DENDRITIC CELLS AS REGULATORS OF IMMUNE REACTIVITY: IMPLICATIONS FOR SKIN TRANSPLANTATION

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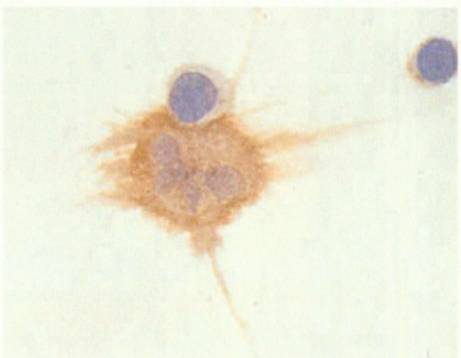

I would like to thank

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CONTENTS:

Introduction

Skin allografts, in contrast to other organ transplants, are acutely rejected despite intensive and toxic for the graft recipient immunosuppressive therapy. Long-term immunosuppression increases the risk for life- threatening infections and cancers. This is why clinical skin allografting practically does not exist. Recent cases of hand transplantation performed in various world centers have renewed the interest in basic studies on the pathomechanism of skin allograft rejection, since skin is the most actively rejected component of the tissue composite graft (1). At the time of organ transplantation, a variety of non-parenchymal cells are transplanted simultaneously with the allograft. Recognition of the importance of these cells as potential immunostimulatory cells led to the concept of "passenger leukocytes" as the principal instigators of rejection. Among them are the dendritic cells (DCs).

Skin dendritic cells play a crucial role in the process of recognition of alloantigens, its processing and initiation of the rejection reaction. DCs are a unique population of leukocytes, and are the most effective antigen presenting cells (APC). They originate from CD34+ bone marrow stem cells and their precursors are seeded via the bloodstream to the tissues where they give rise to immature DCs. Majority of non-lymphoid tissues and organs contain DCs, including skin, heart, liver, lung and mucosal surfaces (2). There are three pools of DCs isolated from skin: a/ epidermal Langerhans' cells (LCs), b/ dermal DCs (DDCs), c/ afferent lymph veiled cells (VC) (3). Cells from each cutaneous compartment can exhibit a distinct morphology, surface phenotype, and function. The bone marrow derived precursors of DCs extravasate in the dermis

and give rise to DDCs and migrate toward epidermis to locate between the keratinocytes as LCs. Human skin is constantly barraged by a wide assortment of infectious agents including bacteria, fungi and viruses. Skin as a complex ecosystem with numerous microbial inhabitants presents highly efficient and evolutionarily conserved mechanisms of the innate immune system preventing colonization of deep tissues by the environmental antigens (defensins, toll receptors, NODs)(4). LCs, as immature DCs have ability to uptake antigen and degrade it to produce peptides capable of binding to MHC class II. In response to tissue damage, inflammatory cytokines and antigen activation LCs mature and migrate from the epidermis toward the dermal initial lymphatic vessels (5) and further with afferent lymph stream, as VCs, they flow to the regional lymph nodes (Fig.1) (6). VCs carry processed antigens or self antigens to lymph nodes where they interact with CD4 T lymphocytes to initiate immune responses, to mobilize lymphocyte and monocyte populations from blood perfusing the node, and to form the antigen-specific cohorts of effector cells. DCs do not appear in efferent lymph and therefore must accumulate in the lymph node as interdigitating cells and die by apoptosis (7).

In order to present antigen to lymphocytes, DCs have to come into physical contact with these cells and form a cluster (Fig.2).

Recently, a term "immunological synapse" has been coined for this type of cluster (8). The onset of signaling and T cell activation by DCs involves formation of a synaptic structure at the DCs-lymphocyte interface, with many features similar to those of the neuronal synapse.

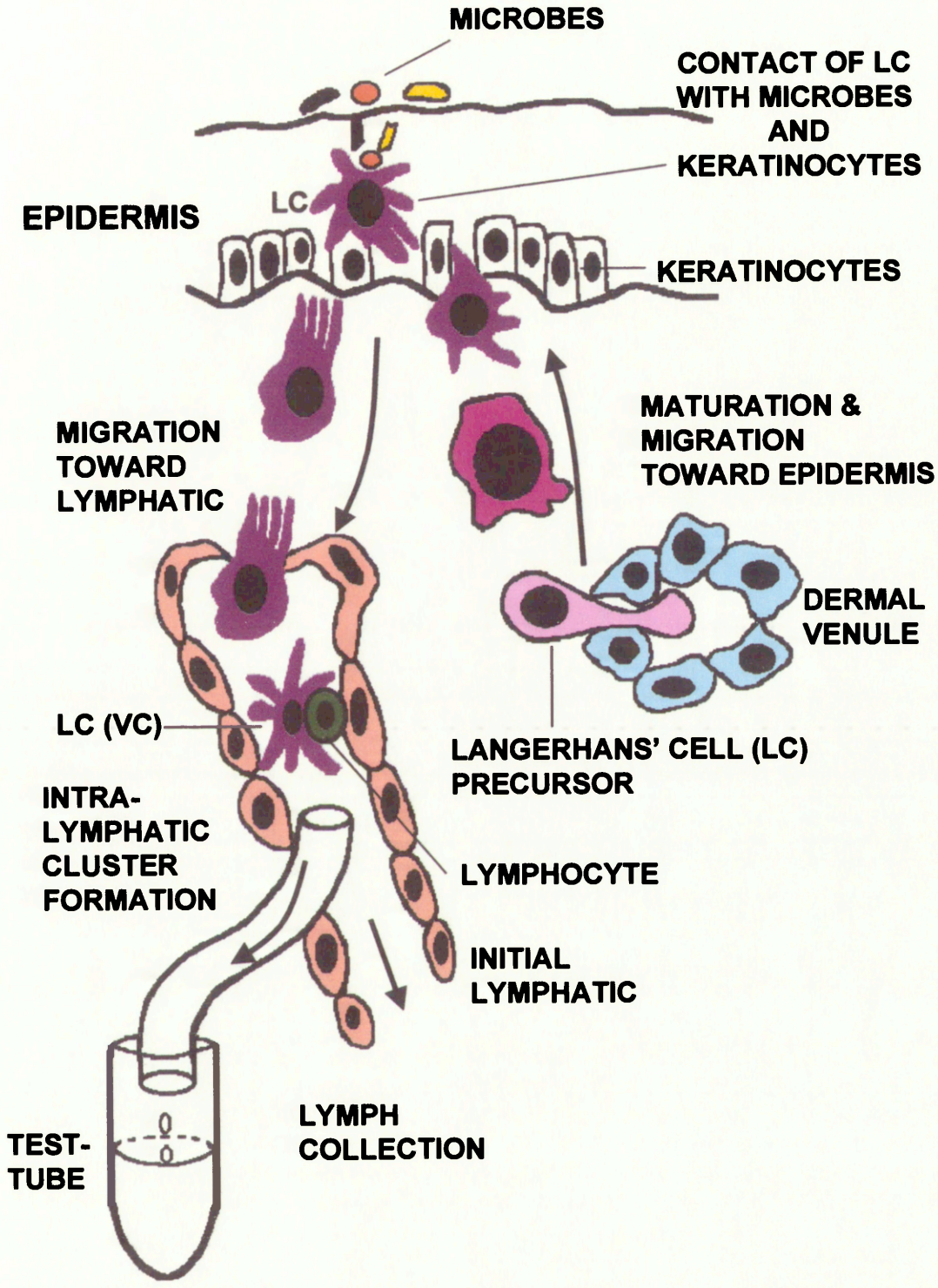

Fig. 1. Migration of Langerhans' cells from venules torward epidermis and initial lymphatic (arrows)

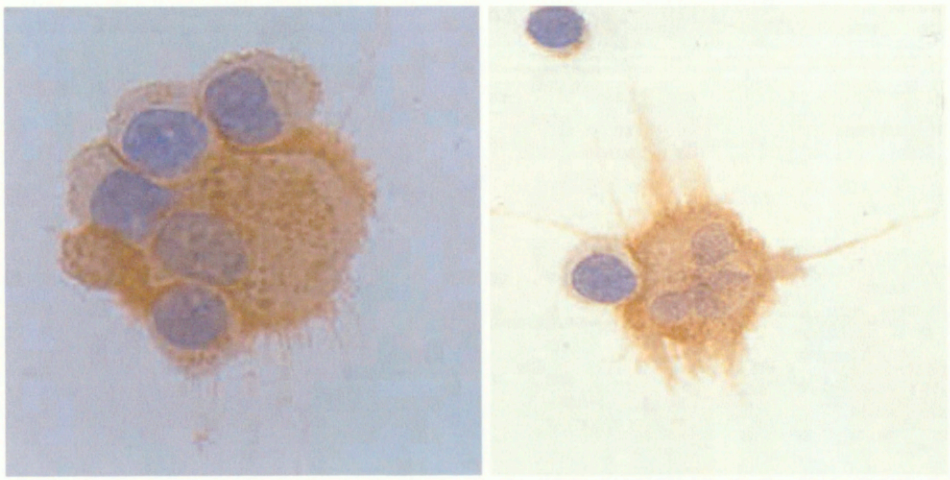

Fig. 2. Clusters of VCs with lymphocytes isolated from afferent lymph, cytospun onto glass slide and stained immunocytochemically with anti-DR-antibody.

In addition to the MHC-TCR contact, this structure includes co-stimulatory and adhesion molecules which interact with counter-receptors. A synapse is composed of the peripheral and central supramolecular activation clusters. The main function of the synapse is to serve as a device for initiating, stabilizing and sustaining TCR signal transduction during DC encounter. The assembly of mature synapse may take several minutes only (9). Interestingly, in skin immune reactions clusters between VCs and lymphocytes can be already seen in the dermal interstitial space and lymph reaching lymph node (10,11). This suggests the VC-lymphocyte cooperation to start already at the site of inflammation and VCs may "select" lymphocytes for migration in afferent lymph to peripheral lymph node (7). In skin allografting the process of allorecognition

and effector reaction is more complex than that after bacterial penetration of skin. The donor provides in the skin graft immunologically active keratinocytes, LCs, dermal macrophages and lymphocytes, whereas the recipient mobilizes own DCs, macrophages and lymphocytes in order to eliminate donor-specific antigens (Fig.3). This complex local reaction is a new biological phenomenon as no MHC disparate skin-to-skin transfer occurs in nature. Each skin allotransplant evokes a host-versus-graft and graft-versus-host reaction.

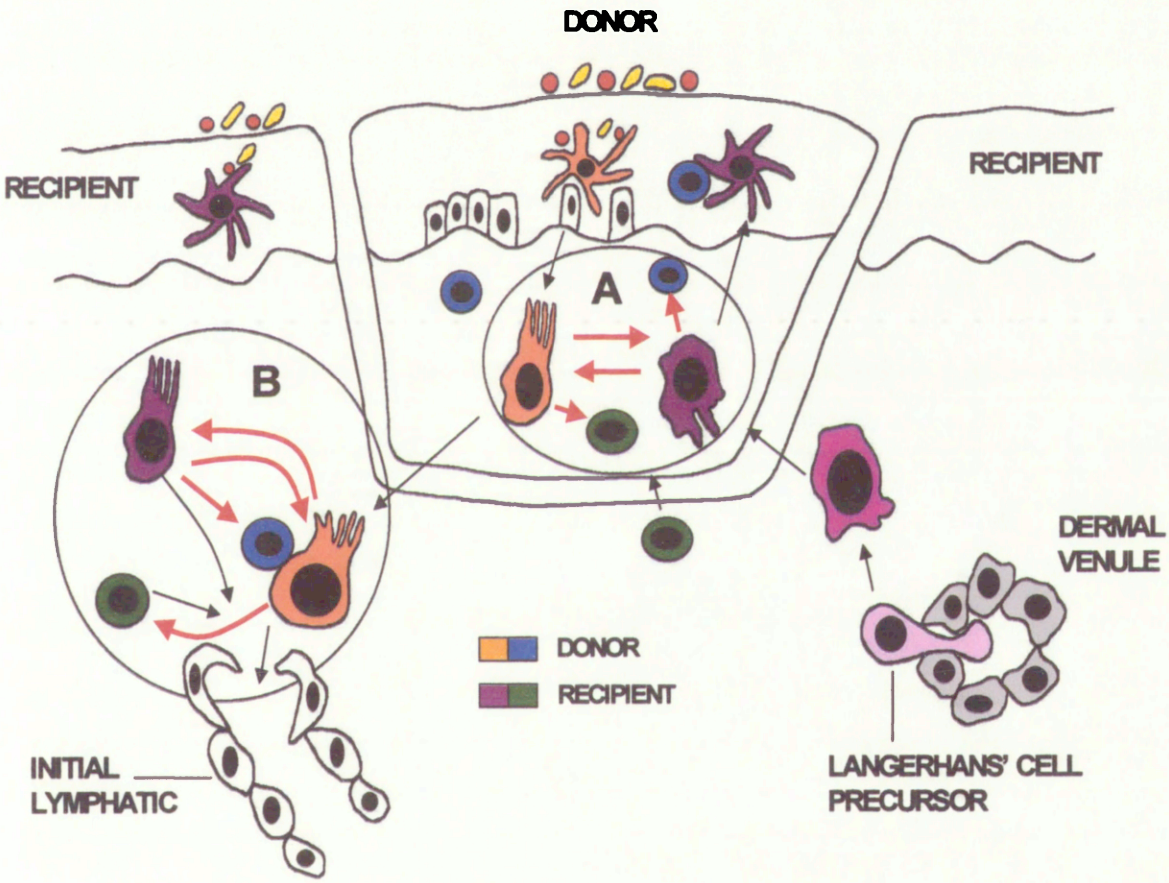

Fig. 3. Stimulators and responders of both donor and recipient origin in (A) – skin graft and (B) – recipient graft bed. Full red arrows indicate cells forming synapses and bidirectional stimulation. Thin black arrows show direction of cell traffic.

The fact that DCs are the most potent inducers of T cell responses led to high interest for their clinical application. In recent years, DCs were increasingly studied for their role as critical adjuvants in vaccines for prevention of microbial infection and for treatment of cancer and autoimmune diseases. DCs can be propagated in vitro from the bone marrow and peripheral blood using various combinations of growth factors (12). However, the in vitro generated dendritic cells undergo maturation in culture and after injection into skin have been shown to remain at the site of injection (13,14). Since a major goal in transplantation research is to understand and exploit the immunogenic properties of passenger DC as well as the tolerogenic properties of immature DCs (15), studies concerning migrating and less matured VCs obtained from afferent lymph draining skin than cultured DCs seem to be more relevant.

Knowledge of migrating DC biology, specially the phenotypic and functional characteristics of afferent lymph VCs, the mechanisms responsible for VC- lymphocyte cluster formation upon stimulation with allogeneic and bacterial antigens, the effect of immunosuppressive drugs on lymph cells clustering towards their possible inhibitory properties, and the localization of DC in skin epidermis and dermis in the inflammatory foci, would facilitate a rational approach for the therapeutic protocols enabling the prolongation of skin allograft survival time.

List of own citated publications, referred in the text by their Roman numerals:

I. **Galkowska H,** Dabrowski M, Olszewski WL. A single step centrifugation methods for enrichment of veiled cells from the canine afferent lymph. J Immunol Method, 1989, 116:207-212 ***IF=2.598***

II. Dabrowski M, **Galkowska H,** Olszewski WL. Functional characteristics of veiled cells from canine prenodal lymph. Immunobiology, 1989, 178: 316-328 ***IF=1.319***

III. **Galkowska H**, Dabrowski M, Olszewski WL. Development of antiserum against veiled (dendritic) cells of canine afferent lymph: implications for transplantation biology. Lymphology, 1992, 25:75-83 ***IF=0.750***

IV. **Galkowska H,** Olszewski WL, Wojewodzka U. Reactivity of antibodies directed against human antigens with surface markers on canine leukocytes. Vet Immunol Immunopathol, 1996, 53 (3-4): 329-334 ***IF=1.659***

V. **Galkowska H,** Olszewski WL, Interewicz B, Porebska A, Wojewodzka U. A novel monoclonal antibody specific for lymph dendritic cells. Lymphology, 1998, 31: 169-173 ***IF=0.750***

VI. **Galkowska H,** Olszewski WL. Immune events in skin. I. Spontaneous cluster formation of dendritic (veiled) cells and lymphocytes from skin lymph. Sc J Immunol. 1992, 35:727-734 ***IF=1.782***

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VIII. **Galkowska H,** Moscicka M, Cybulska E, Wojewodzka U, Mijal J, Olszewski WL. Skin allografts- host cutaneous veiled cells initiate rejection reaction by indirect pathway of allorecognition. Transplantat Proc, 2001, 33: 627-630 ***IF=0.478***

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X. Wojewodzka J, **Galkowska H,** Olszewski WL, Inhibition of formation of synapses between dendritic cells and lymphocytes in skin lymph in an allogeneic reaction by cyclosporin A and Tacrolimus. Transplantat Proc 2003, 35: 2376- 78 ***IF=0.478***

XI. Mijal J, Olszewski WL, **Galkowska H.** Lymph dendritic cells react to bacteria- the mechanism of dermatolymphangioadenitis. Lymphology 2002, 35:103-106 ***IF=0.750***

XII. **Galkowska H,** Olszewski WL, Wojewodzka U, Mijal J, Filipiuk E. Expression of apoptosis- and cell cycle- related proteins in epidermis of venous leg and diabetic foot ulcers. Surgery 2003, 134 (2): 213-220 ***IF=2.631***

XIII. **Galkowska H,** Olszewski WL, Wojewodzka U. Keratinocyte, dermal and infiltrating immune cell capacities remain unimpaired in the margin of chronic venous ulcer. (Histopathology 2004, submitted)

Own studies

Investigation of the in vivo- initiated reaction between migrating LC (VC) and lymphocytes (afferent lymph cell clustering) need an appropriate model providing sufficient numbers of cooperating cells. This is not feasible in rodents with tiny skin afferent lymphatics containing few lymph cells. Therefore, I have chosen a canine model with wide afferent lymphatics and sufficient numbers of circulating lymph cells. The proximal ligation of limb lymphatics and removal of the popliteal lymph node prior to harvesting lymph cells for laboratory studies allowed consecutive lymph sampling by vessel puncture. Results of my own studies accumulated in this folder include:

(i) setting up a model for collection of skin afferent lymph with VCs and lymphocytes,

(ii) phenotypic and functional characterization of these cells using specific and cross- reactive as well as own laboratory raised antibodies,

(iii) studies of VC-lymphocyte clustering kinetic in vitro, immediately after lymph cell collection,

(iv) in vitro effect of Cyclosporin A and Tacrolimus (FK506) on lymph cell cluster formation,

(v) stimulation of cell clustering by skin bacterial antigens,

(vi) preliminary observation on LC localization and contacts with other cells in the inflamed skin in humans.

The results and conclusions from my consecutive publications have been summarized below in paragraphs.

1. Harvesting and enrichment of canine afferent lymph veiled cells (I)

Studies on lymph veiled cells in inbred strains of rodents are limited by low numbers of available cells. Larger animals with high prenodal lymph flows are preferred for that purpose. Among the large animals dogs are particularly suitable, since interruption of their lymphatics brings about an accumulation of VC in the stagnant lymph. A method has been established for obtaining large numbers of VC from afferent lymph for subsequent functional studies. An enrichment of VC was achieved in vivo in dogs following surgical interruption of hind limb lymphatics (16). The stagnant lymph of such dogs contains 10-30-fold more cells compared with normal lymph (17).

Veiled cells present in the afferent lymph of dogs with chronic lymphedema were enriched from 6% to about 50% VC by density gradient centrifugation on 15% metrizamide or discontinuous Percoll gradients. The recovery of VC was about 40% from $0.22 \pm 0.07 \times 10^6$ VC/ml of lymph. The cells were strongly Ia positive and had cytoplasmic S 100 protein. Canine VCs were all positive for S 100 cytoplasmic protein. This property, which has so far been demonstrated for the Langerhans' cells of skin and for interdigitating reticulum cells in lymph nodes, is not present in phagocytic macrophages. This functional property permits the differentiation of the two types of cell lineage. Low density cells from canine lymph were strongly ATP-ase positive and their activities of acid phosphatase, peroxidase and of non-specific esterase

resembled these described for VC from other species (18,19). Canine VC were able to stimulate T cells in allogeneic MLR and in auto-MLR when present at cell concentrations as low as 5% of the responding cells. Moreover they exhibited the PHA presenting function characteristic of autologous lymphocytes.

The present results indicate a close morphological, cytochemical and functional analogy between the veiled cells of dogs and other species.

2) Functional characteristics of canine afferent lymph veiled cells (II)

Veiled cells are believed to be a migrating form of Langerhans' cells from the epidermis, which enter the regional lymph nodes via afferent lymphatics. There, VCs transforming into interdigitating cells may come into a close contact with T cells and act as potent antigen-presenting cells. The main questions of this study were whether canine lymph VCs: a/ share the morphological and cytochemical similarities with VCs from other species, b/ act as accessory cells-mitogen presenters to the autologous and allogeneic lymphocytes, and c/ evoke alloantigen-generated responses.

In the functional assays, VCs displayed the potent accessory-cell activity in the mitogen-induced response of autologous blood- and lymph-derived lymphocytes. In the mixed leukocyte cultures, VCs acted as stimulators of the allogeneic and autologous lymphocyte proliferation. The high spontaneous and mitogen-induced responsiveness of the whole lymph cell population was found to be dependent on the presence of VCs. The small number of VCs (5% of cultured cells) was sufficient to produce the above-mentioned effects.

These data indicate that VCs are cells responsible for the antigen presentation in the skin-associated immune reactions in dogs, which is relevant to the observations on similar cells from the other species.

3) Development of antiserum against afferent lymph veiled cells (III)

Several authors have suggested that the cells responsible for the initiation of allograft rejection are passenger cells. The effectiveness of the Ia-positive veiled passenger cells of the skin collected from afferent lymph in provoking allogeneic response and in initiating renal graft rejection has previously been described. Whether anti-Ia antiserum reduces the number of Langerhans' cells in skin allograft is not known. Also, no data on the possible effects of specific anti-Langerhans' cell serum are available. There have been major problems with raising such antisera due to difficulties in obtaining sufficient numbers of dendritic cells from epidermis or afferent lymph. We have found, however, a method for harvesting large numbers of afferent lymph cells, adequate for immunization of antiserum producers.

The purpose of the present study was to develop anti-veiled cell antiserum and to characterize its effects in vitro and in vivo on skin Langerhans' cells and lymphocytes. Anti-sera used in vitro blocked the Ia and CD1 antigens of VC on smears and inhibited the accessory function of VC in cell response to phytohemagglutinin (PHA) and their stimulatory activity in mixed leukocyte reaction (MLR). In vivo, the local, intracutaneous administration of antisera led to transient depletion of VC from afferent lymph, and to reduction of mononuclear cells in the T-dependent areas in regional lymph nodes. Depletion

of T-dependent areas in lymph nodes with sparing of follicles with use of the anti-veiled cell sera supports that this antiserum was directed primarily if not exclusively against dendritic cells and T lymphocytes.

By arresting the migration of Langerhans' cells from graft into lymphoid tissue or their maturation into immunostimulatory veiled cells, a new strategy for overcoming allograft rejection is raised by using antisera against these immunoreactive cells.

4) Reactivity of antibodies reactive with canine leukocyte surface antigens (IV)

Unlike those of man and mouse, canine leukocytes can be poorly characterized, because only limited numbers of monoclonal antibodies (mAb) to their antigens are available. The first workshop on canine leukocyte antigens has allowed the identification and characterization of a set of anti-canine mAb that enable the extension of immunological research in the dog (20). However, the specificities of these antibodies are still not clear enough to identify the canine equivalent of human CD antigens. For instance, canine CD4 antigen was detected at high levels on granulocytes and MHC class II antigens were found on all canine leukocytes.

In the present study monoclonal and polyclonal antybodies reacting with human leukocytes were screened for their cross-reactivity with canine leukocytes on cytospins using immunocytochemical methods. The cells from canine skin afferent lymph (containing lymphocytes and the migratory form of Langerhans' cells – veiled cells), peripheral blood and blasts raised in culture of lymph cells with PHA were used. A panel of anti-human antibodies was used in

order to investigate their cross-reactivity with canine leukocytes. The labeling was carried out at the microscopic level by immunocytochemical staining. Of 50 antibodies 22 cross-reacted with canine leukocytes from afferent lymph and peripheral blood. Ali leukocytes reacted with MHM23 (CD18). Two anti-HLA-DR antibodies, DK22 and L243, reacted mostly with veiled cells and with PHA-stimulated lymphocytes, whereas TAL1B5 was cross-reactive with all canine lymphocytes. The activation markers CD25, Ki-67, PCNA were identified on PHA-stimulated lymphocytes with ACT1, Ki-67 and PC10 clone produced antibodies. Canine eosinophils reacted with MHM6 (CD23) antibody. A large number of antybodies reacted with canine lymph veiled cells. Canine granulocytes and a subset of lymphocytes were stained with the anti-CD15 antibody only after treatment of cytospins with neuraminidase.

5) A novel monoclonal antibody specific for canine veiled cells (V)

The need for monoclonal antibodies (mAbs) that are specific for DC and block their functions is evident. The purpose of this study was to generate DC specific monoclonal antibodies useful for DC identification and reduction of their ability to present alloantigens to T lymphocytes.

In the present report two novel mAbs have been described that recognize antigens expressed by the canine lymph derived DC and granulocytes. Raised by us mAbs have reduced lymphocyte binding to DC and T celi proliferative response to DC-associated alloantigens in the MLR. Both, whole antibody molecules as well as F (ab') fragments had the modulatory effect on lymph celi binding. It can be suggested that the suppressive effects of these mAbs have

not been due to the blocking of receptors for antigen recognition, but rather to the blocking of the accessory molecules on DC. The epitopes recognized by our novel mAbs are unknown.

6) Spontaneous cluster (immunological synapse) formation by afferent lymph veiled cells and lymphocytes (VI)

A drop of lymph freshly drawn from a skin afferent lymphatic contains 3-6% of clusters formed by veiled cells and lymphocytes that most likely illustrates the in vivo cooperation of these populations. Clustering is the first phase of antigen presentation to lymphocytes. Dendritic cells bind lymphocytes in an antigen-independent pathway and can also bind resting T lymphocytes (21). Little is known about the mechanism involved in the clustering of DC with resting T lymphocytes, including expression of adhesion molecules. Controlling this process may be helpful in mitigation of skin immune reactions.

In this study we tried to elucidate the mechanisms of 'spontaneous' binding of veiled cells from the canine skin afferent lymph with autologous lymphocytes in their own environment, i.e. the lymph, and in the absence of a known antigen. The number of clusters forming ex vivo in the collected lymph samples increased as a function of time and was temperature dependent. Incubation of cells with proteolytic enzymes or monosaccharides did not alter cell interactions. The ability of veiled cells to bind lymphocytes was independent of divalent cations but reduced by xylocaine and retinoic acid. Among steroids only methylprednisolone showed an inhibitory effect on cluster formation. Indomethacin and acetylsalicylic acid had no blocking activity on cell binding.

Also, no effect was seen after treatment with cyclosporine A and azathioprine. An enhanced cluster formation after desialation with neuraminidase was observed. The desialated cells were cultured in order to study their stimulatory and accessory cell functions. No enhancement of autologous mixed leucocyte reaction was seen, but a significantly higher responsiveness to a suboptimal dose of phytohaemagglutinin was observed. The N-ase- mediated non- specific cell attachment could be abrogated by cell washing or treatment with EDTA or xylocaine.

This study indicates that cluster formation by skin lymph veiled cells and lymphocytes is a spontaneous process which cannot be controlled by means usually effective in regulating the in vitro induced clustering of antigen-stimulated cells.

7) Cytokine and adherence molecules involved in the spontaneous veiled cell-lymphocyte cluster formation (VII)

Epidermal LCs form a unique subset of dendritic cells. These cells incorporate, in vivo, the environmental antigens penetrating skin and this may prompt them to undergo such 'spontaneous' clustering with T lymphocytes. Factors governing this process are not clear, however the expression of accessory molecules on DC is thought to play a crucial role. A question arised which cytokines (other than GM-CSF and TNFα) responsible for LC maturation (22) can regulate the process of DC-T cell cluster formation. Because the distinctive role of DC is to initiate primary T cell response, modulating the early step of cell co-operation might help to regulate immune response. Skin is a rich source of

cytokines primarily active locally in the microenvironment of cells in skin. Supernatants from highly enriched murine epidermal LC cultures contain bioactivities of IL1, IL6, GM-CSF and TNFα (23). Furthermore, keratinocytes either produce constitutively, or can be induced to produce a variety of cytokines, among them IL1, IL6, IL8, IL10 and TNFα.

The purpose of this study was to investigate which cytokines, usually present in afferent lymph, enhance 'spontaneous' cluster formation and which adhesion molecules are active in lymph cell binding. The CD54 and CD 58 molecules were found to play a key role in the 'spontaneous' lymph cell clustering. Antibody against fibronectin, a substrate for CD49d and CD49e receptors, reduced DC-lymphocyte binding. Analysis of the effect of cytokines revealed that the pro-inflammatory IL1β rather than IL1α, and TNFα may be responsible for the enhanced lymph cell in vitro clustering. The IL6 had no such augmenting effect. The enhancing effect of endogenous IL1β present in lymph was reduced by the IL1β neutralizing antibody. The effect of exogenous IL1β was limited by IL1 receptor antagonist (IL1Ra). The IL1Ra alone had no effect on cell binding, even when used in the high doses. Neutralizing of IL1Ra in lymph with the specific antibody brought about augmented cluster formation. The enhancing properties of TNFα on cell binding were reduced by the TNFα neutralizing antibody. The IL10 significantly limited lymph DC cluster formation with T cells. In conclusion, these data demonstrate that the present in lymph IL1β and TNFα may be responsible for the observed in vitro enhanced cluster formation of lymph DC with autologous T lymphocytes. Cell binding can be reduced by IL1Ra and by IL10. It provides insight into the potential clinical use of these inhibitors.

In conclusion, our data demonstrate that ICAM1 and LFA3 dependent pathways of cell contacts are associated with 'spontaneous' lymph cell clustering and that the presence of pro-inflammatory cytokines as IL1β and TNFα in peripheral lymph may be responsible for enhancement of this process. IL1Ra and IL10 appear to be the important factors for the inhibition of the early stage of skin immune cell cooperation.

8) Veiled cells initiate skin allograft rejection by indirect pathway of recognition (VIII)

It is generally accepted that there are two pathways of allorecognition. In the "direct" pathway host T cells interact with intact alloantigens on the surface of donor cells. In the "indirect" pathway host T cells recognize processed alloantigen presented by self-antigen presenting cells (APC). It has been suggested that the acute rejection of skin allografts is predominantly mediated by the direct pathway, because the graft contains a significant number of LC and dermal DC. These cells present antigen and provide costimulatory signals to T cells. There is also increasing evidence for a significant role of the indirect pathway in alloresponse (24).

The complexity of cellular interactions in skin allograft rejection prompted to find a model where the role of recipient DC in inhibition of the rejection process could be studied. We have used a canine skin to severe combined immunodeficient (SCID) mouse transplant model. Two weeks after skin grafting, when donor LC had migrated out from the transplant, (25) canine peripheral lymph VC and lymphocytes or peripheral blood mononuclear cells (PBMC)

allogeneic to the graft were injected intraperitoneally and the rejection reaction was followed.

Our obtained in vivo data suggest that accumulation of canine T cells in the graft is mediated by the presence of VC in the injected population and that infiltrating cells may be activated to alloantigen presented by the VC. The importance of VC in allograft destruction is supported by our observation that $CD3^+$ T-cell infiltrates in graft dermis and epidermis were more extensive after injection of the whole population of lymph cells (with VC) than PBMC.

9) Veiled cells mediate uncontrolled skin allograft rejection and are Cyclosporin A-resistant (IX)

The question arises as to whether cutaneous VC, the principal cells responsible for skin allograft rejection, undergo inhibition by other than Cyclosporin A (CsA) immunosuppressive drugs during cell clustering with autologous lymphocytes.

Our in vitro model of cluster formation served as studies on the effect of immunosuppressants on VC-lymphocyte binding. Immunosuppressants like CsA, azathioprine (AZ), dexamethasone (DM), and hydrocortisone (HC), had no effect on VC clustering with autologous lymphocytes in vitro. In contrast, methylprednisolone (MP) and FK506 had a potent inhibitory effect, even at very low doses.

These findings are consistent with the results concerning cluster formation in the presence of CsA by human blood DC with lymphocytes (26). They confirm that FK506 and MP could be superior to CsA in prolongation of

skin allograft survival. Beneficial effect of FK506 on skin allograft survival was observed recently in clinical maintenance of human hand allograft (27).

10) Tacrolimus but not Cyclosporin A inhibits clustering of veiled cells with lymphocytes in allogeneic and autologous cell cultures (X)

Inhibition of immunological synapse formation between DC and lymphocytes would presumably down-regulate alloantigen recognition in skin allografts and response to bacterial or viral infections. Cyclosporine (CsA) and Tacrolimus (FK 506) have been shown to alter DC differentiation and lymphocyte activation (28).

In this study we investigated the effect of CsA and FK 506 on the rate of in vitro formed synapses and on the expression of CD49d antigen, the molecules actively participating in lymph DC- lymphocyte synapse. Cells obtained from lymph draining skin were cultured for 6 days in allogeneic and autologous combinations, in the presence or absence of CsA and FK 506. Thereafter, the cytospins of cultured cells were examined and percentages of the formed synapses and CD49d expression were established. The frequency of synapses was greater in allogeneic than syngeneic cell combinations. Cells treated with FK 506 showed a decreased rate of both autologous and allogeneic synapses as well as lower expression of CD49d. CsA did not inhibit synapse formation either in autologous or allogeneic cell combinations. However, CsA significantly reduced expression of CD49d antigen on both lymph DC and lymphocytes. It suggests that also other molecules were involved in formation of synapse.

We propose that FK506 can inhibit the formation of synapses between DC and lymphocytes, subsequently down-regulate the initial phases of an allogeneic reaction. The suppressive effect of FK506 on synapse formation may explain the effectiveness of this drug for skin allograft survival.

11) Bacteria-stimulated veiled cells form clusters with lymphocytes (XI)

Transplanted allogeneic skin becomes ischemic in course of the rejection process. Ischemia facilitates penetration of microorganisms residing of the skin surface and skin appendices. Bacteria penetrating skin evoke an immediate chemotactic reaction resulting in recruitment of granulocytes, phagocytosis and desintegration of the penetrator. The locally residing Langerhans' cells, macrophages and lymphocytes as well as mobilized from blood immune cells become activated. Presentation of antigen requires direct physical contact of dendritic cell with lymphocytes and formation of immunological synapse (cell-cell cluster). In inflammation, the number of formed clusters is directly proportional to the intensity of the immune process developing in the tissue is lymph drained from.

In this study we investigated the kinetics of lymph VC- lymphocyte cluster formation during a 12h in vitro stimulation with Staphylococcus warneri and the effect of penicilin on this process. The bacteria-stimulated VCs have formed more clusters with lymphocytes than control cells. The proliferative response of lymphocytes to bacteria-stimulated VCs was higher than in cultures with non-stimulated VCs. Benzathine penicillin decreased lymphocyte proliferative

responsiveness irrespective of whether the cocultured VCs were stimulated with bacteria or not.

It seems that during skin allograft rejection veiled cells react to both allogeneic and bacterial antigens. This cumulative process may be more destructive to the graft than rejection of organ transplants without resident bacterial flora.

12) The margin of skin ulcers, an environment for epidermal Langerhans' cells migration and cooperation with keratinocytes and lymphocytes

Langerhans' cells are typically localized in the basal and suprabasal layers of the epidermis and represent the principal hematopoietic barrier to the external environment. The mobilization of LCs to regional lymph nodes as well as the recruitment of their precursors from the circulation into the skin must be tightly regulated events. Certain, so far unknown, conditions should be met in order LC precursors to lodge in the epidermis, cooperate with keratinocytes and migrate upon stimulation towards the initial lymphatics. In lethally irradiated mice transplanted with congenic bone marrow cells epidermal LCs of host origin remained for at least 18 months, whereas DCs in other organs were almost completely replaced by donor cells within 2 months (29). In parabiotic mice with separate organs, but a shared blood circulation, there was no mixing of LCs. However, in skin exposed to ultraviolet light, LCs rapidly disappeared and they were replaced by precursors within 2 weeks.

In inflamed skin multiple changes occur, including the secretion of chemokines and cytokines by keratinocytes, allowing a marked loss of LCs and

the recruitment of LC precursors. In the presented 2 studies, I tried to investigate the environment for LC to lodge in the inflamed skin, and specifically in epidermis, and make a functional contacts with keratinocytes and other cells.

12.1. Normal keratinocyte cell-cycle – related proteins expression in neo-epidermis (XII)

Studies were carried out in the patients with diabetic foot and varicose ulcers of the calf. Skin biopsy specimens were obtained from the border area of ulcers and the topographically corresponding sites of normal skin of patients undergoing orthopedic surgery. At the edge of both types of ulcer keratinocytes were p63+, CD29+, PCNA+ and p53. The mean intensity of p63 and CD29 staining was higher than in controls. The intensity of bcl2 staining was higher at the edge of diabetic ulcers compared with venous ulcers, whereas the intensity of bax staining was similar. The expression of caspase 3 was lower at the edge of venous ulcers and higher in diabetic ulcers compared with controls. The intensity of TUNEL staining was lower at the edge of both types of ulcers compared with controls. Keratinocytes at the edge and distally to both types of ulcers expressed cytokin 16 and 17. There was no expression of cytokeratin 10 at the edge of ulcers.

The impaired epithelialization of chronic leg ulcers is not caused by an inadequate epidermal stem cell proliferation, differentiation or apoptosis. It may rather reflect the distored organization of wound bed, caused by infection and impaired nutrition supply, altering keratinocyte migration.

12.2. Lack of Langerhans' cells in the neo- epidermis and in the granulation tissue (XIII)

Biopsies from leg ulcers of 10 randomly selected patients were examined immunohistochemically for leukocyte phenotypes, vascular adhesion molecules and cytokines and growth factors produced by keratinocytes (KC) and vascular endothelial cells (EC). Granulation tissue contained few fibroblasts and blood capillaries, with high intensity of staining for CD62E and CD106 but not for FGF2 on EC ($p<0.05$). The intensity of staining for scavenging $CD15^+$ $elastase^+$ granulocytes and $CD35^+$ (C3bi) macrophages in ulcer bed was comparable to that in the margin but higher than in distant dermis ($p<0.05$), whereas that for $CD68^+$, HLA DR^+, $TGF\beta^+$ and $CD54^+$ dermal leukocytes was similar in all areas. There was reduced staining for CD4+ and CD8+ cells in ulcer bed ($p<0.05$). There were no $CD1a^+$ Langerhans' cells in the epidermis encroaching upon the granulation tissue and also there was reduced CD1a staining in the adjacent epidermis ($p<0.05$). The expression of cytokines and growth factors by KC was similar in the areas adjacent and remote from ulcer. In dermis adjacent to ulcer the expression of IL1α, IL1β, IL1Ra, EGF and PDGFa was higher than in distant dermis. Although keratinocytes at the edge of venous leg ulcer revealed a normal cytokine and growth factors secretory capacity, there was a limited recruitment of CD1a+ Langerhans cells to dermis and epidermis surrounding the ulcer. The presented in the two last publications observations suggest that keratinocytes at the edge of venous leg ulcers, expressing the normal levels of cell- cycle related markers as well as cytokines and growth factors, do not create conditions for repopulation of neo- epidermis by dendritic cells. This

supports the idea that epidermis is not just a protective barrier, but that keratinocytes and Langerhans' cells interact closely with underlying dermis and are actively involved in the regulation of the wound healing process. It seems that these epidermal immunological processes, and not primarily alterations in fibroblast function alone, are important in the regulation of dermal matrix production during the healing of leg ulcers.

Summary

Formation of cell cluster and molecular immunological synapse between DCs and lymphocytes is indispensable for transfer of antigen information. The experimental model of ligated and subsequently dilated lymphatics of the canine hind limb allowed collecting afferent lymph cells migrating from the skin and forming clusters upon in vivo stimulation. Large volume of lymph and number of collected cells allowed to evaluate the mechanisms of clustering of "fresh" lymph cells without their multiplication in culture. The harvested VCs were characterized and the presented results indicated a close morphological and functional analogy between the canine and human afferent lymph veiled cells. Canine low density VC exhibited the dendritic morphology of the cytoplasm, the presence of a lobulated nucleus and expression of cytoplasmatic S100 protein. The VC were strongly ATP-ase, CD1a and Ia positive, displaying cross-reactivity with anti- human antigen antibodies. Afferent lymph VCs were able to stimulate allogeneic and autologous T lymphocytes in culture when present at cell concentrations as low as 5% of the responding cells.

Developed by us antiserum against afferent lymph cells evoked in vivo a transient depletion of VC from afferent lymph and reduction of mononuclear cells in the T- dependent areas in regional lymph nodes as well as an inhibitory effect in mixed leukocyte reaction (MLR) in vitro. Such effects of anti-VC sera may arrest the migration of VCs into lymphoid tissue leading to overcoming allograft rejection. Raised by us mouse monoclonal antibody against VC had an inhibitory activity in allo-MLR. This antibody as well as other antibodies cross-reactive with canine antigens could be used for VC identification and reduction a spontaneous binding of VC with lymphocytes in vitro.

Formation of clusters by autologous VC and lymphocytes was an active, temperature dependent process. Treatment of lymph cells with a Ca^{2+} channel blockers EDTA and verapamil and incubation of cells with proteolytic enzymes or monosaccharides did not alter celi interaction. Celi binding was significantly decreased by treatment with drugs influencing cell membrane fluidity, xylocaine and retinoic acid. Afferent lymph celi clustering was dependent on the expression of ICAM1 (CD54) and LFA3 (CD58) molecule and blocking of these antigens had an inhibitory effect. In contrast, blocking of other molecules as CD1a and DR had no effect on the rate of cluster formation, whereas blocking of CD18 and CD49 (d,e,f) had an pro-aggregatory effect. The presence of proinflammatory cytokines IL1β and TNFα but not of IL1α and IL6, induced spontaneous in vitro binding of lymph cells. The anti-inflammatory cytokine IL10 and pentoxifylline (drug with anti- TNFα activity) appeared to be the inhibitory factors for the early stage of VC and lymphocyte cooperation. These studies provided insight into the potential clinical application of cluster formation inhibitors.

The rejection-mediating role of VC in skin grafting was shown on SCID mouse chimera model. Intraperitoneal injection of lymph cell population containing VC to recipients of skin allografts prompted T lymphocyte recruitment to allogeneic skin grafts, leading to extensive dermal destruction of transplants in the canine skin/ SCID mouse chimera model, compared to the rather feable effects of lymphocytes alone. These data comply with the role of the recipient professional antigen presenting cells in allorecognition and skin rejection.

Among the in vitro used immunosuppressive drugs only FK506 (Tacrolimus) and methylprednisolone had a potent inhibitory effect on lymph VC- lymphocyte cluster formation. FK506 has also reduced cluster formation in a 6 days culture of lymph cells, both in allogeneic and autologous combinations. CsA had no inhibitory activity on cluster formation. Neither CsA nor FK506 down-regulated the expression of DR antigen on VC and lymphocytes in clusters. In contrast, both drugs reduced expression of CD49d antigen on both types of lymph cells. These findings may expain the low effect of CsA in skin allograft rejection.

Stimulation of VCs with bacteria in vitro led to increased clustering of VCs with lymphocytes and lymphocyte proliferative response. The culturing of lymph cells in lymph resulted in a higher rate of cluster formation compared with that in RPMI culture medium. Interestingly, the presence of benzathine penicillin reduced the responsiveness of lymph cells both in bacteria stimulated and non-stimulated cultures. It seems that during skin allograft rejection veiled cells react to both allogeneic and bacterial antigens. This cumulative process may be more destructive to the graft than rejection of transplants without resident bacterial flora.

The in vivo cooperation of migrating epidermal VCs and lymphocytes, localized in the specific tissue niches and making contacts with immune and non-immune cells, may differ from those observed in vitro. Such differences may occur in the inflamed, contaminated by bacteria and non-healing leg ulcer tissues. At the edge of the venous leg ulcers there was lack of LCs in neo-epidermis and their reduced numbers in the surrounding epidermis, despite a normal level of keratinocyte cell- cycle related proteins. The expression of cytokines and growth factors by keratinocytes was similar in the areas adjacent to and remote from ulcer. In dermis adjacent to ulcer the expression of IL1α, IL1β, IL1Ra, EGF and PDGFa was higher than in distant dermis. Although keratinocytes at the edge of venous leg ulcer revealed a normal cell- cycle proteins as well as cytokine and growth factors secretory capacity, there was a limited recruitment of CD1a+ Langerhans cells to dermis and epidermis surrounding the ulcer. Insufficient levels of locally produced chemokines (30) or low expression of their receptors on migrating cells, and lack of a proper molecular modeling of niches for LC precursors lodgement may be responsible for lack of LC- keratinocyte contacts in epidermis.

Conclusions

A unique model for studies of the in vivo-formed functional clusters between dendritic (veiled, Langerhans) cells was worked out in a large animal (dog) allowing to collect sufficient number of cells and test their functional capacities. Applied experimental model allowed drawing analogies to human conditions. The phenotype and reactivity to stimulating and inhibiting substances has been

elaborated. Specific polyclonal anti-serum and monoclonal antibody had an inhibitory effect on veiled cell reactivity both in vitro and in vivo. The proinflammatory cytokines stimulated and anti-inflammatory cytokine and drugs as pentoxyfillin, FK 506 and methylprednisolon but not cyclosporin A inhibited formation of clusters. Bacterial antigen increased in lymph culture the cluster formation rate. Interestingly, long-term penicillin downregulated this process. In contrast to the in vitro observations, the in vivo cooperation of veiled (Langerhans) cells with lymphocytes and other cells seemed to be additionally governed by the environmental factors. In inflamed tissue there was less recruitment of Langerhans cells despite of high cytokine expression. A proper molecular niche is probably needed.

These studies provided more insight into crucial cellular events in the skin immune system that is antigen presentation between the migrating dendritic cells and lymphocytes forming physical cell cluster and specifically the effectiveness of drugs on this process. The applied model allows further investigations on manipulating cell clustering with immunosuppressive drugs resulting in prolongation of skin graft survival. The recently published data of efficacy of FK506 in prolongation of survival of transplanted human hand corroborate our results on inhibiting effects of this drug on cluster formation (31).

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A single step centrifugation method for the enrichment of veiled cells from canine afferent lymph

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Veiled cells (VC) present in the afferent lymph of dogs with chronic lymphoedema could be enriched from 6% to about 50% VC by density gradient centrifugation on 15% metrizamide or discontinuous Percoll gradients. The recovery of VC was about 40% from $0.22 \pm 0.07 \times 10^6$ VC/ml of lymph. The cells were strongly Ia positive and had cytoplasmic S 100 protein. They were also strongly ATP-ase positive and showed heterogeneity in acid phosphatase, peroxidase and non-specific esterase activity. Low density VC from canine afferent lymph were able to stimulate both blood and lymphatic lymphocytes in autologous mixed leukocyte reaction when present at concentration as low as 5% of cultured cells.

Key words: Veiled cell; Afferent lymph; Density gradient

Introduction

Langerhans' cells (LC), a minor portion of the total epidermal cells (Stingl et al., 1980) have been described as the primary immunocompetent cells in skin playing a role in the presentation of antigen to T cells and the induction of contact sensitivity (Braathen et al., 1984; Katz et al., 1985). They can migrate from the skin and a proportion of these cells acquire the appearance of veiled cells (VC) and can be retrieved from the afferent lymph (Drexhage et al., 1979; Spray et al., 1980; Pugh et al., 1983; Hall and Robertson, 1984; Knight, 1984). Since LC or VC collected from afferent lymph are very potent at evoking immune responses to alloantigens in vivo (Lechler and Batchelor, 1982; Knight et al., 1983; Olszewski et al., 1983, 1984, 1987) they play a major role in transplantation biology.

Studies on VC in inbred strains of rodents are limited by low numbers of available cells. Larger animals with high prenodal lymph flows are preferred for that purpose. Among the large animals dogs are particularly suitable, since interruption of their lymphatics brings about an accumulation of VC in the stagnant lymph.

The purpose of the present studies was to establish a method for obtaining large numbers of VC from afferent lymph for subsequent functional studies. An enrichment of VC was achieved in dogs with chronic lymphoedema following surgical interruption of hind limb lymphatics. The stagnant lymph of such dogs contains 10–30-fold more

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Abbreviations: LC, Langerhans' cells; VC, veiled cells; PBS, phosphate-buffered saline; FCS, foetal calf serum; BSA, bovine serum albumin; AcPh, acid phosphatase; ANAE, α-naphthyl acetate esterase; ATP-ase, adenosine triphosphatase; APAAP, alkaline phosphatase-anti-alkaline phosphatase complex; PHA, phytohaemagglutinin; MLR, mixed leukocyte reaction; AMLR, autologous mixed leukocyte reaction.

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cells compared with normal lymph (Galkowska and Olszewski, 1986).

Materials and methods

Dogs

Normal outbred dogs and dogs with chronic lymphoedema produced by surgical interruption of hind limb afferent lymphatics (Olszewski et al., 1968) were used.

Prenodal lymph

Lymph was collected by cannulation of the hind limb lymphatics. In dogs with lymphoedema it was obtained by direct percutaneous puncture of dilated lymphatics. It was collected into plastic tubes and heparin was added to a concentration of 10 U/ml of lymph (Olszewski and Ryffa, 1984).

Discontinuous Percoll gradient

A stock isotonic Percoll solution was made by mixing 92% of Percoll (Pharmacia) with 8% of 10 × concentrated PBS (Gibco) to a final osmolarity of 285 mosM. Five different concentrations of Percoll in RPMI 1640 medium (Gibco) supplemented with 10% FCS (Gibco) were prepared, ranging from 38% to 66.7% (Fig. 1). After layering 2 ml volumes of different Percoll solutions, 2 ml of cell suspension in RPMI 1640 medium with 10% FCS were placed on the top and spun down at room temperature at 500 × *g* for 30 min. Cells from the interfaces were collected and washed with medium.

The number of cells from the layers was calculated and the cell purity was evaluated on cytospin smears stained with May Grunwald-Giemsa. Cell viability, checked by trypan blue dye exclusion, was over 95%.

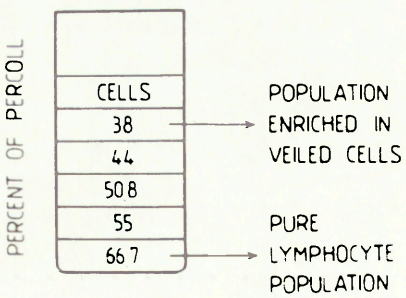

Fig. 1. Separation of lymph cells on a discontinuous gradient of Percoll solution

Metrizamide gradient

15% metrizamide (Nyegaard) in PBS with 0.2% BSA (Sigma) was used. 5 ml of cell suspension were layered over 3 ml of gradient and spun down at 550 × *g* for 10 min. The number of cells from the interface and their purity were checked. The cell viability was over 95%.

Cytochemistry of cells on cytospins

Cells were fixed in cold acetone for 1 min. Acid phosphatase activity was assessed using α-naphthyl phosphate monosodium salt in the acetate buffer (pH 5) and Fast Red TR. The method of Horwith et al. (1977) was used to determine the non-specific esterase activity, using α-naphthyl acetate. Peroxidase activity was assessed using benzidine dihydrochloride (400 mg/10 ml of 40% ethanol and 0.02 ml of 3% hydrogen peroxide solution). ATP-ase localization was performed on smears fixed with formalin in cacodylate buffer with 11.5% sucrose, then incubated with ATP and $CaCl_2$ solution in Tris buffer (pH 9.5) for 45 min at 37 °C. After washing, smears were incubated in 2% $CaCl_2$ solution for 3 min followed by ammonium sulphide solution for 3 min.

Detection of Ia antigens

Cytospins fixed in cold acetone for 1 min were incubated for 30 min with normal swine serum (diluted 1/5) and then with anti-HLA-DR mouse monoclonal antibody (Becton-Dickinson, diluted 1/20) for 30 min at room temperature. Next, the rabbit anti-mouse IgG (Becton-Dickinson, diluted 1/20) was layered over the cells for 30 min and then the cells were covered with swine anti-rabbit IgG-APAAP conjugate (Becton-Dickinson, diluted 1/20) for 30 min. After incubation with AP substrate (Dakopatts) for 15 min smears were lightly stained with Mayer's haematoxylin.

Detection of S 100 protein

Cytospins were fixed in cold acetone for 1 min and rabbit anti-S 100 antiserum (Dakopatts) diluted 1/100 was placed over the cells for 30 min. Then the cytospins were incubated for 30 min

with swine anti-rabbit IgG-APAAP conjugate (diluted 1/20) followed by AP substrate for 15 min.

Stimulatory activity of VC in vitro

The culture medium used consisted of RPMI 1640 medium supplemented with 10 mM Hepes buffer, 20% FCS, 100 U/ml penicillin and streptomycin (Gibco), 2 mM L-glutamine (Gibco). Responding lymphocytes from peripheral blood were separated on a Lymphoprep (Nyegaard) gradient at $500 \times g$ for 30 min. Lymphocytes from blood or lymph were cultured at a density of 2×10^5 cells in 0.2 ml of medium in microculture plates, with and without 5% VC from the Percoll gradient enriched population. Stimulatory cells were preincubated with mitomycin C (Sigma) used at a concentration of 40 μg/ml for 40 min at 37°C. Cultures were performed in triplicate and incubated for 6 days in a humidified atmosphere of 5% CO_2 in air. 20 h before termination of the culture 100 μl of medium were replaced by an equal volume of fresh culture medium and 0.4 μCi of [^{3}H]thymidine (Amersham, 2 Ci/mM) was added. Radioactivity was measured on glass fibre filters in Permafluor cocktail (Packard).

Mitogen presenting function of VC

Lymphocytes from peripheral blood and from lymph were cultured in the presence of PHA (Wellcome HA, 90 μg/ml) with and without 5% VC for 3 days. The VC were treated with mitomycin C and the culture conditions were the same as described above.

Results

Cellular composition of canine afferent lymph

The concentration of cells obtained from normal (non-stagnant) lymph was $0.23 \pm 0.21 \times 10^6$/ml (9–21% VC). The total yield of VC was usually too low for functional studies. They could, however, be used for morphological characterization. Stagnant lymph was rich in cells ($4.7 \pm 3.8 \times 10^6$ cells/ml of lymph) and the total numbers of cells obtained were sufficient for tests. The majority of cells were lymphocytes and about 3–12% were large mononuclear macrophage-like cells

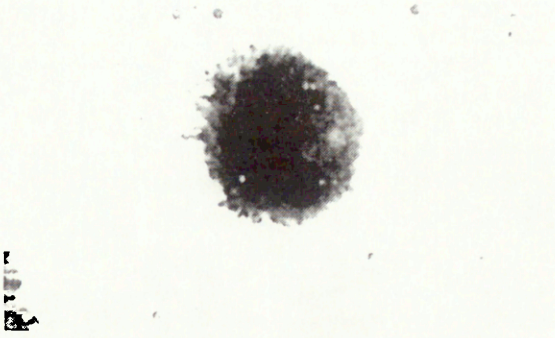

Fig. 2. Macrophage-like, low density VC from lymph after centrifugation and stained with May-Grunwald-Giemsa.

($0.22 \pm 0.07 \times 10^6$/ml of lymph). Fig. 2 illustrates such a cell stained with May-Grunwald-Giemsa.

Density gradient fractionation

The distribution profiles of cells with dendritic morphology on the discontinuous Percoll gradient and on 15% metrizamide are shown in Table I. The top band of the Percoll gradient contained about 52% of low density veiled cells (range 40–72%), whereas lymphocytes were present in the high density band. A large number of VC from the top band was found in the middle of rosettes formed by lymphocytes. The recovery of VC from the Percoll gradient ranged from 23% to 53% (Table I). Fractionation of lymph cells on the metrizamide gradient gave similar results. The purity of VC from the gradient interface ranged from 54% to 70%, with a yield of about 46%.

Cytochemistry of VC from cytospins

Only about 10% of VC were weakly positive for peroxidase and about 40% of VC showed acid phosphatase activity. About 60% of VC showed a reaction for non-specific esterase of differing degrees of activity and 45% of VC were weakly positive. All VC showed strong ATP-ase activity (Table II).

Labelling with anti-Ia monoclonal antibody

All of the VC examined in cytospins were Ia positive and 80% of them showed a strong reac-

TABLE I

ENRICHMENT OF VEILED CELLS (VC) BY CENTRIFUGATION THROUGH A PERCOLL GRADIENT ($n = 10$) OR THROUGH 15% METRIZAMIDE ($n = 3$)

	Purity of VC (%)		Yield (%)
Before fractionation	6.7 ± 2.4	(3–12)	
Percoll layers:			
38%	52.0 ± 9.2	(40–72)	33.6 ± 11.0 (25–53)
44%	19.0 ± 8.0	(6–33)	
50.8%	4.1 ± 3.1	(1–10)	
55%	1.0 ± 1.4	(0– 3)	
66.7%	0		
15% metrizamide interphase	64.6 ± 9.2	(54–70)	46.0 ± 1.0 (45–47)

tion (Table II). Fig. 3 illustrates a VC stained for Ia antigens.

Reaction with anti-S 100 serum

All of the VC examined in cytospins were positive for the S 100 protein (Table II). Fig. 4 shows the reaction products with discrete localization within the cytoplasm.

Stimulatory activity in vitro

The VC collected from the Percoll gradient were able to stimulate allogeneic peripheral blood

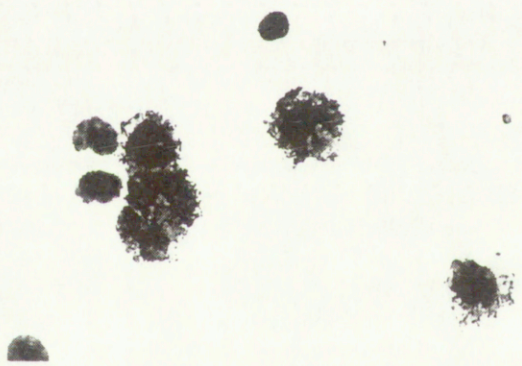

Fig. 3. Ia staining of VC using mouse anti-HLA-DR serum. The VC is strongly Ia positive.

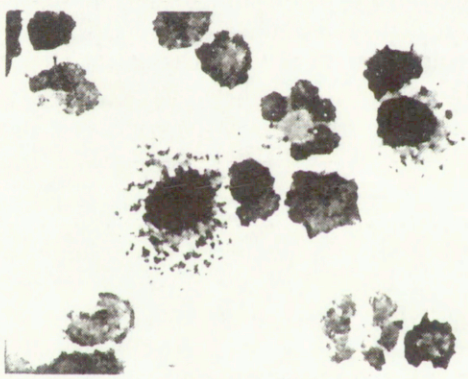

Fig. 4. S 100 staining of VC using rabbit anti-S 100 protein anti-serum. The large macrophage-like cells from lymph are strongly positive.

TABLE II

CYTOCHEMISTRY AND IMMUNOCHEMISTRY OF CANINE VEILED CELLS FROM AFFERENT LYMPH (% of positive VC)

	AcPh	Peroxy-dase	ANAE	ATP-ase	S-100	Ia (HLA-DR)
Before fractionation	50 +	50 +	100 + +	100 + + +	100 + + +	50 + +
						50 + + +
VC enriched	40 +	10 +	45 +	100 + + +	100 + + +	20 + +
			14 + +			80 + + +

Key: strong (+ + +), intermediate (+ +), weak (+) enzymatic activity.

TABLE III

THE EFFECT OF CO-CULTURING BLOOD LYMPHOCYTES WITH 5% ALLOGENIC VEILED CELLS FOR 6 DAYS (MLR)

Experiment	Control	With VC	SI [b]
1	0.41 ± 0.05 [a]	1.44 ± 0.17	2.5
2	0.38 ± 0.11	0.96 ± 0.10	1.5

[a] cpm × 10^{-3} ± SD.
[b] Stimulation index.

TABLE IV

THE EFFECT OF CO-CULTURING BLOOD (BL) AND AFFERENT LYMPH (LL) LYMPHOCYTES WITH 5% AUTOLOGOUS VEILED CELLS FOR 6 DAYS (AMLR)

Responder cells	Experiment	Control	With VC	SI [b]
BL	1	0.3 ± 0.04 [a]	1.4 ± 0.1	3.6
	2	0.1 ± 0.01	0.3 ± 0.05	2.0
LL	1	2.7 ± 0.1	7.5 ± 0.2	1.7
	2	0.9 ± 0.3	3.8 ± 0.6	3.2

[a] cpm × 10^{-3} ± SD.
[b] Stimulation index.

lymphocytes in a 6 day MLR (Table III) when present at 5% of the responding cells. They also stimulated autologous lymphocytes from both blood and lymph in AMLR (Table IV).

Mitogen presenting function

The VC were able to augment the responsiveness of autologous lymphocytes to PHA in a 3 day culture (Table V).

Discussion

A single stage enrichment procedure was found to give a satisfactory yield of veiled cells from the afferent lymph of the hind limbs of normal and lymphoedematous dogs. Highly enriched populations were obtained by density centrifugation on discontinuous Percoll gradient or on 15% metrizamide. Both procedures resulted in the same VC purity and recovery. The advantage of the Percoll gradient over metrizamide was the simultaneous isolation of a pure lymphocyte population. The recovery of VC from both gradients was much higher than reported by Rhodes (1985) from the mesenteric lymph of adenectomized mice after enrichment on 14.5% metrizamide. The use of a 13% metrizamide gradient for enrichment of prenodal canine VC from stagnant lymph resulted in an 18% of VC. Although the functional properties and morphological appearance of veiled cells and lymphocytes from normal and from stagnant lymph were similar, the cell yield from normal vessels was, in many cases, found to be too low for functional evaluations.

Low density cells from canine stagnant lymph exibited many of the characteristic features described for VC obtained from other experimental animals (Drexhage et al., 1979; Hall and Robertson, 1984; Rhodes, 1985). These included the dendritic morphology of the cytoplasm, the presence of a lobulated nucleus and of relatively few lysosomes.

All canine VC, in common with the VC from other species studied (Knight, 1984), were strongly Ia positive displaying cross-reactivity with mouse

TABLE V

THE EFFECT OF CO-CULTURING BLOOD (BL) AND LYMPH (LL) LYMPHOCYTES WITH 5% AUTOLOGOUS VC IN THE PRESENCE OF PHA

Cells	Experiment	Control	PHA	SI [b]	With VC	AI [c]
BL	1	0.2 ± 0.02 [a]	6.5 ± 0.7	31	9.6 ± 0.5	1.5
	2	0.1 ± 0.01	3.3 ± 0.4	32	5.4 ± 0.1	1.6
LL	1	0.1 ± 0.01	13.8 ± 1.0	137	26.6 ± 1.2	1.9
	2	0.07 ± 0.005	8.5 ± 0.9	120	23.3 ± 0.2	2.7

[a] cpm × 10^{-3} ± SD.
[b] Stimulation index for PHA.
[c] Augmentation index for VC.

anti-HLA-DR antiserum. Such cross-reactivity of anti-Ia alloantisera was described by Rowden (1985) and Greenlee et al. (1988) but the reasons for the phenomenon are not clear. Canine VC were all positive for S 100 cytoplasmic protein. This property, which has so far been demonstrated for the Langerhans' cells of skin (Nakajima et al., 1982) and for interdigitating reticulum cells in lymph nodes (Takahashi et al., 1981), is not present in phagocytic macrophages (Rowden, 1985). This functional property permits the differentiation of the two types of cell lineage. Low density cells from canine lymph were strongly ATP-ase positive and their activities of acid phosphatase, peroxidase and of non-specific esterase resembled these described for VC from other speciec (MacPherson and Pugh, 1984; Rhodes, 1985). Canine VC were able to stimulate T cells in allogeneic MLR and in auto-MLR when present at cell concentrations as low as 5% of the responding cells. Moreover they exhibited the PHA presenting function characteristic of autologous lymphocytes.

The present results indicate a close analogy between the veiled cells of dogs and other species (Knight, 1984). Relatively large numbers of the cells are available in stagnant lymph and this will permit functional studies, the raising of antisera against veiled cells for in vivo studies of their immunosuppressive properties, and cell adoptive transfer to skin allograft recipients for the investigations of rejection accelerating activity.

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Functional Characteristics of Veiled Cells from Canine Prenodal Lymph

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Abstract

The surgical interruption of afferent lymphatics in the hind limb of dog leads to peripheral lymph stasis. The stagnated lymph contains large numbers of immunocompetent cells originating solely from the skin. This experimental model allows a study of the functions of the afferent skin-draining lymph cell population, the recovery and assessment of the lymphokines and other mediators liberated by these cells during the culture, and the production of anti-sera against different types of lymph cells.

In the present study, we focused on the functional, morphological and cytochemical evaluation of the non-lymphoid cells, isolated from the whole lymph cell population by means of the gradient centrifugation technique. The non-lymphoid cells were large, with an irregularly-shaped nucleus and numerous cytoplasmic projections, giving them a «veiled» cell (VC) appearance. All VC were strongly positive for DLA-class II antigens and membrane-associated ATP-ase, and 60 % of them exhibited the activity of non-specific esterase.

In the functional assays, VC displayed the potent accessory-cell activity in the mitogen-induced response of autologous blood- and lymph-derived lymphocytes. In the mixed leukocyte cultures, VC acted as stimulators of the allogeneic and autologous lymphocyte proliferation. The high spontaneous and mitogen-induced responsiveness of the whole lymph cell population was found to be dependent on the presence of VC. The small number of VC (5 % of cultured cells) was sufficient to produce the above-mentioned effects.

These results indicate that VC is a cell responsible for the antigen presentation in the skin-associated immune reactions in dog, which is relevant to the observations on similar cells from the other species.

Introduction

Veiled cells (VC) have been described in the afferent lymph of many species, including rabbit, pig, sheep, dog, man (skin lymph) (1–6), and rat, guinea pig and pig (intestinal lymph) (7). VC have also been isolated from the thoracic duct of mesenteric lymphadenectomized rats and mice (8–10).

Abbreviations: *VC* = veiled cell(s); *LC* = Langerhans cell(s); *DC* = dendritic cell(s), *PBM* = peripheral blood mononuclear cells; *LL* = lymph lymphocytes; *SI* = stimulation index; *AI* = augmentation index; *MLR* = mixed leukocyte reaction; *AMLR* = autologous mixed leukocyte reaction; *DFCS* = decomplemented fetal calf serum.

These cells are believed to be a migrating form of Langerhans cells (LC) from the epidermis, which enter the regional lymph nodes via afferent lymphatics (1, 2, 6, 9, 11, 12). The postulated role for migrating VC is the transportation of antigens from the skin or gut mucosa to the paracortex of lymph nodes. There, VC transforming into interdigitating cells may come into a close contact with T cells and act as potent antigen-presenting cells (13–15).VC, like LC and interdigitating cells, are descendants of the myeloid lineage, but their relation to the mononuclear phagocytic system cannot be strictly defined because of many morphological and functional differences (9). VC from most species so far investigated have characteristic cytoplasmic projections and are larger than other afferent lymph cells. They have a relatively small lobulated nucleus and clear cytoplasm (2, 6). The very characteristic structure, present in some VC from the skin-draining lymph, and shared with the epidermal LC, is a cytoplasmic Birbeck granule. The other commonly recognized features of these cells are the high activity of the membrane-associated ATP-ase, also the presence of non-specific esterase and acid phosphatase and expression of MHC class II antigens (6, 8, 16). VC, like LC and dendritic cells (DC), are potent stimulators of lymphocyte responses *in vitro* and *in vivo* (15, 17–20). They were shown to perform accessory cell function in mitogen- and antigen-stimulated lymphoproliferation, together with a stimulatory function in mixed leukocyte cultures (8, 21–23). Due to the immunostimulatory activities of DC, their role in evoking allogeneic reactions in skin and gut transplants, has become a matter of growing interest.

In this study, we analyzed some functions of VC in a dog. The dog model is widely used in transplantation research. Futhermore, in this species, it is possible to bring about the dilatation of afferent lymphatics, resulting in an accumulation of leukocytes as a consequence of surgical interruption of peripheral lymph out-flow from the limb (24). This model is so far impossible to introduce in small laboratory animals. It had not been assayed in pigs, sheep or calves either. Taking advantage of this technique, we were able to study functionally the pure skin-draining lymph cell population. In particular, we found it of interest to concentrate on the VC present in the canine skin-draining lymph, since the functions of these cells have not been yet investigated in this species. The main questions were whether VC: 1) share the morphological and cytochemical similarities with VC from other species, 2) act as accessory cells-mitogen presenters to the autologous and allogeneic lymphocytes, and 3) evoke alloantigen-generated responses.

Materials and Methods

Animals

Dogs with lymphedema following surgical interruption of lymphatics served as donors of lymph (24).

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Dogs with lymphedema following surgical interruption of lymphatics served as donors of lymph (24).

by fresh medium, and subsequently, 0.4 μCi/well of tritiated thymidine (Amersham, 2 Ci/mM) was added. At 72 h, the cultures were harvested with a Skatron harvester, and incorporation of [^{3}H]-thymidine was measured in Beckman β-counter. Mixed leukocyte reactions were carried out for 144 h in U-shaped 96-well microtiter plates under the same culture conditions, and the [^{3}H]-thymidine incorporation was measured as described above.

Results

Isolation of VC on Percoll gradient

In the unfractionated stagnant lymph, VC constituted 6.7 % (living cell suspensions) or 5.75 % (May-Grunwald-Giemsa stained smears) of the entire cell population; the rest being 88.5 % small lymphocytes, 3.25 % monocytes, 1 % blast cells, 1 % neutrophils and 0.5 % eosinophils. In the Percoll gradient, VC were found in the low-density fractions, predominantly in the first interface overlying 38 % Percoll. This corresponded to the approximate density of 1.05 g/ml. The purity of VC in this fraction was 52.0 ± 9.2 % ($n = 20$) ranging from 40–83 %. The VC yield was 33.6 ± 11.0 % (25–53 %), corresponding to the absolute numbers of $1.05 \pm 0.55 \times 10^6$ ($0.45–2 \times 10^6$) recovered cells. For the functional evaluation of VC, the fractions of 74–83 % purity were used. VC frequency diminished in the interfaces overlying lower Percoll fractions, being consecutively 19.0 ± 8.0, 4.1 ± 3.1, 1.0 ± 1.4 and less than 1 %. Small (predominantly T – (4)) lymph lymphocytes accumulated in the high-density fractions, overlying the 55 % and 66.7 % Percoll layers (1.075 g/ml density for the latter). The purity of lymphocytes in the lowest interface was 96.25 ± 2.6 %. The mean lymphocyte number was $52.4 \pm 5.9 \times 10^6$ ($45–60 \times 10^6$) cells, yielding 77.2 ± 16.8 % (60–95 %). These lymphocytes were used as responder lymph lymphocytes (LL) in the *in vitro* experiments.

Morphological and cytochemical identification of VC

In the May-Grünwald-Giemsa-stained specimens, VC appeared as large cells (about 2–3 times the size of the small lymphocyte), with clear cytoplasm, showing no granules. The nucleus/cytoplasm ratio was relatively low. The shape of the nucleus varied between different cells. At least two major kinds of cells could be distinguished: one with lobulated nucleus, and the other with marginally located oval or horse-shoe-shaped nucleus (Figs. 1 and 2). The cytoplasmic projections were also visible, but not to the same extent as in the living cell suspensions. Some VC appeared in clusters with lymph lymphocytes.

About 10 % of VC were weakly positive for peroxidase, and about 40 % showed a weak acid phosphatase activity. About 60 % of VC showed the reaction for non-specific esterase of strong or moderate degree, and the remaining 40 % of VC were weakly positive. Almost 100 % of VC showed

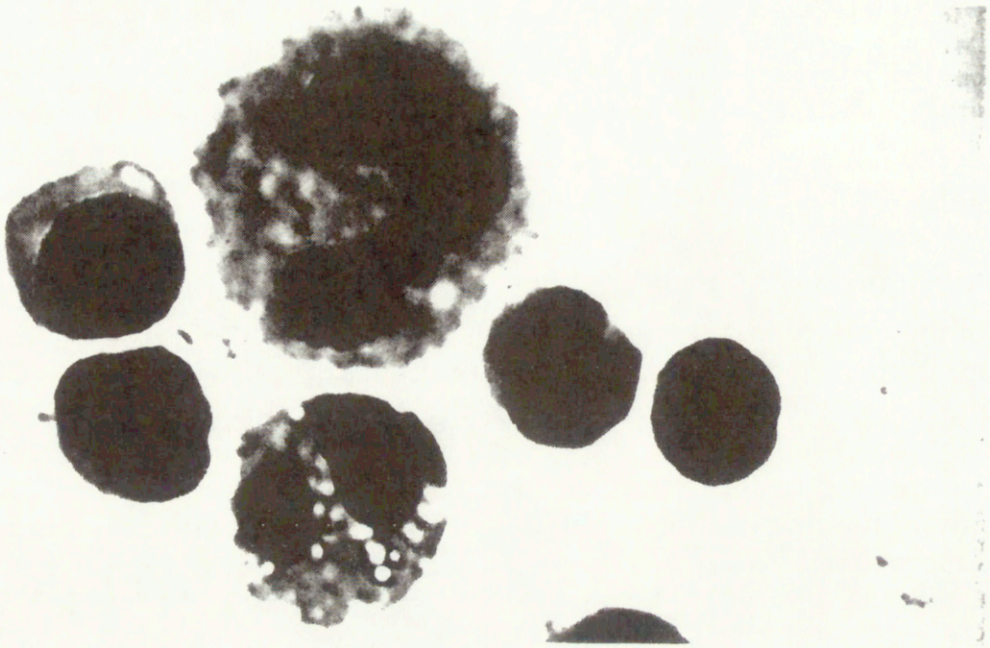

Figure 1. Veiled cell surrounded by lymphocytes and a granulocyte. Note the large size of VC, the irregular shape of its cell membrane, and the lobulated nucleus. May-Grünwald-Giemsa staining 1000×.

the strong activity of the membrane-associated ATP-ase. On the basis of cross-reactivity with the mouse anti-HLA-DR monoclonal antibody (27, 28), all VC were found DLA-class II positive. Among them, 80 % were strongly positive.

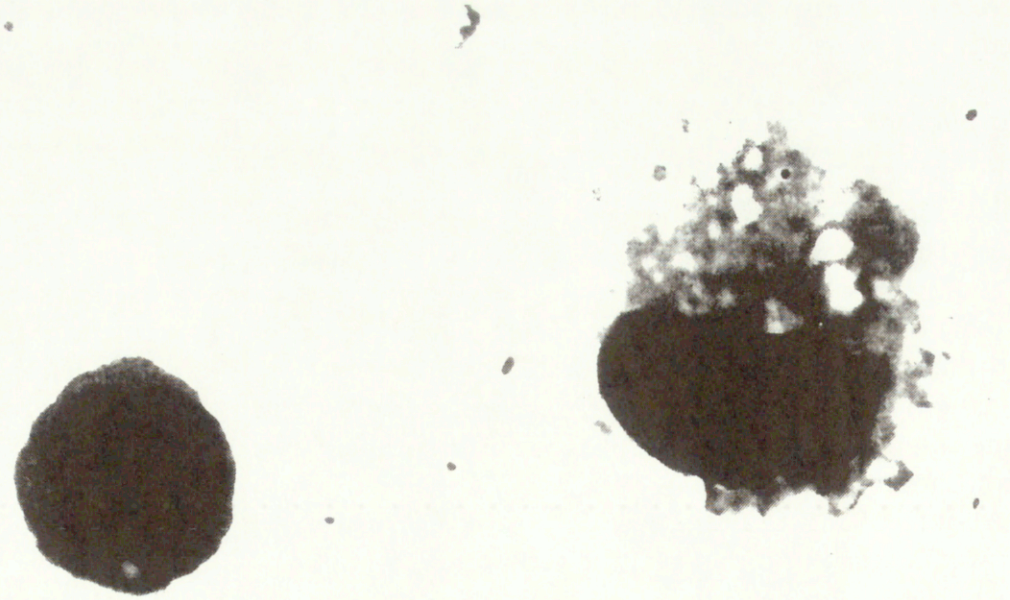

Figure 2. Veiled cell (right) with marginally located kidney-shaped nucleus and well-developed cytopodia. May-Grünwald-Giemsa staining 1000×.

Table 1. The effect of VC on [^{3}H]-thymidine uptake of autologous PBM stimulated with PHA

Exp.	Autotransformation rate	with PHA	SI	with PHA and VC[a]	AI
1	0.8 ± 0.12[b]	3.9 ± 0.07	3.9	2.8 ± 0.50	0.7
2	0.2 ± 0.04	3.4 ± 0.30	16.0	6.2 ± 0.10	1.8
3[c]	0.3 ± 0.02	6.5 ± 0.70	20.7	9.6 ± 0.50	1.5
4	0.2 ± 0.02	0.9 ± 0.24	3.5	1.6 ± 0.30	1.8
5[c]	0.1 ± 0.01	3.4 ± 0.40	33.0	5.5 ± 0.10	1.6
6[c]	0.05 ± 0.02	1.1 ± 0.33	21.0	2.3 ± 0.12	2.1

[a] 10.5×10^3 mitomycin C-treated VC/well.
[b] mean cpm of triplicate cultures $\times 10^{-3} \pm$ SD.
[c] PBM isolated on Lymphoprep and subsequently enriched on Percoll gradient in order to eliminate contaminating granulocytes.

Two $\times 10^5$ responder cells, stimulated with 90 µg PHA/ml, were cultured in a final volume of 0.2 ml in flat-bottom microtiter plates with or without VC for 72 h. Stimulation index (SI) was calculated dividing δ cpm for PHA-stimulated response by autotransformation value. Augmentation index (AI) was calculated dividing cpm for PHA and VC-stimulated response by cpm for PHA-stimulated response.

VC as accessory cells in the lectin-stimulated lymphocyte proliferation

In order to evaluate the influence of VC on PHA-induced lymphocyte responses, different responder cell populations were co-cultured with VC in the presence of mitogen. In 5 out of 6 experiments, PBM supplemented with autologous VC responded higher to PHA than PBM alone (Table 1). The mean augmentation of the response (augmentation index (AI)) was 1.6. Such an effect was mediated by 10.5×10^3 VC added per well, which constituted 5 % of all the cells present in the single well. We found this number optimal, since the addition of larger numbers of VC per well (10 % – 22×10^3, 20 % – 5×10^3) had either little or no further enhancing effect on the level of the responsiveness. Addition of 133×10^3 VC per well (40 % of the cultured cells) was found to be inhibitory (data not shown).

Table 2. The effect of VC on [^{3}H]-thymidine uptake of autologous L stimulated with PHA

Exp.	Autotransformation rate	with PHA	SI	with PHA and VC[a]	AI
1	0.1 ± 0.01[b]	13.9 ± 1.00	138.0	26.6 ± 1.20	1.9
2	0.3 ± 0.08	24.6 ± 3.46	81.0	35.8 ± 1.07	1.5
3	0.07 ± 0.01	8.5 ± 0.90	120.0	23.4 ± 0.20	2.8
4	1.6 ± 0.16	11.9 ± 1.30	6.4	15.1 ± 1.20	1.3

[a] 10.5×10^3 mitomycin C-treated VC/well.
[b] mean cpm of triplicate cultures $\times 10^{-3} \pm$ SD.

For culture and SI and AI calculation details, see footnote to Table 1.

Table 3. The effect of VC on [^{3}H]-thymidine uptake of allogeneic PBM stimulated with PHA

Exp.	Autotrans-formation rate	with PHA	SI	with PHA and VC[a]	AI
1	0.2 ± 0.14[b]	30.0 ± 2.30	149.0	33.7 ± 1.60	1.1
2	0.2 ± 0.02	6.3 ± 0.18	30.5	6.1 ± 0.16	0.9
3[c]	0.2 ± 0.06	9.5 ± 0.96	46.5	8.9 ± 0.28	0.9
4	0.1 ± 0.02	8.7 ± 0.34	86.0	7.6 ± 0.37	0.9
5[c]	0.2 ± 0.13	11.7 ± 0.17	57.5	15.2 ± 0.47	1.3

[a] 10.5×10^3 mitomycin C-treated VC/well.
[b] mean cpm of triplicate cultures $\times 10^{-3} \pm$ SD.
[c] PBM isolated on Lymphoprep and subsequently enriched on Percoll in order to eliminate contaminating granulocytes.

For culture and SI and AI calculation details, see footnote to Table 1.

Similar results were obtained with LL as responders. In 4 experiments, the addition of autologous VC increased the PHA-stimulated proliferation of LL 1.8-fold (Table 2). The level of augmentation was comparable to that seen in PBM cultures. Nevertheless, the absolute level of LL responsiveness was higher than that of PBM.

In contrast to the autologous cell co-culture, no evident enhancing effect of VC was noted in the allogeneic combination. Only in the case of the two high-responders (Exp. 1 and 5), did a slight augmentation of the response to PHA occur in the presence of VC (Table 3). Addition of larger numbers of VC up to 40 % of cultured cells did not produce any additive stimulation (data not shown).

Table 4. [^{3}H]-thymidine uptake of PBM or LL cocultured with autologous VC for 6 days (AMLR)

Responders	Exp.	Autotransformation rate	with VC[a]	SI
PBM	1	0.38 ± 0.02[b]	0.75 ± 0.09	1.9
	2[c]	0.40 ± 0.04	1.46 ± 0.10	3.6
	3	0.24 ± 0.07	0.50 ± 0.20	2.1
	4[c]	0.13 ± 0.01	0.38 ± 0.05	2.9
LL	1	2.79 ± 0.10	7.54 ± 0.20	2.7
	2	0.97 ± 0.30	3.80 ± 0.60	3.9

[a] 10.5×10^3 mitomycin C-treated VC/well.
[b] mean cpm of triplicate cultures $\times 10^{-3} \pm$ SD.
[c] PBM isolated on Lymphoprep and subsequently enriched on Percoll gradient in order to eliminate contaminating granulocytes.

2×10^5 responder cells were cultured in a final volume of 0.2 ml in U-bottom microtiter plates with or without VC for 144 h. Stimulation index (SI) was calculated dividing VC-stimulated response by autotransformation value. [^{3}H]-thymidine incorporation in VC added per well was less than 100 cpm for all experiments.

Table 5. [^{3}H]-thymidine uptake of PBM cocultured with allogeneic VC for 6 days (MLR)

Exp.	Autotransformation rate	with VC[a]	SI
1	0.25 ± 0.04[b]	0.47 ± 0.13	1.9
2[c]	0.41 ± 0.05	1.45 ± 0.17	3.5
3	0.74 ± 0.18	0.91 ± 0.08	1.2
4[c]	0.39 ± 0.11	0.96 ± 0.10	2.5

[a] 10.5×10^3 mitomycin C-treated VC/well.
[b] mean cpm of triplicate cultures $\times 10^{-3} \pm$ SD.
[c] PBM isolated on Lymphoprep and subsequently enriched on Percoll gradient in order to eliminate contaminating granulocytes.

For culture and SI calculation details, see footnote to Table 4.

VC as stimulators in the mixed leukocyte cultures

As presented in Table 4, VC showed the stimulatory activity towards autologous PBM and LL during the 6-day mixed cultures, with the mean SI = 2.6. Higher cpm levels were observed in the cultures with autologous LL, but due to their high autotransformation rate, the relative stimulation was of the same range as in PBM.

The presence of 5 % of VC was also sufficient for the stimulation of proliferation of allogeneic blood-derived lymphocytes (Table 5). This stimulation was better expressed in experiments 2 and 4, when responder populations contained less granulocytes after additional purification on Percoll. This resulted in the responses of 3.5 and 2.5 times over the control value.

Role of VC in the high reactivity of lymph cell population

Afferent lymph cell population was found strongly reactive in terms of spontaneous and lectin-induced proliferation. Depletion of the whole lymph cell population of VC (purified LL fraction contained less than 1 %

Table 6. VC-mediated reconstitution of the responsiveness of LL

	Exp.	Whole population	LL	LL + VC[a]
PHA-induced tritiated thymidine uptake	1	20.1 ± 0.73[b]	13.9 ± 1.00	26.6 ± 1.20
	2	30.3 ± 3.70	24.6 ± 3.46	35.8 ± 1.07
	3	18.8 ± 2.49	8.5 ± 0.90	23.4 ± 0.20
Spontaneous tritiated thymidine uptake	1	5.0 ± 0.57	2.8 ± 0.10	7.5 ± 0.20
	2	2.3 ± 0.19	1.0 ± 0.30	3.8 ± 0.60

[a] 10.5×10^3 mitomycin C-treated VC/well.
[b] mean cpm of triplicate cultures $\times 10^{-3} \pm$ SD.

Whole afferent lymph cell population, purified LL or LL + VC were cultured for 3 days with 90 µg PHA/ml (PHA-induced proliferation) or for 6 days in culture medium alone (spontaneous proliferation).

of VC) produced an evident decrease in its responsiveness to PHA as well as spontaneous proliferation (Table 6). Reconstitution with 10.5×10^3 VC per well (5 % of cells present in the culture, whereas the percentage of VC in the whole population was about 6 %) restored the responsiveness of LL and even exceeded the initial levels.

Discussion

In the present study, we examined the immunological properties of veiled-like cells from stagnated lymph of dogs with chronic lymphedema. The lymph stagnation provides the unique source of pure skin-draining afferent lymph cells which are easily obtainable both instantly and in large quantity. This seems to be the major advantage of this method, since the peripheral lymph collection in humans or large animals is time-consuming and yields only limited numbers of cells. On the other hand, cannulation of ductus thoracicus after mesenteric lymphadenectomy, or isolation of DC from the skin or lymph nodes does not lead to the recruitment of pure peripheral afferent lymph cell population.

The term veiled cell used in this paper should be regarded as a descriptive form for cells which, due to their distinctive features, differed markedly from the other cell types in the afferent lymph. They were large, strongly DLA-class II positive cells with characteristic lobulated nuclei and uniquely well-developed cytopodia. They exhibited the activity of membrane-associated ATP-ase and non-specific esterase. Together with the potent immunostimulatory role of VC, these data generally agree with the description of VC given by other authors (2, 6, 15, 16), and strongly suggest that the cell under study is a member of the veiled cell population.

VC investigated in our study showed the accessory cell activity in mitogen-induced autologous lymphocyte proliferation. This observation is consistent with findings on VC and LC in other species (9, 18, 19, 21) and on DC in dog (23). The accessory activity of VC was evident for the response of lymphocytes isolated from peripheral blood and from afferent lymph. The LL response to PHA alone and to PHA + VC was higher than that of PBM. This is in agreement with the results obtained previously in our laboratory (29). There may be various reasons for this fact, such as the higher purity of lymph-derived lymphocytes (96 % vs 83 % in PBM), greater granulocyte contamination of blood-derived responder cells, or the more efficient cooperation between VC and T helper lymphocyte subset (the OKT-4$^+$ population is more abundant in afferent lymph than in blood (30)). Finally, the more vigorous response of LL as compared to their peripheral blood counterparts could be caused by the persistance of a very low number of VC in LL population. As reported by other authors (9, 18, 23) and confirmed in the present study, even extremely low numbers of LC, VC or DC ($1.25 - 3 \times 10^5$/0.2 ml culture) can stimulate lymphocyte proliferation.

In our, as well as in the other authors' investigations concerning the function of DC or macrophages as accessory cells, the nature of the signal delivered by these cells (stimulation or inhibition), is dependent on the culture conditions (concentration of cells, the dose of mitogen, time of the culture) (15). The stimulatory effect, which is best accentuated when relatively low numbers of accessory cells are used, may become inhibitory when their number in the culture is increased. In our opinion, the inhibitory effect of the addition of a large number of VC (133×10^3/well – 40 % of cultured cells) to the mitogenic assays does not correspond to the inherent suppressor activity of these cells which might be expressed *in vivo*, but is a reflection of non-optimal culture conditions, when the number of added VC indeed exceeds the physiological values.

No evident mitogen-presenting function of VC to allogeneic lymphocytes was found in our study. In two out of five cases, only a slight stimulation of the response was observed. This observation is contradictory to earlier reports (21, 23), and might indicate the requirement of MHC-compatibility in order that maximal stimulation can occur in this culture system. More tests would allow a more accurate evaluation of this fact.

The ability of lymphocytes to respond in AMLR is strictly dependent on the presence of Ia-positive cells in the stimulatory population (31, 32). There have been numerous reports on DC as stimulators of syngeneic and autologous lymphocytes in mixed cultures in the rat (22), mouse (33), dog (23) and human models (32). The moderate stimulation of effector immune mechanisms (transplant rejection) by the non-primed syngeneic DC was also observed *in vivo* in rat (20). Alternatively, in other studies, VC from rabbits and DC from mice produced low or negative syngeneic stimulation, which could be increased if the DC originated from sensitized individuals (15). Our results support the former view, showing that strongly MHC-class II-positive VC fulfill a stimulatory function in mixed cultures with both autologous PBM and LL, without any exogenous antigenic or mitogenic stimuli.

The presence of class II MHC molecules is also crucial for generating responses to alloantigens. VC, strongly expressing these antigens, and used as stimulators in MLR, promoted the proliferation of allogeneic PBM. As aforementioned, a small number of VC (stimulator/responder ratio 1:19) were effective. This observation is compatible with the characteristics of DC in rat, mice, dog and human (22, 17, 23, 18, 32). As demonstrated in these studies, DC are the strongest stimulators of allogeneic MLR, effective in low numbers (DC/responder ratio 1:10–100), and no other cell type (all non-T peripheral blood leukocytes, macrophages, B or null cells) can substitute them in terms of the strength of allostimulation.

One technical problem that arises in the *in vitro* investigation of VC function is the purity of VC added to the lymphocyte cultures. In the experiments shown here, it ranged between 74–83 %. Hence, some activity of contaminating cells cannot be excluded from the results. However, the

predominance of VC-like cells in the stimulating cell population, together with the small number of these cells added per well indicate the superior role of VC in eliciting the reaction of the responder cells. Analogically, the responder cell populations also contained the autonomous accessory cells, namely monocytes in PBM and some contaminating macrophages in the afferent lymph lymphocytes. The actual degree of this contamination, being even less than 4 %, as determined from the stained lymph cell smears, still plays the functional role in the mitogenic assays (the minimal accessory cell number required for the response of lymphocytes to mitogen to occur is less than 1 %). In our assay system, the presence of autonomous accessory cells in the responder populations was reflected by the considerable level of the response to PHA in cultures non-supplemented with VC. However, the addition of VC caused the marked augmentation of PBM proliferation and restored completely the LL response comparing to the whole afferent lymph cell population. The dynamics of these reactions show that VC are very efficient in this respect. Hence, the possibility of some differences in the mechanism of fulfilling the accessory function between VC and «classical» accessory cells is not excluded.

In conclusion, the above-mentioned properties of VC suggest the active involvement of these cells in the skin-associated immune reactions in dog. They also imply that the immunogenicity of the skin is dependent on the presence of LC–VC population, since these cells may play an important role as «passenger» cells in transplantation immunity.

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DEVELOPMENT OF ANTISERUM AGAINST VEILED (DENDRITIC) CELLS OF CANINE AFFERENT LYMPH: IMPLICATIONS FOR TRANSPLANTATION BIOLOGY

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ABSTRACT

A method is described for production of anti-veiled cell serum against veiled cells (VC) or dendritic cells obtained from canine skin lymph. By use of discontinuous Percoll gradient, VC from lymph were enriched to about 50% of the entire lymph cell population. After immunization of rabbits with the priming total dose of 10^7 VC (intramuscular, subcutaneous, intracutaneous) and with the same total booster injection (intravenous), the sera obtained were cytotoxic mainly for VC, with cytotoxin titer 1:16-1:32 and for agglutinin 1:256-1:512, respectively. Antisera used in vitro *blocked the Ia and CD1 antigens of VC on smears and inhibited the accessory function of VC in cell response to phytohemagglutinin (PHA) and their stimulatory activity in mixed leukocyte reaction (MLR).* In vivo, *the local, intracutaneous administration of antisera led to transient depletion of VC from afferent lymph, and to reduction of mononuclear cells in the T-dependent areas in regional lymph nodes.*

Several authors have suggested that the cells responsible for the initiation of allograft rejection are passenger cells (1). The effectiveness of the Ia-positive veiled passenger cells of the skin collected from afferent lymph in provoking allogeneic response and in initiating renal graft rejection has previously been described (2). A great deal of effort has been expended to eliminate these stimulatory cells from allografts. Nonetheless, treatment with total body irradiation, cyclophosphamide or anti-lymphocyte serum have had only limited success in prolonging allograft survival (3,4). Whole organ pretreatment by simple flushing with anti-class II monoclonal antibodies (moAb) has increased survival of canine renal (5) and rat heart allografts (6). Recently, hemoperfusion with anti-Ia or anti-dendritic cell moAbs prior to intact pancreas allografting (7) and *in vitro* treatment of thyroid allografts (8) has produced increased organ survival. However, the Langerhans cell-dependent antigen presenting function of epidermal cells is unaltered after intraperitoneal administration of anti-Ia moAb, as compared with spleen dendritic cells (9). Whether anti-Ia antiserum reduces the number of Langerhans cells in skin allograft is not known. Also, no data on the possible effects of specific anti-Langerhans cell serum are available. There have been major problems with raising such antisera due to difficulties in obtaining sufficient numbers of dendritic cells from epidermis or afferent lymph. We have found, however, a method for harvesting large numbers of afferent lymph cells, adequate for immunization of antiserum producers.

The purpose of the present study was to

develop anti-veiled cell antiserum and to characterize its effects *in vitro* and *in vivo* on skin Langerhans cells and lymphocytes.

MATERIALS AND METHODS

Outbred dogs with chronic lymphedema after surgical interruption of afferent lymphatics were used as lymph cell donors (10).

Collection of Lymph Cells

Lymph (~25-70ml) was collected from dilated skin lymphatics by direct percutaneous puncture. It contained lymphocytes, veiled cells and some granulocytes and macrophages. The total cell number was $2\text{-}5x10^7$. 7% were veiled cells (migrating Langerhans cells), 4% macrophages, 19% granulocytes and 70% lymphocytes.

Enrichment of Veiled Cells (VC)

Five different concentrations of isotonic Percoll in RPMI 1640 medium with 10% fetal calf serum (Gibco) were prepared (38%, 44%, 50.8%, 55%, 66.7%). After layering of 2ml volumes of Percoll solution, 2ml of a lymph cell suspension in RPMI 1640 medium with 10% FCS was placed on the top and spun down at room temperature at 500xg for 30 min. Low density interface cells were collected and washed with medium. This latter population enriched to about 50% VC also contained lymphocytes. High density interface cells were pure lymphocytes (11).

Immunization Procedure

The priming total dose was 10^7 veiled cells from a low density cell population suspended in 3ml of PBS and then emulsified with an equal volume of complete Freund's adjuvant (Difco). The 2ml portions were injected subcutaneously, intracutaneously and intramuscularly into a rabbit. A total booster injection of 10^7 veiled cells in 3ml of PBS was administered intravenously 3 weeks later (3x in 1ml portions, every other day). Rabbits were sacrificed 7 days after the last injection and sera were collected. They were aliquoted, stored in -20°C and decomplemented before using (56°C for 30 min). A part of sera was absorbed with allogeneic lymphocytes from the canine lymph nodes (2:1) for 2 h at 4°C and next for 30 min at 37°C.

Cytotoxic Titer

Serum cytotoxic titer was checked against the veiled cell enriched population and high density cells. Cells adjusted to the concentration of $2x10^7$/ml of RPMI 1640 medium were placed in microculture plate in the volume of 25µl, mixed with 50µl of sera dilutions and incubated at 37°C for 45 min. Then 50µl of diluted 1:15 rabbit complement (Behring) was added and cells were incubated for 45 min. The cell viability was evaluated by 0.2% trypan blue exclusion after mixing 1:1 with the cell suspension. Agglutinin titers were checked by light microscopy simultaneously.

Effect of Antisera on the Expression of Ia and T6 Antigens on Veiled Cells

Cytospins fixed in cold acetone for 1 min were incubated for 30 min with antiserum or with normal rabbit serum as control (both diluted 1:5). Then cytospins were incubated with mouse anti-HLA-DR and anti-T6 (CD1) monoclonal antibodies (Dakopatts, diluted 1:20) for 30 min. Rabbit anti-mouse IgG, alkaline phosphatase-anti alkaline phosphatase (APAAP) complex, alkaline phosphatase (AP)-substrate (Dako) and Mayer's hematoxylin were used for visualizing cross-reactivity with canine DR and CD1 antigens.

Effect of Antisera on the Reactivity of Lymph Cells in Culture

Culture medium consisted of RPMI 1640 medium supplemented with 10mM Hepes buffer, 20% FCS, 100 U/ml penicillin and streptomycin (Gibco) and 2mM L-glutamine (Flow). Whole lymph cells or high

density cells from Percoll gradient ($2x10^6$) were cultured in 0.2ml medium, both with and without normal rabbit serum or antisera, diluted to a final concentration from 1:8 to 1:96 in the presence or absence of PHA (Wellcome HA15, 90µg/ml). Cultures were performed in triplicate and incubated for 72 h in humidified atmosphere of 5% CO_2 in air. Twenty hours before the culture termination, 100µl of medium was replaced by equal volume of fresh medium in culture and 0.4µCi of ^{3}H-thymidine (Amersham, 2Ci/mM) was added. Radioactivity was measured on glass fiber filters in Permafluor cocktail (Packard). To investigate the direct influence of antisera on the veiled cells only, the low density cells ($2x10^4$) were preincubated with the respective dilutions of antisera or normal rabbit serum for 45 min at 37°C in microculture plate. After washing by plate centrifugation and removing of medium the high density cells were added ($2x10^5$) and cocultured with or without PHA for 72 h. To investigate the effect of antisera on activity of VC in a 6 day mixed leukocyte reaction (MLR), whole lymph cells were cultured with or without 5% VC from the Percoll gradient enriched population. Subsequently cells were preincubated with mitomycin C (Sigma) used at a concentration of 40µg/ml for 40 min at 37°C. Control monoclonal antibodies Dakopatts: anti-HLA-DR (M704), anti-T6 (M721), anti-macrophages (M718) were used in a final concentration of 1:50 and 1:100.

Effect of Antisera on VC Outflow from Afferent Lymph

Lymph was obtained from a cannulated afferent lymph vessel of the hindlimb adjacent to the saphenous vein and collected continuously for 90 min (control) before intracutaneous injection of normal rabbit serum or anti-VC serum (1 ml, diluted 1:1 with 0.9% NaCl), and for the next 150 min after injection, at 30 min intervals. The outflow of lymph cells, including VC, was measured.

Effects of Antisera on Lymph Node

Seventy-two and ninety-six hours after two and three, respectively, intradermal injections in the dorsum of the paw of 1ml anti-veiled cell antiserum or normal rabbit serum (dilutions 1:1), popliteal lymph nodes were harvested and fixed in 4% formalin. Histological sections were stained with hematoxylin-eosin and evaluated microscopically.

TABLE 1
Cytotoxin and Agglutinin Titers of Anti-Veiled Cells (A-VC) Antisera (Before and After Absorption) Compared with Normal Rabbit Serum (NRS)

Cells	NRS	A-VC	Absorbed A-VC
Low density (VC-enriched)			
Cytotoxicity (%)	0	20-40	0-10
Cytotoxin titer	0	1:16-1:32	0-1:8
Agglutinin titer	1:8	1:512	1:64-1:128
High density			
Cytotoxicity (%)	0	10	0
Cytotoxin titer	0	1:16-1:32	0
Agglutinin titer	0	1:256-1:512	0-1:16

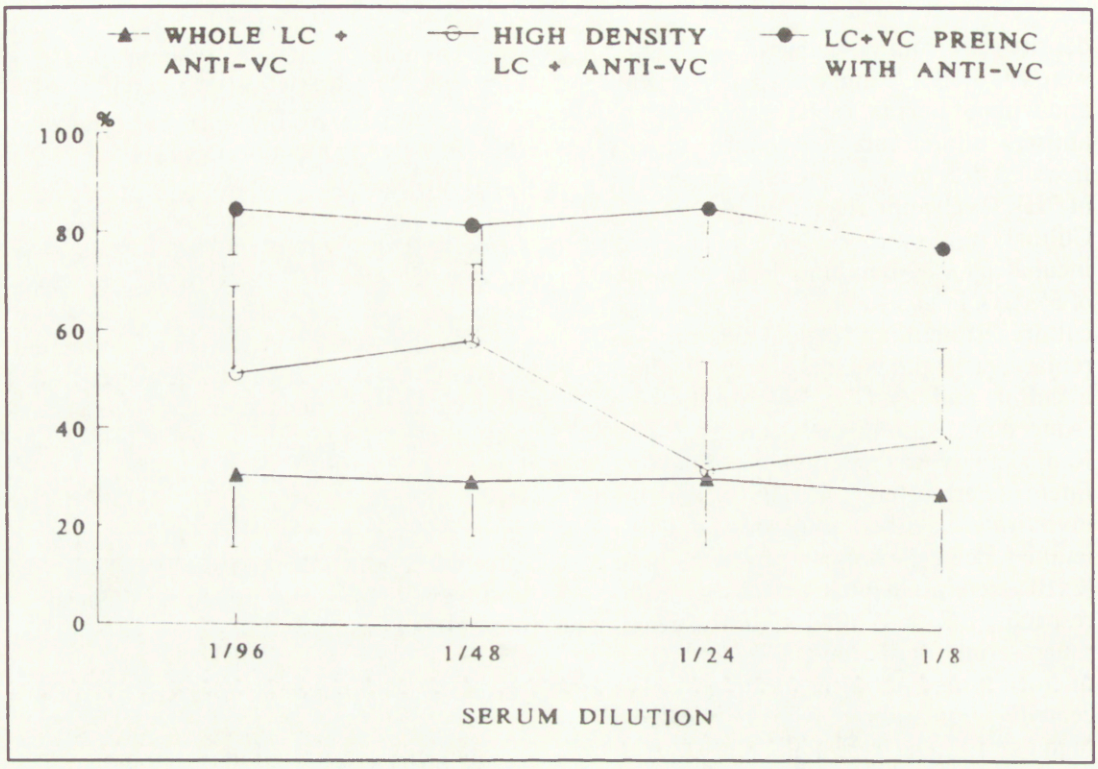


Fig. 1. Effect of anti-VC sera on lymph cell (LC) response to PHA expressed as percent of reactivity in presence of normal rabbit serum in culture. (▲ WLC; ( ) HDC; (●) HDC supplemented with 5% VC preincubated with anti-VC serum for 45 min before the onset of the culture. Results are represented as mean percentages ± SD of control cultures, which were supplemented with adequate dilutions of normal sera.

RESULTS

Cytotoxin and Agglutinin Titers

The raised antisera were mainly cytotoxic for veiled cells (20-40% of dead cells), whereas lymph lymphocytes were comparatively resistant (10% of dead cells) *(Table 1)*. Antisera showed a high titer of agglutinins (1:256-1:512) and of cytotoxins (1:16-1:32). Control normal rabbit serum was not cytotoxic and agglutinated only low density cells (titer 1:8). After absorption with allogeneic lymphoid cells, antisera showed decreased agglutinin titers, but were still cytotoxic for veiled cells (0-10% of dead cells).

Expression of Ia and T6 (CD1) Antigens on Veiled Cells

Preincubation of cell smears with antisera before detection of Ia and T6 antigens with monoclonal antibodies led to blocking of these surface antigens, as compared with normal rabbit serum (data not shown).

Lymph Cell Activity in Culture with Antisera and MoAb

Antisera added to the culture inhibited whole lymph cell response to PHA to about 30% of their response in the presence of normal rabbit serum *(Fig. 1)*. When veiled cells were pretreated with antisera before adding lymphocytes to the culture, the inhibitory effect of antisera was still observed (80% of cell response as compared to normal rabbit serum). Stimulatory activity of VC in MLR was blocked by

TABLE 2
Effect of Anti-Veiled Cells (VC) Sera and Monoclonal Antibodies (moAb) on VC Stimulatory Activity in Mixed Leukocyte Reaction (MLR) (n=3) Data Expressed As Percent of Control Values (Mean±SD)

Agent	Dilution 1:100	1:50
Control serum	115.7±30.0	141.0±21.1
Anti-VC serum	40.3±26.8	66.4±49.2
Anti-Ia moAb	72.4±27.0	65.7±16.8
Anti-T6 moAb	94.4±22.5	109.7±34.3
Anti-MQ* moAb	100.6±21.0	101.4±27.1

* macrophage

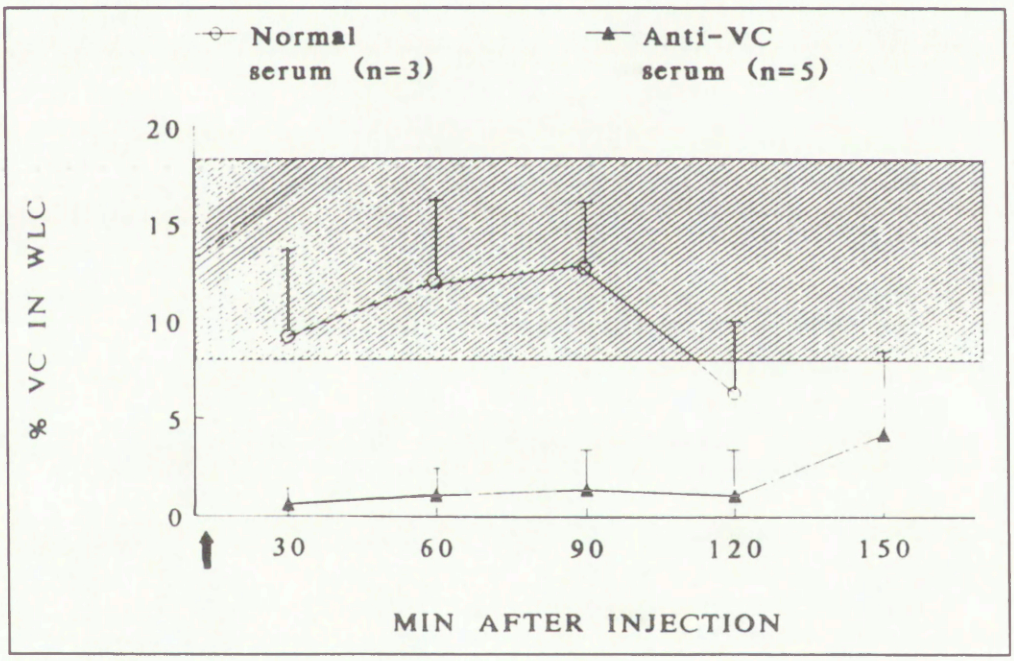


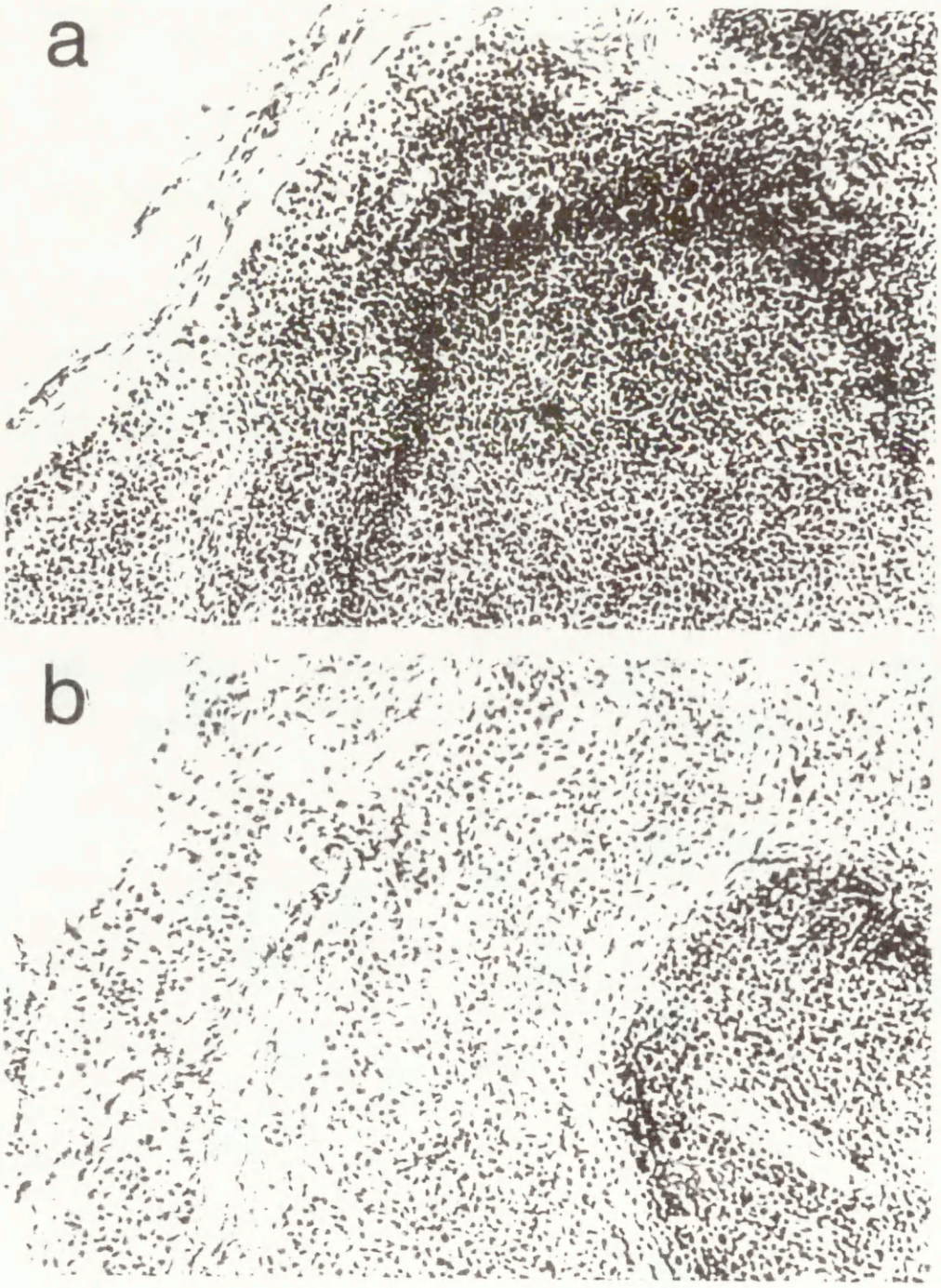
Fig. 2. Effect of anti-VC sera on in vivo *VC outflow from afferent lymph expressed as percent of VC in whole lymph cell population. Collection of lymph samples and cell counts were performed in 30 min intervals before (shaded area) and after the subcutaneous injection of normal serum (○) and anti-VC serum (▲).*

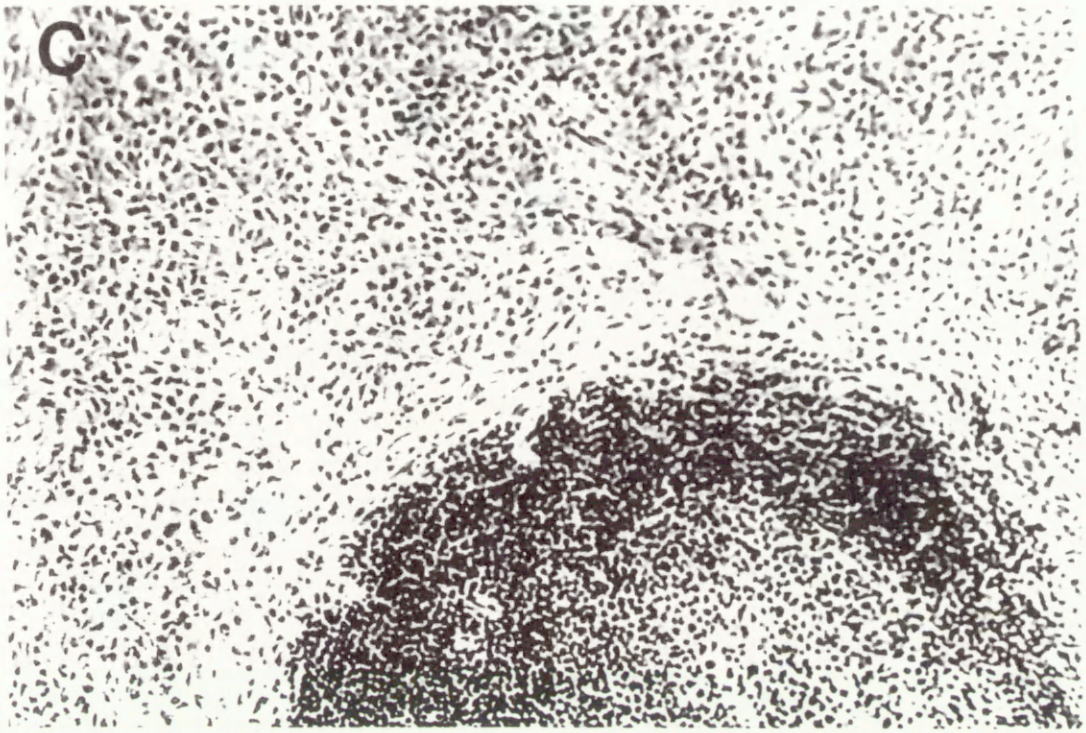
antisera and by anti-Ia moAb as compared with normal rabbit serum and control anti-T6 or anti-macrophage moAbs *(Table 2)*.

Effect of Antisera on VC Outflow In Vivo

Antisera were able to reduce the outflow of VC for 150 min after intradermal injection as compared with normal rabbit serum *(Fig. 2)*, leaving the trafficking of lymphocytes thereafter almost intact.

Fig. 3. Photomicrograph of a lymph node after intradermal injection of anti-veiled cell (VC) serum. a: normal rabbit serum; b: two doses of anti-VC serum; c: three doses of anti-VC serum (x250). Note the marked reduction of mononuclear cells in the T-dependent areas with sparing of the follicles after injection of anti-VC sera (b and c).





Histological Changes in Regional Lymph Node

Intradermal injection of anti-veiled cell antiserum reduced mononuclear cells in the T-dependent areas and spared follicles in the regional lymph node in a dose dependent fashion. Normal rabbit serum did not produce any changes *(Fig. 3)*.

DISCUSSION

The presence of interstitial dendritic cells in non-hematopoietic tissues has many implications for organ grafting (12,13). Mature dendritic cells constitutively express high density class II antigens and stimulate allogeneic T cells very effectively. Depleting dendritic cells from the transplanted organ would render the host immune system relatively unresponsive to the graft. In skin transplantation, the use of Langerhans cell-free cultured epidermal allografts delays but does not obviate graft rejection (14,15). On the other hand, the presence of Langerhans cells is not sufficient to induce rejection. A necessary step involves induction of Ia on other epidermal cells (keratinocytes) and rejection is blocked by treatment of the host with reagents that interfere with the induction of class II antigens (16).

The rationale for using anti-Ia moAb was to eliminate or prevent Ia-positive cells from interacting with resting T lymphocytes. Perry and Williams (17) demonstrated that survival of skin allografts transplanted across multiple minor or class I, but not class II barriers, was significantly prolonged by anti-Ia moAb treatment of recipients, and was associated with the development of specific suppressor T cells. In the case of dogs, Fuller et al (18) postulated that the presence of canine natural antimouse IgG, can markedly influence the biologic effects of *in vivo* administered moAbs.

In this study, we developed a xenogeneic anti-veiled cell antisera by using veiled cells (VC) from afferent stagnant lymph (lymphedematous dog hindlimbs). Sera were cytotoxic for VC *in vitro*, blocked their Ia and CD1 surface antigens and inhibited VC stimulatory and accessory

functions in culture. Ladiges et al (19) reported that Ia-like antigens on canine monocytes involved in MLR were cross-reactive with anti-human class II moAbs. We used as a control cross-reactive mouse anti-human moAbs (Dako) against HLA-DR and CD1 antigens. The anti-veiled cell sera developed by us reduced the outflow of VC from afferent lymph after intradermal administration leaving the trafficking of lymphocytes thereafter largely unaffected. Depletion of T-dependent areas in lymph nodes with sparing of follicles with use of the anti-veiled cell sera supports that this antiserum was directed primarily if not exclusively against dendritic cells and T lymphocytes. Moreover, our demonstration that paracortical areas were depleted is consistent with results of Hay et al (20) after local injection of antilymphocyte serum. By arresting the migration of Langerhans cells from graft into lymphoid tissue or their maturation into immunostimulatory veiled cells, a new strategy for overcoming allograft rejection is raised by using antisera against these immunoreactive cells.

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Short communication

Reactivity of antibodies directed against human antigens with surface markers on canine leukocytes

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Abstract

A panel of anti-human antibodies was used in order to investigate their cross-reactivity with canine leukocytes. The labeling was carried out at the microscopic level by immunocytochemical staining. Of 50 antibodies 22 cross-reacted with canine leukocytes from afferent lymph and peripheral blood. All leukocytes reacted with MHM23 (CD18). Two anti-HLA DR antibodies, DK22 and L243, reacted mostly with veiled cells and with PHA-stimulated lymphocytes, whereas TAL1B5 was cross-reactive with all canine lymphocytes. The activation markers CD25, Ki-67, PCNA were identified on PHA-stimulated lymphocytes with ACT1, Ki-67 and PC10 clone produced antibodies. Canine eosinophils reacted with MHM6 (CD23) antibody. A large number of antibodies reacted with canine lymph veiled cells. Canine granulocytes and a subset of lymphocytes were stained with the anti-CD15 antibody only after treatment of cytospins with neuraminidase.

Keywords: Canine leukocytes; Human CD-antibodies; Cross-reactivity

1. Introduction

Dogs are widely used for transplantation studies. They provide an excellent model for allograft follow up studies and studies of immunosuppressive intervention. The rejection process of allografts is mediated by recipient leukocytes. Unlike those of man and mouse, canine leukocytes can be poorly characterized, because only limited numbers of

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monoclonal antibodies (mAb) to their antigens are available (Abbassi et al., 1991; Hotzl et al., 1991; Gebhard and Carter, 1992; Danilenko et al., 1992; Moore et al., 1992; Yang et al., 1994). Recently, the first workshop on canine leukocyte antigens has allowed the identification and characterization of a set of anti-canine mAb that enable the extension of immunological research in the dog (Cobbold and Metcalfe, 1994). However, the specificities of these antibodies are still not clear enough to identify the canine equivalent of human CD antigens. For instance, canine CD4 antigen was detected at high levels on granulocytes and MHC class II antigens were found on all canine leukocytes. Many groups have been using cross-reactive commercial anti-human reagents to identify canine leukocyte antigens (Marchal et al., 1993; Chabanne et al., 1994). These antibodies were applied for flow cytometry of canine peripheral blood leukocytes and for immunohistological staining of dog thymus, lymph node, kidney and skin.

In the present study we have screened monoclonal and polyclonal antibodies reacting with human leukocytes for their cross-reactivity with canine leukocytes on cytospins using immunocytochemical methods. The cells from dog skin afferent lymph (containing lymphocytes and the migratory form of Langerhans cells — veiled cells) (Galkowska et al., 1989), peripheral blood and blasts after culture of lymph cells with PHA were used.

2. Material and methods

2.1. Dogs

Outbred dogs with chronic lymphedema after surgical interruption of hind limb afferent lymphatics served as the peripheral blood and skin lymph donors (Olszewski et al., 1968).

2.2. Canine cell handling

Cells were isolated as described (Dabrowski et al., 1989). Briefly, lymph was collected by the percutaneous puncture of dilated lymphatics, into tubes with 10 IU ml^{-1} of heparin (Novo). Cells were washed twice and resuspended in MEM (Gibco). Blood was drawn into tubes with heparin and after dilution 1:2 with 0.9% NaCl was layered over Lymphoprep (Nyegaard) and spun down at 250 g for 30 min. Cells from the interface were collected, washed three times and resuspended in MEM. PHA stimulation was performed in tubes. Total volume was 2 ml of RPMI1640 (Gibco) supplemented with 100 U ml^{-1} of penicillin, 100 μg ml^{-1} of streptomycin, 2 mM L-glutamine and 20% of decomplemented fetal calf serum (Gibco). Cells adjusted to 3×10^6 2 ml^{-1} were cultured with 180 μg PHA (HA15, Wellcome) for 48 h in the humidified atmosphere of 5% CO_2 in air at 37°C. After culture cells were washed twice and resuspended in MEM (10^6 ml^{-1}).

2.3. Immunocytochemistry

Antibody binding was detected with the APAAP technique (DAKO) using previously described methodology (Galkowska et al., 1995). Briefly, the cytospins were fixed with

acetone and incubated with normal goat serum (1/5) for 30 min. Next, the cytospins were incubated with mouse mAb (diluted 1/5 or more) for 30 min, with goat anti-mouse IgG (Dako) diluted 1/100 with 10% normal dog serum for 30 min and for another 30 min with mouse APAAP (Dako) diluted 1/50. Then, the AP-substrate was applied for 15 min, and finally the cytospins were stained with Mayer's hematoxilin. The staining was performed at room temperature (22°C) with TBS buffer as washing agent. For staining with polyclonal rabbit antibodies the cytospins were incubated with normal swine serum and after binding of rabbit antibody the biothylated swine anti-rabbit IgG (Dako) diluted 1/300 with 10% normal dog serum was used. Next, the Ap-streptavidin (Dako) diluted 1/800 and AP-substrate were applied. For staining with rat anti-human CD49f antibody the normal rabbit serum, rabbit anti-rat IgG (Dako) diluted 1/50 and rat-APAAP (Dako) diluted 1/50 were used. Treatment with neuraminidase (Vibrio cholerae, Calbiochem, 1 U ml^{-1} of buffer pH 5.5, containing 4 mM $CaCl_2$, 50 mM NaOAc, 154 mM NaCl) was carried out on acetone fixed cytospins, by incubation with 100 μl of enzyme for 1 h at 37°C. Next, the anti-CD15 mAb was applied. Working dilutions of reactivity with canine cells mAb are listed in Table 3.

3. Results and discussion

Of the 50 anti-human Abs, 22 cross-reacted with canine leukocytes and these are listed in Table 1. The non-cross-reacting Abs are shown in Table 2. The canine cellular

Table 1
Anti-human leukocyte antibodies that are cross-reactive with dog leukocytes

CD/antigen	Clone	Isotype	Origin
CD 1a	NA1/34	IgG2a	Dako
CD 14	TUK 4	IgG2a	Dako
CD 15 [a]	C3D-1	IgM	Dako
CD 18	MHM 23	IgG1	Dako
CD 23	MHM 6	IgG1	Dako
CD 25	ACT-1	IgG1	Dako
CD 45RO	UCHL 1	IgG2a	Dako
CD 45RB	PD7/26	IgG1	Dako
CD 49d	HP2/1	IgG1	Immunotech
CD 49e	5AM1	IgG2b	Immunotech
CD 49f	GoH3	IgG2a	Immunotech
CD 54	84H10	IgG1	Immunotech
CD 58	L306.4	IgG2a	Becton Dickinson
CD 68	Ber-MAC3	IgG1	Dako
Ki-67	Ki-67	IgG1	Dako
PCNA	PC 10	IgG2a	Dako
HLA DR	DK 22	IgG2a	Dako
	CR3/43	IgG1	Dako
	TAL.1B5	IgG1	Dako
	L 243	IgG2a	Becton Dickinson
S-100 protein			Dako
fibronectin			Dako

[a] Positive staining after treatment of cytospins with neuraminidase.

specificities for various anti-human antibodies are presented in Table 3. Interestingly, the mAb against CD15 stained canine granulocytes and a subset of lymphocytes only after treatment of cytospins with neuraminidase (Vibrio cholerae). This has been reported previously by Ross and coworkers (Ross et al., 1994) for human Langerhans cells. The activation markers CD25, Ki-67, PCNA were identified on PHA-stimulated lymphocytes with ACT-1, Ki-67 and PC10 clone produced antibodies. Our positive results of the cross-reactivity of MHM23 (CD18) are in agreement with those of Marchal and coworkers (Marchal et al., 1993) and Chabanne and coworkers (Chabanne et al., 1994). We also confirmed that mAbs against OKT6 and Leu6 (CD1) did not cross-react with dog CD1 (Marchal et al., 1993; Chabanne et al., 1994), but we found that NA1/34 (CD1a) Ab reacted with canine lymph veiled cells. These cells also reacted with polyclonal anti-S100 protein Ab, HP2/1 (CD49d), 5AM1 (CD49e) and GoH3 (CD49f) mAbs. Subsets of veiled cells reacted with TUK4 (CD14), 84H10

Table 2
Anti-human leukocyte antibodies that are non-cross-reactive with dog leukocytes

CD/antigen	Clone	Isotype	Origin
CD 1	T 6	IgG1	Ortho Diagnostic
	SK 9	IgG2b	Becton Dickinson
CD 2	MT 910	IgG1	Dako
CD 3	UCHT 1	IgG1	Dako
	T3-4B5	IgG1	Dako
CD 4	MT 310	IgG1	Dako
CD 8	DK 25	IgG1	Dako
CD 11a	MHM 24	IgG1	Dako
CD 11b	2LPM19c	IgG1	Dako
CD 11c	KB 90	IgG1	Dako
CD 29	K 20	IgG2a	Dako
CD 43	DF-T1	IgG1	Dako
CD 44	L 178	IgG1	Becton Dickinson
CD 45	T29/33	IgG1	Dako
CD 45RA	4KB5	IgG1	Dako
	L 48	IgG1	Becton Dickinson
CD 45RO	OPD 4	IgG1	Dako
CD 49d	P4G6	IgG3	Dako
CD 49e	P1D6	IgG3	Dako
CD 54	LB 2	IgG2b	Becton Dickinson
	6.5B5	IgG1	Dako
CD 68	EBM 11	IgG1	Dako
	KP 1	IgG1	Dako
CD 69	L 78	IgG1	Becton Dickinson
CD 71	Ber-T9	IgG1	Dako
Collagen IV	CIV 22	IgG1	Dako
Laminin	4C7	IgG2a	Dako
Dendritic reticulum cell	R4/23	IgM	Dako

(CD54), L306.4 (CD58), Ber-MAC3 (CD68) and UCHL1 (CD45RO) mAbs. Canine eosinophils reacted with MHM6 (CD23) mAb. All leukocytes reacted with MHM23 (CD18), PD7/26 (CD45RB) and anti-fibronectin Abs. The anti-HLA DR antibodies (DK22, L243) reacted mostly with canine veiled cells and with PHA-stimulated lymphocytes. The CR3/43 mAb also reacted weakly with a subset of lymphocytes, and TAL1B5 was cross-reactive with all canine lymphocytes. Our previous (Galkowska et al., 1992) and recent (Galkowska et al., 1995) studies demonstrate the usefulness of some of these Abs cross-reactive with dog antigens in in vitro cellular assays. Applied by us in MLC anti-DR (DK22) mAb brought about a 50% reduction of canine lymphocyte proliferation, whereas control mAb anti-CD1a (NA1/34) and anti-CD68 (EBM11) had no effect. In a 4 h assay for cluster formation by lymph veiled cells with autologous lymphocytes we observed a significant reduction of cell binding in the presence of antibodies anti-CD54 (84H10), -CD58 (L306.4) and fibronectin. Other reactive with canine cells mAb (anti-CD18; CD49d,e,f) had adhesion promoting activity. Control antibodies anti-CD1a, -S-100 protein and -DR(TAL.1B5) had no effect on cell binding.

Table 3
Reactivity of canine leukocyte antigens with anti-human leukocyte antibodies

CD/antigen	Clone	Dilution	Cellular specificity
CD 1a	NA1/34	1/10	VC [a,b] + +
CD 14	TUK4	1/10	Subset VC +
CD 15	C3D-1	1/10	Neutrophils and subset LY [c] + +
CD 18	MHM23	1/500	All leukocytes + + +
CD 23	MHM6	1/10	Eosinophils + +
CD 25	ACT-1	1/10	PHA blasts + +
CD 45RO	UCHL 1	1/10	Subset Ly +, subset VC + +
CD 45RB	PD7/26	1/10	All leukocytes +
CD 49d	HP2/1	1/100	VC + + +, subset LY + +
CD 49e	5AM1	1/10	VC + + +
CD 49f	GoH3	1/10	VC + +
CD 54	84H10	1/10	Subset VC + +
CD 58	L306.4	1/10	Subset VC + +
CD 68	Ber-MAC3	1/10	Subset VC + +
Ki-67	Ki-67	1/20	PHA blasts +
PCNA	PC 10	1/60	PHA blasts + +
HLA	DR DK 22	1/20	VC + +
	CR3/43	1/10	VC + +, subset LY +
	TAL.1B5	1/10000	VC and LY + + +
	L243	1/20	VC + + +
S-100 protein		1/100	VC + + +
Fibronectin		1/600	All leukocytes + + +

[a] VC, veiled cells.
[b] + + +, strong; + +, intermediate, +, weak reactivity.
[c] LY, lymphocytes.

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A NOVEL MONOCLONAL ANTIBODY SPECIFIC FOR LYMPH DENDRITIC CELLS

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INTRODUCTION

Several authors have suggested that the cells responsible for the initiation of allograft rejection might be the passenger cells. The effectiveness of the Ia-positive dendritic cells (DC) collected from skin afferent lymph in provoking allogeneic response and in initiating renal graft rejection has been described (1). A great deal of effort has been provided to eliminate such stimulatory cells from allografts. Clinical and immunohistochemical findings indicate that allografts of cultured epithelia devoid of LC are rejected and epithelial allografts are repopulated by host LC (2). The possibility that the processing of graft antigens by recipient DC might be important as the indirect allorecognition was sugested (3). It is notable that after iso- and allo- grafting of skin the epidermal LC migrate into the dermis. It was also seen in an organ culture model where the cells continued to migrate into the culture medium (4).
The need for mAbs that are specific for DC and block their functions is evident. Many of antigens expressed on skin DC (CD80/B7-1, CD54/ICAM1, CD58/LFA3, CD29, CD40, CD1a, CD45, MHC classII) have broad cellular distribution which limit their usefulness as markers for DC. The mAb OX-62 was raised against DC obtained from rat lymph, but this mAb did not label all classical DC and reacted with skin γ/σ T cells (5). This mAb had no effect on the MLR between CD4 cells and DC stimulators. In humans, the mAb CMRF-44 was raised against an early activation antigen expressed on DC isolated from blood and tonsil, but not detectable on LC (6). The presence of CMRF-44 mAb did not affect DC function in the MLR. Other human DC-associated surface markers CD83 (7) and CD24 (8) also did not act on intercellular adhesion, as cluster formation between antigen presenting cells and T lymphocytes. Althought DC importance as the most effective cell involved in the initiation of primary immune responses has been demonstrated, no DC-specific marker whose expression could be regulated pharmacologically has been identified.
The purpose of our study was to generate DC-specific monoclonal antibodies useful for DC identification and reduction of their ability to present alloantigens to T lymphocytes.

MATERIAL AND METHODS

Dogs

Outbred dogs with chronic lymphedema after surgical interruption of hind limb afferent lymphatics served as skin lymph donors.

Collection of lymph

Lymph was obtained by direct percutaneous puncture of dilated lymphatic and collected into plastic tubes with heparin solution (10 U ml, Novo Industri,

Copenhagen, Denmark). The average lymph cell concentration was $4.4 \pm 3.7 \times 10^5$ /ml. The percentage of cells with dendritic morphology was 6.5 ± 2.6 and of lymphocytes 82.7 ± 9.2. Eosinofils and macrophages comprised 10.7 ± 6.7 % of cell population.

Immunization and fusion

Two fusions were performed using SP2/0 myeloma cells and splenocytes from BALB/c mice immunized with DC from lymph enriched to about 50% on a 12% Metrizamide gradient. Mice were immunized intradermally with 2×10^3 DC mixed with a complete adiuvant of Freund, than twice with DC given i.p. and boosted with DC i.v. on day -3. On day 0 mice were sacrificed and their splenocytes were frozen. The SP2/0 myeloma cells were frozen as splenocytes. For fusion splenocytes were mixed with the SP2/0 cells at a ratio 10:1, centrifuged and fused using 50% polyethylene glycol 1500 (Sigma) in PBS for 90 sec at 37°C. Fusion was stoped and cells were cultured in medium consisting of RPMI 1640, 10% decomplemented FCS (Sigma), 2 mM L-glutamine, 1 mM sodium pyruvate and 2% HAT (Sigma). Than 2×10^4 cells were plated together with the equal number of mouse peritoneal macrophages and cultured for 7 days in 96-well flat-bottomed plates (Nunck, Naperville, IL). Than the cells were fed with HT medium (Sigma) and cloned by limited dilution before screening the culture supernatants.

Hybridoma screening

Two assays were used to select hybridoma of intrest. Secretion of DC specific immunoglobulins was tested by ELISA using the DC-derived antigen, anti-mouse Ig antibodies conjugated with peroxidase (Sigma) and tetramethylbenzidine substrate. Selective binding of antibodies to lymph DC was evaluated by immunocytochemistry (see below).

Purification of mAbs

Two positive for DC of 30 screened hybridomas (DC-III/2 and DC-2) and control anti-virus clone (VII/1) were cultured for Ig production and purification. Immuno Type Kit (Sigma) was used for determination of mAb isotypes. The mAbs were precipitated with 50% saturated $(NH_4)_2SO_4$, centrifugated and dissolved in PBS and than purified using protein A (Pharmacia Biotech, Sweden). For the functional studies the mAb solutions were filtred by Millipore 0.22 μm. Part of mAbs was incubated with pepsin and the $F(ab')_2$ fragments were purified on DE32 cellulose column (Whatman,UK).

Immunocytochemistry

The cytospins of lymph or peripheral blood cells, stored at -70°C, were air dried, fixed in cold acetone for 1 min and incubated with normal goat serum (1/5) for 30 min and than with tested supernatants for 30 min. Next, the cytospins were incubated with goat anti-mouse IgG conjugated with biotin (Dako, Denmark) diluted 1/200 with 10% normal dog serum and for another 30 min with alkaline phosphatase(AP)-conjugated streptavidin (Dako) diluted 1/200. The AP substrate (Dako) was than applied for 15 min, and finally the slides were stained with Mayer's Hematoxylin. The staining was performed at room temperature (22°C) with TBS as washing agent.

Anti-human mAbs cross-reactive with canine DC

The following mAbs cross-reactive with canine leukocytes were used: anti- CD1a, -HLA DR (Dako), anti- CD54 (Immunotech) and anti-CD58 (Becton Dickinson).

Functional effects of mAbs on DC in vitro

Lymph dendritic cell-lymphocyte binding assay:
Binding of cells was quantitated after a 4 h incubation of lymph cells in lymph diluted 1:1 (vol/vol) with 0.15 M NaCl (control) or with appriopriate mAbs solutions at 37°C. Previous experiments revealed that lymph cell clustering is not dependent on the lymph cell concentration and that optimal conjugate formation takes place after 4h incubation. Dilution of lymph 1:1 was found necessary to avoid the inhibitory effect of whole lymph on cell binding. Briefly, lymph cell samples (0.1 ml) were centrifugated for 10 min at 250 g and cell pellets were mixed with 20 μl of appriopriate mAb dilution and after 30 min incubation at 4°C cells were supplemented with 0.1 ml of lymph and 0.08 ml of 0.15 M NaCl and incubated for additional 4h at 37°C. The number of lymph DC with two and more lymphocytes attached was counted per 100 of DC under the light microscope.

MLR proliferation:
The culture medium used consisted of RPMI 1640 medium supplemented with 10 mM HEPES buffer, 20% FCS, 100 U/ml penicillin and streptomycin, 2 mM L-glutamine (Gibco, Paisley, UK). Lymph cells were separated on a Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient at 500 g for 30 min. Responding cells were cultured at a density of 2×10^5 cells in 0.2 ml medium with or without 5% (10^4) allogeneic DC and appriopriate mAb dilutions for 6 days. Stimulatory cells were preincubated with mitomycin C (Sigma) used at a concentration of 40 μg/ml for 40 min at 37°C. Eighteen hours before harvest, cultures were pulsed with 0.4 μCi of (^{3}H)thymidine (Amersham, Bucks., UK) and harvested onto glass fibre filters. Incorporation of radioactive label was assessed in a scintilation counter.

Statistical evaluation
For statistical comparison of results the Student's *t* test was used.

RESULTS

Screening of hybridoma clones
Supernatants from two clones, designated DC-III/2 and DC-2, were found to react with lymph-derived DC, examined by immunocytochemistry. They also labeled lymph and peripheral blood granulocytes. The mAb DC-III/2 was determined to be of IgG1 and mAb DC-2 of IgG2a isotype. Control mAb VII/1, produced simultaneously and non-reactive with canine cells, was determined to be of IgG1 isotype.

Reactivity with lymph and peripheral blood cells
Whole lymph cells, DC-enriched population and peripheral blood lymphocytes and granulocytes were screened for DC-III/2 and DC-2 mAbs reactivity by immunocytochemistry. Both, DC-III/2 and DC-2 mAbs stained lymph DC population. Peripheral blood granulocytes were also labeled with both mAbs, but lyphocytes were unreactive.

Effect of mAbs on lymph DC-lymphocyte binding
Cell-cell binding assays are avaiable that are known to depend on integrin function. In our system autologous lymphocyte binding to lymph DC was blocked by anti-CD54 and -CD58 mAbs, but anti-DR mAb had no such activity (Table 1). Both novel mAbs and their F(ab') fragments significantly blocked lymph cell attachment,

as compared to a control VII/1 antibody (Table 2).

Table 1
The effect of cross-reactive with canine cells mAbs on in vitro lymph DC clustering with lymphocytes

mAb	Dilution	% of clusters
no mAb (0.15M NaCl)		22.6 ± 5.2
anti-HLA-DR	1/20	25.3 ± 8.4
anti-CD54	1/100	17.3 ± 6.6 *
anti-CD58	1/5	11.1 ± 2.9 *

All values are expressed as Mean ± SD, (* $p < 0.05$ vs control)

Table 2
The blocking effect of anti-DC mAbs on in vitro lymph DC clustering with lymphocytes
(% of clusters)

mAb	Whole molecules 1/2	Whole molecules 1/10	F(ab')2 fragments 1/2	F(ab')2 fragments 1/10
control	42.2 ± 9.8	38.6 ± 8.7	29.0 ± 1.0	32.0 ± 11.3
DC-2	15.6 ± 7.3	35.7 ± 4.1	14.3 ± 6.6 *	20.6 ± 7.1
DC-III/2	16.0 ± 8.2	35.5 ± 4.7	11.0 ± 5.3 *	18.7 ± 6.4

All values are expressed as Mean ± SD, (* $p < 0.05$ vs control)

Effect of mAbs on lymph DC allostimulatory activity in MLR

DC are characterized functionally by their potent allostimulatory activity. We observed the reducing effect of both novel mAbs, as well as anti-DR mAb on an allogeneic MLR (Table 3). Control antibodies, anti-CD1 and VII/1 had no such effect.

Table 3
Inhibition of the proliferative response in allo-MLR by mAbs (% of control MLR)

mAb	Dilution 1/5	Dilution 1/20
control VII/1	92.0 ± 7.0	124.5 ± 12.0
DC-2	22.5 ± 9.1 *	42.5 ± 27.5 *
DC-III/2	12.5 ± 4.7 *	59.2 ± 17.1 *
anti-HLA-DR	nd	42.0 ± 33.0 *
anti-CD1	nd	90.0 ± 21.0

All values are expressed as Mean ± SD, (* $p < 0.05$ vs control)

DISCUSSION

After skin transplantation DC leave the epidermis and undergo changes that include increased expression of MHC class II antigens (7). The migratory properties of DC likely interlace with their antigen presenting functions to sensitize T cells in situ. Pope et al. (9) showed that cutaneous DC and memory T lymphocytes migrate from human skin explants, and some of these cells form distinctive conjugates. Such spontaneous conjugates of DC with autologous lymphocytes are also present in normal afferent lymph (10), suggesting their contribution to skin immunologic reactions.

In the present report we describe two novel mAbs which recognize antigens expressed by the canine lymph derived DC and granulocytes.

Raised by us mAbs reduced lymphocyte binding to DC and T cell proliferative response to DC-associated alloantigens in the MLR. Both, whole antibody molecules as well as F(ab') fragments had the modulatory effect on lymph cell binding. It can be suggested that the suppressive effect of these mAbs might have been not due to the blocking of receptors for antigen recognition, but rather due to the blocking of the accessory molecules on DC. The epitopes recognized by our novel mAbs are unknown. Further immunochemical studies should be done, as well as functional studies of mAbs after their in vivo administration.

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Immune Events in Skin. I. Spontaneous Cluster Formation of Dendritic (Veiled) Cells and Lymphocytes from Skin Lymph

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To investigate the mechanism of spontaneous attachment of afferent lymph lymphocytes to dendritic cells, cells from canine skin lymph were used. There were 3.3 ± 2.8% of veiled cells in clusters found in lymph flowing from the cannulated lymph vessel. The number of clusters forming ex vivo in the collected lymph samples increased as a function of time and was temperature dependent. Incubation of cells with proteolytic enzymes or monosaccharides did not alter cell interactions. The ability of veiled cells to bind lymphocytes was independent of divalent cations but reduced by xylocaine and retinoic acid. Among steroids only methylprednisolone showed an inhibitory effect on cluster formation. Indomethacin and acetylsalicylic acid had no blocking activity on cell binding. Also, no effect was seen after treatment with cyclosporine A and azathioprine. An enhanced cluster formation after desialation with neuraminidase was observed. The desialated cells were cultured in order to study their stimulatory and accessory cell functions. No enhancement of autologous mixed leucocyte reaction was seen, but a significantly higher responsiveness to a suboptimal dose of phytohaemagglutinin was observed. The N-ase-mediated non-specific cell attachment could be abrogated by cell washing or treatment with EDTA or xylocaine.

This study indicates that cluster formation by skin lymph veiled cells and lymphocytes is a spontaneous process which cannot be controlled by means usually effective in regulating the in vitro induced clustering of antigen-stimulated cells.

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Skin tissue fluid and lymph contain migrating immune cells, including large dendritic (veiled) cells (DC) and lymphocytes (reviewed in Ref. 1). These cells are continuously transported in the lymph stream to the regional lymph nodes. In this way the information about the presence of foreign or self-unmasked antigens, acquired by the migrating cells in the interstitial space, can be transferred within minutes to the lymph node [2]. The processing of antigen by veiled cells (VC) and its presentation to lymphocytes seems to have already started by the time it reaches the skin parenchyma. A drop of lymph freshly drawn from a skin afferent lymphatic contains 3–6% of clusters formed by veiled cells and lymphocytes which most likely illustrates the in vivo cooperation of these populations [3, 4]. Spontaneous clustering of DC with autologous lymphocytes has been observed ex vivo in many species, in peripheral lymph of pigs [5], man [6] and rodents [7].

Clustering is the first phase of antigen presentation to lymphocytes. Dendritic cells are unique for T-dependent immune responses, which occur in cell aggregates or clusters (reviewed in Ref. 8). They bind lymphocytes in an antigen-independent pathway and can also bind resting T lymphocytes [9]. Little is known about the mechanism involved in the clustering of DC with resting T lymphocytes, including expression of adhesion molecules. The DC incorporate in vivo environmental antigens penetrating integuments

or shed autologous tissue cell antigens. This may prompt DC to undergo 'spontaneous' clustering with lymphocytes.

In this study we tried to elucidate the mechanisms of 'spontaneous' binding of veiled cells from the canine skin afferent lymph with autologous lymphocytes in their own environment, i.e. the lymph, and in the absence of a known antigen. Controlling this process may be helpful in mitigation of skin immune reactions.

MATERIALS AND METHODS

Reagents. The following reagents were used: EDTA (Sigma, St Louis, MO); Xylocaine-HCl (2%) (Astra, Sodertalje, Sweden); Trypsin (2.5%) (Flow Laboratories, Irvine, UK); Collagenase (type II) (Sigma); DN-ase I (type II) (Sigma); Neuraminidase (Cl. perfringens type X) (Sigma); RPMI-1640 medium (Gibco, Paisley, UK); L-glutamine (200 mM) (Flow Laboratories); Penicillin-stretptomycin solution (50 ×) (Flow Laboratories); Hepes buffer (1 M) (Flow Laboratories); fetal calf serum (FCS) (Gibco); phytohaemagglutinin (PHA) HA15 (Wellcome, Dartford, UK), [^{3}H]-thymidine (2 Ci/mM) (Amersham, Bucks., UK); Heparin (Novo Industri, Copenhagen, Denmark); sugars and Retinoic acid (Sigma); verapamil (Isoptin R) (Yugolek, Lubljana, Yugoslavia); dexamethasone (Sigma); hydrocortisone (Sigma); methylprednisolone (Solu-Medrol) (Upjohn, Kalamazoo, MI); cyclosporine A (Sandoz, Basel, Switzerland); Azathioprine (Wellcome); indomethacin (Sigma); acetylsalicylic acid (Sigma).

Dogs. Three outbred dogs with chronic lymphedema after the surgical interruption of afferent lymphatics were used as lymph donors [10].

Collection of lymph. Lymph was obtained by direct percutaneous puncture of dilated lymphatics and collected into plastic tubes with heparin (10 U/ml of lymph). The average concentration of lymph cells was $2.2 \pm 1.3 \times 10^6$/ml, and the per cent of cells with VC morphology was $3.2 + 1.7$. Of the whole population of VC $3.3 \pm 2.8\%$ have been found in clusters with lymphocytes immediately after collection of lymph.

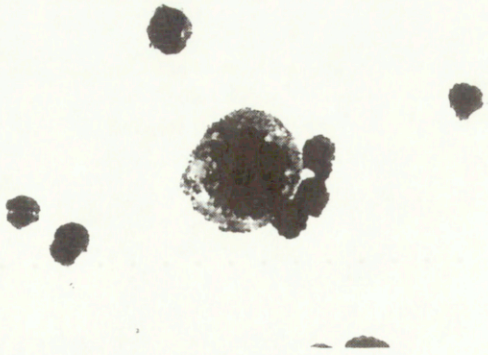

FIG. 1. Cluster isolated from afferent lymph, cytospun onto glass slide and stained with May-Grunwald-Giemsa. × 480.

Veiled cell lymphocyte binding assay. VC lymphocyte binding was quantitated either in lymph immediately after lymph collection or after incubation of lymph cells in lymph mixed 1:1 (vol/vol) with appropriate reagent solutions. The number of VC with two or more lymphocytes attached (Fig. 1) per 100 VC seen in several randomly selected fields was counted under the light microscope (magnification × 400). The period of cell incubation with applied agents was chosen depending on the peak of their effect in vitro.

Effect of temperature and medium on cell binding. Lymph cells (0.1 ml) mixed 1:1 with 0.15 M NaCl or autologous lymph were incubated for 4 h at 37 C in a tissue culture incubator, at 39 C and 22 C in water baths, and at 4 C in the refrigerator. After incubation cells were gently mixed and clusters were counted.

Treatment with EDTA and verapamil. Lymph cells were mixed with EDTA (10 mM and 20 mM) and incubated for 1 h or with verapamil solution (5×10^{-3} M dissolved in 0.15 M NaCl, used at concentrations of 10^{-4} and 10^{-5} M) and incubated for 4 h at 37 C.

Treatment with enzymes. Lymph cells were mixed with the following solutions of reagents prepared in 0.15 M NaCl to the final concentrations: trypsin (0.5 mg/ml and 1 mg/ml); collagenase (0.1 mg/ml and 0.5 mg/ml); DN-ase (20 μg/ml and 100 μg/ml); N-ase (1 U/ml and 2 U/ml). They were then incubated for 60 min at 37 C. In the case of neuraminidase some of the treated samples were washed five times with RPMI-1640 medium supplemented with 10% FCS, or after centrifugation cells were resuspended in EDTA (20 mM) or xylocaine (0.1%) solutions and incubated again for 30 min at 37 C.

Effect of xylocaine and retinoic acid. Lymph cells were mixed with xylocaine solutions (0.05% and 0.2%) in 0.15 M NaCl and incubated for 1 h, or with retinoic acid (dissolved in absolute ethanol to 10^{-2} M and mixed with 0.15 M NaCl to a concentration of 5×10^{-3} M and stored in the dark at 4 C) at final concentrations of 10^{-4} and 10^{-6} M and incubated for 4 h at 37 C.

Effect of monosaccharides and heparin. All sugars were stored frozen at −20 C at 200 mM in 0.15 M NaCl, with the exception of glucose-6-phosphate and mannose-6-phosphate (in H_2O). Heparin was stored at 500 μg/ml in 0.15 M NaCl. Lymph cells were mixed with the following reagents to the final concentrations: sugars, 100 mM (osmolarity 300 mOsm), heparin, 250 μg/ml. They were then incubated for 4 h at 37 C.

Effect of immunosuppressants and anti-inflammatory drugs. All steroid immunosuppressants (hydrocortisone, dexamethasone, methylprednisolone) were dissolved in ethanol to 10^{-2} M, and the stock solutions after mixing with 0.15 M NaCl were used at the final concentrations of 10^{-4} M and 10^{-6} M. Cyclosporine A and azathioprine were dissolved in ethanol to 5 mg/ml as stock solutions and after mixing with 0.15 M NaCl were used at final concentrations of 5 μg/ml and 0.5 μg/ml. Indomethacin was dissolved in ethanol to 10 mg/ml and after mixing with 0.15 M NaCl was used at final concentrations of 5 μg/ml and 1 μg/ml. Acetylsalicylic acid was dissolved in ethanol to 20 mg/ml and used at the final concentrations of 10 μg/ml and 5 μg/ml. Lymph cells were incubated for 4 h at 37 C.

Effect of N-ase-induced cluster formation on lymphocyte response. Lymph cells concentrated to 9×10^6 cells

in 0.15 M NaCl with 2 U ml of N-ase were incubated for 60 min at 37 C. Next, cells were washed four times with RPMI-1640 medium with 10% FCS and cultured in 0.2 ml of culture medium in a microculture plate at a density corresponding to 2×10^5 non-aggregated lymph cells, with or without three doses of PHA (90, 18 and 4.5 μg/ml). Culture medium consisted of RPMI-1640 medium supplemented with 10 mM Hepes buffer, 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cultures were performed in triplicate and incubated for 6 days (autologous mixed leucocyte reaction, AMLR) and 3 days (PHA) in a humidified atmosphere of 5% CO_2 in air. Twenty hours before termination of cultures 100 μl of medium were replaced by an equal volume of fresh culture medium and 0.4 μCi of [^{3}H]-thymidine was added. Radioactivity was measured on glass fibre filters in Permafluor cocktail (Packard, Zurich, Switzerland).

Statistics. To compare the results the Student's *t*-test was used.

RESULTS

Time, medium and temperature dependence of VC-lymphocyte binding

The interaction of lymph cells increased as a function of the time for which cells were incubated together, from 9.5% after 1 h to 20.7% after 4 h of incubation ($P < 0.05$) (Table I). After 20 h of incubation at 37 C the rate of clustering was unchanged (data not shown). Fig. 2 shows data on cluster formation after 4 h of incubation at different temperatures in 0.15 M NaCl or lymph. Clustering of cells suspended in NaCl was similar at 37 C and 22 C (20.7 ± 2.8% and 16.2 ± 3.3%, respectively) and significantly lower at 4 C and 39 C (12.1 ± 4.4% and 11.8 ± 3.8%, respectively). The percentage of clusters formed in the presence of lymph was reduced at temperatures from 4 C to 37 C ($P < 0.05$), as compared with clustering in NaCl. At 39 C the percentage of clusters both in NaCl and lymph was lower than at 37 C ($P < 0.05$).

Treatment with EDTA and verapamil

Treatment of lymph cells with EDTA and verapamil, a Ca^{2+} channel blocker, did not affect the VC-lymphocyte binding rate (Table I).

Treatment with enzymes

Table I shows the effect of enzyme treatment on the ability of lymph cells to interact. Cells subjected to proteolysis with trypsin or collagenase and hydrolysis with DN-ase for 1 h were not changed in their ability to interact. When cells were subjected to cleavage of terminal sialic acid residues with neuraminidase, increased cluster formation was seen. Washing of N-ase-treated cells with RPMI-1640 medium supplemented with 10% FCS five times reduced the per cent of clusters from 52.1 ± 19.5 to 24.5 ± 3.1. N-ase-induced cell binding was found to be dependent on divalent cations or cell membrane fluidity.

TABLE I. Effect of different agents on spontaneous binding of lymph lymphocytes by VC after incubation at 37 C

Time of incubation (h)[d]	Cell treatment[a, b]	% of clusters[c] (mean ± SD)
0	Directly from lymph	3.3 ± 2.8
1 h	NaCl (M)	
	0.15	9.5 ± 6.1#
	EDTA (mM)	
	20	16.5 ± 4.9
	10	15.0 ± 9.8
	xylocaine (%)	
	0.2	0.7 ± 0.5*
	0.05	3.7 ± 1.8
	trypsin (mg/ml)	
	1	11.6 ± 16.8
	0.5	13.8 ± 8.2
	collagenase (mg/ml)	
	0.5	6.5 ± 3.5
	0.1	12.0 ± 1.4
	DN-ase (μg/ml)	
	100	18.0 ± 12.2
	20	16.3 ± 11.0
	N-ase (U/ml)	
	2	69.0 ± 24.5*
	1	48.0 ± 30.5
4 h	NaCl (M)	
	0.15	20.7 ± 2.8#
	verapamil (M)	
	10^{-4}	17.0 ± 7.0
	10^{-5}	23.0 ± 1.4
	retinoic acid (M)	
	10^{-4}	10.0 ± 2.1*
	10^{-6}	13.0 ± 1.4*

[a] Lymph cell samples (0.1 ml) were mixed 1:1 with reagents diluted with 0.15 M NaCl, giving the different final concentrations.

[b] Xylocaine was diluted in 0.15 M NaCl; retinoic acid was dissolved in ethanol, so that the final concentration of solvent was 0.01% in 0.15 M NaCl.

[c] Data are the mean of duplicate determinants of three or more experiments ± SD.

[d] The time at which the peak of agent effect appeared was chosen.

# $P < 0.05$ 4 h versus 0 h and 4 h versus 1 h incubation.

* $P < 0.05$ versus NaCl.

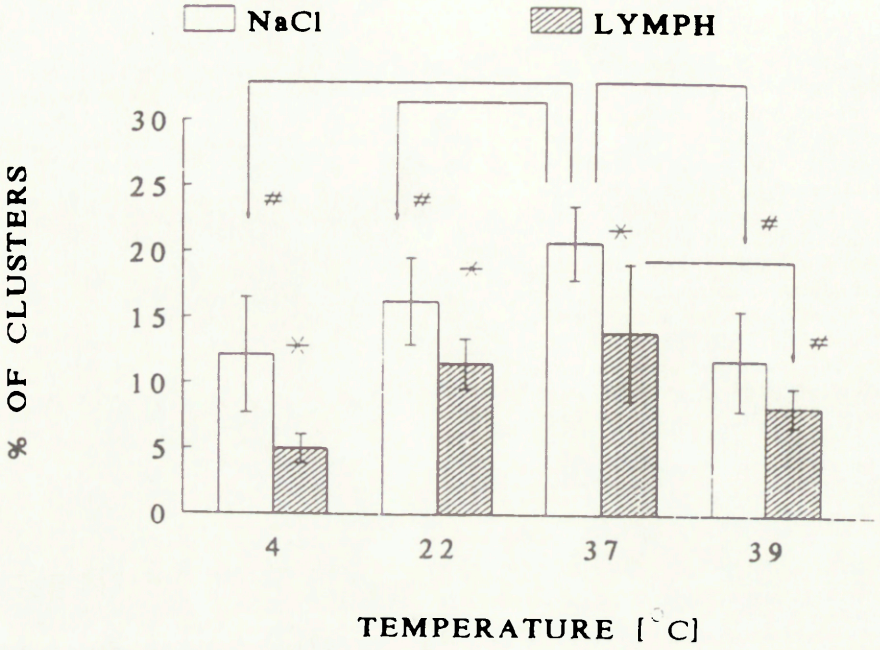

FIG. 2. Effect of temperature and medium on VC lymphocyte interaction after 4 h of incubation. Lymph cell samples (0.1 ml) were suspended in 0.1 ml of autologous lymph or 0.15 M NaCl. $^{\#}P < 0.05$ other temperature versus 37 C; $^{*}P < 0.05$ lymph versus NaCl.

Incubation of cells for 30 min in the presence of EDTA or xylocaine reduced the per cent of fresh clusters to the control level (Table II).

Effect of retinoic acid and xylocaine

Cell binding was significantly decreased in a dose-dependent manner when cells were treated with xylocaine and retinoic acid, drugs which influence cell membrane fluidity (Table I).

Effect of sugars and heparin

In order to investigate the involvement of carbohydrate structures in cluster formation the effect of the following sugars and heparin was studied: galactose, mannose, glucose-6-phosphate, mannose-6-phosphate, methyl-α-mannoside. Neither heparin nor sugars hindered the clustering of lymph cells (data not shown).

Effect of immunosuppressants and anti-inflammatory drugs

Only treatment with methylprednisolone resulted in a decrease in the per cent of clusters formed ($P < 0.05$) (Table III). Other steroids or immunosuppressants including cyclosporine A and azathioprine and anti-inflammatory drugs had no effect on lymph cell binding.

Effect of N-ase-induced clusters on lymphocyte responsiveness to PHA and in autologous MLR

As shown in Fig. 3, N-ase-treated lymph cells cultured for 3 days showed higher responsiveness of lymphocytes to a suboptimal dose of PHA than the control cells. However, the same level of lymphocyte stimulation was observed in a 6-day autologous MRL irrespective of N-ase pretreatment.

DISCUSSION

Our studies were undertaken to characterize the spontaneous, physical interactions between migrating skin lymph veiled cells and lymphocytes observed directly after lymph collection. Understanding of these interactions could widen our knowledge of the cooperation of veiled cells and lymphocytes in vivo.

Spontaneous binding of VC with lymphocytes proceeded ex vivo as a function of time for which cells were incubated together in the absence of any antigen, reaching a maximal level at 4 h. Cell

TABLE II. Effect of different procedures on VC-lymphocyte interaction after treatment with N-ase

	Treatment[a]	% of clusters (mean ± SD)
N-ase 2 U ml	Non-treated	52.1 ± 19.5
N-ase 2 U ml	Washed with RPMI	24.5 ± 3.1
N-ase 2 U ml	EDTA 20 mM	12.3 ± 4.0*
N-ase 2 U ml	Xylocaine 0.1%	10.5 ± 3.5*

[a] Lymph samples mixed 1 : 1 with 4 U ml of N-ase in 0.15 M NaCl were preincubated for 1 h at 37 C, then cells were washed five times with medium or incubated with EDTA or xylocaine for 30 min at 37 C.

* $P < 0.05$ versus washed cells.

TABLE III. Effect of immunosuppressants and anti-inflammatory drugs on lymph cell binding after 4 h of incubation at 37 C

Drugs[a]	% of clusters (mean ± SD)
Control (M NaCl)	
0.15	20.1 ± 3.5
Hydrocortisone (M)	
10^{-5}	21.1 ± 7.1
10^{-6}	23.5 ± 4.1
Dexamethasone (M)	
10^{-5}	18.0 ± 10.7
10^{-6}	18.5 ± 3.7
Methylprednisolone (M)	
10^{-5}	14.1 ± 5.5*
10^{-6}	13.5 ± 4.4*
Cyclosporine A (μg ml)	
5	19.3 ± 5.8
0.5	22.5 ± 5.5
Azathioprine (μg ml)	
5	20.3 ± 5.5
0.5	20.0 ± 4.0
Indomethacin (μg ml)	
5	16.3 ± 7.8
1	19.0 ± 7.5
Acetylsalicylic acid (μg ml)	
10	17.8 ± 4.8
5	23.0 ± 6.0

[a] All drugs were dissolved in ethanol, so that the final concentration of solvent was not higher than 0.001%.

* $P < 0.05$ versus control.

attachment occurred even at 4 C, and this may suggest the involvement of the CD2 and LFA-3 adhesion pathway in VC-lymphocyte binding, since the LFA-1-ICAM pathway functions only at 37 C [11]. It was found that sheep VC express a high level of LFA-3 and a small amount of CD2 [12]. Experiments performed at 39 C revealed reduced cell attachment in the presence of both lymph and NaCl. This could be explained as an effect of hyperthermia, since the canine skin temperature is 33-34 C (our unpublished observation). It is known that hyperthermia can affect cell adherence in the target-cytotoxic cell system in vitro. Lower cell binding was observed in the presence of lymph as compared with NaCl, after incubation at temperatures from 4 C to 37 C. It cannot be ruled out that some humoral factors present in lymph are involved, and further studies remain to be carried out.

The present study indicates that the spontaneous binding of lymphocytes to autologous VC is mediated by a divalent cation-independent pathway. Neither EDTA nor verapamil, a Ca^{2+} channel blocker, interfered with spontaneous cell binding in our system. In contrast, active cluster formation by canine blood DC with concanavalin A-stimulated lymphocytes in a 3-day culture [13] was blocked by verapamil. This may suggest that the spontaneous binding is instead a physical property of cell surface molecules.

Our data are consistent with the notion that treatment of DC and Langerhans cells with proteolytic enzymes does not alter their attachment functions [14], whereas the spontaneous binding of lymph node cells or thymocytes with peritoneal exudate macrophages [15] and attachment of non-primed lymphocytes to alveolar macrophages in vitro [16] are affected by trypsin.

We showed that the ability of VC to spontaneously bind lymphocyte was inhibited by treatment of cells with xylocaine, a drug which interferes with lymphocyte immune functions, by an affect on the cell membrane fluidity, cell adhesion or aggregation [17]. Retinoic acid also abolished VC-lymphocyte binding. This may explain the observed influence of retinoic acid on the efficiency of antigen presentation by spleen DC [18] and on the lymphocyte response to epidermal cells in MLR [19].

In several systems of cell-cell interaction [20, 21] carbohydrate structures have been identified as acceptor structures. Our studies showed that VC-lymphocyte binding is not a lectin-dependent process.

Glucocorticoids have been reported to exert immunosuppressive effects in vivo and in vitro on both lymphocyte and Langerhans cells from the epidermis [22-24]. We attempted to analyse the effect of clinically widely used corticosteroids on cluster formation. Only methylprednisolone used in both physiological and pharmacological doses caused a statistically significant decrease in the percentage of in vitro spontaneously formed

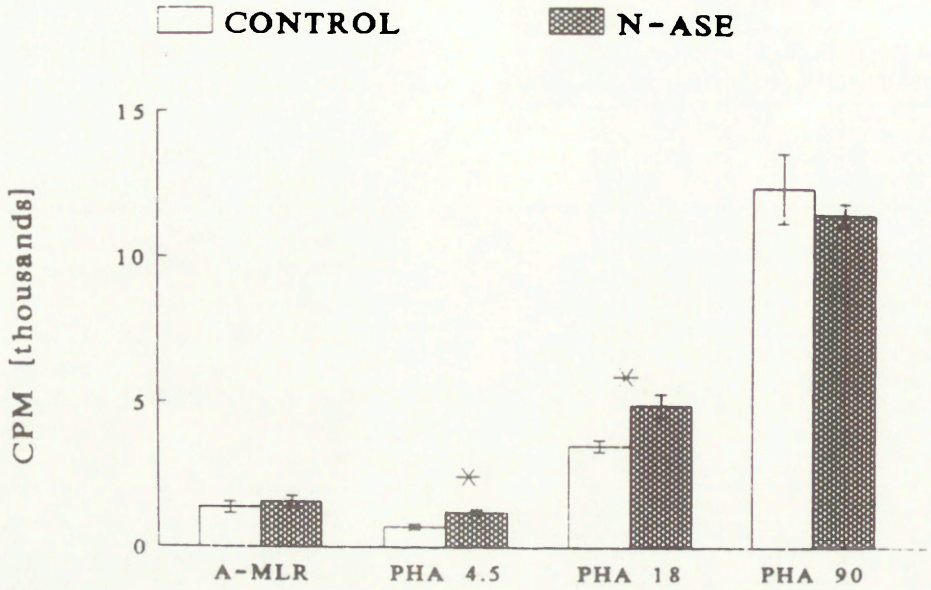

FIG. 3. Effect of N-ase treatment on stimulatory and accessory function of VC in culture. Two × 10^5 lymph cells treated or non-treated (control) with 2 U/ml of N-ase were cultured in a final volume of 0.2 ml alone (AMLR, 6 days) and with or without PHA (4.5; 18; 90 μg/ml, 3 days). Results are expressed as mean ± SD of three experiments. $^*P < 0.05$ versus control.

clusters of VC with autologous lymphocytes. This raises the possibility that other glucocorticosteroids may not be as potent as methylprednisolone, which affects the generation of cytotoxic lymphocytes in vitro by influencing the initial stage of the immune response [25].

The immunosuppressants cyclosporine A (CyA) and azathioprine, which also affect Langerhans cells in vivo and their accessory cell function [26] as well as VC functions [27, 28], did not prevent spontaneous cluster formation in our system. This is consistent with results of cluster formation by canine [13] and human [29] blood DC with lymphocytes; however, Furue & Katz [30] mention that cultured murine Langerhans cells pulsed with CyA do not form clusters with T cells. These discrepancies can be explained by the different experimental settings. We investigated the spontaneously forming clusters of cells freshly obtained from lymphatic and most likely primed in vivo, whereas in other reports cells were cultured and challenged with known antigens in vitro.

We found that the anti-inflammatory drugs indomethacin and acetylsalicylic acid had no effect on cluster formation.

Spontaneous binding of lymphocytes to VC in our system was enhanced by neuraminidase. It was reported that N-ase produces a variety of effects, among them enhanced cell interactions [31] and changed migration of lymphocytes [32]. However, no increase in attachment of lymphocytes to desialated unprimed macrophages has been observed [16, 33]. Enhancement by N-ase of VC-lymphocyte non-specific interactions did not affect the level of autologous MLR. Hirayama *et al.* [34] also did not observe an enhanced MLR when stimulatory DC or T cells were treated with N-ase. It is possible that N-ase modifies molecules other than Ia molecules which allow cell-to-cell contact. However, the expression of various functional epitopes of the adhesion molecules (CD2, LFA-3, LFA-1 and ICAM-1) on monocytes and T cells was not enhanced by N-ase [35].

Lack of enhanced response in AMLR after N-ase treatment in our experiments could indicate that the specific antigenic signal for clustering had been acquired in vivo and no additional response should be expected in culture, although more clusters were formed due to desialation. Furthermore, we found that the N-ase-mediated non-specific cell attachment could be abrogated by intensive washing of cells and by treatment with EDTA or xylocaine, which act on cell membranes. This clearly differentiates the mechanisms of spontaneous and N-ase-mediated clustering.

The reason for clustering of lymph cells in the skin tissue fluid in vivo remains unclear. A large array of environmental antigens (bacterial, viral, fungal) penetrate the skin following microtrauma. Self-antigens of dying tissue cells may also contribute to stimulation of DC. This is the subject of further studies. Interestingly, T cells that accumulate in afferent lymph draining the hind leg of sheep are all of memory phenotype [36].

We suggest that spontaneous binding of VC with autologous lymphocytes in skin lymph contributes to the distinctive capacity of skin Langerhans cells to activate memory/effector T cells in situ [37]. Controlling this process may be helpful in mitigation of skin immune reactions.

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Cytokines and Adherence Molecules Involved in Spontaneous Dendritic Cell-Lymphocyte Clustering in Skin Afferent Lymph

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The skin afferent lymph dendritic cell (DC) spontaneously forms clusters with autologous T cells. The role of adhesion molecules and cytokines in this process was investigated. Analysis of the expression of adhesion receptors on the canine peripheral lymph DC revealed the presence of CD54, CD58, CD18 as well as CD49d and CD49e molecules and cell surface fibronectin. The CD54 and CD58 molecules were found to play a key role in the 'spontaneous' lymph cell clustering. Antibody against fibronectin, a substrate for CD49d and CD49e receptors, reduced DC-lymphocyte binding. Analysis of the effect of cytokines revealed that the pro-inflammatory IL1β rather than IL1α, and TNFα may be responsible for the enhanced lymph cell *in vitro* clustering. The IL6 had no such augmenting effect. The enhancing effect of endogenous IL1β present in lymph was reduced by the IL1β neutralizing antibody. The effect of exogenously added IL1β was also limited by the IL1 receptor antagonist. The IL1Ra alone had no effect on cell binding, even when used in the high doses. Neutralizing of IL1Ra in lymph with the specific antibody brought about augmented cluster formation. The enhancing properties of TNFα on cell binding were reduced by the TNFα neutralizing antibody. The IL10 significantly limited lymph DC cluster formation with T cells. In conclusion, these data demonstrate that the present in lymph IL1β and TNFα may be responsible for the observed *in vitro* enhanced cluster formation of lymph DC with autologous T lymphocytes. Cell binding can be reduced by IL1Ra and by IL10. It provides insight into the potential clinical use of these inhibitors.

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INTRODUCTION

In vitro and *in vivo* studies have demonstrated that epidermal Langerhans cells (LC) form a unique subset of antigen-presenting dendritic cells (DC) [1]. These cells are continuously transported in the afferent lymph stream to the regional lymph nodes. The antigen-bearing LC can be found in the lymph nodes draining skin after epidermal application of contact sensitizers [2]. These cells migrate out from the skin grafts and enter the host afferent lymph vessels only a few hours after skin transplantation [3]. Freshly isolated LC process antigens and present them to lymphocytes *in vitro*. LC play a crucial role in the initiation of the immune responses to antigens penetrating skin from the environment or liberated from own ageing or damaged skin cells. The *in vivo* site and mode of interactions between antigen-bearing skin DC and T lymphocytes are not fully understood.

Skin lymph freshly drawn from the normal afferent lymphatics contains some dendritic cells in clusters formed spontaneously with lymphocytes [4]. This reflects the *in vivo* co-operation of the two cell populations. The molecular basis for the antigen-unspecific DC–T cell aggregation is still unknown. Epidermal DC incorporate, *in vivo*, the environmental antigens penetrating skin and this may prompt them to undergo such 'spontaneous' clustering with T lymphocytes. Factors governing this process are not clear, however the expression of accessory molecules on DC is thought to play a crucial role. The increased expression of adhesion molecules was observed during *in vitro* culture of epidermal DC [1,5]. Also, during transit to draining lymph nodes, LC are induced to increased expression of ICAM1 [6]. A question arises which cytokines (other than GM-CSF and TNFα) responsible for DC maturation [7], can regulate the process of DC–T cell cluster formation. Because the distinctive role

of DC is to initiate primary T cell response, modulating the early step of cell co-operation might help to regulate immune response. Skin is a rich source of cytokines which are primarily active locally in the microenvironment of cells in skin. Supernatants from highly enriched murine epidermal LC cultures contain bioactivities of IL1, IL6, GM-CSF and TNFα [8]. Furthermore, keratinocytes either produce constitutively, or can be induced to produce a variety of cytokines, among them IL1, IL6, IL8, IL10 and TNFα [9, 10]. In our previous studies IL1 as well as IL1 inhibitor were identified in normal human skin afferent lymph [11]. The question arises as to whether the cytokines present in afferent lymph can be responsible for 'spontaneous' cluster formation of lymph DC with autologous T lymphocytes, and which adhesion molecules participate in this process.

The purpose of this study was to investigate which cytokines, usually present in afferent lymph, enhance 'spontaneous' cluster formation and which adhesion molecules are active in lymph cell binding.

MATERIALS AND METHODS

Dogs. Outbred dogs with chronic lymphedema after surgical interruption of hind limb afferent lymphatics served as skin lymph donors [12].

Collection of lymph. Lymph was obtained by direct percutaneous puncture of dilated lymphatic and collected into plastic tubes with heparin solution (10 U/ml).The average lymph cell concentration was $4.4 \pm 3.7 \times 10^6$/ml.

Reagents. The following reagents were used: Heparin (Novo Industri, Copenhagen, Denmark); recombinant human IL1α, IL1β, IL6, IL10, TNFα (Genzyme, Cambridge, MA, USA); polyclonal rabbit anti-IL1β, anti-IL6 and anti-TNFα antibodies (Genzyme); recombinant human IL1Ra (receptor antagonist) and anti-IL1Ra neutralizing antibody (R&D Systems, Minneapolis, MN, USA); anti-human antibodies (Abs): anti-CD1a, -CD18, -DR (Dakopatts, Denmark), anti- CD54, -CD49 d,e,f (Imunotech, France) and anti- CD58 (Becton Dickinson, San Jose, CA, USA); polyclonal rabbit antibodies anti-fibronectin (FBN) and anti-S100 protein (Dakopatts); pentoxifylline (Hoechst, Milan, Italy). The doses of reagents were chosen depending on the peak of their effect. Monoclonal antibodies against canine CD4 and CD8 antigens were rat IgG, a kind gift of Dr S. Cobbold, Cambridge, UK.

Lymph dendritic cell-lymphocyte binding assay. Binding of cells was quantitated after incubation of lymph cells in lymph diluted 1 : 1 (vol/vol) with 0.15 M NaCl (control) and with appriopriate reagent solutions at 37°C. Previous experiments revealed that lymph cell clustering is not dependent on the lymph cell concentration and that optimal conjugate formation takes place after 4 h incubation [4]. Dilution of lymph 1 : 1 was found necessary to avoid the inhibitory effect of whole lymph on cell binding [4]. Figure 1 presents lymph DC and the cell cluster freshly isolated from afferent lymph, visualized in phase contrast microscopy. The number of lymph DC with two and more lymphocytes attached was counted per 100 of DC under the light microscope. For blocking cell surface antigens with antibodies, lymph cell samples were centrifugated for 10 min at 250 *g* and cell pellets were mixed with 20 μl of appriopriate antibody dilution. After 30 min incubation at 4°C cells were supplemented with 0.1 ml of lymph and 0.08 ml of 0.15 M NaCl and incubated for an additional 4h at 37°C.

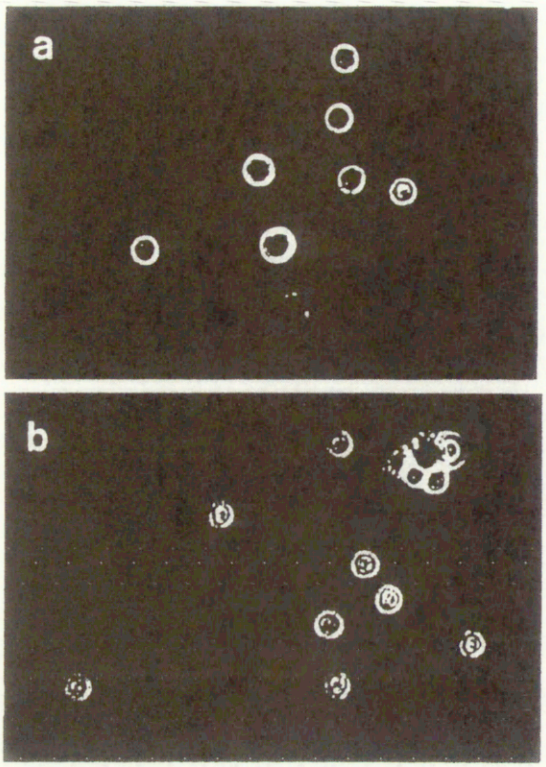

Fig. 1. Dendritic cell (DC) (a) freshly isolated from afferent lymph and (b) cluster of DC with lymphocytes (phase-contrast microscopy, ×500).

Labelling of lymph cells with antibodies. The cytospins of lymph cells were fixed in cold acetone for 1 min. For staining with mouse monoclonal Abs cytospins were incubated with normal goat serum (1/5) for 30 min and then with monoclonal Abs for 30 min. Next, the cytospins were incubated for 30 min with goat anti-mouse IgG (Z 420 Dako) diluted 1/100 with 10% normal dog serum and for another 30 min with APAAP (alkaline phosphatase anti-alkaline phosphatase D 651 Dako) diluted 1/50. The AP substrate was then applied for 15 min, and finally the slides were stained with Mayer's hematoxylin. The staining was performed at room temperature (22°C) with TBS as washing agent. For staining with polyclonal rabbit Abs the cytospins were incubated with normal swine serum and after incubation with Abs the swine anti-rabbit IgG (E 353 Dako) diluted 1/300 with 10% normal dog serum was used. Next, the AP- streptavidin (D 396 Dako) diluted 1/800 and AP substrate were applied. The staining with rat anti-dog Abs was carried out by flow cytometry analysis. Cells (10^6) were incubated with 50 μl of 10% normal rabbit serum for 20 min at room temperature (22°C) and then with 50 μl anti-dog Abs for 1h on ice. Next, cells were washed twice and then incubated for 1h on ice with FITC conjugated rabbit anti-rat IgG (F 234 Dako) diluted 1/20 with 10% normal dog serum. After cell washing FACS analysis was performed in a FACStar sorter (Becton-Dickinson).

Table 1. Reactivity of anti-human Abs cross-reactive with the canine peripheral lymph DC (% of positive DC)

Antigen	Antibody	Reactivity with DC
CD54 (ICAM1)	84 H 10	37% + +[a]
CD58 (LFA3)	L 306.4	50% + +
CD18 (LFA1ß)	MHM 23	80% + + +
CD49d (VLA4α)	HP 2/1	95% + + +
CD49e (VLA5α)	SAM 1	80% + + +
CD49f (VLA6α)	Go H3	70% + +
CD1a	NA 1/34	60% + +
HLA DR	TAL 1B5	100% + + +
Fibronectin	A 245	100% + + +
S 100 protein	Z 311	100% + + +

[a] Strong reactivity (+ + +), intermediate (+ +), weak (+).

Statistical evaluation. For statistical comparison of results the Student's *t*-test was used.

RESULTS

Lymph cell populations

The per cent of cells with DC morphology was 6.5 ± 2.6 and of lymphocytes 82.7 ± 9.2. The lymphocyte population was composed of 58.4 ± 2.5% $CD4^+$ and 8.7 ± 4.6% $CD8^+$ T cells. Eosinophils and macrophages comprised 10.7 ± 6.7% of cell population.

Effect of Abs against adherence molecules on lymph cell clustering

The detailed phenotypic profile of canine afferent lymph DC stained with anti-human Abs is showed in Table 1. Spontaneous cell attachment in afferent lymph was significantly reduced by anti-human cross-reactive with dog monoclonal antibodies against CD54(ICAM1) and CD58(LFA3) (17.3 ± 6.6% and 11.1 ± 2.9% versus 22.6 ± 5.2, respectively, $P < 0.001$) (Table 2). In contrast, moAbs against CD18 and CD49 (VLA4,VLA5,VLA6) had the pro-aggregatory properties. Higher concentrations of these moAbs (1/10–1/1000 for CD18 and 1/10–1/200 for CD49) induced the homotypic cell aggregation (data not shown). Other control moAbs, against CD1a and DR, had no effect on cluster formation. Polyclonal Abs against fibronectin, in contrast to the control Abs anti-protein S100, was inhibitory for lymph cell binding (14.5 ± 1.9% and 21.7 ± 4.6% versus 22.6 ± 5.2%, respectively) (Table 2).

Effect of cytokines on lymph cell clustering

Exogenous IL1α, IL6 and IL1Ra applied separately, even in high doses, had no significant effect on dendritic cell-lymphocyte *in vitro* attachment (Table 3). When exogenous

Table 2. The effect of blocking of cell surface molecules with antibodies on *in vitro* lymph cell clustering

Antibodies reactive with[a]	Dilution	% of clusters (mean ± SD)[b]	*P*[*]
control	—	22.6 ± 5.2	
CD54	1/100	17.3 ± 6.6	< 0.012
CD58	1/5	11.1 ± 2.9	< 0.001
CD1a	1/5	23.2 ± 4.7	
HLA DR	1/20	25.3 ± 8.4	
CD18	1/2000	30.0 ± 3.7	< 0.008
CD49d	1/2000	30.5 ± 3.1	< 0.005
CD49e	1/500	33.2 ± 8.6	< 0.001
CD49f	1/500	29.7 ± 4.7	< 0.011
Fibronectin	1/2	14.5 ± 1.9	< 0.001
Protein S-100	1/2	21.7 ± 4.6	

[a] Lymph cell samples (0.1 ml) were centrifugated and pellets were incubated with 20 µl of antibody dilutions for 30 min at 4°C. Cells, thereafter, were supplemented with 0.1 ml of lymph and 0.1 ml of 0.15 M NaCl and incubated for 4 h at 37°C.

[b] Data are the mean of duplicate determinants of three or more experiments ± SD. Different dilutions of used antibodies were studied and these giving the effect are presented.

[*] *P* versus control.

Table 3. Effect of exogenous cytokines on spontaneous *in vitro* binding of lymphocytes by dendritic cells from skin peripheral lymph

Reagents[a]	Concentration	% of clusters[b]	
lymph	50% in 0.15 M NaCl	22.3 ± 4.3	
+ IL1α (pg/ml)	1000	22.5 ± 2.0	
	500	23.1 ± 9.0	
	50	18.5 ± 6.3	
+ IL1Ra (ng/ml)	400	25.8 ± 9.5	
	100	23.5 ± 10.2	
+ IL6 (ng/ml)	200	22.6 ± 5.2	
	50	28.7 ± 9.2	
	25	23.7 ± 3.5	
+ IL1ß (pg/ml)	100	30.2 ± 8.1	*$P < 0.03$
	50	22.2 ± 4.3	
+ IL1ß + IL1Ra	100 + 400	22.3 ± 6.2	†$P < 0.05$

[a] Lymph cell samples (0.1 ml) suspended in lymph were diluted 1 : 1 with 0.15 M NaCl (control) and mixed with 0.1 ml of reagents diluted with 0.15 M NaCl, then incubated for 4 h at 37°C.

[b] Data are the mean of duplicate determinants of three or more experiments.

[*] *P* versus control with 50% lymph.

† *P* versus IL1ß.

Table 4. Effect of endogenous IL1β and IL1Ra on *in vitro* peripheral lymph dendritic cell cluster formation with autologous lymphocytes

Reagents[a]	Concentration	% of clusters (mean ± SD)[b]	
lymph	50% in 0.15 M NaCl	29.6 ± 5.8	
+ anti-IL1β (μg/ml)	100	22.5 ± 4.4	*$P < 0.02$
	50	24.6 ± 7.9	
+ anti-IL1Ra (μg/ml)	10	42.8 ± 9.0	*$P < 0.003$
	2	33.8 ± 6.9	

[a] Lymph cell samples (0.1 ml) suspended in lymph were mixed 1 : 1 with reagents diluted with 0.15 M NaCl and incubated for 4 h at 37°C.

[b] Data are the mean of duplicate determinants of three or more experiments ± SD.

* P versus control with 50% lymph.

IL1β was added to the system, a significantly enhanced cell clustering was seen (30.2 ± 8.1% versus 22.3 ± 4.3%, $P < 0.03$). This effect was abrogated by treatment with IL1Ra, a competitive inhibitor of IL1 receptor (30.2 ± 8.1% versus 22.3 ± 6.2%, $P < 0.05$) (Table 3). The enhancing effect of endogenous IL1β in lymph was abrogated by adding of anti-IL1β antibody (22.5 ± 4.4% versus 29.6 ± 5.8%, $P < 0.02$) (Table 4). Binding of lymph cells was also enhanced by treatment with anti-IL1Ra antibody neutralizing the endogenous lymph IL1Ra (42.8 ± 9.0% versus 29.6 ± 5.8%, $P < 0.003$) (Table 4). As shown in Table 5, lymph cell attachment was significantly increased in the presence of exogenous TNFα (46.5 ± 6.0% versus 30.9 ± 7.1%, $P < 0.001$). The enhancing effect of endogenous TNFα in lymph was abrogated by anti-TNFα antibody (21.5 ± 6.4% versus 30.9 ± 7.1%, $P < 0.03$). In order to investigate the effect of endogenously produced TNFα, the pentoxifylline, an inhibitor of TNF formation, was used. The inhibitory effect of pentoxifylline on lymph cell clustering was dose-dependent (Table 5). Exogenous IL10 significantly reduced lymph cell binding from 30.9 ± 7.1% to 22.2 ± 3.8% ($P < 0.005$) (Table 5).

DISCUSSION

Dendritic cells play a dominant role in autologous and allogeneic mixed leucocyte reaction (MLR) [13, 14]. Cluster formation of DC with T lymphocytes represents an essential, but insufficient, step in T cell activation. In the induction stage of cell contact, naive T cells bind to DC by an antigen-independent mechanism [4, 15], but only specific T cells can be fully activated. In our present study we have explored the molecular basis of autologous T cell adhesion to DC observed in the peripheral lymph [4] and the possible role of cytokines and adhesion molecules in this process. The physical contact of DC with lymphocytes depends on engagement of adhesion

Table 5. Effect of TNFα on *in vitro* lymph cell cluster formation

Reagents[a]		Concentration	% of clusters[b]	P*
lymph		50% in 0.15 M NaCl	30.9 ± 7.1	
+ TNFα	(ng/ml)	5.0	46.5 ± 6.0	< 0.001
		2.5	34.6 ± 8.1	
+ anti-TNFα	(μl/ml)	50	21.5 ± 6.4	< 0.03
		10	31.3 ± 5.3	
+ Pentoxifylline	(μg/ml)	500	10.8 ± 4.9	< 0.001
		100	21.2 ± 5.8	< 0.001
		10	21.7 ± 3.0	< 0.003
+ IL10	(ng/ml)	100	22.2 ± 3.8	< 0.005
		50	26.5 ± 9.5	

[a] Lymph cell samples (0.1 ml) suspended in lymph were mixed 1 : 1 with 0.1 ml of reagents diluted with 0.15 M NaCl and incubated for 4 h at 37°C.

[b] Data are the mean of duplicate determinants of three or more experiments.

* P versus control with 50% lymph.

molecules. Analysis of the expression of adhesion receptors on the canine peripheral lymph DC revealed that these cells express CD54(ICAM1), CD58(LFA3) and CD18(LFA1β), as well as CD49d and CD49e (VLA4α, VLA5α). Moreover, expression of cell surface fibronectin was detected. The CD54 and CD58 molecules were found to play a key role in the 'spontaneous' binding of lymph cells, since antibodies directed against them significantly reduced *in vitro* cluster formation. Blocking effect of the ICAM1 antibody on cluster formation was observed for human blood DC in an allogeneic MLR [16] but not for the tonsil DC [17]. Our findings that T cell clustering with lymph DC is dependent on the CD58 molecule remain in contrast to the situation observed with blood DC [16]. There are controversial reports concerning tonsil DC [17,18]. An inhibitory effect of anti-ICAM1 antibody was observed in allogeneic MLR with tonsil DC [17] and of anti-LFA3 antibody in primary proliferative response of cultured Langerhans cells [19]. Antibodies against ICAM1 and LFA3 reduced T cell proliferation in MLR with blood DC [20]. It may result from different incubation time and concentration of antibodies. It is also possible that different antibodies may recognize different epitopes of a certain molecule and therefore may not be sufficient to influence cell cluster formation.

In case of anti-LFA1β (CD18) antibody its pro-aggregatory activity was seen. Our results confirm data reported by Xu *et al.* [20], but do not concert with the findings of Scheeren *et al.* [16]. This may result from different testing conditions. It is also possible that Abs applied may have recognized different epitopes of the CD18 molecule. We also observed the enhanced lymph cell clustering in the presence of anti-VLA4α, -VLA5α and -VLA6α antibodies. Such adhesion promoting activity has been demonstrated for CD44 [16] and CD45 [20] in case of blood DC. Koopman *et al.* [21] observed stimulation of homotypic T cell adhesion by two of four used anti-CD44 antibodies. This stimulatory activity did not represent a dose-dependent problem due to a low CD44 molecule expression or to a low binding affinity. The induction of other adhesion pathways (LFA1-ICAM1 and CD2-LFA3) by triggering of CD44 receptor was postulated. Our results concerning the pro-aggregatory activity of anti-CD18 and -CD49 (d,e,f) moAbs also seem to be not related to the amount of antibody. Other control Abs, anti- CD1a, -S 100 protein or HLA DR had no effect on lymph cell cluster formation. Anti-DR antibodies are unable to disrupt cluster formation in a 4 h assay [16], however, they reveal strong inhibition of MLR. Fibronectin is a multifunctional molecule and can act as the substrate for cell adhesion by VLA4 and VLA5 receptors [22]. Antibodies against fibronectin are more effective in inhibition of T cell adhesion to FBN than the anti-VLA5 Abs [23]. In our study a reduced binding of lymph DC with T lymphocytes was observed after treatment with anti-FBN Abs. It remains in agreement with observations of Klingemann *et al.* [24] on the inhibitory effect of anti-FBN antiserum on canine blood DC-lymphocyte cluster formation.

Analysis of the effect of cytokines, which are constitutively present in skin afferent lymph, revealed that the pro-inflammatory IL1 and TNFα can be responsible for 'spontaneous' cell clustering. The IL6 had no such effect on cluster formation. Our finding concerning IL1 supports the notions that IL1 could enhance clustering of mouse DC with T cells in MLR [25] and that IL1 administered *in vivo* was found to be important in the initiation of primary immune response in skin [26]. Our data demonstrate that IL1β rather than IL1α is responsible for the enhanced cluster formation of autologous DC and T cells. Effect of IL1β was reduced by the IL1β neutralizing antibody and by the IL1 receptor antagonist. IL1Ra alone had no effect on cluster formation in lymph but administered together with exogenous IL1β blocked the enhancing effect of IL1β on cell attachment. The presence of IL1Ra in lymph could be responsible for the lower cluster formation in whole lymph observed by us, compared with lymph diluted to 50% of normal concentration [4]. Neutralizing of IL1Ra in lymph with the antibody brought about the augumented cluster formation. This indicates that IL1Ra, which is a competitive inhibitor of the IL1β receptor [27], can prevent activation of cells by IL1β even in the induction phase of cell co-operation. Collectively, these results argue for a critical role of IL1β and its inhibitor in DC-induced primary T cell response. Therefore, IL1Ra may be therapeutically useful in treatment of skin diseases. Administered *in vivo* IL1Ra was able to reduce the severity of several inflammatory diseases in animals and humans [28–30].

Our data also indicate that human TNFα has properties enhancing *in vitro* cell custering and this effect is reduced by treatment with the neutralizing anti-TNFα antibody. Koide *et al.* [25] observed little effect of human TNF on murine cell clustering in the MLR. Murine TNFα, which maintans the viability of murine Langerhans cells *in vitro*, did not induce the functional maturation of these cells and cluster formation in the MLR [31]. These discrepancies can be explained by differences observed between the murine and human TNF *in vitro* and *in vivo* activities [32]. Pentoxifylline has recently been shown to modulate TNFα *in vitro* and *in vivo* production [33, 34]. In our system the inhibitory effect of pentoxifylline on lymph cell cluster formation was dose-dependent. These results also argue for a modulatory role of TNFα in DC clustering with T cells.

Interestingly, IL10 can act in the early step of DC- T cell co-operation, on cell cluster formation. IL10 was reported to inhibit the primary allogenic T cell response to mouse [35] and human [36] LC *in vitro*. Recently, Wang *et al.* [37] showed that the presence of localized IL10 secretion at the site of the allogeneic tumor cell challenge resulted in a marked inhibition of CTL generation *in vivo*. *In vitro* inhibition of allor-eactivity by IL10 was observed using both spleen-derived DC and macrophages, which shows the potential clinical use of this cytokine.

A question remains regarding the nature of changes induced in DC by cytokines. It is known that cytokines can

influence expression of adhesion molecules in LC culture. The increased cell adhesion could be due to: (1) up-regulation of previously exsisting molecules; (2) induction of new molecules; or (3) changes in allinity of receptors associated with altered receptor density, epitope expression or membrane mobility. We do not demonstrate changes in the expression or the affinity of adhesion molecules in our 4 h assay, but such changes might be expected to occur. Recent studies showed that expression of several co-stimulatory molecules on LC undergoes regulation by cytokines in culture. ICAM1 expression on mouse LC was up-regulated by IL1 and down-regulated by IL10 after 72 h culture [38]. TNFα was able to augment the expression of B7 [39], which is a critical accessory molecule, but seems not to play a pivotal role in LC-T cell clustering [40]. Mechanism of IL10 action on DC function is not yet clear. Peguet-Navarro *et al.* [36] suggested that the suppressive effect of IL10 was not related to the expression of ICAM1 and LFA3 antigens on human LC. Enk *et al.* [35] showed that the inhibitory effect of IL10 on mouse LC was observed when fresh but not cultured LC were used, and this effect was not mediated by MHC class II expression. It suggests that IL10 can influence other unknown molecule(s) or can change the affinity of existing molecules.

In conclusion, our data demonstrate that ICAM1 and LFA3 dependent pathways of cell contacts are associated with 'spontaneous' lymph cell clustering and that the presence of pro-inflammatory cytokines as IL1β and TNFα in peripheral lymph may be responsible for enhancement of this process. IL1Ra and IL10 appear to be the important factors for the inhibition of the early stage of skin immune cell cooperation. Whether the cytokines regulate cluster formation through modulation of lymph DC adhesion molecule will be investigated in our further studies in the future.

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ELSEVIER

Skin Allografts—Host Cutaneous Veiled Cells Initiate Rejection Reaction by Indirect Pathway of Allorecognition

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T-CELL recognition of alloantigen is a central and primary event initiating allograft rejection, which leads to the destruction of engrafted tissues. Skin allografts are acutely rejected in large animals despite intensive immunosuppressive therapy. Skin migrating immune and resident cells as dermal dendritic cells (DC), lymph veiled cells (VC), epidermal Langerhans cells (LC), lymphocytes (LY), and endothelial cells as well as keratinocytes may play a role. It is generally accepted that there are two pathways of allorecognition. In the "direct" pathway host T cells interact with intact alloantigens on the surface of donor cells. In the "indirect" pathway host T cells recognize processed alloantigen presented by self-antigen presenting cells (APC). It has been suggested that acute skin allograft rejection is predominantly mediated by the direct pathway because the graft contains a significant number of LC and dermal DC. These cells present antigen and provide co-stimulatory signals to T cells. There is also increasing evidence for a significant role of the indirect pathway in alloresponse.[1,2] The complexity of cellular interactions in skin allograft rejection prompted us to find a model where the role of recipient DC in initiation of the rejection process could be studied.

We have used a canine skin to severe combined immunodeficient (SCID) mouse transplant model. Two weeks after skin grafting, when donor LC had migrated out from the transplant,[3] canine peripheral lymph VC and LY or peripheral blood mononuclear cells (PBMC) allogeneic to the graft were injected intraperitoneally, and the rejection reaction was followed. Results of our studies suggest that allogeneic VC promote recruitment of T cells to skin allograft and subsequently graft rejection.

MATERIALS AND METHODS

Mice

FOX CHASE C.B.-17 SCID mice were obtained from IFFA CREDO (L'Arbresle, Cedex, France) and bred at Medical Research Center (Warsaw, Poland) in a pathogen-free environment. All animals used were 6-weeks old and had less than 5 mg/L of serum IgG by radial immunodiffusion assay (Nanorid, The Binding Site, Birmingham, UK). Each mouse was screened for the absence of CD3− peripheral blood T lymphocytes by flow cytometry with rat anti-mouse-CD3/PE monoclonal antibody (Ab) (Serotec LTD, Kidlington, England).

Dogs

Normal mongrel dogs (n = 3) and three dogs with lymphedema following surgical interruption of lower leg lymphatics served as donors of skin grafts and cells from peripheral lymph and blood. The average concentration of lymph cells was 2.2 ± 1.3 × 10^6 mL; the percent of cells with DC morphology was 3.2 ± 1.7. Peripheral lymph cells consisted of about 80% CD3+ T lymphocytes.

Skin Grafting

Canine skin sheets obtained from the groin under general anesthesia were cut into 10/15-mm pieces and stored in cold 0.9% NaCl until engraftment. SCID mice were anesthetized by intramuscular injection of ketamine and intraperitoneal injection of 3.6% chloral hydrate. The skin graft was placed on the dorsum. The dressing was removed after 10 days.

Canine Leukocyte Isolation and Inoculation

PBMC were isolated on Lymphoprep (Nycomed, Oslo, Norway) gradient as described previously[4] and suspended in 0.9% NaCl for injection. Peripheral lymph cells were obtained from lymph collected by percutaneous puncture of dilated lymphatics and centrifugation. Pure (100%) population of lymph LY and enriched (over 50%) population of lymph VC were obtained on 12% metrizamide gradient (Nycomed), as described previously.[5] To neutralize host natural killer cells and optimize the engraftment of canine cells, mice were injected with 10 μL IV of anti-asialo-GM-1 (Wako Chemicals Richmond, Va) in a volume of 0.2 mL saline, 24 hours before intraperitoneal cell inoculation.

Experimental Setting

Experiments were carried out in four groups. In group 1 (n = 9) 2 × 10^7 canine allogeneic whole lymph cells, in group 2 (n = 3) lymph LY-enriched population, in group 3 (n = 3) lymph VC-enriched population, and in group 4 (n = 3) PBMC were injected intraperitoneally into recipients of skin grafts 2 weeks after transplantation. In the control group (n = 3), canine syngeneic whole lymph cell population was given.

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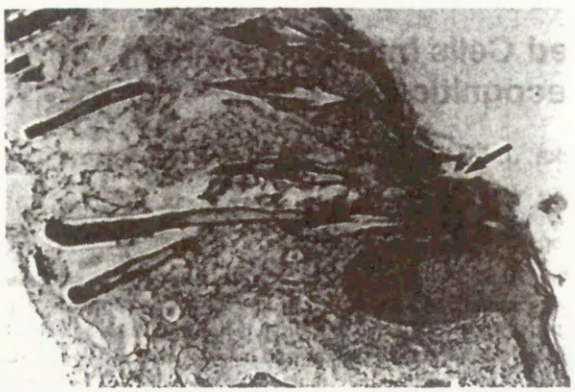

Fig 1. Histologic appearence of canine skin graft to SCID mouse harvested 3 weeks after engraftment and stained with H&E. Arrow points to the border between canine (thick) and mouse (thin) epidermis (×100).

Histology and Immunohistochemistry

Skin grafts were harvested 7 days after cell inoculation. One half of the skin specimen was snap frozen in dry ice-cold acetone and stored at −70°C until use for cryostat sections. The other half was formalin fixed, paraffin embedded, and stained with hematoxylin and eosin. CD3+ canine T-cell infiltrates were identified with antihuman CD3 polyclonal Ab (Dako, Denmark) diluted 1/100. Veiled cells were visualized with specific monoclonal Ab DCIII/2 raised in our laboratory.[5] Cryostat sections were blocked for 20 minutes in 50% normal swine serum before incubation with primary Ab (omitted in controls). Positive cells were detected with LSAB2-alkaline phosphatase complex and fast red according to the manufacturer's instructions (Dako).

RESULTS

Histopatologic Characterization of Canine Grafts Placed on SCID Mice

Initial studies revealed that canine skin grafts placed on SCID C.B.-17 mice healed in 2 weeks. Full thickness canine epidermis was clearly distinguishable from thin mouse epidermis (Fig 1). No infiltrates were observed in the bed and border of the graft 1 month after transplantation (Fig 2).

Histopatologic Characterization of Canine Skin Grafts Placed on SCID Mice Inoculated With Allogeneic Canine Leukocytes

In group 1, transfer of whole lymph cell population (LY and VC) caused after 7 days mononuclear cell and granulocyte infiltration (+−) in graft's bed. Some infiltrating cells at the border of canine and mouse papillary dermis (−), and at the epidermodermal junction (−) were seen (Fig 3a). No evident destruction of epidermis was observed, however, there were foci of acanthosis. Minor mobilization of mononuclear cells and granulocytes (−) was seen around the graft venules.

In group 2, allogeneic LY accumulated together with some granulocytes in graft's bed (+) and at the epidermodermal junction (+). Epidermis remained intact (Fig 3b).

Fig 2. Higher magnification (×200) of graft shown on Fig 1 depicting intact canine dermal-epidermal junction and lack of infiltrates in the dermis.

In group 3, transfer of VC-enriched population resulted in intense mononuclear cell and granulocyte infiltrates in dermis (++) and around microvessels (+++). Diffuse infiltrates of mononuclear cells and granulocytes were observed at the epidermo-dermal border (+++) (Fig 3c). Extensive necrosis of epidermis in the midportion of the graft was seen. On immunohistochemical pictures (data not presented), canine veiled cells (DC-III/2+) were seen in canine dermis but not epidermis. Graft infiltrating cells were predominantly canine CD3+ cells, however, there were also many mouse granulocytes.

In group 4, skin grafts in mice inoculated with PBMC were slightly infiltrated by mononuclear cells (+/−). There were no perivascular cell accumulation and epidermis looked normal (Fig 3d).

In the control group, after injection of syngeneic whole lymph cells, the graft remained entirely normal (data not presented).

DISCUSSION

SCID mice are permissive for the growth of xenogeneic lymphoid cells[6,7] and tissues.[8] Murray et al[9] developed a chimeric human/SCID mouse model to study human skin allograft rejection. They allowed the skin graft to heal prior to intraperitoneal infusion of allogeneic PBMC in a large number (3×10^8 cells) and observed accumulation of dermal T cells expressing CD25 marker and destruction of dermal microvasculature. The changes in dermal microvessels were independent of human B cells and antibodies.[10]

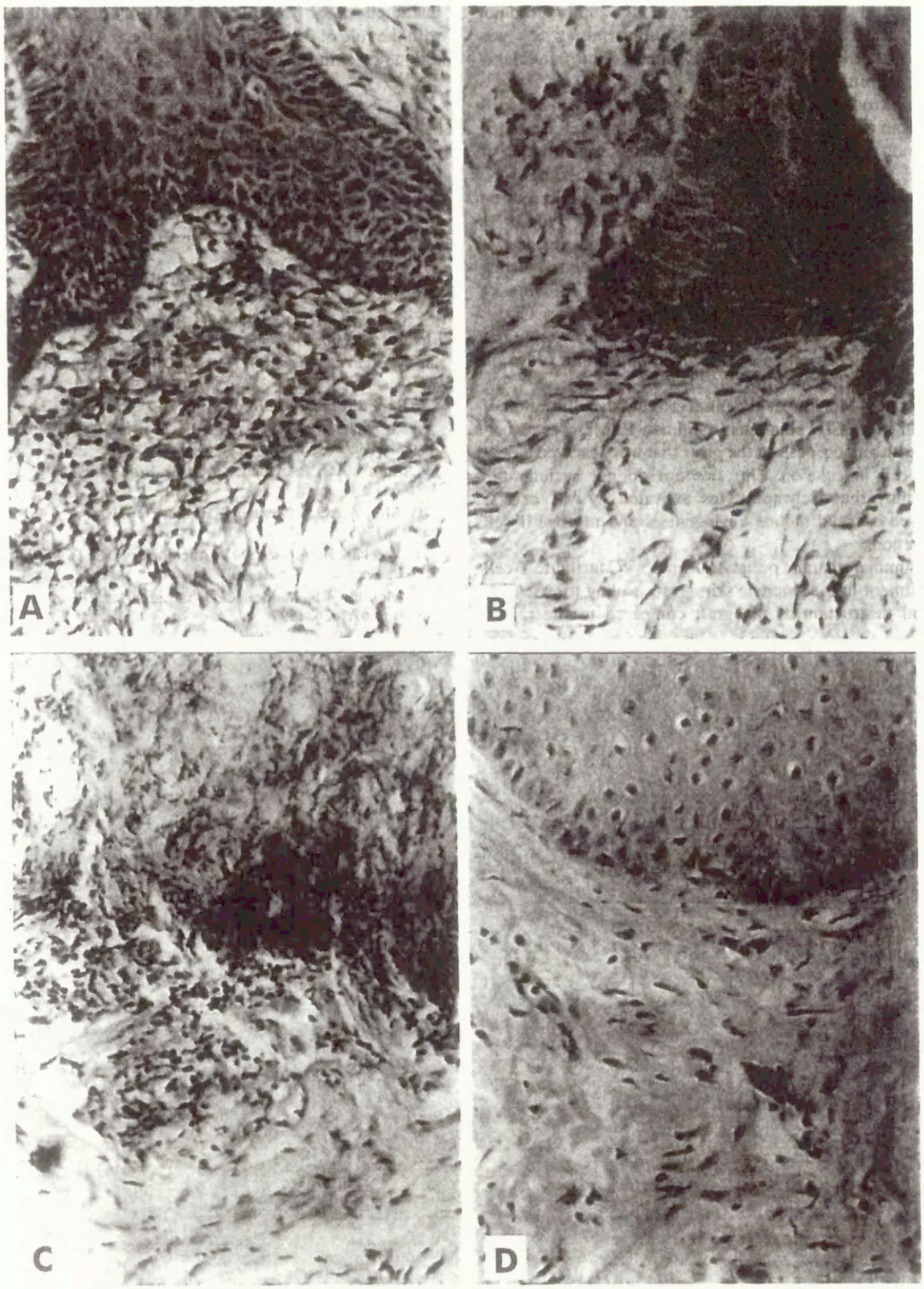

Fig 3. Histologic appearence of canine skin harvested from SCID mouse 2 weeks after engraftment and 7 days after injection of 2 × 10^7 IP canine allogeneic leukocytes, stained with H&E (×200). Skin graft harvested from an animal that received: **(a)** whole lymph cells, **(b)** lymph lymphocytes, **(c)** lymph veiled (dendritic) cells, and **(d)** peripheral blood mononuclear cells. Note diffuse infiltrates at the epidermo-dermal junction in **(c)** with total destruction of epidermis.

Interestingly, in this SCID/chimera model, authors[9] did not observe extensive necrosis of the epidermis, which was reported after transplantation of human skin to an allogeneic recipient.

We introduced a novel SCID mouse/canine skin allograft model to test the effect of recipient VC on T-cell mediated allograft injury. Our data suggest that the accumulation of canine T cells in the graft is mediated by the presence of VC in the injected population and that infiltrating cells may be activated to alloantigen presented by the VC. Wecker et al[11] observed that SCID mice reconstituted with unfractionated lymph node cells (containing DC) generated strong skin allograft rejection. The importance of DC in allograft destruction is supported by our observation that CD3+ T-cell infiltrates in graft dermis and epidermis were more extensive after injection of the whole population of lymph cells (with VC) than PBMC.

Recently, Moulton et al[12] observed that the infiltration of human skin/SCID chimeras by human PBMC was associated with angiogenesis in the graft that was observed 7 days after cell inoculation. Our findings do not exclude the possibility that T lymphocytes activated by VC or VC themselves could induce angiogenesis and massive T-cell infiltration.

In summary, canine peripheral lymph VC facilitate T-cell recruitment into allogeneic skin graft, leading to extensive dermal destruction of the graft compared to the rather feable effects of lymph LY or PBMC. These data comply with the role of the recipient professional APC in allorecognition and skin rejection.

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ELSEVIER

Cutaneous CsA-Resistant Veiled (Dendritic) Cells Are Responsible for Uncontrolled Skin Allograft Rejection

H. Galkowska, M. Moscicka, E. Cybulska, U. Wojewodzka, J. Mijal, and W.L. Olszewski

SKIN ALLOGRAFTS, in contrast to other organ transplants, are acutely rejected despite intensive immunosuppressive therapy with cyclosporin A (CsA). The mechanism of resistance to this drug remains unclear.[1] Skin tissue fluid and lymph contain migrating immune cells, among them large veiled cells (VC) and lymphocytes (LY). A drop of lymph freshly drawn from a skin afferent lymphatic contains 3 to 6% of clusters formed by VC and T cells, which signifies the in vivo cooperation of these subpopulations.[2] Spontaneous clustering is the first phase of antigen recognition and alloantigen presentation to T cells by dendritic cells (DC). Inhibition of cell clustering would presumably down-regulate this process. The question arises as to whether cutaneous VC are the principal cells initiating skin allograft rejection and whether other than CsA immunosuppressants can inhibit clustering of VC with autologous LY.

To investigate the role of recipient VC in initiation of the rejection process, we have used canine skin to severe combined immunodeficient (SCID) mouse transplant model. Two weeks after skin grafting, when donor LC had migrated out from the transplant,[3] canine peripheral lymph VC and LY allogeneic to the graft were injected IP, and the rejection reaction was followed. Our in vitro model of cluster formation served as studies on the effect of immunosuppressants on VC-LY binding. Our results suggest that, allogeneic to the skin graft, VC promoted recruitment of autologous T cells to the graft's dermis and subsequently graft destruction. Immunosuppressants like CsA, azathioprine (AZ), dexamethasone (DM), and hydrocortisone (HC) had no effect on VC clustering with autologous LY in vitro. Methylprednisolone (MP) and FK506 had a potent inhibitory effect, even at very low doses.

MATERIALS AND METHODS

Mice

FOX CHASE C.B.-17 SCID mice were obtained from IFFA CREDO (L'Arbresle, Cedex, France) and bred at Medical Research Center (Warsaw, Poland) in a pathogen-free environment. All animals used were 6 weeks old and had less than 5 mg/L of serum IgG by radial immunodiffusion assay (Nanorid, The Binding Site, Birmingham, UK). Each mouse was screened for the absence of $CD3^+$ peripheral blood T lymphocytes by flow cytometry with rat antimouse CD3/PE monoclonal antibody (Ab) (Serotec LTD, Kidlington, England).

Dogs

Normal mongrel dogs (n = 3) and three dogs with lymphedema following surgical interruption of lower leg lymphatics served as donors of skin grafts and cells from peripheral lymph. The average concentration of lymph cells was $2.2 \pm 1.3 \times 10^6$ mL; the percent of cells with DC morphology was 3.2 ± 1.7. Peripheral lymph cells consisted of about 80% $CD3^+$ T lymphocytes.

Skin Grafting

Canine skin sheets obtained from groin under general anesthesia were cut into 10/15-mm pieces and stored in cold 0.9% NaCl until engraftment. SCID mice were anesthetized by IM injection of ketamine and IP injection of 3.6% chloral hydrate. The skin graft was placed on the dorsum. The dressing was removed after 10 days.

Canine Leukocyte Isolation and Inoculation

Peripheral lymph cells were obtained from lymph collected by percutaneous puncture of dilated lymphatics and centrifugation. Pure (100%) population of lymph LY and enriched (over 50%) population of lymph VC were obtained on 12% metrizamide gradient (Nycomed) as described previously.[4] To neutralize host natural killer cells and optimize the engraftment of canine cells, mice were injected IV with 10 uL of anti-asialo-GM-1 (Wako Chemicals, Richmond, Va, USA) in a volume of 0.2 mL saline, 24 hours before IP cell inoculation.

Experimental Setting

Experiments were carried out in three groups. In group 1 (n = 9) 2×10^7 canine allogeneic whole lymph cells were suspended in 0.9% NaCl; group 2 (n = 3) was lymph LY-enriched population; in group 3 (n = 3) lymph VC-enriched population were injected IP into recipients of skin grafts 2 weeks after transplantation. In control group (n = 3) canine syngeneic whole lymph cell population was given.

Histology and Immunohistochemistry

Skin grafts were harvested 7 days after cell inoculation. One half of skin specimen was snap-frozen in dry ice-cold acetone and stored at

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Table 1. The Effect of Immunosuppressants on Lymph Cell Binding after 4 Hours of Incubation at 37°C

Drugs	Concentration	% of Clusters (mean ± SD)	*P* value
Saline (control)	0.15 M	20.1 ± 3.5	
HC	10^{-5} M	21.1 ± 7.1	
	10^{-6} M	23.5 ± 4.1	
DM	10^{-5} M	18.0 ± 10.7	
	10^{-6} M	18.5 ± 3.7	
MP	10^{-5} M	14.1 ± 5.5	< .05
	10^{-6} M	13.5 ± 4.4	< .05
CsA	5 ug/mL	19.3 ± 5.8	
	0.5 ug/mL	22.5 ± 5.5	
AZ	5 ug/mL	20.3 ± 5.5	
	0.5 ug/mL	20.0 ± 4.0	
FK 506	4 ug/mL	9.1 ± 2.4	< .05
	0.4 ug/mL	11.5 ± 2.8	< .05
	0.04 ug/mL	12.0 ± 3.2	< .05

Abbreviations: HC, hydrocortisone; DM, dexamethasone; MP, methylprednisolone; CsA, cyclosporine A; AZ, azathioprine; FK 506, tacrolimus.

−70°C until use for cryostat sections. The other half was formalin-fixed, paraffin-embedded, and stained with hematoxylin and eosin. $CD3^+$ canine T cell infiltrates were identified with antihuman CD3 polyclonal Ab (Dako, Denmark) diluted 1/100. Veiled cells were visualized with specific monoclonal Ab DCIII/2 raised in our laboratory.[4] Cryostat sections were blocked for 20 minutes in 50% normal swine serum before incubation with primary Ab (omitted in controls). Positive cells were detected with LSAB2-alkaline phosphatase complex and fast red according to the manufacturer's instructions (Dako).

VC-LY Cell Binding

VC-LY binding was quantitated 4 hours after incubation of cells at 37°C in lymph mixed 1:1 with appropriate drug solutions or 0.15 mol NaCl for control. The number of VC with two or more LY attached per 100 VC seen in several randomly selected fields was counted by light microscopy. Immunosuppressants were dissolved in ethanol, and stock solutions after mixing with 0.15 mol NaCl were used in both physiological and pharmacological doses.

RESULTS

Histopathologic Characterization of Canine Grafts Placed on SCID Mice

Initial studies revealed that canine skin grafts placed on SCID C.B.-17 mice healed in 2 weeks. Full-thickness canine epidermis was clearly distinguishable from thin mouse epidermis. No infiltrates were observed in the bed and border of the graft 1 month after transplantation.

Histopathologic Characterization of Canine Skin Grafts Placed on SCID Mice Inoculated With Allogeneic Canine Leukocytes

In Group 1, transfer of whole lymph cell population (LY and VC) after 7 days induced mononuclear cell and granulocyte infiltration (++) in graft's bed. Some infiltrating cells at the border of canine and mouse papillary dermis (+) and at the epidermo-dermal junction (+) were seen. No evident destruction of epidermis was observed; however, there were foci of acanthosis. Minor mobilization of mononuclear cells and granulocytes (+) was seen around the graft venules. In Group 2, allogeneic LY accumulated together with some granulocytes in graft's bed (+) and at the epidermo-dermal junction (+). Epidermis remained intact. In Group 3, transfer of VC-enriched population resulted in intense mononuclear cell and granulocyte infiltrates in dermis (++) and around microvessels (+++). Diffuse infiltrates of mononuclear cells and granulocytes were observed at the epidermo-dermal border (+++). Extensive necrosis of epidermis in the midportion of the graft was seen. On immunohistochemical pictures, canine veiled cells (DC-III/2+) were seen in canine dermis but not epidermis. Graft infiltrating cells were predominantly canine $CD3^+$ cells; however, there were also many mouse granulocytes. In the control group, after injection of syngeneic whole lymph cells, the graft remained entirely normal.

Effect of Immunosuppressants on In Vitro Cluster Formation

Treatment with MP resulted in a decrease in the precent of formed clusters (Table 1). Other steroids, CsA, and AZ had no effect on LY binding. In contrast, FK506 had a potent inhibitory effect, even at low doses.

DISCUSSION

SCID mice are permissive for the growth of xenogeneic lymphoid cells[5,6] and tissues.[7] Murray et al[8] developed chimeric human/SCID mouse model to study human skin allograft rejection. They allowed the skin graft to heal prior to IP infusion of allogeneic PBMC in a large number (3×10^8 cells) and observed accumulation of dermal T cells expressing CD25 marker and destruction of dermal microvasculature. The changes in dermal microvessels were independent of human B cells and antibodies.[9] Interestingly, in this SCID/chimera model the authors[8] did not observe extensive necrosis of the epidermis, which was reported after transplantation of human skin to an allogeneic recipient.

We introduced a novel SCID mouse/canine skin allograft model to test the effect of recipient VC on T cell-mediated allograft injury. Our data suggest that the accumulation of canine T cells in the graft is mediated by the presence of autologous VC in the injected population and that infiltrating cells may be activated to alloantigen presented by the VC. Wecker et al[10] observed that SCID mice reconstituted with unfractionated lymph node cells (containing DC) generated strong skin allograft rejection. The importance of DC in allograft destruction is supported by our observation that $CD3^+$ T cell infiltrates in graft dermis and epidermis were more extensive after injection of the whole population of lymph cells (with VC) than pure lymphocytes.

Antigen presentation by DC to LY proceeds through direct contact of both cell types. Inhibition of cutaneous VC and lymphocyte clustering could presumably down-regulate the alloantigen recognition in the "indirect" pathway of recognition. We attempted to analyze the effect of widely used immunosuppressants on cluster formation by autologous VC and LY in vitro. Among corticosteroids only MP used in both physiological and pharmacological doses produced a statistically significant decrease in the percentage of spontaneously formed clusters. CsA and AZ did not prevent cell clustering. The FK506 had a potent inhibitory effect on cluster formation in vitro. These findings are consistent with results of cluster formation in the presence of CsA by canine[11] and human[12] blood DC with LY. It confirms that FK506 and MP could be superior to CsA in prolongation of skin allograft survival. Beneficial effect of FK506 on skin allograft survival was observed in rats[13] and recently in clinical maintenance of human hand allograft.[14]

In summary, canine peripheral lymph VC, resistant to CsA and sensitive to FK506 and MP in vitro, facilitate autologous T cell recruitment into allogeneic skin graft, leading to extensive dermal destruction of the graft compared to the rather feable effects of lymph LY. These data comply with the role of the recipient DC in allorecognition and skin rejection.

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Inhibition of Formation of Synapses Between Dendritic Cells and Lymphocytes in Skin Lymph in an Allogeneic Reaction by Cyclosporine and Tacrolimus

J. Wojewodzka, H. Galkowska, and W.L. Olszewski

ABSTRACT

Skin, an important component of composite tissue allografts is considered to be among the most immunogenic of tissues. The mechanisms of resistance of skin allografts to pharmacological immunosuppression remain unknown. We investigated this problem at the level of antigen presentation by graft dendritic cells (DC) to recipient lymphocytes (L). Cells obtained from lymph draining skin were examined for formation of synapses, necessary for antigen presentation, in the presence of cyclosporine (CsA) or tacrolimus (FK 506). In culture the frequency of DC-L synapses was greater in allogeneic than syngeneic combinations. Cells treated with FK 50% showed a decreased rate of formation of autologous or allogeneic DC-L synapses and lower expression of CD49d. The suppressive effect of FK 506 on DC-L synapse formation may explain the effectiveness of this drug for skin allograft survival.

DONOR dendritic cells (DC) play a dominant role in initiating the rejection of skin allografts. They migrate from the graft to recipient lymph nodes, contacting recipient lymphocytes (L). The first phase of antigen presentation by DC to L demand a direct contact of both cell types: clustering and synapse formation (Fig 1).[1] Inhibition of synapse formation between DC and L would presumably down-regulate alloantigen recognition in skin allografts and response to bacterial or viral infections. Cyclosporine (CsA) and tacrolimus (FK 506) have been shown to alter DC differentiation and L activation.[2] We propose that these drugs inhibit the formation of synapses between DC and L, subsequently down-regulating the initial phases of an allogeneic reaction. Previously we have shown that CD49d molecules actively participate in DC-L synapse formation.[3] In this study we investigated the effect of CsA and FK 506 on the expression of CD49d molecules on DC and L and the rate of formation of synapses.

MATERIALS AND METHODS

Collection of Lymph Cells

Outbred dogs with chronic lymphedema following interruption of afferent lymphatics served as the lymph cell donors. Lymph was collected into tubes with heparin (10 U/mL) by percutaneous puncture. The average concentration of lymph or cells was 1.9 ± 0.7 × 10^6 /mL, including 4.4 ± 2.2% cells with DC morphology and 3.3 ± 2.8% DC-L clusters.

In Vitro Formation of DC and L Synapses

Pure populations of L and enriched population of DC were obtained using a 13% metrizamide gradient. Both populations were mixed at a ratio of 4 × 10^5 DC 2 × 10^6 L for culture as autologous or allogeneic combinations in inactivated fetal calf serum or cell-free lymph. The number of DC with 1 or more attached L per 100 DC was estimated under a microscope using a ×400 magnification.

Immunocytochemistry of DC-L Synapses

The cytospins of autologous and allogeneic L and DC before and after 6-day culture were labelled with anti-human dog cross-

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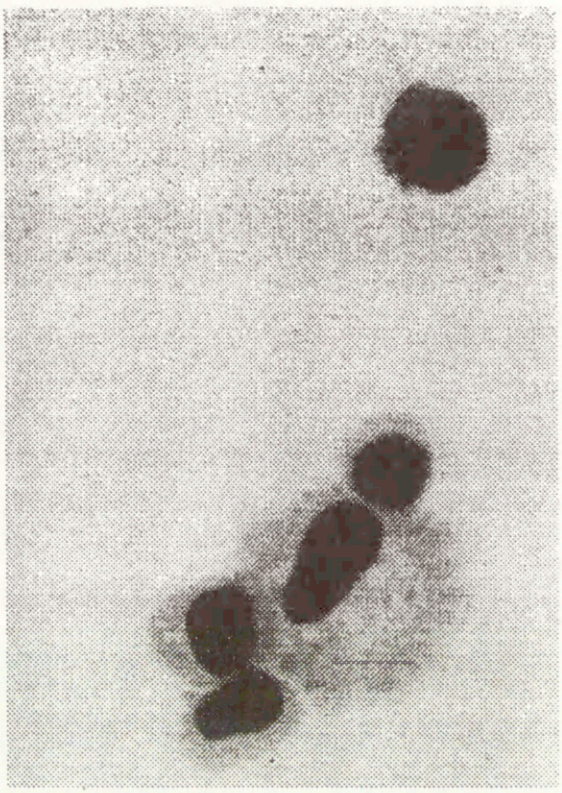

Fig. 1. Cluster between DC (large cell in the middle) and 3 L.

reactive monoclonal antibodies: HLA-DR (BD PharMingen, San Diego, Calif, USA) and anti CD49d (Dako, Glostrup)

The Effect of CsA and FK 506 on Formation of DC and L Synapses

DC were cultured in vitro with allogeneic or autologous L for 6 days in the presence of CsA (0.4 μg/mL) or FK 506 (0.4 μg/mL). The number of synapses and the expression of CD49d or HLA DR on DC and L was measured.

RESULTS

The In Vitro Effect of CsA or FK 506 on the Formation of Synapses

DC and L incubated in vitro in the presence of CsA did not show fewer synapses either in autologous or allogeneic combinations than did the controls, when cultured either in RPMI or autologous lymph. In contrast, FK 506 inhibited formation of synapses in both autologous and allogeneic groups cultured in RPMI as well as lymph. Control autologous DC and L cultured in RPMI formed 38.8 ± 0.96% of synapses compared with 28.0 ± 2.8% for the FK 506 Tacrolimus-treated cells. The percentage of allogeneic DC-L in RPMI synapses was 30.0 ± 14.0, also lower than controls ($P < .05$). FK 506 decreased formation of autologous DC-L synapses in normal lymph from 46.5 ± 14.7% to 16.0 ± 5.7% ($P < .05$) and allogeneic DC-L from 64.0 ± 6.7% to 41.5 ± 9.2% ($P < .05$).

The Effect of CsA and FK 506 on Expression of CD49d and HLA DR

Neither CsA nor FK 506 down-regulated the expression of HLA DR on DC and L in clusters. In contrast, both drugs significantly reduced expression of CD49d antigen on both DC and L, more effectively in lymph than in RPMI medium (Fig. 2). This finding correlated with lower rate of formation of synapses.

DISCUSSION

This study suggests an increased frequency of DC-L synapses with allogeneic compared with syngeneic combinations in culture. FK 506 decreased the formation rate of DC-L synapses in culture and resulted in a reduced expression of CD49d on DC and L synapses. Analysis of expression of surface molecules on canine lymph DC and L revealed that these cells express CD1a, CD14, CD18, CD49d, CD54, CD58, CD86, HLA DR, L, CD3, CD4, and CD8 antigens. We found previously[3] that there were more CD49d and HLA DR alloantigen-stimulated DC and L in synapses than in the nonbinding populations. These molecules may play a role in synapse formation. While CsA did not decrease the numbers of synapses, it down-regulated the expression of CD49d. In contrast, FK 506 down-regulated both synapse formation and CD49d expression.

Because CsA down-regulates the responsiveness of L, it was anticipated that it would have an inhibitory effect on synapse formation. In our studies, in vivo treatment of DC or L with CsA at concentrations used for immunosuppression did not affect the formation rates of cutaneous lymph DC-L, corroborating the data of Woltman et al demonstrating a lack of CsA effect on in vitro autologous cell binding.[2] These findings may explain the low effect of CsA in skin allograft rejection. In contrast, FK 506 showed potent inhibitory effects on lymph cell binding. Although both FK 506 and CsA exert their effects at the same early stage in the L activation process, FK 506 binding to L during preincubation is stronger and irreversible,[4] a finding that may explain the differences in synapse formation rate. Interestingly, both drugs decreased the expression of CD49d suggesting that other molecules play additional roles in the mechanism of synapse formation.

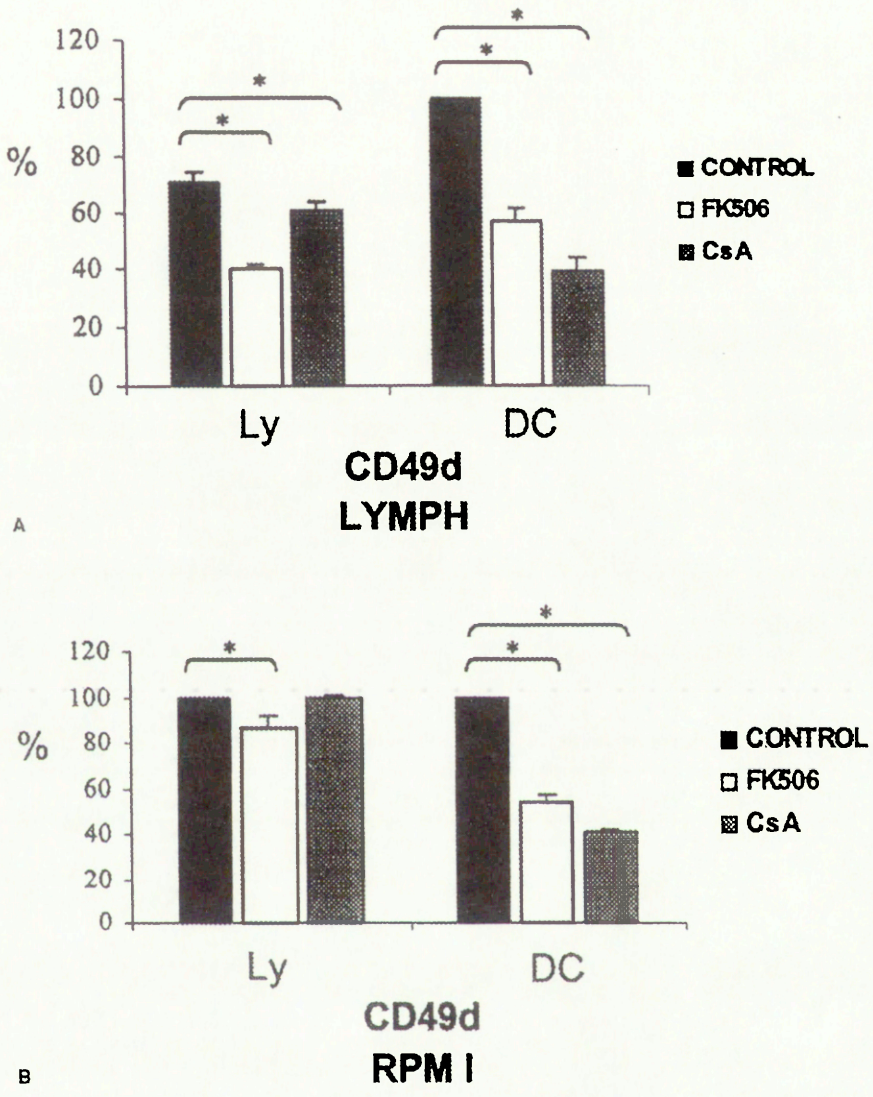

Fig. 2. Percentage of CD49d$^+$ DC and L forming synapses after in vitro culture with CsA and FK 506 in lymph (A) or RPM1(B).

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LYMPH DENDRITIC CELLS REACT TO BACTERIA – THE MECHANISM OF DERMATOLYMPHANGIOADENITIS

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INTRODUCTION

Bacteria penetrating skin evoke an immediate chemotactic reaction resulting in recruitment of granulocytes, phagocytosis and desintegration of the penetrator. A local inflammatory reaction develops with all typical clinical signs. The locally residing Langerhans cells, macrophages and lymphocytes as well as blood mobilized immune cells become activated. Some of these populations enter initial lymphatics and are transported with lymph stream to the regional lymph nodes. They carry with them the ingested or adsorbed bacterial antigens. The lymph nodes are the sites where node lymphocytes are primed with antigen and populations of committed cells arise. This is recognized clinically as enlargement of lymph nodes. It has been known for some time that the process of antigen priming of lymphocytes starts already in the lymph draining from tissues penetrated by bacteria. This function is executed by the migrating dendritic cells, which adsorb bacterial cells and travel to the regional lymph nodes, where they initiate immune reaction resulting in production of memory clones of lymphocytes against specific bacterial antigens. This process comprises processing of antigen and its presentation to lymphocytes. Presentation of antigen requires direct physical contact of dendritic cell with lymphocytes and formation of a cluster (immunological synapse). This type of clusters is seen in normal afferent lymph draining from skin. In inflammation, the number of formed clusters is directly proportional to the intensity of the immune process developing in the tissues where lymph is drained from. The questions arise which chemokines and their receptors, adhesion molecules and their ligands participate in the antigens presentation process. Moreover, is tissue fluid and lymph chemical environment important in the cluster formation process? Are there differences in dendritic cell reaction to extracellular and intracellular bacteria resulting in differences in antigen presentation? And finally, do antibiotics inhibit the process of dendritic cell activation and cluster formation?

In this study we investigated the kinetics of Langerhans (veiled) dendritic cell-lymphocyte cluster formation during stimulation with Staphylococcus warneri and the downregulating effects of penicillin on this process.

MATERIAL & METHODS

Skin dendritic veiled cells (VC) and lymphocytes (L) were harvested from canine hind-limb afferent lymph. C were isolated from other lymph populations and $0.4x10^6$ cells were cultured with live $2.5x10^6$ Staphylococcus warneri for 12 hours. Microscopical examination revealed bacterial cells attached to the surface of VC. Group 1.The bacteria-stimulated VC were suspended with autologous L in the RPMI medium or cell-free canine 100% lymph and incubated for 48h. The ,VC were mechanically detached from L and cultured for 4 and 24hours for spontaneous cluster re-formation. Usually, 1-3 lymphocytes attached to a VC. The number of clusters formed in time was measured. Group 2. ^{3}H-TdR was added to the bacteria-stimulated VC- lymphocyte 72 hours culture to measure the level of lymphocyte activation by stimulated VC. Group 3.BrdUrd (bromodeoxyuridine) in a concentration of 20ug/ml was added to the 48 hours bacteria-

stimulated VC and lymphocyte culture. The number of BrdUdr-positive cells was counted on smears stained with anti-BrdUrd monoclonal antibody. Group 4. In some experiments, benzathine penicillin in a dose of 120 units/200 ul well was added to the VC cultured with Staphylococcus warneri. ^{3}H-TdR was ten added to the 72 hours bacteria-stimulated VC-lymphocyte culture. The level of DNA incorporated tracer was measured and expressed in cpm.

RESULTS

Group 1. The percentage of clusters formed in time between bacteria-stimulated VC and L was evidently higher than in the controls. The culture of VC-L in lymph resulted in higher rate of cluster formation than in RPMI medium (Fig.1).
Group 2. The lymphocyte responsiveness to bacteria-stimulated VC was higher that to the unstimulated VC, both in lymph and RPMI medium (Fig.2).
Group 3. The percentage of BrdUrd-positive cells was higher in cultures of bacteria-stimulated VC-L than when unstimulated VC were used. The positive cells analysed on smears were large cells resembling lymphocyte in the S-stage.
Group 4. The incorporation rate of radioactive H-TdR was lower in cultures supplemented with benzathine penicillin irrespective whether the VC were stimulated or not stimulated by Staphylococcus warneri. The culture in 100% lymph or RPMI medium also did not influence the lymphocyte responsiveness (Fig.3).

CONCLUSIONS

The bacteria-stimulated afferent lymph VC form more clusters with lymph lymphocytes. The responsiveness of lymphocytes by bacteria-stimulated VC is higher than in cultures with non-stimulated VC. Benzathine penicillin decreases lymphocyte responsiveness irrespective of whether the cocultured VC were bacteria stimulated or controls. This last finding requires elucidation.

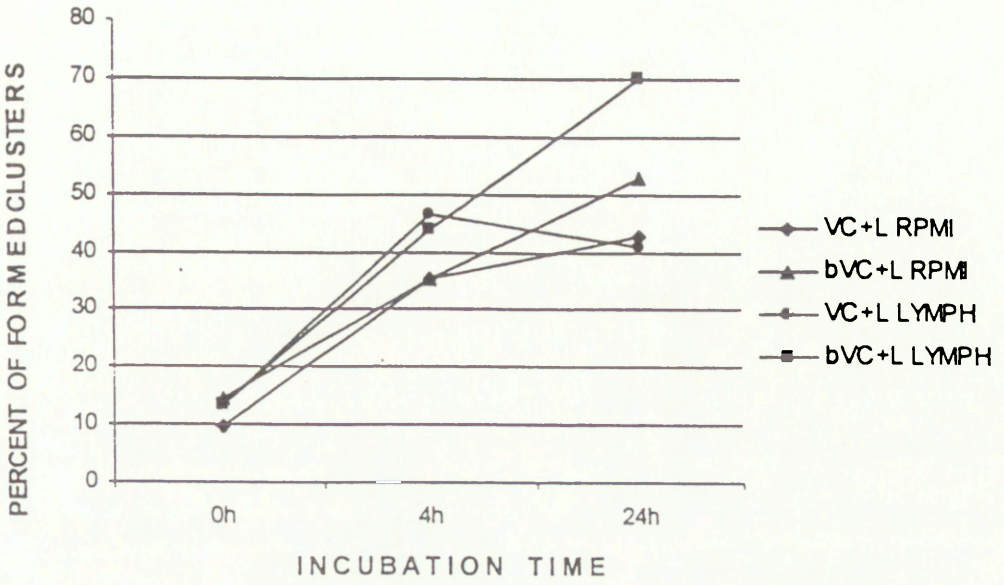

Fig. 1. VC-lymphocyte (L) cluster formation in culture with and without Staphylococcus warneri (b) and lymph.

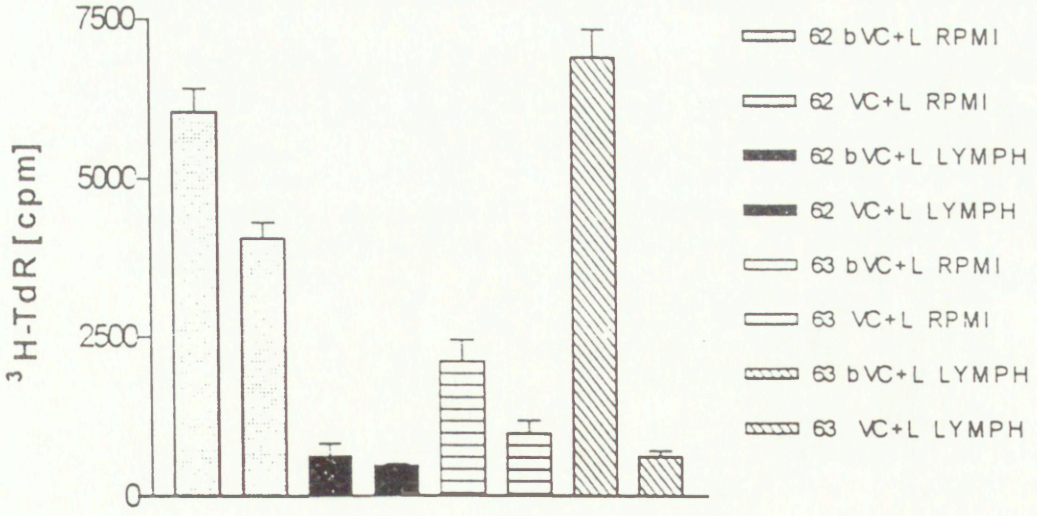

Fig. 2. The responsiveness rate of lymphocytes (L) cultured with bacteria-stimulated (b) and control VC in 100% lymph or RPMI medium. Data of two different experiments are presented (62 and 63).

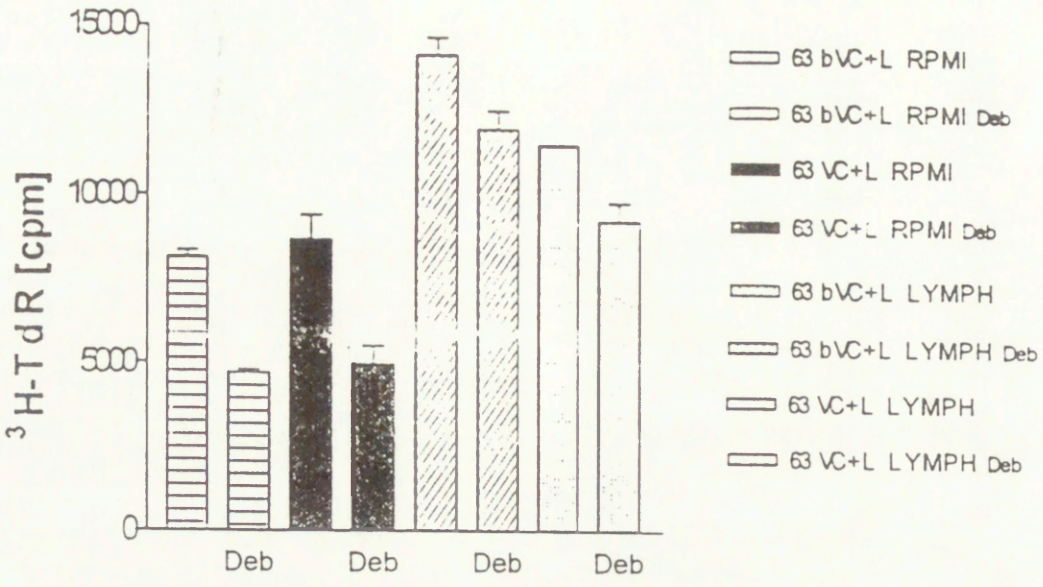

Fig. 3. The responsiveness rate of lymphocytes (L) cultures with bacteria-stimulated (b) and control VC in lymph and RPMI medium with and without benzathine penicillin (deb).

Expression of apoptosis- and cell cycle-related proteins in epidermis of venous leg and diabetic foot ulcers

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Background. *Epithelialization of cutaneous ulcers is a long-lasting process. To study the pathomechanism of impaired epithelialization, we evaluated the role of cell cycle- and apoptosis-related proteins in the regenerating epidermis. We characterized immunohistochemically the expression of cell cycle regulators p63, CD29, PCNA, p53, pro- and antiapoptotic proteins bcl2, bax, caspase 3 and DNA breaks, as well as keratin 10, 16 and 17.*

Methods. *Studies were carried out in 12 patients with diabetic foot, and 10 patients with varicose ulcers of the calf. Skin biopsy specimens were obtained from the border area of ulcers and the topographically corresponding sites of normal skin of patients undergoing orthopedic surgery. Biopsy specimens were stained by use of specific primary antibodies, a kit based on biotin-avidin-peroxidase complex technique, and DAB substrate. Results were expressed as a mean staining intensity.*

Results. *At the edge of both types of ulcers, keratinocytes were p63+, CD29+, PCNA+ and p53−. The mean intensity of p63 and CD29 staining was slightly higher than in controls. The intensity of bcl2 staining was higher at the edge of diabetic ulcers compared with venous ulcers, whereas the intensity of bax staining was similar. The expression of caspase 3 was lower at the edge of venous ulcers and higher in diabetic ulcers and the intensity of TUNEL staining was lower at the edge of both types of ulcers compared with controls. Keratinocytes at the edge and distally to both types of ulcers expressed cytokeratin 16 and 17. There was no expression of cytokeratin 10 at the edge of ulcers. Together, there was a slight tendency for higher expression of cell cycle–related proteins in venous and of apoptosis-related proteins in diabetic ulcers epidermis; however, the differences were minor.*

Conclusions. *The impaired epithelialization of chronic leg ulcers is not caused by an inadequate epidermal stem cell proliferation, differentiation, or apoptosis. It may rather reflect the distorted organization of wound bed, caused by infection and impaired nutrition supply, altering keratinocyte migration. To accelerate healing of an ulcer, modeling of the granulation tissue by regulatory cytokines but not stimulation of keratinocyte growth seems to be indicated. (Surgery 2003;134:213-20.)*

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MOST STUDIES DEALING WITH CUTANEOUS wound healing have focused on granulation tissue remodeling.[1,2] Little attention has been paid to the question of epidermal regeneration and the functional relationship of the matrix-forming cells and epidermal cells migrating on the granulation tissue. The proper conditions for formation of epidermodermal junctions and unimpaired proliferation of keratinocytes are important for covering of the wound surface with epithelium. The question remains whether in skin ulcers with large uncovered areas, the protracted epithelialization is due to a decreased proliferative and migratory capacity of epidermal cells.

Epidermis is continuously renewed, and this process is ensured by antigen p63+, integrin β1+ (CD29) epidermal stem cells, residing in the basal layer.[3,4] Epithelial stem cells are defined as a minor subpopulation of basal cells with the greatest proliferative capacity.[5] These cells give rise to the transient (transit) amplifying cells with reduced p63 and CD29 expression, which are an actively proliferating (PCNA+) pool of basal keratinocytes. Postmitotic differentiating cells with the lowest proliferative capacity and that express cytokeratin 10 represent the differentiation compartment. In suprabasal layers the terminally dif-

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ferentiating keratinocytes are continuously lost from the skin surface undergoing senescence or apoptosis. These processes are controlled by similar molecular regulators, but there is evidence suggesting that they are alternative, independent phenomena.[6,7]

Molecular regulators of apoptosis are also involved in the epidermal cell differentiation. Bcl2 has been found to be associated with the proliferating keratinocytes and antiapoptotic activity. Conversely, proapoptotic protein bax is upregulated during keratinocyte differentiation. Apoptosis is executed by caspases, which are also activated during keratinocyte differentiation.[8] In apoptotic cell nuclei DNA is fragmented by endonucleases, and DNA breaks can be detected in situ by dUTP-biotin nick end labeling (TUNEL). In keratinocytes TUNEL labeling is not always a specific marker of apoptosis because in diseased skin most epidermal layers can be TUNEL positive.[9] Another protein, p53, is considered to keep DNA integrity and to have antiproliferative, proapoptotic effects.[10]

During wound epithelialization, keratinocytes localized at the wound margin undergo changes, including cell migration, proliferation, and differentiation. Acute skin injury was shown to induce keratinocyte activation and hyperproliferation associated with cytokeratin 16 and 17 expression.[11] Cytokeratins are fundamental components of the keratinocyte cytoskeleton, and distinct sets are expressed by cells in the different layers of epidermis in a manner specific for cell differentiation. The persistence of activated keratinocytes in epidermis can implicate abnormal epidermal-dermal interactions during protracted wound healing.

This study was designed to evaluate the functional capacity of keratinocytes, specifically the role of proteins regulating keratinocyte cell cycle, apoptosis, and differentiation in the pathomechanism of protracted epithelialization. The venous and diabetic ulcers were chosen for investigation. Although the pathomechanism of development of ulcers is different in these 2 conditions, in both, the reepithelialization of the granulation tissue is delayed, and epidermal proliferative capacity may be impaired.

MATERIAL AND METHODS

Patients. The biopsy material investigated in this study originated from (a) 12 patients mean age 62 years with type 2 diabetes, complicated by foot ulcers grade 2 to 4 according to Wagner's classification, of 3 to 6 months duration, (b) 10 patients mean age 67 years, with varicose ulcers of the calf of more than 1 year duration, (c) 8 patients without diabetes, mean age 62 years, undergoing elective orthopedic surgery, providing control skin. All patients were treated in a uniform fashion with hydrocolloid dressings. At the time the biopsy specimens were obtained, no signs of inflammation were seen around the ulcers, and no significant numbers of isolates were cultured from the bottom of the ulcers. Occasionally, simple colonies of *Proteus sp* and *Staphylococcus aureus* were identified. This study was approved by the Ethical Committee of the Medical Research Center, Warsaw, Poland.

Skin biopsy. Skin biopsy specimens were obtained from the border areas of ulcers and from the topographically corresponding sites of control subjects. A local anesthetic of 2% lignocaine was used on the patients. A wedge of tissue, approximately 10 mm long and comprising the ulcer edge, base, and surrounding nonaffected skin was excised. One fragment of specimen was snap-frozen in dry ice-cold acetone and stored at −70° C until processed, whereas the other was fixed in 4% phosphate-buffered formalin and paraffin-embedded. The 4-μm frozen sections were dried at room temperature (RT) overnight and stored at −20° C until processed.

Antibodies and immunohistochemistry. The primary antibodies specific to the following antigens were applied (code number and working solution in parentheses): p63 (sc-8431, 1/50), CD29 (sc-9970, 1/50) from Santa Cruz Biotech (Santa Cruz, Calif); cytokeratin 16 (NCL-CK16, 1/40), cytokeratin 17 (NCL-CK17, 1/40) from Novocastra Lab (Newcastle Upon Tyne, UK); PCNA (M879, 1/75), cytokeratin 10 (M7002, 1/100), p53 (M7001, 1/25), bax (A3533, 1/20), bcl2 (M887, 1/50), caspase 3 (A3537, 1/25) from Dako (Glostrup, Denmark). The paraffin-embedded 4-μm sections were deparafinized, dewaxed, and rehydrated in TRIS-buffered saline solution (TBS). They were stained for the presence of the following antigens: PCNA, CK10, p63, CD29, and p53. Before staining for p53 antigen, sections were boiled 2 times for 5 minutes in 10-mmol/L citrate buffer in a microwave. All paraffin-embedded sections were blocked with 50% normal swine serum for 20 minutes before incubation with primary antibodies.

The frozen sections were allowed to thaw at RT before being unwrapped and then fixed in cold acetone for 10 minutes at RT. To remove endogenous peroxidase, all sections were incubated in 3% H_2O_2 for 5 minutes and washed in TBS before staining. Next, tissue sections were incubated with the primary antibodies for 30 minutes, except for anti-p63 and CD29 antibodies, which required 60 minutes incubation. Control sections were processed parallelly without the primary antibody. A standard LSAB (Dako) labeling technique with

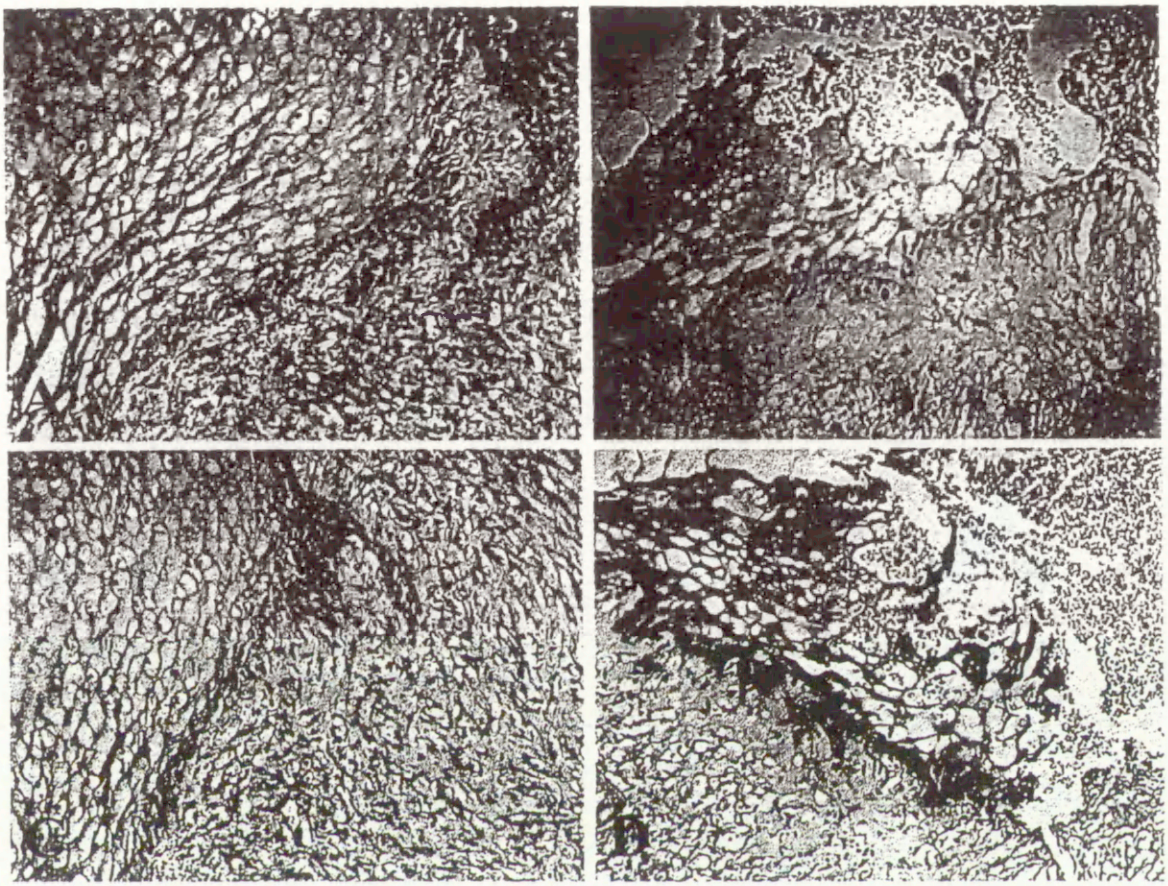

Fig 2. Immunohistochemical localization of p63 and CD29 proteins in epidermis at the edge of chronic leg ulcers. **A**, p63 in diabetic ulcer; **B**, p63 in venous ulcer; **C**, CD29 in diabetic ulcer; **D**, CD29 in venous ulcer. Positive staining is evidenced by the brown reaction product. (Original magnification ×20.)

keratin, CK 16 and 17 (Fig 4). There was no expression of CK 17 and CK 16 in control calf epidermis. There was a weak staining of CK 16 and a slight expression of CK 17 in control foot epidermis. Keratinocytes of the edge of diabetic and calf ulcers did not express CK10, the keratin characteristic for differentiated cells. There was only a slight expression of CK 10 in the regions proximal and distal from the diabetic ulcer. Interestingly, keratinocytes of proximal and distal regions from venous ulcers coexpressed CK10, 16, and 17. Figure 5 shows the expression of CK16 in keratinocytes at the edge of foot *(A)* and calf *(B)* ulcers, and the lack of CK10 (C and D, respectively).

DISCUSSION

Regeneration of epidermis during normal wound healing takes place by migration, proliferation, differentiation, and apoptosis of keratinocytes on the wound borders. These processes are also involved in homeostatic mechanisms in healthy skin, a rapidly renewing tissue. Only a few studies on human keratinocyte proliferation, differentiation, or apoptosis have been performed in chronic wounds.[12,13] This study provided the following information: (a) expression of p63 and CD29 by keratinocytes at the edge of both the venous and diabetic ulcers, (b) high level of expression of PCNA and low of p53 in both types of ulcers, (c) higher intensity of bcl2 staining in diabetic than venous ulcers, (d) higher expression of caspase 3 and DNA breaks in diabetic ulcer keratinocytes, (e) presence of cytokeratin 16 and 17, and no expression of cytokeratin 10 at the edge of both types of ulcers. The localization of PCNA+ proliferating pool of keratinocytes was similar to that observed by others[12,13] in chronic venous ulcers and in normal wound healing.

The proliferative compartment of skin epidermis consists of stem and transient amplyfying ker-

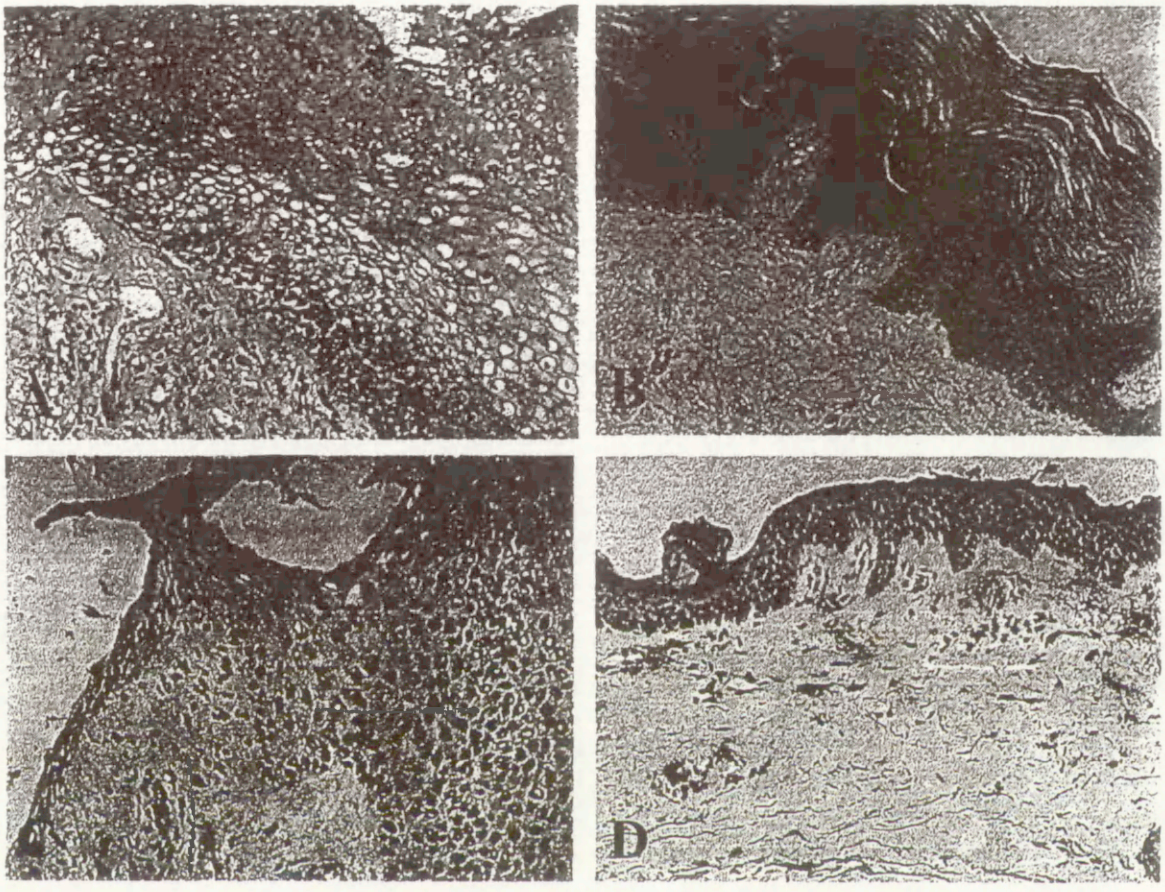

Fig 3. Immunohistochemical localization of PCNA antigen and DNA breaks (TUNEL assay). **A**, PCNA at edge of diabetic ulcer; **B**, PCNA in control foot skin (only single positive cells); **C**, TUNEL stain at edge of venous ulcer (single positive cells in basal layer and around blood vessels); **D**, TUNEL stain in control calf epidermis (numerous positive cells around blood vessels). (Original magnification ×20.)

atinocytes. Stem cells are restricted to p63+ keratinocytes that possess the ability whether or not to proliferate and duplicate their DNA. Integrin β1 (CD29) is another marker of keratinocyte stem cells.[4] Amplifying and postmitotic differentiating keratinocytes are characterized by a reduced expression of both markers. A reduced expression of p63 by the migratory keratinocytes was observed at the margin of normal cutaneous wounds in human beings.[14] In this study we observed only a slight intensity of p63 staining in keratinocytes at the ulcer edge and in proximal and control epidermis. The keratinocytes at the edge of venous ulcers and in the proximal areas of the diabetic ulcers showed increased intensity of CD29 staining compared to control tissue. This confirms the hyperproliferative character, especially of foot sole epidermis, because it has been proposed that β1 integrin expression by suprabasal keratinocytes in transgenic mice leads to hyperproliferation and perturbed differentiation of keratinocytes.[15]

A mechanism that suppresses undesirable keratinocyte proliferation by controlling cell number is apoptosis and cellular senescence. Because epidermal terminal differentiation results in the loss of the ability to proliferate and ultimately in dead structures, it might be considered as a form of senescence or apoptosis.[7] Two proteins, bcl2 and p53, are molecular regulators of keratinocyte proliferation, differentiation and apoptosis. The deregulation of bcl2 in oncogenesis leading to synthesis of a large amount of this protein resulted in uncontrolled cell proliferation.[16] In this study we observed a lack of bcl2 in control tissue and a weak expression at the edge of calf ulcers and in the surrounding epidermis. In contrast, there was the increased intensity of bcl2 staining in epidermis surrounding the diabetic foot ulcer. The p53 pro-

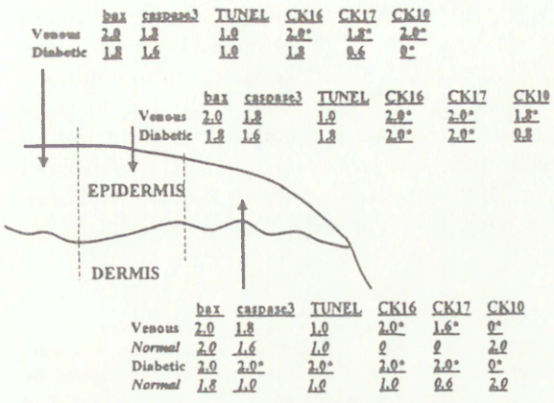

Fig 4. Mean staining intensity of bax, caspase 3, DNA breaks (TUNEL) and cytokeratins 10, 16 and 17 in different areas of regenerating epidermis of venous and diabetic foot ulcers and topographically corresponding control skin (calf or foot). (*$P < .05$ venous or diabetic versus normal, score: 0 = no staining, 1 = weak staining, 2 = moderate to strong staining)

tein was absent in keratinocytes at the edge of both types of ulcers, whereas in the proximal and distal areas of calf ulcers the intensity of p53 staining was increased compared with control tissue and corresponding regions of foot ulcers. The suppression of p53 at the edge of ulcers can be consistent with the active proliferative state of keratinocytes, whereas the increased expression of p53 in epidermis surrounding calf ulcers can serve to downregulate cellular proliferation. Our results are consistent with the observed inhibition of p53 protein expression on day 1 and 2 after acute cutaneous injury in swine and the significantly increased expression on day 5.[17] In mice the bcl2 and p53 protein expression in wound epithelium was found tending to oppose levels, when p53 has increased, bcl2 decreased.[18] Such kinetic of both proteins can be associated with the proproliferative activity of bcl2 and antiproliferative effect of p53.

There is a growing number of proteins that can contribute to cell survival or suicide and some of

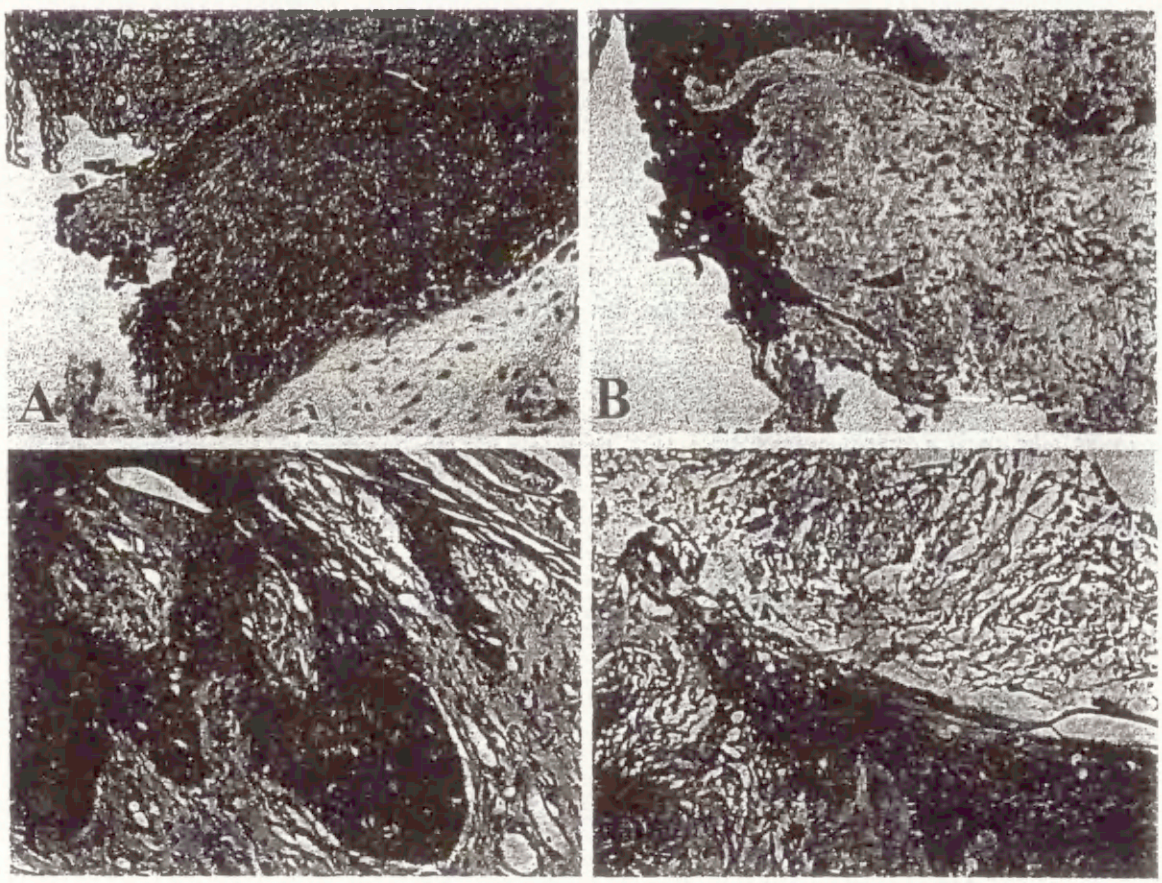

Fig 5. Immunohistochemical localizatin of cytokeratin 16 and 10 at the edge of chronic leg ulcers. A, CK16 in diabetic ulcer; B, CK16 in venous ulcer; C, CK10 in diabetic ulcer (lack of positive cells); D, CK10 in venous ulcer (a slight expression). (Original magnification ×20.)

them like protein bax and caspases have a proapoptotic activity.[19] In cultured keratinocytes upregulation of bax was associated with induction of TUNEL+ DNA breaks.[20] This study showed the same moderate staining intensity of bax throughout ulcer epidermis and controls in both types of skin. The expression of caspase 3 was increased at the edge of diabetic foot ulcer compared to that observed in the proximal and distal epidermis and in control. The low intensity of caspase 3 staining at the edge of calf ulcer correlated with the weak intensity of TUNEL assay. In case of foot ulcer both parameters were increased. This can suggest that in hyperreactive epidermis caspase 3 is activated during keratinocyte differentiation and is required for normal loss of the nucleus. Moreover, TUNEL is not always a specific marker of apoptosis in keratinocytes[9] and may detect free DNA ends associated with cell proliferation.

Normal skin injury was shown to have a profound effect on keratinocyte hyperproliferation, associated with induction of CK16 and reduction of CK10 in regenerating epidermis until the ninth day after wounding.[11] In this study we observed the expression of hyperproliferative CK16 and CK17 at the ulcer margins, as well as in proximal regions of venous calf and diabetic foot ulcers. These findings are similar to those found in healing of necrotic-, necrotic/granulating-, and reepithelializing venous ulcers.[12] In contrast to calf skin demonstrating coexpression of CK16 and CK17 with CK10 in epidermis surrounding venous ulcer, we did not observe CK10 expression in epidermis surrounding diabetic foot ulcers. Such coexpression of CK10 and CK16 was seen in the control foot sole skin. This observation suggests that keratinocytes in this type of epithelium are hyperproliferative.

Altogether, there was a slightly higher tendency of keratinocytes at the edge of venous ulcers to express the cell cycle–related proteins and of those of diabetic ulcers to stain more intensively for apoptosis-regulating proteins. These differences were rather minor. The keratinocytes at the edge and adjacent regions of both types of ulcer had a high proliferative activity. Nevertheless, covering of the granulation tissue by keratinocytes was sluggish. No evident relationship between the rate of healing and the proliferating capacity of keratinocytes was observed.

This study demonstrated that the impaired epithelialization of chronic ulcers, both venous ulcers of the calf and diabetic foot ulcers, is not caused by the lack of epidermal stem cells, inadequate proliferation, differentiation or apoptosis at the edge of wounds. Failure of chronic wound healing may rather reflect the distorted organization of wound bed, caused by infection and impaired nutrition supply, altering keratinocyte migration at the ulcer edge. It seems that modeling of the granulation tissue creating conditions for formation of epidermodermal junctions by means of cytokines regulating fibroblast activity and matrix construction might be the future therapeutic modality.

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KERATINOCYTE, DERMAL AND INFILTRATING IMMUNE CELL CAPACITIES REMAIN UNIMPAIRED IN THE MARGIN OF CHRONIC VENOUS ULCER

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Aim: The present study was undertaken to determine the functional capacity of skin cells in chronic venous ulcers and specifically (i) which immune cell phenotypes are recruited in the ulcer bed, margin and distant dermis, (ii) how active is local production of cytokines and growth factors necessary for proliferation and migration of keratinocytes (KC), vascular endothelial cells (EC) and infiltrating leukocytes, and (iii) whether chronic scavenging is replaced by bed remodeling. *Methods and results:* Biopsies from leg ulcers of 10 randomly selected patients were examined immunohistochemically for leukocyte phenotypes, vascular adhesion molecules and cytokines and growth factors produced by KC and EC. Granulation tissue contained few fibroblasts and blood capillaries, with high intensity of staining for CD62E and CD106 but not for FGF2 on EC ($p<0.05$). The intensity of staining for scavenging $CD15^+$ $elastase^+$ granulocytes and $CD35^+$ (C3bi) macrophages in ulcer bed was comparable to that in the margin but higher than in distant dermis ($p<0.05$), whereas that for $CD68^+$, HLA DR^+, $TGF\beta^+$ and $CD54^+$ dermal leukocytes was similar in all areas. There was reduced staining for CD4+ and CD8+ cells in ulcer bed ($p<0.05$). There were no $CD1a^+$ Langerhans' cells in epidermis encroaching upon the granulation tissue and the reduced CD1a staining in the adjacent epidermis ($p<0.05$). The expression of cytokines and growth factors by KC was similar in the areas adjacent and remote from ulcer. In dermis adjacent to ulcer the expression of IL1α, IL1β, IL1Ra, EGF and PDGFa was higher than in distant dermis. *Conclusion:* There is chronic accumulation of scavenging cells with lack of remodeling of the granulation tissue and, at the same time, preserved

secretory potential of KC and dermal EC in non-healing venous leg ulcers. These finding justify surgical excision of ulcer granulation tissue in long lasting ulcers and full thickness skin grafting.

Introduction

It is widely believed that venous leg ulcers are the consequence of long-lasting venous hypertension, but the exact pathogenesis of ulceration is still unclear. The theories regarding impaired healing of chronic ulcers include pressure damage of capillaries with leakage of fibrinogen leading to formation of the perivascular cuffs, [1] release of toxic metabolites, proteolytic enzymes, and free radicals [2] and cytokine- mediated fibrinous matrix cuff formation. [3] Venous hypertension results in preferrential accumulation of leukocytes in the disesased leg resulting in oxygen stress and subsequent vessel injury. This can aggravate further condition and the process becomes locked in a self-amplifying cascade with persistently elevated levels of proinflammatory cytokines preventing progress into the healing phase. More recently, a theory has been put forward that growth factors are trapped in matrix cuff and are not available to stimulate healing. [4]

Healing of skin ulcer is a complex process requiring a collaborative effort of different tissues and cells. [5,6] In normal healing the wound margin and bed tissue become infiltrated by macrophages, neutrophils, and few lymphocytes.

Communication between the infiltrating leukocytes and integumentary cells (e.g. fibroblasts, keratinocytes, Langerhans' cells and endothelial cells) and activation of these cells are mediated by cytokines and growth factors. The role of these factors in chronic venous ulcer healing is poorly understood. It is likely that inappropriate numbers of infiltrating cells in the ulcer bed and the reduced cytokine signals may be contributory factors to the maintenance of venous ulcer chronicity.

Therefore, the focus of this study was to determine (i) which phenotypes of cells partipating in scavening and heaing are recruited at the edge of ulcer; (ii) are the producing cytokines and growth factors keratinocytes necessary for their proliferation and migration; (iii) are there signs of ulcer bed remodelling or only of chronic scavenging. We randomly selected a group of patients with chronic venous leg ulcer and investigated immunohistochemically in the biopsied ulcer margin and bottom the phenotypes of infiltrating leukocyte and the the expression of various cytokines and growth factors. Expression of vascular adhesion molecules responsible for leukocyte extravasation was also analyzed. This study demonstrates a protracted scavenging process in ulcer bed tissue and, at the same time, preserved secretory potential of keratinocytes and dermal capillary EC without abality to proliferate and migrate into and onto ulcer tissue.

Material and methods

PATIENTS

Ten patients (3 males and 7 females) in the mean age of 67 years (69-78) with venous ulcers in course of the postthrombophlebitic syndrome were randomly selected. A chronic leg ulcer was defined as existing longer than one year. Ulcers were located at the lower medial aspect of calf. Their mean diameter was 5.5 ± 2.1cm (range 3-12 cm). There were no signs of acute skin inflammation around the ulcer. The ulcer surface was pale and covered with watery exudate. Topically hydrocolloid dressings were applied. No topical or systemic antibiotics were given. Bacteriology of ulcer surface swab revealed presence of Staphylococcus aureus, E. coli and occasionally Acinetobacter spp. and Proteus spp. Color Doppler investigation showed recanalization of large veins and their valvular incompetency. Single incompetent perforators were mapped. There were no perforators in the bottom of ulcers. This study had approvement of the Local Ethics Committee and patients gave written consent.

SPECIMEN COLLECTION AND IMMUNOHISTOCHEMISTRY

Skin biopsies were obtained under local anasthesia with 2% lignocaine. They comprised the ulcer base, edge and surrounding skin. One half of the specimen was snap frozen in dry ice cold acetone and stored at – 70 ^{0} C until processed. The other half was fixed in 4% phosphate buffered formalin and paraffin embedded for histological and immunohistological studies. Snap frozen cryostat sections of 4 μm thickness were mounted on poly-L-lysine-treated glass slides, air dried overnight and stored at –20 ^{0} C until used, whereas the paraffin embedded sections were mounted on silane coated slides from Dako (Glostrup,

Denmark) and stored at room temperature (RT). The primary antibodies specific to the following antigens were applied (code number and working solution in parentheses): CD1a (M721, 1/50), CD68 (M718, 1/50), CD15 (M733, 1/30), CD35 (M710, 1/100), CD4 (M716, 1/10), CD8 (M783, 1/30), HLA-DR (M746, 1/30), Elastase (M752, 1/100), fVIII (von Villebrand factor, M616, 1/100), CD54/ICAM1 (intercellular adhesion molecule 1, M7063, 1/50), CD62E/ELAM1 (endothelial cell adhesion molecule 1, E-selectin, M7105, 1/30), CD106/VCAM1 (vascular cell adhesion molecule 1, M7106, 1/50), EGF-R (epidermal cell growth factor- receptor, M886, 1/20) from Dako; IL1α (interleukin 1α, LP-710, 1/20), IL1β (interleukin 1β, LP-712, 1/50), PDGFa (platelets- derived growth factor a, ZP-214,1/30), IL6 (interleukin 6, LP-716, 1/30), IL8 (interleukin 8, 80-3644-02, 1/20), TNFα (tissue necrosis factor α, IP-300, 1/100) from Genzyme (Cambridge, MA,USA); IL1Ra (interleukin 1 receptor anagonist, AB-280-NA,1/30), GM-CSF (granulocyte macrophage colony stimulating factor, AF-215-NA,1/50), FGF 2 (fibroblasts growth factor 2, AB-33-NA,1/50), TGFβ (transforming growth factor β, AB-100-NA, 1/30), VEGF (vascular endothelial cell growth factor, AB-293-NA, 1/100) from R&D Systems (Abingdon, UK); EGF (epidermal growth factor, AB-1910,1/100), TGFβ -R (TGFβ- receptor, MAB-2152, 1/50) from Chemicon (Temecula, CA,USA). Deparaffinized, dewaxed, rehydrated in TRIS- buffered saline (TBS) paraffin-embedded sections were stained for the presence of elastase and VEGF. For staining of some intracellular cytokines (IL1α, IL1β, PDGFa) the frozen sections were permeabilized with 0.1% saponin (Serva, Boehringer Ingelheim, Heidelberg, Germany). All procedures were carried out at

RT, except of staining for VEGF (sections were boiled in 0.01M sodium citrate two times for 5 min in microwave). Paraffin embedded sections and these stained in the presence of saponin were blocked with 50% normal swine serum for 20 min. Next, the endogenous peroxidase was blocked in all specimens by incubation in 3% H_2O_2 for 5 min, sections were rinsed with TBS and then incubated with primary antibody for 30 min (for VEGF sections were incubated overnight with primary antibody at 4 ^{0} C). A standard labelling technique using biotin-streptavidin-immuno-peroxidase complex was applied. For paraffin embedded sections LSAB+ kit and for frozen LSAB2 kit from Dako were used. The biotinylated secondary antibody and streptavidin- peroxidase conjugate were applied for 18 min. A chromogenic substrate DAB (diaminobenzidine, Sigma, St Louis, MO, USA) was incubated for 5 min. Next, tissue sections were counterstained with 0.2% Mayers hematoxylin (Sigma) for 1 min. For evaluation the ulcer specimen section was divided into 3 areas: 1/ the ulcer bed; 2/ epidermis and dermis adjacent to the ulcer; 3/ epidermis and dermis distal to the ulcer. The mean staining intensity was assessed semiquantitatively using a score: 0= no staining; 1= weak staining; 2= moderate staining; 3= strong staining. The two scores of 3 segments were averaged for each specimen and these values were used for comparisons.

STATISTICAL ANALYSIS

Wilcoxon signed rank test was used to compare the values of mean staining intensities. $P< 0.05$ was considered statistically significant.

Results

ULCER BED TISSUE

The ulcer bed contained few fibroblasts with large pyknotic nuclei and few blood capillaries. The very upper layer was made up of acellular matter (Fig.1). The deeper layers did not show collagen bundles. The surface of the ulcer bed and keratinocytes covering peripheral regions of ulcer granulation tissue were covered by $CD15^+$ granulocytes with high elastase expression (Fig.2). There were no $CD1a^+$ Langerhans' cells detected and only few $CD4^+$ and $CD8^+$ lymphocytes (less than in the adjacent and remote dermis, $p<0.05$, Fig.3) were seen. The concentration of $CD15^+$ elastase-containing neutrophils and $CD35^+$ (C3bi) macrophages was evidently higher in the ulcer bed tissue than in remote normal dermis (Fig.2 and 4, $p<0.05$). The staining intensity for $CD68^+$ macrophages, HLA DR^+, $TGF\beta^+$ and $CD54^+$ cells was similar to the adjacent and remote dermis (Fig.2 and 3). The expression of factor VIII, CD54 and VEGF antigens on endothelial cells was also comparable to the adjacent and normal dermis (Fig.3). There was, however, stronger expression of ELAM1 and VCAM 1 antigens ($p<0.05$). In contrast to the adjacent and normal dermis there was a weak expression of IL8 on EC in ulcer bed. Expression of IL 1α, IL 1β, IL1Ra, IL6, TNFα, PDGFa, GM-CSF, EGF and TGFβ-R by endothelial cells was similar in the ulcer bed tissue and normal dermis (Fig.5). In contrast to the remote dermis there was no expression of FGF2 on endothelial cells in the granulation tissue ($p<0.05$).

EPIDERMIS

There was evidently lower staining intensity for $CD1a^+$ Langerhans' cells in the adjacent to ulcer than the remote epidermis (Fig.2, $p<0.05$). These cells were not seen in neoepidermis encroaching upon the ulcer surface (Fig.6). The expression of all studied cytokines and growth factors in keratinocytes was similar in both the adjacent and remote to the ulcer areas (Fig.5).

DERMIS

There was intensive recruitment of $CD15^+$ and elastase containing neutrophils in dermis adjacent to ulcer compared to distal dermis (Fig.2, $p<0.05$). This applied also to CD35 and $CD4^+$ cells but not $CD68^+$, $CD8^+$, $CD54^+$, $TGF\beta^+$ and HLA DR^+ cells (Fig.2 and 3). There were some perivascular $CD1a^+$ cells in the adjacent and remote dermis. The expression of factor VIII, CD54, VEGF and CD62E antigens by EC was similar in both areas, whereas of CD106 was significantly higher in dermis adjacent to the ulcer ($p< 0.05$). The expression of IL1α, IL1β, IL1Ra, EGF and PDGFa by EC was higher in dermis adjacent to ulcer than in normal tissue (Fig.5 and 7, $p<0.05$). The staining intensity of IL6, TNFα, TGFβ, TGFβ-R, FGF2 and GM CSF was similar in the adjacent and remote dermis.

Discussion

In skin wounds epidermal and dermal cells produce mediators, recruiting inflammatory and immune cells, as well as progenitory cells, perpetuating the healing process. The present study provided following information: (i) lack of Lagerhans cells in the new epidermis and normal expression of proinflammatory cytokines (IIα, IL1β, IL1Ra, IL6, TNFα) and growth factors (VEGF, GM-CSF, PDGFa, FGF2, TGFβ, EGF, EGF-R) by keratinocytes; (ii) presence of elastase producing granulocytes and activated $CD35^+$ macrophages, paucity of lymphocytes and TGFβ producing cells, lack of Langerhans' cells in the ulcer bed, suggesting continuing scavenger phase of healing despite of a long duration of ulcer; (iii) downregulation of FGF2 and upregulation of ELAM1 and VCAM1 antigens in new capillary endothelial cells in the ulcer bed, suggesting continuing inflammatory, not proliferative phase of healing; (iv) increased frequency of granulocytes, activated macrophages and $CD4^+$ lymphocytes in dermis adjacent to the ulcer, higher expression of proinflammatory cytokines (IL1α, IL1β, IL1Ra) and some growth factors (EGF, PDGFa) by endothelial cells, but not of other factors (IL6, TNFα, FGF2, GM-CSF) participating in normal healing process.

Unexpectedly, we did not find any $CD1a^+$ cells in a new epidermis encroaching upon the ulcer surface. Rosner et al.[7] observed four times fewer number of $CD1a^+$ cells in the ulcer margin compared to clinically intact epidermis. The reduced level of Langerhans' cells in skin and dendritic cells in lymphoid organs of mouse treated in vivo with VEGF was described. [8] The VEGF was also directly responsible for inhibition of dendritic cell maturation from $CD34^+$

precursors in vitro [9] and was chemotactic for monocytes.[10] Our present study has demonstrated only slightly increased expression of VEGF in epidermis at the ulcer edge and concomitantly lack of Langerhans cells. The VEGF is constitutively produced by keratinocytes and TNFα activated neutrophils. [11] Recently, Tian et al. [12] observed more intensive VEGF staining of keratinocytes at the edge of nonhealing leg ulcers compared to healing ulcers. It is likely that the increased production of VEGF at the edge of nonhealing ulcer can affect an influx and/or differentiation of Langerhans' cell precursors. Upregulated expression of VEGF and its mRNA in basal and suprabasal layers of epidermis in chronic leg ulcer margin was also observed by Lauer et al. [13] In contrast, Peschen et al. [14] observed a weak expression of VEGF throughtout all epidermis of chronic leg ulcers.

Another growth factor, GM-CSF, is a pleiotropic cytokine important for Langerhans' cell recruitment and development in epidermis, for proliferation of keratinocytes and infiltration of dermis by inflammatory cells. [15] I was shown that GM-CSF accelerates healing of chronic leg ulcers, [16,17] but not acute wounds.[18] Our study showed no changes in the expression of this cytokine throughout the dermis and epidermis of chronic leg ulcers. These results are consistent with data reported by Li et al. [19] and suggest that there is no upregulation of GM-CSF in the chronic leg ulcer tissue. The role of GM-CSF in wound healing is controversial, since a local application of recombinant GM-CSF reduced the number of fibroblasts in the granulation tissue and collagen deposition in the experimental surgical wounds.[20]

Generally, our findings concerning phenotyping of infiltrates in ulcer base and dermis adjacent and remote from the ulcer are in concordance with the results of other studies in venous leg ulcers. [7,21,22] Infiltrating cells, granulocytes and macrophages, were the dominant cell population present in the ulcer bed and the adjacent dermis. However, other authors did not emphasize the excessive frequency of elastase producing granulocytes in these lesions. It is likely, that persistance of neutrophils and their destructive enzymes, like elastase, appears responsible for chronicity of venous leg ulcers. There were only few lymphocytes in the ulcer bed and $CD4^+$ cells predominated in the adjacent dermis. The role of T cells in the healing of venous ulcer healing is limited. Data showed [23] that human wound-associated T lymphocyte populations were modulated during acute wound healing. It indicated a role of elevated $CD8^+$ cell density in downregulation of healing and wound closure. On the other hand, Rosner et al. [7] did not observe differences in the ratio of CD4/CD8 T cells between the different regions of leg ulcer.

Observed by us the increased expression of E-selectin and VCAM1 on endothelial cells in ulcer base and the adjacent dermis provides support of the notion that these regions are actively involved in recruitment of leukocytes. Rosner et al. [24] also observed the increased expression of E-selectin in ulcer bed. They have also indicated the upregulation of CD54 but not VCAM1.

The recruitment and activation of immune and inflammatory cells into skin are dependent on cytokines and growth factors. In this study, for the first time, we have demonstrated the expression of cytokines and growth factors by

capillary endothelial cells in ulcer dermis. The staining intensity of these factors was unchanged or increased at the ulcer margin compared to the ulcer base. The expression of all studied cytokines and growth factors in keratinocytes at the ulcer margin was not statistically different from that in distal epidermis. Tian et al. [12] compared the scored results of cytokines and growth factors expression in keartinocytes at the ulcer edge from the nonhealing and healing biopsies. There was no significant difference in the expression of IL1α, IL6, TNFα, FGF2 and EGF, except VEGF and PDGFa, expressed more intensely in biopsies from nonhealing ulcers. However, there was significantly higher intensity of staining in keratinocytes in the intact distal epidermis, in nonhealing compared to healing ulcers. Harris et al. [25] did not find statistically significant differences in the levels of IL1α, IL1β, IL6, FGF2, GM-CSF and PDGF-AB between wound fluids from patients with healing and non-healing leg ulcers, divided on clinical criteria. When Trengove et al. [26] have analyzed wound fluids from each individual patient, in both nonhealing and healing phase of chronic leg ulcer, they observed significantly increased levels of proinflammatory cytokines in non-healing phase and unchanged levels of growth factors during healing process. From all these studies it is apparent that impaired healing of venous ulcers is rather not due to a failured production or secretion of cytokines and growth factors. However, the lack of response to these factors in the ulcer tissue may be due to the reduced effectiveness of these factors caused by their enzymatic degradation, lack of receptors, presence of antagonists or soluble receptors.

Reepithelialization of wounds is due to proliferation and migration of keratinocytes at the wound edge. Previously, a highly proliferative epidermis in psoriasis was linked to IL8, [27] cytokine chemotactic for granulocytes. A variety of skin-derived cells, i.e., keratinocytes, fibroblasts, granulocytes and enothelial cells can synthesize IL8. We demonstrated only a weak IL8 reactivity in the endothelial cells in ulcer bed tissue. Recently, Iocono et al. [28] observed significantly increased level of IL8 in wound fluid from unhealing compared to healed burn wounds.

We observed the significant deficiency of FGF2 in the ulcer base. Cooper et al. [29] demonstrated the decreased levels of this factor in wound fluid from chronic pressure ulcers compared to the acute wounds. The role of FGF2 in impaired wound healing remains controversial. A large clinical trial of FGF2 in leg ulcers was stopped because no therapeutic effect was detected.[30]However, some investigators reported a marked increase of fibroblasts and capillaries in FGF2 treated chronic wounds.[31]

It is possible that the observed by us a weak expression of EGF and FGF-R in neoepidermis may enhance keratinocyte proliferation.[32] It was also shown that clinical effects of exogenous EGF on an accelerated wound repair can be due to an increased rate of keratinocyte migration after EGF treatment. [33,34] Our previous study concerning proliferative capacity of keratinocytes at the edge of venous leg ulcer demonstrated that the impaired epithelialization is not caused by the lack of epidermal stem cells, inadequate proliferation, differentiation or keratinocyte apoptosis at the edge of ulcer. [35]

The TGFβ integrates an action of several cell types involved in the tissue repair process and is an important endogenous regulator of wound healing. [36] We observed the unchanged intensity of staining for TGFβ in the neoepidermis and keratinocytes distal to the ulcer, as well as in immune cells present in dermis. Cowin et al. [37] observed a weaker than in normal unwounded skin TGFβ1, β2 and β3 staining within the epidermis and dermis surrounding the chronic venous ulcers.

In our study there was no reactivity of skin cells with anti- PDGFb antibody (data not shown) and no expression of PDGFa in the neoepidermis. However, the increased PDGFa staining in EC in ulcer base and dermis adjacent to the ulcer was observed. Pierce et al. [38] showed a markedly reduced PDGFa expression in fibroblasts and capillaries as well as a lack or a weak expression of PDGFb in chronic wounds. Upregulated expression of PDGFa was seen in keratinocytes from nonhealing venous leg ulcers. [12] Such discrepancy may reflect differences between the used antibodies and the various phases of healing in studied tissues.

In conclusion, keratinocytes at the edge of venous leg ulcer revealed normal secretory capacity, however, there was a limited recruitment of immune cells partcipating in wound healing to dermis surrounding ulcer and ulcer bed. The mechanism of inhibited immune cell extravasation in the ulcer base and dermis adjacent to the ulcer, despite of E-selectin and VCAM1 expression on EC, remains unclear. There was no marked upregulation of cytokines and growth factors in the ulcer tissue and no remodeling of the ulcer bed tissue was

observed. It is likely that healing of venous leg ulcer may be improved by modulating of the persisting inflammatory process. It can be reached by activation of Langerhans' cells at the wound edge, controlling factors that stimulate extravasation of immune celi precursors from circulation to the ulcer bed (like chemokines), controlling factors that inhibit inflammatory response and down-regulate neutrophil infiltration or neutralize their elastase production [39] and activate angiogenesis, fibrogenesis and extracellular matrix formation. Recently, the succesful engraftment of bone marrow- derived cells in the treatment of non-healing chronic wounds was described. [40]

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Acknowledgement

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Fig.6 Immunohistochemical staining with anti-CD1a antibody shows lack of Langerhans' cells in epidermis (E) enroaching upon the granulation tissue (G) (a) and their marked expression in epidermis distal to the ulcer (b). Arrows indicate individual Langerhans' cells among numerous immunostained cells.

Fig.7 Immunohistochemical staining of PDGFa shows numerous positive dermal (D) blood capillaries (BC) at the edge of ulcer under neo-epidermis (E).

Figure legends:

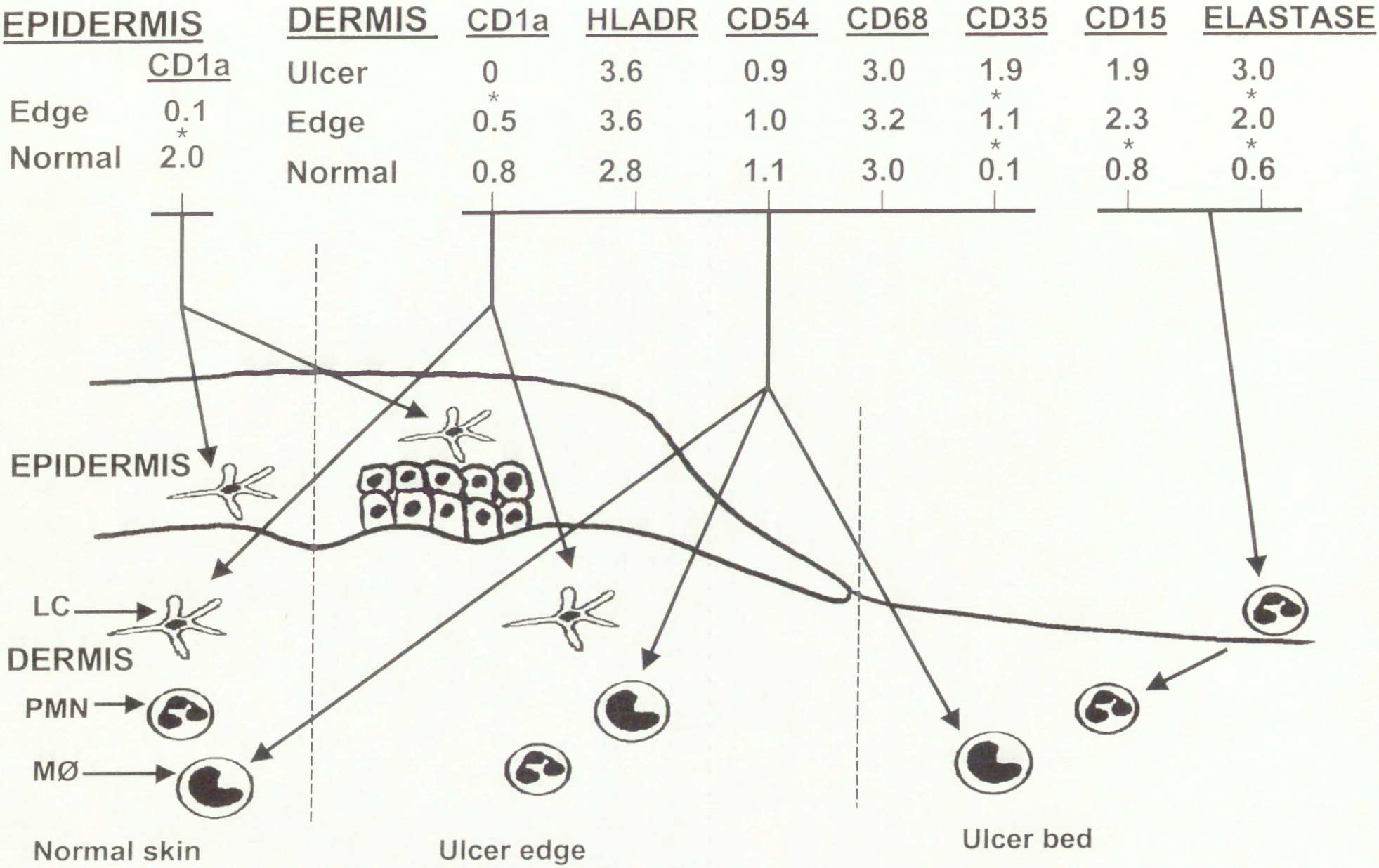
Fig.1 Histologic section of ulcer dermis (D) and epidermis (E) adjacent to the venous leg ulcer shows granulation tissue (G), acellular matter (AM) and few blood capillaries (BC) in the upper layer of ulcer tissue.

Fig.2 Mean staining intensity of antigens characteristic for immune and infiltrating cells in different areas of the margin biopsies of chronic venous ulcers. (* $P < 0.05$ ulcer or edge versus normal area, score:0= no staining, 1= weak staining, 2= moderate staining, 3= strong staining).

Fig.3 Mean staining intensity of antigens characteristic for lymphocytes, macrophages and capillary endothelial cells in dermis from the margin biopsies of chronic venous leg ulcers. (* $P < 0.05$ ulcer or edge versus normal area, score: 0= no staining, 1= weak staining, 2= moderate staining, 3= strong staining).

Fig.4 Immunohistochemical staining shows excessive infiltration of elastase producing neutrophils (a) and CD35 positive macrophages (b) in the granulation tissue (G). Arrows indicate individual cells among numerous immunostained cells.

Fig.5 Mean staining intensity of cytokines and growth factors in keratinocytes and endothelial cells in different areas of the margin biopsies of chronic venous leg ulcers. (* $P<0.05$ ulcer or edge versus normal area, score: 0= no staining, 1= weak staining, 2= moderate staining, 3= strong staining).



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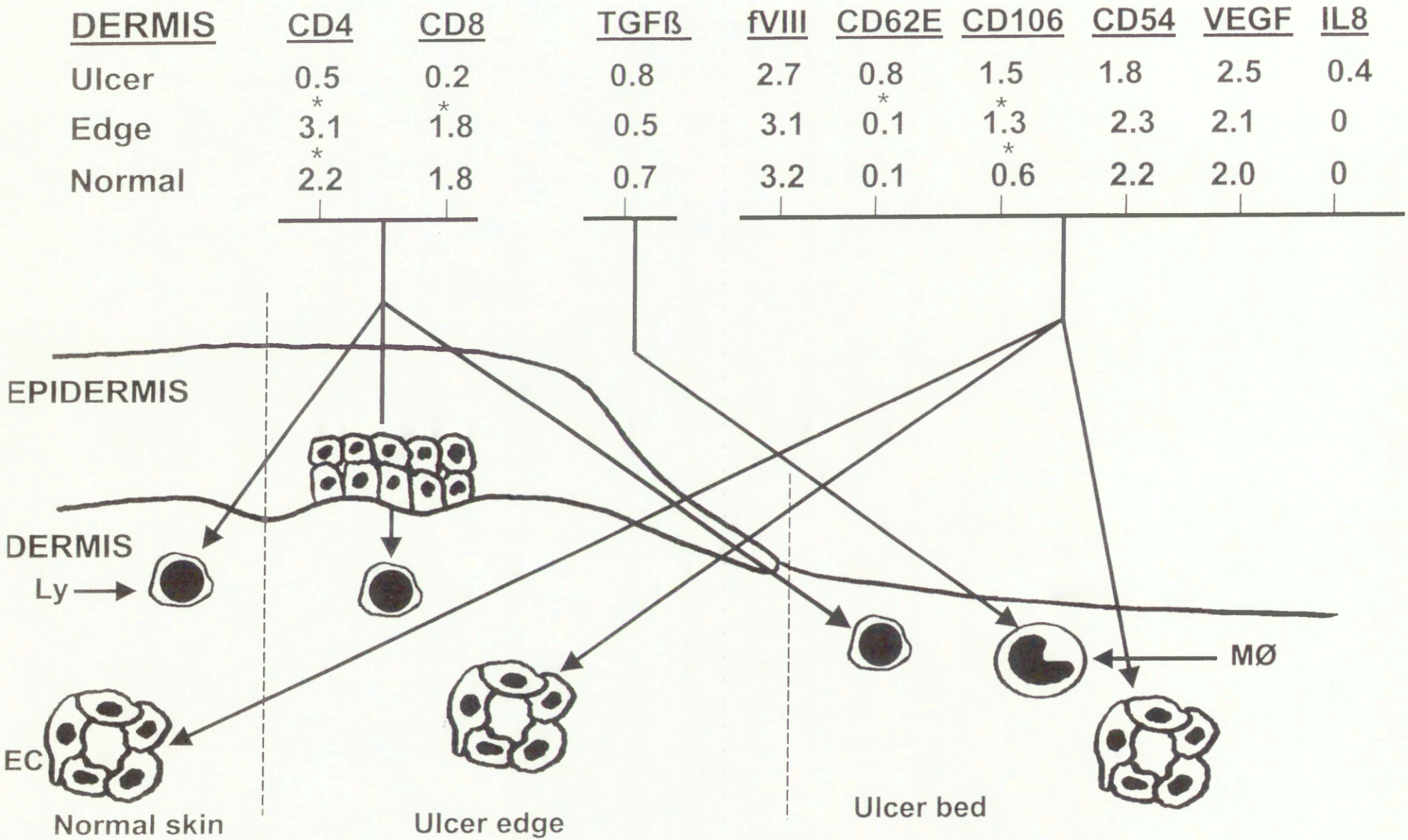

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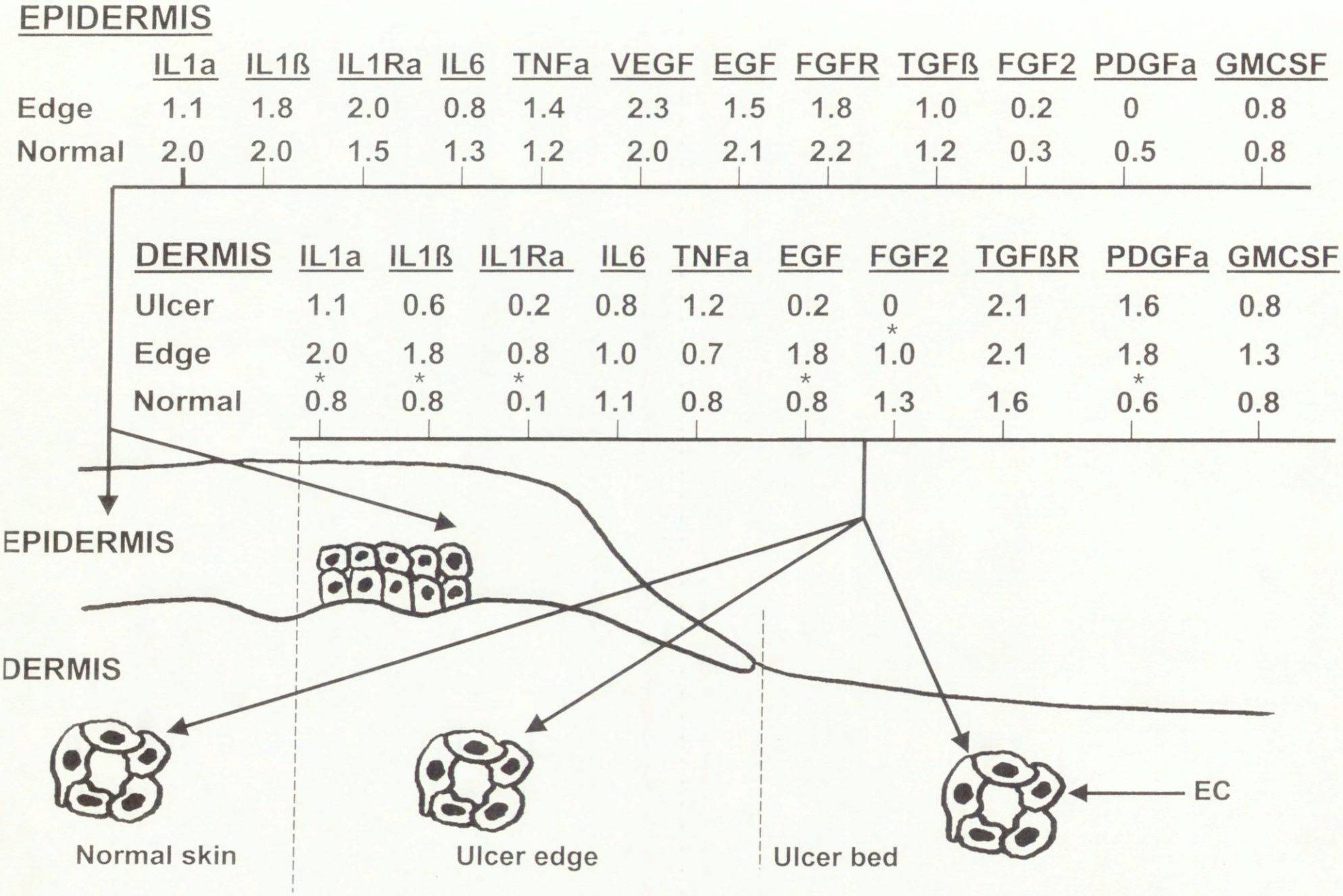

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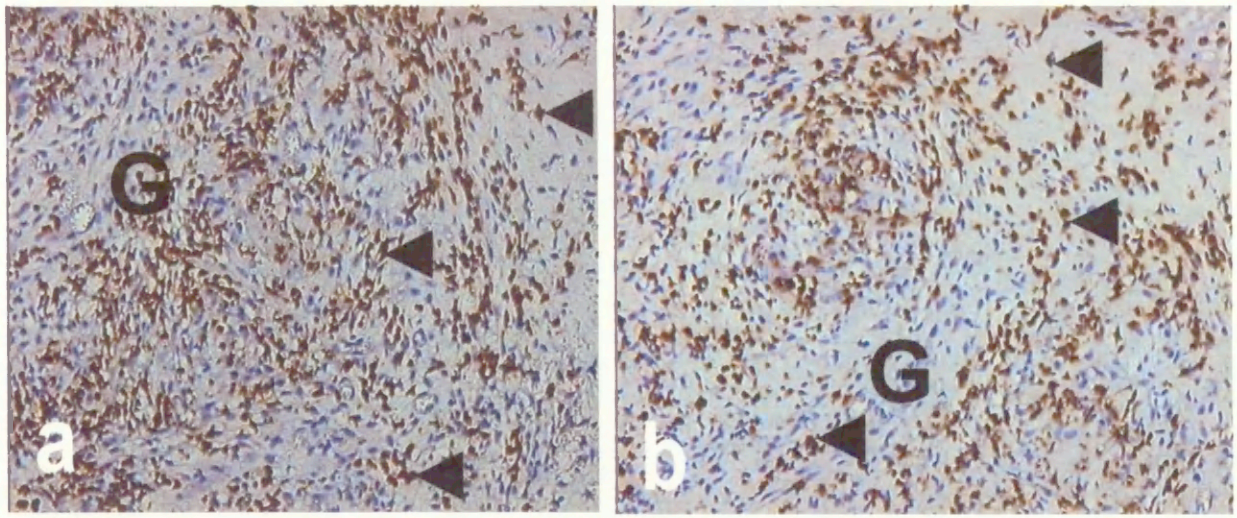

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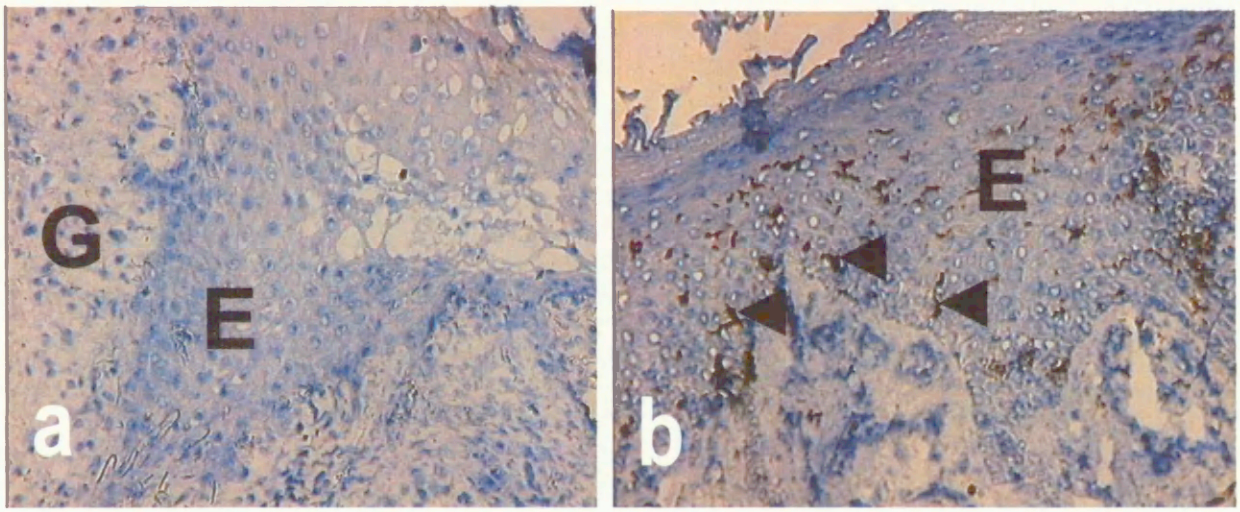

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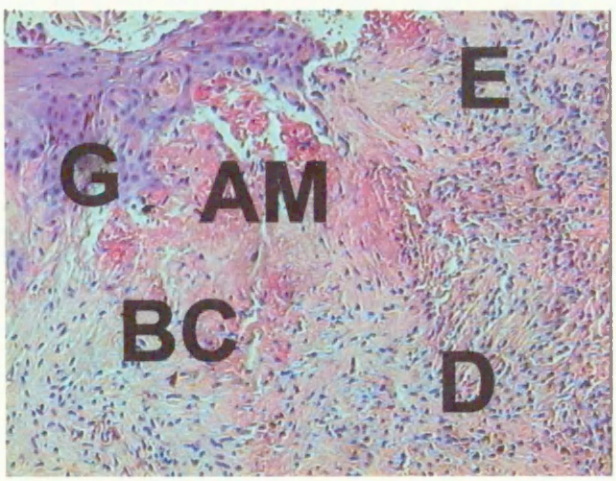

Fig. 1.

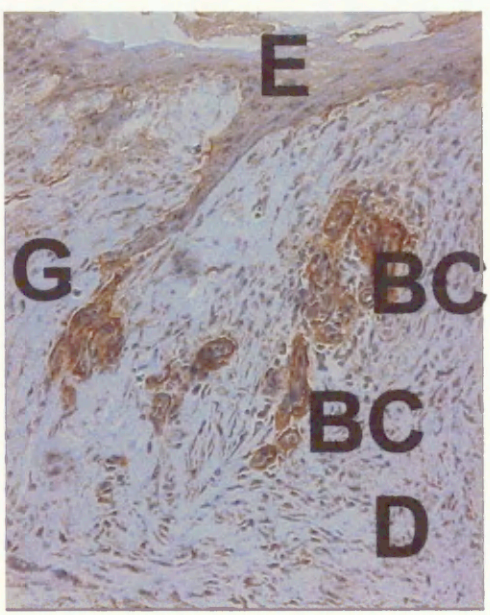

Fig. 7.

Dendritic Cells as Regulators of Immune Reactivity: Implications for Skin Transplantation

Hanna Galkowska

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Abstract:
Skin allografts, in contrast to other organ transplants, are acutely rejected despite intensive and toxic for the graft recipient immunosuppressive therapy. Long-term immunosuppression increases the risk for life- threatening infections and cancers. This is why clinical skin allografting practically does not exist. Skin Langerhans (dendritic) cells play a crucial role in the process of alloantigen recognition, its processing and initiation of the rejection reaction. These cells mature and migrate from the epidermis toward the dermal initial lymphatic vessels and further with afferent lymph, as veiled cells, they flow to the regional lymph nodes. Since a major goal in transplantation research is to understand and exploit the immunogenic properties of "passenger cells" as well as the tolerogenic properties of immature dendritic cells, studies concerning migrating less matured veiled cells obtained from afferent lymph draining skin seem to be relevant. Knowledge of mechanisms responsible for immunological synapse formation by veiled cells upon stimulation with allogeneic and bacterial antigens and of immunosuppressive drugs effect on this process, as well as of localization of Langerhans cells in skin epidermis and dermis in the inflammatory foci, would facilitate a rational approach for the therapeutic protocols enabling the prolongation of skin allograft survival time.

Key words: Dendritic Cells; Afferent Lymph; Epidermis; Antigen Presentation; Skin Transplantation

Introduction

Skin allografts, in contrast to other organ transplants, are acutely rejected despite intensive and toxic for the graft recipient immunosuppressive therapy. Long-term immunosuppression increases the risk for life-threatening infections and cancers. This is why clinical skin allografting practically does not exist. Recent cases of hand transplantation performed in various world centers have renewed the interest in basic studies on the pathomechanism of skin allograft rejection, since skin is the most actively rejected component of the tissue composite graft [1]. At the time of organ transplantation, a variety of non-parenchymal cells are transplanted simultaneously with the allograft. Recognition of the importance of these cells as potential immunostimulatory cells led to the concept of "passenger leukocytes" as the principal instigators of rejection. There are among them the dendritic cells (DCs).

Skin dendritic cells play a crucial role in the process of recognition of alloantigens, its processing and initiation of the rejection reaction. DCs are a unique population of leukocytes, and are the most effective antigen presenting cells (APC).

They originate from CD34+ bone marrow stem cells and their precursors are seeded via the bloodstream to the tissues where they give rise to immature DCs. Majority of non-lymphoid tissues and organs contain DCs, including skin, heart, liver, lung and mucosal surfaces [2]. There are three pools of DCs isolated from skin: a/ epidermal Langerhans' cells (LCs), b/ dermal DCs (DDCs), c/ afferent lymph veiled cells (VC) [3]. Cells from each cutaneous compartment can exhibit a distinct morphology, surface phenotype, and function. The bone marrow derived precursors of DCs extravasate in the dermis and give rise to DDCs and migrate toward epidermis to locate between the keratinocytes as LCs. Human skin is constantly barraged by a wide assortment of infectious agents including bacteria, fungi and viruses. Skin as a complex ecosystem with numerous microbial inhabitants presents highly efficient and evolutionarily conserved mechanisms of the innate immune system preventing colonization of deep tissues by the environmental antigens (defensins, toll receptors, NODs) [4]. LCs, as immature DCs have ability to uptake antigen and degrade it to produce peptides capable of binding to MHC class II. In response to tissue damage, inflammatory cytokines and antigen activation LCs mature and migrate from the epidermis toward the dermal initial lymphatic vessels [5] and further with afferent lymph stream, as VCs, they flow to the regional lymph nodes (Figure 1) [6]. VCs carry processed antigens or self antigens to lymph nodes where they interact with CD4 T lymphocytes to initiate immune responses, to mobilize lymphocyte and monocyte populations from blood perfusing the node, and to form the antigen-specific cohorts of effector cells. DCs do not appear in efferent lymph and therefore must accumulate in the lymph node as interdigitating cells and die by apoptosis [7].

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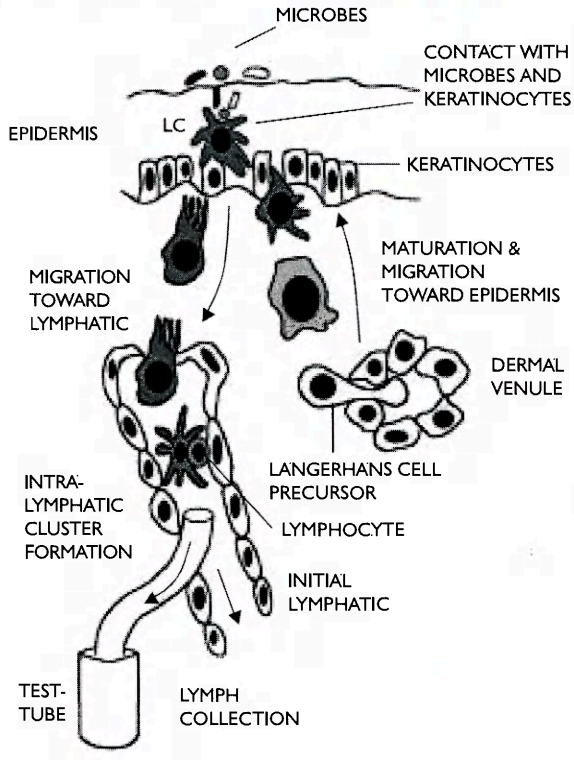

Figure 1. Migration of Langerhans' cells from venules toward epidermis and initial lymphatic (arrows).

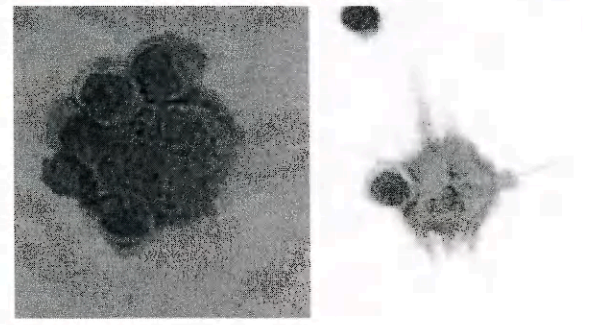

Figure 2. Clusters of veiled cells with lymphocytes isolated from afferent lymph, cytospun onto glass slide and stained immunocytochemically with anti- DR- antibody.

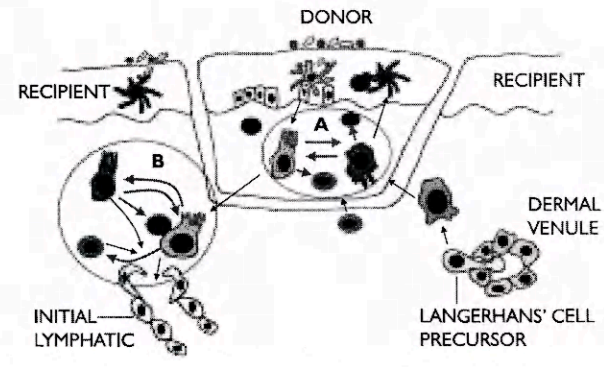

Figure 3. Stimulators and responders of both donor and recipient origin in A- skin graft and B- recipient graft bed. Full arrows indicate cells forming synapses and bidirectional stimulation. Thin arrows show direction of cell traffic.

In order to present antigen to lymphocytes, DCs have to come into physical contact with these cells and form a cluster (Figure 2). Recently, a term "immunological synapse" has been coined for this type of cluster [8]. The onset of signaling and T cell activation by DCs involves formation of a synaptic structure at the DCs-lymphocyte interface, with many features similar to those of the neuronal synapse. In addition to the MHC-TCR contact, this structure includes co-stimulatory and adhesion molecules which interact with counter-receptors. A synapse is composed of the peripheral and central supramolecular activation clusters. The main function of the synapse is to serve as a device for initiating, stabilizing and sustaining TCR signal transduction during DC encounter. The assembly of mature synapse may take several minutes only [9].

Interestingly, in skin immune reactions clusters between VCs and lymphocytes may be already seen in the dermal interstitial space and lymph reaching lymph node [10,11]. This suggests the VC-lymphocyte cooperation to start already at the site of inflammation and VCs may "select" lymphocytes for migration in afferent lymph to peripheral lymph node [7]. In skin allografting the process of allorecognition and effector reaction is more complex than that after bacterial penetration of skin. The donor provides in the skin graft immunologically active keratinocytes, LCs, dermal macrophages and lymphocytes, whereas the recipient mobilizes own DCs, macrophages and lymphocytes in order to eliminate donor-specific antigens (Figure 3). This complex local reaction is a new biological phenomenon as no MHC disparate skin-to-skin transfer occurs in nature. Each skin allotransplant evokes a host-versus-graft and graft-versus-host reaction.

The fact that DCs are the most potent inducers of T cell responses led to high interest for their clinical application. In recent years, DCs were increasingly studied for their role as critical adjuvants in vaccines for prevention of microbial infection and for treatment of cancer and autoimmune diseases. DCs can be propagated in vitro from the bone marrow and peripheral blood using various combinations of growth factors [12]. However, the in vitro generated dendritic cells undergo maturation in culture and after injection into skin have been shown to remain at the site of injection [13,14]. Since a major goal in transplantation research is to understand and exploit the immunogenic properties of passenger DC as well as the tolerogenic properties of immature DCs [15], studies concerning migrating and less matured VCs obtained from afferent lymph draining skin than cultured DCs seem to be more relevant.

Investigation of the in vivo- initiated reaction between migrating LC (VC) and lymphocytes

(afferent lymph cell clustering) need an appropriate model providing sufficient numbers of cooperating cells. This is not feasible in rodents with tiny skin afferent lymphatics containing few lymph cells. Therefore, a canine model with wide afferent lymphatics and sufficient numbers of circulating lymph cells was chosen. The proximal ligation of limb lymphatics and removal of the popliteal lymph node prior to harvesting lymph cells for laboratory studies allowed consecutive lymph sampling by vessel puncture.

Harvesting, enrichment and function of canine afferent lymph veiled cells

Harvesting and enrichment of VC was achieved in vivo in dogs following surgical interruption of hind limb lymphatic vessels [16]. The stagnant lymph of such dogs contains 10-30-fold more cells compared with normal lymph [17] and VCs present in the afferent lymph can be enriched from 6% to about 50% by density gradient centrifugation on 15% metrizamide or discontinuous Percoll gradients. These cells were strongly Ia positive, had cytoplasmic S 100 protein, were strongly ATP-ase positive and their activities of acid phosphatase, peroxidase and of non-specific esterase resembled these described for VC from other species [19,20]. Canine VC were able to stimulate T cells in allogeneic MLR and in auto-MLR when present at cell concentrations as low as 5% of the responding cells. Moreover, they exhibited the PHA presenting function characteristic of autologous lymphocytes [21]. These results indicate a close morphological, cytochemical and functional analogy between the veiled cells of dogs and other species.

Antisera against afferent lymph veiled cells

Several authors have suggested that the cells responsible for the initiation of allograft rejection are passenger cells. The effectiveness of the Ia-positive veiled passenger cells of the skin collected from afferent lymph in provoking allogeneic response and in initiating renal graft rejection has previously been described. Whether anti-Ia antiserum reduces the number of Langerhans' cells in skin allograft is not known. By arresting the migration of Langerhans' cells from graft into lymphoid tissue or their maturation into immunostimulatory veiled cells, a new strategy for overcoming allograft rejection could be raised by using antisera against these immunoreactive cells. There was a major problem with raising such antisera due to difficulties in obtaining sufficient numbers of dendritic cells from epidermis or afferent lymph and no data on the possible effects of specific anti-Langerhans' cell sera were available. Canine afferent lymph cells were adequate for immunization and development of anti-veiled cell antiserum. Raised by us and used in vitro anti-serrum blocked the Ia and CD1 antigens of VC on smears and inhibited the accessory function of VC in cell response to phytohemagglutinin (PHA) and their stimulatory activity in mixed leukocyte reaction (MLR) [22]. In vivo, the local, intracutaneous administration of antisera led to a transient depletion of VC from afferent lymph, and to reduction of mononuclear cell density in the T-dependent areas of regional lymph nodes. Depletion of T-dependent areas in lymph nodes with sparing of follicles with use of the anti-veiled cell sera supports that this antiserum was directed primarily if not exclusively against dendritic cells and T lymphocytes.

Immunocytochemical markers of canine dendritic cells

Unlike those of man and mouse, canine dendritic cells can be poorly characterized, because only limited numbers of monoclonal antibodies (mAb) to their antigens are available. The first workshop on canine leukocyte antigens has allowed the identification and characterization of a set of anti-canine mAb that enable the extension of immunological research in the dog [23]. However, the specificities of these antibodies are still not clear enough to identify the canine equivalent of human CD antigens. For instance, canine CD4 antigen was detected at high levels on granulocytes and MHC class II antigens were found on all canine leukocytes.

We screened monoclonal and polyclonal antybodies reacting with human leukocytes for their cross-reactivity with canine leukocytes on cytospins using immunocytochemical methods [24]. Of 50 antibodies 22 cross-reacted with canine leukocytes from afferent lymph and peripheral blood. A large number of antybodies also reacted with canine lymph veiled cells. We have raised and described two novel mAbs that recognize antigens expressed by the canine lymph derived DC [25]. They reduced lymphocyte binding to DC and T cell proliferative response to DC-associated alloantigens in the MLR. Both, whole antibody molecules as well as F (ab') fragments had the modulatory effect on lymph cell binding. It can be suggested that the suppressive effects of these mAbs have not been due to the blocking of receptors for antigen recognition, but rather to the blocking of the accessory molecules on DC. The epitopes recognized by our novel mAbs are unknown.

Spontaneous cluster (immunological synapse) formation by afferent lymph veiled cells and lymphocytes

Epidermal Langerhans cells form a unique subset of dendritic cells. These cells incorporate, in vivo, the environmental antigens penetrating skin and this may prompt them to undergo 'spontaneous' clustering with T lymphocytes in afferent lymph. Dendritic cells bind lymphocytes in an antigen-independent pathway and can also bind resting T cells [26]. Controlling this process may be helpful in mitigation of skin immune reactions. Therefore, we have tried to elucidate the mechanisms of 'spontaneous' binding of veiled cells from the canine skin afferent lymph with autologous lymphocytes in their own environment, i.e. the lymph, and in the absence of a known antigen [27]. The number of clusters forming ex vivo in the collected lymph samples increased as a function of time and was temperature dependent. Incubation of cells with proteolytic enzymes or monosaccharides did not alter cell interactions. The ability of veiled cells to bind

lymphocytes was independent of divalent cations but reduced by xylocaine and retinoic acid. Among steroids only methylprednisolone showed an inhibitory effect on cluster formation. Indomethacin and acetylsalicylic acid had no blocking activity on cell binding. Also, no effect was seen after treatment with cyclosporine A and azathioprine.

Factors governing this process are not clear, however the expression of accessory molecules on DC is thought to play a crucial role. A question arised which cytokines (other than GM-CSF and TNFα) responsible for LC maturation [28] can regulate expression of adhesion molecules and process of DC-T cell cluster formation. Skin is a rich source of cytokines primarily active locally in the microenvironment of cells in skin. Supernatants from highly enriched murine epidermal LC cultures contain bioactivities of IL1, IL6, GM-CSF and TNFα [29]. Furthermore, keratinocytes either produce constitutively, or can be induced to produce a variety of cytokines, among them IL1, IL6, IL8, IL10 and TNFα. Our studies showed [30] that the CD54 and CD58 molecules play a key role in the 'spontaneous' lymph cell clustering. Antibody against fibronectin, a substrate for CD49d and CD49e receptors, reduced DC-lymphocyte binding. Analysis of the effect of cytokines revealed that the pro-inflammatory IL1β rather than IL1α, and TNFα may be responsible for the enhanced lymph cell in vitro clustering. The IL6 had no such augmenting effect. The IL1Ra alone had no effect on cell binding, even when used in the high doses. Neutralizing of IL1Ra in lymph with the specific antibody brought about augmented cluster formation. The enhancing properties of TNFα on cell binding were reduced by the TNFα neutralizing antibody. The IL10 significantly limited lymph DC cluster formation with T cells. It provides insight into the potential clinical use of these inhibitors.

Veiled cells initiate skin allograft rejection by indirect pathway of recognition

It is generally accepted that there are two pathways of allorecognition. In the "direct" pathway host T cells interact with intact alloantigens on the surface of donor cells. In the "indirect" pathway host T cells recognize processed alloantigen presented by self-antigen presenting cells (APC). It has been suggested that the acute rejection of skin allografts is mediated predominantly by the direct pathway, because the graft contains a significant number of LC and dermal DC. There is also increasing evidence for a significant role of the indirect pathway in alloresponse [31].

The complexity of cellular interactions in skin allograft rejection prompted us to find a model where the role of recipient DC in inhibition of the rejection process could be studied. We have used a canine skin to severe combined immunodeficient (SCID) mouse transplant model [32]. Two weeks after skin grafting, when donor LC had migrated out from the transplant, [33] canine peripheral lymph VC and lymphocytes or peripheral blood mononuclear cells (PBMC) allogeneic to the graft were injected intraperitoneally, and the rejection reaction was followed. These data suggest that accumulation of canine T cells in the graft is mediated by the presence of VC in the injected population and that infiltrating cells may be activated to alloantigen presented by the VC. The importance of VC in allograft destruction is supported by our observation that $CD3^+$ T-cell infiltrates in graft dermis and epidermis were more extensive after injection of the whole population of lymph cells (with VC) than PBMC.

Tacrolimus but not Cyclosporin A inhibits clustering of veiled cells with lymphocytes in allogeneic and autologous cell cultures

The question arises as to whether cutaneous VC, the principal cells responsible for skin allograft rejection, undergo inhibition by other than Cyclosporin A (CsA) immunosuppressive drugs during cell clustering with autologous lymphocytes. Immunosuppressants like CsA, azathioprine, dexamethason, and hydrocortisone had no effect on VC clustering with autologous lymphocytes in vitro. In contrast, methylprednisolone (MP) and FK506 had a potent inhibitory effect, even at very low doses [34]. These findings are consistent with the results concerning cluster formation in the presence of CsA by human blood DC [35]. They confirm also that FK506 and MP could be superior to CsA in prolongation of skin allograft survival [36].

Inhibition of immunological synapse formation between DC and lymphocytes would presumably down-regulate alloantigen recognition in skin allografts and the response to bacterial or viral infections. CsA and Tacrolimus (FK 506) have been shown to alter DC differentiation and lymphocyte activation [37]. We investigated the effect of CsA and FK 506 on the rate of in vitro formed synapses and on the expression of CD49d antigen, the molecule actively participating in forming of lymph DC- lymphocyte synapse [38]. Cells obtained from lymph draining skin were cultured for 6 days in allogeneic and autologous combinations, in the presence or absence of CsA and FK 506. Thereafter, the cytospins of cultured cells were examined and percentages of the formed synapses and CD49d expression were established. The frequency of formed synapses was greater in allogeneic than syngeneic cell combinations. Cells treated with FK 506 showed a decreased rate of both autologous and allogeneic synapses as well as lower expression of CD49d. CsA did not inhibit synapse formation either in autologous or allogeneic cell combinations. However, CsA significantly reduced expression of CD49d antigen on both lymph DC and lymphocytes. It suggests that also other molecules were involved in formation of synapse.

Bacteria-stimulated veiled cells form clusters with lymphocytes

Transplanted allogeneic skin becomes ischemic in course of the rejection process. Ischemia facilitates penetration of microorganisms residing of the skin surface and skin appendices. Bacteria penetrating skin evoke an immediate chemotactic reaction resulting in recruitment of granulocytes, phagocytosis and desintegration of the penetrator. The locally residing Langerhans' cells, macrophages and lymphocytes as

well as mobilized from blood immune cells become activated. Presentation of antigen requires direct physical contact of dendritic cell with lymphocytes and formation of cluster (immunological synapse). In inflammation, the number of formed clusters is directly proportional to the intensity of the immune process developing in the tissue is lymph drained from.

We investigated the kinetics of lymph VC- lymphocyte cluster formation during a 12h in vitro stimulation with Staphylococcus warneri and the effect of penicilin on this process [39]. The bacteria-stimulated VCs have formed more clusters with lymphocytes than control cells. The proliferative response of lymphocytes to bacteria-stimulated VCs was higher than in cultures with non-stimulated VCs. Benzathine penicillin decreased lymphocyte proliferative responsiveness irrespective of whether the cocultured VCs were stimulated with bacteria or not. It seems that during skin allograft rejection veiled cells react to both allogeneic and bacterial antigens. This cumulative process may be more destructive to the graft than rejection of organ transplants without resident bacterial flora.

The margin of skin ulcers, an environment for epidermal Langerhans' cells migration and cooperation with keratinocytes and lymphocytes

Langerhans' cells are typically localized in the basal and suprabasal layers of the epidermis and represent the principal hematopoietic barrier to the external environment. The mobilization of LCs to regional lymph nodes as well as the recruitment of their precursors from the circulation into the skin must be tightly regulated events. Certain, so far unknown, conditions should be met in order LC precursors to lodge in the epidermis, cooperate with keratinocytes and migrate upon stimulation towards the initial lymphatics. In lethally irradiated mice transplanted with congenic bone marrow cells epidermal LCs of host origin remained for at least 18 months, whereas DCs in other organs were almost completely replaced by donor cells within 2 months [40]. In parabiotic mice with separate organs, but a shared blood circulation, there was no mixing of LCs. However, in skin exposed to ultraviolet light, LCs rapidly disappeared and they were replaced by precursors within 2 weeks.

In inflamed skin multiple changes occur, including the secretion of chemokines and cytokines by keratinocytes, allowing a marked loss of LCs and the recruitment of LC precursors.

We studied the environment for LC to lodge in the inflamed skin, specifically in epidermis, and to make a functional contact with keratinocytes and other cells. Studies were carried out in the patients with diabetic foot and varicose ulcers of the calf [41]. Skin biopsy specimens were obtained from the border area of ulcers and the topographically corresponding sites of normal skin of patients undergoing orthopedic surgery. At the edge of both types of ulcer keratinocytes were p63+, CD29+, PCNA+ and p53. The mean intensity of p63 and CD29 staining, markers of epidermal stem cells, was higher than in controls. Data have showed that impaired epithelialization of chronic leg ulcers is not caused by an inadequate epidermal stem cell proliferation, differentiation or apoptosis. It may rather reflect the distored organization of wound bed, caused by infection and impaired nutrition supply, altering keratinocyte migration. We also studied the presence of epidermal and dermal LCs at the margin of venous ulcers [42, unpublished data]. Biopsies from leg ulcers of 10 randomly selected patients were examined immunohistochemically for leukocyte phenotypes, vascular adhesion molecules and cytokines and growth factors produced by keratinocytes (KC) and vascular endothelial cells (EC). There were no $CD1a^+$ Langerhans' cells in the epidermis encroaching upon the granulation tissue and also there was reduced CD1a staining in the adjacent epidermis ($p<0.05$). Granulation tissue contained few fibroblasts and blood capillaries, with high intensity of staining for CD62E and CD106 but not for FGF2 on EC ($p<0.05$). The intensity of staining for scavenging $CD15^+$ $elastase^+$ granulocytes and $CD35^+$ (C3bi) macrophages in ulcer bed was comparable to that in the margin but higher than in distant dermis ($p<0.05$), whereas that for $CD68^+$, HLA DR^+, $TGF\beta^+$ and $CD54^+$ dermal leukocytes was similar in all areas. There was reduced staining for CD4+ and CD8+ cells in ulcer bed ($p<0.05$). The expression of cytokines and growth factors by KC was similar in the areas adjacent and remote from ulcer. In dermis adjacent to ulcer the expression of IL1α, IL1β, IL1Ra, EGF and PDGFa was higher than in distant dermis.

Although keratinocytes at the edge of venous leg ulcer revealed a normal cell- cycle proteins as well as cytokine and growth factors secretory capacity, there was a limited recruitment of CD1a+ Langerhans cells to dermis and epidermis surrounding the ulcer. Insufficient levels of locally produced chemokines [43] or low expression of their receptors on migrating cells, and lack of a proper molecular modeling of niches for LC precursors lodgement may be responsible for lack of LC- keratinocyte contacts in epidermis.

Conclusions

A unique model for studies of the in vivo-formed functional clusters between dendritic (veiled, Langerhans cells) was worked out in a large animal (dog) allowing to collect sufficient number of cells and test their functional capacities. Applied experimental model allowed drawing analogies to human conditions. The phenotype and reactivity to stimulating and inhibiting substances has been elaborated. Specific polyclonal anti-serum and monoclonal antibody had an inhibitory effect on veiled cell reactivity both in vitro and in vivo. The proinflammatory cytokines stimulated and anti-inflammatory cytokine and drugs as pentoxyfillin, FK 506 and methylprednisolon but not CsA inhibited formation of clusters. Bacterial antigen increased in lymph culture the cluster formation rate. Interestingly, long-term penicillin downregulated this process. In contrast to the in vitro observations, the in vivo cooperation of veiled Langerhans cells with lymphocytes and other cells seemed to be additionally governed by the environmental factors. In inflamed

tissue there was less recruitment of Langerhans cells despite of high cytokine expression. A proper molecular niche is probably needed.

These studies provided more insight into crucial cellular events in the skin immune system that is antigen presentation between the migrating dendritic cells and lymphocytes forming physical cell cluster and specifically the effectiveness of drugs on this process. The applied model allows further investigations on manipulating cell clustering with immunosuppressive drugs resulting in prolongation of skin graft survival. The recently published data of efficacy of FK506 in prolongation of human hand survival corroborate our results on inhibiting effects of this drug on cluster formation [44].

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