigen; a few CD4 and CD8 T lymphocytes were also found. LC were unevenly distributed in the epithelium; they were located mainly in the basal and intermediate layers. More T cells were labeled in the subepithelial connective tissue than in the epithelium; most of these cells were also HLA-DR positive. An intense infiltrate was observed in the stroma, with positive HLA-DR cells. No CDla positive cell was detected in the stroma.

In normal cutaneous or mucosal epithelia, the epithelial cells were always negative with each antibody tested.

Cutaneous HPV lesions. Most of the cutaneous biopsies exhibited either a mild or moderate inflammatory infiltrate containing CD4, CD8 and a few CDla positive cells. No T cell subset predominated.

The morphology and distribution of LC were abnormal as compared to those of normal skin : their dendritic shape was lost in many cases; their density was drastically reduced. The highest percentage of lesions characterized by an absence of LC in the epithelium was in plantar warts (7/17, 41 %) where the most intense cytopathic effect and the highest production of viral antigen and/or viral particles were to be observed (Table 1).

Vulgaris warts from various localizations (hands, legs face, trunk) showed a less intense cytopathic effect than plantar warts; they produced a low amount of viral antigen but contained HPV DNA. In such cases, reduced numbers of LC were detected in the epithelium either with CDla or anti-HLA-DR monoclonal antibodies.

Table 1. Distribution of LC phenotype in cutaneous and mucosal HPV-induced papillomas (CD1 and DR positive cells).

Tissues	Number lesions	CDla antigen Epithelium	CDla antigen Stroma	HLA-DR Antigen Epithelium	HLA-DR Antigen Stroma
Cutaneous lesions					
Plantar sole	17	10 (+/-)	10 (+/-)	0 (-)	6 (+/-)
Vulgaris warts	57	43 (+/-)	38 (+/-)	11° (+/-)	15° (+/-)
Extragenital Bowen	5	5 (+)	4 (+/-)	5 (+)	5 (+)
Cutaneo -mucosal lesions					
Condyloma acuminata	11	10 (++/+)	6 (+/-)	6 (++/+)	7 (++/+)
Buschke-Löwenstein	2	2 (++/-)	1 (+/-)	1 (++/-)	2 (++/+)
Bowenoid papulosis	5	4 (+)	3 (+/-)	4 (+/-)	4 (+)
Mucosal lesions					
Juvenile LP	29	23 (++/+)	8 (+)	24 (+)	25 (++/+)
Adult LP	10	6 (++/+)	6 (+)	8 (+)	9 (++/+)
CIN grade 2 3	18	15 (++/+)	4 (+)	16 (++/+)	18 (+++/++)
Normal tissues					
Epidermis	12	12 (++)	-	+	-
Oral or trachea mucosa	5	5 (+++)	-	++	+++
Cervical mucosa	5	5 (+++)	-	++	+++

A semi-quantitative grading scheme was used according to the number of positive cells in the field : +++ : 20-50 positive cells; ++ : 10-20 positive cells; + : less than 10 positive cells per section; - : Absence of positive cells in the section.
The numbers are percentages of positive biopsies.
(°) Only 18 samples were tested for HLA-DR antigen

Bowen's disease lesions of extragenital origin presented a mild infiltrate and a reduced density of LC. HLA-DR antigen was not expressed on epidermal keratinocytes of any specimen. Perilesional skin of the patients remained unchanged as compared to normal skin samples taken from similar sites.

Cutaneo-mucosal HPV lesions : In condyloma acuminata, bowenoid papulosis and Buschke-Lowenstein, the density of Langerhans cells was reduced in the epithelium and a moderate infiltrate was observed. In some cases, only a few CDla positive cells were detected at the junction of epithelium and stroma (Fig 2). Condyloma acuminata exhibited a less severe cytopathic effect than in cutaneous biopsies. The other specimens were mainly characterized by a hyperproliferation of basal layers.

Mucosal HPV lesions : A mild local cellular immune response was found in most laryngeal papillomas whereas an intense inflammatory reaction was observed in the

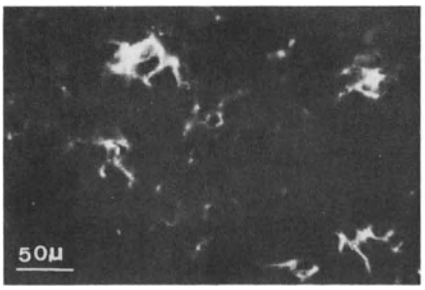

Fig.1 High numbers of LC in normal epidermis.

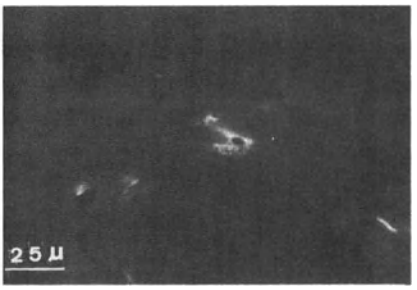

Fig.2 Rare LC in Buschke Löwenstein.

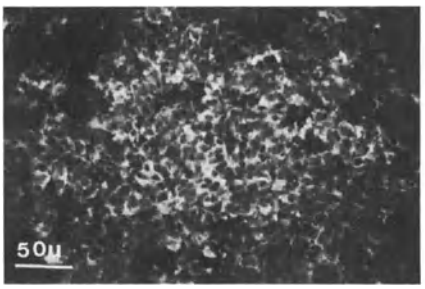

Fig.3 Expression of HLA-DR antigen by stromal cells in a CIN.

Fig.4 HLA-DR expression by epithelial cells in a laryngeal papilloma.

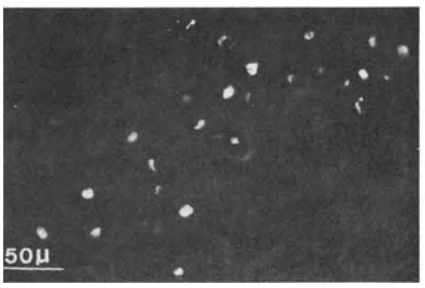

Fig.5 Viral antigen in a hand wart section.

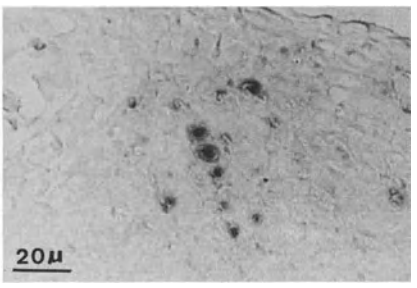

Fig.6 Foci of labeled nuclei with HPV-DNA type 11 in a Buschke Löwenstein.

stroma of CIN (Fig 3). In both groups of mucosal lesions intermediate reactions were seen.

LC were found in the epithelium in a larger proportion of laryngeal juvenile papillomas and CIN than in cutaneous lesions (Table 1).

Compared to normal tissue, the distribution of LC was abnormal; only a few LC in the epithelium of most laryngeal papillomas from children or from adults. LC were present in the corium of some biopsies. Most LC were confined to the junctional zone between the epithelium and the corium and displayed an abnormal round shape, without dendritic processes in some cases. The corium contained high densities of CD4 and CD8 T cells and HLA-DR-positive cells.

In CIN, LC did not display any morphological changes under light microscopy. They were located in the basal and intermediate layers; low numbers of LC were sometimes seen in the stroma. In most cases, the epithelium contained reduced numbers of CDla and HLA-DR positive cells as compared to the normal tissue. A population of CD8 positive cells was usually admixed with LC.

In some cases of adult laryngeal papillomas and CIN, HLA-DR antigen was expressed in foci of lesional epithelial cells, in basal and intermediate cell layers, usually associated with a dense dermal infiltrate (Fig 4).

Most of the mucosal specimens displayed a cytopathic effect limited to foci of infected cells.

Virological studies

Viral antigen was detected in large amounts and in a high proportion of plantar and vulgaris warts (Fig 5) whereas it was absent in Bowen's disease lesions. Viral DNA was found in each series of papillomas in a higher percentage than viral antigen (Table 2). Viral DNA was typed with Southern technique and/or in situ hybridization under stringent conditions. Plantar warts

contained HPV type 1 in 12 cases or HPV 2 in 5 cases. In vulgaris warts HPV type 1 was identified in 6 cases and HPV 2 in 39 cases and 10 were negative. Bowen's disease lesions and bowenoid papulosis were infected with HPV type 6/11 in one case and HPV 16 in 1 case; 2 others could not be identified.

Table 2. Presence of viral antigen and viral DNA in the lesions

Tissues	HPV Antigen	DNA
Cutaneous lesions		
Plantar sole	16/17 (94.1 %)	17/17 (100 %)
Vulgaris Warts	32/56 (57.1 %)	45/55 (81.8 %)
Extragenital Bowen	0/5 (0 %)	1/5 (20 %)
Cutaneo-mucosal lesions		
Condyloma acuminata	2/11 (18.1 %)	8/11 (72.7 %)
Buschke Löwenstein	0/2	1/2
Bowenoid papulosis	0/5 (0 %)	3/5 (60 %)
Mucosal lesions		
Juvenile LP	14/27 (51.8 %)	12/25 (48 %)
Adult LP	2/10 (20 %)	4/5 (80 %)
CIN grade 2,3	0/18 (0 %)	13/16 (81.2 %)
Normal tissues		
Epidermis	-	-
Oral or tracheal mucosa	-	-
Cervical mucosa	-	-

LP : Laryngeal papillomas

Viral antigen was usually present in a low amount (1 or 2 nuclei per section), in 14/27 (52 %) of juvenile or adult laryngeal papillomas. It was present in higher amounts in condyloma acuminata but only in 2/11 (18 %) of these papillomas. It was undetectable in Bowen's disease, bowenoid papulosis and CIN biopsies. On the contrary, viral DNA was detected in a large proportion of the lesions (Fig 6). Condyloma acuminata were infected by HPV type 1 in 4 cases, HPV 2 in 1 case, HPV 6/11 in 3 cases; one sample was negative. Laryngeal papillomas contained HPV type 6/11 in 15 cases, and HPV type 16 in 1 case from adult; 13 samples were negative and one could not be identified. CIN contained HPV type 6/11 in 3 cases and

16 in 3 cases. Multiple infections were seen in 7 cases.

DISCUSSION

Human papillomavirus infection of skin or mucosa is characterized by a disturbance in epithelial cell kinetics, epithelial differentiation and epithelial coherence. The LC are the immunocompetent cells of the epithelia which could play an important role in immunological surveillance.

The analysis of a large number of non-regressing lesions induced by HPV demonstrates a local cellular immune response in most of them. A mild or moderate infiltrate of CD4, CD8 T lymphocytes and LC was present in the stroma whereas an LC depletion was observed in the epithelium.

In benign non-regressing cutaneous lesions, there was no marked inflammatory infiltrate (12,15). On the contrary, a dense infiltrate of CD4, CD8 T lymphocytes and mononuclear phagocytes has been described in regressing plane warts (16-20).

In premalignant and malignant lesions, Bowen's disease Buschke Löwenstein and bowenoid papulosis the inflammatory reaction was usually more intense than in benign lesions. The infiltrate was dense in some laryngeal papillomas and CIN (21-23). Furthermore, in these mucosal lesions, CD8 T cells were found to predominate (20, 24, 25). In few cases, rare NK cells were also observed (19, 20, 23).

LC density was reduced in the epithelium of most benign and malignant lesions studied as compared to normal tissues. This observation was independent of the location of the lesions on skin or on mucosa, of the degree of malignancy and of HPV type.

The LC distribution was influenced by the amount of viral antigen in keratinocytes (15). The depletion of LC is the most drastic in plantar warts which showed the

most intense cytopathic effect and severe antigenic alterations or loss of some membrane antigens in the suprabasal compartment of the infected epithelia (26). In lesions with a high amount of virus, these modifications are directly related to the viral lytic activity of keratinocytes. This phenomenon could perhaps account for the LC depletion. Up to now, no virus particle has ever been detected in LC whereas some of these cells may either have abnormal round shapes or may have lost HLA-DR antigen (15). Variations in LC morphology have been described in hyperproliferative lesions (27) or after UV irradiation (28). This suggests that drastic modifications of the LC environment may alter the number, the morphology and the phenotype of the dendritic cells. Similar findings have been reported for other epidermal lesions (29) where immunohistochemical studies of LC in various skin tumors showed that LC density depends on the degree of epidermal differentiation (29).

An increase or a decrease of LC density have been reported by other investigators in various cutaneous lesions (30-33) and in CIN (24, 34-38). The discrepant findings can be explained by the small number of biopsies under study, the variability of the host response and the sensitivity of the test used to evaluate the LC density (39).

Functional LC are required as antigen-presenting cells for initiation of immune response and for stimulation in epidermal cell-lymphocyte reactions (40-41). LC might play a major role in the HPV induced lesions for antigen presentation to the immune system at the site of viral invasion. In spontaneously regressing plane warts, LC were admixed with keratinocytes as shown by ultrastructural studies (19). This picture is similar to that described in the process of allergic contact dermatitis (42). Similarly, in normal cervix direct

contact between LC and lymphocytes has been reported (43) All these features indicate that LC are involved in the local host defense at the site of viral invasion.

It has been suggested that cellular immunity might be elicited by the virus infected cell or a cell fragment containing viral antigen rather than the extracellular virion.

Mucosal lesions contain HPV types which differ from those usually detected in skin warts by their degree of cytopathogenicity and their oncogenic potential. Furthermore, in some cases of laryngeal papillomas and CIN, epithelial basal cells express HLA-DR, suggestive of a local γ-interferon production which would seem to enhance the level of both HLA class 1 and class 2 antigen expression on the cell surface (22). Non immunological causes, such as hormonally regulated mechanisms have also been suggested as provoking in vivo synthesis of HLA-DR antigen by epithelial cells. As juvenile laryngeal papillomas may disappear spontaneously during puberty, this is indicative of an eventual role of a hormonal factor (44).

Epithelial cell membrane alterations have also been found in mucosal lesions mainly β2-microglobulin expression which is lost in the upper layer cells (26). As T cells and LC recognize antigen only when associated with cell surfaces and together with HLA products, we may suppose that loss of β2-microglobulin expression on epithelial cells hampered the antigen recognition and thus a proper response and thereby an immunologic tumor rejection (45).

Taken together, our findings suggest a local intra-epithelial immune deficiency in persistent or premalignant papillomas. The defective immune response could result from reduced lymphocyte migration into the epithelium and the lack of activation of the existing lymphocytes due either to the depletion of LC or

functional disorders. However, the acquisition of HLA-DR moieties by infected epithelial cells in some laryngeal papillomas and CIN (22, 23) as well as in regressing flat warts (19) widens the repertoire of their functional capacity mainly in antigen presenting function to immunocompetent cells as suggested by Nickoloff (46). This capacity is probably compromised when epithelial cells lose their HLA class 1 antigen (26).

In conclusion, in HPV-induced lesions, the elimination of virus-infected cells is probably mediated by cytotoxic T cells as in other lesions; it requires the collaboration of activated LC to become operative. The reduction of LC population within lesional epithelium and the low level or absence of cytotoxic T cells may contribute to the deficient surveillance leading either to persistence, recurrency or malignancy. In this process, the infected epithelial cells release mediators such as γ-interferon, known to enhance both HLA class 1 and 2 antigen expression and therefore may play an important role. In the evolution towards a malignant state, both oncogenic HPV types and other factors such as UV irradiation for epidermal lesions, smoking for laryngeal papillomas and bacterial or viral infections in CIN are also determinant.

The balance between the antigenicity of infected epithelial cells and the reactivity of the host is multifactorial and is still poorly defined.

Premalignant or malignant lesions did not significantly differ from benign lesions by their local reactivity at the site of viral invasion. Plantar warts and vulgaris warts are the only group with specific features.

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POX AND IRIDOVIRUS INTERACTIONS WITH SKIN LANGERHANS CELLS

16

VACCINIA VIRUS AND DENDRITIC (LANGERHANS) CELLS: NEED FOR ELUCIDATION OF THE ROLE OF DENDRITIC CELLS IN VACCINATION.

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In the review on smallpox by Behbahani (1) it is indicated that Edward Jenner used materials from individuals infected with cowpox or horsepox (grease) for immunization. The live vaccines subsequently used were produced from four basic strains: a) The Lister Institute or Elstree strain in the United Kingdom that originated from a Prussian soldier with smallpox during the 1870 Franco-Prussian war and was introduced into Britian from Cologne as calf lymph in 1907, b) the Wyeth or the New York Board strain in the United States of America that was started in 1876 from an unidentified vaccine imported from Britain in the 1850s, c) the EMG 3 strain in the USSR which was derived from a commercial vaccine of unknown origin from Ecuador, and d) a vaccine strain strain designated Tian Tan strain that is being used in China.

Behbahani (1) indicates that "the vaccine virus now called vaccinia virus in its present form is different from both the original cowpox virus and the smallpox virus". The terms "smallpox vaccine" and "vaccinia virus" were used synonymously until the 1930s. A virus isolated from a person with cowpox was found to be closely related serologically and biologically to vaccinia virus but could be distinguished from it (2). Indeed, vaccinia virus was found to be genetically related to smallpox virus rather than to cowpox. Esposito and Knight (3) reported that three vaccinia virus strains could be differentiated from

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other orthopoxviruses in the terminal region of the HindIII DNA fragment. The Chinese vaccination virus strain Tian Tan varies from vaccinia virus strain WR in restriction enzyme sites in the viral DNA (4). Comparing the DNAs of cowpox virus, vaccinia WR strain and the Elstree (Lister) virus revealed similarity to DNAs of monkeypox and camelpox.

Complications after vaccinia virus skin vaccination like encephalitis, eczema vaccinatum was noted at 10 per 10^6 vaccinees and 1 per 10^6 is the estimated risk of death. Complications like superinfection in a variety of skin conditions approach 1000 per 10^6 vaccinees. Immunodeficient individuals develop progressive vaccinia infections (5). Basal cell carcinoma (6) or malignant histiocytoma (7) were reported to arise at the site of smallpox vaccination. Indeed, during the worldwide vaccination campaign against smallpox the contraindication for vaccination was skin abnormalities. Vaccination against smallpox has now been abandoned due to the the success of the World Health Organization program to eradicate smallpox. The reasons why changes in the skin lead to abnormal responses and enhanced pathogenicity of vaccinia virus remain unexplained.

The involvement of skin dendritic (Langerhans) cells in infection of the skin with vaccinia virus was first studied by Nagao and Inaba (8). There is now a need to understand the role of dendritic (Langerhans) cells in smallpox vaccination due to the development of a new generation of recombinant vaccinia viruses that carry foreign virus genes [e.g. hepatitis B surface antigen (9), influenza virus hemagglutinin (10), HIV-1 envelope protein (11) and rabies virus antigens (12)]. If such recombinant vaccines (13) are to be used in human populations, the interaction with dendritic cells in the skin and the ability of dendritic cells to protect against the recombinant vaccinia virus must be explained.

To date, apart from the electron microscopy data on the uptake of vaccinia virions by skin dendritic cells (8) only one study on the response of these cells to vaccinia virus was reported (14). Three vaccinia virus strains (Noguchi, Lister and WR) were studied by inoculation of the virus into the footpad skin of male 4 to 8-week-old C57BL and A strain mice. To

eliminate the dendritic cells from the footpad skin, prednisolone was injected into the footpad two days prior to virus injection. This treatment was found to cause a local depletion of dendritic (Langerhans) cells without an effect on the immune system. Injection of the three vaccinia strains into the footpad skin led to a local increase in the number of dendritic cells. The response of dendritic cells to the Noguchi and WR strains was higher than that caused by the Lister strain. After three weeks the number of dendritic cells in the virus-infected footpads returned to the normal level.

Inactivation of the skin dendritic cells by prednisolone was done to study the role of these cells in the response to vaccinia virus (14). It was reported that the Lister strain retained its non-pathogenic phenotype after injection into the prednisolone-treated mice. However, the WR and Noguchi strains which are nonpathogenic in untreated mice, killed the prednisolone-treated mice. Although it is not possible to exclude an effect by prednisolone on other cell types, it seems reasonable to suggest that in the absence of dendritic cells in the skin there may be expression of residual viral pathogenicity that was not detectable in their presence (15).

The molecular mechanisms of antigen presentation by dendritic cells are not yet known. However, the observation of Nagao and Inaba (8) that vaccinia virions are taken up by the skin dendritic cells suggests that the virion proteins are probably degraded in these cells and the antigenic peptides are present on the cell surface inside the MHC protein. Thus, the ability of dendritic cells to present foreign viral proteins produced in keratinocytes should be studied so as to evaluate the usefulness of the recombinant vaccinia viruses in immunization.

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LANGERHANS CELLS IN THE SKIN OF NORMAL AND LUMPY SKIN DISEASE VIRUS-INFECTED CATTLE

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Lumpy skin disease (LSD) is a dermotropic virus infection of cattle caused by a member of the capripox virus family. It presents as dermal thickenings 0.5-5.0 mm in diameter that involve all the skin layers. In addition the regional lymph nodes, mucous membranes and lungs are invariably affected (1). It was reported that during the acute stage of the disease, necrotic areas of the lesions are infiltrated by neutrophils, macrophages and occasionally, eosinophils. As the lesions progress these cells are gradually replaced by round cells (lymphoblasts, lymphocytes, plasma cells and macrophages) and by fibroblasts, while the keratinocytes are swollen and eosinophilic inclusions are present in different cell types (1). The histopathological description is reminiscent of an earlier one concerning sheeppox in which considerable dermal oedema and the relatively early appearance of large numbers of cells of unique appearance, termed "cellules claveleuses" were described (2). These cells show rounded or oval nuclei and cytoplasm of increased basophilia. Their cytoplasmic boundaries are ill-defined but there are often long, irregular processes giving the cell a somewhat stellate appearance (2). From the description of this unique lesion in sheeppox one might conclude that dendritic or even Langerhans cells (LC) were actively involved in its histopathogenesis.

In studies on the role of LC in vaccinia virus (VV) infection in mouse skin (3), it was found that VV induces a marked increase in LC density at the site of infection. These findings are in agreement with the

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earlier report by Nagao and Inaba (4) on the involvement of LC in VV infection in the mouse skin. LC act as carriers of the virions, presumably for antigen processing and presentation to T cells in the lymph nodes, properties classically ascribed to dendritic cells (5,6). It was therefore of interest to determine whether dendritic cells are involved at the site of keratinocytic proliferation due to infection and replication of LSD virus. This is a preliminary study that shows that LC are present in the skin lesions of normal and LSD virus-infected cattle, albeit with some qualitative differences in the latter situation.

An Israeli isolate of LSD virus was passaged three times in bovine calf kidney cells and used to infect six-month-old calves at tenfold dilutions (10^0-10^{-6}). The animals were inoculated with 0.1 ml of the infected cell suspensions at multiple sites intradermally on the flank. Skin biopsies (0.5 mm^2) were removed from the lesions three weeks after inoculation and immersed in PBS containing 0.82% EDTA for 2.5 hr at 37°C. After that time the epidermal layers were separated to allow staining of the cell sheets with an anti-bovine Ia monoclonal antibody designated TH 14B, which is a murine IgG_{2a} specific for a highly conserved monomorphic antigenic determinant present on one of three detectable class II antigens in cattle (7). These antibodies stain bovine dendritic cells (7). A fluorescein-isothiocyanate (FITC)-labelelled goat anti-mouse Fab (Bio-Makor, Rehovot, Israel) was reacted with the cell sheets and immunoflourescence was viewed in a Nikon epiflourescent microscope.

Typical LC with long dendritic processes were seen in the LSD-affected skin (Fig. 1a,b) and in normal skin (Fig. 1c). These preliminary observations indicate that LC do not completely disappear during the infectious process of LSD virus. However, during histogenesis of the lesion, the LC tended to clump together (Fig. 1a) and lose their intense flourescence (Fig. 1b). The function and role of dendritic Langerhans cells in the development of the skin lesions is under study.

Acknowledgements

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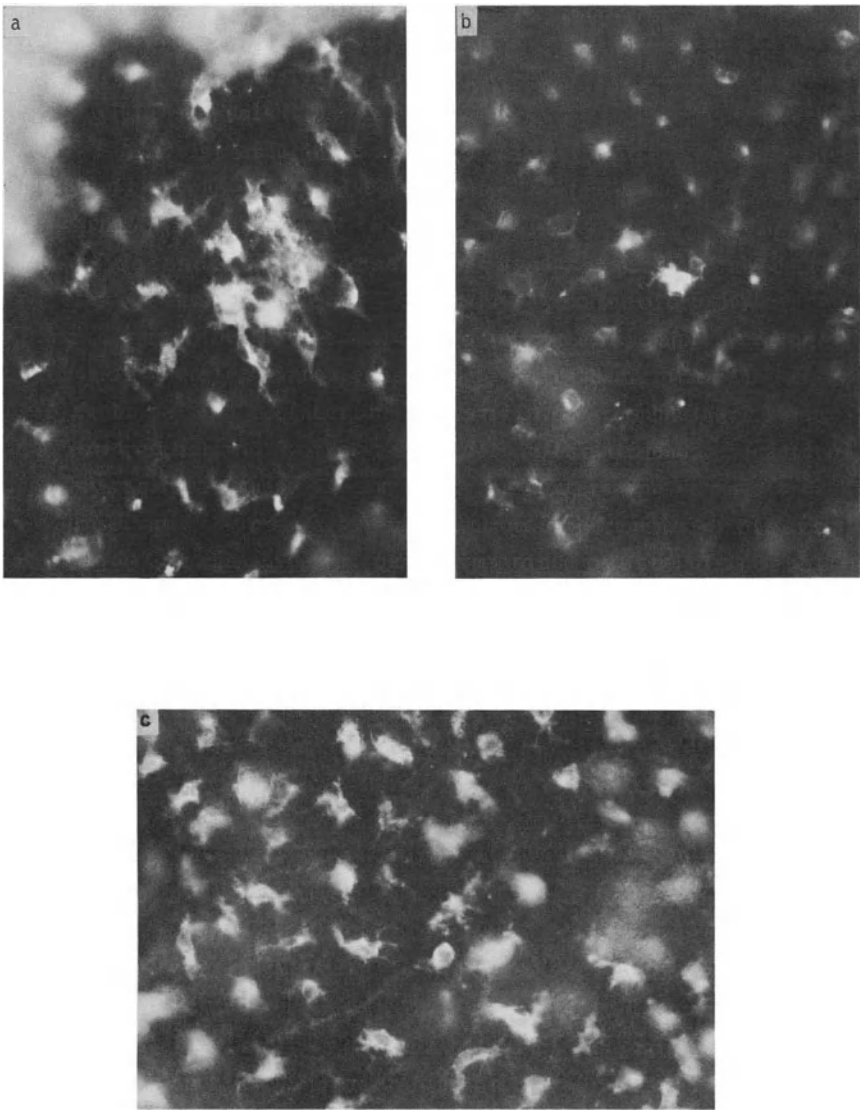

Fig. 1. Langerhans cells in LSD virus-infected skin lumps (a,b) and in normal bovine skin (c).

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LANGERHANS CELL AND INTERDIGITATING DENDRITIC CELL INFECTION IN AFRICAN SWINE FEVER

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ABSTRACT

African swine fever (ASF) presently occurs in domestic pigs as a virulent disease, and a moderately virulent disease. The virulent form causes a necrotizing disease and high mortality in 7 to 10 days with negligible immunoglobulin production. The moderately virulent disease does not produce extensive necrosis, has low mortality, and detectable antibody to ASF is produced starting at 6 days post-inoculation (DPI).

Using avidin-biotin alkaline phosphatase (ABC-AP) immunostaining systems on paraffin sections we have identified ASF-infected interdigitating dendritic cells (IDCs) in the lymph node as early as 2 days post-infection. Infection of interdigitating cells was confirmed with anti-S-100 double staining and by finding viral factories in these cells by electron microscopy. Similar electron microscopic results were obtained in vitro with Langerhan's cells (LCs) collected from skin explant cultures. The infection and necrosis of these antigen-presenting cells may explain the lack of a humoral immune response in virulent ASF, despite their apparently normal lymphocyte functions.

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INTRODUCTION

African swine fever, a disease of pigs caused by an iridovirus, presently occurs as a virulent disease with high mortality for domestic pigs in Africa and as a moderately virulent disease with low mortality in the Iberian peninsula, Sardinia, and some parts of Africa. The virulent form is a fulminating necrotizing disease of the mononuclear phagocytic system with extensive hemorrhage, anemia, and death in 7 to 10 days with negligible immunoglobulin production. In contrast, in the moderately virulent disease there is minimal necrosis and many pigs recover by 10-14 days post-inoculation (DPI). Immunoglobulin production in these pigs follows a normal pattern, with IgM detectable by 4 DPI, and IgG by 5 or 6 DPI (1). ASF antibody is not neutralizing and viremia persists for one to two months in the presence of a high antibody titer.

In both of the above forms of ASF, lymphocyte functions in vitro are not significantly affected (2). In the virulent disease, the foci of cell necrosis in the paracortex of the lymph nodes and lack of an immune response in the presence of an apparently functional lymphoid system, suggests a failure of antigen-presenting cells may underly the immunologic deficiency.

MATERIALS AND METHODS

Tissues and immunostaining.

Mandibular lymph nodes were collected from normal and ASF infected Yorkshire crossbred pigs receiving moderately virulent (Dominican Republic II) or virulent

(Lisbon '60) isolates of ASF by the oral-nasal route. Pigs were killed on 0,1,2,3,4,5, and 7 days post-inoculation (DPI) using an overdose of pentabarbital. All tissues and cells were fixed in paraformaldehyde-lysine-periodate (PLP) fixative (3).

As an aid in localizing ASF-infected dendritic cells for electron microscopy, matching paraffin-embedded sections were stained by the avidin-biotin complex-alkaline phosphatase (ABC-AP) immunostaining procedure. Dendritic cells were stained by an indirect procedure with rabbit anti-bovine S-100 antibody (Accurate Chem. Westbury, NY) and a Vector anti-rabbit ABC-AP kit (Vector labs, Burlingame CA); and ASF antigen was stained using biotinylated hyperimmune swine anti-ASF antiserum and the Vector ABC-AP system. Both were incubated overnight at 4 C using 1:400 primary antisera. For double ABC-AP staining, S-100 was done first, then ASF. For chromagens, blue alkaline phosphatase substrate kit III (for S-100) and "Vector Red" (for ASF) were purchased from Vector labs.

Langerhans cell collection and infection.

For in vitro investigation of ASF-infected dendritic cells, normal LCs were collected from skin sheets from the back of a normal pig. The pig's back was depiliated and scrubbed. Following euthanasia, approximately 300 square cm of skin (1 mm thick) were shaved off using and microtome knife with a handle. The skin sheets were collected in Eagle's minimal essential medium with high antibiotics and fungizone (MEM-AB), held on ice for 30 minutes, and then floated on fresh

MEM-AB in Petri dishes overnight. The skin was carefully removed, and the media and floating cells gently decanted. The lightly adherent LCs were flushed into a centrifuge tube, gently pelleted and resuspended in 1 ml of MEM-AB and seeded into 10 wells of a 96 well tissue culture plate. These cells were approximately 90% LCs with a few adherent lymphocytes as determined by light and electron microscopy.

To determine infectivity of LCs with ASF, vero cell-adapted ASF virus was added to half the wells at a multiplicity of infection of approximately 1. The cells were incubated for 18 hrs at 37 C and fixed in PLP for examination by electron microscopy. Non-infected wells were fixed similarly for controls.

Electron microscopy.

Cell pellets of skin-derived LCs fixed in PLP and selected subcapsular areas of lymph node paracortex were postfixed in 1% osmium tetroxide, dehydrated in graded acetone and embedded in Effapoxy (Fullam, Latham, NY). Thin sections were examined in a Zeiss EM-10C electron microscope.

RESULTS

Interdigitating dendritic cells.

As early as 2 or 3 DPI, cells were found staining for ASF antigen in the paracortex of the mandibular lymph node. These cells were identified morphologically, by light microscopy, as interdigitating dendritic cells (IDCs). Compared to uninfected controls and hog cholera infected lymph nodes, staining of these IDCs for S-100

antigen was markedly reduced in ASF by DPI-3. Using double staining ABC-AP procedures for S-100 and ASF antigens, S-100 positive interdigitating dendritic cells were found with strongly staining ASF antigen in the cytoplasm. By electron microscopy, these cells had morphologic characteristics of IDCs and some were found to contain intracytoplasmic viral factories, free ASF viral particles in the cytoplasm, and budding virus from the plasma membrane (Fig. 1). In ASF-infected lymph nodes, most IDCs appeared to have attenuated cytoplasmic extensions and contact with lymphocytes was reduced compared to controls (Fig. 2).

Langerhans cells.

The skin-derived LCs from uninfected cultures had ultrastructural characteristics found in similar cells of other species. Numerous cytoplasmic extensions were present and were sometimes found in close contact with lymphocytes. The cytoplasm contained few organelles, and the nuclei were highly convoluted (Fig. 3). Birbeck granules were not found, as is the case for most domestic species. The in vitro infection of LCs with ASF virus was nearly 100% in 18 hours. The LCs were markedly swollen, vacuolated, and contained viral factories and free ASF virus in the cytoplasm (Fig. 4). Most infected cells were degenerate and beginning to lyse. Lymphocytes were found to be unaffected.

DISCUSSION

It seems likely that, as a result of ASFV infection, the ability of IDCs, and perhaps LCs, to

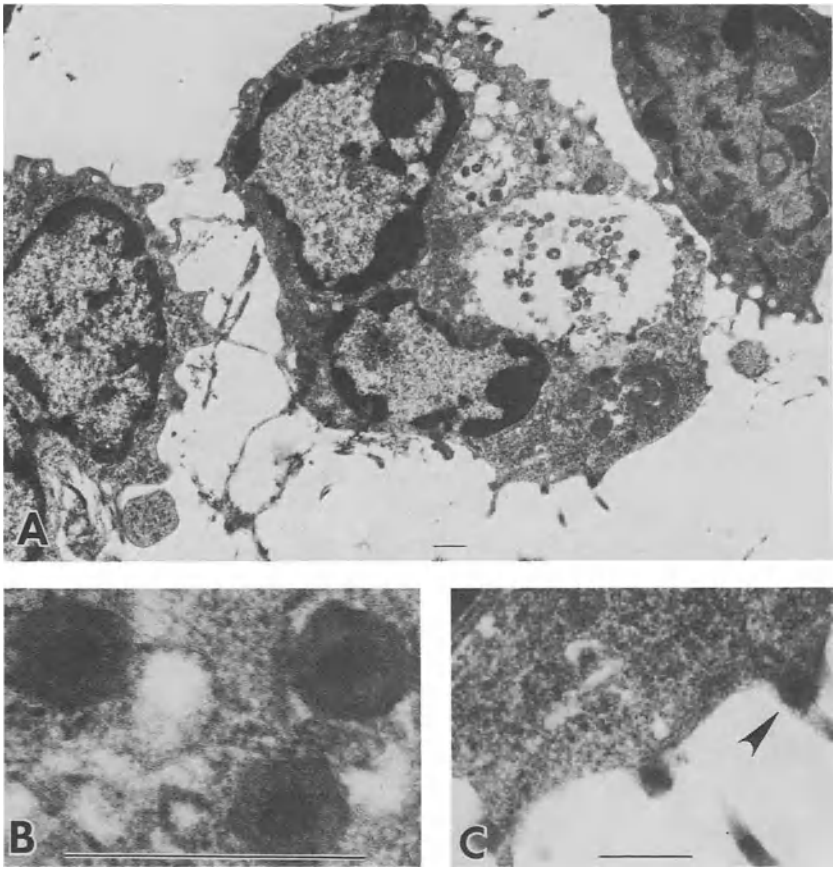

Fig. 1. Electron micrograph of ASF virus infected pig mandibular lymph node paracortex at 3 DPI.
A. An ASFV infected interdigitating dendritic cell with typical convoluted nucleus and absence of lysosomes.
B. Enlargement of the cytoplasmic viral factory with assembling viral nucleocapsids.
C. Enlargement of a viral particle budding from the cytoplasmic membrane. Bar = 1 μM

Fig 2: EM of normal pig lymph node paracortex with an interdigitating dendritic cell surrounded by lymphocytes. Note close contact of cells and interdigitating processes. Bar = 1 µM

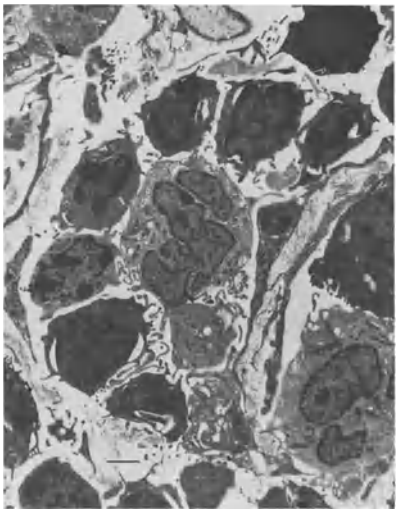

Figure 2

Fig 3: EM of normal pig Langerhans cells isolated in vitro for 24 hrs. Note convoluted nuclei and cytoplasmic processes. Bar = 1 µM

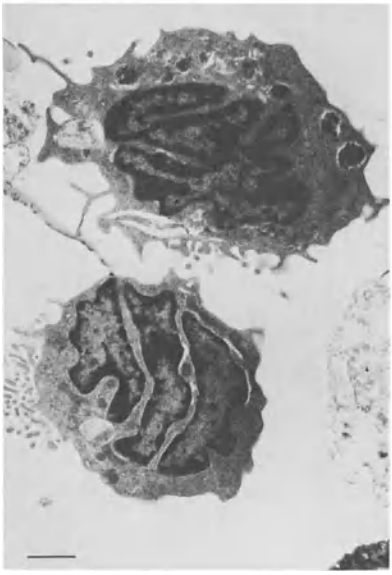

Figure 3

Fig 4: EM or ASFV infected pig Langerhans cell 18 hr PI in vitro. There is marked cell swelling and degeneration. Insert: viral factory in cytoplasm with nucleocapsids. Bar = 1 µM

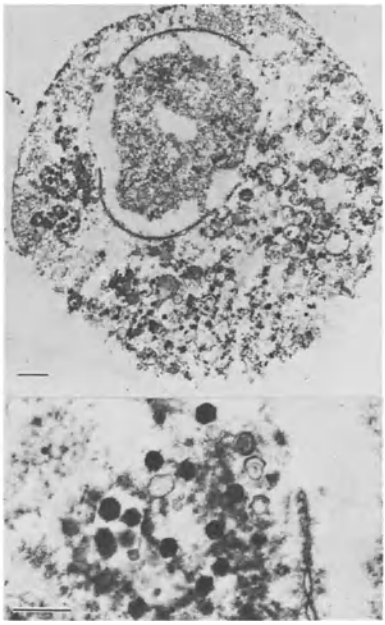

Figure 4

function as antigen presenting cells is compromised. We suggest that this may be the basis for the failure of an immune response in pigs infected with the virulent ASF virus.

There are few studies demonstrating viral infection of dendritic cells in vivo. The most notable of these is the human immunodeficiency virus (HIV). In this infection, Langerhans cells in the skin (4), interdigitating dendritic cells in the lymph node (5), and peripheral blood dendritic cells have been shown to be infected in vivo. Peripheral blood dendritic cells have also been infected in vitro with HIV (6). Furthermore, in chronic AIDS patients, Langerhans cells in the skin and mucous membranes (7), and interdigitating dendritic cells in lymphoid organs (8) have been shown to be decreased. It has been suggested that HIV infects dendritic cells early and is later transmitted to T-helper cells through their close association in the paracortex of the lymph node(5). The infection of Langerhans cells, interdigitating cells, T-helper cells, and possibly follicular dendritic cells (9), and the chronic depletion of these cell types results in a type of chronic combined immunodeficiency in the case of AIDS.

In contrast to AIDS, ASFV infects LCs and IDCs but not T cells. We propose that the lack of an immunoglobulin response and high mortality in the highly virulent ASF infection is due to viral infection of these dendritic cells and/or a blockade of their functional capacity, with eventual cell necrosis. In the

moderately virulent form of ASF, IDCs are also infected but there is an immune response indicating that the IDC function may not be completely compromised. Recovery or death of the pig may depend on the degree of incapacitation of the IDCs by ASFV infection. African swine fever infection in the pig may serve as an animal model to determine the effect of a functional depletion of IDCs and better define the role of this important cell in immunity. Since LCs are considered to be the precursors to IDCs, and have now been shown to be infected in vitro, these cells may play a more important role in ASF than previously thought.

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PERSPECTIVES

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PERSPECTIVES OF DENDRITIC CELL RESEARCH

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The dendritic (Langerhans) cells in the skin belong to a family of dendritic cells, the progenitors of which are made in the bone marrow. The precursor cells are present in the blood and the mature cells are present in all epithelia of the body. The studies presented in this book focus on the skin dendritic cells, their properties and involvement in normal physiological conditions like aging and in abnormal genetic states or after environmental damage (like virus infections). The available information on the skin dendritic (Langerhans) cells does not provide us with answers to many questions concerning the evolutionary aspects of dendritic cell development in avian and reptile species, the role of dendritic cells in the development of skin cancer, the importance of dendritic cells in normal skin in humans, the mechanism of antigen presentation by the dendritic cells and the relationship between dendritic cells in epithelia of different organs in the body. These questions, which might provide new insight into treatment of autoimmune diseases as well as new approaches to immunization against pathogenic agents like viruses are currently under study. This review of current developments in the research on aspects of dendritic cells which were not dealt with in the present book, may provide the reader with additional information on the current reseach avenues.

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Dendritic cells and environmental chemicals

Cigarette smoking was associated with a significant decrease in the number of Langerhans cells in normal cervical epithelium, having a dose response relationship between the number of cigarettes smoked daily and the effect on cell counts (1). Cigarette smoking was reported (2) to be associated with a two-fold increase in the dendritic/Langerhans cell lineage with bronchiolar epithelium in the lungs. Most of the Langerhans cells found in the lung parenchyma of smokers were observed in close association with areas of alveolar type II pneumocyte hyperplasia. This might explain why most adult patients who develop Langerhans cell granulomatosis are smokers (2).

Application of irritant agents (sodium lauryl sulfate or croton oil) to human skin in-vivo resulted in a progressive depletion in the number of epidermal Langerhans cells from 3.1 ± 0.2% of total epidermal cells to 1.2 ± 0.1% after 8 days (3). The capacity of irritants such as croton oil to abrogate the epidermal antigen-presenting (Langerhans) cells may be related to the tumor-promoting potential of these agents. In this respect the effect of croton oil resembles the effect of the tumor promoter 12-0-tetradecanoylphorbol 13-acetate (TPA) on skin dendritic (Langerhans) cells in murine ear epidermis causing depletion of these cells (4). Similar results were also reported by Baxter et al. (5).

Contrary to the above, it was also shown (6) that chemical compounds related to the monobenzyl ether of hydroquinone (MBEH) such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) that are used as preservative/antioxidants in topical medications, cosmetics, food and rubber products can increase the population of Thy-1$^+$ dendritic (Langerhans) cells in the skin.

Subcutaneous injections of testosterone propionate (TP) for 14 days resulted in a significant decrease in LC density both in orchiectomized males and normal females as well as in adrenalectomized mice (7). Topical corticosteroids, which have anti-inflammatory action and modulation of cutaneous immune

reactions in man, were reported (8) to reduce the number of Langerhans cells.

The effects of local hyperthermia treatment (43°C for 45 min) on contact sensitivity and the number of Langerhans cells in the mouse skin was reported (9). It was found that the density of Langerhans cells and the contact sensitivity were significantly decreased by hypothermia.

Cyclosporin A (CsA) and the blocking of antigen presentation by dendritic cells

Painting mouse skin with the contact sensitizer FITC one day after oral treatment with CsA resulted in inhibition of delayed hypersensitivity. The numbers of dendritic cells in the lymph nodes more than doubled after skin painting of the CsA-treated mice (10). It was suggested (10) that antigen presentation by dendritic cells might be prevented by CsA together with direct effects on T and B cells. Additional studies (11) revealed that overnight in-vitro inoculation of Ia^+ Langerhans cells from 16 to 18-month-old BALB/c mice with a mixture of cytokines (IL-1, IL-2, IL-3, IL-4, IL-6, TNFα, IFNα, GM-CSF) caused a significant increase in the Ia expression on Langerhans cells. Addition of CsA to the medium abolished the effect of the cytokines except IL-2 and IL-6 (11).

To resolve the mode of action of CsA the effect of the drug on mRNA of cytokines in monocytes and T cells was studied (12). It was concluded that CsA affects the production of lymphokines by T lymphocytes.

Loss of epidermal Langerhans cells in the onset of type 1 diabetes in humans

Research on antigen-presenting Langerhans cells in the skin of diabetic patients revealed a significant decrease in these cells in 10 patients immediately after the onset of diabetes (13). This result suggests that other antigen-presenting cells may be involved in the pathogenesis of type 1 diabetes (13). Indeed, two morphologically distinct populations of Ia^+ cells were found to be scattered within the Ia^- islet tissue (14). Large irregularly

shaped Ia^+ cells were identified as dendritic cells by antibody 33D-1 that is specific for the mouse dendritic cell antigen. The second type consisted of small, round Ia^+ and 33D-1 antibody-negative cells (14). These cells are involved in rejection of murine islet allografts and pretreatment with anti-dendritic cell antibody prevents the rejection of the Langerhans islet allografts (14).

Prospects

Interest in antigen-presenting dendritic cells stems from their involvement in normal and pathological conditions. The current focus is on the immunological role of the dendritic cells (15). Much less is known about their involvement in embryogenesis and in the regulation of epithelial tissues. Studies on avian thymic accessory cells (16) revealed some aspects of dendritic cell interaction with thymic epithelial cells and thymocytes.

Much is still to be studied on regulatory functions of the dendritic cells, their mode of action under normal conditions and in disease processes. Molecular studies on the genes that function in the progenitor, precursor and mature dendritic cells are needed to resolve the questions regarding the immunological and regulatory nature of these cells.

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