DENDRITIC CELLS IN FUNDAMENTAL AND CLINICAL IMMUNOLOGY

Volume 2

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DENDRITIC CELLS IN FUNDAMENTAL AND CLINICAL IMMUNOLOGY

Volume 2

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PREFACE

These Proceedings contain the contributions of the participants of the Third International Symposium on Dendritic Cells that was held in Annecy, France, from June 19 to June 24, 1994. This symposium represented a follow-up of the first and second international symposia that were held in Japan in 1990 and in the Netherlands in 1992.

Dendritic cells are antigen-presenting cells, and are found in all tissues and organs of the body. They can be classified into: (1) interstitial dendritic cells of the heart, kidney, gut, and lung;(2) Langerhans cells in the skin and mucous membranes; (3) interdigitating dendritic cells in the thymic medulla and secondary lymphoid tissue; and (4) blood dendritic cells and lymph dendritic cells (veiled cells). Although dendritic cells in each of these compartments are all $CD45^+$ leukocytes that arise from the bone marrow, they may exhibit differences that relate to maturation state and microenvironment. Dendritic cells are specialized antigen-presenting cells for T lymphocytes: they process and present antigens efficiently *in situ,* and stimulate responses from naive and memory T cells in the paracortical area of secondary lymphoid organs. Recent evidence also demonstrates their role in induction of tolerance. By contrast, the primary and secondary B-cell follicles contain follicular dendritic cells that trap and retain intact antigen as immune complexes for long periods of time. The origin of follicular dendritic cells is not clear, but most investigators believe that these cells are not leukocytes. Follicular dendritic cells present native antigen to B cells and are likely to be involved in the affinity maturation of antibodies, the generation of immune memory, and the maintenance of humoral immune responses. Three areas appeared as being the object of much impetus: (1) the establishment of methods allowing *in vitro* generation of large amounts of functional dendritic cells; (2) the role of accessory molecules in the development of an immune response; and (3) the role of (follicular) dendritic cells in AIDS pathogenesis.

The symposium was attended by more than 300 participants and 25 invited speakers. The format of the meeting was developed in order to permit optimal interactions and ample discussions between participants. Each of the seven sessions included at least three invited speakers and a total of 45 oral presentations were selected from abstracts. On the last evening of the symposium, five prizes were awarded to the five best posters according to a selection made by the Scientific Committee in terms of data, discussion, and presentation. The participants agreed that the award ceremony should be a part of future symposia.

We would like to express our deepest gratitude for the valuable support of "Ministère de l'Enseignement Supérieur et de la Recherche," INSERM, Fondation Marcel Mérieux and Schering-Plough. We gratefully acknowledge the support of "Agence

Nationale de Recherche sur le SIDA" (A.N.R.S.), the European Community (DGWII), "Ligue Nationale de Recherche contre le Cancer," Claude Bernard University (Lyon), Immunotech, Dynal, Polylabo, and Sigma. We also thank the companies listed below for their contributions to our symposium.

In closing, we wish to convey our warmest thanks for the various support we obtained (financial and otherwise), without which we could not have implemented this symposium.

The next symposium will be organized by Dr. Ricciardi-Castagnoli and Dr. Lanzavecchia, most probably in 1997, in Italy.

Organizing Committee

Dr. Jacques Banchereau
Schering-Plough

Dr. Daniel Schmitt
INSERM U346

Dr. Louis Valette
Fondation Marcel Mérieux

Acknowledgments

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DENDRITIC CELLS IN FUNDAMENTAL AND CLINICAL IMMUNOLOGY

Volume 2

CHARACTERIZATION OF HUMAN CD34+ DERIVED DENDRITIC / LANGERHANS CELLS (D-Lc)

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INTRODUCTION

Dendritic cells (DC) are professional antigen presenting cells which are found in all lymphoid and non lymphoid organs. The various DC are thought to represent different steps of maturation of the same lineage which function as sentinel of the immune system. In the periphery, DC such as Langerhans cells (Lc) capture the antigen, then migrate via the lymphatics and home to the T cell rich area of lymph nodes where they are called interdigitating cells (IDC). There, they present processed antigen to naive T cells and generate an antigen specific primary T cell response (1). DC are from bone marrow origin (2) and we and others have shown, in human, the cooperation of GM-CSF and TNFα in the generation from hematopoietic progenitor cells (HPC) of high numbers of DC related to Lc (3, 4, 5) In the present study we show that i) These in vitro generated dendritic/ Langerhans cells (D-Lc) are very potent in priming naive T cells and in capturing, processing and presenting soluble antigen to antigen-specific T cell clones. ii) B70/B7-2 is identical to CD86 and is the major functionnal ligand for CD28. iii) Two subsets of D-Lc precursors were identified, which generate either Birbeck granules (BG) positive Lc or BG^- $CD1a^+$ DC.

MATERIALS AND METHODS

Umbilical cord blood samples were obtained according to institutional guidelines. Cells bearing CD34 antigen were isolated from non-adherent mononuclear fractions through positive selection by indirect immune "panning" using anti-CD34 monoclonal antibody (MoAb) (Imu-133.3, Immunotech, Marseille, France). Cultures were established in the presence of GM-CSF and TNFα in complete medium (10% FCS). After 12 days the cultures contained 40 to 80% $CD1a^+$ D-Lc (5).

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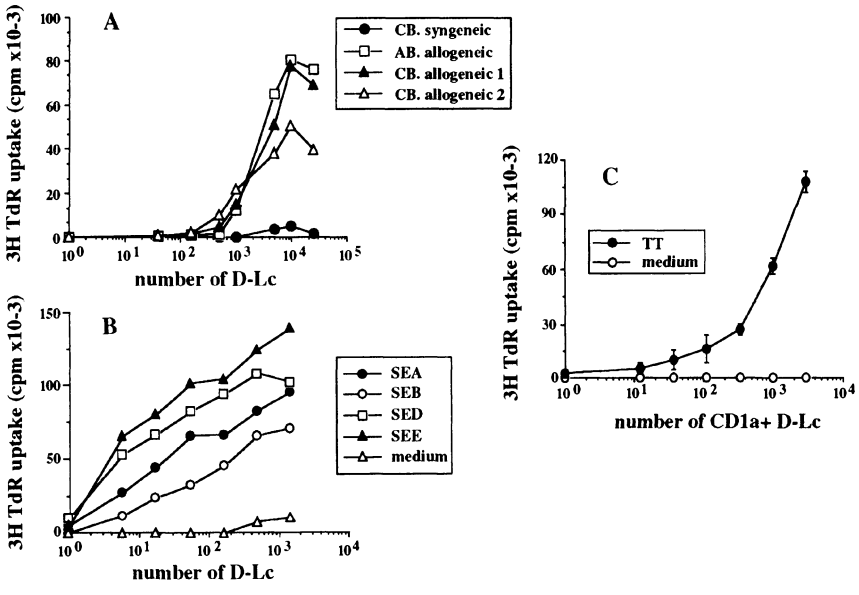

Fig.1: CD4+ T cells were purified from adult or cord blood by negative selection (≥95%). TT specific T cell clones were established from peripheral blood maternal cells. CD34+ progenitors derived D-Lc were used, after irradiation (40 Gy), as stimulator cells for A) resting allogeneic or syngeneic adult (AB) or cord (CB) blood CD4+ T cells ($2.5x10^4$ per well), B) resting syngeneic cord blood CD4+ T cells ($2.5x10^4$ per well) in presence of superantigens (1ng/ml), C) CD4+ T cells from TT specific T cell clones ($1x10^4$ per well).

RESULTS

1- Interaction between D-Lc and CD4+ T cells

As shown in Fig.1A, D-Lc induced a strong proliferation of both adult and naive cord blood allogeneic CD4+ T cells. Maximal T cell proliferation, observed for $5x10^3$ D-Lc and above, resulted in a 300 fold increase of ^{3}H TdR incorporation over the background, as measured after 5 days. 150 stimulator D-Lc (ratio D-Lc:T=1:170) induced a 10 fold increase over background of ^{3}HTdR incorporation of all the allogeneic CD4+ T cells. At $5x10^3$ (ratio D-Lc:T=1:5) and above, D-Lc were also found to induced significant proliferation of syngeneic CD4+ cord blood T cells, but at a magnitude 20 times lower than that observed for allogeneic CD4+ T cells. As shown in Fig.1B, all superantigens stimulated the proliferation of naive CD4+ T cells in the presence of syngeneic D-Lc. As few as 6 D-Lc induced a 10-20 fold increase of T cell proliferation in presence of any superantigen tested and half maximal T cell proliferation was generally observed with less than 60 D-Lc (ratio D-Lc:T=1:417) (Fig.1B). Electron microscopy demonstrated that CD1a+ D-Lc were able to capture a soluble antigen (gold labelled immunoglobulins) through receptor mediated endocytosis (not shown). Furthermore HLA matched tetanus toxoid (TT) specific T cell clones, were found to proliferate in response to low concentration of TT (40ng/ml) presented by CD1a+ D-Lc (Fig.1C). Thus, in vitro generated D-Lc can prime naive T cells and process native antigens what might be useful for priming antigen specific naive T cells for eventual cellular immunotherapy.

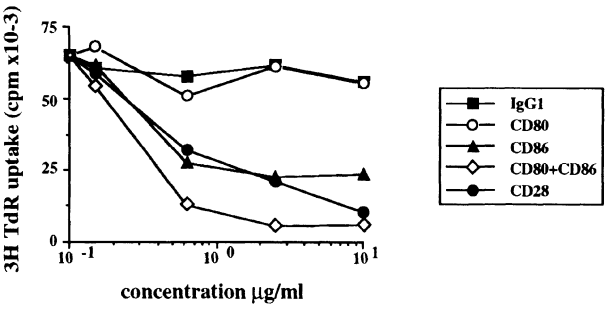

Fig.2: CD34$^+$ progenitors derived D-Lc were used, after irradiation (40 Gy), as stimulator cells for CD4$^+$ T cell proliferation in presence of anti CD80, CD86 and CD28 monoclonal antibodies.

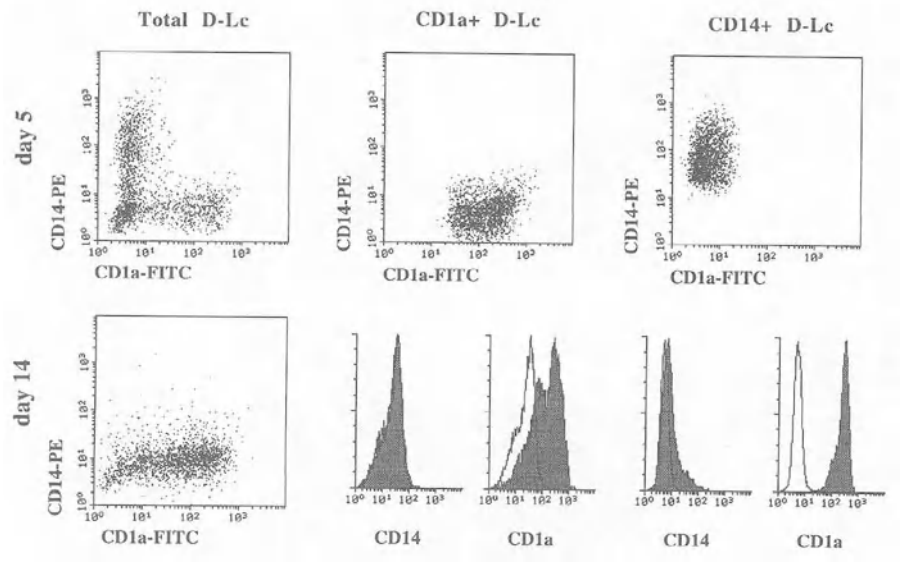

Fig.3: After 5-6 days of culture in presence of GM-CSF+TNFα, cells were collected and labelled with FITC-conjugated OKT6 (CD1a) (Ortho Diagnostic) and PE-conjugated Leu-M3 (CD14) (Becton Dickinson). Cells were separated into CD1a$^+$CD14$^-$ and CD1a$^-$CD14$^+$ fractions using a FACStar (Becton-Dickinson). Sorted cells were seeded in GM-CSF+TNFα and reanalysed for CD1a and CD14 expression at day 14.

2- B70/B7-2 is identical to CD86 and is the major functional ligand for CD28

In addition to expressing CD80 (B7/BB1), a subset of D-Lc expressed B70/B7-2 (6). Binding of the CTLA4-Ig fusion protein was completely inhibited by a combination of antibodies (MoAbs) against CD80 and B70/B7-2 indicating the absence of expression of a

third ligand for CD28/CTLA-4. Interestingly, MoAbs against CD86 (7) completely prevented the binding of CTLA4-Ig in presence of MoAbs against CD80 and bound to a B70/B7-2 transfected fibroblast cell line demonstrating that the B70/B7-2 antigen is identical to CD86 (not shown). CD28 triggering was essential during D-Lc induced alloreaction as it was inhibited by MoAbs against CD28 (Fig.2). However, none of the anti-CD80 MoAbs demonstrated any activity on the D-Lc induced alloreaction. In contrast, a MoAb against CD86 (IT-2) was found to suppress the D-Lc dependent alloreaction by 70% and this inhibitory effect was enhanced to ≥90% when a combination of anti-CD80 and anti-CD86 MoAbs was used. The present results demonstrate that D-Lc express, in addition to CD80, the other ligand for CTLA-4, CD86 (B70/B7-2) which plays a primordial role during D-Lc induced alloreaction.

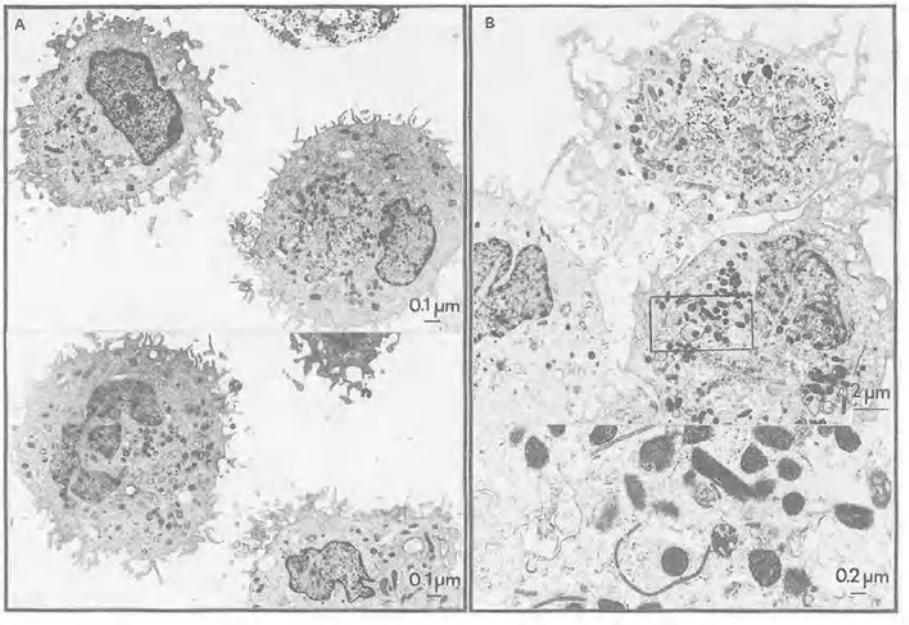

Fig.4: $CD14^+$ (Panel A) and $CD1a^+$ (Panel B) precursors FACS-sorted at day 5 were analysed in transmission electron microscopy at day 12

3- Identification of two dendritic cell subsets

Kinetics of expression of CD1a and CD14 was studied, using double cell surface staining, during the generation of D-Lc from $CD34^+$ progenitors. At day 0 neither CD1a nor CD14 could be detected on $CD34^+$ cells, but at day 5 two subsets of cells were identified, one $CD1a^+$ $CD14^-$ and one $CD1a^-$ $CD14^+$. At day 14 all cells were $CD1a^+CD14^-$ (Fig.3). The two subsets of precursors were sorted at day 5 and recultured under the same conditions. Although they lacked proliferating capacity, the cells mature and at day 14 the two subsets were $CD1a^+CD14^-$ (Fig.3B). The two cell types expressed high levels of accessory

molecules including CD80 and CD86 and were equally able to stimulate naive cord blood T cell proliferation (not shown). Of high interest, the CD1a$^+$ precursors gave rise at day 12 to Lc containing Birbeck granules (BG), while, the CD14$^+$ precursors lead to CD1a$^+$ DC lacking BG (Fig.4). In a typical experiment, the D-Lc derived from the total population and the CD1a$^+$ precursors were found to contain 20% and 69% of BG$^+$ cells, respectively. In contrast, BG were never detected at any time tested (day 9 to 13) in CD14$^+$ derived DC. It has to be noted that TNFα was required for the emergence of the CD1a precursor at day 5 but not for the maturation of the DC subsets (day 5 to 14).

DISCUSSION

The D-Lc generated in vitro are characterized by i) a dendritic morphology, ii) a capacity to induce allogeneic naive T cell proliferation iii) high expression of accessory molecules and MHC class II antigen but lack of FcγRI and CRI. So far, no major difference in phenotype and function could be identified between the two DC subsets and additionnal studies are onging. While the CD1a$^+$ precursor correspond to Lc precursor, the physiologic counterpart of the CD14$^+$ precursor derived DC remains to be discovered. In particular, as numerous studies have reported the generation of DC from monocytes cultured in presence of GM-CSF and mainly GM-CSF+IL-4 (8, 9, 10, 11, 12), the CD14$^+$ precursor derived DC might be related to monocytes derived DC (Fig.6) which are BG$^-$. These studies suggest that two unrelated DC subsets, with different hematopoietic origin, might exist in vivo. The understanding of the function of those two subsets will represent a new challenge.

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STEM CELL FACTOR ENHANCES DENDRITIC CELL DEVELOPMENT

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INTRODUCTION

Stem cell factor (SCF), also known as steel factor, c-kit ligand, mast cell growth factor) by itself is only weakly effective or ineffective in regulating hematopoiesis but interacts with specific cytokines to enhance the development of multiple lineages. [1-10] Within a particular lineage, SCF may regulate the development of progeny by interacting with progenitor cells found at various stages of differentiation.[11-13] SCF increases the number and size of the primary colony forming unit (CFU) by increasing the number of cells leaving quiescence and entering the cell cycle [14,16] and by permitting the CFU to undergo an additional number of divisions before entering a nonmitotic state.[1,11,16] The latter effect can be assessed as an increase in the replating potential of the CFU.[16]

Although cells of the dendritic cell (DC) series have long been described as potent stimulators of T and B cell responses,[17] the lack of DC specific markers and their trace distribution throughout the body has made them difficult to study. Recent strategies have provided a means for obtaining larger numbers of DCs and have furnished insight into the close ontogenic relationship between monocyte-macrophages (mono-mΦs) and DCs. We and others have shown that tumor necrosis factor (TNF) and granulocyte-macrophage colony stimulating factor (GM-CSF) synergistically control the development of DCs from a common mono-DC progenitor.[18-21] Because of its role in enhancing the development of multiple lineages, we hypothesized that SCF would also synergize with TNF+GM-CSF to increase DC hematopoiesis. In this brief report, we discuss our findings related to the effects of SCF on DC development from CD34+, cord blood-derived, progenitor cells.

RESULTS AND DISCUSSION

The proliferative potential of CD34+ progenitor cell cultures initiated with TNF+GM-CSF versus TNF+GM-CSF+SCF increased ~ twofold (Figure 1C).

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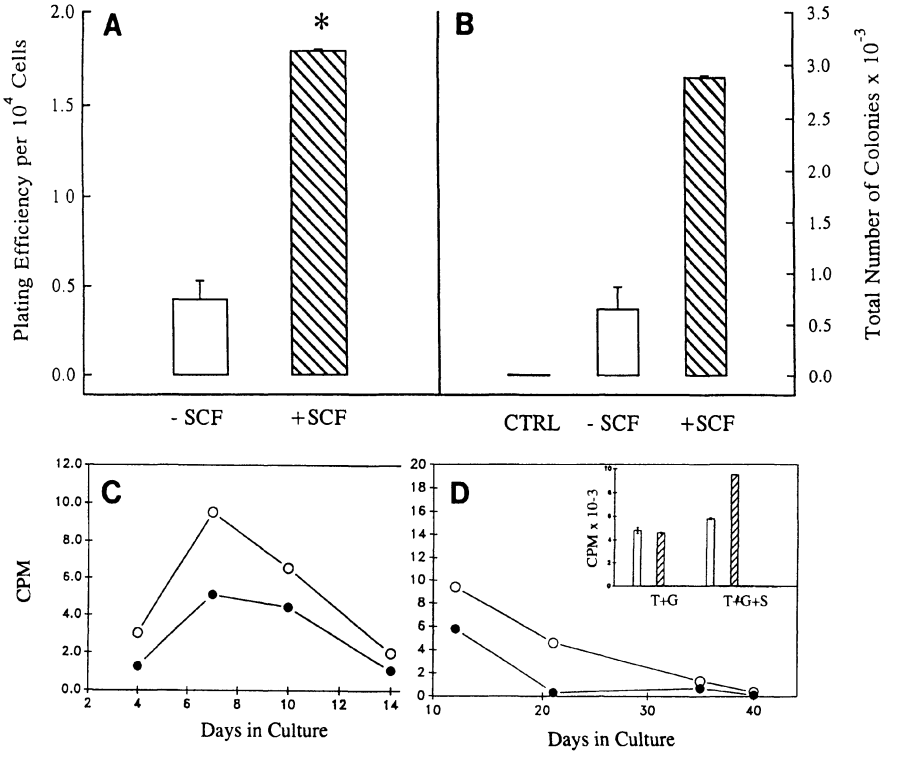

FIGURE 1. The effects of TNF+GM-CSF and TNF+GM-CSF+SCF on DC related primary plating efficiency and proliferative events. (A) plating efficiency = total # of colonies / # of cells plated X 100, (B) total number of colonies, CTRL = 5% normal human serum, (C) proliferative events in liquid cultures, as measured by thymidine uptake. Open circles = +SCF, filled circles = - SCF, (D) proliferative events associated with long term mono-DC growth, as measured by thymidine uptake. Inset: day 12 events in cultures continuously supplemented with either TNF+GM-CSF or TNF+GM-CSF+SCF (hatched bars) versus cultures receiving these cytokines at the onset of culture only (open bars). Main: kinetic analysis of proliferation beyond day 12. Open circles = supplemented with TNF+GM-CSF+SCF every 48 hrs., closed circles = TNF+GM-CSF+SCF at onset of culture only.

A temporal analysis employing thymidine uptake revealed that these increases occurred earlier in culture (by day 4), and reached statistical significance by day 7 ($P=0.046$), when a peak response was noted. Manual cell counts performed on day 10 also established ~ twofold increases ($P=0.05$) in the absolute number of cells present in TNF+GM-CSF+SCF cultures.

With TNF+GM-CSF+SCF, colonies developing in methylcellulose cultures were both larger (Figure 2B) and more numerous (Figures 1A,B). The efficiency for generating CFU increased from .42 to 1.79% ($P=0.044$) in the presence of SCF (Figure 1A). Most (~70%) of the colonies were mono-DC CFU and colony type and distribution were not affected by the addition of SCF. In situ analysis revealed that, as we previously reported with TNF+GM-CSF treatment,[18] the DCs generated in the presence of SCF were CD14 and nonspecific esterase negative, class II MHC positive, and nonphagocytic. As shown previously for TNF+GM-CSF, PMN and lymphocyte content was also low (<10%) with TNF+GM-CSF+SCF.

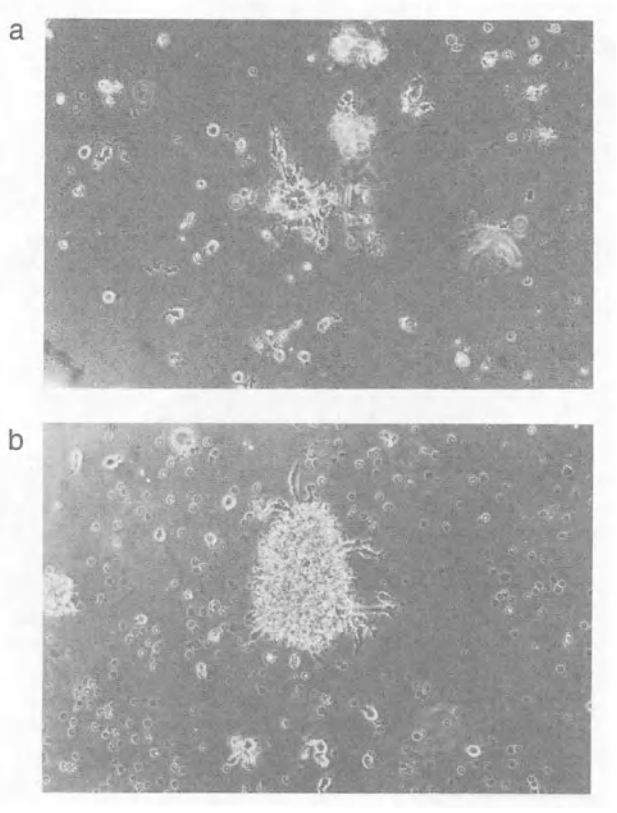

Figure 2. Mono-DC CFUs maturing in methylcellulose with TNF+GM-CSF (A) and TNF+GM-CSF+SCF (B) treatment on day 7. CFU developing with SCF were larger. DCs appear to be emigrating from the mono-DC CFU. Mono-CFU were also present in these cultures, but as classically described, these are much more compact and do not exhibit the projections associated with the mono-DC CFU. Original magnification = x 50.

DCs arising with TNF+GM-CSF+SCF were potent stimulators of naive T cells in the mixed leukocyte reaction (MLR). The capacity to produce a potent MLR was retained at stimulator to responder ratios as low as 0.03:1, consistent with a DC-mediated response. TNF+GM-CSF and TNF+GM-CSF+SCF cultures exhibited equal stimulatory capacity, supporting that SCF does not alter the proportion of mono-DCs.

Long term mono-DC cultures were maintained with a continuous supply of TNF+GM-CSF+SCF, but not with TNF+GM-CSF (Figure 1D). In cultures supplemented with TNF+GM-CSF+SCF, a prolonged proliferative potential and the propagation of numerous mono-DC containing clusters were noted beyond day 20, signifying an extended capacity for self renewal. No such differences were noted in TNF+GM-CSF cultures being fed with TNF+GM-CSF only ($P=0.43$), although enhanced viability of mature DC progeny was noted. Higher proliferative levels were still noted at three weeks ($P=0.004$), finally declining to unfed levels after a month. Cells removed from long term TNF+GM-CSF+SCF cultures and recultured with either TNF+GM-CSF or TNF+GM-CSF+SCF displayed a secondary plating potential for the DC lineage.

We previously noted that CD34+ progenitors treated with GM-CSF alone yielded mostly mono-mΦs and PMN cells, and little DC progeny. In contrast, PMN were scant when cells removed from long term TNF+GM-CSF+SCF cultures were recultured with GM-CSF. The combination of GM-CSF and G-CSF also failed to increase PMN cell content, suggesting the existence of a post GM-CFU progenitor which is capable of producing mono-DCs, but not granulocytes.

Thus, enhanced stimulation of the DC pathway can be achieved by supplementing cytokines controlling DC development (TNF+GM-CSF) with SCF. Although increased numbers of mono-DC CFU and mature DC progeny were obtained with the addition of TNF+GM-CSF+SCF at the onset of the culture period, the continuous supplementation of cultures with these cytokines was required to prolong DC hematopoiesis. SCF did not deviate the commitment to the DC lineage instituted by TNF+GM-CSF. The mechanism for the enhancement appears to follow the same general patterns previously reported for SCF-mediated lineage enhancement, i.e., increases in colony size, number, and plating capacity.

The combination of TNF+GM-CSF+SCF provides a powerful in vitro strategy which should aid in defining important control points during DC development, and consequently, in understanding abnormalities associated with DC development and function. Other investigators have indicated that the plating efficiency for all colony types (erythroid, GM-CFU, GEMM CFU) arising from cord blood progenitors stimulated maximally with SCF+Epo+IL-3+GM-CSF is 1-2%.[2,4] Relative to these reports, the plating efficiency of DC-related colonies is notable (1.8%). Thus, although DCs are normally present at low levels (<0.1%) in tissue and peripheral blood, the capacity to greatly amplify the DC lineage exists, under the appropriate conditions. Interestingly, large numbers of DCs may be localized in areas of chronic inflammation such as the rheumatoid joint, which contains an abundance of hematopoietically active cytokines, including GM-CSF, TNF and SCF [26-29].

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SYNERGISTIC INTERACTION BETWEEN *c-kit* LIGAND (SCF), GM-CSF AND TNF PROMOTES OPTIMAL DENDRITIC LANGERHANS CELL PROLIFERATION FROM PRIMITIVE PROGENITORS IN HUMAN BONE MARROW

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INTRODUCTION

Stem cell factor is the ligand for the membrane receptor product of the c-kit proto-oncogene. This receptor with tyrosine kinase activity is expressed on the most immature haemopoietic progenitors as well as on later commited cells. Many recent studies have defined its activities upon clonogenic progenitors of the erythroid and myeloid series though SCF on its own has little effect under stringent conditions of serum exclusion. Nevertheless SCF appears to be essential to the maturation of commited as well as of pluripotent cells when stimulated in culture by GM-CSF, IL-3, G-CSF or erythropoietin.[1,2]

We have previously described a clonogenic assay for dendritic cell progenitors in human bone marrow and blood. These highly characteristic colonies consist of cells which are CD1a$^+$ve, express class II especially DQ and are active in alloantigen presentation in MLC. We have termed these progenitors CFU-DL.[3] In the absence of serum this assay allows the assessment of the actions of rG/F singly and in combination and we and others have shown that TNF and GM-CSF interact to promote CFU-DL growth from CD34$^+$ cells.[4,5] To investigate the role of SCF in DC development we have now carried out further work on human bone marrow cells studying the dose responses of these clonal progenitors to IL-3, GM-CSF , TNFα and M-CSF in the presence and in the absence of SCF.

METHODS

Freshly thawed cryopreserved non-adherent bone marrow mononuclear cells from normal donors (BM-NADC) were used in every experiment. Serum replete and serum deprived cultures in methylcellulose were performed as previously described[4] scoring for colonies on the inverted microscope at 14 days of incubation. The different colony types were recognised morphologically and dendritic morphology was confirmed on air dried

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cytospin preparations with anti-CD1a antibody NA1/34 (by APAAP). Many of these colonies also contain macrophages but were scored together with the pure dendritic cell colonies as CFU-DL in this study. Dose response curves were determined to varying levels of individual cytokines in the presence of fixed concentrations of other cytokines and expressing colony numbers as a percentage of 'optimal' growth observed in serum for each experiment.

RESULTS

In a series of experiments dose response data were obtained for varying concentrations of GM-CSF, TNFα and IL-3 and the effects on colony scores of adding SCF at 25U/ml were observed.

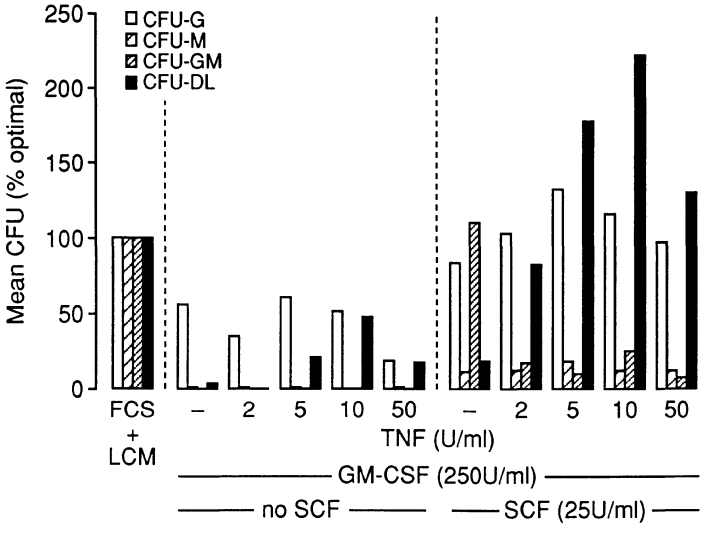

Figure 1. Increasing concentrations of TNFα at a fixed concentration of GM-CSF of 250U/ml in the presence or absence of SCF. Vertical axis : mean percentage of optimal CFU growth (FCS + PHA-LCM) at 14 days of culture. CFU-G: granulocyte colony forming cell, CFU-M: macrophage colony forming cell, CFU-GM: granulocyte/macrophage colony forming cell, CFU-DL: dendritic Langerhans colony forming cell. Mean of three experiments.

Effects of varying concentrations of TNFα in serum deprived cultures

As in previous studies GM-CSF alone elicited 50% of optimal granulocyte growth but vitually no CFU-DL and the addition of TNFα at 10U/ml stimulated CFU-DL to 50% . In the presence of SCF (25U/ml) the dendritic colonies were markedly enhanced to 215% of optimal and there were also striking increases in granulocyte and mixed CFU-GM colony scores. A similar enhancement by SCF of CFU-DL as well as of CFU-G and CFU-GM was observed over a range of concentrations of GM-CSF (50-500 U/ml) in the presence of

TNFα. Neither GM-CSF alone nor SCF with TNFα but without GM-CSF could elicit significant dendritic cell growth. In contrast to GM-CSF and TNFα, IL-3 had little detectable effect on DC growth at concentrations in culture of 50 to 500 U/ml provided that GM-CSF, TNFα, SCF or all three cytokines were present in the cultures.

In further experiments (data not shown) a range of concentrations of SCF (5 to 50 U/ml) alone failed to elicit significant growth of any cell type but though addition of TNFα had no effect, GM-CSF at 50 to 500 U/ml was observed to stimulate growth of dendritic cell colonies up to 20% of optimal even in the absence of aded TNFα. This was not observed if M-CSF (20 to 2000 U/ml) was substituted for GM-CSF. The size and nature of CFU-DL colonies were affected by SCF. Colonies were generally larger and a number of mixed granulocyte- or erythroid-dendritic cell colonies were observed under these culture conditions.

DISCUSSION

These findings demonstrate that human rSCF strikingly enhances the growth of dendritic cells from an early bone marrow progenitor. It is incapable of promoting clonal expansion of CFU-DL alone or with TNFα but has a synergistic effect on growth induced by GM-CSF and TNF. The smaller effect observed when only GM-CSF and SCF were present in culture is interesting since earlier findings suggested an obligatory role for TNF in serum deprived conditons.[4] The possiblity of accessory cell derived TNF in these cultures cannot be excluded as these studies were on unfractionated BM-NADC. This synergy of SCF with later acting growth factors is similar to that previously reported for both the myeloid and erythroid series.[1,2]

The larger more cellular dendritic colonies seen with SCF were also frequently of mixed granulocyte or erythroid character. This implies that SCF may be acting by recruitment of a yet more primitive progenitor which has a greater differentiation repertoire and a larger proliferative potential than those CFU stimulated to maturation in its absence.[1,6] In such a case the synergy observed would be of a different type to that already postulated for TNF and GM-CSF which, it has been suggested, may be due to modification of GM-CSF receptor expression.[7]

Further studies on accessory cell depleted populations and single $CD34^+$ cells will help to define these issues but it is clear that rSCF should be capable of increasing the yields of mature DC in-vitro from bone marrow, cord or peripheral blood haemopoietic stem cells.

ACKNOWLEDGEMENTS

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PROGENITOR RECRUITMENT AND *IN VITRO* EXPANSION OF IMMUNOSTIMULATORY DENDRITIC CELLS FROM HUMAN CD34$^+$ BONE MARROW CELLS BY c-*kit*-LIGAND, GM-CSF, AND TNFα

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SUMMARY

Several cytokines have been identified that support the development of dendritic cells from murine and human precursor populations, most notably GM-CSF, TNFα, and IL-4.[1-7] We have been interested in human bone marrow as a source of defined CD34$^+$ progenitors to generate large numbers of autologous dendritic cells for use as adjuvants in immune based therapy. In serum-replete conditions with c-*kit*-ligand, GM-CSF, and TNFα, dendritic cells constitute $\sim$10-15% of the myeloid progeny (equivalent to $\sim 1.7 \times 10^6$ dendritic cells per single ml of starting bone marrow); and they develop together with granulocytic intermediates and monocytes in the same cultures.[8] CD14$^-$ dendritic cells share expression of class II MHC and costimulatory ligands with CD14$^+$ monocyte progeny, but only the CD14$^-$ HLA-DR$^+$ dendritic cells are highly stimulatory of resting unprimed T cells.[8] We have further identified a novel colony that develops in the presence of GM-CSF and TNFα alongside typical CFU-GM, which is comprised of dendritic cells mixed with $\leq$15% monocytes (CFU-DC/mono).[9] c-*kit*-ligand recruits and expands early progenitors responsive to the dendritic cell-differentiating effects of GM-CSF and TNFα, effecting a 100- to 1000-fold greater expansion of CFU-DC/mono by 14d and 21d respectively than does the combination of GM-CSF and TNFα without c-*kit*-ligand.[9] Conclusive proof that dendritic cells have been generated under these *in vitro* conditions has been founded on the morphologic, phenotypic, and functional criteria that are critical to their identification as potent accessories for primary T cell responses, and their distinction in particular from monocytes.

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BONE MARROW CD34$^+$ PRECURSORS GENERATE LARGE NUMBERS OF IMMUNOSTIMULATORY DENDRITIC CELLS [8]

CD34$^+$ bone marrow progenitors were grown in IMDM-20%FCS with exogenous GM-CSF and TNFα. Adherent cells with stellate, elongated cytoplasmic projections, as well as motile, veiled, nonadherent cells were distinguished in suspension culture by 7d, but only if TNFα were present. The latter were characteristic of mature blood dendritic cells. These developed alongside more uniformly round cells and aggregates, which were the only cell types seen in GM-CSF supplemented cultures without TNFα. After 12-14d, all but the firmly adherent cells were harvested and analyzed (80-90% of the total yield). Up to half of the nonadherent cells were HLA-DR$^+$. Only the CD14$^-$ HLA-DR$^+$ cells stimulated strong primary responses by resting allogeneic T cells, however, similar to those effected by blood dendritic cells (Table 1). This was irrespective of the homogeneous expression of costimulatory ligands (CD11a,b,c, CD18, CD54, CD58, and CD80) by both CD14$^-$ and CD14$^+$, HLA-DR$^+$ myeloid progeny. These cells were also motile in suspension culture, displayed the unique cytoplasmic and nuclear morphology that characterizes mature dendritic cells, and were double esterase negative. Dendritic cells constituted ~10-15% of the total; thus one could expect ~1.7 x 10^6 mature dendritic cells after 12-14d expansion from CD34$^+$ precursors in a single ml of starting bone marrow.

Table 1. CD14$^-$ HLA-DR$^+$ dendritic cells, but not CD14$^+$ HLA-DR$^+$ monocytes, derived from human CD34$^+$ bone marrow progenitors grown in GM-CSF and TNFα ± c-*kit*-ligand, are potent stimulators of resting unprimed T cells in an allogeneic MLR.[1]

Dose	*Stimulators (1500r)* Blood dendritic cells	CD14$^-$ DR$^+$	CD14$^+$ DR$^+$	CD14$^+$ DR$^-$
5 x 10^3	173459 ± 5148	183351 ± 10008	15476 ± 2930	951 ± 494
1.5 x 10^3	145602 ± 9776	143162 ± 6043	2140 ± 49	585 ± 189
0.5 x 10^3	116742 ± 5843	108367 ± 11106	691 ± 443	342 ± 73

[1] Cultures were initiated with 1.5 x 10^5 allogeneic T cell responders per well. Results are expressed in cpm ± S.D. of ^{3}HTdR incorporated during 12h pulse on d5. Representative of five experiments.

DENDRITIC CELLS AND MONOCYTES SHARE A COMMITTED CLONOGENIC PROGENITOR [9]

Clonogenic assays of human CD34$^+$ bone marrow progenitors were undertaken in semisolid medium to determine the colonial relationship of dendritic cells to monocytes and granulocytes. The addition of TNFα to c-*kit*-ligand and GM-CSF led to the appearance of novel colonies comprised predominantly of dendritic cells mixed with ≤15% monocytes, which we have termed CFU-DC/mono; these developed in addition to CFU-GM. To substantiate the enrichment of dendritic cells in these CFU-DC/mono, colonies were manually plucked from the methylcellulose and the phenotypic and morphologic features of mature blood dendritic cells were confirmed. Similar dendritic cells were not identified in CFU-GM elicited by c-*kit*-ligand and GM-CSF, with or without TNFα. Conversely, granulocytes and their intermediates were not present in the putative dendritic cell-containing colonies. The cells from both types of colonies were tested in primary allogeneic MLRs to confirm the presence or absence of stimulatory activity for resting T cells, similar to that exerted by mature blood dendritic cells (Table 2). Potent accessory activity was recovered from CFU-DC/mono, but not from CFU-GM, and was attributed to the

dendritic cells rather than the monocytes in these colonies. This was justified by the numerical predominance of dendritic cells in these CFU-DC/mono. Moreover, $CD14^+$ $HLA\text{-}DR^+$ monocytes generated under similar cytokine conditions in suspension culture were at least 1.5 to 2 logs less stimulatory than $CD14^-$ $HLA\text{-}DR^+$ dendritic cells; and CFU-GM which contained monocytes were inactive in stimulating resting unprimed T cells.

Table 2. CFU-DC/mono are comprised predominantly of dendritic cells with potent stimulatory activity for resting unprimed T cells in an allogeneic MLR.[1]

Stimulators (1500r) / *Dose*	Blood dendritic cells	Blood monocytes	CFU-DC/mono	CFU-GM
1×10^3	243185 ± 19614	4370 ± 3056	284161 ± 29345	7271 ± 569
0.3×10^3	153392 ± 10585	544 ± 313	192566 ± 6305	5005 ± 3294
0.1×10^3	107068 ± 10389	187 ± 25	108915 ± 2734	181 ± 8

[1] Cultures were initiated with 1×10^5 allogeneic T cell responders per well. Results are expressed in cpm ± S.D. of ^{3}HTdR incorporated during 12h pulse on d4-5. Representative of three experiments.

c-*kit*-LIGAND RECRUITS AND EXPANDS CFU-DC/MONO [9]

c-*kit*-ligand substantially expanded the progeny of $CD34^+$ bone marrow progenitors during 12-14d culture in IMDM-20%FCS, GM-CSF, and TNFα. To determine the mechanism by which c-*kit*-ligand exerted its effect, we assessed replating efficiency as a measure of progenitor expansion. $CD34^+$ bone marrow cells were cultured in suspension, harvested at weekly intervals, then returned to suspension culture as well as tested for CFU-GM and CFU-DC/mono recruitment and expansion. By 14 and 21d in liquid culture, there were 100 and 1000-fold more CFU-DC/mono respectively, than in parallel cultures without c-*kit*-ligand (Figure 1).

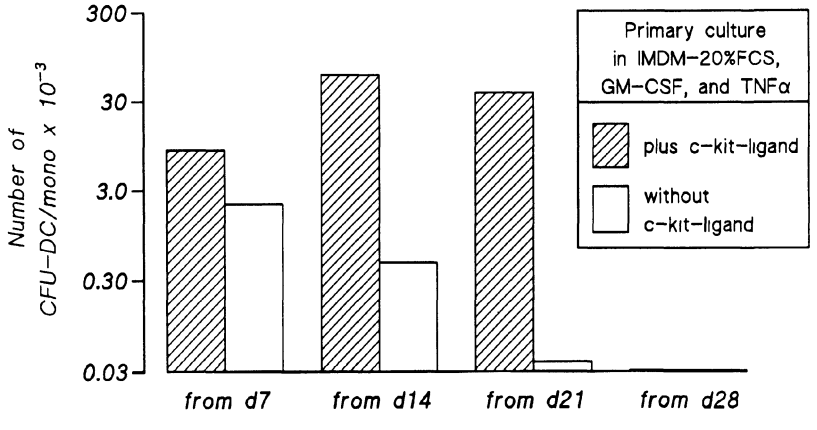

Figure 1. c-*kit*-ligand recruits and expands CFU-DC/mono in synergistic combination with GM-CSF and TNFα. $CD34^+$ bone marrow progenitors were grown in suspension culture with GM-CSF and TNFα, with and without c-*kit*-ligand. The resultant cells were evaluated for expansion of CFU-DC/mono at weekly intervals using clonogenic assays in 0.36% agarose/IMDM-20%FCS, GM-CSF, and TNFα.

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THYMIC DENDRITIC CELLS: SURFACE PHENOTYPE, DEVELOPMENTAL ORIGIN AND FUNCTION

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INTRODUCTION

Are the dendritic cells (DC) found in the thymus similar in origin and function to those in other lymphoid tissues? Thymic DC are generally similar in morphological appearance to DC elsewhere, and share many cell surface markers. Thymic DC are short-lived cells which are, like DC elsewhere, of bone marrow origin[1,2]. However, it had not been clear whether they are continuously generated within the thymus itself, or arrive preformed via the bloodstream. Our work delineates a separate stream of DC generated within the thymus. Thymic DC form multicellular complexes with developing T cells, giving rise to the rosette structures found in collagenase digests of thymic tissue[3,4]. The functional result of this DC-thymocyte interaction is believed to be the destruction of any self-reactive cells (negative selection)[5]. This outcome is different from that of the DC-T lymphocyte interactions in lymph nodes, where any T cells reactive with presented foreign antigens are stimulated to proliferate and differentiate[6]. Investigation of the basis of this difference has pointed to the state of maturation of the interacting T-lineage cells, rather than to any differences in the antigen-presenting DC[7]. Without questioning that the maturation state of the interactive T cells is an important factor, our work has raised the additional possibility that certain DC may be specialized for delivering negative signals to T cells.

ISOLATION AND SURFACE PHENOTYPE OF DC

We have developed a new procedure for the isolation of DC from lymphoid tissue, including thymus[8]. It is no less tedious and expensive than alternative approaches. However, it is designed to isolate most DC from tissues, including those tightly bound in multicellular complexes, and it avoids prolonged incubation at 37° which may alter DC surface phenotype and function. The procedure involves collagenase digestion of tissue fragments at 22°, addition of EDTA to dissociate T cell-DC rosettes, selection of light density cells, and then extensive depletion of cells other than DC using a cocktail of

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monoclonal antibodies (mAb) and immunomagnetic beads. This enriched preparation (30-90% DC) is then labelled and sorted for cells bearing characteristic DC markers (high class II MHC; high CD11c) to obtain pure DC. This approach has been applied to both mouse and human tissue[9].

When isolated in this way, then cultured for a short time at 37° to allow recovery of normal morphology, thymic DC show a characteristic DC appearance of irregular shape with cytoplasmic extensions[8,9]. These thymic DC also express surface antigens characteristic of murine DC,[6,8-10] including high levels of class I and class II MHC, high levels of CD11c, and the interdigitating marker NLDC145 (Fig. 1). However, a surprising feature of thymic DC prepared in this way is their staining for a range of markers characteristic of lymphoid cells, including CD8, CD4, Thy 1, CD2 and BP-1; it is reassuring that they are negative for CD3 and TCR, and their TCR and Ig genes are not rearranged[8-11].

Some of these lymphoid markers on mouse thymic DC, such as CD4, are present only at low levels, whilst others, such as CD8α, may be at levels similar to lymphoid cells[8,11]. Some markers, such as CD8α, are present on all thymic DC although the actual level does vary[11]. Some markers, such as the early B-cell marker BP-1[12], are present only on thymic DC, whilst others, such as CD8α, are also found at high levels on a subpopulation of DC in peripheral lymphoid tissue[8,11] (Fig. 1). The presence of CD8 on a minor subpopulation of DC had previously been noted by others[13,14]. Our isolation procedure extracts these strongly tissue-bound $CD8^+$ DC very effectively, so in our preparations they appear as a major subpopulation of splenic DC (Fig. 1). The form of CD8 on DC is predominantly the CD8αα homodimer, rather than the CD8αβ heterodimer characteristic of most T cells, since DC stain strongly for Ly 2 but only weakly for Ly 3[8].

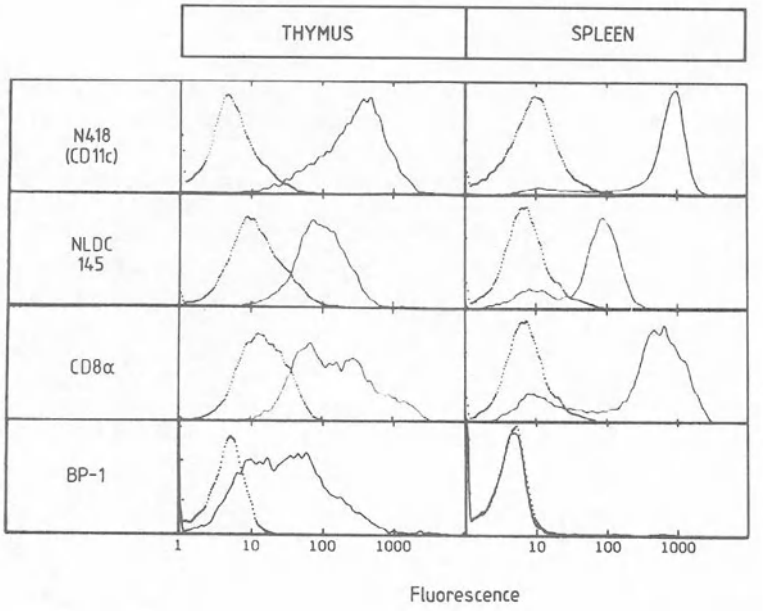

Figure 1. A comparison of DC isolated from the thymus and the spleen. DC were isolated, but not sorted, as described elsewhere[8]. They were stained in two fluorescent colors for class II MHC and the marker listed. On flow cytometric analysis, DC were gated as cells showing high staining for class II MHC, and relatively high forward and side light scatter. DC from both sources are $CD11c^+$. All thymic but not all splenic DC stain with the interdigitating marker NLDC145. All thymic DC are $CD8^+$ but the level varies from low to high, whereas splenic DC are divisible into $CD8^+$ and $CD8^-$ subpopulations. Only thymic DC stain for BP-1, although not all are positive. Full details are given elsewhere[11].

Are these characteristic lymphoid molecules on the DC surface synthesized by the DC themselves? Thymic DC and splenic DC express mRNA for CD8α (but not for CD8β)[8], and thymic DC (but not splenic DC) express mRNA for BP-1[11], suggesting these surface molecules are true DC products. However, some lymphoid markers are picked up from thymocytes. One indicator of this is the extensive drop in staining for the marker if isolated DC are incubated overnight in culture medium (Fig. 2). Thus, although CD8α and BP-1 expression is maintained after short-term culture of thymic DC, in line with the molecules being integral components of the DC membrane, CD8β, CD4 and Thy 1 all decrease, suggesting these molécules have only a transient association with the DC surface.

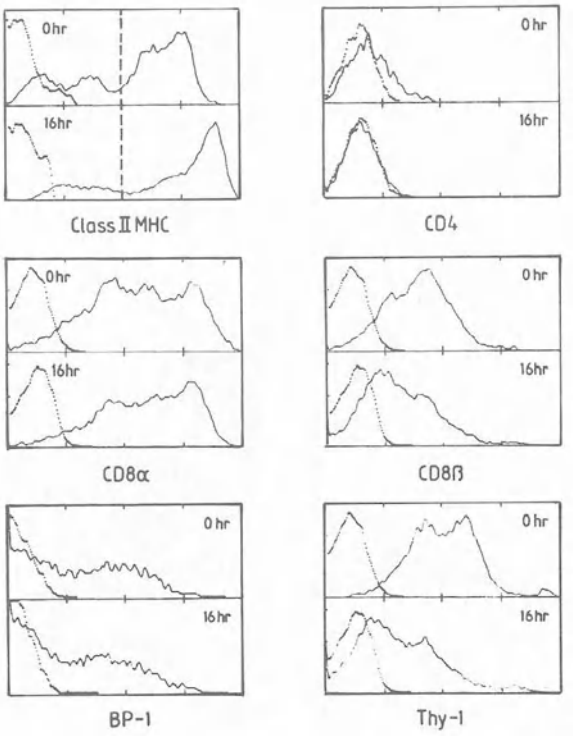

Figure 2. Changes in surface markers on thymic DC after overnight culture. DC were isolated from the thymus, but not sorted, as described elsewhere[8]. The DC were analyzed as in Fig. 1, the gating used for class II MHC^{hi} cells being shown in the first panel; in all subsequent panels only the profiles of the class II MHC^{hi} cells are shown. One third of each DC preparation was stained and analyzed directly. The remainder was incubated at 37° overnight in a culture medium which included serum and the supernatant of activated T cells to conserve DC viability. The cultured cells were then stained and analyzed. CD4, CD8β and Thy 1 all drop on overnight culture. Class II MHC, CD8α and BP-1 are retained. Further details are given elsewhere[11].

We have studied this issue closely in the case of Thy 1 expression on thymic DC. To determine the source of this Thy 1, we reconstituted irradiated C57BL mice with a mixture of congenic bone marrow cells derived from Thy 1.1 and Thy 1.2 mice. When the resultant chimeric thymuses are examined 15 days later the T-lineage cells are either Thy 1.1^+ or Thy 1.2^+, with no T-lineage cells positive for both markers, as expected.

However the thymic DC have both Thy 1.1 and Thy 1.2 on their surface (Fig. 3). In addition, at this time point, when the number of thymocytes in the thymus is much less than normal, the DC also stain less strongly than normal for Thy 1. As a control, a similar experiment was run reconstituting the thymus with congenic bone marrow cells from Ly 5.1 and Ly 5.2 mice. In this case the DC isolated from the chimeric thymuses were clearly either Ly 5.1^+ or Ly 5.2^+, with no DC showing staining for both markers (Fig. 3). Thus while the Ly 5 of the DC surface is dictated by the genotype of the DC, the Thy 1 of the DC surface reflects the type and number of thymocytes in the environment. We conclude that the Thy 1 of the thymic DC is derived from T-lineage thymocytes, probably when the rosette structures are disrupted. Most of the CD4 and a little of the CD8 (as monitored by CD8β) is probably of similar thymocyte origin, although most of the CD8α appears to be true DC product.

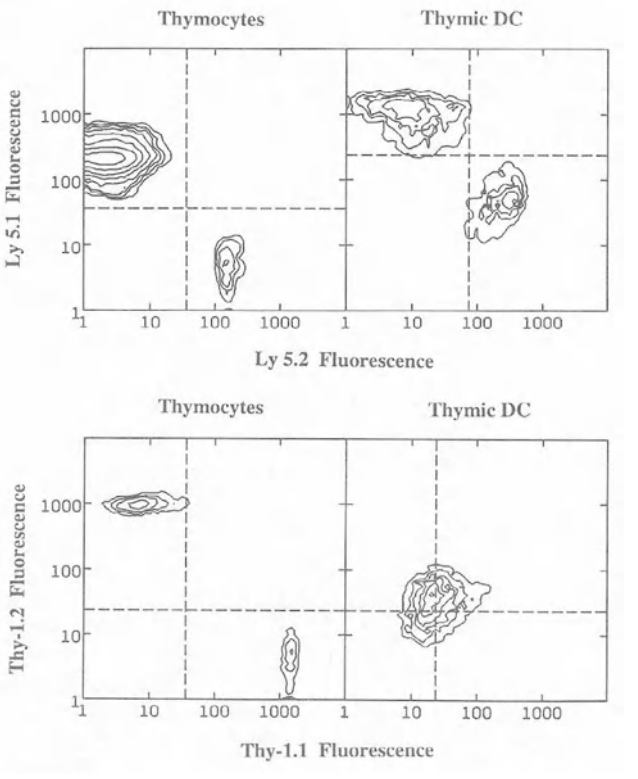

Figure 3. Thy 1 and Ly 5 allotypes on the surface of DC from chimeric, reconstituted thymuses. Mice (C57BL/6 Ly 5.1-Pep^{3b}) were irradiated (550 rads, two doses 3 hr apart), then reconstituted by intravenous transfer of $2x10^6$ bone marrow cells, being a mixture of $1x10^6$ cells from each of two near-congenic strains. For the Thy 1 experiments the donor mice were C57BL/6J (Thy 1.2) and C57BL/Ka Thy 1.1. For the Ly 5 experiments, the donor mice were C57BL/6J (Ly 5.2) and C57BL/6 Ly 5.1-Pep^{3b}. Fifteen days later chimeric reconstituted thymuses were pooled. After collagenase digestion, a sample was taken for direct staining of thymocytes. From the remainder, the DC were isolated as described elsewhere[8]. They were stained in three fluorescent colors for class II MHC (allophycocyanin conjugated) together with either Ly 5.1 (biotinylated, phycoerythrin-avidin second stage) and Ly 5.2 (fluoresceinated), or Thy 1.2 (phycoerythrin conjugated) and Thy 1.1 (fluoresceinated). The flow cytometric data was then gated for DC showing high levels of class II MHC and the Ly 5 or Thy 1 distribution then determined. A sample lacking only the Ly 5 or Thy 1 mAb in the stain was used for establishing the limits of the background staining, used to define the quadrants. The Ly 5 and the Thy 1 data are from different experiments, since a single preparation did not give sufficient DC progeny.

THE SURFACE PHENOTYPE OF HUMAN THYMIC DC

The procedure for isolation of mouse thymic DC is applicable, with only modification of the mAb used for depletion of non-DC, to human thymic DC[9]. When human and murine thymic DC are isolated side-by-side and compared, a surprising species difference emerges (Fig. 4). Whilst murine thymic DC express high levels of CD8 and stain relatively weakly for CD4, human thymic DC are the converse, expressing very high levels of CD4 but only marginal levels of CD8. The biological significance of this is far from clear. However, it does suggest that human thymic DC should be susceptible to infection with the HIV-1 virus. Studies on this aspect are discussed by Paul Cameron elsewhere in this volume.

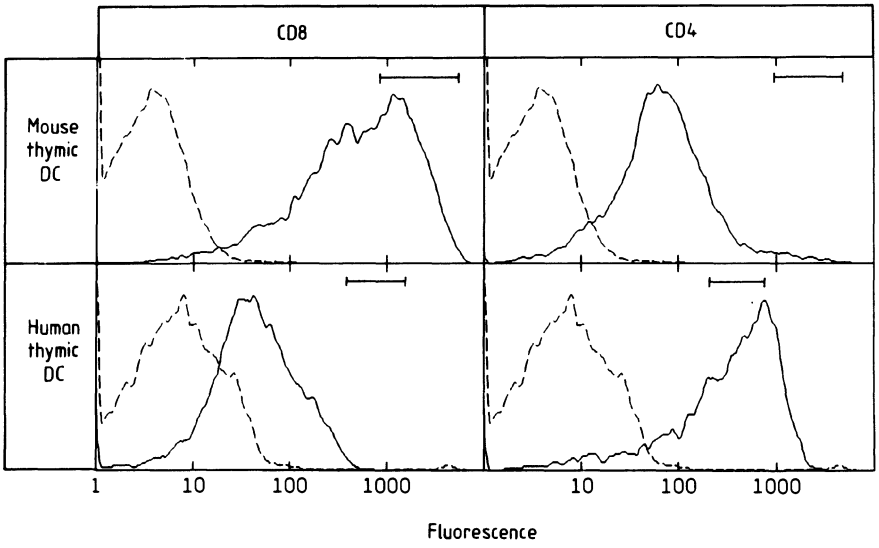

Figure 4. A comparison of DC from the mouse and the human thymus. DC were isolated from young mouse or infant human thymus, as described elsewhere[8,9]. The procedures used were similar, as discussed in text. They were then stained in two fluorescent colors for the appropriate class II MHC and CD4 or CD8α, the flow cytometric data then being gated for class II MHC^{hi} cells as in Figs. 1 and 2. The highest sensitivity was used for the CD4 and CD8α stains; the staining level for thymocytes is given as a bar, for comparison. Mouse thymic DC are $CD8^{hi}$ $CD4^{lo}$ whereas human thymic DC are $CD8^{lo}$ $CD4^{hi}$. Full details are given elsewhere[9].

DEVELOPMENTAL ORIGIN OF THYMIC DC

Our interest in the development of thymic DC arose from our identification of a rare population of early precursor cells in the adult mouse thymus[15,16]. These precursors resemble bone marrow multipotent hemopoietic stem cells in most surface markers, but differ from them in expressing the Sca-2 antigen and in expressing moderate levels of CD4[15,16]. This population had been shown to produce both T cells and B cells when transferred intravenously into an irradiated recipient, but did not produce detectable erythroid or myeloid cells[16]. Thus these precursors have the properties expected of a lymphoid-committed precursor, although it is not yet clear whether each cell has both T and B developmental potential, or whether the population consists of a mix of separate T-committed and B-committed precursors with identical surface phenotype. We have

recently shown that this same thymic "lymphoid" precursor population serves as a source of thymic DC[17].

Our assay for precursor function involves the intravenous or intrathymic injection of pure precursor cells into irradiated congenic mice differing at the Ly 5 locus. At various times after transfer, lymphoid organs are removed, dissociated and then usually analyzed directly by immunofluorescent staining and flow cytometry for the entry of donor-derived cells into various hemopoietic lineages[15,16]. However, the low level of DC makes such direct analysis impossible. Accordingly, it is necessary to pool many recipient organs and enrich the DC, prior to staining and analysis for expression of donor-type Ly 5 together with various DC markers[17]. For intrathymic transfer studies only one thymus lobe is injected, so DC preparations prepared from the pooled non-injected lobes serve as a closely matched control[17]. The type of results obtained from such experiments are shown in Fig. 5.

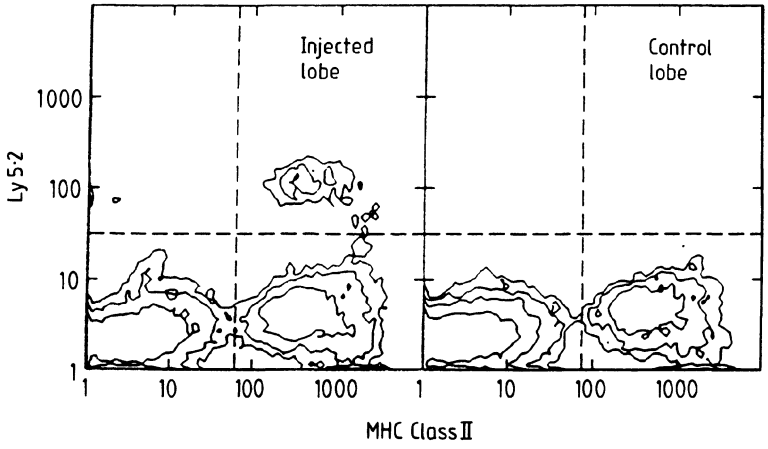

Figure 5. The development of thymic DC from the intrathymic precursor population. The precursor cells were isolated from donor mice (C57BL/Ka Thy 1.1 (Ly 5.2)) by extensive depletion of most T-lineage and unrelated lineage cells, then sorting for cells which were Thy 1^{lo} $HSA^{moderate}$ class II MHC$^-$, as described elsewhere[15,17]. The purified precursors (10^4) were then injected into the left thymic lobe of irradiated (750 rad) recipients (C57BL/6 Ly 5.1-Pep^{3b}). Fifteen days later the injected lobes of 8 recipients were pooled, and the non-injected lobes of the same recipients also pooled. DC were purified from each, as described elsewhere[8] and stained in two fluorescent colors for class II MHC and for donor-type Ly 5.2. The class II MHC staining was very bright and sensitivity was reduced to obtain the on-scale reading. A population of donor-derived DC was discerned in the injected lobes, but not in the control, non-injected lobes. These Ly 5.2^+ class II MHC^{hi}, donor-derived DC were also shown to be $NLDC145^+$ in this experiment, and in other experiments to be $CD11c^{hi}$, $CD8\alpha^+$, $CD8\beta^-$, $CD4^-$, $CD3^-$, and to have DC morphology. Full details are given elsewhere[17].

Intrathymic transfer of the purified thymic precursor population produces DC progeny 7-21 days later, as well as T-cell progeny[17] (Fig. 5). The DC progeny express all the markers characteristic of normal thymic DC, including high class II MHC, CD11c, NLDC145, BP-1 and CD8α but not CD8β[17]. The ratio of T-cell to DC progeny is around 1000:1, about the normal ratio of these cell types in the thymus[11,17]. The number of DC progeny declines after 21 days, indicating they have a short intrathymic lifespan, as do the T-lineage progeny[11]. It is notable that no granulocyte or macrophage progeny are detected when these intrathymic precursors are transferred, in contrast to bone marrow

stem cells which produce myeloid cells as well as T cells and DC when injected into the thymus[17].

These results indicate that these short-lived thymic DC are produced endogenously from an intrathymic precursor cell. We suggest this is a mechanism for ensuring that negative selection within the thymus is restricted to self-antigens, in contrast to previous models where preformed DC entering the thymus from the bloodstream might present exogenous and foreign antigens to the developing T cells.

The results also suggest that thymic DC could be of lymphoid rather than myeloid origin, which would accord with their expression of certain lymphoid markers. Since the thymic precursor cells also form $CD8\alpha^+$ DC in the spleen if transferred intravenously (Wu, experiments in progress), these precursors may give rise to a distinct sublineage of DC. Such a lineage would be concentrated in the thymus but would also be found in other lymphoid organs. However, clonal evidence is needed to establish if these $CD8\alpha^+$ DC differ in origin from "myeloid" DC.

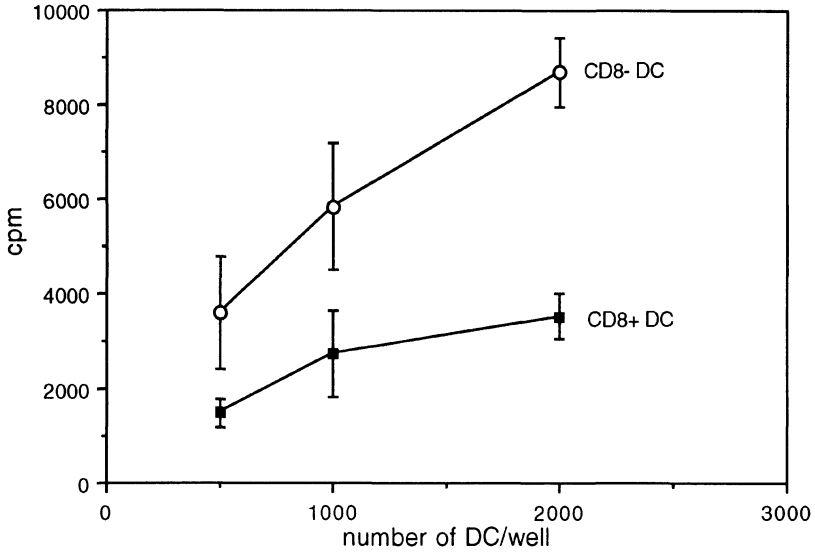

Figure 6. The stimulation of $CD4^+$ T cells by allogeneic DC subpopulations. $CD4^+$ T cells were isolated from the lymph nodes of CBA mice by depletion of $CD8^+$ and $s\text{-}Ig^+$ cells. DC were isolated as described elsewhere[8] but finally sorted into $CD11c^{hi}$ $CD8^-$ and $CD11c^{hi}$ $CD8^+$ fractions (avoiding class II MHC staining which might block antigen presentation). The $CD4^+$ T cells ($2x10^6$) were mixed with various numbers of DC and cultured in 200µl of medium (RPMI-1640), containing 10% fetal calf serum and $2x10^{-5}$M 2-mercaptoethanol) in V-well plates in a 10% CO_2-in-air incubator. After 3 days 0.5µCi ^{3}H-thymidine was added and 12 hr later the cultures harvested onto glass-fibre paper. Incorporation of thymidine was assessed on a gas-flow counter with around 100-fold lower sensitivity than liquid scintillation counting. Values are the mean of triplicate cultures ± SD. Background counts of T cells or DC alone were <50 cpm. Similar differences between $CD8^+$ and $CD8^-$ DC were seen at day 2 and day 4 of culture.

FUNCTIONAL DIFFERENCES BETWEEN DC POPULATIONS

A standard assay for DC function is their capacity to serve as efficient allogeneic stimulator cells in a mixed lymphocyte-type culture[6]. We have found splenic DC prepared by our procedure, and sorted as $CD11c^{hi}$ cells, do stimulate purified $CD4^+$ allogeneic T cells very effectively; thymic DC also stimulate, but give a weaker proliferative response. However when the $CD8^+$ and $CD8^-$ splenic DC are separated, the $CD8^-$ DC are always the most effective stimulators, and this subpopulation accounts for most of the stimulation

achieved by unseparated DC. $CD8^+$ splenic DC do stimulate, but at the lower level characteristic of thymic DC (which are all $CD8^+$). This result is readily seen in a thymidine uptake assay from day 2 to day 4 of the response (Fig. 6), and is also apparent from direct counts of blast cell formation. There is no evidence that this represents a difference in the maturation state of the DC; both subpopulations express similar high levels of class II MHC and both bind the CTLA-4-Ig construct. However, forward and side light scatter measurements and other assays suggest there is an increased number of dying, apoptotic cells in the cultures stimulated by $CD8^+$ DC (Süss, unpublished data).

Our current interpretation is that $CD8^+$ DC both stimulate and kill interacting $CD4^+$ T cells, producing an aborted response, while $CD8^-$ DC produce a normal stimulatory response. One explanation could be that $CD8^+$ DC act as "veto" cells, in line with the experiments of Sambhara and Miller[18], who found that transfection of an antigen presenting cell with CD8 caused it to induce apoptosis in the interacting T cells. However to date we have no evidence that CD8 per se is the cause of the relatively poor proliferative response of the T cells, and CD8 may merely be a secondary marker of a particular physiological state of the DC.

CONCLUSIONS

Thymic DC continuously develop within the adult mouse thymus itself, from a distinct intrathymic precursor which is similar to or the same as the thymic precursor of T-lineage cells. Thymic DC synthesize and express on their surface two characteristic lymphoid markers, CD8α and BP-1. However, many other T-cell markers found on the DC surface are simply picked up from thymocytes. A subpopulation of DC in peripheral lymphoid organs resembles thymic DC in some respects, such as expression of CD8α. Such $CD8\alpha^+$ DC might represent a distinct DC sublineage. There is evidence that $CD8\alpha^+$ DC may regulate the proliferative response of $CD4^+$ T cells.

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RECOMBINANT GM-CSF INDUCES CYTOKINE PRODUCTION IN MOUSE DENDRITIC CELL CLONES

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INTRODUCTION

GM-CSF is, in vitro, one of the most effective signal for the growth and differentiation of immature dendritic cells (1-2).

Previous studies from our laboratory, showed that GM-CSF enhances the capacity of the immortalized mouse dendritic cell clone CB1 to induce contact sensitivity in vivo and antigen presentation in vitro (3). Other spleen-derived dendritic cell clones were generated (4) with the retroviral vector MIBψ2 N11 (5); among them, the D2SC/1 clone exhibited a more mature phenotype than CB1, in terms of surface markers expression; it could specifically prime T cells in vivo (see contribution in this issue by M. Lutz et al.) and was not GM-CSF-dependent for antigen presentation in vitro.

Thus these two clones could represent different stages of dendritic cell differentiation and may be used as models for studying the immunobiology of dendritic cells.

In this study, we investigated the response of the CB1 and D2SC/1 dendritic cell clones to GM-CSF in terms of cytokine production.

RESULTS AND DISCUSSION

The difficulty in generating pure dendritic cell cultures, hindered the studies on cytokine production by these cells (6-12).

The availability of immortalized and cloned dendritic cells enabled us to investigate the pattern of cytokines secreted in response to GM-CSF or LPS. After activation with GM-CSF, only two out of ten ELISA-tested cytokines were detected in the culture supernatants.

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IL-1β was already induced in the CB1 cells one hour after mouse recombinant GM-CSF stimulation, LPS being uneffective (Fig.1A). In contrast, LPS but not GM-CSF induced IL-1β production in D2SC/1 clone (Fig.1B).

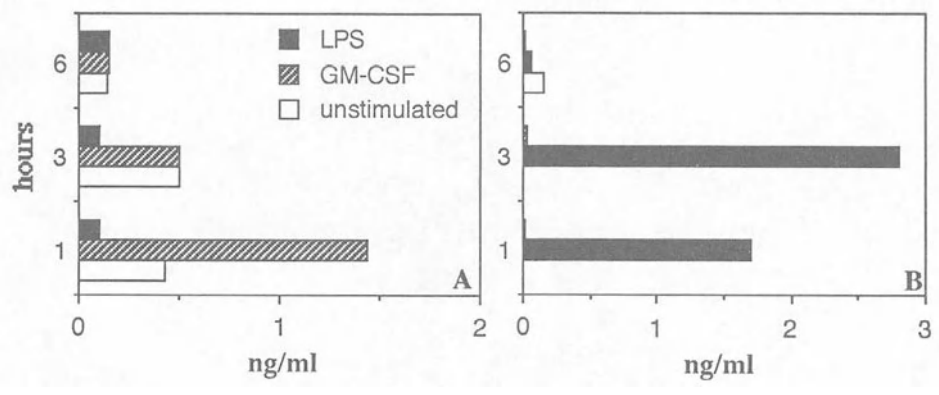

Figure 1. Production of IL-1β by CB1 (A) and D2SC/1 (B) clones after stimulation with 2 ng/ml of GM-CSF or 0,1 μg/ml LPS, as determined by ELISA.

IL-1β has been shown to be expressed in Langerhans cells but its role in the activation of resting T cells is still controversial (13).

The heterogeneity of the two cell clones was again confirmed by the production of TGFβ1, that was induced in D2SC/1 clone after GM-CSF stimulation (Fig.2A) but not in the CB1 clone (Fig.2B). This is consistent with the D2SC/1 clone having a more mature phenotype. These results, together with other studies (14), suggest functional heterogeneity among the spleen dendritic cell population.

No IL-1α, IL-4, IL-10 and IL-12 production was detected in both dendritic cell clones after low doses of GM-CSF induction. In contrast, TNFα was inducible but only with LPS stimulation (Table 1). Interestingly, IL-12 was produced after Herpes Simplex Virus type I cell infection and this production could be further enhanced by the addition of GM-CSF (K. Sandberg, personal communication).

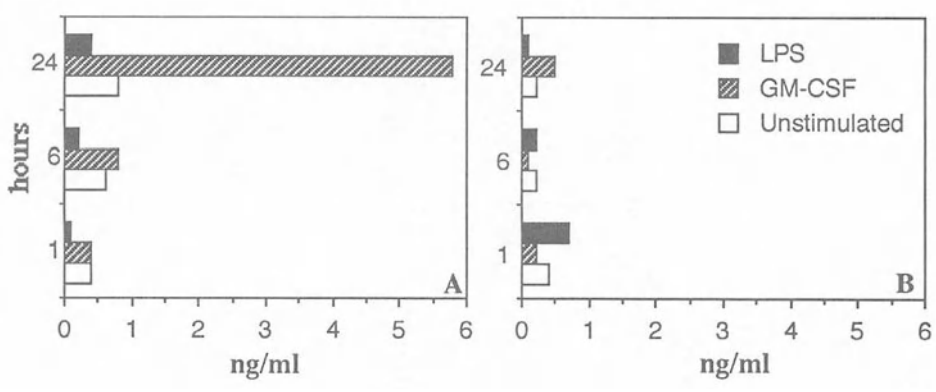

Figure 2. Production of TGF-b1 by D2SC/1 (A) and CB1 (B) dendritic cell clones after stimulation with 2 ng/ml of GM-CSF or 0,1 μg/ml LPS as determined by ELISA.

Table 1: Production of cytokines by dendritic cell clones

Cytokines	Stimuli	
	rGM-CSF (2ng/ml)	LPS (0,1μg/ml)
IL-1α	-	-
IL-4	-	-
IL-10	-	-
IL-12	-	-
TNFα	-	+
TNFβ	-	-
TGFβ1	+**	-
IL-1β	+*	+**

* CB1
** D2SC/1

CONCLUSIONS

In conclusion, we have shown the existence of heterogeneity between spleen dendritic cell clones by measuring their capacity to respond to GM-CSF, a cytokine known to mediate the growth and the differentiation of dendritic cells (1-2).

The pattern of cytokine expression by dendritic cell clones, in response to GM-CSF and LPS, is limited compared to macrophages (4). Further signals should be investigated to define the stimuli that drive activation of dendritic cells.

ACKNOWLEDGEMENTS

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REVIEW OF THE CHARACTERISTICS OF SIX CELL LINES WITH LANGERHANS CELL PHENOTYPE

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INTRODUCTION

Langerhans cells (LC) are bone marrow derived cells present in the epidermis, dermis and mucous membranes.[1,2] They are usually identified by the expression of surface CD1a and HLA-DR and electron microscopically by the presence of Birbeck (BG) or Langerhans cell granules.[3] Since the time consuming isolation procedures and the small yield of cells obtained by the laborious methods used for LC isolation, have hampered the research on LC, there has therefore been a search for pure, monoclonal, cell lines in order to facilitate LC research. Moreover, since recent studies of highly selected subpopulations of peripheral blood mononuclear monocyte-macrophages demonstrated the generation of CD1a+ cells from these subpopulations, LC was suggested belonging to the monocyte-macrophage lineage.[4,5] The present review is based on reports on the generation of six stable, selfreplicant, adherent, dendritic, CD1a+, HLA-DR+, CD23/FcERII+ cell lines with phenotypic features of LC .[6-10].

MATERIAL AND METHODS

Cells: Two cell lines, were initially derived from a histiocitic lymphoma cell line and four cell lines DD1, DD2, DD3 and DD4 were derived from peripheral blood mononuclear cells obtained from a normal donor, after being cocultured with supernatant of one of the initial cell lines.[6-10]

Immunofluorescence assays: The surface antigens CD1a, HLA-DR, CD14, CD45, CD11a, CD11b and CD36 were studied. The panel of monoclonal antibodies used for the cellular immuno phenotyping were either PE or FITC conjugated. Two color immunofluorescence analysis was performed after appropriated color compensation for each one of the markers, in a Coulter EPICS profile analyzer.

RNA Analysis-RT PCR: Total RNA isolation and RT-PCR was performed as previously described.[11] Beta Actin primers (Stratagene, La Jolla, CA) were used to test the quality of the cDNA samples. A battery of sense and antisense primers specific for the CD1a were synthesized[7] and used in a "Hot Start" PCR protocol with cDNA templates. A nested PCR was conducted in order to increase the specificity of the PCR.[12] The CD23 β sense primer CGGGGACGCAATAGAGTCAGAGGC, and the antisense primers

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GTCCTGTTCCTTTTGGCTGTGGATGC were used in the first PCR. In the nested PCR the β primer and the antisense primer GGAGCCCTTGCCAAAATAGTAGCAC were used.

RESULTS AND DISCUSSION

It was generated initially two cell lines, RAN1 and LAS1, using several rounds of selection for adherence and limiting dilutions in order to obtain monoclonality. When cultured in liquid media, RAN1 and LAS1, strongly adhere to polystyrene and grow in flat colonies with close cellular contact. However, using PMA, the cell line RAN1 adopted dendritic morphology. The dendritic morphology is a classical characteristic of the Langerhans cell in the epidermis, but they appear round when cultured in vitro. These two characteristics are present both by the RAN1 as well as LAS1 cell line (Table 1).[6-8]

The CD1a expression is one of the most characteristic markers for Langerhans cells[13]. Using FACS analysis it was clearly demonstrated that the six cell lines express significant amounts of surface CD1a. Moreover, the cell lines were also analyzed for evidence of CD1a at the RNA level, searching for CD1a transcripts using a nested PCR procedure that allow to identify CD1a distinctly from the other members of the CD1 gene family.[12,14] The six cell lines showed in an Ethidium bromide gel the product with the CD1a expected size. The cell lines also express HLA-DR that is another characteristic marker for Langerhans cells,[6-10] and the simultaneous coexpression of CD1a and HLA-DR has been widely considered as the most reliable marker for identification of LC.[13,15-17] The marker CD14 that defines a subpopulation of monocytes is not expressed by LC. Moreover, the cell lines have a negative expression of this marker (Table 1).[8-10]

The cell lines also have the low affinity Fc receptor for IgE /CD23 (Table 1).[6-10] The CD23 was initially recognized in epidermal cell suspension enriched with LC only after induction with IL-4 and/or IFNγ.[18] However, we found that by PCR analysis the cell lines clearly have transcripts for the β isoform of CD23. The expression of the β CD23 isoform by some lymphoid cells requires a stimulus, because β CD23 is not constitutively expressed. The inducers for β CD23 expression are IL-4 for B lymphocytes,[19] GM-CSF and TNF for macrophages[20] or a viral infection for some B and T cell lines.[11,19] The event that mediated the constitutive expression of β-CD23 by these cell lines is not known.

The CD45 marker has been identified in LC and FACS analysis of the cell lines demonstrated a weak or negative expression of this marker. However, using antibodies that identified the isoforms CD45RO and CD45RA, it was demonstrated that the cell lines express principally the isoform CD45RO.[6-10] To date this finding has not been described for LC, but it might possibly represent a useful marker for defining the activation status of LC or subpopulations of LC.[21]

Table 1. Characteristics of the six cell lines

Cell Line	Adherence	Dendritic	CD1a	HLA-DR	CD45RO	Fc IgG receptor	β CD23
RAN1	+	+	+	+	+	+	+
LAS1	+	+	+	+	+	+	+
DD1	+	+	+	+	+	+	+
DD2	+	+	+	+	+	+	+
DD3	+	+	+	+	+	+	+
DD4	+	+	+	+	+	+	+

The coexpression of the LC markers CD1a and HLA-DR by the cell lines together with the dendritic appearance under special culture conditions indicate that they have phenotypic features of LC. In addition, these cell lines have been in culture for more than a year without significant change or loss of the morphological features or the surface markers suggesting that the cell lines are in a stable state of differentiation.

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IMMORTALIZED MURINE DENDRITIC CELLS: PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION

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INTRODUCTION

Problems with the isolation of pure populations of dendritic cells (DC) have hindered investigations of their various functional attributes. Permanent cell lines have, however, been established from murine macrophages by means of novel recombinant retroviruses.[1,2] A new ectropic retrovirus carrying the v-myc oncogene of the avian MH2 leukemia virus together with the LTRs of the mouse AKR virus, is capable of activating transcription and secretion of M-CSF and expression of M-CSF receptors. An autocrine loop is set up in infected macrophages.[3,4] Recent developments in the induction of division and differentiation of murine DCs from either blood or bone marrow progenitors have provided an opportunity to establish permanent DC cell lines.[5] Growth and division of DC precursors in culture on exposure to GM-CSF is dramatic and the pathway of differentiation appears to be titled away from the macrophage phenotype. The objective of this study was to expose developing murine bone marrow-derived DC progenitors to the immortalizing retrovirus 3RV from the N-11 cell line.[2] This was an attempt to create permanent DC cell lines which maintain both phenotypic and functional characteristics of the DC lineage.

METHODS

Clusters of differentiating DCs were derived from bone marrow progenitor cells according to the protocol of Inaba et al.[5] The mice strains Balb/c, CB.17 (scid/scid) and CD.1 were employed. Following either negative lineage-specific antibody/complement ablation or positive selection with immunomagnetic techniques and anti Sca-1 antibodies the cells were cultured with added GM-CSF (100-200 U/ml for 14-21 days). Clusters were isolated and exposed to supernatants from the virus producing microglial cell line N-11 (courtesy of Dr. M. Righi). After 49-56 days of growth the cells were cloned by limiting dilution and the individual clones were characterized. No GM-CSF was added to the virally-transformed cells. Adherent splenic macrophages were isolated and immortalized as previously described.[3] Cells

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were phenotyped as cytospins with an extensive panel of antibodies against murine macrophage, lymphocyte and DC markers. Functional attributes were tested by means of the conventional assay for transfer of contact hypersensitivity.[6] Hapten-derivitized (FITC) Balb/c clone DDC/B301 cells were injected subcutaneously into naive recipient mice followed by ear challenge at 5 days post sensitization. Standard ear swelling measurements were taken and compared to controls either lacking derevitized hapten or to animals sensitized by normal epicutaneous routes.[6]

RESULTS AND CONCLUSIONS

Efficient immortalization of cells occurs with the 3RV retrovirus and cell lines with both stable macrophage and DC phenotypes were produced in a three mouse strains. The lines were productive of virus and had doubling time in the 15-20 hr region. The DC lines DDC/B210K from CD.1 mice and DDC/B301 from Balb/c showed short dendritic processes and reactivity with the various antibodies associated with the DC phenotype (Table 1). In comparison the splenic CD.1 line SM-140 showed typical macrophage phenotypic markers. All markers persisted over a period of at least 6 months. The assay of antigen presenting function showed a significant ability of the cloned DCs to induce contact hypersensitivity reactions in injected naive recipients. While the ear swelling results were somewhat less than that achieved via the epicutaneous/Langerhans cell route, the Balb/c clone DDC/B301 induced significant ear swelling on challenge in animals given mitomycin-treated hapten-labeled cells (Fig. 1). These results indicate that it is now possible and relatively easy to immortalize not only macrophages using this retroviral system, but also to capture and "freeze" DCs at stages of their differentiation. At present it is not clear what relationship the induction of M-CSF associated with the 3RV virus plays to the known susceptibility of DC precursors to GM-CSF manipulations.

Table 1. Immunostaining of Murine DC and cloned cells.

Phenotyping: Clones DDC/B210K / B301 versus SM-140

MARKER	Bone marrow progenitors	GM-CSF stimulated	SM-140 Splenic Macs	DDC/B210/L	DDC/B301
MHC classII (Ia)	neg	pos	+/-	weak pos	pos
NLDC-145*	neg	pos	neg	pos	pos
MIDC-8*	neg	pos	neg	pos	pos
BM8*	neg	pos	neg	pos	pos
M1-8*	neg	pos	neg	pos	pos
HSA (J11d.2)	+/-	pos	neg	pos	pos
CD11b (Mac-1)	neg	+/-	pos	+/-	+/-
M342	neg	pos	neg	pos	pos
MOMA	neg	+/-	pos	pos	pos
F4/80	neg	neg	pos	+/-	neg
CD18* (2E6)	ND	pos	pos	pos	pos
33D1*	neg	neg	neg	neg	neg
Lgp-55	ND	ND	neg	pos	pos
Sca-1	pos	neg	neg	neg	neg
Esterase	neg	+/-	pos	neg	neg

*NLDC-145/MIDC-8 = Dr. Kraal, BM8 = Dr. Malorny, M1-8 = Dr. Maruyama,
M342 /33D1 /2E6 = Dr. Steinman Lgp-55 (ICAM2 homolog) = Dr. Golde
After 14 days of GM-CSF 28% NLDC-145 / MIDC-8 +ve cells = Virus infection day
Thy-1, L3T4, Lyt1, Lyt2 negative in all clones
SM-104 = Virally immortalized splenic macrophage cell line

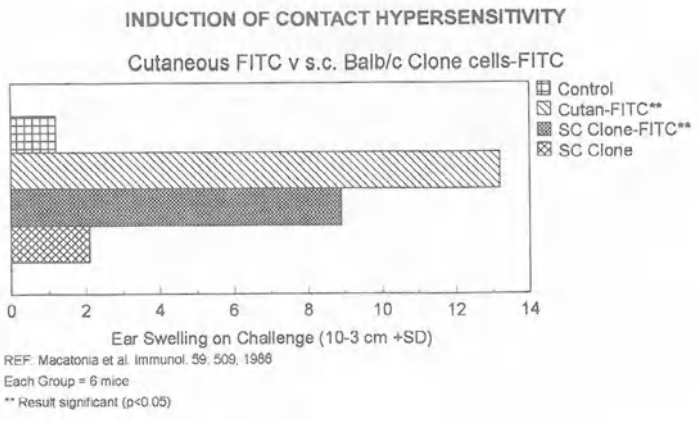

Figure 1. Ear swelling responses following subcutaneous injected cloned DCs and FITC or epicutaneous exposure to FITC.

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PRODUCTION AND PROPERTIES OF LARGE NUMBERS OF DENDRITIC CELLS FROM HUMAN BLOOD

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INTRODUCTION

Dendritic cells (DC) form a system of antigen presenting cells that are specialized to stimulate resting T cells and to initiate T-dependent immune responses ("nature's adjuvant") [for review see [1]]. Despite the difficulties in purifying this trace (≤ 1% at most sites) cell population a good deal is known about how DC sensitize T cells both in tissue culture and whole animal models. The limited numbers of DC hindered, however, molecular studies and the use of these cells for adoptive immunotherapy. In 1992 we described a simple method to grow large numbers of DC from murine blood[2] or bone marrow[3]. In this liquid culture system GM-CSF induced MHC class II-negative progenitor cells to develop proliferating cellular aggregates, and from these, many typical DC were then released. These DC exhibited a characteristic morphology, mobility, phenotype, and strong T cell stimulatory capacity. Importantly, DC grown in such cultures have been shown to process antigen, to home to the T-dependent regions, and to sensitize mice *in vivo* thus illustrating their potential as immunogens [2,4]. In the human system GM-CSF + TNFα was then shown to induce the formation of substantial numbers of DC from CD34+ cord blood progenitor cells[5,6]. Putative DC (as judged from morphology and phenotype, functional assays were not performed) yet only in small numbers could also be generated from CD34+ human bone marrow progenitors, again in the presence of GM-CSF + TNFa[7]. Neither neonatal blood nor bone marrow are, however, an ideal source of DC for potential clinical applications (e.g. immunotherapy). Peripheral blood would be a most suitable source, yet the enrichment of substantial numbers of CD34+ cells is impractical due to their low incidence (< 0.1% of mononuclear cells as opposed to 1-4 % in bone marrow[8]). Given our prior work showing that DC can be grown from murine blood without tedious enrichment procedures[2], we set out to extend the methodology to progenitors from man. We first used cord blood and peripheral blood collected from cancer patients during G- or GM-CSF therapy and hematopoietic recovery as both sources are known to contain substantial numbers of hematopoietic progenitor cells[9,10]. Following the establishment of a simple method to

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generate aggregates of growing DC we then modified it for use with blood from normal healthy adults. By using this systematic approach we found that human blood can indeed be used as a source to grow large numbers of potent immunostimulatory DC without purification of CD34+ cells and by using many of the same criteria as in our initial mouse work[11].

RESULTS and DISCUSSION

Generation of proliferating DC aggregates from cord blood mononuclear cells

It has been reported that GM-CSF if supplemented with TNFα generates up to 1 x 10^7 DC from 1 x 10^6 purified (> 95%) CD34+ cord blood hematopoietic precursor cells[5,6]. A limitation of the protocol is the difficulty to obtain larger numbers of CD34+ cells as these are rather scarce (~ 1.6 % of low density cells) even in cord blood[9], and not more than 80 ml of cord blood can be easily obtained. When we tried to generate proliferating DC aggregates (hereafter also termed "balls") by using the simple method described for murine blood[2] - i.e. culturing PBMC or their MHC class II negative fraction in the presence of GM-CSF - we were initially not successful. We then realized that the varying, yet substantial percentage of nucleated erythroid cells in the cord blood-derived low density fraction was toxic and had to be reduced prior to plating. A large number of pilot experiments finally revealed that the simplest approach was to prepare low-density (< 1.077 g/ml) cord blood cells, and to coat them with anti-glycophorin A mAb (in pilot experiments we had also included anti CD3 + HLA-DR mAb but this proved unnecessary) followed by two panning steps for removal of most erythroid cells[11]. The resulting cell suspension was then plated in 16-mm tissue culture wells at 1 to 2 x 10^6 cells in 1 ml standard medium supplemented with GM-CSF (400 - 800 U / ml) +/- TNFα (10 - 50 U / ml) and fed every other day by aspirating 0.3 ml of medium and adding back 0.5 ml of fresh medium with cytokines. Similar to what we had described in the mouse[2] - yet in contrast to the culture systems using purified CD34+ cells as a starting population[5,6] - small adherent cell aggregates appeared after 4 to 7 days. These early balls already displayed a hairy appearance and were typically developing on a nest of spindle-shaped cells similar to what we had noted in the mouse. At this stage contaminating non-adherent cells could be removed by a careful rinse with warm medium, though this was not essential. The balls enlarged over the next 7 to 10 days, and started to release typical "veiled" DC (not shown). DC balls only formed if GM-CSF was supplemented to the culture medium. The addition of TNFα, though not required for the formation of DC balls, increased their size, doubled the yield of mature DC, and significantly enhanced the T cell stimulatory activity of the DC progeny[11]. It was advantageous to wash out and omit TNFα during the last 1-2 days of culture as the DC balls then fell apart more easily and released single, mature DC. These were identified on the basis of cytology, phenotype, and function. Under the inverted microscope they displayed the characteristic thin and highly mobile cytoplasmic sheets ("veils"), and electron microscopy revealed the typical ultrastructure of DC, notably the organelle-free veils and a paucity of lysosomes and phagosomes[11]. A LC granule was found only in 1 out of 100 cell profiles. The phenotype (data not shown) was similar to that of cultured human LC[2] (included in most staining experiments for direct comparison), i.e. a MHC class II rich "null" leukocyte [CD1a+, HLA-DR++, 9.3F10+, CD3-, CD14-, CD19-, CD20-). Anti-Lag (LC granule associated antigen) mAb[13] reacted only with 1-2% of cells that were, interestingly, localized in the center of (rare) residual DC balls[11]. The cord blood derived-DC were potent stimulators of resting T cells in the 1° allogeneic MLR as well as

oxidative mitogenesis[11]. The protocol allowed us to generate 1 - 5 x 10^6 DC from 40 ml of cord blood at a purity of 20 - 50% (after a metrizamide flotation step ≥ 80%).

Generation of proliferating DC aggregates from PBMC collected from cancer patients during hematopoietic recovery

We next turned to PBMC obtained from cancer patients in full remission (leukemias/lymphomas and solid tumors) who were treated with high-dose chemotherapy and either G-CSF or GM-CSF. We expected this to be a useful model as it is well known that hematopoietic progenitors including CD34+ cells are mobilized in substantial numbers into the blood during hematopoietic recovery[10]. Results were comparable in all experiments. PBMC from which the bulk of CD3+ and strongly HLA-DR+ cells had been removed by 2 panning steps (see Methods) were plated at 1(or 2) x 10^6 cells in 16-mm tissue culture wells in 1 ml of standard culture medium containing 5-10% FCS or 5% cord serum, and supplemented with human rGM-CSF (400 or 800 U/ml). On day 2 the non-adherent cells were transferred to fresh wells. Cultures were fed every other day thereafter and for a total of 16 days. Characteristic proliferating DC balls appeared by the 5th day in the transferred wells (similar to what we had observed in the mouse), as evident under the inverted phase contrast microscope. Initially these balls were loosely adherent, but became nonadherent as they expanded in size over the course of a week (day 5 to 11) (Fig. 1A) and developed characteristic processes including veils at their edges.

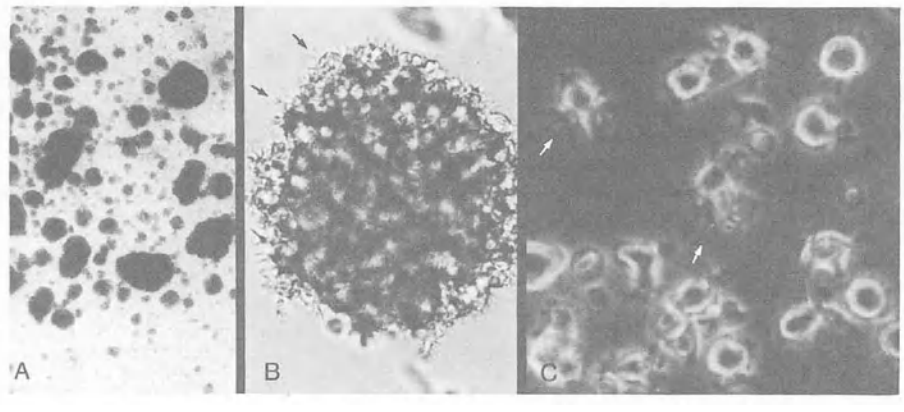

Figure 1. Development of DCs in liquid cultures of $CD3^-$ & $HLAD\text{-}DR^-$ blood mononuclear cells obtained from cancer patients after high-dose chemotherapy and G-CSF treatment, and supplemented with GM-CSF + TNFα. DC aggregates have grown to substantial size on d7, and have become nonadherent (A). The peripheral cells display a characteristic dendritic or veiled appearance that is particularly evident following isolation of the aggregates by 1g sedimentation [2] (B). On d12 the aggregates were dissociated by pipetting to obtain single DCs (C, arrows indicate some veils). A, x 25; B, x 200; C, x 500.

Some balls appeared also in the original wells, but typically these did not enlarge to the same extent as those arising in the nonadherent fractions. The wells had to be subcultured, e.g. 1 well split into 2- 3 wells, as cell density increased. Occasionally adherent non-DC balls appeared that reduced the purity of the DC cultures by producing macrophages and / or granulocytes. Typically, these balls consisted of round cells without any veils, were more adherent, and were usually left behind at the day 2 transfer step of the protocol. If these adherent colonies reappeared, we simply transferred the growing non-adherent DC balls to

another well. Two alternative approaches were used to isolate the mature DC from the growing cultures. One method consisted of removing cells / cell aggregates that were nonadherent and separate the balls from nonballs by 1 g sedimentation (Fig. 1B). DC were then released in large numbers from the balls over an additional 1-2 days of culture (Fig. 1C). The DC in the nonball fraction could be isolated by flotation on dense metrizamide as described [14]. In the second method we simply waited until the balls had become very large and loose, harvested all the nonadherent cells, left them on ice for 20 min., resuspended then vigorously with a pipette to disaggregate the balls, and floated the mature DC on metrizamide columns. The DC were identified on the basis of their typical morphology at the light and ultrastructural level, their phenotype of a MHC class II rich "null" leukocyte [HLA-DR++, 9.3F10+, CD3-, CD14-, CD19-, CD20-), and potent immunostimulatory function[11]. Interestingly, the DC progeny were - in contrast to the DC grown from cord blood - either CD1a negative or slightly positive. They also uniformly lacked anti-Lag (LC granule associated antigen) mAb[3] immunoreactivity and LC granules were not detectable on ultrathin sections. GM-CSF proved to be essential for the development of DC. G-CSF, M-CSF, IL-3, or no cytokine did not permit the development of DC balls. GM-CSF at 400-800 U/ml was optimal, regardless of whether blood donors had been treated with either GM-CSF or G-CSF. Irradiation (3000 rad) of the inoculum prevented the formation of DC balls indicating that they emerged by growth. Addition of TNFα at 10-50 U/ml usually though not always increased DC yields up to two-fold, and regularly and substantially improved the function of the DC progeny[11]. Human rIL-1α (at 50 LAF units/ml) was added in some experiments during the last 24 hours, and further increased function[11]. Starting from 60 ml of blood, and after culturing in the presence of GM-CSF + TNFα for 16 days, the yield of mature DC was 6-12 x 10^6 at 60-80% purity. This is at least 20 times the yield of mature DC in 60 ml of fresh normal blood which would be at most 5% (3-6 x 10^5) of this, and is remarkable given the fact that HLA-DR+ cells and thus DC were removed by panning at the onset of the cultures. It is conceivable that the above protocol could also be directly applied to non-cancer patients after administration of G-CSF, which recently has been shown to mobilize progenitors also into blood of normal adults with minimal toxicity[15].

Generation of proliferating DC aggregates from PBMC of normal healthy adults

We next applied the protocol described above to PBMC obtained from normal healthy adults. We plated 1-2 x 10^6 PBMC that had been depleted of most HLA-DR+ and CD3+ cells (either by anti-CD3 + HLA-DR panning as described above or by preparing E rosette negative PBMC[14] followed by anti-HLA-DR panning) in 16-mm culture wells in 1 ml culture medium (see above) supplemented with GM-CSF (400 - 800 U/ml) +/- TNFα(10 - 50 U/ml). Nonadherent cells were transferred to new wells after 1d. In more than 20 standardized experiments we observed that some small adherent hairy balls formed in the wells between days 8 and 16. These balls, however, quite surprisingly then deteriorated over the next few days leaving behind nonviable cells or, less often, transformed into what appeared to be macrophages. As an adherent stromal layer, which we had found critical for the development of DC balls from mouse blood, had not formed early on we reasoned that we had possibly removed cells from the PBMC that are required for the formation of such a layer. We, therefore, next used unfractionated PBMC as in our initial studies with mouse blood[2]. We plated 1 x 10^6 PBMC in 16-mm wells in GM-CSF (800 U / ml) +/- TNFα (50 U/ml), and after 1 day transferred the nonadherent cells containing most lymphocytes into new wells. Careful observation in 12h intervals under the inverted microscope disclosed that within 2 days in the adherent fraction *many* small adherent balls had developed that

displayed a strikingly hairy appearance characteristic of early proliferating murine and human DC balls. Within another 2 days, however, these balls had transformed into round cells and finally gave rise to a monolayer of macrophages. This occurred in cultures supplemented with GM-CSF as well as in cultures with GM-CSF + TNFα. On days 12 to 16 then some adherent DC balls emerged again but only in ~ 30 to 50 % of the original wells (containing the adherent fractions left after transfer of cells on day 1), notably in those supplemented with GM-CSF + TNF α, and increased in size over the ensuing days (Fig. 2).

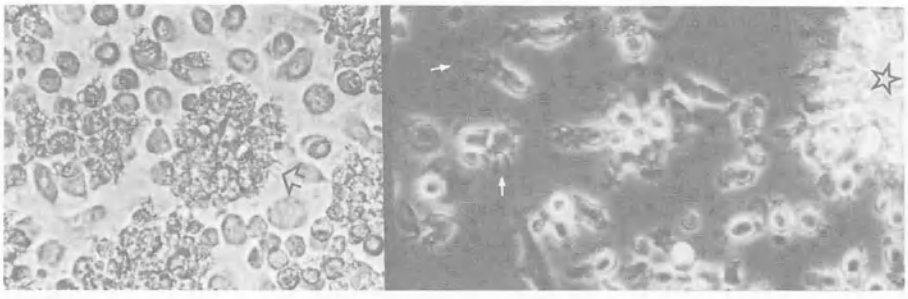

Figure 2. Development of DCs in liquid cultures of normal, adult blood mononuclear cells supplemented with GM-CSF + TNFα. Low power view of expanding DC aggregates (d16 culture) loosely affixed to an adherent monolayer (left). The aggregates can be easily dislodged, and then release typical DCs (right, arrows indicate veils, asterisk marks a residual DC aggregate). x 350

These aggregates when dislodged by careful rinsing and transferred to fresh wells released single cells during overnight culture that qualified as DC due to their typical morphology (Fig. 2), phenotype of an MHC class II rich "null" leukocyte [CD1a+, HLA-DR++, CD3-, CD14-, CD19-, data not shown] , and T cell sensitizing capacity[11]. The yield was 4% of all PBMC cells originally plated in the wells where DC balls finally emerged. This is 8 times the number of DC contained in fresh blood (≤ 0.5% of PBMC).

Despite the appearance of DC balls in part of the wells after a prolonged culture period the puzzling phenomenon remained that we *regularly* observed early on (24 - 48h) in *all* wells a *large* number of small yet *transient* DC balls. We had already noticed in cultures of PBMC collected during hematopoietic recovery (see above) that early, small DC balls can transform into non-DC balls. This is in congruence with recent evidence that DC, macrophages and granulocytes arise from a common precursor[7,16]. We, therefore, reasoned that PBMC of normal healthy adults might contain a DC precursor that initially formed small DC balls but under our culture conditions then further on developed mainly along the macrophage pathway. As IL-4 is known to block macrophage colony formation at 500 - 1000 U/ml[17] we cultured PBMC in GM-CSF + IL-4 at 1000 U/ml in order to indirectly promote development along the DC pathway. In the presence of GM-CSF *and* IL-4 two striking changes were observed. First, the initial hairy balls did not transform into macrophages but rather rapidly increased in size over the next few days, became nonadherent, displayed characteristic veils at their periphery, and finally started to release mature DC. Secondly, it was evident that within the first 2 days of culture most of the single adherent cells (i.e. monocytes) scattered in between the small adherent balls had become nonadherent and transformed into cells that displayed processes including veils and thus resembled DC. DC balls also formed in the nonadherent fraction that had been transferred into fresh 16-mm wells on day 1, but they were obscured by the presence of

many lymphocytes and less frequent as compared to the original well. Large, growing DC balls only formed in the presence of *both* GM-CSF *and* IL-4. After 20 standardized and reproducible experiments performed in 16 mm wells we sought to simplify handling by using larger 35 mm wells. A series of experiments revealed that the simplest protocol was to plate 5 - 10 - 20 x 10^6 PBMC/ 30 mm well in 3 ml standard medium, to discard the nonadherent fraction already after 2 hours, and to culture the adherent fraction in medium supplemented with GM-CSF (800 U/ml) + IL-4 (500 U/ml). In adherent fractions of the cultures then identical changes occurred as described above for the 16 mm wells (Fig. 3A,B,C).

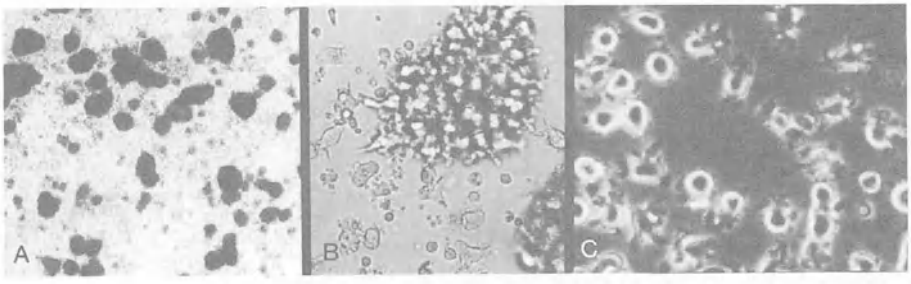

Figure 3. Development of DCs in liquid cultures of normal, adult blood mononuclear cells supplemented with GM-CSF + IL-4. On d3 small adherent DC aggregates are readily visible under the inverted phase contrast microscope (A). On d7 the DC aggregates have become nonadherent, very large, and loose (B) (compare Fig. 1A). The nonadherent fraction of the cultures was harvested and vigorously resuspended to obtain single DCs in large numbers (C). A, B, x 25; C, x 350.

It was important to remove the nonadherent fraction very carefully (by taking it off and one slight rinse with warm medium). Pilot experiments had shown that the nonadherent fractions which contained the bulk of contaminating lymphocytes then developed almost no DC balls, and, therefore, could be discarded. If, however, three washes rather than one slight rinse were applied DC balls appeared mainly in the nonadherent fraction. The adherent fractions then contained only few growing DC balls although most of the remaining adherent cells again transformed into veiled DC (as observed in 16 mm well cultures, see above) yet without significant growth. When PBMC were irradiated (3000 rad) at the onset of culture such single veiled DC still appeared, but rapidly growing DC balls were no longer seen. A variable percentage of cell nuclei (5 - 10%) inside the DC balls reacted with anti-Ki 67 mAb[18] identifying them as cycling cells[11]. These data suggest that DC balls evolved from a loosely adherent, proliferating precursor similar to what we had found in murine blood [2]. After 5 to 7 days of culture the DC balls had become very large and loose yet did not seem to grow further. The nonadherent fractions were then harvested from cultures, vigorously resuspended to disaggregate the DC balls, and analyzed. The cell fractions contained 40 - 60% DC and contaminating lymphocytes (mainly T cells). DC could be further enriched (≥ 80 %) by flotation on dense metrizamide. The blood-derived DC exhibited [11] a characteristic DC morphology at the light and electron microscopic level, and a phenotype as well as T cell stimulatory capacity comparable to that of cultured LC[2] and dermal DC[19], both of which constitute well-defined DC subsets. With respect to the phenotype it was of note that the blood-derived DC expressed CD1a, CD4, and FcεRI. LC granules were not detectable by electron microscopy, and only a rare cell - usually located in the center of residual DC balls - expressed the LC granule-associated antigen Lag[13]. Anti-CD68

immunostaining revealed a perinuclear spot as found in DC isolated from blood as opposed to the strong, diffuse granular cytoplasmic staining of macrophages[11]. Pilot experiments have shown that T cells can be removed from PBMC by E rosetting [14] in order to increase the purity of DC fractions (≥ 80%) obtained after culture in GM-CSF + IL-4. The yield of mature DC was 6 - 15 % of all PBMC that were initially plated. Thus, from one buffy coat (~ 500 x 10^6 PBMC) we obtained 30 to 80 x 10^6 DC within 5 to 7 days of culture, which is 12 to 30 times the number of DC contained in fresh blood (≤ 0.5% of PBMC [14,20]).

Relevance of culturing DC from peripheral human blood

Human DC until recently could be grown in substantial numbers solely from cord blood, and only after tedious purification of rare CD34+ hematopoietic precursor cells[5,6]. We have now achieved to grow human DC - without any need for isolating precursor cells - from PMBC obtained from three sources: cord blood, peripheral blood of cancer patients collected after high-dose chemotherapy and treatment with G-CSF or GM-CSF to promote hematopoietic recovery, and, most importantly, peripheral blood of healthy adults. Large numbers of human DC can now be grown by a simple and reproducible protocol from small blood samples. This will allow further elucidation of DC function, notably by molecular approaches. It will also be feasible now to explore the immonogenic potential of DC in humans, e.g. for adoptive immunotherapy in cancer patients[1,21,22].

A remarkable finding was that irrespective of the blood source used the bulk of mature DC appeared to derive from growing DC balls similar to what we had described in the mouse[3]. In the murine system several detailed studies have already elucidated that GM-CSF supports the in vitro development of DC from MHC class II negative to more committed, rapidly proliferating, MHC class II positive precursors that finally give rise to fully mature, non-dividing DC which are released at the periphery of the DC balls[2,3,16]. Further investigation of the culture systems we described here is required to formally prove that the mature human DC are progeny of proliferating precursor cells in the center of the DC balls. It will also be interesting to verify that human DC progenitors like mouse ones phagocytose particulates such as bacillus Calmette-Guerin organisms, so that the DC progeny are then able to sensitize T cells to mycobacterial antigens[4].

Based on our current observations we suspect that the DC precursors that give rise to the DC balls in cord blood, blood of cancer patients during hematopoietic recovery, and blood of healthy adults are different. In cord blood the DC balls likely derive from CD34+ cells as preliminary experiments (N. Romani, unpublished observations) have shown that depletion of CD34+ cells from the initial inoculum virtually abolishes the formation of DC balls. This would also readily explain the need to add TNFα which is known to induce GM-CSF responsiveness of CD34+ cells by upregulating the GM-CSF-R β chain[23,24]. With regard to blood derived from cancer patients under G- or GM-CSF therapy we are inclined to believe that besides CD34+ cells more committed precursors are involved, as DC balls develop rapidly and intensely even if in the HLA-DR / CD3-depleted inoculum CD34+ cells were barely detectable by FACS analysis. In peripheral blood of normal human adults the issue of the nature of the DC precursor(s) seems most complex yet also most intriguing. The observation that only in part of the wells, and after a prolonged culture period (≥ 2 weeks) in GM-CSF + TNFα DC balls emerge might indicate that these DC balls are derived from an early precursor (possibly rare CD34+ cells in peripheral blood). The most puzzling observation in our study, however, was that many small, adherent, hairy "candidate" DC balls formed within the first 24 to 48 hours likely originating from loosely adherent, HLA-DR positive precursor cells. These early "candidate" DC balls, however, then rapidly transformed into macrophages in both GM-CSF and GM-CSF + TNFα supplemented

cultures. If IL-4, which has been known for quite a while to specifically block macrophage colony formation[17], was added this transformation did not occur, and large DC balls developed and released typical DC. We do not yet know whether IL-4 indeed acts by the mechanism we have selected it for, i.e. by blocking the macrophage developmental pathway and thus indirectly promoting DC development from a common precursor.

Apart from the fact that the precursor(s) that give(s) rise to large, *proliferating* DC balls in response to GM-CSF + IL-4 seems to be HLA-DR positive, CD14 negative to weakly positive, and loosely adherent the exact nature is currently not yet known. Another remarkable observation in our cultures was that the single, firmly adherent cells developed by a *non-proliferative* pathway into cells resembling DC in morphology as well as phenotype in response to GM-CSF + IL-4, whereas they transformed into typical macrophages in GM-CSF alone. FACS sorting experiments (U. O`Doherty and D. Brang, unpublished observations) indicate that these cells derive from CD14++ monocytes (Fig.4) which after short-term culture in GM-CSF + IL-4 become nonadherent and similar in morphology to typical DC, yet are 50 - 70% less potent T cell stimulators when directly compared to DC isolated from fresh blood. The transformation of monocytes into cells that qualify as DC under the aegis of GM-CSF + IL-4 has recently also be reproted by Sallusto and Lanzavecchia[25] who made this discovery when using a protocol that had been used previously by Porcelli et al.[26] to induce CD1b on monocytes. It is likely that these monocyte-derived DC are related to the "monocyte-derived accessory cells" generated by Peters et al.[27] from culturing firmly adherent monocytes in serum-free medium supplemented by IL-4 (yet without exogenous addition of GM-CSF). GM-CSF has recently been shown[28] to induce synthesis and surface expression of native CD1 molecules by CD14+ monocytes. Rossi et al.[29] had previously reported that CD1a+ cells are generated from adherent PBMC in Iscove´s modified Dulbecco´s medium with FCS which might be due to to endogenously produced GM-CSF. In any case, CD1a+ cells prepared according to the protocol by Rossi et al.[29] are CD14+ and very weak in stimulating a 1° MLR (A. Lenz, unpublished observations), and thus are clearly not related to cells that derive from monocytes under the aegis of GM-CSF *plus* IL-4. The latter seem to qualify as DC by all in vitro criteria such as morphology, phenotype, and T cell stimulatory function. Nevertheless, we suspect from ongoing experiments that these "monocyte-derived accessory cells" nevertheless do not exhibit all the important functional properties of DCs, notably their migratory and homing properties. Specifically, they seem to readhere to surfaces upon culture in medium *without* GM-CSF + IL-4 in sharp contrast to mature DC isolated from human blood or grown from proliferating precursors with GM-CSF + IL-4.

Provided that the effects of the combined action of GM-CSF + IL-4 on DC precursors and monocytes take place *in vivo* as observed in vitro the implications would be manifold and far-reaching. For example, the presence of GM-CSF and IL-4 in lesions of atopic dermatitis, a disease involving Th2 mediated immune responses, might contribute to the abundance of lesional DC / LC as well as to the intensity and notorious chronicity of this disease process[30]. In this context it is also notable that the DC that we have grown from PBMC under the aegis of GM-CSF and IL-4 exhibit a phenotype virtually identical to cultured epidermal LC[12] and dermal DC[19]. Particularly striking is the expression of the high affinity receptor for IgE (FceRI). This receptor is also expressed by LC[31,32] and is thought to play a decisive pathogenic role in atopic dermatitis by capturing even tiny amounts of allergen via surface bound IgE for subsequent antigen processing[30]. The DC cultured by the protocol described here now offer a model which is readily accessible in contrast to human LC that can be enriched from human skin samples only in small numbers. It might be worthwhile to consider the possibility that the combined *in vivo* administration of GM-CSF + IL-4 might enhance immune responses by inducing a burst of DC

development. As emntioned above it seems also very promisising to use DC grown in vitro for adoptive immunotherapy to induce resistance against tumors and infectious agents.

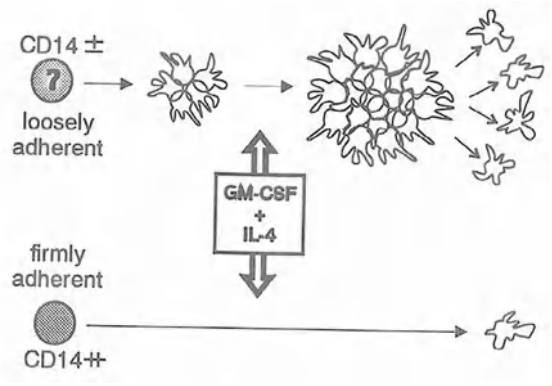

Figure 4. Diagram of the proposed pathway of DC development in liquid cultures of normal blood mononuclear cells supplemented with GM-CSF + IL-4. A very loosely adherent, CD14 negative or weakly positive DC progenitor (1) gives rise to small initial, adherent aggregates of veiled cells (2), which, however, then transform into macrophages (3) in the presence of GM-CSF alone. The addition of IL-4 presumably suppresses the monocyte differentiation potential [17] of the DC progenitor, so that the initial veiled aggregates do not transform into macrophages but rather proliferate (4), increase in size, and start to release mature DCs (5). In such cultures DCs also seem to derive from firmly adherent, CD14 ++ monocytes by a *non*-proliferative pathway.

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A SIMPLIFIED METHOD FOR GROWING DENDRITIC CELLS FROM RAT BONE MARROW

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INTRODUCTION

Bone marrow (BM) derived dendritic cells (DC) are the most potent stimulator cells of primary mixed leukocyte reactions (MLR) and also serve as powerful antigen-presenting cells in the priming of CD4+ T cells *in vivo* The investigation of mechanisms by which DC function, both *in vivo* and *in vitro* requires optimal conditions for culture of these cells so that large numbers can be produced for analysis With the availability of recombinant (r) GM-CSF, several reports have shown that it is possible to grow many DC from murine [1] and human[2] BM Although murine rGM-CSF has been used in the rat to promote the survival of lymph-borne DC for up to 72 hours[3], until this report cytokines have not been used to enhance the DC yield from fresh rat BM cultures

MATERIALS AND METHODS

BM from 7 to 9 week old Lewis (LEW) rats was depleted of Fc+ and plastic adherent cells by panning for one hour on petri dishes coated with a mixture of 10% normal goat serum and 5% normal human AB serum in PBS Nonadherent cells (NAC) were removed and cultured (3×10^6/ml) in flasks coated with a 1% solution of 300 bloom porcine skin gelatin in PBS, using medium supplemented with an optimal concentration of murine rGM-CSF Cultures were fed every second day by exchanging half the medium for fresh rGM-CSF containing medium, without discarding any floating cells Varying numbers of DC-rich cultured BM cells and fresh LEW spleen cells (positive controls) were used as stimulators in mixed lymphocyte reactions against a constant number of responder cells (9×10^4)

The DC yield from BM cultures was monitored by double staining with the mAbs OX-6 (MHC class II) and OX-62 (rat dendritic cells) for flow cytometric analysis (cells were confirmed to be OX-19 to avoid the possibility of cross-reaction between OX-62 and γ/δ T-cells) Scanning and transmission electron microscopy (EM) were performed using standard techniques

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RESULTS

GM-CSF significantly ($p<0.01$) improved cell proliferation five days after commencing cultures of unfractionated rat BM. Because the propagation of murine DC on type-1 collagen-coated tissue culture plates has been found to promote "immature" liver-derived DC maturation in the presence of GM-CSF[4], we analysed the effect of coating tissue culture flasks with gelatin on BM proliferation. Gelatin-coating increased the proliferation of unfractionated BM by 1.3-1.4 times ($p=0.05$, d5 culture) and augmented NAC yield in d6 cultures from 8.2 ± 0.7% to 13.5 ± 2% of the initial number of cells cultured ($p<0.02$).

Plastic adherent and Fc+ cells were depleted by panning on petri dishes coated with normal serum. NAC from unfractionated cultures showed 3.5 times greater allostimulatory ability than unfractionated LEW splenocytes, while NAC from cultures depleted of plastic adherent and Fc+ cells were 31 times stronger than fresh spleen cells. OX-62 staining revealed that DC yield could be increased from 5.2% to 8.5% of NAC by depleting Fc+ and plastic adherent cells before culture.

GM-CSF also promotes monocyte, macrophage and granulocyte growth in cultures of BM, so to further enrich DC purity, NAC from d5 cultures of unfractionated BM and adherent and Fc+ cell depleted BM were panned in serum-coated dishes before analysis (Fig. 1). Results were consistently better when NAC were depleted of Fc+ and plastic adherent cells (C and D vs. A and B), demonstrating that this technique could efficiently remove contaminating monocytes (ED-1 and ED-2 positive cells) from cultured BM. The stimulatory ability of d5 BM cultures also improved, NA and adherent fractions were 132 (D) and 32 (B) times stronger than fresh spleen cells, respectively.

To ascertain the optimum DC yield from this system, a detailed growth curve was performed (summarised in Table 1), sequentially documenting the yield of cells double-labelled with OX-6 and OX-62. Initial depletion of Fc+ and plastic adherent cells from 300×10^6 fresh unfractionated BM cells, yields 0.9×10^6 DC. After 7d culture and redepletion of Fc+ and plastic adherent cells, a maximum of 6.5×10^6 DC can be obtained. The allostimulatory ability of these cells increases up to d9, at which stage they are between 597 (PVG responder) and 665 (ACI responder) times more potent allostimulators in MLR than equivalent numbers of unfractionated LEW splenocytes.

Transmission EM demonstrated many cells with long dendritic processes, lobed nuclei and profuse mitochondria, while scanning EM revealed classical and distinct cytoplasmic veils.

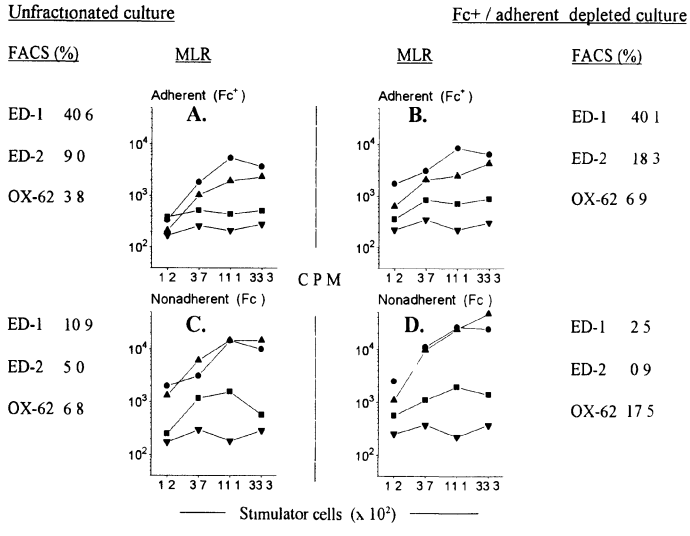

Figure 1. Depletion of Fc+ and adherent cells from NAC obtained after 5d culture with GM-CSF in gelatin-coated flasks enriches the DC population to 17.5% (D), with an associated increase in allostimulatory ability while reducing ED-1 contamination to 2.5%. Examination of the adherent cells remaining in the panning plates (A, B) revealed that 40% of these cells were ED-1 while less than 7% were OX-62$^+$. Throughout these experiments NAC from cultures of Fc+ and adherent cell depleted BM (B, D) showed consistently more OX-62$^+$ cells with higher levels of stimulatory ability in MLR than was achieved with unfractionated BM (A, C). Representative data from three experiments are shown. (MLR = mixed lymphocyte reaction. FACS = flow cytometry.)

Table 1. BM, depleted of Fc+ and plastic adherent cells, was placed in culture and analysed 1d to 9d later Although the number of NAC gradually diminished, DC ($OX6^+/OX62^+$) increased to a maximum of $6\ 5x10^6$ by d7 The allostimulatory ability of these cells continued to increase until day 9, at which stage they were approximately 600 times more potent than fresh LEW splenocytes at stimulating an MLR

Day	NA cells recovered from	Mean yield of NA	$OX6^+/OX62^+$ cells	Ratio of ability in MLR to that	
	(x 10^6)	(x 10^6)	(x 10^6)	ACI responder	PVG responder
1	69	29	0 46	68	37
3	72	44	2 6	124	99
5	38	14	2 8	178	135
7	27	14	10 8	177	180
9	7 2	2 5	0 52	665	612

DISCUSSION

The recent availability of a specific mAb for rat DC (OX-62[5]) makes it possible to reliably monitor the development of cultured DC, allowing accurate optimisation of techniques to propagate DC from the rat Unfortunately GM-CSF also promotes the growth of monocytes, which can express class II Ag as a surface marker and hence, simple determination of the number of MHC class II+ cells in culture, as has been performed previously[3 6], does not adequately differentiate between monocytes and DC Therefore, in this paper putative DC were double labelled with OX-62 and OX-6, allowing accurate determination of cell phenotype DC yield was a function of the time spent in culture (up to d7) and allostimulatory ability strongly correlated with the intensity of surface marker expression

It is remarkable that as simple a procedure as panning on normal serum-coated petri dishes prior to culture of rat BM in rGM-CSF supplemented medium, with repanning of the nonadherent cultured cells prior to analysis, allows one to generate a population of cells which are 46% double-positive for OX-6 and OX-62 and have over 100 times the allostimulatory ability of fresh spleen cell controls Furthermore culture of these cells in gelatin-coated flasks significantly increases the yield of DC obtained This procedure can readily be adopted in any cell culture laboratory

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TRANSITION OF T_3-INDUCED MONOCYTE-DERIVED VEILED/DENDRITIC CELLS INTO MACROPHAGE-LIKE CELLS BY LIPOPOLYSACCHARIDE

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INTRODUCTION

Dendritic cells are excellent antigen-presenting cells and play a crucial role in the initial phases of normal and autoimmune responses. Although the precursor of the dendritic cells has not yet been fully characterized, the transformation of cells residing in the blood monocyte pool into veiled/dendritic cells was reported by us and others[1,2]. Iodinated compounds such as metrizamide, thyroid hormones triiodothyronine (T_3) and thyroxine (T_4), and to a lesser extent reverse T_3 and highly iodinated thyroglobulin enhance the transition of monocytes to veiled/dendritic cells[2]. The transition induced by T_3 and T_4 is dependent on the production of granulocyte-macrophage colony stimulating factor, tumour necrosis factor-α and interleukin-6, since antibodies specific for the different cytokines had blocking effects. Antibodies directed to granulocyte colony stimulating factor, macrophage colony stimulating factor and interleukin-1β had no effect[2].

The aim of the present study was to determine whether the monocyte-derived veiled/dendritic cells are able to convert into macrophage-like cells. Therefore, the cells were cultured in the presence of lipopolysaccharide (LPS) and thereafter the morphology, the presence of surface markers and the acid phosphatase reactivity of the cells were studied.

MATERIALS AND METHODS

Isolation and Culture of Monocytes

Peripheral blood monocytes from healthy volunteers were isolated by Ficoll-Isopaque density gradient centrifugation, followed by Percoll density gradient centrifugation. The cell suspension contained 60-80% non-specific esterase-positive

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monocytes. The cells were incubated with serum-free Iscove's Modified Dulbecco's culture fluid culture alone or were stimulated under non-adhering conditions for 30 min with T_3 at a concentration of 2×10^{-10} M. Cells were washed and further cultured in the serum-free culture fluid for a period of 16 h (overnight) under non-adhering conditions.

Determination of the Percentage of Veiled/Dendritic Cells

The percentage of veiled/dendritic cells was determined by criteria based on morphology and staining pattern. In wet preparations, these cells are large, with actively moving veiled cytoplasmic processes and/or dendritic extensions. In cytospin preparations, the cells have long cytoplasmic protrusions, a reniform nucleus, strong MHC class-II positivity, but absent or weak acid phosphatase reactivity.

Enrichment and Functional Activity of Veiled/Dendritic Cells

After the overnight culture period, cells were placed for 2.5 hr in the top chamber of a Boyden chamber system divided by a polycarbonate microfilter (5μm) from the bottom chamber containing chemoattractant fMLP. The top chamber contained very high percentages of veiled/dendritic cells. The cells were tested for their capability of stimulating T cells in an allogeneic mixed leucocyte reaction (MLR).

Incubation of Cells with LPS

Monocytes and veiled/dendritic cells (Boyden chamber method) were incubated for 24 and 48 hr in the presence of LPS (*E. coli*-derived, 10 ng/ml). Cells were stained with the following antibodies RFD1 (dendritic cells), RFD7 (macrophages), RFD9 (macrophages), My4 (CD14, monocytes) and KIM6 (CD68, macrophages). Acid phosphatase reactivity of the cells was determined.

RESULTS

Veiled/Dendritic Cells: Enrichment and MLR

Exposure of blood monocytes to medium alone resulted in $13.2 \pm 2.7\%$ veiled/dendritic cells. A higher yield of veiled/dendritic cells was achieved after pulsing of blood monocytes with T_3, resulting in $30.5 \pm 5.7\%$ veiled/dendritic cells. Enrichment of the T_3-pulsed cells with the use of a Boyden chamber resulted in cell fractions with $67 \pm 4.3\%$ veiled/dendritic cells.

T_3-induced veiled/dendritic cells (Boyden chamber method) were very potent MLR stimulators (max. of 135000 c.p.m.) compared to nonpulsed monocytes (max. of 2250 c.p.m.)

Effect of LPS on Monocytes and Monocyte-derived Veiled/Dendritic Cells

In the monocyte fraction, a high percentage of cells are positive for My4 (CD14) and low numbers of $RFD1^+$, $RFD7^+$, $RFD9^+$ and KIM6 ($CD68^+$) cells were observed (Table 1). During culture of the monocytes with LPS, the percentage of cells positive for RFD7, RFD9 and especially for KIM6 increased, whereas the number of $My4^+$

cells slightly decreased after 24 hr. The percentage of RFD1$^+$ cells slightly increased during LPS-treatment. No veiled cells were observed in the monocyte fraction before and after LPS exposure (Table 1).

After pulsing monocytes with T_3, overnight culture and Boyden chamber-enrichment 72% of the cells had veils (Table 1) and displayed a weak acid phosphatase reactivity. The veiled/dendritic cell fraction contained a high percentage of RFD1$^+$ cells. A small portion of the cells were positive for RFD7, RFD9 and KIM6. Forty percent of the cells were positive for My4. After culture in the presence of LPS, the number of cells with veils decreased and the percentage of cells positive for RFD7, RFD9 and especially for My4 and KIM6 increased and showed a strong acid phosphatase reactivity. Furthermore, the number of cells positive for the DC marker RFD1 decreased after LPS treatment (Table 1).

Table 1. Percentage of monocytes and T_3-induced purified veiled/dendritic cells positive for different surface markers (n=1-5)

type of cell	LPS exposure (hr)	cells with veils	RFD1$^+$ cells	My4$^+$ cells	RFD7$^+$ cells	RFD9$^+$ cells	KIM6$^+$ cells
monocytes	0	0	4±2	70±4	6±2	6±2	8±2
	24	0	17±4	63±9	11±7	13±6	39
	48	0	16±7	71±5	25±16	34±11	55
VC/DC	0	72±4	50±8	40±4	6±2	4±2	4±4
	24	37±2	30±4	50±20	22±22	33±28	63
	48	24±12	19±14	76±26	43±26	43±31	83

CONCLUSIONS

The present study shows that stimulation of human peripheral blood monocytes with iodinated compounds such T_3 enhanced their ability to mature into cytologically and functionally characteristic veiled/dendritic cells. The T_3-induced monocyte-derived veiled/dendritic cells become phenotypically macrophage-like cells when cultured in the presence of LPS. The functional activity of these cells, such as their stimulatory activity in an allogeneic MLR and their capacity for phagocytosis remains to be determined.

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IMMUNOPHENOTYPICAL AND FUNCTIONAL CHARACTERIZATION OF BONE MARROW DERIVED DENDRITIC CELLS

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INTRODUCTION

Mature dendritic cells of bone marrow origin are the most efficient antigen presenting cells (APC's) and are required for primary antigen dependent T cell responses.[1] The successful initiation of this response depends on at least two signals to be delivered by the APC. The first is provided by the interaction of the MHC class II-peptide complex and the T-cell receptor, the second signal by the action of costimulatory molecules.[2,3] Recently among the latter B7 receptors have been studied most extensively as counterreceptors of CD28 (on most T_H cells) and CTLA4 (on activated T cells).
Large numbers of dendritic cells can be generated from mouse bone marrow cultures supplemented with GM-CSF.[4,5] Those bone marrow derived dendritic cells (BMDC) are fully potent APC's and immunphenotypical analysis showed that these BMDC like mature DC from epidermis or spleen express large amounts of MHC class II as well as accessory ligands necessary for T cell stimulation.[4] To our knowledge for BMDC the expression and functional role of B7-1 and B7-2 have not been described yet. Here we show a phenotypisation of BMDC by FACS analysis and provide evidence for the involvement of the B7-receptors as costimulatory molecules on BMDC.

MATERIAL AND METHODS

Bone Marrow Culture

BMDC were generated as described with slight modifications.[4] Briefly: Bone marrow cells from 4 to 10 week old female BALB/c mice were depleted of cells carrying T-, B-cell markers or MHC class II by antibody cocktails and magnetic beads. 5 x 10^6 of the resulting cells were placed in 6-well plates (Falcon) in 5 ml of medium containing 20U/ml rmGM-CSF (Hermann Biermann GmbH, FRG). After 3 to 4 days in culture (dic) the cells were fed, aspirating 80% of the supernatant and adding back fresh GM-CSF containing medium (9ml/well). After 6 dic cell aggregates were separated by 1 x g sedimentation (50% FCS/RPMI 1640), dissociated and cultured overnight in 2ml medium with GM-CSF.

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Following harvest cells were allowed to adhere 2 h to the surface of Petri dishes (Greiner) to remove macrophages. The remaining cells were used as stimulator cells in MLR and antigen dependent T cell stimulation or FACS analysed.

Responder T Cells And T Cell Stimulation

Naive T cells were obtained by depleting non T cells from spleen dissociates of 6 to 12 week old female C3H mice with biotinylated anti B-cell, -macrophage and -dendritic cell mAb (αB220, αmouse-Ig, αMAC-1, αMHC class II) and streptavidin coupled beads (Dynal) in a magnetic field. PPD-dependent T cell lines LNC-2 and LNC-4 were a gift of *E. Schmitt* (University of Mainz, FRG).
For MLR 1 x 10^4 BMDC´s were exposed to 1200 rad x-ray irradiation and coincubated in the presence of blocking monoclonal antibodies, isotype controls, 1 µg/ml indomethacin and 1 x 10^5 allogeneic T cells in 96-well round bottom culture plates (Nunc). [^{3}H]Thymidin uptake was measured at 144 h after a 24 h pulse with 0,5µCi/well. For stimulation of the T cell lines 1 x 10^3 (optimal stimulation) or 1 x 10^2 irradiated BMDC´s (suboptimal stimulation) were cultured with blocking or isotyp control mAb´s (30µg/ml), 1µg/ml indomethacin, 100µg/ml PPD and 3 x 10^4 LNC-2 or LNC-4 in 96-well round bottom culture plates. [^{3}H] Thymidin uptake was measured at 96 h.

Fluorescence-Activated Cell Sorter Analysis (FACS)

After blockage with mouse-γ-globulin (2mg/ml) to minimize nonspecific binding, BMDC´s were stained directly with fluorescein (FITC)- or phycoerythrin (PE)-conjugated mAb´s or unlabeled first mAb´s and fluorescence labeled second Ab. MAb´s were commercial avaible or purified from hybridoma supernatants (*ATCC*). In order to discriminate contaminating granulocytes from dendritic cells a double labeling technique was employed with FITC- or PE-conjugated αMHC class II or αGR-1 mAb´s. Samples were analysed in a FACScan (Becton Dickinson). Low expression represents 2 - 3 x, intermediate expression 4 - 20 x , high expression > 20 x background staining with isotype control antibodies.

RESULTS

Table 1 displays the antigeneic profile of the BMDC with high MHC class II expression. Among the antigens not analysed on these cells before, B7 antigens were of special interest. Both B7-1 and B7-2 were expressed. In comparison to only low expression of B7-1 very strong expression of B7-2 was observed (Table 1).
To determine the functional capacity of the costimulatory antigens B7-1 and B7-2 the BMDC´s were used as stimulator cells in allogeneic MLR as well as in the antigen dependent stimulation of T cell lines (optimal or suboptimal doses of stimulator cells) with or without blocking mAb´s. Stimulation of the primary T cell response (MLR) was inhibitable by αB7-1 or αB7-2 (Table 2). It seems that both receptors are important in primary stimulation. In secondary T cell responses the receptors appeared to be less important because optimal numbers of stimulator cells could not be inhibited by αB7-1 or αB7-2 antibodies (Table 2). Under conditions with limiting numbers of stimulator cells αB7-1 and αB7-2 mAb´s inhibit the antigen dependent proliferation of LNC-2 cells (T_H1). Different from LNC-2 cells the proliferation of LNC-4 cells (T_H0) could only be blocked by αB7-2 antibody. (Table 2)

Table 1. Relative fluorescence intensities obtained from FACS analysis on bone marrow dendritic cells.

Flourescense intensity	Specificity
high	MHC class II and I, HSA, CD18, CD44, CD54, B7-2
intermediate	CD11b, CD48
low to intermediate	CD25, CD32/16, 33D1, F4/80, MAC-2, NLDC-145
low	CD11a, CD49d, N418 (CD11c), B7-1 (CD80)
negative	CD4, CD23, CD61, CD102, CD122, MAC-3

Table 2. Inhibition of primary and secondary T cell stimulation by monoclonal antibodies.

	% inhibition				
monoclonal antibody	MLR	LNC-2 optimal stimulation	LNC-2 suboptimal stimulation	LNC-4 optimal stimulation	LNC-4 suboptimal stimulation
MHC class II	85	92	99	86	97
B7-1 (1G10)	50	0	50	0	0
B7-2 (GL-1)	72	0	43	0	83
B7-1 + B7-2	n.d.	16	73	0	95

CONCLUSIONS

Dendritic cells derived from murine bone marrow in the presence of GM-CSF express both B7 antigens with high expression of B7-2. Usage of both costimulatory receptors depends on the choice of T cell responders. In the primary T cell response represented by MLR both ligands of CD28 or CTLA4 participated in the generation of costimularory signals with an apparent predominance of the B7-2 ligand. In secondary T cells responses additional signals to those triggered by the MHC class II / TcR interaction were as expected less important and were only necessary if stimulator cell were used in limited numbers. Under this condition the APC's seemed to need their whole stimulatory capacity including the B7 antigens. Additional studies are necessary to clarify if the stimulation of different subsets of T helper cells requires the different usage of the B7 antigens by BMDC.

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EXPRESSION OF B7 COSTIMULATOR MOLECULES ON MOUSE DENDRITIC CELLS

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INTRODUCTION

Dendritic cells are specialized antigen-presenting cells. They are potent, activate quiescent T cells including naive T cells, and function *in situ* to stimulate different T-dependent immune responses. How do dendritic cells develop, and how do they act as such effective APCs? We like to divide their mechanism of action into three areas. 1] At the level of "signal one," dendritic cells express high levels of MHC class II products, they can retain antigen following a pulse for days, and they have a vacuolar system that consists of many endosomes rather than scavenging lysosomes. 2] At the level of "signal two," dendritic cells express many accessory molecules e.g., ICAM-1 & 3 [CD54 & CD50], LFA-1 & 3 [CD11a & CD58], B7-1 & 2 [CD80 & CD86]. 3] A third area relates to *in situ* properties. Dendritic cells are positioned at locations where antigens enter the body, they efficiently capture antigens *in vivo*, and they can migrate to the T cell areas to activate naive T cells. Here we emphasize accessory molecules especially the B7 system, a strong stimulator of IL-2 production [Table 1].

Table 1. The B7 costimulator on dendritic cells

Is B7 expressed at high levels on dendritic cells relative to other APCs?
How is B7 regulated on dendritic cells?
Does B7 contribute to dendritic cell function?
Is B7 expressed on dendritic cells *in situ?*

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UPREGULATION OF B7 EXPRESSION DURING DENDRITIC CELL MATURATION

We used a large panel of monoclonals made in rats or hamsters [1]. Here we stress two IgG_{2a} antibodies, 1G10 anti-B7-1 [2] and GL1 anti-B7-2 [3]. We first measured expression on many populations with a FACS, e.g., spleen cells that have a low buoyant density [Fig. 1]. Low density spleen cells are the most abundant source of dendritic cells in mice. On the left is a double label with N418 which identifies a leukocyte integrin [likely CD11c] that is abundant on dendritic cells [4 5]. On the right is double labeling with B220, a CD45 isoform on B cells. Arrows mark the dendritic cells. At day 0, the freshly isolated cells stain weakly for B7-2 and not at all for B7-1. After culture, there is a large increase in B7, primarily B7-2. Dendritic cells upregulate B7-2 to much higher levels than B cells.

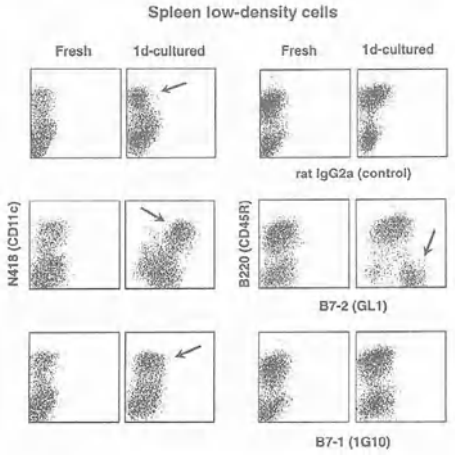

Figure 1. Expression of B7-1 and B7-2 [x-axes] on mouse spleen cells with a low buoyant density. The double labels [y-axes] distinguish the dendritic cells [arrows] as $N418^+$ B220 , and the B cells as N418 $B220^+$. See text for further details.

A similar upregulation occurs when epidermal cells are studied fresh or after a period in culture. The upregulation can be monitored at the level of CTLA4-Ig binding, as described by Larsen et al [6], or with individual monoclonals to B7-1 and B7-2. The staining on cultured epidermal dendritic cells is much more pronounced for B7-2 than B7-1 [1]. To prove definitively that B7-2 was the main CTLA4 ligand, we did blocking studies. The GL-1 antibody to B7-2 blocked CTLA4-Ig binding >90% to spleen or epidermal dendritic cells.

When dendritic cells are isolated from skin and spleen, their capacity to stimulate T cells is not fully developed, i.e., the cells are immature. During culture, the morphology, phenotype, and function becomes typical of dendritic cells. This *maturation* phenomenon was described by Schuler in mouse skin [7] and has been evident in many tissues, e.g., rat lung [8], mouse kidney [9], and human blood [10]. Major changes involve B7-2, ICAM-1, CD40 [Figure 2].

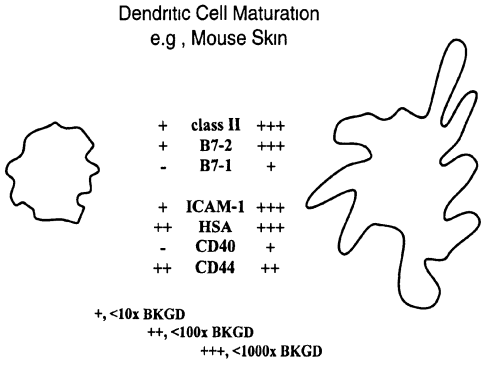

Figure 2. Changes in the surface expression of several molecules involved in dendritic cell function when epidermal cells are placed in culture. Freshly isolated dendritic cells [left] are smaller with less extensive processes than cultured cells [right].

While this article emphasizes mouse dendritic cells, high levels of accessory molecules are found in other species, especially human cutaneous dendritic cells as illustrated by the work from Romani [11,12], Teunissen [13], Pope [14] and colleagues.

THE REGULATION OF B7 EXPRESSION ON DENDRITIC CELLS

It is known that GM-CSF can mediate dendritic cell maturation, especially the acquistion of strong T cell stimulatory activity [15,16]. When we tried to block the upregulation of B7 with polyclonal anti-GM-CSF, the block was only partial. Dendritic cells may be exposed to GM-CSF during their isolation, so that we might have added the antibody too late.

LPS increases B7 levels on macrophages and on B cells [17]. We took advantage of the availability of LPS-responder and nonresponder mice, i.e., C3H/HeN and C3H/HeJ. In both strains, dendritic cell B7-2 increased markedly during culture, whether LPS was or was not added [1]. LPS did induce B7-2 on peritoneal macrophages and B cells, but only in C3H/HeN responder mice. The levels of B7-2 on stimulated macrophages and B cells were much lower than on dendritic cells, however [1]. It seems therefore that the levels of B7 and the regulation of B7 expression on dendritic cells differ from other APCs.

FUNCTION OF B7 ON DENDRITIC CELLS

B7-2 contributes significantly to dendritic cell function, at least as assessed by blocking studies. We used graded doses of spleen dendritic cells to stimulate T cells in the mixed leukocyte reaction [MLR]. GL-1 anti-B7-2 blocked the MLR, with the extent of block being much greater at low APC doses [Table 2]. 1G10 anti-B7-1 blocked only weakly, sometimes not at all. CTLA-4 Ig, which binds both to B7-2 and B7-1, blocked more than B7-2 antibody [Table 2], but the combination of B7-1 and B7-2 mAbs could block as well as CTLA-4 Ig [1].

Table 2 Blocking of dendritic cell function at the level of B7

Blocking Antibody	Response to graded doses of dendritic cells in the Allogeneic MLR 10^4	$3x10^3$	10^3	$3x10^2$	Syngeneic MLR 10^4	$3x10^3$	10^3
None	129.6	76.7	27.8	5.6	22.9	5.7	0.5
Human IgG_1, 10 μg/ml	121.4	66.9	20.9	4.8	24.9	6.1	0.5
1 μg/ml	121.3	80.6	28.7	5.5	22.6	5.9	0.6
CTLA-4 Ig, 10 μg/ml	54.1	9.7	1.1	0.3	2.1	0.2	0.2
1 μg/ml	80.5	20.5	2.2	0.7	9.8	0.6	0.2
Rat IgG_{2a}, 10 μg/ml	121.3	52.8	6.2	1.6	15.3	1.5	0.2
1 μg/ml	125.4	62.0	11.4	4.6	26.8	2.6	0.2
GL-1 mAb, 10 μg/ml	92.6	27.8	5.9	1.4	11.2	1.2	0.2
1 μg/ml	102.6	31.0	8.0	1.4	12.8	1.6	0.2

Dendritic cells from C57BL/6 x DBA/2 F1 [allo MLR] and BALB/C x DBA/2 [syn MLR] mice were added to $3x10^5$ C x D2 T cells. 3H-thymidine uptake was measured at 74-84h. Data are means of triplicate cultures where the standard deviations of the mean were <10% of the values.

EXPRESSION OF B7 IN SITU

B7 expression was monitored by labeling frozen sections of many different organs with an immunoperoxidase method. Two monoclonals to B7-1 did not stain any organ. When we used GL1 antibody to B7-2, staining of many different tissues was noted [1]. In nonlymphoid organs, the most B7-2 was noted on liver Kupffer cells and heart interstitial cells. Perhaps the nonlymphoid organ that would be of greatest interest to this meeting is stratified squamous epithelium, the location of Langerhans cells. We studied the dorsal epithelium of tongue. Many MHC class II^+ profiles were noted in the characteristic suprabasal position, but only infrequent profiles stained for B7-2 in adjacent sections [Figure 3]. This finding is consistent with the FACS data above, i.e., expression of B7-2 is weak on freshly isolated, epidermal dendritic cells.

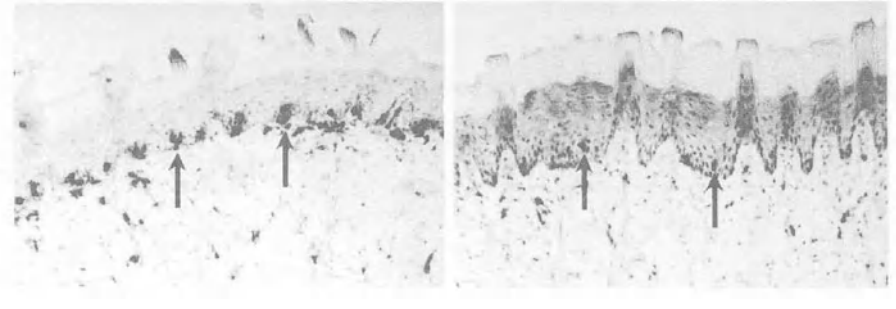

Figure 3. Staining of the dorsum of the tongue for MHC class II [left] and B7-2 [right]. There are many suprabasal dendritic cells [left, arrows], but few of these stain clearly for B7-2 [right, arrows].

Lymphoid organs [1] showed considerable staining for B7-2 [but not as mentioned, for B7-1]. In *lymph node*, the staining was strong along the capsule, the location of many macrophages, while the corresponding cells in *spleen*, the marginal zone metallophils, also expressed much B7-2. Follicular B cells did not show clear staining, but many dendritic profiles stained strongly in the deep cortex of node and periarterial regions of spleen, i.e., the T cell areas. In *thymus* [Figure 4], anti-MHC class II stained strongly in the medulla and more weakly in the cortical epithelium. B7-2 also was abundant in the medulla, but in the cortex, only scattered presumptive macrophages stained weakly.

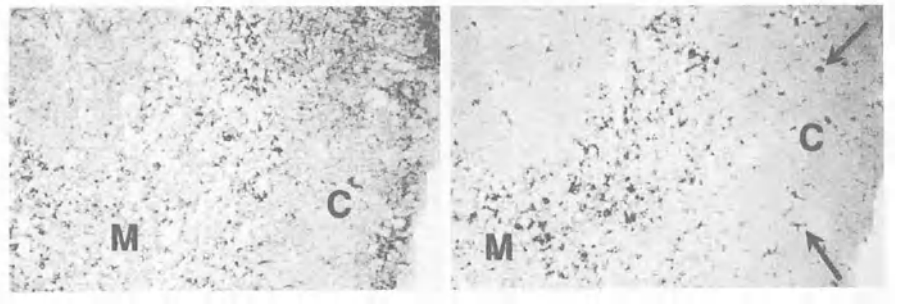

Figure 4 Staining of the thymus at low power with anti-MHC class II [left] and anti-B7-2 [right]. The medullary [M] and cortical [C] regions are evident.

So there is an apparent paradox. Many dendritic cells that are resident in lymphoid tissues like spleen, lymph node and thymus, seem to express B7-2 whereas the cells we isolate from spleen are B7-2 weak. What we think is going on is diagrammed below. The dendritic cells that are isolated from spleen are shown as stellate cells at the periphery of the white pulp nodule. We say this because of antigens identified with two other monoclonals. Isolated dendritic cells are positive for N418 but negative for M342. The M342 antibody, isolated by R. Agger, stains dendritic cell endosomes in periarterial sheaths [18]. Isolated dendritic cells lack M342 but develop it quickly upon overnight culture, in the same way that B7-2 rises markedly in culture.

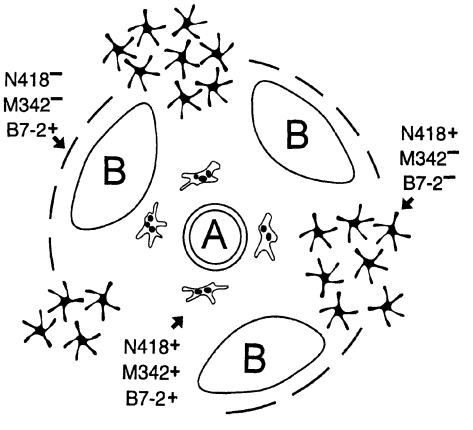

Figure 5 Diagram of a splenic white pulp nodule illustrating three different populations of cells: marginal zone metallophils at the periphery of the B cell follicles [B], dendritic cells in the periarterial [A] sheaths, and nests of dendritic cells that are readily isolated from spleen and that localize primarily we propose to the periphery, where the T cells enter the white pulp.

SUMMARY

Dendritic cells express most known accessory molecules [ICAM's, LFA's, B7's, and CD40] for binding and stimulating T cells. B7 is the most abundant of these, and B7-2 very much predominates relative to B7-1. B7 expression is regulated, not by LPS, but by some signal [s] that parallels maturation. B7 contributes to the T cell stimulatory function of dendritic cells, as do the other accessory molecules. B7-2 is expressed on dendritic cells and macrophages at several sites *in situ*, especially dendritic cells in the T cell areas.

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DEVELOPMENT OF RAT DC BY IN VITRO CULTURE OF BONE MARROW CELLS

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INTRODUCTION

Dendritic cells (DC) represent a subpopulation of leukocytes of bone marrow (BM) origin, involved in crucial immunological reactions. DC play a fundamental role in the primary immune response by stimulating quiescent T cells. In this study we describe an in vitro culture system to raise DC from unfractionated bone marrow (BM) cells of LEWIS rats in the presence of low doses of mouse recombinant GM-CSF, that was successfully used in previous work to culture mouse DC[1,2,3].

RESULTS AND DISCUSSION

Development of rat DC in rmGM-CSF supplemented BM-cultures

In the course of studies on the generation of various accessory cells by lymphokine driven differentiation of bone marrow cells, we detected the DC-inducing capacity of recombinant mouse GM-CSF on unfractionated LEWIS rat BM cells. The development of cells with dendritic morphology was found to depend on the initial cell concentration, the GM-CSF concentration added to the culture and the culture period. Maximal DC numbers were detectable in the presence of a low dose of rmGM-CSF (2ng GM-CSF/ml) following 8 days of continuous in vitro culture. An increase of the GM-CSF dose caused a decrease in yield of viable cells in the 8 day primary culture. Histological analysis revealed the development of monocytes, macrophages and granulocytes apart from differentiating DC. Moreover around day 4 of culture we occasionally noticed a few cells with a morphology strikingly resembling follicular DC. Unlike coinduced BM-macrophages outgrowing BM-DC exhibited high levels of MHC class II molecules constitutively. Approximately 15-30% of the BM-cells collected at day 8 expressed this marker as evaluated by FACscan analysis. This trait enabled the purification of DC by immunomagnetic bead-selection which yielded

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BM-DC with a purity of 85-95%. With respect to the starting BM-cell population positively selected DC amounted to 3-10%.

Ultrastructural, cytochemical and functional characterization of rat BM-DC

Transmission electron-microscopic analysis of highly immunobead-enriched rat DC confirmed the previous findings. The cells exhibited characteristic dendritic morphology showing many large veils and pseudopodia that extended from the cell's body. The cytoplasm had few prominent cytoplasmic inclusions and typical round mitochondria.

To further define the population of rmGM-CSF-induced DC cytochemical staining procedures were performed. The activity of nonspecific esterase known to be strongly expressed in monocytes and macrophages was monitored using the substrate α-naphthylbutyrate. As compared to macrophages, mature class II-selected BM-DC demonstrated a much lower activity of this enzyme.

A function performed almost exclusively by cells of the dendritic linage is the ability to stimulate naive allogeneic T lymphocytes in a MLR[4]. Immunomagnetically selected day 8 BM-DC were therefore cocultured in varying numbers with nylon wool filtered, allogeneic LEWIS.AVN rat spleen T cells ($1.5x10^6$ cells/ml). In addition the BM-DC's antigen presentation capacity was assessed by coculturing graded numbers of enriched DC with ovalbumin as antigen (Ova; 50 ng/ml) and a class II restricted, Ova-specific LEWIS rat T cell clone (1/D6.III, $1x10^5$ cells/ml). Syngeneic whole spleen cells (SC) were used as a control presenter population in the two experiments. The two assays demonstrated that at the optimal cell dose class II^+ BM-DC proved to be much more efficient stimulators than syngeneic SC (Figure 1).

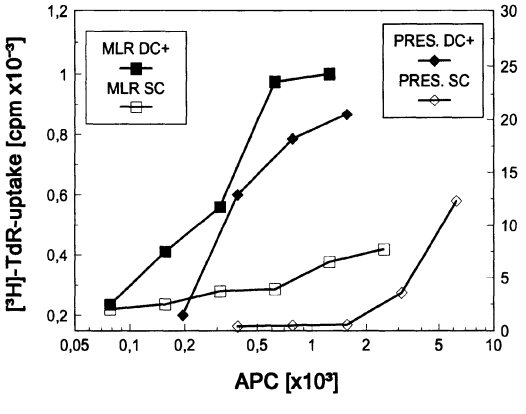

Figure 1. Immunoselected BM-dendritic cells (DC+) compared to control spleen cells (SC) in two test systems: primary allogeneic MLR and antigen presentation.

Investigation of the phagocytic capacity of rat DC

In contrast to macrophages DC are recognized to be phagocytosis negative. Recently two reports clearly demonstrated that immature DC take up particulate antigen before they differentiate into the mature phagocytosis negative phenotype[5,6]. Since DC undergo progressive differentiation in the GM-CSF supplemented BM culture this system offered the

possibility to explore the phagocytic capacity of progenitor DC. Day 8 BM-DC were given 1μm polystyrene-beads 24h before harvesting. Class II expression was monitored by mAb OX6-binding (10nm Au-conjugate) and the cells were immunobead selected employing a bead-coupled (4.5μm) mAb against the RT1.D isotype. Figure 2 shows the uptake of particles by strongly class II^+ DC. In further experiments the cultures were incubated with latex-beads ranging from 1-10μm and stained for class II expression. Transmission electron-microscopic analysis demonstrated the uptake of particles of up to 10 μm in diameter by cells with dendritic morphology (not shown). Furthermore strongly class II^+ DC were detectable in close contact with class II^- cells that exhibited the characteristic dendritic morphology. Both cells were phagocytosis positive suggesting that a DC progenitor exists which is $phagocytosis^+$ but class II^-.

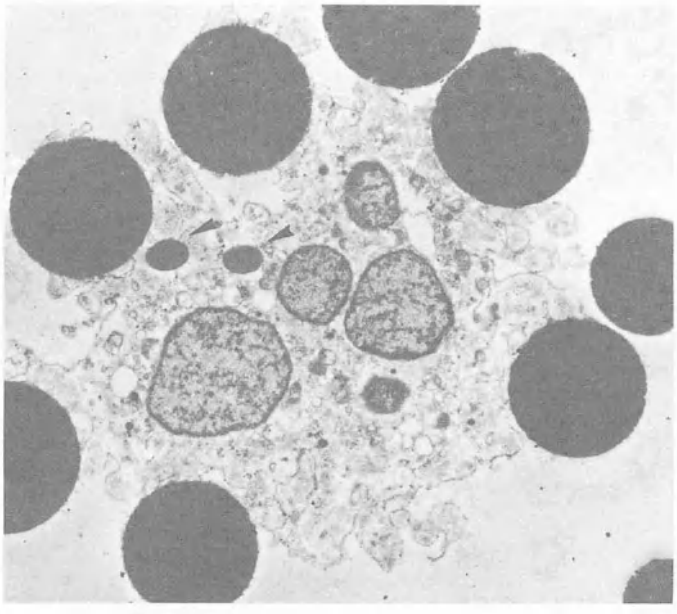

Figure 2. Transmission electron-micrograph (6500x) of a BM dendritic cell immunoselected with mAb 14-4-4S coupled beads (4,5μm) having engulfed two polystyrene particles (arrow heads).

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DENDRITIC CELLS DIFFERENTIATED FROM HUMAN MONOCYTES THROUGH A COMBINATION OF IL-4, GM-CSF AND IFN-γ EXHIBIT PHENOTYPE AND FUNCTION OF BLOOD DENDRITIC CELLS

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SUMMARY

Our previous studies demonstrated that monocytes, when cultured under certain conditions, are able to differentiate into DC-like cells (MoDC) presenting a high accessory activity and low phagocytic function In the present study, we demonstrate that under the effect of a triple combination of IL-4, IFN-γ and GM-CSF human blood monocytes are able to differentiate into the cells expressing an identical phenotype and functional features of blood dendritic cells MoDC stimulated T cell proliferation 20 -30 times higher than untreated monocytes, similar to blood DC They expressed abundant HLA-DR molecules, but only trace amounts of the monocyte/macrophage markers CD16 (FcR III), CD32 (FcR II), and CD14 Phagocytosis of Ig- and complement-opsonized bacteria was reduced by 93%

INTRODUCTION

Based on the analysis of bone marrow cell differentiation we earlier postulated that DC precursors in the bone marrow pass through a monocytic state before developing into DC (1) Our previous observations revealed that human blood monocytes can as well be induced to develop into DC-like cells (2) Such cells are dendritic in morphology and express a high accessory activity towards T-cell proliferation, although CD14 remains expressed on the cells as a strong reminiscence of their monocyte origin In order to determine further factor(s) that induce the differentiation of monocytes into DC, we investigated the effect of various

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cytokines and the second messenger molecule cyclic AMP (3) in serum-containing or serum-free culture systems.
It was found that IL-4 downregulated CD14 expression, a phenomenon also reported by others. However, our further studies revealed this effect of IL-4 to depend on the presence of serum. In a serum-free culture, GM-CSF was found to be required to synergize with IL-4 to reduce CD14 expression (4).
In this report, we describe that MoDC differentiation from Mo by IL-4 and GM-CSF will further be enhanced by IFN-γ. After culture of monocytes with this triple combination, we examined the phenotype, phagocytosis, and accessory activity in an allo-MLR and antigen-specific stimulation of T cells. In order to compare the MoDC with blood DC, we used APC and T cells from the same donors. The results show here that the MoDC exhibited a phenotype and an accessory activity very close to those characteristic of blood DC.

RESULTS AND DISCUSSION

Monocytes were treated with a triple combination of IL-4, GM-CSF, and IFN-γ, and their activity was examined both in the allo-MLR and TT stimulation. In a lower concentration of IFN-γ (50 U/ml) in combination with 50 U/ml IL-4 and 100 U/ml GM-CSF better results were obtained than with higher ones. Expression of CD14, CD16, CD32, and HLA-DR antigens by monocytes was measured in various cytokine combinations. IL-4 was the most potent cytokine in reducing CD14 expression while IFN-γ or GM-CSF had only a marginal effect. However, combining GM-CSF with IFN-γ acted in an additive way. IFN-γ appeared to be more effective in increasing the expression of HLA-DR than IL-4 or GM-CSF. Combining the three lymphokines resulted in a further enhancement of this effect (Fig. 1). FACS analysis revealed complete abrogation of CD14 expression (not demonstrated),

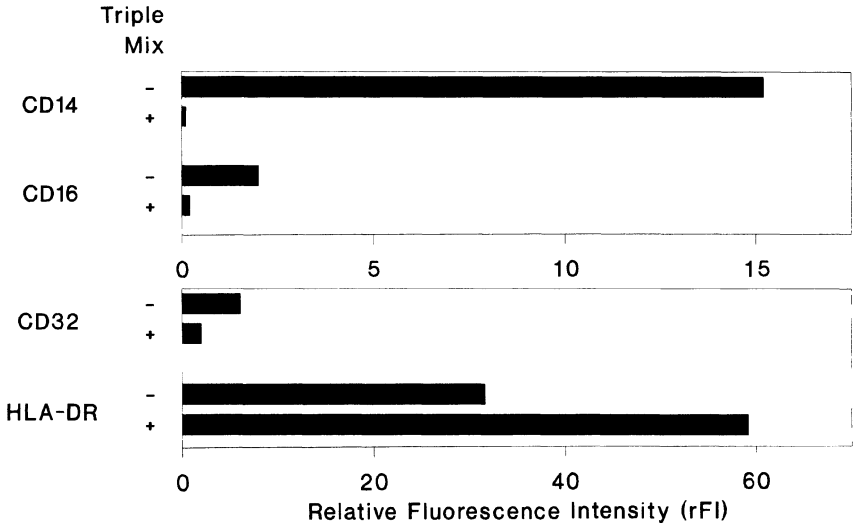

Fig. 1: Effect of a triple combination of cytokines on the expression of surface molecules CD14, CD16, CD32, and HLA-DR.. Monocytes were cultured in the presence of IL-4 (50 U/ml), GM-CSF (100 U/ml). and IFN-γ (50 U/ml) for 6 days. They were labeled by indirect immunofluorescence and analyzed in a FACS. Mean fluorescence intensity was determined. The data were collected and expressed as rFIs. The graph is representative of 8 independent experiments.

whereas expression of HLA-DR strongly increased throughout the entire population, and CD32 underwent a significant decrease It is noteworthy that up- or downregulation of the respective markers was confined to the entire population which excludes the possibility that contaminating DC were responsible for the described effects

As FcR for IgG, CD16 and CD32 play an important role in the phagocytosis by monocytes Since CD16 and CD32 were strongly reduced on monocytes treated with the triple combination, we determined the phagocytic activity of monocytes by using Ig- and complement-opsonized *E. coli* Phagocytosis was strongly reduced, in some cases almost completely lost (not demonstrated)

Table 1: Accessory activity of blood DC as compared with monocyte-derived DC (MoDC) Monocytes and blood DC were isolated from eight donors Monocytes were treated with a triple combination of cytokines (see Fig 1) to generate MoDC Non-treated monocytes served as controls Accessory activity was tested either in the allogenic MLC or antigen specific stimulation (TT) Accessory activity of non-treated monocytes was defined as 100% The resulting stimulation was expressed as percent increase

	Allogeneic MLC % increase		Antigenic Stimulation % increase	
Donor #	DC / Mo (2 d)	MoDC / Mo untreated	DC / Mo (2d)	MoDC / Mo untreated
1	210	491	206	260
2	232	1432	333	1532
3	1007	1257	-	-
4	379	1801	845	617
5	320	347	-	-
6	48	876	41	385
7	194	330	-	-
8	3378	265	1983	50
Cells per well	$5x10^3$	$1\ 25\ x10^3$	$5x10^3$	$0\ 625x10^3$
Mean ±SEM	721± 393%	850 ± 207%	682 ± 352%	569 ± 257%
Statistics	n = 8 (p = 0 8)		n = 5 (p = 0 8)	

To determine the accessory activity of antigen-presenting cells (APC), we have examined T-cell proliferation in an allo-MLR and after tetanus-toxoid stimulation Cumulated data are collected in table 1 As shown, MoDC displayed a strongly increased accessory activity in the allo-MLR and TT stimulation, at average 6-8 times higher than untreated monocytes For comparison, the accessory activity of isolated blood DC was also about 6-8 times higher than that of monocytes Proposed concentrations of the three mediators in different medium/serum conditions are given in table 2

CONCLUSION

While single cytokines are able to induce up- or downregulation of single phenotypic markers in monocytic cells, a triple combination of IL-4, GM-CSF and IFN-γ has been found to induce monocytes to differentiate into cells which lack classical markers characteristic of monocytes and macrophages, including phagocytosis Such cells express abundant class II antigen and a high potency of inducing T-cell proliferation in the allo-MLR and antigen-specific stimulation We thus regard these cells as dendritic cells and propose a monocytic origin of dendritic cells as a physiological way of differentiation, similar to the differentiation pathway of Langerhans cells which develop into lymphoid DC and as well appear to derive from monocytes

Table 2: Effectivity of cytokines dependent on the medium/serum conditions The values have been obtained from determinations of CD14, HLA-DR, C5aR, accessory activity (MLC), and morphology of the cells

	IL-4 (U/ml)	GM-CSF (U/ml)	IFN-γ (U/ml)
RPMI 1640, 5% human serum	50	100	50
IMDM plus lipids	3-10	6-20	3-10
CG-medium (Vitromex)	3-10	6-20	3-10
MCDB-302 (Sigma)	2,5-5	5-10	2,5-5

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FUNCTIONAL CD40 ANTIGEN ON B CELLS, DENDRITIC CELLS AND FIBROBLASTS

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INTRODUCTION

During antigen specific immune responses, antigen specific naive B cells undergo a cascade of events including activation, expansion, mutations, isotype switch, selections and differentiation into either antibody secreting plasma cells or memory B cells. These antigen-dependent events occur in different areas of secondary lymphoid organs, as well as other non-lymphoid organs. It requires the interaction of B cells with antigens and numerous cell types including T cells, dendritic cells (DC) and follicular dendritic cells (FDC). These cells interact with B cells through different cell surface molecules and through the release of polypeptidic mediators called cytokines.

With the idea of establishing a versatile culture system allowing the identification of B cell surface antigens acting either as receptors for growth and differentiation factors or as counterreceptors to surface molecules of interacting cells, purified B cells were cultured with a variety of monoclonal antibodies in the presence of a murine fibroblast cell line which had been transfected with human Fc receptor (FcγRII/CDw32). It was hypothetized that the immobilization of the antibody on the FcγR expressing line would represent an *in vitro* system as close as possible to the situation that occurs *in vivo* when B cells interact with cellular partners through surface antigens, a situation resulting in intense crosslinking of surface molecules. When we used the FcγRII/CD32 transfected cell line to search for antibodies inducing strong B-cell DNA synthesis and, more importantly, B-cell growth as measured by an increase in viable cell numbers, only antibodies to CD40 scored positive, leading to the denomination, CD40 system (Fig. 1) (1). Herein, we will describe the properties of the CD40 antigen and of its ligand, as well as the functional consequences of its crosslinking on cells that express it such as B lymphocytes, dendritic cells and fibroblasts.

The CD40 antigen and its ligand

The CD40 antigen is a 45-50 kDa glycoprotein of 277 AA, including a 193 AA extracellular domain, which is a member of the tumor necrosis factor receptor family (2, for review). It was independently identified in 1985 and 1986 by monoclonal antibodies reacting with carcinomas and B cells and showing costimulatory effects for B lymphocytes. The extracellular domain is composed of four imperfect repeats of ≈ 40 residues, anchored by a superimposable pattern of six cysteines. This organisation is found in the other members of the superfamily including: the p75 low-affinity, nerve growth factor receptor ; the p55 and p75 receptors for tumor necrosis factor ; the receptor for the lymphotoxin α / lymphotoxin ß membrane complex ; CD27 and CD30 ; OX 40 ; 4-1-BB ; FAS/CD95 ; two

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viral holomogs of the TNF receptors. The X-ray crystal structure of the complex formed by soluble binding domains of p55 TNFR and a lymphotoxin α trimer suggests that CD40 looks like a slightly bend rod. Three CD40 subunits are likely to encage the CD40-L trimer and scrupulously avoid contact between each other. The mouse CD40 gene, composed of nine exons that span 16.3 kb of genomic DNA, is located on the distal region of chromosone 2 which is syntenic to human chromosome 20q11q13 where the human CD40 gene is located.

In 1992, expression cloning using CD40 Fc fusion protein allowed the isolation of a CD40-ligand (CD40-L) from activated T cells (3). The human CD40-L is a polypeptide of 261 AA including a 215 AA extracellular domain with five cysteins. CD40-L is a member of the Tumor Necrosis Factor family that includes TNFα, LTα, LTß, CD27-L/CD70, CD30-L, 4-1BB-L, OX40-L, FAS-L. CD40-L is also expressed on basophils.

B lymphocytes express functional CD40

Proliferation

Monoclonal antibodies to CD40 were identified by their ability to costimulate with either anti-IgM antibodies or phorbol esters for the proliferation of purified B cells Triggering B cells through their CD40 antigen rather favors their response to IL-4 and IL-10 while antigen receptor triggering favors response to IL-2.

While antigen receptor triggering never resulted in increased numbers of viable cells, B lymphocytes cultures in the CD40 system (FcγRII/CD32 L cells and anti-CD40) expand within ten days. These latter cultures can be kept for up to three weeks provided regular enrichment of culture medium. The CD40 system permits the entry into cycle of a major fraction of B cells and the proliferation of various B cell subpopulations including naive $sIgD^+$, $sIgM^+$ B cells, germinal center $CD38^+$, $sIgD^-$ B cells, memory $CD38^-$, $sIgD^-$ B cells, as well as $CD5^+$ and $CD5^-$ B cells. Cotriggering of the antigen receptor with either anti-Ig or SAC particles results in enhanced proliferation. Addition of IL-4 or IL-13 to B cells cultured in the CD40 system results in their sustained proliferation and allows in the generation of factor-dependent long-term normal B cell lines as well as B cell clones. Cells cultured in the CD40 system with IL-4 express CD19, CD20, CD40, sIg, high levels of CD23 and HLA class II antigen. Interestingly, $sIgD^+$ B cells exhibit a higher rate of growth than $sIgD^-$ B cells and surprisingly long-term cultures yield a significant proportion of IgD^+ cells. In fact, the $sIgD^+$ cells represent the long-term IL-4 dependent proliferating B cell pool, part of which loose their sIgD.

Both viral and human IL-10 enhance the proliferation of CD40 activated B cells and appear to be almost as efficient as IL-4 during early cultures (4). The combination of IL-4 and IL-10 results in additive cellular proliferation. In our hands, IL-2 poorly enhances the proliferation of CD40-activated B cells. However, IL-10 upregulates the expression of CD25/Tac on anti-CD40 activated B cells and accordingly addition of IL-2 strongly enhances B cell proliferation. B cells cultured in IL-10 form loose aggregates which then yield cultures mostly composed of single large cells identified as plasma blasts.

Differentiation

B cells cultured in the CD40 system or with CD40-L transfected cells, produce marginal amounts of immunoglobulins. However human B cells cultured in the CD40 system together with SAC particles proliferate strongly and, strikingly, produce large amounts of IgM, IgG and IgA without IgE. Naive $sIgD^+$ $sIgM^+$ B cells secrete only IgM, whereas $sIgD^-$ $sIgM^{+/-}$ B cells secrete IgG and IgA and some IgM, indicating that dual triggering of sIg and CD40 results in B cell differentiation. Under these conditions, B cells secrete large amounts of IL-6 and IL-10 which neutralization by appropriate antibodies results in inhibition of Ig secretion.

Addition of IL-4 or IL-13 to CD40-activated B cells results in a slight increase in the production of IgM and IgG and in the secretion of IgE following isotype switching. Addition of IL-10 to CD40-activated B lymphocytes results in the production of considerable amounts of IgM, IgG and IgA without IgE as a result of a differentiation into plasma cells. IL-10 induces CD40-activated total tonsil B cells to secrete IgG1, IgG2 and IgG3. CD40-activated naive $sIgD^+$ $sIgM^+$ B cells were found to secrete essentially IgM but also IgG1 and IgG3 in response to IL-10 suggesting that this cytokine may act as a switch

factor for certain IgG subclasses. CD40-activated naive $sIgD^+$, $sIgM^+$ B cells cultured with IL-10 also produce low levels of IgA and addition of TGFß induces large amounts of IgA while inhibiting IgM and IgG production. Thus, the engagement of CD40 on B cells turns on their isotype switching machinery, the specificity of which is subsequently provided by cytokines.

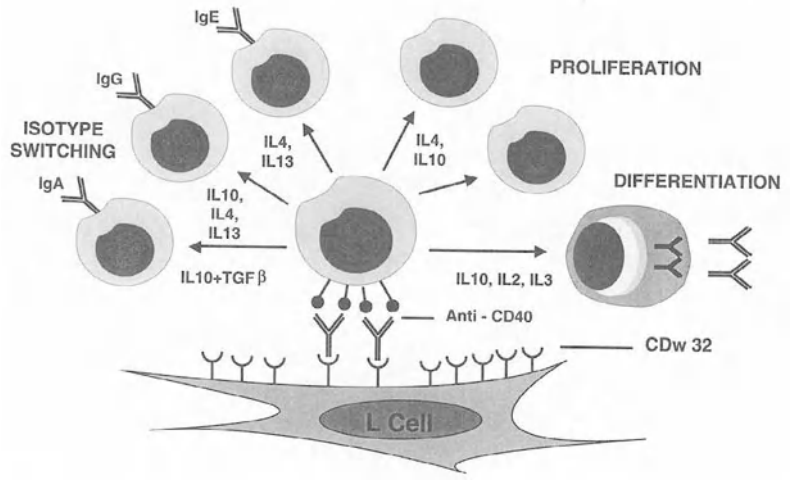

Fig. 1. Characteristics of the CD40 system.

Dendritic cells express functional CD40

Immunostaining of tonsil and spleen sections indicates that interdigitating dendritic cells express CD40 with an intensity higher than that of B cells. Likewise dendritic cells isolated from blood have also been shown to express CD40 (5). Interestingly, skin Langerhans cells only weakly express CD40 but its intensity strongly increases following culturing (6). Dendritic cells which are generated *in vitro* by culturing $CD34^+$ hematopoietic progenitors with GM-CSF and TNFα also express high levels of CD40 (7). These cells are dependent on GM-CSF for their survival but they can also be rescued by culture over CD40-Ligand transfected L-cells (8).

CD40 activation of cultured dendritic cells induced important morphological changes with a reduction of cytoplasm content and a remarkable increase of dendrite development as well as an altered phenotype. In particular, CD40 triggering induced maintenance of high levels of MHC class II antigens and upregulation of accessory molecules such as CD58, CD80/B7 and CD86/B70 (9), which may contribute to enhanced antigen presenting capacity. CD40 engagement also induces maturation of dendritic cells as illustrated by upregulation of CD25, usually expressed on interdigitating dendritic cells of secondary lymphoid organs. Finally, CD40 activation turns on secretion of TNFα and the chemotactic factors, IL-8 and MIP1α. These results found with a fibroblast cell line stably expressing CD40-L are likely to be physiologically relevant as dendritic cell induced T cell activation results in upregulation of CD40-L on T cells. Thus, T cell activated dendritic cells may contribute to the recruitment in the secondary lymphoid organs of other immune cells, such as CD8 T cells or B cells that will then receive help from CD4 cells. Accordingly addition of dendritic cells to cultures of B cells with CD40-L transfected L cells results in enhanced B cell proliferation, B cell differentiation and switch to IgA (Dubois et al., this volume ; Fayette et al., this volume).

Fibroblasts express functional CD40

Skin and synovial fibroblasts in primary cultures homogeneously express surface CD40 are detected by flow cytometry. CD40 was expressed on synoviocytes isolated from patients suffering from both rheumatoid arthritis and osteoarthritis. The levels of expression were much lower than those observed on B cells and detecting CD40 on fibroblasts required

the use of a biotinylated antibody and a phycoerythrin conjugated streptavidin to be identified. mRNA analysis demonstrated the presence on these cultured synovial fibroblasts of a typical 1.3 kb CD40 mRNA. To determine whether fibroblast CD40 was functional, fibroblasts were cultured in the presence of irradiated CD40-Ligand transfected L cells. CD40-L activated synoviocytes demonstrate increased DNA synthesis on measured by incorporation of tritiated thymidine at various time points of a 7 day culture. Cell cycle analysis and enumeration of viable cells after seven days of cultures further demonstrated the CD40-Ligand induced proliferation of synoviocytes. The observed proliferation was strictly dependent on CD40 crosslinking as blocking antibodies specific for both CD40-Ligand (Mab LL2) and CD40 (Mab 89) completely inhibited the CD40-L stimulatory effect. Finally, CD32 transfected L cells were unable to enhance synoviocyte proliferation. Interestingly, addition of IFNγ to cultures of CD40-L activated fibroblasts results in increased proliferation possibly because IFNγ enhances CD40 expression on fibroblasts.

This finding raises the question as to whether activated T cells of rheumatoid synovium may contribute, through CD40-L expression, to the uncontrolled proliferation of synoviocytes that ultimately results in cartilage and bone destruction typical of rheumatoid arthritis.

The present observation may be also be relevant to immune responses was activated CD40-L positive T cells in germinal centers may contribute to the activation/proliferation of the myofibroblast-like follicular dendritic cells.

Possible role of CD40 in antigen driven immune responses (Fig. 2)

At site of tissue injury, pathogens/antigens are captured by dendritic cells which then migrate through lymphatics into the paracortical areas of draining secondary lymphoid organs where DC present processed antigen to T cells and possibly unprocessed antigen to B cells, though this latter case remains to be demonstrated. The DC-T cell interactions results in specific T cell activation. In particular, prompt upregulation of CD40-Ligand permits further activation of the dendritic cells resulting in enhanced antigen presentation capacity as well as the release of chemotactic factors that may attract other antigen specific cells, such as $CD8^+$ T cells and/or B cells. DC-activated $CD4^+$ T cells also secrete cytokines that may participate either into their autocrine proliferation and differentiation or in the paracrine proliferation and differentiation of recruited $CD8^+$ T cells or B cells. Interactions between $CD4^+$ T cells and B cells result in two different outcomes. One leads to the generation of plasma blasts that will migrate into medullary cords to become antibody secreting plasma cells. This activation of B cells is felt to be CD40-independent as it occurs in CD40-Ligand deficient patients who suffer from the hyper-IgM syndrome. Note that secondary humoral responses involving memory B cells may also occur during extrafollicular reactions in a CD40 independent fashion. $CD4^+$ T cell dependent activation of B cells also leads to the generation of B cell blasts that will migrate into primary follicles to initiate the germinal center reaction. This activation is thought to be strictly dependent on CD40 ligation as hyper-IgM syndrome patients do no display germinal centers in their lymphoid organs. The generated B cell blasts become centroblasts (in contact of FDC ?), enter into extensive proliferation and undergo somatic mutations through presently undeciphered mechanisms. The centroblasts further differentiate into non-proliferating centrocytes that are selected by antigen present as immune complexes on FDC. Selected B cells can then present processed antigen to antigen specific T cells that have proliferated during the extrafollicular reaction and have migrated within germinal centers. This T/B cell interaction is likely to involve CD40 and will result in the expansion of B cells bearing high affinity antigen receptors and in their isotype switch. It is possible that CD40-L positive may signal CD40 positive FDC at that stage.

CD40 triggering is also likely to be involved in the further differentiation of switched centrocytes into either memory B cells or plasma blasts. Cytokines such as IL-10 may be involved in this branching but circulating antigen itself may skew the differentiation towards plasma blasts that will produce sufficient antibodies to eliminate it. Indeed, the double triggering of CD40 and antigen receptor (with either anti-Ig or SAC particles) has been shown to induce B lymphocytes to differentiate into high rate Ig secreting cells. Following elimination of free antigen, B cells stop differentiating into plasma blasts and remain in a stand-by situation that is memory.

It should be noted that activated B cells and dendritic cells can produce a soluble form of CD40 (10) which may contribute to an interruption of immune responses by preventing activation of CD40 bearing cells.

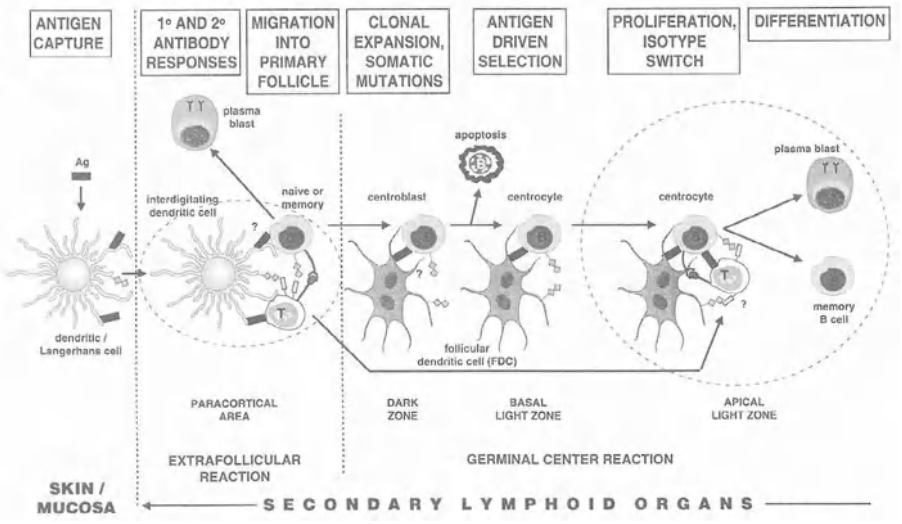

Fig. 2. B-lymphocyte immunopoiesis.

Conclusion

CD40 appears to be functional on many different cell types such as progenitor B cells, mature B cells, dendritic cells, monocytes/macrophages, thymic stromal cells and fibroblasts. It therefore remains to be determined whether other CD40 bearing cells such as follicular dendritic cells, CD34 hematopoietic progenitors, as well as various tumor cell types, do respond to CD40 ligation. Furthermore, as other members of the TNF-R superfamily have been found to bind several ligands (NGF-R, TNF-RI, TNF-RII, 4-1BB), the existence of a second ligand for CD40 should be considered.

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CHARACTERIZATION OF HUMAN BLOOD DENDRITIC CELLS : CYTOKINE PROFILES

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INTRODUCTION

Human blood dendritic cells (DC) have been found to carry terminal complement complexes (TCC) on their surface, the function of which is unknown[1]. A monoclonal antibody (mAb) described by Würzner *et al.*[1], designated X-11, recognizes blood DC by binding to a neoepitope on C9 of the TCC or the membrane attack complex. The deposition of TCC on homologous host cells can lead to lysis and, at sublytic concentrations, to the mediation of many inflammatory processes[2,3]. We have used this mAb to characterize the functional properties of X-11$^+$ DC *in vitro* and to determine the cytokine profiles of these cells isolated directly from blood.

MATERIALS AND METHODS

Peripheral blood mononuclear cells (PBMC) were separated from normal donors using Hypaque-Ficoll. X-11$^+$ cells were removed from PBMC by positive selection with X-11 mAb (Serotec, U.K.) and sheep anti-mouse coated magnetic particles (Dynal, Norway). Following magnetic separation, X-11$^+$ rosettes were found to constitute 0.001-0.01% of PBMC, an estimated 1-10% of blood DC. Using the more conventional method of Percoll gradient separation for DC isolation, we found that X-11$^+$ cells could only be isolated from the Percoll DC-enriched fraction and not from other Percoll fractions. Further enrichment of DC by culture for 48h resulted in loss of the X-11 marker, thereby precluding the use of this antibody in isolations of DC from cultured cells.

PBMC and PBMC depleted of X-11$^+$ cells (PBMC-X-11) were placed in culture at 2 x 10^6 cells/ml in the presence or absence of PHA (5 μg/ml) for up to 7 days. At various times cells were harvested and analyzed on a FACScan flow cytometer for large activated cells using forward scatter and for the expression of CD3 and CD25 (IL-2Rα) using FITC-conjugated and PE-conjugated antibodies, respectively. Supernatants from cell cultures were assayed for the secretion of TNF using the L929 bioassay[4] and for IL-6 using the 7TD1 cell line[5]. Cytokine secretion was also determined in cultures stimulated with TNFα

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(2.5 ng/ml), GM-CSF (100 U/ml), LPS (10 μg/ml), IFN-γ (100 U/ml) and IL-2 (100 U/ml).

Messenger RNAs (mRNA) were isolated from positively selected X-11$^+$ cells and analysed for cytokine expression by reverse transcription-PCR (RT-PCR). mRNA isolation, cDNAs synthesis, and cytokine-specific PCRs were carried out as described by Sprecher and Becker[6]. Primers were either synthesized according to published sequences[6] (β-actin, IL-1α, IL-1β, TNFα) or were purchased from Stratagene (IL-4, IL-8, IFN-γ).

RESULTS AND DISCUSSION

In order to examine the functional properties of X-11$^+$ cells, these cells were depleted from PBMC prior to stimulation *in vitro* with PHA. Cultures depleted of X-11$^+$ cells showed a 2-fold lower proportion of activated T-cells as determined by FACScan analysis using forward scatter and CD3 expression, compared to PBMC that had not been depleted. In support of these findings, cells expressing the CD25 activation marker were reduced 2.6-fold in number in X-11-depleted cultures when compared to undepleted cultures.

The effect of reduced activation by PHA in the absence of X-11$^+$ cells was examined in terms of cytokine secretion. Mononuclear cell cultures depleted of X-11$^+$ cells produced reduced levels of TNF when compared to undepleted PBMC in both untreated and PHA-treated cell cultures (Figure 1). While PHA provided the most potent stimulus for TNF production, other treatments such as TNFα, GM-CSF, LPS, IFN-γ and IL-2 showed similar trends i.e. reduced TNF levels in the absence of X-11$^+$ cells. Similarly, IL-6 levels were lower in response to PHA and other stimuli in PBMC-X-11 cultures when compared to cultures containing X-11$^+$ cells. Together these data suggest that X-11$^+$ cells, although a minor component of PBMC, exert a potent effect on cell activation and cytokine secretion.

To determine the cytokine profiles of X-11$^+$ cells, positively selected X-11$^+$ cells were analysed by RT-PCR. IL-1β and IL-8 mRNAs were detected in X-11$^+$ cells, apparently constitutively produced, whereas, IL-1α, IL-4, IFN-γ and TNFα mRNAs were not

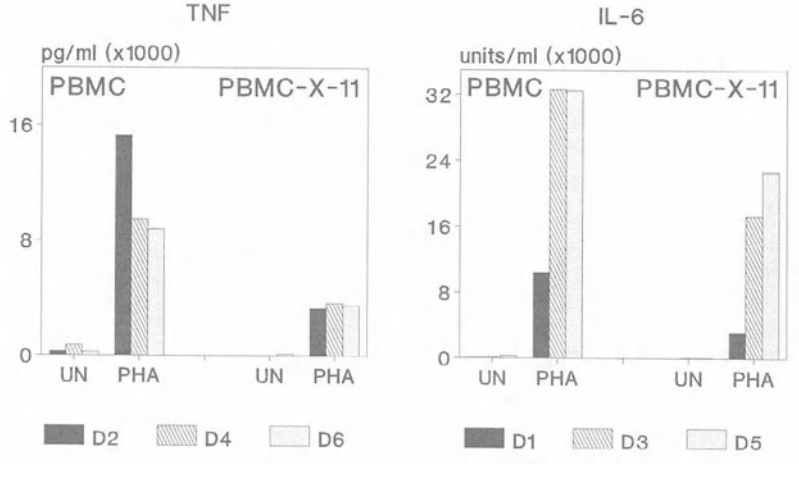

Figure 1. Cytokine secretion in PBMC and PBMC depleted of X-11$^+$ cells (PBMC-X-11) in untreated cultures (UN) or in response to PHA.

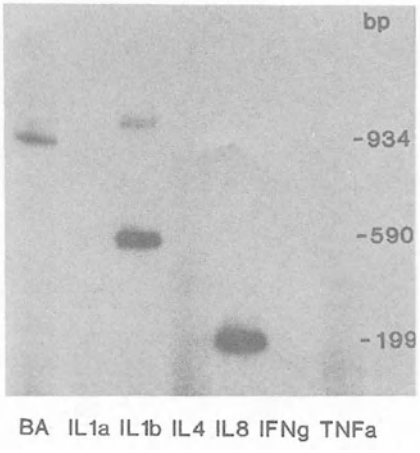

Figure 2. Cytokine mRNA profiles of X-11$^+$ cells determined by RT-PCR. PCR product sizes are indicated in base-pairs (bp).

(Figure 2). Control monocyte and T-cell preparations showed cytokine profiles distinct from those obtained for X-11$^+$ cells, and thus excluded the possibility of contamination of the X-11$^+$ cell preparation with these cell types.

In conclusion, X-11$^+$ cells may constitute a small subpopulation of human blood DC that possess properties which may distinguish them from their X-11-negative DC counterparts. Functionally, X-11$^+$ cells displayed a potent activity with respect to the potentiation of cellular activation in the presence of PHA. This was shown by their depletion resulting in near-complete abrogation of IL-2α receptor (CD25) expression on mononuclear cells and an early but reversible inhibition of T-cell activation. Consistent with this there was a reduced cytokine response in X-11-depleted cultures. Of particular interest in the future will be the further characterization of the DC phenotype that is defined by the presence of TCC on the cell surface.

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PURIFICATION OF HUMAN LUNG DENDRITIC CELLS BY THREE COLOR FLOW CYTOMETRY

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INTRODUCTION

Dendritic cells (DC) are potent Antigen Presenting Cells (APC) distributed among many tissues, including non lymphoid organs: epidermis, heart, liver, lung and gut and lymphoid organs: blood, lymph, tonsil, thymus (1). DC can present antigen to T cells and then induce strong T cell-mediated immune responses (2).
We are interested in the purification of lung DC not only because they play an important role in primary immune responses to inhaled allergens but also because the identification of novel functional lung DC molecules may lead to new targets for immunosuppression.

MATERIAL AND METHODS

Preparation of lung cells

Lung pieces obtained from operative specimens were dissociated, passed through a wire-mesh and treated for 60 min at 37°C with collagenase and DNAse. Cells were then layered on a Ficoll gradient. After centrifugation, mononuclear cells were harvested and cultured overnight at 37°C in petri dishes.

Negative sorting of non adherent lung cells

Non adherent cells were harvested and stained with Phycoerythrin(PE)-labelled anti-CD3 (T cells), CD20 (B cells), CD56 (NK cells) and CD14 (monocytes) mAbs (all mAbs at 10 µg/ml). After 30 min incubation at 4°C and two washes, 2 µg/ml Propidium Iodide (PI) was added. The cells were analysed and then sorted on a FACStar plus (Becton Dickinson). After sorting, purified lung DC were stained with different monoclonal antibodies and the phenotype of the purified DC was compared to the phenotype of lung epithelial cells and activated blood monocytes.

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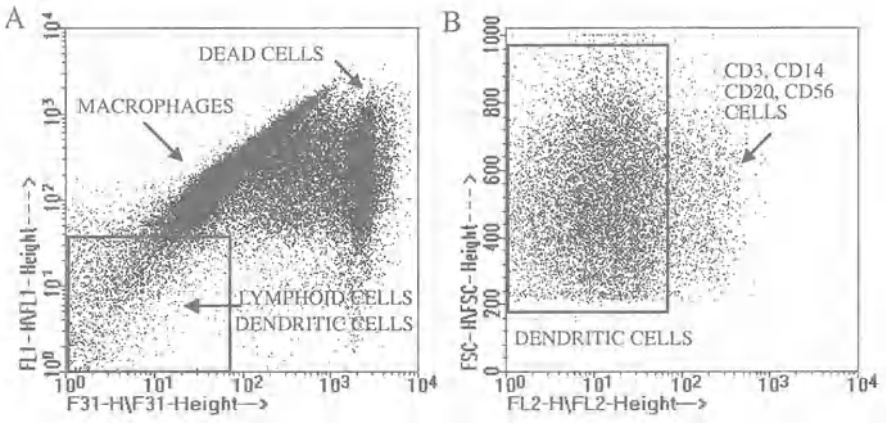

Figure 1. Sorting of human lung dendritic cells.

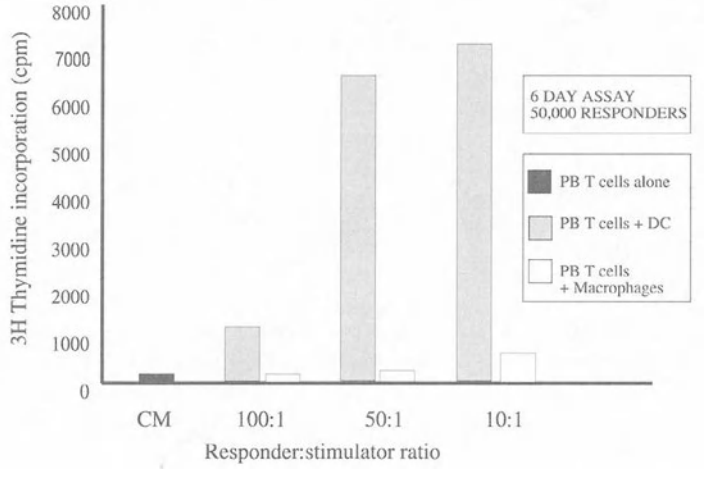

Figure 2. MLR between lung dendritic cells or lung macrophages and peripheral T cell responders

Table 1. Phenotype of lung dendritic cells compared to monocytes and lung epithelial cells.

CD	Dendritic Cells	Activated Monocytes	Epithelial Cells
CD3	< 2,000*	< 2,000	< 2,000
CD14	5,000	96,000	< 2,000
CD40	67,000	5,000	5,000
CD80	37,000	< 2,000	< 2,000
HLA DR	1,500,000	50,000	41,000

* number of antigens / cell

Induction of a Mixed Lymphocyte Reaction (MLR) using dendritic cells and PB T cell responders

Purified blood T cells (50,000 / well) were cultured with different numbers of irradiated lung DC (3,500 rad) or lung alveolar macrophages purified by cell sorting. Thymidine incorporation was measured after 6 days of culture using standard techniques.

RESULTS AND DISCUSSION

In the lung, DC can be obtained from the parenchyma rather than the bronchoalveolar lavage fluid which contains alveolar macrophages (3). After dissociation of the lung, treatment with collagenase and DNAse then overnight adherence at 37°C in petri dishes, lung cells were stained with PE-labelled anti-CD3, -CD20, -CD56 and -CD14, to identify the contaminating leukocytes, and PI in order to stain the dead cells. The analysis of lung cells by flow cytometry revealed several populations: auto fluorescent cells which represent macrophages, PI-positive dead cells and PE-positive lymphoid cells. A first gating (Fig 1a) was made in order to eliminate the auto fluorescent alveolar macrophages and the dead cells. A second gating (Fig 1b) enabled the removal of contaminating lymphocytes and monocytes. The sorted negative population contained the lung DC with a purity varying from 60 to 95 %. The purified sorted population was then analysed for surface molecules which are characteristic for DC and compared to activated human monocytes and a lung epithelial cell line. The quantification of the number of surface molecules, revealed that lung dendritic cells expressed high levels of HLA Class II, CD80 and CD40, compared to human monocytes or lung epithelial cells (Table I). The population was not contaminated by T cells or monocytes since sorted cells do not express CD3 or CD14. The phenotype of these cells is in accordance with the previously reported phenotype of other sources of DC either from spleen or from blood (4). In the lung, after inhalation of allergens, DC directly present the antigen to T cells. While we have not yet investigated the antigen-presenting capacity of the purified lung DC population, a MLR was assessed between lung DC and peripheral T cell responders and compared to the MLR induced by purified lung macrophages from the same organ. Results (Fig 2) show that purified lung DC are able to induce a strong MLR compared to lung macrophages as described in the literature for other types of dendritic cells (2, 4).

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IDENTIFICATION OF A NOVEL CELL SURFACE PROTEIN EXPRESSED BY MURINE EPIDERMAL LANGERHANS CELLS AND SOME LYMPHOID DENDRITIC CELLS

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Studies of murine epidermal Langerhans cells and their relationship to other accessory cells, including lymphoid as well as nonlymphoid dendritic cells and macrophages, are hampered by the paucity of monoclonal antibodies that differentiate dendritic cells from other accessory cells. At present, only a few monoclonal antibodies (including 33D1 (1), N418 (2) and NLDC-145 (3)) react with antigens that are characteristically (but not necessarily uniquely) expressed on the cell surfaces of dendritic cells. These antigens, however, are absent or are variably expressed by epidermal Langerhans cells and are not universally expressed by dendritic cells in all tissues. Recently, we identified a monoclonal antibody (G8.8) that reacts with a novel cell surface glycoprotein that is expressed by epidermal Langerhans cells (and keratinocytes), as well as subpopulations of lymphoid dendritic cells.

Monoclonal antibody G8.8 (rat IgG2a) was raised against glycoproteins isolated from a murine thymic epithelial cell line (TE-71) with a cell surface phenotype most like medullary thymic epithelial cells (4). The antigen recognized by monoclonal antibody G8.8 is a cell surface glycoprotein that migrates with an apparent MW of 38-40 kD in SDS polyacrylamide gels under both reducing and nonreducing conditions (4), and that does not correspond to any known adhesion molecule or leukocyte differentiation antigen. Immunohistochemical studies confirmed that G8.8 antigen was expressed by thymic epithelial cells in situ (4). However, an initial survey of other tissues revealed that G8.8 antigen was also expressed by epithelial cells in liver, gut, and skin (4).

Initially, we hoped to utilize G8.8 antibody to selectively deplete keratinocytes from epidermal cell suspensions comprised of keratinocytes as well as Langerhans cells. Single cell suspensions were prepared from the epidermis of BALB/c mice by limited trypsinization (5) and epidermal cells were assessed for simultaneous expression of G8.8 and I-A antigens using two color flow cytometry. These studies confirmed that G8.8 antigen was expressed on the cell surfaces of all keratinocytes, but also indicated that G8.8 antigen was present in comparable amounts on the surfaces of Langerhans cells. Furthermore, G8.8 antigen persisted on the cell surface of Langerhans cells maintained in culture for up to 72 hours, and was uniformly present on Langerhans cells that emigrated from skin explants in vitro (6). These observations prompted a survey of other dendritic cells for G8.8 antigen expression.

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Immunohistochemical studies of skin-associated lymph nodes indicated that G8.8 expression was limited to dendritic cells in T-cell dependent (extrafollicular) regions. To further characterize the phenotype of G8.8 reactive cells in skin-associated lymph nodes, dendritic cells were enriched by metrizamide density centrifugation (7) and examined by flow cytometry for simultaneous expression of G8.8 and I-A antigens, G8.8 and N418, or G8.8 and NLDC-145. These studies demonstrated that G8.8 and I-A antigens were expressed concordantly by low density cells derived from skin-associated lymph nodes. Furthermore, all cells that expressed G8.8 also expressed both N418 and NLDC-145. However, a subpopulation of N418 reactive cells, and a subpopulation of NLDC-145 reactive cells, did not express G8.8 antigen. In subsequent experiments, dendritic cells were enriched from mesenteric lymph nodes by density gradient centrifugation, and from spleen and thymus using density gradient centrifugation and overnight adherence to plastic (8,9). Flow cytometric studies of these various cell preparations demonstrated that G8.8 antigen was also expressed by a subpopulation of N418 reactive cells from mesenteric lymph node, spleen and thymus.

Recently, technology that permits propagation of dendritic cells from murine blood and bone marrow has been developed (10,11). To characterize the distribution of G8.8 on these putative precursors of tissue dendritic cells, BALB/c blood and bone marrow leukocytes were cultured in the presence of recombinant murine GM-CSF for seven days. In contrast to what we observed in the case of epidermal Langerhans cells and lymphoid dendritic cells, significant levels of G8.8 were not detected on the surfaces of dendritic cells propagated from blood or bone marrow.

The monoclonal antibody G8.8 reacts with a novel trypsin-resistant cell surface glycoprotein that is expressed by Langerhans cells and subpopulations of dendritic cells in lymph nodes, spleen, and thymus. However, G8.8 antigen is absent from the surfaces of dendritic cells propagated from murine blood and bone marrow leukocytes, unlike previously described dendritic cell markers such as antigens reactive with monoclonal antibodies 33D1, N418, and NLDC-145 (10,11). The observation that G8.8 antigen is expressed by subpopulations of dendritic cells in tissues whereas it is absent from the surfaces of putative dendritic cell precursors (dendritic cells propagated from blood or bone marrow in vitro) suggests that G8.8 may identify dendritic cells in different maturational or functional states. Future studies designed to further characterize subpopulations of dendritic cells reactive with G8.8, and to determine the function of the antigen recognized by this monoclonal antibody are in progress.

ACKNOWLEGEMENTS

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CHARACTERISATION OF ADHESION MOLECULES ON THE SURFACE OF DENDRITIC CELLS ISOLATED FROM HUMAN BLOOD

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BACKGROUND

Dendritic cells in lymph nodes and non-lymphoid tissue are derived from the bone marrow[1]. Since mature dendritic cells are thought to be non-dividing the maintenance and expansion of their numbers in tissue is likely to be dependent upon recruitment of cells from the circulation. Leucocyte migration into tissue depends upon surface adhesion-promoting molecules interacting with counter-receptors on vascular endothelium[2] and similar mechanisms may regulate the extravasation of blood dendritic cells. The aim of this study was to compare by flow cytometry the expression of adhesion molecules on preparations of highly purified human blood dendritic cells with those on lymphocytes, monocytes and polymorphonuclear cells (PMNs).

MATERIALS AND METHODS

(i) Isolation of dendritic cells, lymphocytes, monocytes and PMNs

Mononuclear leucocytes, isolated by centrifugation of diluted blood on Lymphoprep were incubated on plastic overnight at 37°C. The non-adherent cells were centrifuged over metrizamide gradients to give a population of low density dendritic cells. These cells were further enriched by incubation on IgG-coated plates to remove contaminating Fc receptor positive monocytes. A second panning removed antibody-labelled non-dendritic cells by negative selection using plates coated with anti-mouse IgG to remove CD3, CD14, CD16 and CD19 positive cells. The purity of dendritic cells was >79% as assessed by their EM morphology. Mononuclear leucocytes (purity 97%) and polymorphonuclear cells (purity >95%) were isolated by centrifugation on Histopaque gradient and washed twice before labelling[3].

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(ii) Flow cytometry

Flow cytometric analysis was performed with the low flow rate setting on a FACScan (Becton Dickinson) equipped with Lysys II version 1.1 software. Dendritic cells were identified by their high HLA-DR expression (quantum red-labelled) and low expression of CD3, CD14, CD16, CD19 and CD56 (phycoerythrin-labelled antibodies). Adhesion molecules on dendritic cells (Table 1) were identified by using direct or indirect FITC immunofluorescence and the expression of these molecules on other leucocytes recorded in parallel samples. Results were expressed as the number of FITC-positive cells and the level of adhesion molecule expression as the mean fluorescence intensity (MFI).

Table 1. Distribution and expression of adhesion molecules on dendritic cells and other leucocytes

	% POSITIVE				MFI			
	Dendritic cells	Monos	Lymphs	PMNs	Dendritic cells	Monos	Lymphs	PMNs
VLA-1	*40	0	0	0	*124	7	3	9
" 2	28	†75	6	†1	121	51	†28	†24
" 3	1	40	8	7	89	44	†25	†21
" 4	37	†95	†84	11	103	61	†42	†50
" 5	46	†96	45	†20	101	105	†27	†22
" 6	49	72	46	†9	*124	51	29	21
CD29	74	†98	58	†30	97	125	†39	†22
CD11a	85	98	96	†99	*190	109	68	39
CD11b	86	97	†18	†99	159	134	†35	†64
CD11c	81	98	†30	†95	*233	80	27	30
CD18	94	100	97	†99	168	138	†90	†55
CD15	55	78	†3	†100	134	102	†48	†2044
CD44	58	†99	92	†100	292	†744	309	277
CD54	80	87	†15	†12	*293	35	23	18
CD62L	*24	87	73	95	59	†105	78	97

Results are the mean of 8 experiments and are expressed as the percentage of leucocytes bearing adhesion molecules and as the mean fluorescence intensity (MFI) of expression of adhesion molecules.
*$p<0.05$ when comparing dendritic cells with all 3 classes of leucocytes, *i.e.* monocytes (monos), lymphocytes (lymphs) and polymorphonuclear cells (PMNs).
†$p<0.05$ when compared with dendritic cells only.

RESULTS AND DISCUSSION

Table 1 shows that the number of dendritic cells bearing VLA-1 was greater than that of the other leucocytes and that they possessed a higher expression of this molecule and VLA-6. The ß1 integrins (VLA-2 and VLA-6) bind to constituents of the extracellular matrix though VLA-4 is also the counter-receptor for VCAM-1 which is induced on endothelial cells by the activation of inflammatory cytokines[4]. There was a higher expression of CD11a and CD11c on dendritic cells in relation to monocytes, lymphocytes and PMNs though the number of cells bearing these adhesion molecules was similar for the various classes of leucocytes. Although it is well documented that CD11a recognises ICAM-1, which is constitutively expressed on endothelial cells and upregulated by the action of cytokines such as TNF, little is known of the ligand for CD11c. ICAM-1 (CD54) is present on the surface of many cell types and its expression was elevated on dendritic cells in comparison with the other leucocytes.

L-selectin (CD62L) mediates lymphocyte homing to peripheral lymph nodes, enhances lymphocyte and neutrophil adhesion to activated endothelial cells and promotes the 'rolling' of neutrophils along the walls of the microvasculature[2,5]. This molecule was detected on only a minority of dendritic cells in contrast to its widespread distribution on monocytes, lymphocytes and PMNs. Activation of both lymphocytes[5] and PMNs[2] results in the shedding of L-selectin from their surfaces and the finding of small numbers of L-selectin positive dendritic cells may be a reflection of their functional status. In addition to binding to glycosaminoglycans, CD44 also appears to be a 'homing' receptor in that it promotes the attachment of lymphocytes to addressins on the endothelial cells in lymphoid tissue. There was no difference between dendritic cells and lymphocytes in the prevalence and expression of CD44 though there were less dendritic cells bearing this adhesion molecule in relation to monocytes and PMNs. It is proposed that the distinct expression of adhesion molecules on dendritic cells will favour their binding to blood vessel walls and to their characterisation in the circulation.

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IN SITU EXPRESSION OF ACTIVATION MARKERS BY LANGERHANS' CELLS CONTAINING GM-CSF

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Langerhans' cells (LC) are dendritic, marrow-derived cells. When they are activated, they migrate from the epidermis to the paracortical zone of the draining lymph node, where they are called interdigitating dendritic cells (IDC). Langerhans' cell histiocytosis (LCH) is characterized by the accumulation of large mononucleated cells, associated with inflammatory cells. The LCH cells contain Birbeck granules within their cytoplasm, and express CD1a. This suggests that they belong to the LC lineage.

We performed immunohistochemical staining of 10 LCH frozen biopsies with 14 markers. The staining of LCH cells was compared to the staining of normal intraepidermal LC and of IDC, in order to precise their resting or activated phenotype.

RESULTS

LCH cells of the 10 frozen biopsies were stained by all the 14 markers. Replacement of the primary antibody by an irrelevant mouse monoclonal antibody of the same isotype, and omission of the primary antibody yielded negative results. Polymorphonuclear eosinophilic cells, which showed intense cytoplasmic reactivity due to endogenous peroxidase, were ignored.

Normal LC were identified by their dendritic morphology and their intraepidermic suprabasal localization. The 14 markers that we studied were divided in three groups, according to the staining of normal LC (Table 1). Markers of group 1 (DR, DQ, CD1a, CD1c and ICAM-3) were present on the majority of normal LC. Markers of group 2 (CD1b, CD4, LFA-1, LFA-3, CD32 and CD68) were only present on few normal LC, which were usually located nearby LCH granulomas, or nearby inflammatory infiltrates of control skin samples. Markers of group 3 (CD11b, CD24 and B7/BB1) were not detected on normal LC.

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In the control lymph nodes, the staining of IDC within the paracortical area was difficult to assess because of the simultaneous staining of either T cells or macrophages. However, in the two dermatopathic lymphadenopathies, many IDC were present, and were positively stained with anti-CD1a, CD1b, CD1c, CD4, LFA-1, CD11b, CD24, LFA-3, CD68, DR, B7/BB1 and ICAM-3 (Table 1). In the 3 normal lymph node anti-CD1a and CD1b stained only IDC. The antibodies anti-CD24, LFA-3 and B7/BB1 stained IDC and at least some follicular dendritic cells. Anti-CD11b and CD68 stained many macrophages, and we were not able to detect the staining of the few IDC. Anti-CD1c, CD4, LFA-1 and ICAM-3 stained many lymphocytes and we were not able to detect the staining of the few IDC.

DISCUSSION

We performed an extensive *in situ* immunohistochemical study of 10 Langerhans' cell histiocytosis (LCH) frozen biopsies with 14 monoclonal antibodies. These markers are known to be expressed either by resting or by activated normal Langerhans' cells (LC) *in vitro* (1-2). The staining of the LCH cells was positive for all the monoclonal antibodies, whatever the clinical expression. Our results confirm that LCH mononucleated cells belong to the LC lineage.

Most of the previous immunological studies of normal LC are performed *in vitro*. For this purpose, LC are extracted from epidermal sheets with trypsin proteolysis. This technique may either destroy, or reveal epitopes (3). As expected, in our *in situ* study, normal LC and interdigitating dendritic cells (IDC) were positive for DR, DQ and CD1a. The expression of ICAM-3 on LC was recently reported (4), and is confirmed by our results. We observed a positive staining of the majority of normal LC with anti-CD1c antibody, and of some normal LC with anti-CD1b. A previous study failed to stain intraepidermal LC with 5 anti-CD1b and 2 anti-CD1c monoclonal antibodies (5). The authors, however, revealed no information about their immunohistochemical technique. We detected a slight expression of LFA-3 on normal LC. This antigen is undetectable at the surface of fresh extracted LC, because it is trypsin sensitive (6). In our hands, normal LC were not stainedwith anti-CD24 nor with

MARKERS	LCH CELLS	NORMAL LC	IDC
DR	10/10	16/16	2/2
DQ	8/8	8/8	ND
CD1a	10/10	16/16	5/5
CD1c	9/9	14/14	2/2
ICAM-3	7/7	11/11	2/2
CD1b	9/9	8*/13	5/5
CD4	5/5	2/5	2/2
LFA-1	10/10	7*/15	2/2
LFA-3	8/8	11*/11	5/5
CD32	8/8	5*/8	ND
CD68	8/8	3*/13	2/2
CD11b	7/7	0/8	2/2
CD24	8/8	0/7	5/5
B7/BB1	8/8	0/14	5/5

Table 1:
In situ immunohistochemical staining of LCH cells, normal LC and IDC with the 14 monoclonal antibodies

* Only few cells were stained
ND: not determined

anti-B7/BB1, but these two markers were expressed by IDC. These results are concordant with *in vitro* studies, where these markers are undetectable on fresh LC (1-2, 7), but are present on activated LC. However immunohistochemical staining of LC in two normal skin samples by an anti-B7 monoclonal antibody is reported (8). We did not detect CD11b, and we hardly detected CD32 on normal LC. CD11b and CD32 are present *in vitro* on freshly extracted LC. We suppose that extraction of LC with trypsin reveals hidden epitopes.

When LC are activated by an antigen, they migrate from the epidermis to the draining lymph node, and become IDC. Several *in vitro* studies describe the antigenic changes during *in vitro* activation and differentiation of LC (for review see in 3). In our study, only normal LC located near the LCH infiltrates were stained by anti-LFA-1 or anti-CD4 antibodies. LFA-1 is a culture induced LC marker (9). CD4 is an activation marker of LC (10) *in vivo*. Some normal LC located near inflammatory dermal infiltrates of two control skin samples were stained by anti-CD68. A recent study demonstrated that some activated LC express CD68 *in vivo* (11). The expression of LFA-1, CD4 and CD68 by some normal LC in our study may reflect the activation of normal LC located nearby LCH infiltrates or nearby inflammatory dermal infiltrates.

LCH cells expressed LFA-1, CD4, CD24, CD68 and B7/BB1, which are supposed to be activation markers of LC. Indeed, in the present study, IDC of dermatopathic lymphadenopathies expressed these five markers, whereas the majority of normal intraepidermal LC did not. Therefore, our study suggests that LCH cells are activated LC.

We demontrated that LCH cells are activated LC. We previously shown that LCH cells contain GM-CSF (12). GM-CSF activates LC *in vitro* (13). These data suggest that the presence of GM-CSF is associated to the activation of LC *in vivo*.

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PHENOTYPIC VARIATION AND FUNCTIONAL DIFFERENCES WITHIN DENDRITIC CELLS ISOLATED FROM AFFERENT LYMPH

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INTRODUCTION

Dendritic cells are pivotal in the stimulation of naive T cells, however a constraint to the understanding of their biology is that most methods of isolation from tissues or blood involve culturing the cells. While effective methods of isolation have been developed it is known that culturing cells of this lineage results in changes in their capacity to stimulate T cell responses. These functional changes have been related to the level of expression by the cells of a number of surface molecules and their rate of turnover as well as changes in ability to pinocytose and process soluble antigens.

Lymphatic cannulation after surgical removal of the prescapular lymph node enables afferent lymph to be obtained that contains 10-20% dendritic cells, the afferent lymph veiled cells (ALVC). This provides a source of uncultured dendritic cells in a natural, physiological state that are migrating back from the body surface to the draining lymph node. If these cells are exposed to native antigen *in vivo* or *in vitro* they are effective at processing the molecules and presenting processed peptide to T cells.

The objective of this study was to make a detailed investigation of the molecules expressed by dendritic cells in afferent lymph and to investigate the functional activity of the dendritic cell subpopulations identified.

MATERIALS AND METHODS

Afferent lymph was obtained following prescapular lymphadenectomy[1]. The mononuclear cells were obtained after centrifugation over Histopaque 1083 (Sigma). Mononuclear cells were prepared from blood in the same way.

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The murine monoclonal antibodies (mAb) used are referred to by their bovine CD specificity, if known, or their bovine WC (workshop cluster) specificity if the homologous human molecule has not been defined[2]. MAb that have not been given a CD or WC code are referred to by their individual mAb code. One and two colour immunofluorescent staining were by published methods[3]. Three colour staining used two mAb of different isotypes, a biotinylated mAb and murine isotype specific secondary anti-sera together with fluorochrome coupled streptavidin. Staining was assayed on a FACScan (Becton Dickinson).

CD4 or CD8 cells in PBM were labelled with murine mAb and FITC conjugated goat-anti mouse sera and sorted with a FACStar-Plus (Becton Dickinson). ALVC were labelled with WC6 mAb and CD11a mAb before sorting and irradiating (2000 rads). Triplicate samples were used for alloproliferation assays and a 5 day incubation with 10^5 T cells or PBM per well. Media and other conditions were as used previously[3].

RESULTS AND DISCUSSION

ALVC were identified in afferent lymph by their 0^0 and 90^0 light scattering properties and by their high intensity of expression of the bovine WC6 antigen identified with mAb CC98[2,4]. Two colour immunofluorecsent staining using mAb CC98, to identify dendritic cells in afferent lymph, and a number of other mab showed that all ALVC expressed MHC class I and class II antigens at a high intensity and did not express a number of other molecules typically found on T cells or monocytes/macrophages. These included CD3, CD4, CD8, WC1, L-selectin and CD11b. However, ALVC were not a homogeneous population and a number of antigens were expressed at variable intensities on the cell surface. These included CD11a, CD21, CD1b, CD5, and the molecules recognised by mAbs to undefined, novel antigens (mAbs IL-A24, CC81, CC156).

Three colour staining indicated two major populations of ALVC that could be identifed as expressing, or not, CD11a. Within these two populations variable expression of other antigens was evident (Table 1).

The ability of ALVC to stimualte T cell responses was tested in an alloproliferative assay. Both sorted $CD4^+$ and sorted $CD8^+$ T cells from PBM proliferated when cultured with allogeneic ALVC. Sorted $WC1^+$ γ/δ TCR^+ T cells did not proliferate indicating that different signals are required to stimulate these cells compared to MHC class I and class II restricted $CD4^+$ or $CD8^+$ T cells and that these signals are not provided by ALVC.

Sorted $CD11a^+$ and $CD11a^-$ subsets of ALVC differed in their capacity to stimulate alloresponses in sorted $CD4^+$ or $CD8^+$ T cells. The $CD11a^-$ cells were 10-50 times more effective than the $CD11a^+$ cells.

In summary, these studies describe a detailed phenotypic characterisation of ALVC and have defined two distinct subsets evident within the dendritic cells in afferent lymph that exhibit major differences in their capacity to stimulate alloresponses in MHC class I and MHC class II restricted T cells. It remains to be established whether these two ALVC populations represent different stages of maturation, or cells that have acquired distinct properties under the influence of the local environment, or cells that differ in their capacity to influence the pathway of T cell differentiation. Published data shows that Langerhans cells migrating from the skin loose their ability to process native antigen and upregulate their capacity to stimulate T cells[5]. This might imply that the $CD11a^+$ ALVC subset differentiates into $CD11a^-$ cells. However, it has previously been shown that $IL\text{-}A24^+$ ALVC, which correspond to the $CD11a^-$ population, were effective at processing soluble antigen for presentation to a T cell clone but the $IL\text{-}A24^-$ population was not[4]. Thus, the data from these

two series of experiments are not consistent with a simple differentiation hypothesis. Future studies of the antigens expressed by the ALVC subsets and the molecules expressed by responding $CD4^+$ and $CD8^+$ T cells should establish the molecular basis for these differences and forward our understanding of the function of these cells.

Table 1. Phenotype of two afferent lymph veiled cell subsets.

Antigen or mAb	Intensity of staining of ALVC that are:	
	$CD11a^+$	$CD11a^-$
CD3	-	-
CD4	-	-
CD8	-	-
WC1	-	-
MHC class I	+	+
MHC class II (DR)	++	++
MHC class II (DQ)	++	++
WC6	++	++
CD44	+	+
CD29	+	+
FcγRII	+/-	+/-
CD1b	+	++/+
CD21	+	+/-
CD5	+	-
WC10	+	-
mAb CC81	+	-
mAb IL-A24	-	+
mAb CC156	-	+

Staining of gated $WC6^+$ ALVC that were $CD11a^+$ or $CD11a^-$ shown. Intensity of staining indicated, - none, +/- weak, + positive, ++ strong, ++/+ high and low intensity staining evident.

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EXPRESSION OF SOMATOSTATIN ON LANGERHANS CELLS

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INTRODUCTION

Langerhans cells are known to express some neurological markers, such as S100 protein, neuron specific enolase (1) and many neuropeptides (2). Expression, effects or production of neurotransmitters were also described on other immune cells, especially on lymphocytes, mastocytes, monocytes, macrophages and dendritic cells (3).

Somatostatin (SOM) is a neurotransmitter, which is known to inhibit a few hormones and cell proliferation (4). SOM was already localized in the skin on nerve fibers, dermal dendritic cells. In this study, we searched for the expression on Langerhans cells of somatostatin , a neuromediator, by immunohistochemistry, flow cytometry, confocal microscopy and electron microscopy.

MATERIALS AND METHODS

Punch biopsies of normal human skin from men and women were studied by immunohistochemistry. Samples were fixed for 3 hours in a Zamboni solution, rinced in PBS containing 0,3M saccharose and freezed. Samples were incubated with anti-SOM polyclonal antibodies (Amersham) overnigth at 4°C. Enzymatic stainings were performed with streptavidin/biotin conjugated to peroxydase. Immunofluorescent stainings were revealed with FITC or rhodamin anti-IgG antibodies. UV microscope (Zeiss) and confocal microscope (Zeiss) were used.

Epidermal cell suspensions were obtained from normal human skin samples, through action of trypsin (0,05%, 18h, 4°C). They were purified in LC (60-80% LC) by successive centrifugations, using Ficoll-Hypaque medium. Cell pellets were incubated with anti-SOM antibodies for one hour at 4°C (revealed with FITC antibody) and with anti-CD1a antibody (revealed with phycoerythrin). Pellets were fixed in paraformaldehyde and studied with a cytofluorometer FACSCAN. Dead cells and debris were excluded from analysis. The threshold of positivity was established on the basis of the negative control sample (first antibody replaced with PBS).

LC-enriched epidermal cell suspensions were prepared from freshly removed normal human skin. LC enrichment was performed through gradient sedimentation. Cells were resuspended in RPMI-1640 containing 5% fetal calf serum and 5% normal human serum. Cell suspensions were incubated with primary antibodies diluted at 1/100 for 1h at 4°C. After two washes, suspensions were incubated with gold labelled goat anti-rabbit or goat anti-mouse IgG (H+L) 10nm for 1h on ice. Cells were fixed

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with 2% glutaraldehyde and processed for electron microscopy. Controls were performed by replacing the first antibody by normal rabbit or mouse serum. Langerhans cells were identified by CD1a antigen expression and Birbeck granules.

RESULTS

Immunohistochemical study showed a diffuse but weak staining in the epidermis. Langerhans cells were stained by anti-CD1a and anti-SOM antibodies. This result was confirmed on Langerhans cell suspension. Confocal microscopy showed clearly a double staining. By flow cytometry, most of the Langerhans cells had immunoreactivity for SOM. Immunoelectron microscopy preliminary results seemed to confirm that Langerhans cells express SOM..

DISCUSSION

We showed the expression of SOM on Langerhans cells.using different methods SOM was already known to be express on dermal or sweat duct dendritic cells (5) and this expression is enhanced in psoriatic lesions (6). SOM may represent a treatment modality in psoriasis, through dendritic cells or through an effect on keratinocyte proliferation (7). Further studies have to be performed to know whether SOM is produced by dendritic cells or Langerhans cells only express SOM-receptors.

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DERMAL DENDRITIC CELLS ARE IMPORTANT MEMBERS OF THE SKIN IMMUNE SYSTEM

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INTRODUCTION

Dendritic cells (DCs) were originally described as a distinct cell type by Steinman et al. in mouse spleen suspensions[1]. Since then much has been learned about this bone-marrow derived member of the immune system. DCs belong to a lineage of cells that constitute a defined network of antigen-presenting cells (APCs) different from macrophages. DCs are distributed in trace amounts in barrier zones of antigen entry into the body such as the skin, gut, lung and blood. They contribute to the surveillance function of the immune system and are key initiators of primary immune responses[2]. Many insights into the physiology of DC eminate from studies with skin-derived epidermal Langerhans cells. These cells are located in the suprabasal layer of the epidermis, and are characterized by high levels of CD1a on their dendritic processes, and the presence of Birbeck granules. The current concept about the initiation of immune responses in the skin is that antigen which percolates through the epidermis is captured by Langerhans cells, processed and presented on MHC class II molecules on their surface. As motile cells they reach the afferent lymph node and prime naive T cells to the antigen of interest[3]. Several reports have recently challenged the concept that dendritic APCs in the epidermis are the only important APCs in skin immune responses. In contact hypersensitivity reactions a functional role for dermal APCs has been demonstrated[4, 5]. Furthermore a role in UV-B dependent tolerance in mice has been shown[6]. Recently a dendritic cell population has been identified in human dermis by means of immunohistochemistry. These cells are bone-marrow derived, express high levels of MHC class II molecules and factor XIIIa which discriminates them from conventional Langerhans cells[7]. To gain insight into phenotypic and functional characteristics of dermal dendritic cells (DDCs), an isolation strategy was developed which uses characteristic features of DCs.

ISOLATION OF DERMAL DENDRITIC CELLS

Besides their unique functional properties regarding antigen presentation, DCs have characteristics which can be used for isolation purposes. A major property is their **motility** which enables them to carry antigen in vivo to lymph nodes where the respective T cells are encountered. Their **morphology** with multiple membrane processes gives them a specific buoyant densitiy. Furthermore they are **non-adherent to plastic** and have a charcteristic size and granularity which gives them **specific foreward/sidescatter properties** in the flow cytometer. Together with the **absence of certain surface markers** of other

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mononuclear cells (T cells, B cells and natural killer; NK cells) this can be used for live sorting of DCs.

We took into account the above mentioned characteristics and devised the following isolation scheme for cultured dermal dendritic cells (cDDC)[8]. Keratome skin was obtained from healthy volounteers after informed consent. Epidermis and dermis was separated with dispase at 37°C. Afterwards small dermal pieces were placed in culture. Because of their motility, dendritic cells were the first cells to appear at the border of the tissue pieces. Plastic non-adherent cells in suspension were harvested after two days and layered over a column of 14% metrizamide. cDDC constituted about 80% of the mononuclear cells in the interface, with the rest of the cells resembling T cells. For some experiments cDDC were further isolated by cell sorting procedures. The cells were stained with a cocktail of moAbs against T cells, B cells, and NK cells, and the moAb negative cells possessing high foreward/sidescatter properties were positively sorted. These cells contained about 95% cDDC with typical morphology and abundant expression of MHC class II molecules as visualized with immunoperoxidase methods on cytospins.

ULTRASTRUCTURE AND PHENOTYPE OF DERMAL DENDRITIC CELLS

Birbeck et al. discovered the characteristic morphological marker of the epidermal dendritic cells, the Langerhans cell or Birbeck granule[9].Theses are rod-shaped structures with a central, periodically striated lamella which have been postulated to be involved in antigen presentation. They are abundant in fresh human LC (fLC) and are significantly reduced or absent in cultured human LC (cLC)[10, 11].

To further study dendritic cell populations from normal human skin, we compared the ultrastructure of human cLC with cDDC by conventional transmission electron microscopy and scanning electron microscopy. Both cell types were characterized by their numerous, thin elongate cytoplasmic processes, which suggested a veil like appearence. Furthermore they exhibited features of metabolically active cells with scattered mitochondria, a recognizable Golgi apparatus, some lysosomes, phagolysosomes, lipid droplets and a well developed endoplasmatic reticulum. They had large often indented nuclei with heterochromatin preferentially deposited at the nuclear membrane. By scanning electron microscopy it was demonstrated that the dendritic processes were actually thin sheet-like structures, so called "veils". Few cLC possessed Birbeck granules as expected, whereas in about 30-80% of cDDC depending on the isolation procedure Birbeck granules were easily demonstrated[12]. Since it is known that only a rare dermal Langerhans cell expresses Birbeck granules in vivo, these structures most likely have been acquired during culture. Furthermore a certain subpopulation of cDDC (15-20%) acquired low evels of CD1a expression. It is therefore likely that certain cDDC have the intrinsic capacity to develop features of Langerhans cells. Perhaps the stimulus to migrate in-vitro in some ways stimulates their normal in-vivo signalling mechanism by which they leave the dermis as Birbeck granule negative cells, and enter the epidermis as Birbeck positive dendritic Langerhans cells. These data provide a histogenetic link between dendritic cells in the dermis and epidermis.

The phenotype of certain types of DCs has been extensively studied. As professional APCs, they typically express high levels of MHC class II molecules as well as adhesion and costimulatory molecules. There has also been some debate about the expression of certain monoyte/macrophage markers. An extensive phenotypical analysis of cDDC (Fig.1) was done by flow cytometry and immunoperoxidase staining of cytospin preparations[8]. All cDDC expressed factor XIIIa, high levels of HLA-DR and HLA-DQ, CD1c, the Fc gamma receptor II (CD32), various adhesion molecules and costimulatory molecules such as B7-1 and B7-2 and HB-15 (CD83). About 80 % expressed the early myeloid markers CD13 and CD33. Interestingly there were subpopulations (10-15%) which expressed either low levels of CD14 or CD1a indicating differentiation of certain cells towards a Langerhans or monocyte/macrophage phenotype. Similar phenomena can be found when DCs are generated from CD34 positve blood or bone marrow progenitors under the influence of GM-CSF and TNF-a. It seems that in normal human dermis there are probably the same CD34 postive precursors[13] which then differentiate in culture in the presence of certain cytokines such as GM-CSF or TNF-a towards a CD14 postive and CD1a positive population as seen with bone marrow derived DC. Blocking data with anti-GM-CSF and anti-TNF-a antibodies will show

if this hypothesis is correct. These data support the conclusions drawn from the ultrastructural data that certain DDC can develop a Langerhans cell-like ultrastructure and phenotype.

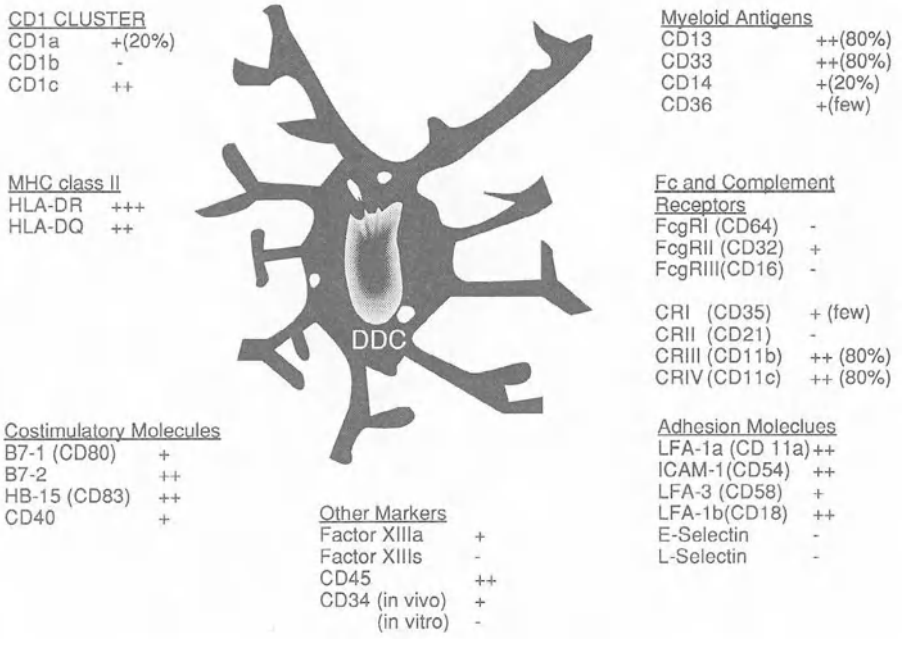

Fig.1: Phenotype of Dermal Dendritic Cells.

FUNCTION OF DERMAL DENDRITIC CELLS

The hallmark of the members of the DC family are their outstanding functional capacities in vitro and in vivo. We tested therefore cDDC in various proliferation assays with a wide range of antigenic stimuli. Responders were always highly purified resting T cells which were devoid af accessory cell function as shown by unresponsivness to phytohemagglutinin (PHA). Stimulator populations were plastic adherent monocytes, cLC and blood-derived dendritic cells (BDC) which were compared to cDDC. Stimulator/Responder ratios were 1:1000, 1:100 and 1:10. cDDC were as potent as cLC and BDC in an allogeneic mixed leucocyte reaction (MLR) and 20-50x more potent than monocytes. The same was true in autologous reactions when PHA or superantigens such as staphyllococcal enterotoxin A or B (SEA, SEB) were used. Furthermore cDDC were able to process and present nominal antigens such as tetanus toxoid (F.O Nestle unpublished observation). These data established DDC as members of the dendritic cell family not only by ultrastructural or phenotypical, but also by functional criteria. When different subpopulations of cDDC were differentially sorted on an EPICS ELITE flow cytometer, it was demonstrated that cDDC with a more monocytoid differentiation (CD14+) were weaker APCs in various proliferation assays, even though these cells didn't differ in other aspects such as dendritic morphology, non-adherence to plastic and expression of high levels of MHC class II. As has already been discussed for Langerhans cells[14], subpopulations of DDC with a specific phenotype, functional activitiy and state of activation may therefore exist.

DERMAL DENDRITIC CELLS IN DISEASE

There has been much speculation about the role of DDC in various pathological conditions based on their increased occurence in such diverse diseases as Kaposi's sarcoma[15], psoriasis[16], cutaneous T cell lymphoma[17], and dermatofibroma[18]. Apart from immunohistologic studies a thorough investigation of these cells in pathologic disease states has been hampered by the lack of an appropriate isolation method. We choose psoriasis as a model disease since DDC are highly increseasd in this disease, and the predominant investigative pathway into the autoimmune phenomena of psoriasis has primarily been in the direction of studies of T cells with little attention to the role of APCs in the dermis. We therefore isolated LC and DDC from keratome samples of involved psoriatic skin, as well as BDC from blood of psoriasis patients, and tested the APC funtion for autologous resting T cells[19]. Sudies involving superantigens were also included because bacterial infections are frequent trigger factors of psoriatic lesion formation, and superantigens have been implicated in the pathogenesis of psoriasis[20]. When we compared the expression of MHC class II antigens, various adhesion and costimulatory molecules of psoriatic DDC and normal DDC, no obvious differences in surface marker expression were found. However, we discovered that relative to normal DDC and BDC from psoriatic patients, psoriatic DDCs are capable of stimulating autologus T cells in the absence of any additional mitogen approximately eight times greater than equal numbers of other dendritic cells bearing identical levels of MHC class II on their surface. It is of interest that the cytokines produced during this DDC:T cell interaction were mainly IL-2 and IFN-γ, but very little IL-4 and no IL-10. We have previously shown that in lesional skin of psoriasis a TH-1 type of cytokine profile predominates in vivo and discussed the pathophysiological significance[21]. The new finding that lesional DDC from psoriatic skin are highly autostimulatory and drive autologous resting T cells into a TH-1 type of cytokine profile suggests that some of the autoimmune phenoma observed in psoriasis is mediated by DDC which carry a currently undefined autostimulatory antigenic peptide associated with their MHC class II molecules. It could well be that a superantigen-like molecule is involved because of the high frequencies of responding T cells in the autologous MLR. Obviously, much more work remains to be performed to rigorously test this hypothesis.

SUMMARY

In conclusion we have shown that motile cells with a dendritic morphology can be isolated from dermis of normal and diseased human skin. DDC bear high amounts of MHC class II molecules on their surface, and are very potent antigen presenting cells. Subpopulations of these cells acquire certain ultrastructural features of Langerhans cells in-vitro such as Birbeck granule formation and CD1a expression. These newly defined members of the dendritic cell family of APCs may be precursors of epidermal Langerhans cells and may play a role in skin immune responses. Furthermore in inflammatory dermatoses such as psoriasis, a role in the autostimulation and cytokine production of T cells could be demonstrated. Given their number, distribution, and in-vitro functional capacity, it is appropriate at this time to conclude that DDCs are indeed important members of the skin immune system.

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EXPRESSION OF CD44 ANTIGEN BY LANGERHANS CELLS AND THY1+ DENDRITIC EPIDERMAL CELLS - ONTOGENETIC VARIATION AND ITS ROLE IN MIGRATION

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INTRODUCTION

Two major isoforms of CD44 can be found on cells of hematopoietic or epithelial origin. These CD44 isoforms differ in their core protein structure and extracellular glycosylation (1).

The hematopoietic form of CD44 has recently been shown to recognize hyaluronate, which explain the above mentioned adhesion specificities (2).

Several studies have documented the cytoskeletal association of CD44 in cell lines. Changes in the cytoskeletal association of CD44 may influence the migration and function of cellls (3).

Epidermal Langerhans cells (LC) are MHC class II antigen bearing, antigen-presenting cells in the epidermis. LC are originating from cells in bone marrow (4).

Although Thy-1+ dendritic epidermal cells (Thy-1+DEC) are heterogeneous cell population, most Thy-1+DEC express Vγ3 / Vδ1 TCR like day 16 fetal thymocytes. These Thy-1+DEC are thought to be derived from day 16 fetal thymocytes (5).

Although the expression of CD44 on murine LC has been reported, little is known about its characteristics (6).

The expression of CD44 on Thy-1+DEC has not been studied. Thus, this study was conducted to investigate the expression of CD44 on LC and Thy-1+DEC and to investigate its expression during ontogeny. Furthermore, the role of CD44 in the migration of LC and Thy-1+DEC into the epidermis was studied.

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RESULTS AND DISCUSSION

In this paper, we revealed 1) that both Ia+LC and Thy-1+DEC expressed CD44, 2) that expression of CD44 in Ia+LC and Thy-1+DEC was slightly but significantly increased in situ, when the whole skin was organ-cultured for 72 hrs, 3) that expression of CD44 in Ia+ LC and Thy-1+DEC was high in fetal life and gradually decreased during the ontogeny, although we confirmed that the immigration of Ia+LC was inhibited by anti-TNF-α or anti-IL-6 antibody, and that the immigration of Thy-1+ DEC was inhibited by anti-TNF-α or anti-Ly48 (S11) antibody. 4) and that anti-CD44 antibody (1M7) did not inhibit the immigration of Ia+LC nor Thy-1+DEC into the epidermis in our in vitro migration assay.

The hyaluronate receptor, CD44 has been suggested to be closely associated with actin filament through its cytoplasmic domain, which is consistent with a role in cell motility and migration (7).

LC and Thy-1+DEC are mobile cells. LC originate from cells in bone marrow and Thy-1+DEC originate from day 16 fetal thymic cells. Thus, it would be posible to speculate that, in fetal life, high expression of CD44 is closely related to their migration into the skin. In fact, high CD44 expression in fetal life on both LC and Thy-1+DEC was noticed which decreased from new born to adult life. This may suggests the participation of CD44 in both LC and Thy-1+DEC migration into the epidermis. The results of the migration of LC and Thy-1+DEC into the epidermis, however, revealed that MoAb IM7(CD44) failed to inhibit their migration into the epidermis. Although this results are against the possibility that CD44 are engaged in the migration of epidermal dendritic cells (LC and Thy-1+DEC), we still can not absolutely exclude its possibily. Because the MoAb used in this study can only partially inhibit the antigen presentation to T cells (8).

Further studies are needed to conclude definitely about the role of CD44 in the migration of LC and Thy-1+DEC into the epidermiss.

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DISTRIBUTION OF HUMAN COLONIC DENDRITIC CELLS AND MACROPHAGES - FUNCTIONAL IMPLICATIONS

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INTRODUCTION

Luminal antigens that have penetrated into the intestinal lamina propria, interact with different components of the immune system including dendritic cells and macrophages Dendritic cells are potent immunostimulatory cells and can take up and present both intestinally and orally administered antigens to naive T cells[1,2] Intestinal macrophages also ingest antigen, but often have an immunosuppressive function they either inhibit the effect of dendritic cells (mouse[3]), or have no detectable action (human[4]) Mucosa-associated macrophages in other systems have similar suppressive effects on immune responses in vitro and in vivo[5-7]

Our previous studies used single cell suspensions enriched for dendritic cells or macrophages to examine their phenotypic and functional characteristics, this paper extends the observations on their phenotype using immunocytochemistry and immuno-electron microscopy and uses this information to determine their anatomic distribution in vivo by immunohistochemistry (both single- and double-labelling techniques)

MATERIALS AND METHODS

Specimens of colon obtained from patients undergoing surgery for cancer were disaggregated into mononuclear cell suspensions, macrophage- and dendritic cell-enriched populations were obtained as described[4]

Immunocytochemistry, immuno-electron microscopy and immunohistochemistry

Antibodies used are listed in Table 1 Immunocytochemistry was performed as described[4]

Immuno-electron microscopy The cell pellet ($2x10^6$ cells) or tissue ($1mm^3$) was microwave-fixed in periodate-lysine-paraformaldehyde (PLP) for 5 min on medium-low utilising a heat sink and then washed in phosphate buffer The material was dehydrated in increasing alcohol concentrations, then infiltrated using Unicryl for 2x1h It was embedded in Unicryl for 12 h, then polymerised with UV light at 4^oC for 2 days

Labelling (S100 and CD68) Sections were prepared on Formivar-coated nickel grids, which were placed on 25µl droplets of PBS for 30 min and then onto 5% BSA for 2h at room temperature The grids were transferred to 1 10 S100 antibody in PBS/ 1% BSA for 4h and washed in PBS/BSA to remove unbound antibody They were placed into droplets of 1 100 10 nm colloidal gold-labelled secondary antibody for 2h Grids were washed in distilled water to remove unbound conjugate A second label for CD68 used 40nm gold particles conjugated to the secondary antibody to distinguish between the primary antibodies The procedure was as above except that sections were placed on a 1 40 CD68 antibody (EBM/11) /PBS/ 1% BSA for 3h Sections were stained using lead citrate and micrographs taken on a Philips CM10 electron microscope

Immunohistochemistry Tissue blocks were fixed in 1% PLP for 4h, then overnight in sucrose phosphate, before embedding in OCT (Tissue-Tek, Miles Inc, Indiana, USA) In later experiments formalin-fixed, paraffin-embedded blocks were studied (S100) Tissue sections were hydrated with TRIS-buffered saline (TBS), pH 7 2, then blocked for 15 minutes with horse serum

Avidin-biotin complex peroxidase (ABC) technique sections were incubated for 1h with primary antibody or isotype controls (see Table 1), then washed in TBS To reduce endogenous peroxidase activity,

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sections were incubated for 30 min in methanol containing 0.1% hydrogen peroxide, then washed in TBS. Biotinylated sheep anti-mouse immunoglobulin was applied for 30 min, and the sections washed in TBS. Sections were incubated with peroxidase-conjugated avidin biotin complex for 30 min and washed in TBS. The reaction was developed for 10 min using 0.5 mg/ml 3,3'-diaminobenzidine, 10 mM imidazole and 0.3% hydrogen peroxide in TBS. Sections were washed in TBS, air-dried and mounted using a non-aqueous medium (D-PX) or the second cycle of labelling was commenced.

Alkaline phosphatase anti-alkaline phosphatase technique: a second primary antibody or its isotype control was applied for 1h, followed by washing in TBS. Alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts) was added for 30 min. After washing in TBS for 10min, sections were incubated with alkaline phosphatase-mouse anti-alkaline phosphatase complex for 30 min, then washed in TBS. Whenever increased staining intensity was desired, the last two steps were repeated using incubations of 10 min. The reaction was developed by adding the substrate (naphthol AS-MX phosphate, Fast Red TR and levamisole) for 20 min. Slides were washed in water, counterstained with Mayer's haematoxylin and mounted.

RESULTS

Immunocytochemistry of subpopulations of disaggregated lamina propria cells

The results of immunocytochemistry for macrophages (γ-globulin-adherent cells), and dendritic cells (obtained by density gradient centrifugation of cells from which macrophages had been removed) are shown in Table 1. The antibody, S100, labelled disaggregated dendritic cells in a proportion of experiments (3 of 11). These cells also demonstrated perinuclear staining by anti-CD68 as has been observed previously[8,9]. This contrasted with the extensive cytoplasmic staining seen in macrophages.

Immuno-electron microscopy

Both dendritic cell- and macrophage-enriched cell suspensions were labelled by anti-class II MHC antibodies (L243). 10nm particles (indicating S100 labelling) were present in the cytoplasm associated with filamentous structures and the nuclei of cells in dendritic cell-enriched suspensions in three of six experiments. S100 was not seen in cells that had the ultrastructural features of macrophages. CD68 labelled cytoplasmic components of macrophages including lysosomes and vesicles.

Anatomic distribution of mucosal dendritic cells and macrophages using immunohistochemistry

Differences in phenotype between intestinal dendritic cells and macrophages are listed in Table 1. These markers were used to determine the distribution of both cell types in the colonic lamina propria by single- and double-labelling immunohistochemical techniques. Two distinct patterns were observed: macrophages were concentrated in a band in the region beneath the luminal epithelium, whilst dendritic cells formed a reticular framework throughout the lamina propria and beneath the basement membrane of the colonic crypts. Double-labelling experiments established that the macrophage marker, 25F9, and the putative dendritic cell marker, S100, were present on mutually exclusive populations of cells.

Table 1. Monoclonal antibodies used and results of immunocytochemistry.

Name	Determinant	Class	Reference	Dendritic cell	Macrophage
L243	Class II MHC	IgG_{2a}	J Immunol 1980; 125:293	++	++
S100	Interdigitating cell	IgG_{2a}	Am J Pathol 1985; 119:73	±	-
25F9	Mature macrophage	IgG_1	J Immunol 1985; 134:1487	-	++
EBM/11	CD68/macrophage	IgG_1	J Clin Pathol 1988;41:510	±	++
All antibodies were derived from the mouse.					

DISCUSSION

Dendritic cells reside in the interstitium of tissues where they take up and process soluble and particulate antigens; they develop potent stimulatory activity whilst migrating to draining nodes. In various models, dendritic cells in afferent lymph are associated with antigen and can initiate specific T cell responses[2,10].

By contrast, macrophages have a suppressive function, both in vitro [5,7] and in vivo[6,11]. Recent studies show that alveolar macrophages inhibit the maturation of dendritic cells into accesory cells; this effect is

mediated by secreted products of macrophages, eg TNFα and nitric oxide[12] and can be abrogated by a range of cytokines including GM-CSF, TNFα, TGFβ1 and IL-4[13]

Human dendritic cells express high levels of class II MHC antigens, but do not have specific markers Although present on a range of cells, S100 is expressed also on members of the dendritic cell lineage[14] The failure to demonstrate consistent S100 staining on disaggregated dendritic cells most probably represents loss of cytoplasmic antigen during their preparation Alternatively, some $S100^+$ cells in tissue sections may be of neuronal origin, but this is unlikely given the presence of cell bodies in the lamina propria Similarly, they do not appear to be myocytes because no labelling is seen in the muscularis mucosa Finally, the demonstration of $S100^+$ cells in dendritic cell-enriched populations using immuno-electron microscopy, its association with filamentous structures and its absence on macrophages suggest that it is a useful dendritic cell marker

EBM/11 recognises an epitope on a cytoplasmic glycoprotein designated CD68 This antigen originally was thought to be specific for mononuclear phagocytes, but subsequent data indicate that it labels cultured dendritic cells in a perinuclear distribution 25F9 was described as a marker of macrophage maturation, but may delineate a population of macrophages with evidence of previous endocytosis[15]

Our studies have shown distinctive patterns of distribution macrophages are concentrated in a band beneath the luminal epithelium, whilst dendritic cells are found in lower numbers and form a reticular framework throughout the lamina propria and beneath the basement membrane of the crypts This pattern is seen in other in other sites eg Langerhans cells of the skin[16] and dendritic cells of the airways of the lung[17]

In summary, the observed functional and distributional characteristics of colonic macrophages and dendritic cells suggest that they subserve different functions macrophages are likely to have a role as a first line of defence, dendritic cells may act as "immune adjuvants" by recruiting T cell responses

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MORPHOLOGIC AND ANTIGENIC FEATURES OF DENDRITIC CELLS IN IMMUNE-MEDIATED DERMATOSES: A HYPOTHESIS OF DIFFERENTIATION*

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INTRODUCTION

Both Langerhans cells (LC) and dermal dendritic macrophages (DMΦ) deserve attention as antigen presenting cells in the skin, as reviewed elsewhere.[1] The morphologic, antigenic and functional features of the formers are extensively dealt with in many chapters of this volume. Dendritic MΦ are characterized by the expression of CD36 antigen and contain many lysosomes;[2] they can stimulate mixed cell reactions in vitro, but it is not known whether they stimulate immune responses in vivo or rather inhibit them, perhaps by activating suppressor cell circuits.[3,4] The differentiation pathways of LC and DMΦ are incompletely known. The formers, in particular, are considered to derive from circulating precursors which express CD14 antigen "dimly"[5] or not at all;[6] it is not clear whether these precursors should be considered as true monocytes or a specialized cell type.[7,8] It has been proposed that LC can differentiate from DMΦ, but the evidence in favour is weak.[9]

Research in vitro and animal model systems provides extensive information on the possible differentiation pathways of bone marrow and circulating cell types and the factors which influence that differentiation, but it cannot tell us definitely what happens in the human skin. We have therefore analyzed biopsies from patients in different clinical conditions, characterized by immune cell infiltration of the skin, to find out possible differentiation steps of dendritic cell precursors.

MATERIAL AND METHODS

Skin biopsies from 31 patients affected by acute or chronic inflammatory skin diseases were used in part for diagnostic purposes and in part prepared with routine methods for immunohistochemistry and electron microscopy for this - and other[10-12] - research; additional biopsies of clinically healthy skin excised at plastic surgery were used as controls

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List of abbreviations. APAAP: alkaline phosphatase/anti-alkaline phosphatase. CD: cluster of differentiation. DMΦ: dendritic macrophage(s). GVHD: graft-versus-host disease. HLA-DR: human leukocyte antigen, DR locus. LC: Langerhans cell(s). LE: lupus erythematosus. MΦ: macrophage(s). TNF: tumor necrosis factor.

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(Table 1). An indirect APAAP method was used to identify HLA-DR, differential antigens and TNF-α. Primary antibodies were purchased from Dakopatts (Glostrup, Denmark), Ortho (Raritan, NJ) and Genzyme (Boston, MA). The Italian Law and ethical guidelines of the Italian National Medical Council were followed thoroughly.

RESULTS

In all cases, both LC in the dermis and DMΦ were increased in number above controls. Moreover, we detected perivascular HLA-DR+ cells, either roundish or with short dendrites, which were in variable numbers CD14+ (Table 1). By electron microscopy, cells with corresponding location and shape were characterized by loose chromatin, flat cisternae of rough endoplasmic reticulum, relatively wide Golgi apparatus, smooth vesicles close to the plasma membrane and few, primary lysosomes (Fig. 1). No cell was found with intermediate features between LC and DMΦ. A few, round cells reacting for TNF-α were detected in the dermal infiltrate and the basal epidermal layer of all cases.

Table 1. Subjects under study and immunolabeled cells per 100 cells in the dermis

clinical condition	males	females	age range (years)	positive cells (mean ± standard deviation) for		
				CD1a	CD14	CD36
healthy skin	7	10	21 - 68	0.41 ± 0.71	2.65 ± 1.27	7.76 ± 2.49
acute GVHD	2	5	18 - 32	29.71 ± 5.28	2.14 ± 1.35	21.14 ± 3.02
erythema multiforme	2	1	19 - 60	27.67 ± 4.04	5.00 ± 2.65	16.67 ± 2.52
cutaneous LE	6	7	18 - 51	23.00 ± 5.04	8.62 ± 1.84	14.31 ± 2.60
atopic dermatitis	2	6	21 - 42	30.00 ± 4.87	16.00 ± 6.78	20.63 ± 6.37
lichen planus	1	3	35 - 59	26.00 ± 5.48	13.50 ± 5.20	15.00 ± 3.56

DISCUSSION

This research has shown that, in different immune mediated diseases, dermal LC and DMΦ increase in comparable numbers and cells with features interpretable as immature dendritic appear in relatively large numbers, whereas elements interpretable as mature MΦ transdifferentiating to mature LC cannot be found even in cases of very rapid increase in LC number, like acute GVHD. These data lead to propose that circulating monocytic precursors give rise to LC and DMΦ and that these precursors comes to express HLA-DR intensely and CD14 with variable intensity depending on the disease, whether acute or chronic, along their differentiation pathway. On the basis of morphology and differential antigen expression it cannot be excluded that one precursor gives rise to both types of skin dendritic cells; alternatively, the precursors of each of these cell types should be very similar to each other.

The terminal differentiation of dendritic cells in vitro is driven by cytokines, among which TNF-α plays a relevant role.[13,14] The finding of TNF-α positive cells in the infiltrated dermis and basal epidermis - in agreement with previous reports on other clinical conditions[15] - suggests that this cytokine is of importance also in vivo and that some putative mononuclear cells (besides keratinocytes)[16,17] participate to its production in inflammatory dermatoses.

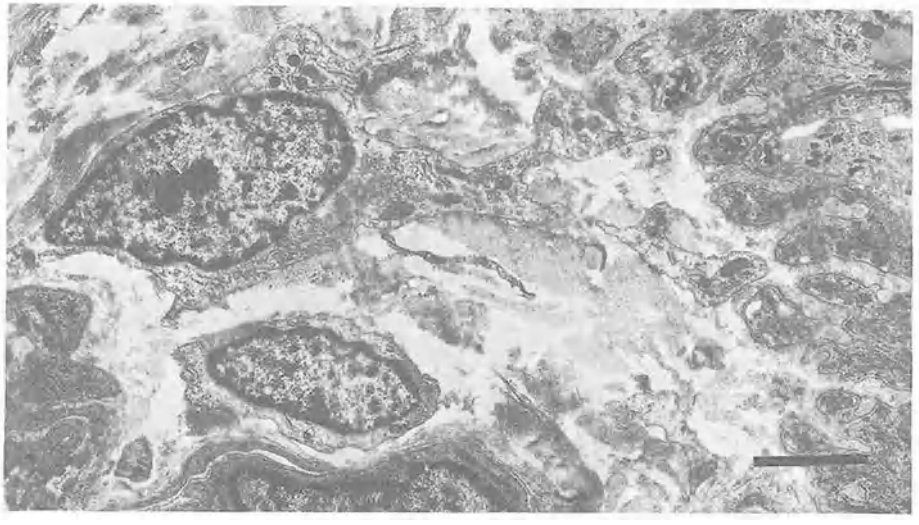

Figure 1. Two immature dendritic cells between papillary dermal capillaries and the epidermis of a case of acute GVHD. Light microscopical immunohistochemistry on comparable sections showed that cells like these were occasionally CD14+. Scale bar = 3 μm.

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TUMOR NECROSIS FACTOR RECEPTORS OF THE MONOCYTE DERIVED LANGERHANS CELL PHENOTYPE "MoLC"

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INTRODUCTION

For the generation of dendritic Langerhans cells from progenitors TNF-a is used as a differentiation factor in combination with a growth factor , GM-CSF (1). Growth factors primarily act by suppression of apoptosis (2). During monocyte development they probably regulate the response to TNF-a by inhibiting the cytotoxic signal which is associated with the last 100 aminoacids of the C-terminus of TNF-RI (p55) (3), and activating only the p75 TNF-R.

The expression and function of p55 TNF-R on monocytes is controversal. During HL60 differentiation into macrophages p55 mRNA and function diminish and TNF-a was unable to crosslink p55 on blood monocytes (4).

Since during monocyte differentiation into dendritic Langerhans cells (MoLC) we could localize both TNF-receptors with monoclonal antibodies (mAb) with similar densities we were interested to know wheather p55 can be activated on MoLC by TNF-a or antibody mediated crosslinking. This system would allow us to study the role of IL-6 which is the growth factor released and used by MoLC (5).

MATERIALS AND METHODS

Monocytes were isolated from buffy coats by Ficoll-Paque (5) or by elutriation. The cells were cultured in IMDM with 2% FCS (5). Phenotypic development was followed by immunofluorescence measured by cytofluorometry with mAb against CD1 (Ortho, OKT6), mAb 29C6 against Fc-E-RI which was supplied by Hoffmann-La Roche (6) and with mAb against B7 (Dianova). TNF-RI and RII were detected by mAb 18 and 20 (p55) and 13 and 36 (p75) created in the laboratry of D. Wallach. For blocking and activation of TNF-receptors 10 μg of a single mAb (mAb 36 against p75) or of each of two antibodies (mAb 18 and 20 against p55) were used. Concentrations of TNF-a, rhIL-6 and IFN-gamma were 10 ng/ml. Conditioned medium from MoLC was added as native IL-6 to final concentration of 12 ng/ml. Dead monocytes were detected by Trypan blue uptake and indicated as a percentage of the total cell number.

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RESULTS

MoLC phenotype

Upon culture in IMDM with 2% FCS monocytes detached within 24 hours forming clusters and gaining morphology of veiled dendritic cells. The cells expressed CD1a , B7 and high affinity receptors for IgE (Fig. 1a,b,c). There was no difference between Ficoll and Elutriator purified cells. Both TNF-receptors were detectable with similar densities (Fig. 1d).

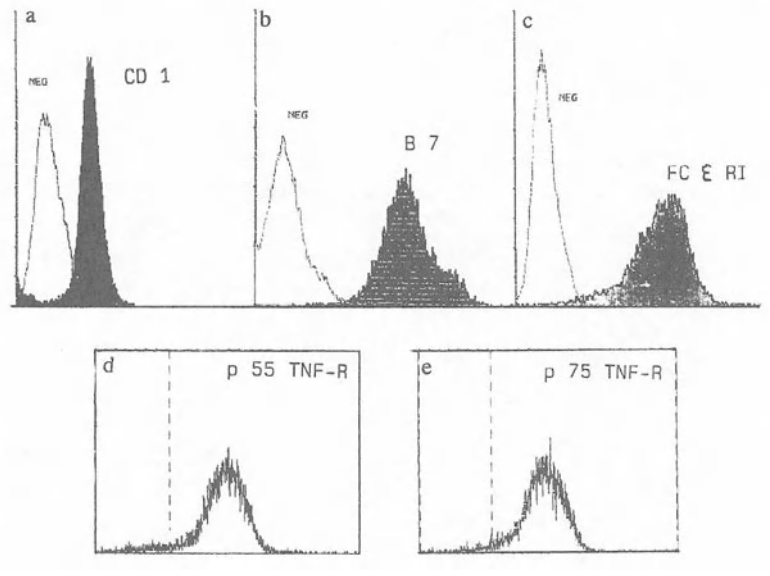

Figure 1 The MoLC phenotype. Expression of CD1a (a) B7 (b), FcE-RI (c), and the TNF-a receptors (d and e)

Effect of TNF-a

In dense monocyte cultures and high concentrations of Il-6 we could not detect any effect of TNF-a. In low density cultures CD1a expression remained normally very low and could be increased by TNF-a (Fig. 2).
B7 expression was not modified by TNF-a alone, but its induction by IFN-gamma was increased by TNF-a (Fig. 3a,b,c).

Effect of TNF-a when p75 TNF-R was blocked by mAb 36

All the receptor blocking and activating experiments were performed with low density cultures (low IL-6) with or without CM of monocytes from high density cultures. Respect to the untreated sample TNF-a effected a decrease of CD1 expression from 60 to 8%.(Fig.4) The decrease of viability was much less effective, and visible only in the absence of conditioned medium (Fig. 5a).

Effect of p55 TNF-R crosslinking when p75 TNF-R was blocked by mAb 36

A decrease of CD1 expression similar to the one obtained with TNF-a (from 60 to 21%) was obtained by activation of p55 TNF-R. The cells adhered. The depression of viability was more effective after crosslinking of the receptor with antibodies (50% of dead cells). Traces of

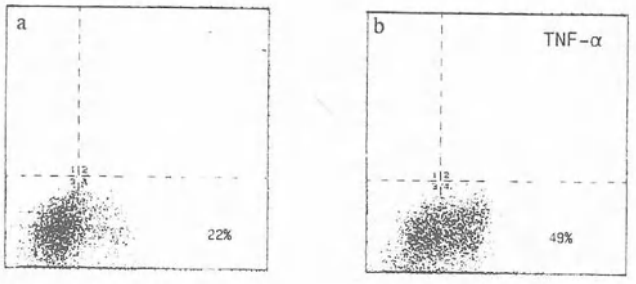

Figure 2 Effect of TNF-a on the expression of CD1a
a) untreated sample, b) TNF-a (10ng/ml)

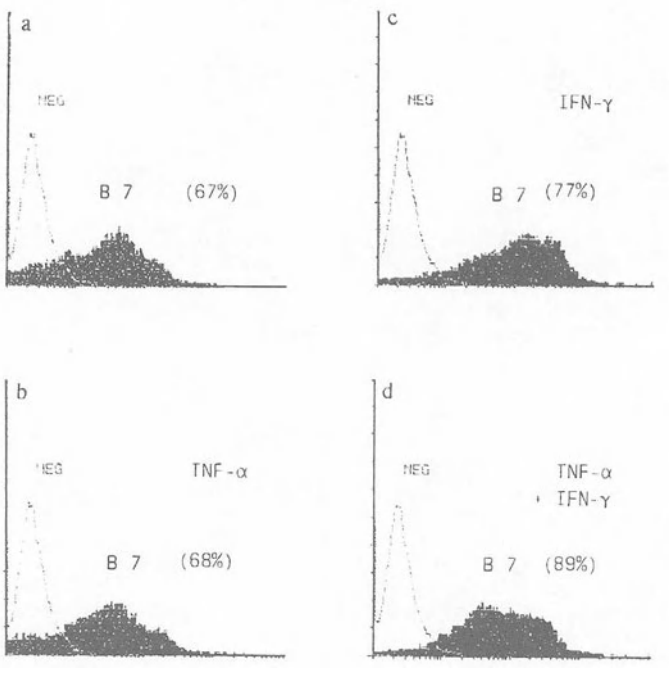

Figure 3 Effect of TNF alpha and IFN gamma
on the expression of B7 a) unteated, b) TNF-a,
c)IFN-γ, d) TNF a and IFN-γ (each 10ng/ml)

IL-6 were released also by low density cultures (0,5-1 ng/ml). However the addition of IL-6 rich conditioned medium or rhIL-6 completely abolished the cytotoxic effect. Neither TNF-a nor mAb crosslinking of p55 TNF-R showed any effect on cell survival in the presence of CM at final IL-6 concentration of 12 ng/ml.

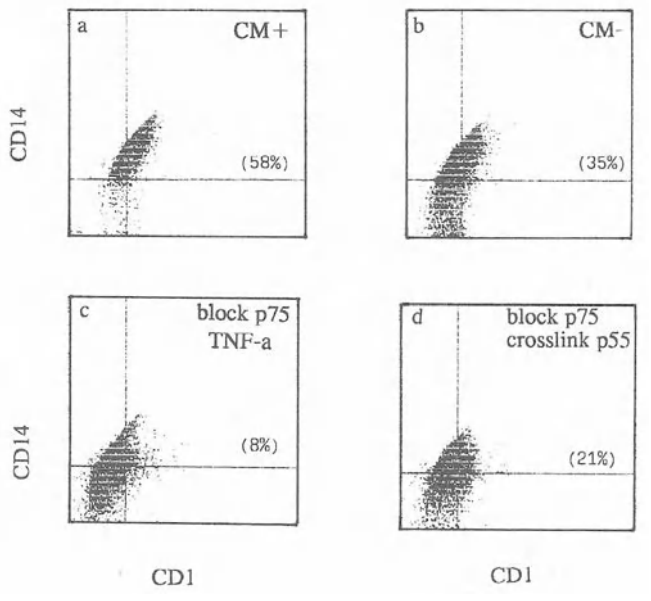

Figure 4 Effect of TNF-a or crosslinking of the p55 TNF-R when p75 TNF-R is blocked by mAb 36
a) IL-6 concentration 12ng/ml, b) IL-6 concentration 1ng/ml, c) as b) block of p75, TNF-a treatment,
d) as b) block of p75, crosslinking of p55

DISCUSSION

Both TNF-receptors are expressed by MoLC. Our experiments show that not only p75 but also the p55-R can be activated. It seems that MoLC answer in two ways to this activation:
1) p55 activation changes the direction of differentiation. The decrease of CD1 expression obtained by TNF-a or mAb crosslink of p55 when p75 is neutralisized by mAb 36 indicates that mAb 36 was able to block p75 signals and indirectly proves that TNF-a induction of LC phenotype development is mediated by the p75 signal. This regards CD1 expression and at least in cooperation with IFN-gamma B7 expression.
2) p55 signal depresses the viability of MoLC. The activation of p55 by a combination of mAbs against different epitopes (3) increased the number of dead cells. This effect however is negatively correlated to cell density and IL-6 concentration. Thus p55 cytotoxic signal needs the withdrawal of growth factors. The signal was completely abolished when the IL-6 concentration is corrected to 12 ng/ml.

MoLC development is based on autocrine stimulation which involves IL-6 with its high affinity receptor (5). From the present results we deduce that IL-6 acts as a growth factor by blocking the cytoxic signal of p55 TNF-R.

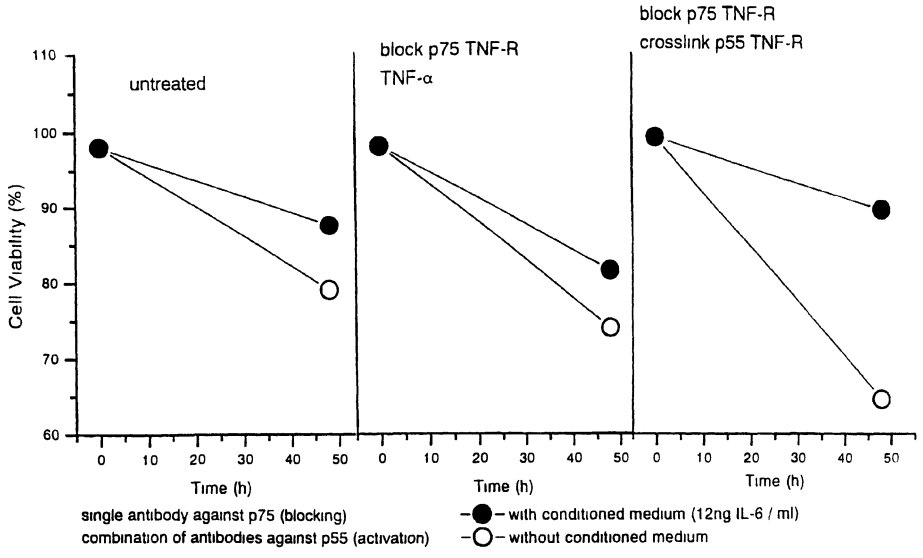

Figure 5 Effect of TNF-a or crosslinking of p55 TNF-R when p75 TNF-R is blocked by mAb 36

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EXPRESSION OF THE HIGH AFFINITY RECEPTOR FOR IMMUNOGLOBULIN E (IgE) BY DENDRITIC CELLS IN NORMALS AND ASTHMATICS

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INTRODUCTION

Immunoglobulin E (IgE) plays an important role in the pathophysiology of asthma and other allergic diseases. In mucosal tissues, IgE binds to specific receptors on the surface of mast cells, eosinophils and other inflammatory cells providing an important trigger mechanism for the release of inflammatory mediators.

The high affinity IgE receptor (FcεRI) is a tetrameric hetero-oligomer comprising an α chain, a β chain and 2 disulphide-linked γ chains. The α chain is necessary for IgE binding and receptor internalisation whereas the β chain seems to play a role in signal transduction[1]. Maternal inheritance of IgE responsiveness has been proposed to be linked with chromosome 11[2] and it has been suggested that the gene for the FcεRI β subunit could be a candidate for this atopy locus[3].

FcεRI was originally described on mast cells and basophils, where it can mediate cell activation in the presence of bound specific IgE and allergen. Recently, it has been reported that Langerhans cells in the skin can also express FcεRI[4,5].

The aims of the present study were (1) to investigate the expression of the α subunit of FcεRI (FcεRI-α) in normal and asthmatic airways and (2) to analyse the cellular provenance of this receptor in the airways, with particular emphasis on the dendritic cell.

MATERIALS AND METHODS

Spirometric readings (FEV1) and a provocation test with methacholine (PC20) were carried out on 10 allergic asthmatics and 9 normal volunteers. Endobronchial biopsies from each subject were fixed in cold acetone then processed into glycolmethacrylate

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(GMA) resin[6]. 2μm sequential sections were immunostained with the following primary murine antibodies: mAb 15.1 directed against FcεRI-α, mAb AA1 specific for mast cell tryptase, mAb OKT6 (Ortho Diagnostics) specific for CD1a and a mAb of IgG1 isotype (Sigma) as negative control. Bound mAb was detected using biotinylated rabbit anti-mouse (Dako Ltd) followed by a streptavidin-biotin-peroxidase complex. Peroxidase was detected using amino-ethylcarbazole (AEC) as a substrate. Sections were counterstained with Mayer's haematoxylin.

Sections were examined by light microscopy. Regions of mucosa were delineated using a VIDS and the corresponding areas calculated using AMS software (Cambridge, UK). Cells immunoreactive for AA1, 15.1 or CD1a were enumerated in both epithelium and submucosa excluding mucosal glands and blood vessels. Comparison between cells in adjacent sections was performed using a computerised system of cell recognition. The Mann-Whitney non-parametric test was used for statistical analysis. Correlation coefficients were calculated using the Spearman non-parametric test. p values of <0.05 were considered significant.

RESULTS

Asthmatic airways contained more FcεRI-α positive cells than normal airways, although this difference did not reach statistical significance (Table 1). Expression of FcεRI-α did not correlate with any of the physiological indices measured.

Table 1. Subject characteristics and expression of FcεRI in the airways.

Subject no	Sex	Age (yr)	Baseline FEV1 (% pred)	PC20 (mg/ml)	15.1+ cells (cells/mm^2)
Asthmatics					
Mean		31.8	86.5	1.06	39.1
+/- SD		9.7	17.9	1.6	45.4
Normals					
Mean		24.7	108.5	>32	14.7
+/- SD		13.5	8.3		18.9

Sections adjacent to those stained with 15.1, were stained with antibodies against either CD1a or tryptase to show the relationship between FcεRI-α and dendritic cells or mast cells respectively. This revealed that 15.1 immunoreactivity was localised to both cell types. Control sections showed no staining. The percentage of dendritic cells that were 15.1 positive was higher in asthmatic subjects (from 60 to 80%) than in normals (from 30 to 50%), however this failed to reach statistical significance. In contrast, a similar analysis used to compare 15.1 positive mast cells showed no difference between asthmatic and normal subjects.

The number of CD1a positive dendritic cells was significantly higher in asthmatic than in normal subjects (7 vs 0 cells/mm^2; $p=0.02$; Figure 1). However, no correlation could be found between the number of dendritic cells and any physiological indices. No significant difference was found in the number of AA1+ mast cells between asthmatic and normal subjects.

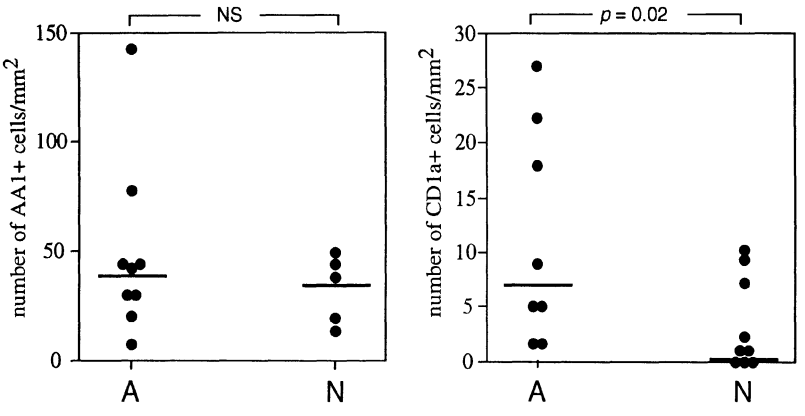

Figure 1. Numbers of dendritic cells (CD1a+) and mast cells (AA1+) in the airways of asthmatics (A) and normals (N). Horizontal lines represent median values.

DISCUSSION

The present study has demonstrated the presence of the α-chain of FcεRI in the airways of both normal and asthmatic subjects. FcεRI-α was expressed not only on mast cells but was also found to be present on CD1a positive dendritic cells in the airways. This extends the finding that skin Langerhans cells express FcεRI[4,5], although its role on dendritic cells in both the skin and the lungs remains to be elucidated.

Expression of FcεRI-α was found to be higher in the airways of asthmatics compared to normals. However this difference did not reach statistical significance. Furthermore, a trend was observed for higher numbers of FcεRI-α+ dendritic cells in the airways of asthmatics than normals, but again this did not reach statistical significance. It may be that increased expression of IgE receptors is not necessary for enhanced dendritic cell function in atopic individuals. For example, it has recently been suggested that FcεRI in atopics may be primed and hence transmit intercellular signals more readily than in normals[9].

Numbers of CD1a positive dendritic cells were significantly higher in asthmatics than normals. This is in agreement with other studies in which greater numbers of dendritic cells were found in both the upper[7] and lower[8] airways of atopic subjects. This increase in dendritic cell number could contribute to the slight, but not significant, increase in total cells expressing FcεRI-α in the asthmatic airway.

Overall the results of the present study do not support the hypothesis of an upregulation of FcεRI in asthmatic tissues. However, in light of the suggestion that the gene for the β subunit of FcεRI is putatively linked to atopy, further studies of the expression and function of FcεRI-β in asthmatics and normals are required.

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A COMPARATIVE STUDY ON LANGERHANS CELLS IN LYMPH NODES WITH DERMATOPATHIC LYMPHADENOPATHY AND HISTIOCYTOSIS X CELLS

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INTRODUCTION

Dermatopathic lymphadenopathy (DPL) is characterized by the existence of a large number of dendritic cells (DC) , namely Langerhans cells (LC) and/or interdigitating cells (IDC), in the paracortical areas. It is generally accepted that histiocytosis X (HCX) are proliferative diseases of LC, to the extent that recently HCX are also termed Langerhans cell histiocytosis. We would now like to show some differences between these LC in DPL, and HCX cells.

MATERIALS AND METHODS

Ten cases of lymph nodes with DPL and 15 cases of HCX were examined. Light microscopically, they were immunostained with S-100 protein, OKT-6 (CD 1a), Leu 3a (CD 4), and/or double stained with the former two antibodies and PCNA. Immunoelectron microscopically, they were stained with CD 1a, CD 4, and S-100 protein.

RESULTS AND DISCUSSION

We have reported that Birbeck granules (BG) possessing LC are positive for both CD 1a and S-100 protein, and IDC which do not possess BG, are only positive for S-100 protein but negative for CD 1a[1]. In two synthetic photographs of the paired mirror sections of DPL, one stained with CD 1a, the other with S-100 protein, it became apparent that over 90% of the DC in the lymph nodes of the DPL were LC which were positive for both CD 1a and S-100 protein plus a few IDC which were only positive for S-100 protein.

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Immunoelectron microscopically, these DC which were positive for CD 1a possessed BG, although the BG were generally few in number. The HCX cells were positive for the CD 1a, CD 4, and S-100 protein antibodies. However, the LC in the DPL were only positive for both CD 1a and S-100 protein, but negative for CD 4. According to the double staining for CD 1a and PCNA or S-100 protein and PCNA, the mean positive rates of PCNA with CD 1a or S-100 protein were 32.8 + 19.9 % in HCX. However, almost all DC were

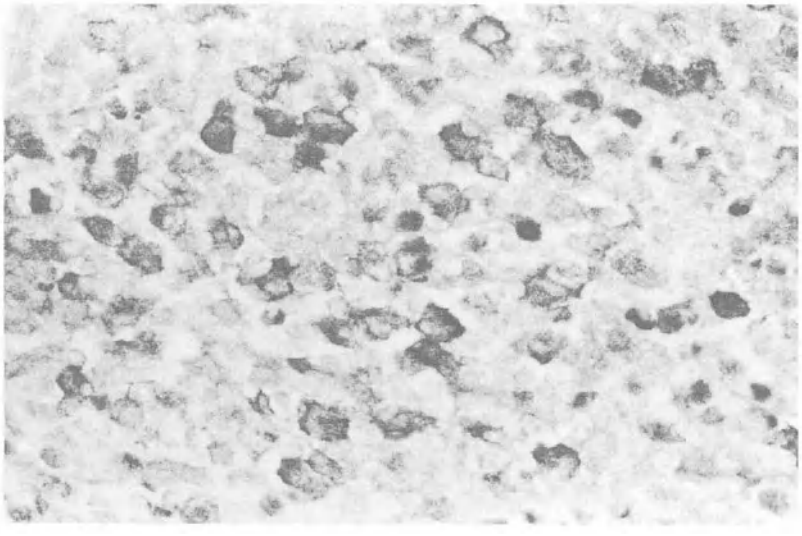

Figure. 1. A case of DPL. Double immunostaining using OKT-6 and PCNA. The plasma membranes of LC are stained with OKT-6, but the nuclei are negative for PCNA.

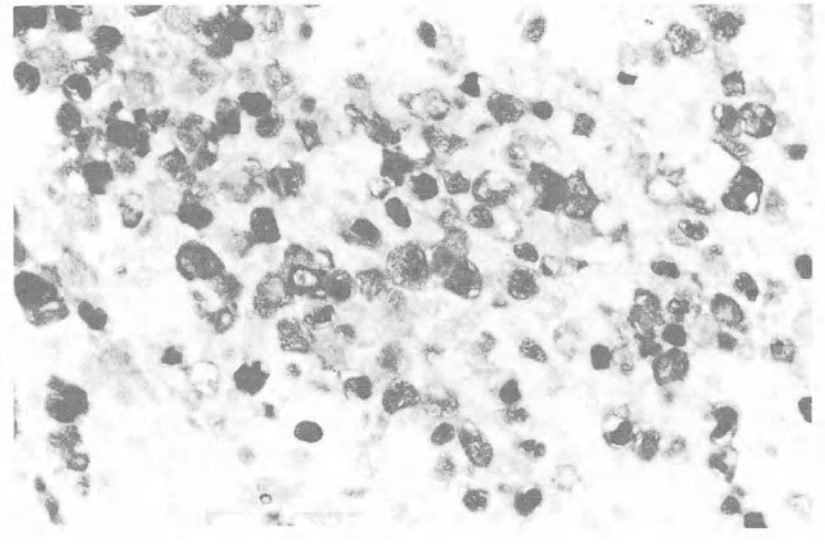

Figure. 2. A case of HCX. Double immunostaining using S-100 protein and PCNA. Many LC which are positive for both S-100 protein and PCNA are observed.

negative for PCNA in the paracortical areas with DPL, although a few veiled cells in the marginal sinuses, similar to a few LC in the epidermis and the dermis of skin lesion were positive for PCNA (Figures 1 and 2). These findings mean that LC in the lymph nodes with DPL are mature cells which do not divide in the lymph nodes, while HCX cells are proliferating cells. According to the generally accepted belief, DC migrate from peripheral nonlymphoid tissues via afferent lymphatics and/or blood into lymph nodes and transport antigens to the T regions of lymph nodes[2]. As stated above, several LC which were positive for CD 1a or S-100 protein were found in the dermis of skin lesion with DPL and LC in the epidermis were generally fewer in number than in the normal epidermis. It is speculated that the LC in the dermis may move down from the epidermis. From our results, we can readily assume that LC in the lymph nodes with DPL may migrate from the skin lesion.

LC and IDC represented slightly different immunological characteristics. And the distribution of both cells in vivo was also different. IDC, in which BG are never observed, and which are only S-100 protein positive, but CD 1a negative, were also found in the mesenteric lymph nodes and spleens. However, LC which were positive for both CD 1a and S-100 protein were only found in superficial and hilar lymph node draining tissues with predominantly squamous epithelia[1]. So we have speculated that LC and IDC might have some different functions because BG bearing LC could only be found in some lymphoid tissues, but not in all lymphoid tissues in vivo. Similarly, some differences were found between CD 4 negative LC in DPL and CD 4 positive HCX cells. Fossum[3] has described that DC are phenotypically heterogeneous and this heterogeneity reflects, at least in part, maturational development of DC along their migratory routes. The presence or absence of the expression of CD 4 molecule between LC in DPL and HCX cells may represent the difference of the degree of development of both cells. The causes of HCX are unknown, although immunodeficiency or some kinds of infections, etc. may be responsible for them. HCX cells are pathological cells which can proliferate easily when compared with LC in DPL.

SUMMARY

It was elucidated that the majority of DC were LC which were positive for CD 1a, but negative for PCNA, and possessed BG in the lymph nodes with DPL. On the other hand, HCX cells were almost always positive for PCNA. From this point of view, it can be speculated that LC in the lymph nodes of the DPL are non dividing mature cells and migrate from the skin lesion. HCX cells which were positive for CD 4 may be more immature cells than LC in DPL, and may be pathological cells which can divide in the foci.

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ROLE OF THE INTERACTION OF FIBRONECTIN WITH EPIDERMAL LANGERHANS CELLS IN REGULATING THEIR MIGRATORY PATHWAY

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INTRODUCTION

Evidence deriving from studies in the mouse showed that Langerhans cells (LC) capture antigens encountered in the epidermis and migrate subsequently from the epidermis to the skin-draining lymph nodes where they efficiently present antigens to T cells[1,2,3]. To migrate from epidermis to regional lymph nodes, antigen-bearing epidermal Langerhans cells must move through extracellular matrix (ECM) of various composition. The basement membrane (BM) of human skin presents a barrier rich in laminin (LM), type IV collagen, nidogen and heparan sulfate proteoglycans. The ECM of the superficial dermis contains interstitial collagens I and III essentially. Fibronectin (FN) is encountered mainly in afferent lymphatics. The effects of these ECM molecules on the LC behavior remain poorly understood.

In the present study, adhesion assays were performed in order to contrast the ability of BM- and dermis-ECM components to successively stimulate the adhesion of epidermal LC. Our observations suggest that interactions with the ECM environment may play a crucial role in the directed migration of LC from the epidermis to the proximal lymph nodes.

MATERIAL AND METHODS

Epidermal cell suspensions were prepared as described by limited trypsinization followed by gradient sedimentation, elimination of basal keratinocytes[4] and application to a second sedimentation gradient, the density of which was decreased from 1.077 to 1.068 g/cm^3. A purity of 70-86% viable, unlabeled LC (as judged by the number of CD1a positive cells) was regularly achieved. Cells in Medium 199 (Gibco BRL SARL, Cergy Pontoise, France) containing 1% BSA were added to LM (from mouse EHS tumor), FN, type I and type IV collagens (Sigma Chemical Co., St. Louis, MO, USA) for 45 min at 37°C.

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Determination of the number of adherent LC were performed as described[4]. The cells adherent to one substrate were recovered and then allowed to attach to fresh wells coated with the same or the three different ECM proteins by the same method.

RESULTS AND DISCUSSION

In the primary adhesion assay, of the LC population, 40% ± 7% adhered specifically to mouse LM, 20.82% ± 7% to type IV collagen, 62.2% ± 10.96% to FN, and 19.93% ± 3.21% to type I collagen. The use of mouse LM resulted in only 40% ± 7% of the LC population which attached to LM instead of 76% ± 15% which attached to human LM[4]. Only a subset of $\alpha 6^+$ LC seems to interact with mouse LM. This subset could correspond to the LC which express more than 70gg/ 100μm (threshold determined arbitrarily) and which represent 50% of the starting population.

In the secondary adhesion assay, the recovered LM-attached cells did not show any changes in adhesion capacity to type IV collagen, type I collagen and FN. Adhesion to type IV collagen did not significantly affect readhesion to FN and type I collagen, while a decrease of 30% of adherent LC to LM was observed compared to the original LM-adherent population. Readhesion of recovered type I collagen-attached cells resulted in a decrease of 39% of LC binding to LM whereas readhesions to FN and type IV collagen were not affected. In contrast, readhesion of recovered FN-attached cells to fresh dishes resulted in a decrease of 56% of LC binding to LM, in an increase of 48% and 60% of LC binding to type I and type IV collagens respectively. In each case, non adherent cell fractions recovered from the primary adhesion assays exhibited, in the secondary adhesion assays, binding capacities similar to control cells, indicating that adherence to ECM molecules did not select preexisting LC subsets in the primary adhesion assays. It appears that the interactions of LC with the BM components can be normally followed by interactions with the dermis-ECM molecules and that following contact with dermis ECM molecules, the binding capacity of epidermal LC to the BM laminin is highly reduced.

Table 1. Schematic representation of the modifications of the percentages of adherent LC observed in the secondary adhesion assay.

	Secondary adhesion to			
	LM	Coll IV	Coll I	FN
LM-attached LC	↗	→	→	→
Coll IV-attached LC	↘	↗	→	→
Coll I-attached LC	↘	→	↗	→
FN-attached LC	↘	↗	↗	→

A selection of an LC subpopulation lacking expression of ß1 integrin LM receptors seems unlikely since 96% of LC expressed the LM receptor $\alpha 6ß1$, distributed as 74.68 ± 29.3 gold granules per 100 μm of cell membrane (gg/100μm) as determined by immunogold labeling with anti $\alpha 6$ subunit mAb GoH3 (kindly provided by P. W. Modderman. Central Laboratory of the Netherlands Red Cross Blood Transfusion service, Amsterdam) performed

on transmission electron microscopy, followed by a quantitative analysis[5]. 55.5% of the cells expressed α5 (P1D6, purchased from Telios Pharmaceutical, Inc. San Diego, CA) (26.8 ± 12.8 gg/100μm). Consequently, most of the cells which expressed α5ß1, a receptor involved in binding of LC to FN[4], also expressed α6ß1, and the quantitation of receptor expression on freshly isolated cells demonstrated that α6ß1 was always more represented than α5ß1 on the surface of a same cell. Even if the quantitation of receptors demonstrates that some LC can express α5ß1 and not α6ß1, this subpopulation does not exceed 3% and consequently does not alone represent the LC adherent fraction (17.4%) which readheres to LM after a first contact with FN. Furthermore, the majority of the recovered LM-attached cells readhered to LM demonstrating that α6ß1 was still functional, and the quantitation study showed that whereas LM-recovered cells, when replated either on LM or FN, still expressed high level of α6 (65.9 ± 26.4 and 53.3 ± 18.7 respectively), FN-recovered cells replated either on FN or LM, displayed a reduced expression of α6 (43.9 ± 17.3 and 44 ± 16 respectively, $p< 0.001$), demonstrating that FN is able to modulate the expression of the α6ß1 integrin on epidermal LC. Thus, it seems more likely that contacts of epidermal LC with FN induce a specific down regulation of the α6ß1 expression correlated with modification of the binding capacities of LC to LM. The observation that cultured LC, which fully resemble LC that have migrated to lymph nodes[6], do not express α6 anymore (unpublished observation) might support this hypothesis. By contrast, the binding capacities of recovered FN-attached cells to type I and type IV collagen were increased suggesting that contact of epidermal LC with FN could stimulate adhesion to collagen.

The mechanism(s) involved in this phenomenon remain(s) to be investigated: differential effects on ß1 integrin activation, effects on cytoskeletal organization....Whatever the mechanism is, the contact between epidermal antigen-bearing LC and ECM components might contribute to regulate the direction of LC migration from the epidermis to the dermis by modulating LC adhesion affinity to each other.

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EXPRESSION OF NEUROPEPTIDES ON HUMAN EPIDERMAL LANGERHANS CELLS

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INTRODUCTION

The response of nervous system to stimuli consists of the release of neuropeptides (NP) which act on biological functions and especially on immunity (1).but their effects on immune cells are not well known.

Only twenty NP have been localized in the skin among the two hundred already characterized. Their local pharmacological effects are well listed, but our knowledges about their probable role on immunocompetent cutaneous cells i.e. Langerhans cells (LC) are very poor.

The aim of this study was to search for the presence of NP on human epidermal cells, especially LC. Substance P (SP), Neurotensin (NT), Vasoactive Intestinal Polypeptide (VIP), Neuropeptide Tyrosine (NPY),Nerve Growth Factor receptor (NGFr) and Gastrin Releasing Peptide receptor (GRPr) were researched by immunohistochemistry, flow cytometry and electron microscopy.

MATERIALS AND METHODS

Eleven punch biopsies of normal human skin from men and women (20 to 56 years old) were studied by immunohistochemistry. Samples were fixed for 3 hours in a Zamboni solution, rinced in PBS containing 0,3M saccharose and freezed.Samples were incubated with anti-NP antibodies overnigth at 4°C. Enzymatic stainings were performed with streptavidin/biotin conjugated to peroxydase. Immunofluorescent stainings were revealed by FITC.

Epidermal cell suspensions were obtained from normal human skin samples, through action of trypsin (0,05%, 18h, 4°C). They were purified in LC (60-80% LC) by successive density centrifugations. Cell pellets were incubated with anti-NP antibodies for one hour at 4°C (revealed with FITC antibody) and with anti-CD1a antibody (revealed with phycoerythrin). Pellets were fixed in paraformaldehyde and studied with a cytofluorometer FACSCAN. Dead cells and debris were excluded from analysis.The threshold of positivity was established on the basis of the negative control sample (first antibody replaced with PBS).

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LC-enriched epidermal cell suspensions were prepared from freshly removed normal human skin. LC enrichment was performed through gradient sedimentation. Cells were resuspended in RPMI-1640 containing 5% fetal calf serum and 5% normal human serum. Cell suspensions were incubated with primary antibodies diluted at 1/100 for 1h at 4°C. After two washes, suspensions were incubated with gold labelled goat anti-rabbit or goat anti-mouse IgG (H+L) 10nm for 1h on ice. Cells were fixed with 2% glutaraldehyde and processed for electron microscopy. These experiments were repeated three times. Controls were performed by replacing the first antibody by normal rabbit or mouse serum. Langerhans cells were recognized by CD1a antigen and Birbeck granule expressions.

RESULTS

Cytofluorometry showed that CD1a+ cells expressed SP, VIP, CGRP, NT, GRPr but immunoelectron microscopy confirmed immunoreactivity only to NT and GRPr.

Using immunohistochemistry, we have localized GRPr on normal human skin. This receptor is expressed on vessels, sweat glands, nerves and on epidermal cells. GRPr was strongly expressed on human suprabasal epidermal dendritic cells. NT was expressed on epidermal cells except basal layer cells. In the epidermis, NGFr was localized on basal keratinocytes. This receptor was also seen on dermal dendritic cells. CGRP was expressed on basal and suprabasal epidermal cells. The epidermis was not stained with SP, VIP and NPY.

Table 1. Summary of the immunohistochemical stainings.

NP	Epidermal stainings
NT	+ Except basal layer cells
GRPr	+ Especially dendritic cells
CGRP	+ Basal and suprabasal cells
NGFr	+ Only on basal keratinocytes
SP VIP NPY	-

DISCUSSION

We showed SP, VIP, CGRP, NT and GRPr-immunoreactivity on CD1a+ cells. CGRP was already found on LC of the human oesophagus (2). Nerve fibers containing CGRP were found to be in contact with human epidermal LC (3). To determine the functions of CGRP on murine LC, mixed epidermal cell-lymphocyte cultures were processed and demonstrated that CGRP inhibits the murine LC capacity of antigenic presentation (3). SP, VIP, CGRP, NT and GRP have effects on monocytes or macrophages (4) (5) (6).

Thus, these NP probably influence LC functions. Further studies are necessary to determine whether these NP have functional significance. It will be interesting to study the effects of these NP on human LC in mixed epidermal cell-lymphocyte reaction.

GRP is a 27-amino-acid NP which was first isolated in the swine stomach. It shares the same ten carboxy-terminal amino-acids than bombesin (BOM). GRP and BOM have been localized in the skin of batrachians by *in situ* hybridation of their mRNA. They are found in cutaneous granular glands (7). In human normal skin, immunofluorescence studies could not reveal the expression of BOM but we showed GRPr expression in human skin, especially on suprabasal epidermal dendritic cells.

NT-immunoreactivity was visualized on epidermal cells except basal cells. The reason why basal cells are not NT-immunoreactive remains to be determined. NT may be only expressed on differentiated cells.

Cytofluorometry studies demonstrated that CD1a- cells were stained by antibodies directed to NT, GRPr, CGRP, SP and VIP. More experiments may be carried out to determine the functional significance of the presence of SP on CD1- cells, especially because SP has no known action on normal human keratinocytes (8) (9). VIP is known to stimulate proliferation of human keratinocytes (4). The effects of NT, GRP and CGRP on human keratinocytes have never been studied.

NP seem to be expressed in human keratinocytes and Langerhans cells. However, their exact role still remains to be determined. Further studies have to be performed to know if Langerhans cells express NP or NP-receptors.

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MONOCYTE DERIVED DENDRITIC CELLS (MODC) PRESENT PHENOTYPE AND FUNCTIONAL ACTIVITIES OF LANGERHANS CELLS / DENDRITIC CELLS

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INTRODUCTION

Dendritic cells (DC) have long been regarded as an independent cell lineage. Nonetheless increasing evidence over the last years lead to the understanding that DC are of myeloid origin. It has been demonstrated that peripheral blood monocytes can be differentiated into antigen presenting accessory cells[1]. CD1a has been found on myeloid leukaemic cells[2] and DC were demonstrated to derive from the same $CD34^+$ cell as macrophages[3] ($M\Phi$). Furthermore, Histiocytosis-X cells, long known to be the semi-maligne cell variant of Langerhans cells (LC)[4] are known to express myeloid markers such as CD14 [5].

We have demonstrated earlier the achievement of a Langerhans cells phenotype from peripheral blood monocytes, indicating the possibility to follow a suggested pathway from monocytes via Langerhans cells (LC) to Dendritic cells[6,7] in-vitro. It could be shown that monocyte derived accessory cells (MoAC) of a LC like phenotype ($CD1a^+$ / $CD14^{dim}$) have a non-specific esterase and phagocytic activity comparable to LC[8] described by others. Further studies were now performed to follow the differentiation pathway and continue experiments on the lymphocyte stimulation capacity in an allogeneic primary mixed leukocyte reaction (MLR).

MATERIALS AND METHODS

Monocytes were purified and cultured as described before[7]. Additional supplements used were LPS (Sigma), ConA (Sigma), rhuIL-4, rhuIL-10, rhuGM-CSF and rhuTNFα (all PBH, Hannover, FRG).

For flow cytometry, cells were prepared as described in detail elsewhere[9]. Monoclonal antibodies (mAbs) used were: α-CD1a (NA1/34), Ortho; α-CD14 (IOM 2), Immunotech.; α-HLA-DR (CR3/43), Dako; α-HLA-DP, Becton Dickinson; α-HLA-DQ (IOT2d) and α-CD80 (MAB 104), both Immunotech.. In the case of indirect immunofluorescence, the following polyclonal antisera were used: goat α-Mouse-PE (R 9670), Sigma and goat α-Mouse-FITC (115-095-062), Immunotech.

Peripheral blood T-lymphocytes were isolated for the MLR by passaging monocyte depleted lymphocytes over a nylon wool collumn. 4×10^4 monocyte derived accessory cells (MoAC), in-vitro cultured for three days, were co-cultured with 10^6 of the freshly isolated T-lymphocytes (nylon wool purified T-lymphocytes contained less than 5% non-T-Lymphocytes/monocytes as measured by flow cytometry). After 72h, DNA synthesis was measured by a 24h pulse of 2μCi [^{3}H] thymidine. In order to exclude background

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proliferation by lymphocytes, MoAC were irradiated with 25Gy (2500rad) using photons (8MeV) from a linear accelerator in several MoAC preparations.

RESULTS

Three day old MoAC expressed various levels of CD1a and CD14, depending on the donor, FCS and medium batches. All such factors depended upon each other and especially FCS and medium quality were of greatest importance. Media and FCS batches containing LPS or other so far undefined inhibitors led only to MΦ-precorsors but not to MoAC. Furthermore, some donors – although beeing healthy – contained monocytes which would just differentiate towards macrophages. As a results, CD1a and CD14 undergo no modulation in some donors, whereas MoAC of most other donors expressed small amounts of CD1a within 3 days and downregulate CD14 over a 6 day period. Comparing MoAC and macrophages, MΦ express 10fold more CD14, 10 fold less HLA-DR and no CD1a.
Treatment with GM-CSF & IL-4 stronly enhanced CD1a expression and supressed CD14. On the other hand, GM-CSF alone had no effect on CD1a expression and with donor-monocytes unwilling to express some CD1a without cytokines, even GM-CSF & IL-4 were mostly not able to induce CD1a on more than 2/3 of the cells.
Additional supplementation of IL-10 fully abolished the effects of GM-CSF & IL-4, but cells remained at least fully viable in trypan blue exclusion tests. Cultures treated with IL-10 alone lost much of their viability and downregulated most markers. Exogenous supplementation of low doses (<100U) TNFa did not alter differentiation pathways; >100U were at least slightly suppressive to MoAC differentiation and >500U enhanced macrophage development.
MLR results strongly supported earlier similarities between MoAC and Langerhans cells/ Dendritic cells (Fig.1). While controls with freshly isolated monocytes or fully differentiated macrophages were allways incompetent to stimulate lymphocytes (<5000 cpm; Data not shown), MoAC were potent lymphocyte stimulators in an allogeneic MLR. Although results varied between the different donors, MoAC were allways more effective than 3 day old monocytic cells differentiating towards macrophages (20% human AB serum), which had an intermediate but distinctive stimulating capacity.
Cytokine composition in MoAC culture medium was crucial for MLR outcome. GM-CSF & IL-4 significantly enhanced lymphocyte stimulation by MoAC, but did not significantly alter lymphocyte stimulation by monocytes differentiating to macrophages at day 3 of in-vitro culture (Fig.1). Finally, it was evident, that MoAC differentiation depended on media, serum (as MLR results varied with the serum amount) and endogenous factors (as demonstrated by a complete medium change before MLR; data not shown). MoAC had their stimulation maximum at a FCS concentration of about 10%.

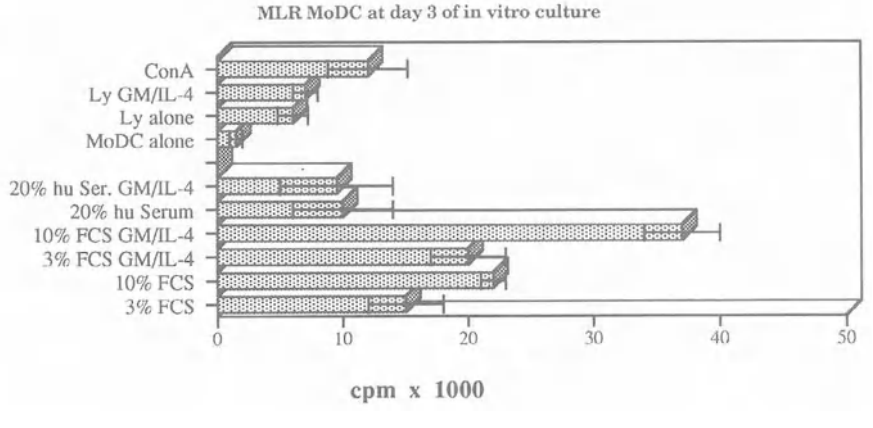

Fig.1: Autologous MLR using 3 day old monocytic cells. Controls were performed using ConA (positive), lymphocytes (Ly) alone and with Cytokines or MoDC alone (background). MoDC without and with cytokines show a distinct lymphocyte stimulating cypacity.

DISCUSSION

In order to study monocyte-derived accessory cells (MoAC), we used an in-vitro differentiation sytem, which we described earlier[7]. With adequate media and exogenous supplements such as high quality FCS, CD1a expression in the human system is consistently reproducible and stable for up to 7 days in culture. The cells express HLA-DR, -DP, -DQ and modest levels of CD80. Our experiments performed during the last 2 years strongly indicate that monocyte accessory differentiation is a multifactory event. If cultures contain LPS for example, MoAC achievement is impossible and monocytes from a number of doners (<10%) seem absolutely unwilling to become MoAC, probably beeing primed to MΦ in-vivo. Cytokine supplementation experiments show that IL-4 and GM-CSF are required but not sufficiant for CD1a expression and contribute to the success of MoAC differentiation. Furthermore, exogenous GM-CSF & IL-4 mostly just modulate CD1a, CD14 and MHCII expression and further cytokines seem to influence the MoAC differentiation, as indirectly supported by flow cytometric experiments with various FCS concentrations (data not shown). Data presented by another group indicate that IFNγ contributes to the MoDC differentiation (Peters et al., this issue). Analyses of supernatants give evidence to the hypothesis of a cytokine network in MoAC differentiation, with participation of IL-1β and IL-6.

Immunostimulation of T-cells by MoAC in a primary MLR is comparable with other Langerhans cells/Dendritic cells in the human system described so far. GM-CSF & IL-4 enhanced the stimulating capacity of MoAC, while they did not support or even suppressed MΦ differentiating cells. Moreover, MLR data strongly underline the idea of third factors for differentiation, as FCS concentrations influenced the MLR too.

Our data provide further evidence to considerations that the precursors of DC, a long time believed to be a minor and independent population, are of myeloid-monocytic origin. Furthermore the data strongly support the view that monocytes may be differentiated into accessory cells of DC-phenotype and -function. Such cells should therefore be termed **MoDC** (a term also used by G. Schuler in Annecy), especially as DCs consist of a wide variety of subpopulations and phenotypes (e.g. eLC, cLC, IDC) and published data show that no in-vitro differentiation established so far may fulfill all criteria for DC at once[10, 11, 12]. Finally, our report is in parallel with two earlier publications[13,14] as well as the most recent workshop report from G. Schuler (this issue), whereas the development of DC from monocytes has not been pointed out explicitly before.

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DEVELOPMENT AND DISTRIBUTION OF T CELL-ASSOCIATED DENDRITIC CELLS IN ORGANS AND TISSUES OF MICE DEPLETED OF BLOOD MONOCYTES BY ADMINISTRATION OF STRONTIUM-89

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INTRODUCTION

In the development and differentiation of T cell-associated dendritic cells (epidermal Langerhans cells, interdigitating cells in the paracortex of lymph node, and veiled cells in the dermis and within the afferent lymphatics), the importance of granulocyte-macrophage colony-stimulating factor (GM-CSF) has been pointed out. This fact was based on the results of previous studies of in vitro response of dendritic cells to GM-CSF[1] and studies on osteopterosis (op) mice lacking functionally active macrophage colony-stimulating factor (M-CSF).[2] Recently, a few groups of investigators have succeeded in generating dendritic cells in cultures of bone marrow cells with GM-CSF in mice[3] and humans.[4] However, the relationship between dendritic cells and blood monocytes still remains unclear. To elucidate it, it is necessary to investigate the behavior of the dendritic cells in a condition extremely depleted of blood monocyte.

Strontium-89 (^{89}Sr) is a bone-seeking radioisotope emitting β-rays that mostly dissipate in soft tissues after a very short distance.[5,6] It is rapidly taken into growing bones by exchange with calcium, thus effectively irradiating and destroying bone marrow cells, particularly precursors of blood monocytes. Unlike administration of corticosteroids or whole body irradiation, a single intravenous administration of ^{89}Sr induces severe monocytopenia in mice without any significant influence on tissue macrophages for over 8 weeks.[7] In such severely monocytopenic mice, extramedullary hematopoiesis occurs in the spleen with lapse of time after ^{89}Sr administration, sustaining monocyte production to compensate for reduced bone marrow hematopoiesis. To prevent this, the use of splenectomized mice is recommended.[5]

In order to elucidate the relationship between blood monocytes and dendritic cells, we produced severe long-lasting monocytopenia in splenectomized mice by intravenous injection of ^{89}Sr and examined then number, morphology, and distribution of their dendritic cells in various organs and tissues.

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MATERIALS AND METHODS

Nonpregnant, 8-week-old female BALB/c mice were splenectomized. Four weeks after splenectomy, these mice were injected intraperitoneally with $^{89}SrCl_2$ 55.5 MBq/g body weight or 200 mg of nonradioactive $^{88}SrCl_2$ in saline. ^{89}Sr, specific activity 3,330-5,000 MBq/mmole, was purchased from Amersham (Amersham, UK) in chloride form and stored in a lead safe at 4°C unitl use. A small amount of blood was sampled from the tail vein of all the animals every week for cell counting, and 1,000 white blood cells were counted on blood films for the differential count. Six weeks after administration, all the animals were killed with ether anesthesia and the liver, lymph nodes, thymus, intesines, heart, lungs, kidneys, uterus, ovaries, and skin were excised. These tissues were fixed with periodate-lysine-paraformaldehyde (PLP) fixative at 4°C for 4 hours and submitted to the procedures for preparing cryostat sections described elsewhere.[2] Epidermal sheets were prepared from the skin as described previously.[2] For detection of dendritic cells, immunostochemistry was performed according to the method described previously,[2] using anti-mouse monoclonal antibody against dendrtic cells NLDC-145, and the number of NLDC-145-positive cells was counted in each tissue. Statistical significance of the data was evaluated by the Student's t-test. Electron microscopy was also carried out as described previously.[2] In addition, tissue macrophages were also detected immunohistochemically using BM8, anti-mouse monoclonal antibody specific for tissue macrophages.[2]

RESULTS AND DISCUSSION

In the ^{89}Sr-treated splenectomized mice, the white blood cell count remained below 4,000 from 2 weeks after administration ($p<0.05$) and blood monocytes disappeared nearly completely. Such severe monocytopenia continued until 6 weeks after administration. We counted the number of NLDC-145-positive dendritic cells per mm^2 in the epidermis, thymic medulla, and paracortical area of the lymph nodes of the ^{89}Sr- or ^{88}Sr-treated mice and compared them(Fig 1). No statistically significant difference in the number of dendritic cells in the tissues were found. The cell distribution was essentially the same in both groups. In the ^{89}Sr-treated mice, NLDC-145-positive cells were detected in other tissues such as the intestinal mucosa, renal interstitium, lungs, uterus, and ovaries. These results indicate that dendritic cells normally develop in the tissues of severely monocytopenic mice. In previous studies of severely monocytopenic mice by ^{89}Sr administration, peritoneal resident macrophages, alveolar macrophages, or Kupffer cells were found to survive for a long period of time without supply of blood monocytes.[5-7] In the present study, we found no statistically significant differences in the number of BM8-positive macrophages in the liver, brain, kidneys, endometrium, and ovaries between the ^{89}Sr-treated and ^{88}Sr-treated mice, providing evidence in support of the previous view that tissue macrophages are an independent cell population distinct from monocytes and monocyte-derived macrophages. The present study adds evidence to the view that the dendritic cells, like the tissue macrophages, are a cell population distinct from the monocytic cell lineage. Our previous study revealed that colony forming unit-spleen (CFU-S) and colony stimulating unit-macrophage (CFU-M) completely disappear in the bone marrow of ^{89}Sr-treated splenectomized mice from 4 weeks on. However, CFU-S is detected in peripheral blood, though very minor.[7] Taken together, these findings suggest that the dendritic cells are derived from dendritic precursor cells that are in the developmental stage earlier than CFU-M.

CONCLUSION

From the above data, it is considered that the T cell-associated dendritic cells can develop in the epidermis, thymic medula, and in the T cell-dependent areas of peripheral lymphoid tissues of splenectomized mice severely depleted of blood monocytes by ^{89}Sr administration. This suggests that the dendritic cells are derived not from blood monocytes but from dendritic precursor cells that are in the developmental stage earlier than CFU-M.

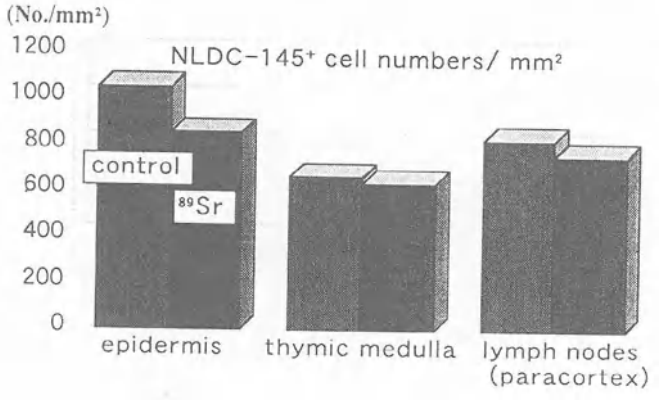

Figure 1. Comparison of the number of NLDC-145-positive dendritic cells per 1 mm^2 in the epidermis, thymic medulla, and paracortex of lymphnodes of ^{89}Sr or ^{88}Sr-treated splenectomnized mice.

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ESTABLISHMENT AND CHARACTERIZATION OF ANTIGEN-PRESENTING CELL LINES (XS SERIES) DERIVED FROM NEWBORN MOUSE EPIDERMIS

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INTRODUCTION

Activation of naive T cells occurs primarily through antigen presentation by a distinct class of leukocytes, termed dendritic cells (DC) (reviewed in 1). Langerhans cells (LC) are a skin-specific member of this family and play a pivotal role in the induction of T cell-mediated immunity against various antigens that are present in or penetrate into skin, which include reactive chemicals (contact hypersensitivity), alloantigens (skin graft rejection), microorganisms (protective anti-infectious immunity), and tumor-associated antigens (protective antitumor immunity) (reviewed in 2). A major limitation in studying the biology of LC has been the absence of stable, long-term cell lines. To overcome this limitation, we have established a series of antigen presenting cell lines (XS series) from newborn BALB/c mouse epidermis (3,4). Here we describe the establishment and characterization of XS lines.

RESULTS

Establishment of XS lines

Epidermal cells isolated from newborn BALB mice were enriched for LC and cultured in complete RPMI supplemented by mouse recombinant GM-CSF (5 ng/ml) and culture supernatants (10%) from Pam 212 keratinocytes. Small colonies of round cells appeared on top of the confluent keratinocytes after 1-2 weeks, and they were harvested by pipetting and plated into new wells. Interestingly, these round cells that were loosely attached to keratinocyte surface became firmly adherent, extending several elongated dendrites or numerous short pseudopodia on new plates. At this time, virtually all cultures contained two morphologically distinct cell populations, one with a dendritic or round shape (expanded thereafter as XS lines) and one with a "fibroblastoid" appearance (NS lines). These two populations were separated by differential trypsin treatments and

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cultured independently, in which XS lines were fed with complete RPMI supplemented with GM-CSF, Pam 212 supernatants and NS cell supernatants (10%), and NS lines were expanded in complete RPMI in the absence of added growth factors. To date, epidermal cells procured from a total of 100 newborn mice have been plated independently onto 60 wells, and we have established 55 independent XS lines and 6 NS lines, all of which have been expanded over a 12 month-period.

Immunological Features of XS Lines

XS20 and XS52 have been analyzed extensively for their surface phenotypes. Both lines displayed similar phenotypes; they expressed Ia, CD45, CD32/16 (Fcγ receptor), CD11b (C3 receptor) and heat stable antigen, suggesting that they belong to the DC lineage. More specifically, the feature of Ia^{low}/B7-1$^-$/E-cadherin$^+$ suggests that XS lines resemble LC freshly-isolated from skin (fLC) rather than cultured LC (cLC), which are known to be Ia^{high}/B7-1$^+$/E-cadherin$^-$ (5-7).

In the mixed leukocyte reaction, XS20 and XS52 both exhibited a modest, but significant capacity to activate naive, allogeneic T cells isolated from CBA mice. When compared with splenic DC or cLC, however, XS lines were much less potent in this capacity to activate naive T cells. By contract, XS lines were even more potent than splenic DC or cLC in their capacity to present a protein antigen to primed T cells, as measured by using a KLH-specific Th1 clone, HDK-1. Moreover, XS cell capacity to present KLH was diminished significantly by exposure to a relatively low fluence (50 J/m^2) of UVB, showing a high sensitivity to this treatment. Importantly, it is known that fLC are less potent in the activation of naive T cells, whereas more potent in the presentation of complex protein antigens than are cLC or splenic DC (2). Likewise, LC have been shown to become increasingly resistant to the effects of UVB when cultured under conventional conditions (8). Therefore, it appears that XS lines also resemble fLC more closely than cLC in their antigen-presenting profile.

In RT-PCR, XS20 and XS52 both expressed constitutively mRNAs for IL-1β and macrophage inflammatory protein-1α (MIP-1α), which have been reported previously to be expressed by fLC (9,10). By contrast, neither XS20 nor XS52 expressed mRNA for IL-6, which has been shown to be produced by cLC but not by fLC (11). These results, again, indicate the strong resemblance between XS lines and fLC. XS lines also expressed PCR signals for IL-1α, IL-7, CSF-1, TNFα and IFNα and faint signals for IL-10 and IL-12 (p40). mRNAs were not detected for IL-2, IL-3, IL-4, GM-CSF, SCF, PDGFα, FGFβ, LIF, IFNγ and IFNβ.

In ^{3}H-thymidine uptake assays, XS20 and XS52 both proliferated maximally in the presence of added GM-CSF or CSF-1. By contrast, other cytokines, including IL-1 through IL-12, TNFα, MIP-1α, G-CSF or SCF showed only minimal, if any, growth-promoting activities. XS lines also responded well to culture supernatants from NS01, a fibroblastoid cell line. We have observed by testing these cytokines in combinations that: a) TNFα inhibits GM-CSF-dependent proliferation but not NS supernatant-dependent proliferation, b) IFNγ, at concentrations as low as 0.4 ng/ml, inhibits XS cell growth significantly, and this inhibition was evident for both GM-CSF-driven and NS supernatant-driven proliferation, and c) IL-1α, IL-1β, IL-3, IL-4 or IL-7 showed minimal effects on XS cell growth, even when tested with GM-CSF, M-CSF or TNFα. Thus, XS lines resemble LC in their proliferative responses to GM-CSF but differ substantially from LC by their responses to other cytokines.

DISCUSSION

Long-term antigen-presenting cell lines (XS series) established from newborn mouse epidermis exhibit several features of LC freshly-isolated from skin. These features include: a) surface phenotype, b) antigen-presenting profile and susceptibility to UVB radiation, c) cytokine mRNA profile, and d) proliferative response to GM-CSF. Although future studies are required to determine the extent to which XS lines also resemble epidermal LC in their in vivo behavior, we believe that the availability of these lines as well as the methodologies for their establishment should enhance our capability to study the biology of LC at biochemical and molecular levels.

Success in generating XS lines was made possible by the use of supernatants from NS lines. With respect to identity of NS lines, we now believe that they represent a unique subset in the fibroblastic stromal cells, based on the following observations: a) NS lines are fibroblastoid in shape, b) large amounts of type I collagen were detected in their cytoplasm, and c) fibroblast lines derived from the dermal portion of newborn skin also secreted, although in smaller amounts, XS cell growth-promoting activity. As to the identity of NS factor(s), we now have some evidence that indicate that CSF-1 is fully responsible for XS cell growth-promoting activity of NS supernatant (Takashima; unpublished observation). Thus, these observations suggest the presence of a unique pathway in which fibroblastic stromal cells do support the proliferation of LC by the elaboration of CSF-1.

In this regard, Kämmpgen et al. have reported very recently that GM-CSF-R, but not CSF-1R, is detectable in cLC and splenic DC (12). Importantly, this observation was made using LC cultured for 3 days and splenic DC cultured overnight, both of which represent "fully-matured" populations. On the other hand, we have observed recently that XS lines, as well as fLC, do express CSF-1R, as measured by RT-PCR and specific antibodies (Takashima, unpublished observation). Therefore, our current hypothesis is that LC in situ express CSF-1R, which is downregulated upon their isolation and subsequent culturing.

Our data suggest that LC are capable of producing several different cytokines, thereby producing a unique cytokine mileau in epidermis, and are also capable of responding to various cytokines produced by neighboring cells. Moreover, the finding that XS lines remain immature even after 12 months in culture, would provide a useful system by which we can study the molecular mechanisms for LC maturation.

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MIGRATION OF RAT DENDRITIC CELLS AND MACROPHAGES FROM THE PERITONEAL CAVITY TO THE PARATHYMIC LYMPH NODES

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INTRODUCTION

Previous studies demonstrated the presence of a small number of dendritic cells (DC), approximately 1%, in the peritoneal cavity of rats (1). Upon stimulation with either specific (e.g. Bacillus Calmette-Guérin, BCG) or non-specific stimuli (e.g. Thioglycollate Broth, TG) the number of DC had increased to 3% (2). Different studies demonstrated the superior capacity of peritoneal DC compared to peritoneal macrophages (Mϕ) to present the antigen glutamine-tyrosine (GT) to GT-primed T cells in an in vitro assay (1,2,3), which is in agreement with other studies (4). Intraperitoneal stimulation with different stimuli had no effect on the antigen presenting capacity of DC, whereas that of the Mϕ decreased, and especially after BCG Mϕ had a suppressive effect on T cell proliferation. From these studies it was concluded that in the peritoneal cavity the DC was the major APC and although Mϕ can act as APC, this function of Mϕ strongly depends on the state of inflammation.

In the present study we examed the in vivo migration of both DC and Mϕ to the draining lymph node, in order to enlighten the role of both APC types in antigen presentation in vivo. We used congenic rats that only differ in one molecule of the leucocyte common antigen that can be detected with a monoclonal antibody.

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MATERIALS AND METHODS

Animals

For the experiments male isolator reared PVG-RT7^a and SPF PVG-RT7^b rats were used, 2 months of age, weighing 160-180 g. PVG-RT7^b rats were used as donor rats since all leucocytes carried the LCA marker that can be detected with the mAb His 41 (5). PVG-RT7^a rats were used as acceptor rats. All rats were obtained from Harlan-CPB (Zeist, The Netherlands), they were kept under standard laboratory conditions and were given food and water ad libitum.

Migration study

Peritoneal Mϕ and spleen DC were obtained from PVG-RT7^b rats. Peritoneal cells were obtained by peritoneal lavage (1). Mϕ were purified from this cell suspension by adherence onto the plastic culture dish surface during a culture period. Adherent cells were detached by incubation with 0.5% EDTA on ice. DC were isolated from the spleen using the method described by Knight et al. (6). Briefly, a cell suspension was made of the organ by incubation with collagenase and DNase in medium. DC were enriched from that cell suspension by a culture period based on the non-adherence of DC, followed by a density gradient using 14.5% Nycodenz. Thereafter remaining B-cells were removed using anti-rat IgG Dynabeads. Viability of all cell suspension was at least 85%.

PVG-RT7^a rats received 1x10^6 cells, either peritoneal Mϕ or spleen DC, in 1 ml PBS i.p. Control rats received 1 ml PBS i.p. After 4 days the rats were killed and the parathymic lymph nodes, that drain the peritoneal cavity, were taken out and frozen in liquid nitrogen. The presence of migratory cells was detected on cryostat sections.

Immunocyto- and histochemistry

Cytocentrifuge preparations were either processed for May-Grünwald/Giemsa (MGG) staining or for indirect immuno- and enzymcytochemistry (1). The mAb used were: MRC Ox6 (7), specific for MHC class II antigens; MRC Ox19 and Ox52 (8), specific for T cells. Acid phosphatase activity was demonstrated as per Burnstone (1), and was double stained with MHC class II antigens by sequential incubation.

Migratory cells were detected on cryostat sections of frozen lymph nodes by indirect APAAP staining (9). Sections were fixed with aceton for 10 min at 4°C and subsequently incubated with the mAb His 41 (a gift from Dr F. Kroese, University of Groningen), rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark) and APAAP immune complexes. Control slides, were the first step was omitted, were completely devoid of alkaline phosphatase staining. No staining for the mAb His 41 was observed in tissues of PVG-RT7^a rats.

RESULTS

Cellular Composition of Cell Fractions

Table 1 sumarizes the criteria applied to discriminate DC from Mϕ. Especially the spot like staining observed in DC for acid phosphatase activity as well as for the mAb ED1 distinguished these cells from Mϕ that stain for acid phosphatase activity and ED1 throughout the cytoplasm. All DC stained with the mAb Ox6 demonstrating MHC class II molecules whereas only 13% of the peritoneal Mϕ did. Purity of DC was at least 85% and for peritoneal Mϕ at least 90%.

Table 1. Morphological and immuno- and enzymatic histogogical characteristics of Mϕ and DC.

	cell type	
criterium	peritoneal Mϕ	spleen DC
cell surface	irregular	irregular, many cell processes
nucleus	bean-shaped, eccentrically situated	bean-shaped, eccentrically situated
MHC class II positivity	varying	constitutively
acid phosphatase activity	all over the cytoplasm	spot like next to the nucleus
staining for mAb ED1	all over the cytoplasm	spot like next to the nucleus
purity of the fraction	> 90%	> 85%

Migration to the Parathymic Lymph Nodes

Four days after i.p. administration both peritoneal Mϕ and spleen DC had arrived in some of the parathymic lymph nodes that drain the peritoneal cavity. Most migratory cells had accumulated in one of the several parathymic lymph nodes, few in a second, while the other lymph nodes did not contain any migratory cells. Besides the presence of both migratory Mϕ and DC in the subcapsular sinus, the migratory cell types populated different areas of the parathymic lymph nodes. Peritoneal Mϕ were present in the follicles and in the medulla (Fig. 1A). Spleen DC were present in the paracortical area (Fig. 1B).

After administration of PBS no staining for the mAb His 41 was observed in the parathymic lymph nodes.

CONCLUDING REMARKS

In this study we introduce a congenic rat model to study the migratory properties of Mϕ and DC. With a mAb against the $RT7^b$ leucocyte common antigen (CD45) very small numbers of migratory cells ($1x10^6$ injected cells) could be detected.

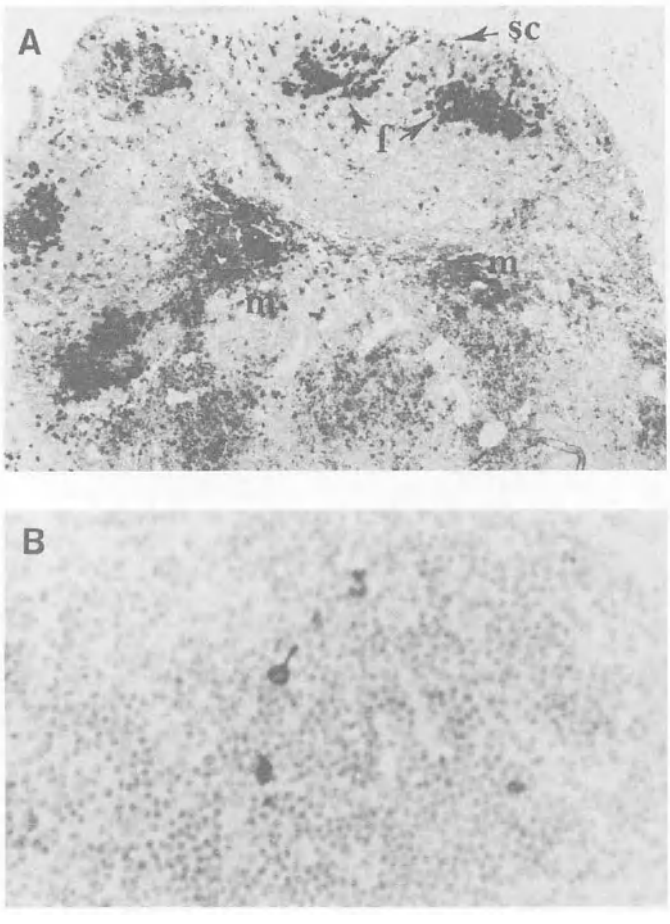

Figure 1. Presence of migratory peritoneal Mϕ and spleen DC in the parathymic lymph nodes 4 days after i.p. administration of the cells, as demonstrated by APAAP-staining of cryostat sections. (A) Overview of one of several parathymic lymph nodes containing peritoneal Mϕ. Migratory Mϕ are present in the subcapsular sinus (sc), follicles (f) and medulla (m). (B) Detail of the paracortex of a parathymic lymph node demonstrating DC with long cell processes amongst the T cells.

DC injected into the peritoneal cavity migrate to the paracorical area of the parathymic lymph nodes. This is in agreement with earlier studies demonstrating the migration of DC injected into the footpad to the paracortex of the draining popliteal lymph nodes (10). Furthermore, DC have shown to be capable of priming T cell in the draining lymph node in vivo (11).

Peritoneal Mϕ do not migrate to the paracortical area of the draining lymph nodes but migrate to the subcapsular sinus, the medulla and into the follicles. The migration of Mϕ to the subcapsular sinus and the medulla are in agreement with earlier studies (12).

However, the presence of i.p. injected Mϕ in the follicles of the draining lymph nodes only 4 days later was suprisingly. Possibly, these migratory Mϕ become tingible body Mϕ.

In a detailed kinetic study the migratory properties of Mϕ will be compared with those of DC. First the migration of both cell types from the footpad to the popliteal lymph node will be compared. Using the peritoneal cavity as the injection site it is also possible to study the kinetics of these antigen presenting cell types not only to the draining lymph nodes, but also at the site of inflammation. Finally, other lymphoid tissues as well as the omentum will be included in this study.

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IN VITRO MIGRATION CAPACITY OF EPIDERMAL LANGERHANS CELLS

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INTRODUCTION

Epidermal Langerhans cells (LC) reside in the suprabasal portion of squamous epithelia, and function as potent antigen presenting cells[1] Previous studies[2-4] showed that epicutaneous hapten application induces a significant accumulation of antigen-bearing dendritic cells in the regional lymph nodes This suggests that LC are capable of migrating, and probably carrying foreign antigens from the epidermis to the lymph nodes in order to present these antigens to resting T cell However, the precise mechanism of this migration is far from being clear

In this study, we performed two *in vitro* migration assays to study some of the characteristics of LC as migrating cells One of the assays is the invasion assay (Matrigel assay), and we examined the capacity of LC for migration through a reconstituted basement membrane The other one is the phagokinetic track assay, and we examined the capacity of LC for random migration on various extracellular matrix (ECM)

MATERIALS AND METHODS

Epidermal cells were prepared from human breast and abdominal skins by trypsinization, and LC (CD1a positive cells) were enriched by density centrifugation using Lymphoprep 6-20% LC-enriched suspensions were used for the invasion assay, and more than 85% LC-enriched suspensions were used for the phagokinetic track assay In order to study the effect of hapten on LC migration, these LC suspensions were incubated with Trinitrobenzenesulfonic acid (TNBS) and FITC, or SLS for 10min at 37°C

Cell invasion ability was assessed by using a modified Boyden chamber with two membranes[5] Briefly, the upper membrane (13mm-diameter polycarbonate filter) was coated with 50μg of Matrigel, and the second membrane (13mm-diameter cellulose nitrate filter) was used to capture migrating cells Fibroblast-conditioned medium prepared from human dermal fibroblasts was used as a source of chemoattractants The chambers were incubated for 20h at 37°C At the end of the incubation, the second membrane (cellulose nitrate filter) was removed, fixed, and HLA-DR positive cells on the upper surface of this membrane were counted

The random migration of LC was assessed by a modification of the method of Albrecht-Buehler[6] and O'Keefe et al [7] Colloidal gold-coated coverslips were prepared and were placed

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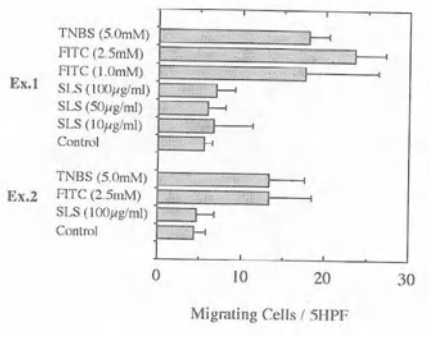

Figure 1. Effects of TNBS, FITC and SLS on LC invasion through Matrigel.

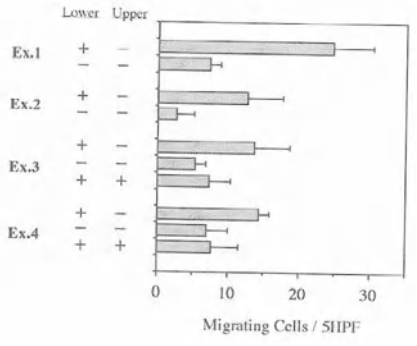

Figure 2. Effect of fibroblast conditioned medium on LC invasion through Matrigel.

in 24-well culture plate. They were then coated with type I and type IV collagen (COLL), fibronectin (FN) or laminin (LM). Highly enriched LC suspensions were inoculated on each well, and were cultured for 20h at 37°C. Cultures were then fixed, and the migration tracks were observed and were quantified by an image analyzer. The area occupied by migration tracks in the high power field was calculated, and this value was termed the migration index.

RESULTS

The invasion assay revealed that LC were capable of migrating through the Matrigel-coated membrane. As shown in Figure 1, *in vitro* TNBS and FITC treatments significantly increased in LC invasion. By contrast, SLS treatment did not induce an increased invasion. The number of migrating cells decreased in the absence of fibroblast conditioned medium. Moreover, it also decreased when this medium was placed in both the upper and lower compartments (Figure 2). This means that fibroblast conditioned medium is likely to stimulate chemotaxis of LC rather than their chemokinesis.

In vitro hapten treatment also induced a marked increase in LC random migration on several ECM. As shown in Figure 3, the migration index of hapten-treated LC on type IV COLL significantly increased. By contrast, SLS treatment did not show such an effect. We confirmed that migrating cells on type IV COLL expressed both CD1a and HLA-DR (data not shown), indicating that hapten-induced cell migration is that of LC. Moreover, when we used LC-depleted epidermal cell suspensions, hapten treatment did not increase in cell migration (data not shown). Figure 4 gives the migration index of TNBS-treated LC on various ECM. LC migration on type I COLL and FN were also markedly increased. By contrast, little migration was observed on LM nor in the absence of ECM.

DISCUSSION

The present study revealed that ECM plays some important roles in regulating LC migration. This conclusion is based on the finding that hapten-modified LC were capable of migrating on type I COLL, type IV COLL and FN, but not on LM nor in the absence of ECM. We previously found that LC express several β1 integrins[8,9], and α5β1 and α6β1 function as FN receptor and LM receptor of LC respectively[10]. These data let us speculate that LC get in contact with various ECM via integrins, and this allows LC to reside in the epidermis, and also function as one of the driving mechanics for migration through ECM-rich components, such as the basement membrane and the dermal connective tissue.

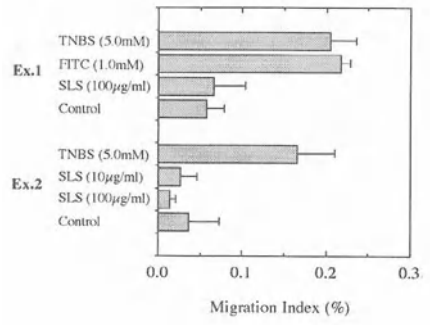

Figure 3. Effects of haptens and SLS on LC random migration on type IV COLL (25μg/ml).

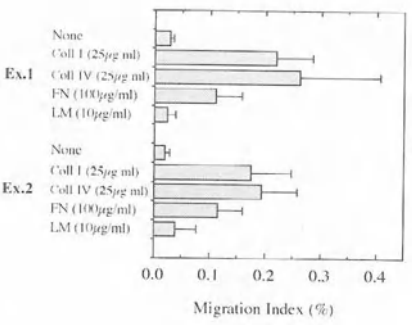

Figure 4. Effects of type I and type IV COLL, FN and LM on TNBS-treated LC migration.

The present study also revealed that *in vitro* hapten treatment stimulated both the chemotactic invasion and the random migration of LC. Moulon et al.[11] recently showed that this *in vitro* incubation of freshly isolated LC with haptens allows LC to prime hapten-specific T cell. Our present data suggest that this *in vitro* contact between LC and haptens also could be one of the triggers to initiate and/or stimulate LC migration. In addition, we found that fibroblast conditioned medium prepared from human dermal fibroblasts stimulated LC chemotactic invasion, suggesting that some soluble factors derived from the dermis (fibroblasts) could function as chemoattractants and may regulate the direction of LC migration.

LC migration seems to be regulated by many factors, such as ECM, some soluble factors (including cytokines) derived from the dermis and probably from the epidermis as well, and adhesion molecules expression on LC themselves. Our *in vitro* methods described here must be one of the useful tools to indicate the role of these factors on LC migration.

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EPIDERMAL LANGERHANS CELL MIGRATION : SIGNALS AND MECHANISMS

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INTRODUCTION

Following skin sensitization a proportion of local Langerhans cells (LC) are stimulated to migrate from the epidermis via afferent lymphatics to draining lymph nodes[1]. We have reported recently that tumour necrosis factor α (TNF-α), a keratinocyte-derived cytokine, may be an important mediator of this process and have proposed that the induced production, or increased production, of TNF-α by keratinocytes in response to skin sensitization might provide an initial stimulus for the movement of LC from the epidermis[2,3]. In support of this is the demonstration that keratinocyte mRNA for TNF-α is upregulated following topical exposure of mice to skin-sensitizing chemicals[4]. To investigate further the requirement for TNF-α in the movement of LC from the skin to draining lymph nodes we have examined the influence of a neutralizing anti-TNF-α antibody on this process.

Also of interest are the nature of the changes induced in LC following receipt of the stimulus to migrate. Altered expression of adhesion molecules by LC, or surrounding cells, may play a role in this process. Accordingly, it has been proposed that expression by epidermal LC and keratinocytes of the homophillic adhesion molecule E-cadherin is necessary for the retention of LC within normal epidermis[5]. We have examined the expression of E-cadherin in normal murine epidermis and by lymph node dendritic cells (DC).

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EXPERIMENTAL

We have reported previously that intradermal exposure of mice to homologous recombinant TNF-α results in the accumulation of DC in draining lymph nodes[2] and the loss of a proportion of LC (approximately 25%) from the epidermis[3]. The kinetics of these responses are compared in Figure 1. Intradermal injection of TNF-α was found to induce a rapid reduction (within 30 minutes of exposure) in the frequency of Ia^+ LC in the epidermis local to the site of injection (Figure 1a). A subsequent increase in draining lymph node DC numbers was observed as early as 2 hours following administration of TNF-α, with further increases being detected at 4 hours (Figure 1b). Similar treatment of mice with 30μg of bovine serum albumin, in which the TNF-α was suspended, failed to influence either epidermal LC frequency or lymph node DC numbers compared with untreated animals (data not shown). These results indicate that TNF-α can provide a signal for the migration of LC from the skin to draining lymph nodes. The rapid kinetics of responses to intradermally administered TNF-α are consistent with the proposal that LC migration following skin sensitization results from the production by keratinocytes of TNF-α.

To investigate further the requirement for TNF-α in LC migration, we have examined whether it is possible to inhibit the accumulation of DC in draining lymph nodes following topical sensitization with a neutralizing anti-TNF-α antibody. Administration to mice of a polyclonal rabbit anti-mouse TNF-α antibody (ip) 2 hours prior to sensitization on the dorsum of both ears with 1% oxazolone resulted in a marked inhibition (approximately 85%) of allergen-induced DC accumulation in draining (auricular) lymph nodes measured 18 hours later. Treatment with normal rabbit serum was without effect. Skin irritation has been reported also to cause increased synthesis of TNF-α by keratinocytes[4] and the accumulation of DC in draining lymph nodes[6]. Prior

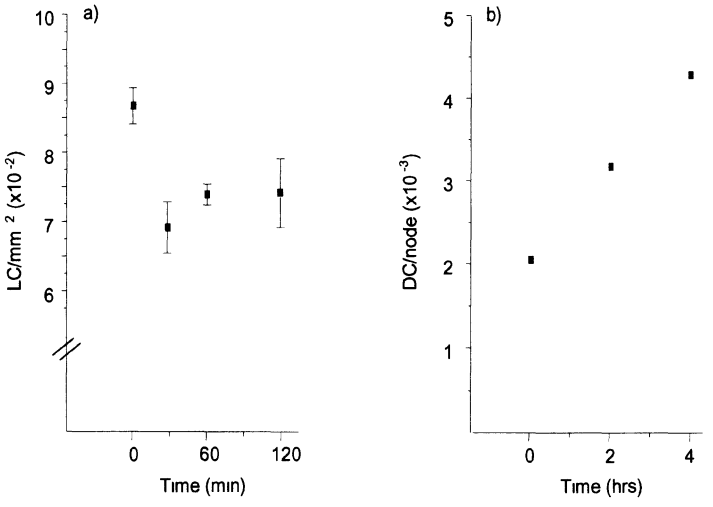

Figure 1. Influence of TNF-α on (a) epidermal LC frequency and (b) DC accumulation in draining lymph nodes. Groups of BALB/c mice received 30μl intradermal injections into both ear pinnae of 50ng murine TNF-α in 0.1% BSA. Control mice were untreated or received 0.1% BSA alone. (a) LC frequency was assessed following indirect immunfluorescence staining of epidermal sheets for Ia expression (LC/mm^2 ± SE)[3]. (b) The frequency of DC in low buoyant density fractions of draining lymph node cell suspensions was assessed by direct morphological examination[2].

administration to mice of anti-TNF-α served to inhibit (by 99%), the accumulation of DC in draining lymph nodes examined 18 hours following exposure to the skin irritant sodium lauryl sulphate. The conclusion drawn is that various types of cutaneous trauma will result in local production of TNF-α by keratinocytes and that, under certain circumstances, this cytokine stimulates the migration of LC from the skin to draining lymph nodes.

One question that remains is the nature of the changes induced in responsive LC by TNF-α that facilitate migration from the epidermis. That altered expression of E-cadherin may be involved is suggested by the fact that cultured LC express lower levels of E-cadherin compared with freshly isolated LC, and bind to keratinocytes with reduced avidity[5]. Using a biotin-streptavidin-peroxidase staining technique, in conjunction with a monoclonal antibody directed against murine E-cadherin (ECCD-2 : rat IgG1) we have confirmed in epidermal sheets prepared from the ears of naive BALB/c mice, that both LC and keratinocytes express E-cadherin with LC displaying relatively high levels of this molecule. Expression of this molecule was restricted to epidermis and, in frozen skin sections, cells within the dermis, including dermal DC, exhibited undetectable levels of E-cadherin. Under conditions where LC expressed high levels of this molecule, DC isolated from the lymph nodes of sensitized or non-sensitized mice, failed to express measurable E-cadherin. It is possible, therefore, that following receipt of the signal to migrate, loss of E-cadherin expression by LC will facilitate the release of LC from keratinocytes prior to their migration from the epidermis.

CONCLUSION

In summary, it is proposed that TNF-α provides an important signal for LC migration from the skin and that this cytokine may be an important mediator of cutaneous immune responses. The mechanisms of LC migration may involve altered expression of adhesion molecules, such as E-cadherin, by LC themselves and/or by adjacent keratinocytes. The possibility that TNF-α mediates such changes is being investigated currently.

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POPULATION DYNAMICS AND FUNCTIONS OF RESPIRATORY TRACT DENDRITIC CELLS IN THE RAT

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INTRODUCTION

The epithelial surfaces of the respiratory tract are continuously exposed to environmental antigens, and the maintenance of immunological homeostasis in this vital organ system requires efficient local mechanism for antigen surveillance, both in the steady state and during episodes of inflammatory stimulation.

Recent work from a number of laboratories (as reviewed recently[1,2]) have highlighted the important role of populations of Dendritic Cells (DC) in the airway wall and peripheral lung, in this surveillance process.

The review below focusses upon recent data relevant to the functional and surface phenotype and turnover of these DC populations in the rat model, and their responsiveness to inhaled inflammatory stimuli.

LUNG DENDRITIC CELL POPULATIONS IN NORMAL ADULT ANIMALS

Distribution

Normal adult rats display discrete populations of DC at different levels of the respiratory tree. The cells are readily recognized in frozen tissue sections on the basis of strong immunostaining for class II MHC (Ia), lack of staining for markers defining mature tissue macrophages, and characteristic dendriform morphology.

In the upper respiratory tract, DC appear at regularly spaced intervals in stained frozen sections of nasal mucosal tissue, and are particularly prominent at the base of the nasal turbinates.[3] A second population has recently been described within the epithelium lining the conducting airways, closely associated with the underlying epithelial basement membrane.[4,5] The application of a technique which was developed specifically for sectioning isolated airway segments through the epithelium and parallel to the underlying basement membrane,[6] demonstrates that the distribution of this population is as an evenly spaced "network", analogous to the Langerhans cells of the epidermis.[6,7] In normal adult rats, the intraepithelial density of these DC is inversely related to airway diameter, ranging from 500-800 per mm^2 in the large airways such as the trachea, down to <100 per mm^2 in the small bronchioles.[7,8] By comparison, the staining pattern observed with sections from small human bronchiole segments[9] appears to closely resemble that of large airways in the rodent.

In the peripheral lung, a third population of DC has been described in alveolar septal

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walls.[4,5,7,10] The distribution of these is again not random, the majority appearing within the connective tissue at interseptal "junctional" zones, where adjacent alveolar septal walls meet.[7] In addition, a fourth DC population has been documented amongst the "free" cells present in epithelial lining fluids overlying the alveolar surface; these comprise in the order of 0.1% of the cell population harvested by the bronchoalveolar lavage (BAL) technique from normal rats,[11] and similar figures have been reported for human.[12]

Surface phenotype

Ia expression on DC in adult animals is generally held to be constitutive, and accordingly surface phenotypic analyses of these cells *in situ* generally rely upon the use of dual colour immunohistochemical procedures employing anti-Ia as one of the MoAb pairs, at least for initial population characterisation.

Employing this approach, the two major DC populations in the rat respiratory tract, designated airway (ADC) and parenchymal lung DC (LDC) respectively, have been characterised in detail. These populations are readily distinguishable from macrophages on the basis of their failure to express the ED2[13] and RMA[14] markers, while expressing variable levels of the markers defined by ED4 and ED9.[13] The majority of the ADC express the pan-macrophage marker ED1 whereas LDC do not,[7,13] and in contrast, expression of ICAM-1,[8] CD44,[5] and CD11a[4,7] is more pronounced on LDC. Additionally, IgG-FcR expression is seen on up to 50% of LDC, but is absent from the LDC population.[8] Collectively, these findings suggest different baseline levels of activation or differentiation in the two populations, a conclusion reinforced by further observations below.

More recent studies from our laboratory have also defined a subpopulation of airway intraepithelial DC which stain with the MoAb Ox62 (anti-subsets of Tγ/δ cells and DC), but which are CD3$^-$ (and hence not Tγ/δ) and also Ia$^-$. This population is predominant in neonates (see below), and comprises in the order of 25-30% of resident airway intraepithelial DC in normal adult rats.[3]

Antigen presenting cell (APC) function

DC from both the airway epithelium and lung parenchyma have been demonstrated to acquire inhaled antigen *in situ*, and to subsequently present it in immunogenic form to primed T-cells *in vitro*.[5,13,15] Additionally, respiratory tract DC function as effective accessory cells in mitogen(15) or MLR[8,15] assays *in vitro*. However, systematic analysis of the APC functions of these cells revealed that their capacity to express APC activity *in vitro* required an initial pre-incubation phase, either subsequent to or during the isolation procedure - if they were tested immediately after single-step isolations which took 4 hours or less to complete, they functioned poorly, but APC activity upregulated on a log-scale if they were held in culture (especially in the presence of GM-CSF) for 24 hrs or longer.[13,15] Additionally, the direct expression of APC activity by lung DC in T-cell cultures, or their functional maturation during pre-culture, was inhibited by the presence of lung-derived macrophages (in particular mature Alveolar Macrophages - AM),[5,15] and this macrophage-mediated inhibition could be prevented via blocking of the nitric oxide synthase pathway.[15] This suggests that, analogous to other peripheral tissue DC populations such as epidermal LC,[16] respiratory tract DC are specialised for acquisition and processing of antigen, but are normally restrained from presenting these antigens effectively to T-cells until they migrate into an environment conducive to their functional maturation,[15] in particular regional lymph nodes. It also appears that this restraint involves more than simply the lack of (GM-CSF) maturation signals, and includes the presence of local "suppressive" factors (including NO from adjacent macrophages) which actively inhibit their differentiation.[15] Consistent with this observation, selective suppression of AM functions *in vivo* leads to upregulation of the APC activity of lung DC, and "hyperresponsiveness" to inhaled antigens.[15,17]

Turnover of respiratory tract DC populations in the steady state

Recent studies from our lab have sought to determine the basal rate of turnover of lung (especially airway) DC populations in SPF rats. These experiments employed a radiation chimera model involving congenic rats expressing allotypic variants of CD45 which are recognisable by appropriate MoAbs, permitting parallel quantitation of "waning" host airway

DC and "waxing" donor DC, following x-irradiation and reconstitution with congenic bone marrow. Thus, x-irradiation (1,000 rads) triggers a decline of 85% in the resident tracheal epithelial DC population within 2 days which further declines to <10% original levels by day 7, in the absence of bone-marrow reconstitution.[18] Following graft establishment, incoming congenic DC repopulate the airway epithelium over a 3-5 day period.[18] These experiments indicate that the half-life of the airway DC population is normally in the order of 36 hrs; parallel experiments supplied half-life estimates of >72 hrs and >6 days respectively for parenchymal lung DC and epidermal LC in the same animals.[18]

This extremely rapid turnover time for rat airway DC is rivalled only by the population from the gut wall,[19] and these observations are suggestive of an important role for DC in antigen surveillance at the "front-line" mucosal surfaces.[18]

POPULATION DYNAMICS OF RESPIRATORY TRACT DC DURING INFLAMMATION

Changes in surface phenotype

Recent studies in our laboratory have compared surface expression of a variety of markers on normal airway epithelial DC, with those in animals acutely and chronically exposed to "pro-inflammatory" dusts. Airway DC in chronically exposed animals display marked physical changes, notably increase in overall size together with increased length and degree of branching of processes, and more intense immunostaining for Ia;[7] in addition, increases in both the percentage of positive staining (and intensity thereof) was noted for the β-chain of CD11a/CD18, and the differentiation markers defined by MoAbs Ox41, ED8, and ED9.[7] Comparable changes were seen in acute inflammation induced by bacterial LPS.[7]

Recruitment of airway DC during acute inflammation

Exposure of rats to aerosolised bacterial LPS triggered a transient increase in airway intraepithelial DC numbers, which peaked (at levels up to 50% above controls) 24 hrs later.[7] In more detailed follow-up studies employing inhalation of a heat-killed suspension of the more powerful pro-inflammatory organism *Moraxella catarrhalis*, it was demonstrated that the acute granulocytic response was mirrored by a parallel recruitment to the airway epithelium of large numbers of DC, which over the 2-24 hr period of the acute response were maintained at levels equivalent to up to x3 fold above baseline.[20] The available evidence suggests that the majority of these cells rapidly cycle through the inflamed epithelium, carrying a "snapshot" of the local (antigenic) environment to the T-cell system in the regional lymph nodes.[20]

Recent reports[11] also indicate that instillation of inflammatory stimuli directly into the alveolar spaces triggers a small but significant influx of DC onto the alveolar surface, which also appear to subsequently "home" to regional lymph nodes.[21]

AIRWAY AND LUNG DC IN THE NEONATE

Recent reports have documented the gradual postnatal accumulation of Ia+ DC in both the lung wall[22] and the airway epithelium.[3] It is evident that "seeding" of the Ia- precursors of these populations commences in the fetus, and postnatal "maturation" of the DC networks throughout the respiratory tree involves both the recruitment of new precursors, as well as the progressive up-regulation of Ia expression on resident cells.[3] This upregulation of Ia expression occurs most rapidly at sites of maximal stimulation from inhaled agents, notably at the base of the nasal turbinates,[3] and progresses steadily down the respiratory tree, presumably "driven" by inhaled inflammatory stimuli and the cytokines generated by the latter.[3,10]

It is of interest to note that at the same time that Ia expression on neonatal airway DC is low-weak, corresponding expression on epidermal LC or nasal epithelial DC in the same animals is extremely strong.[3] This infers either that the genes regulating Ia expression on neonatal DC are not constitutively "switched on" and require specific local inductive signals (e.g. cytokines) from the tissue environment into which they migrate, or alternatively that

different tissues in the neonate produce varying levels of inhibitors of Ia gene expression which are able to downmodulate surface Ia expression on incoming DC.[3]

CONCLUSIONS AND SPECULATION

The information provided above from studies on the rat permit formulation of the following general model to describe the functioning of the DC networks in the respiratory tract:

- under normal steady-state conditions DC form a contiguous network throughout epithelial surfaces of the respiratory tract, their density reflecting the intensity of local stimulation from inhaled irritants;
- their prime function is to sample incoming antigens, for presentation to the T-cell system after their subsequent migration to draining lymph nodes;
- during their sojourn in the epithelium, they are (normally) actively prevented from maturation into competent APCs by signals from adjacent macrophages; this is a host-protective mechanism to limit the frequency of potentially damaging T-cell activation events within delicate respiratory epithelial tissues;
- under inflammatory stress, the DC networks have the capacity for rapid and (if necessary) sustained upregulation, presumably to increase the efficiency of local antigen surveillance.

It may be speculated that any significant deterioration in the fine control of the functions of these dynamic DC networks could have major implications in relation to local immunological homeostasis, and such changes may constitute aetiologic factors in a variety of inflammatory diseases of the respiratory tract, in particular those which include excessive local T-cell activation in disease pathogenesis.

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REGULATION OF THE HUMORAL RESPONSE BY THE ANTIGEN-PRESENTING CELLS *IN VIVO*

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The specificity of the immune response is determined by the interaction of the T cell receptor with its appropriate ligand, i.e. the antigenic peptide in the context of the MHC complex. More recently, a second signal, the costimulatory signal, has been described, which is required for optimal activation of T cells.

Both signals are provided by specialized cells, called antigen-presenting cells, that include B lymphocytes, macrophages and dendritic cells. Among these populations, only dendritic cells are able to activate antigen-specific, resting T cells *in vitro*.

Murine $CD4^+$ helper cells consist of at least two non overlapping subsets called Th1 and Th2 cells which differ by their lymphokine secretion pattern, and induce distinct effector mechanisms.

The aim of this study was to investigate whether the nature of the antigen-presenting cells present during the first encounter with the antigen could influence the T helper phenotype development *in vivo*.

Several groups of mice were immunized by a single injection of antigen (human gamma globulins, HGG) pulsed *in vitro* on distinct populations of APC. Balb/c mice were injected with 3×10^5 antigen-pulsed splenic dendritic cells, small resting B cells or peritoneal macrophages on day 0, and received 5 μg of HGG 5 days later (in order to activate native antigen-specific B cells). The amplitude and isotypic profile of the primary humoral response were analyzed 8 days later. Figure 1 shows that antigen-pulsed dendritic cells and macrophages, but not resting B cells, induced the secretion of high levels of specific antibodies. Although the level of antibodies is lower in mice immunized with macrophages, as compared to dendritic cells, early after priming, both responses are of same amplitude later in the course of immunization.

Of note, the responses induced by dendritic cells and macrophages display distinct isotypes: antigen-pulsed dendritic cells induce the synthesis of IgG1 and IgG2a antibodies, whereas macrophages promote the production of IgG1 but not of IgG2a antigen-specific antibodies, as previously described (1).

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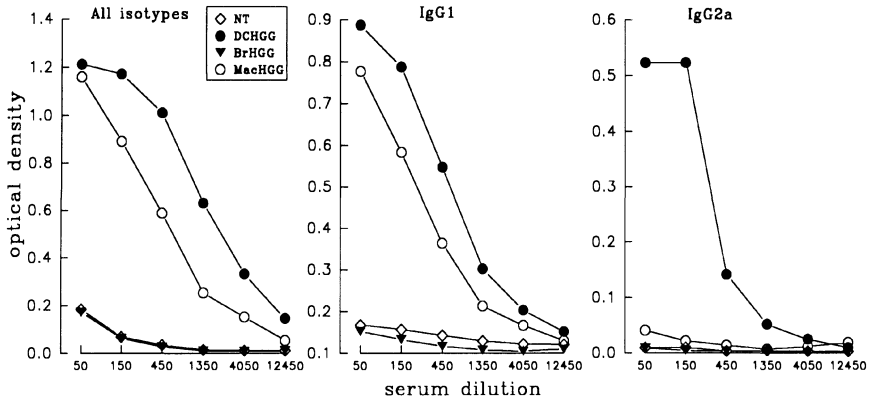

Figure 1: *HGG-pulsed dendritic cells and macrophages, but not resting B cells, induce a primary humoral response in vivo, characterized by distinct isotypic profiles.* BALB/c mice (5 per group) were injected with $3x10^5$ pulsed dendritic cells (DC), macrophages (Mac) or resting B cells (Br, a Sephadex G10 non adherent spleen cells, depleted of T lymphocytes), or left untreated (NT). All mice were boosted with 5µg of HGG in saline 5 days later. The mice were bled 10 days after antigen boost and antigen-specific antibodies of all isotypes, as well as of IgG1 and IgG2a isotypes were measured by ELISA. Each point represents the mean of 5 treated mice.

Since Th1 and Th2-derived lymphokines reciprocally regulate the determination of Ig isotype responses, these data indirectly suggest that T helper cell differentiation can be controlled at the level of antigen presentation. To diretly measure T cell activation *in vivo*, antigen-pulsed dendritic cells were injected into the footpads of naive mice, according to a protocol described by Inaba *et al.* (2). 5 days later, the lymph nodes were harvested, cultured with or without antigen, and the lymphokines analyzed in the supernatants. Our data show that lymph node cells from mice injected with dendritic cells proliferated upon restimulation *in vitro* and secreted high levels of interferon-gamma and IL-2, showing that Th1-type helper cells were activated (data not shown). Experiments are in progress to compare the lymphokine production of lymph node cells from mice immunized with HGG-pulsed macrophages, resting B lymphocytes or dendritic cells.

The observation that resting B cells do not prime mice *in vivo* is in agreement with previous reports, showing that resting B cells do not activate naive T cells *in vitro* or *in vivo*, but rather induce a state of unresponsiveness (3). Therefore, we tested whether the animals injected with HGG-pulsed resting B cells could respond to the same antigen in an immunogenic form. Antigen-pulsed dendritic cells were injected in mice pretreated with unpulsed resting B cells, in animals injected with HGG-pulsed resting B cells and in control animals. All mice received 5 µg of antigen on day 5 and were bled 11 days later.

The data in Figure 2 show that mice injected with HGG-pulsed resting B cells display a decreased humoral response, as compared to control group or even to the group pretreated with resting B cells in the absence of antigen. By contrast, preimmunization of mice with antigen-pulsed dendritic cells strongly enhances the humoral response induced by a second injection of the same cells (data not shown), showing that the diminished response is not due to overstimulation. Thus, the injection of an antigen on resting B cells can induce a state of hyporesponsiveness to the antigen, a phenomenon which could be related to the poor costimulatory activity of these cells (4).

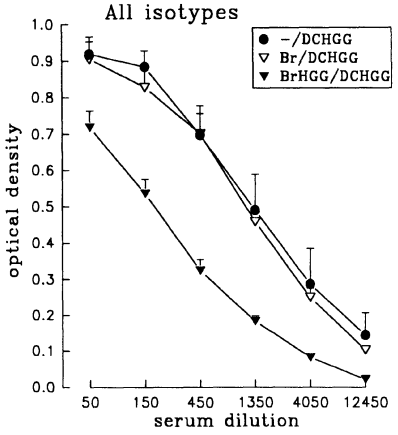

Figure 2: *HGG-pulsed resting B cells induce a state of hyporesponsiveness to the Ag.* 5 BALB/c mice were injected intravenously with 10^6 HGG-pulsed resting B cells (Br), unpulsed Br or were left untreated. 8 days later, all mice received an injection of $3x10^5$ HGG-pulsed dendritic cells (DC) and 5 days later an injection of 5µg of HGG in saline. Mice were bled 11 days later and antigen-specific antibodies of all isotypes were measured in individual sera.

Taken together, our observations suggest that the nature of the cell which presents the antigen to naive T cells regulates the development of the resulting immune response, and in particular of the Th1/Th2 balance. Priming of antigen-specific T cells *in vivo* requires the presentation of the antigen on dendritic cells or macrophages. By contrast, mice primed with an antigen on resting B cells become hyporesponsive to the same antigen presented by professional APC. Of note, this observation may explain the beneficial effect of blood transfusions on allograft survival. The role of costimulatory molecules of B7 family remains unclear (5), although this work shows a correlation between the expression of B7 molecules and the capacity to prime mice. Indeed, peritoneal macrophages express low but significant levels of B7-1 and B7-2, dendritic cells express high levels of both, whereas resting B cells (G10 non adherent) express barely detectable levels of these molecules (1). The induction of Th1 versus Th2-like responses may reflect quantitative differences in the amount of B7-1 and B7-2 on dendritic cells versus macrophages, or alternatively could be determined by differential levels of class II expression, release of soluble factors like IL-12, or expression of other costimulatory molecule(s). Very recently, several reports have shown that B7-1 could have an inhibitory effect on immune responses, suggesting that the differential expression of B7-1 and B7-2 on the APC could influence T helper differentiation (6). The injection of B7-1 or B7-2 specific antibodies in mice primed with antigen-pulsed dendritic cells may help to clarify this point.

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ANTIGEN PRESENTING AND PRIMARY IN VITRO SENSITIZING CAPACITY OF CD1a$^+$ DENDRITIC CELLS GENERATED FROM HUMAN BLOOD

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INTRODUCTION

Dendritic cells (DCs) are highly specialized antigen presenting cells (APCs) initiating primary T-lymphocyte associated immune responses[1]. DCs are located in many non-lymphoid tissues and a specialized form of DCs - the Langerhans cells (LCs) - is found in the skin. Here they fulfill their in vivo functions by capturing antigens in the epidermis and presenting these antigens to T-lymphocytes in a HLA-restricted way[2]. Until now the small numbers of functional active LCs which could be isolated from human skin were the limiting factor in this field of research[3].

For our studies we had developed a simple method to generate CD1a$^+$ HLA-DR$^+$ dendritic LC equivalents from the peripheral blood of healthy human volunteers by supplementing GM-CSF and TNF-α to the medium. After a culture period of 6 days we were able to maximally obtain numbers of 70% CD1a$^+$ HLA-DR$^+$ LC equivalents from human blood. These in vitro generated, CD1a$^+$ enriched DC-populations were used to study their antigen presenting and primary in vitro sensitizing capacities in combination with purified autologous T-lymphocytes.

MATERIAL AND METHODS

Reagents: Culture media (SFM-medium and RPMI 1640) and supplements were purchased from Gibco BRL. All reagents were purchased from Sigma, except ^{3}H-thymidine (Amersham) and monoclonal antibodies (Dianova) except anti-CD1a (Coulter).

Cells: CD1a$^+$ HLA-DR$^+$ enriched DC population were isolated from human blood. Briefly, the monocytic cell fraction was isolated by sequential density gradient centrifugation over Ficoll and Percoll. These cells were incubated non adherently in teflon bags under serumfree conditions in the presence of GM-CSF (100 U./ml) and TNF-α (50 U./ml) for 6 days. Then cells were harvested, and analyzed cytofluorometrically upon HLA-DR and CD1a-expression as well as functionally upon their antigen-presenting capacities. Purified autologous

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T-lymphocytes and allogeneic peripheral blood leukocytes (PBLs) were isolated as described elsewhere[4].

Antigen-presentation-tests: $CD1a^+HLA\text{-}DR$ enriched DCs were seeded at various cell-titers into 96 well plates. Nylon wool purified autologous T-lymphocytes or allogeneic PBLs were added to these cultures in different cell-numbers. The same time the mitogen (ConA or PHA) or the superantigen SEB was added. Control responses were: T-lymphocytes, PBLs and DCs alone; DCs and T-lymphocytes or PBLs; DCs in presence of the above stimuli without T-lymphocytes. After an incubation period of 3 to 4 days cultures were pulsed with ^3H-thymidine and proliferation was determined by liquid scintillation counting. Each culture was set up as triplicate and results are given as mean cpm (SD<10%). Primary in vitro sensitization test: DCs were coupled with the trinitrophenyl-hapten (TNP-) according to the method of Shearer[5]. TNP- or not hapten-modified DCs were cultured with autologous T-lymphocytes and T-lymphocyte proliferation was determined[6].

RESULTS

We have developed a simple method to generate $CD1a^+HLA\text{-}DR^+$ DCs from the peripheral blood of healthy human volunteers. If the culture medium was supplemented with the two cytokines GM-CSF and TNF-α about 40 - 70 % cells of the cultured monocytic cell fraction expressed the phenotype of human LCs.

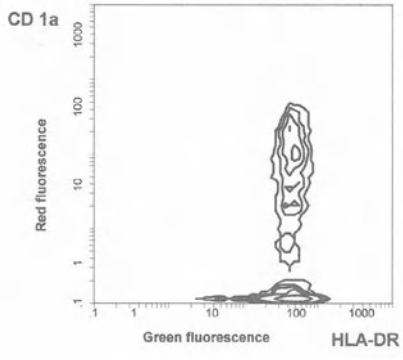

Figure 1. Monocytic cells cultured serumfree in teflon bags for 6 days in the presence of GM-CSF and TNF-α express the cellsurface molekules CD1a and HLA-DR in combination.

These CD1a+ enriched cell population showed enhanced accessory functional activity in the primary allogeneic T-cell response. Beside this activity they also revealed excellent accessory functions in the presence of the two mitogens ConA or PHA towards autologous T-lymphocytes. The antigen presentation of the staphylococcal superantigen SEB was more than ten times increased with regard to controls (fresh monocytes; monocytes cultured without GM-CSF and TNF-α).

Furthermore the primary in vitro sensitizing capacity to naive T-lymphocytes by these cells could also be demonstrated. $CD1a^+$ enriched DCs were coupled with the hapten TNP before they were cocultured with purified autologous T-lymphocytes. After 6 days of cocultivation a primary signifikant T-lymphocytes proliferation could be measured with regard to controls. The grade of this proliferative activity depends on the number of T-lymphocytes as well as the applicated number of hapten modified DCs. In this primary sensitization approach

our DCs developed a potential of antigen presentation according to that of fresh or short time cultured Langerhans cells[7].

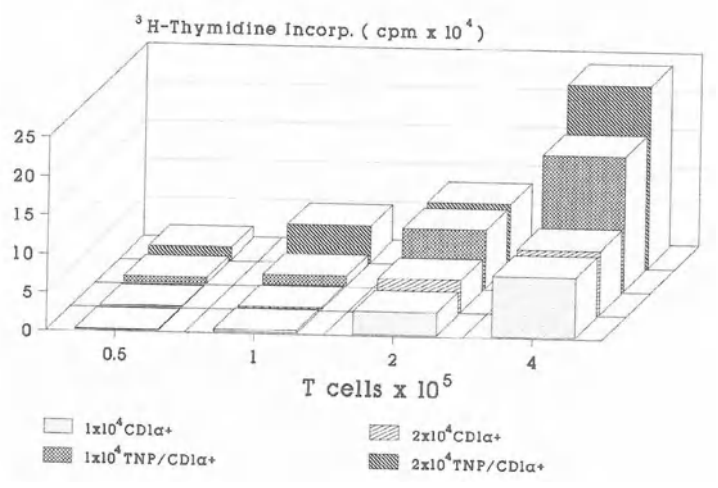

Figure 2. Various cell titers of CD1a$^+$ enriched DCs as APCs were cultured with purified autologous T-lymphocytes for 6 days. In the primary sensitization reaction the CD1a$^+$ DCs were coupled with the hapten TNP (TNP/CD1a$^+$). According to the applicated numbers TNP-coupled DCs we were able to induce a signifikant primary T-lymphocyte activation with regard to the non modified DCs (CD1a+).

In summary our results demonstrate not only the usefulness of in vitro generated CD1a$^+$ DCs in order to study their antigen presenting capacities, but also the possibility for the evaluation of an in vitro approach to study the early steps of T-lymphocyte sensitization in the view of developing a predictive in vitro test for sensitizing substances.

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ANNEXIN EXPRESSION IN HUMAN DENDRITIC CELLS

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INTRODUCTION

Dendritic cells are the most potent antigen-presenting cells for the induction of primary antigen-specific T-cell responses in vitro and in situ (1). The first step in antigen-presentation is endocytosis of the antigen followed by antigen degradation to antigen peptides, binding to the major histocompatibility complex molecule and transport to the cell surface or exocytosis. The endocytotic capacity of dendritic cells has been regarded as minimal, but recent work on dendritic cells from mouse spleen has shown that the fluid phase traffic through late endosomes is as active in spleen dendritic cells as in other types of antigen-presenting cells (2). To further investigate the antigen endocytosis and exocytosis pathway in human peripheral blood dendritic cells we mapped the distribution of annexins in the dendritic cell cytoplasm.

The annexins are a new family of Ca^{2+}-dependent and phospholipid-binding proteins that are structurally related. They have the ability to promote vesicle aggregation and membrane fusion by their binding to membrane phospholipids and are thought to be a major pathway for communication between the cellular membrane and the cytoplasmic environment (3). It has been shown in several systems that members of the annexin family mediate calcium-regulated traffic during endocytosis and exocytosis (4, 5).

MATERIALS AND METHODS

Cell separation: Isolation of blood mononuclear cells was accomplished using a slightly modified method previously described by Freudenthal and Steinman (6). Final low density cell populations contained 50-85% large irregularly shaped cells having a dendritic cell phenotype when analysed using antibodies against CD2/CD19 (DAKO-CD2, MT910, FITC-Conjugated/ DAKO-CD19, HD37, RPE-Conjugated), CD45/CD14 (DAKO-CD45, T29/33, FITC-Conjugated/ DAKO-CD14, TÜK4, RPE-Conjugated) and HLA DR (DAKO-HLA-DR, CR3/43) together with a flow cytometer (EPICS Profile 4235637). The Metrizamide gradient separation was sometimes followed by a 2 hours incubation of the cell suspension with antibodies against CD3, CD14, CD19 and CD56 at 4°C. The cell

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suspension was washed three times and panned on goat-anti-mouse coated petri dishes for 30 minutes on ice, and non-adherent cells washed away and examined as above.

Immunofluorescence staining of annexins: Cytocentrifuge preparations of dendritic cells were labelled with rabbit polyclonal antibody against purified annexin proteins isolated from human neutrophils (annexin I, IV, V and VI diluted 1: 25 and annexin III diluted 1:10) following standard procedures.

Antigen endocytosis and exocytosis: Dendritic cells were pulsed for 6, 30 or 60 minutes with 0.33 mg/ml dinitrophenyl (DNP)-FITC-albumin (DNP/albumin molecular ratio 5,8 and FITC/albumin molecular ratio 5,8) at 37°C, endocytosis was stopped simultaneously by adding excess of 4°C RPMI 1640. All cells were washed three times by centrifugation and then reincubated at 37°C for 0, 6, 30 or 60 minutes to initiate exocytosis (transport to the surface bound to major histocompatibility complex class II antigen). Exocytosis was stopped in the same way as endocytosis. Cytocentrifuge preparations were made during all stages of endocytosis and exocytosis. The slides with DNP-FITC-albumin endocytosed and/or exocytosed dendritic cells were stained with annexin antibodies as above.

Confocal microscopy: The slides were examined in a confocal fluorescent imaging system, the Phoibos 1000 microscope (Molecular Dynamics, Sunnyvale, CA) with a 100x (numerical aperture = 1.4) objective. This technique and image space software allow colocalization assessment of adjacent structures within a 0.2 μm range.

RESULTS

Human dendritic cells were stained with polyclonal antibodies against different annexins (I, III, IV, V and VI) to see whether dendritic cells express annexins and where the different annexin types were located in the dendritic cells. Dendritic cells express annexins I, III, IV; V and VI, but with different distribution patterns and strength. Dendritic cell bodies are stained by all annexin antibodies, but only antibodies against I and VI stain the dendrites. Annexin I and VI seem to be connected to the cytoskeleton and outline the plasma membrane, besides being distributed in the cytosol. Dendritic cells which have endocytosed DNP-FITC-albumin show an intimate association between endosomes containing antigen and those containing annexin V (Figure. 1).

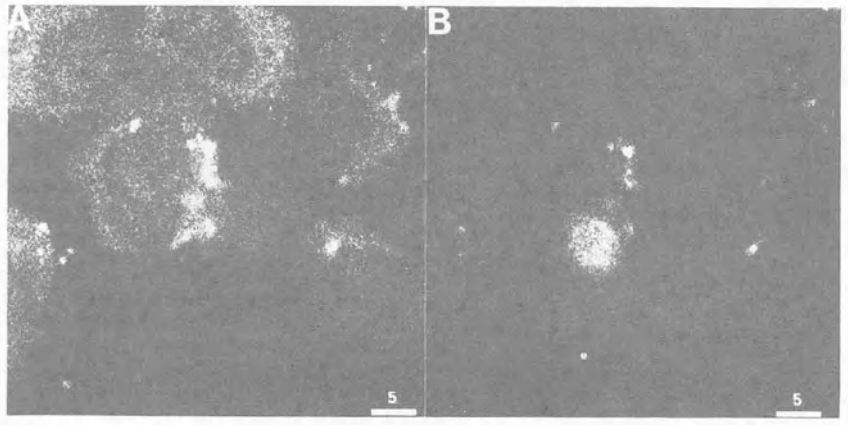

Figure 1. Confocal images (A and B) showing the same section in dendritic cells negatively selected from human peripheral blood. The dendritic cells were incubated for 6 min with 0.33 mg/ml DNP-FITC-albumin at 37°C and 30 min reincubation at 37°C and then fixed and stained for annexin V. (A) Confocal image showing the annexin V distribution in dendritic cells. (B) Confocal image showing the endocytosed DNP-FITC-albumin in dendritic cells. Bars, 0.5 μm.

DISCUSSION

The results of these studies show that there is a colocalisation between endosomes and/or exosomes and annexin V in human peripheral blood dendritic cells. Annexin V has previously been shown to be involved in the formation of aggregates of endocytosed *Chlamydia trachomatis* in cultured HeLa and McCoy cells (4). Previous studies of mouse spleen dendritic cells or Langerhans cells have used long pulses of antigen that cause the antigen to accumulate in the terminal degradative organelles rather than be involved in traffic through late endosomes and the related processing organelles (7, 8). However Levine and Chain showed that fluid phase endocytosis in dendritic cells is as active as in other antigen-presenting cells and that pathways other than the lysosomal one are operative (2). Furthermore peripheral blood dendritic cells concentrate HLA DR molecules in an intracellular compartment of small vesicles in a juxtanuclear position where acid phosphatase activity is found (9). If the HLA DR storage vesicles of dendritic cells can be delivered to the endocytic route, as in B cell lines, HLA DR should play a significant role in antigen presentation (10).

In our studies using an antigen concentration of 0.33 mg/ml and pulsing for short periods annexin V was found to colocalise with the vesicles containing endocytosed DNP-FITC-albumin. Detection of annexin V thus provides a candidate for further studing the endocytotic activity of dendritic cells providing more physiological information concerning the route of antigen-handling in dendritic cells.

ACKNOWLEDGEMENTS

The authors wish to thank miss P Nybom for help with the Phoibos instrument.

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SPLEEN ACCESSORY CELL ANTIGEN PROCESSING AND *IN VITRO* INDUCTION OF SPECIFIC LYMPHOCYTE PROLIFERATION IN BALB/c MICE INFESTED WITH NYMPHAL *Ixodes ricinus* TICKS

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INTRODUCTION

Ticks are hematophagous organisms that transmit numerous human and veterinary diseases. They also induce an anti tick immune response during their blood meal. Thus, rabbits repeatedly infested with *I. ricinus* nymphs and adults produce IgG antibodies reacting against some salivary gland and integumental proteins from female ticks[1]. In contrast, experimental infestations of BALB/c mice with nymphal *I. ricinus* do not generate anti tick antibodies (IgG) or only occasionally. However mice immunized with salivary gland extract (SGE) develop antibodies that recognize tick integumental and salivary gland proteins (Fig 1).

In this work we attempted to establish the ability of T lymphocytes from tick infested mice to respond *in vitro* to tick SGE and integumental extracts (IE), after spleen accessory cells (SAC) antigen processing.

MATERIALS AND METHODS

Animals and infestations

Eight to 12 weeks old BALB/c mice purchased from IFFA CREDO (Arbresle, FRANCE) were used for these experiments. Mice were infested with 15 nymphal *I. ricinus* ticks each. Ticks were reared in our laboratory.

Tick protein extracts

Integuments and salivary glands were dissected from partially fed female ticks. Antigenic extracts were prepared following the procedures previously described[1]. Protein concentration was determinated by the Coomassie blue method. SGE and IE were finally sterilized through a 0.2 µm millipore filter and stored at -20°C until use.

Antigen processing test

A pool of SAC were obtained from 5 BALB/c mice killed 9 days after infestation. $5x10^5$ spleen cells (red blood cells free) per well were incubated in a 96-well flat bottomed plate at 37°C in a 5% CO_2 saturated atmosphere. Two hours later non adherent cells were removed and the wells washed three times with 10% FCS in PBS (pH 7.4). Adherent cells were incubated 20 min with or without chloroquine (0.25 mM/well). Tick extracts (20 µg/well) were added and adherent cells were incubated for an additional three and half hours. In control wells, only culture medium was added. Each well was washed four times. $4x10^5$ lymph node cells of these same infested mice were added. After 96 hours incubation, 0.1 µCi/well of [^{3}H] thymidine was added. The uptake of tritiated thymidine was determinated 24 hours later by liquid scintillation counting.

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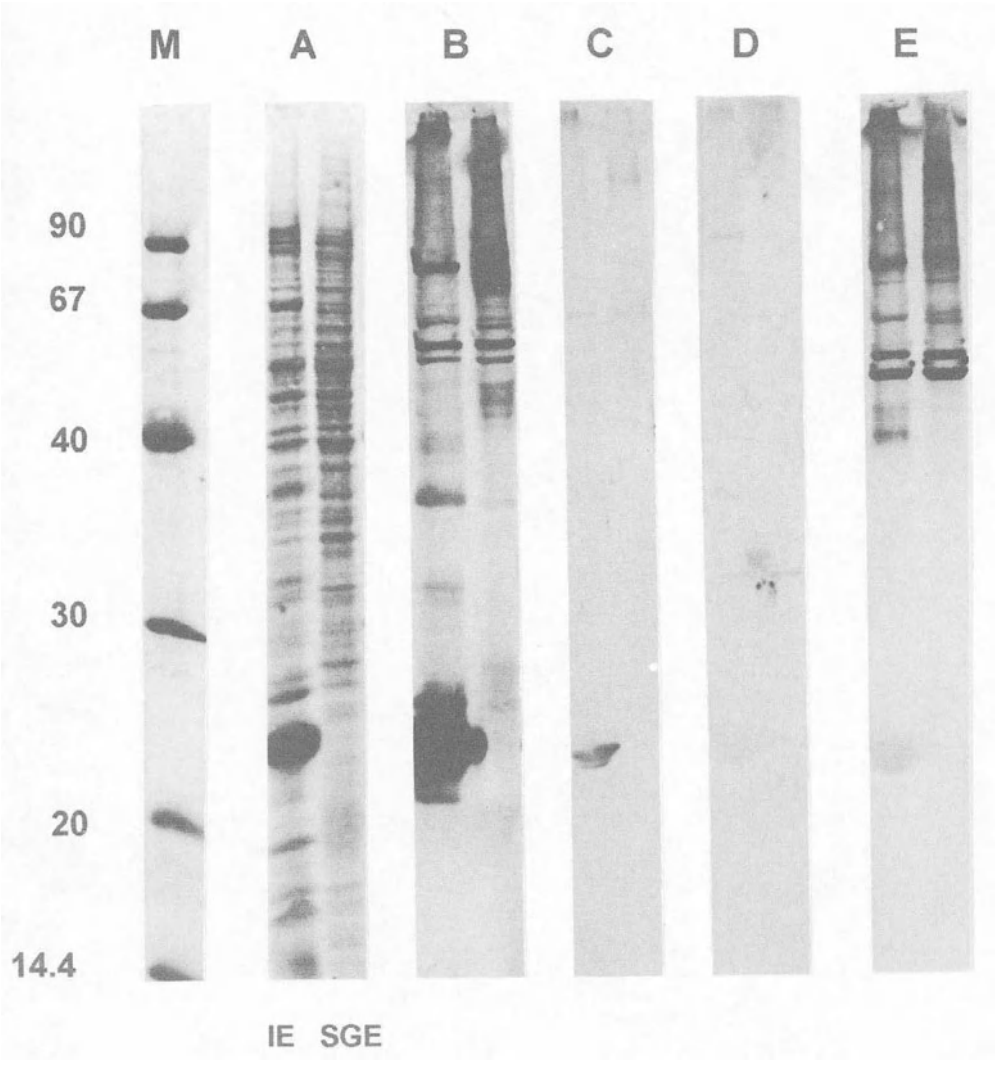

Figure 1. Integument or salivary gland extracts from partially fed females were separated on SDS-PAGE 12%, transferred on nitrocellulose paper and stained with colloïdal gold (panel A). Immunoblot probed with sera of pluriinfested rabbits with adults (panel B) or by sera of mice puriinfested with nymphs (panels C and D) show that mice do not generate anti-tick antibodies or only occasionally. In contrast, mice immunized with salivary gland extract develop antibodies which recognize tick integumental as well as salivary gland proteins (panel E). M: molecular weight markers of 90, 67, 40, 30, 20, and14.4 kDa. IE: integumental extract. SGE: salivary gland extract.

RESULTS AND DISCUSSION

Table 1: BALB/c mice infested with nymphal *I. ricinus* ticks: Chloroquine effect on *in vitro* stimulation of lymph node cells by mice spleen accessory cells pulsed with tick extracts.

	Uptake of tritiated thymidine (mean cpm of quadruplicate ± SD)	
	Chloroquine 0.25 mM	Without Chloroquine
Controls (culture medium)	5417 ± 1241	4648 ± 1602
SGE (20µg/well)	4794 ± 1547	14443 ± 2675 *
IE (20µg/well)	4681 ± 2745	4066 ± 2913

(*) Signigicant difference using Student's t-test ($p<0.01$)

SGE but not IE is able to stimulate T lymphocytes from mice infested with ticks. In this assay, spleen adherent cells from infested mice process SGE immunogens. Pretreatment of accessory cells with chloroquine abolishes the *in vitro* stimulation of lymph node cells.

Nine days after tick fixation, SAC process and present salivary gland antigens with efficiency to lymphocytes from draining lymph nodes near tick fixation sites. Treatment of SAC with chloroquine fully inhibits this phenomenon. Under the same conditions but pulsed with IE, lymphocytes are not stimulated. Our observations suggest that, during tick feeding, mice antigen presenting cells (APC) selectively trap immunogens contained in tick saliva and process them before triggering anti tick specific immune response. Nine days after tick fixation, spleen cells do not respond when stimulated with SGE (results not shown). This unresponsiveness is apparently due to the regionalisation of T specific lymphocytes and not to APC inefficiency. APC treatment with chloroquine during antigen pulsing period abolishes their subsequent capacity to present foreign antigen to T cells but left the MLR largely intact [2]. Our work corroborates this observation. Chloroquine raises intracellular pH interfering with processing and presentation of antigen by MHC class II molecules [3].

In BALB/c mice infested with *I. ricinus* nymphs some antibodies reacting against one integumental antigen of 25 kDa was detected using western blot (Fig 1). We have shown that IE probably does not contain immunogen proteins which can be processed and presented by mice APC to T cells. IgG antibodies developed against tick saliva probably cross react with IE proteins.

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FUNCTIONAL DICHOTOMY OF DENDRITIC CELLS ISOLATED FROM BLOOD AND LYMPH NODES

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INTRODUCTION

Previous studies have shown that dendritic cells (DC) isolated from mouse lymph nodes or spleen can induce primary proliferative responses to contact sensitizers when exposed to the chemical either *in vivo* or *in vitro*[1,2]. In this study we have attempted to induce primary immune responses to contact sensitizers *in vitro* using peripheral blood DC. Neither human nor mouse blood DC stimulated a response to picryl sulphonic acid by syngeneic lymphocytes. This contrasted with mouse lymph node or spleen DC where good primary proliferative responses to picryl sulphonic acid have been obtained. Here we have examined adhesion molecule expression on mouse lymph node and blood DC to determine whether functional variation is related to surface molecule expression.

METHODS

Peripheral blood mononuclear cells (PBMC) from human or mouse blood were obtained by density gradient centrifugation. PBMC were cultured overnight in tissue culture flasks and DC obtained by centrifugation over a metrizamide gradient. Murine lymph node DC were obtained by centrifugation of a single cell suspension over a metrizamide gradient[1]. DC were pulsed *in vitro* with picryl sulphonic acid and cultured with syngeneic lymphocytes in 20µl hanging drop proliferation assays[1]. Murine DC from blood and lymph

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node were examined using FACS analysis for the expression of MHC class II, B7, VCAM-1, LECAM-1, CD44, LFA-1 and ICAM-1.

RESULTS

Human blood DC pulsed with a range of concentrations of picryl sulphonic acid and cultured with syngeneic lymphocytes did not induce significant proliferative responses. Prior culture of human blood DC with GM-CSF did not cause a proliferative response to picryl sulphonic acid. The inability of human blood DC to stimulate primary immune responses to sensitizer contrasts with mouse lymph node DC pulsed with allergen which induce good proliferative responses *in vitro*. Comparisons between mouse blood and lymph node DC were made to see if this was due to a species or tissue difference. Mouse lymph node DC pulsed with picryl sulphonic acid stimulated good proliferative responses. Blood DC pulsed with the sensitizer and cultured under the same conditions did not stimulate primary immune responses (Fig.1A & B), however, like lymph node DC, blood DC were able to stimulate an allogeneic response although the responses to blood DC were considerably lower than those obtained with lymph node DC. Blood DC pulsed with sensitizer were also unable to stimulate a secondary response in sensitized lymph node lymphocytes. There was no evidence that blood DC produced an inhibitory effect as shown by the co-culture of blood and lymph node DC pulsed with picryl sulphonic acid (Fig.1C).

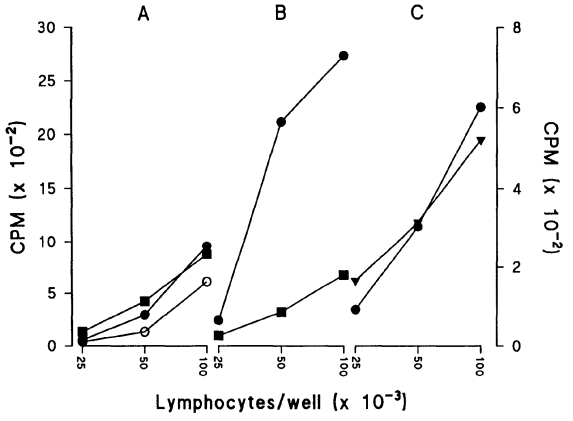

Figure 1. Stimulation of naive murine lymph node lymphocytes by DC isolated from lymph nodes or peripheral blood and pulsed with 50μg/ml picryl sulphonic acid *in vitro* (mean ± s.e). Uptake of [^{3}H] thymidine in cultures of 25-100 x 10^3 lymphocytes after 3 days in culture. (A) Lymphocytes only (○) +lymph node DC (●) or + blood DC (■); (B) Lymphocytes + lymph node (●) or blood (■) DC pulsed with picryl sulphonic acid; (C) Lymphocytes + lymph node and blood DC pulsed with picryl sulphonic acid (●) and lymphocytes + lymph node DC only pulsed with picryl sulphonic acid (▼). (A & B use the left hand Y axis; C uses the right hand Y axis)

Murine blood and lymph node DC were analysed for adhesion molecule expression. Levels of LFA-1, CD44 and LECAM-1 were comparable on both types of DC but lower levels of MHC class II, ICAM-1 and B7 were found on blood DC than lymph node DC but differences in adhesion molecule expression were not sufficient to inhibit the allogeneic response.

DISCUSSION

Unlike those in mouse lymph nodes, DC isolated from human or mouse blood and pulsed with sensitizer *in vitro* did not stimulate primary immune responses to contact sensitizers although primary responses to other antigens e.g. *Chlamydia trachomatis* and HIV-1[1,3,4], have been obtained. The absence of a proliferative response to contact sensitizers suggested possible differences in surface antigen expression. Adhesion molecules play an important role in the initiation of primary immune responses. Antibodies to LFA-1, LFA-3 or ICAM-1 can interfere with the stability of DC/T cells clusters and reduce proliferative responses[5,6]. Blood and lymph node DC both expressed LFA-1, ICAM-1, MHC class II, B7, LECAM-1 and CD44. Lower levels of adhesion molecules were found on blood DC however these cells were able to stimulate an allogeneic response although less efficiently than lymph node DC. The development of a contact sensitivity response is highly dependent on adhesion molecules[7,8,9] and the lower expression on blood DC may have contributed to the lack of a proliferative response to contact sensitizers.
The data suggest that DC isolated from different tissues may have different functions and may reflect DC at different stages of maturity.

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ANTIGEN PROCESSING CAPACITY OF DENDRITIC CELLS FROM MICE OF DIFFERENT MHC BACKGROUNDS: DOWN-REGULATION UPON CULTURE AND EVIDENCE FOR HETEROGENEITY OF DENDRITIC CELL POPULATIONS

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INTRODUCTION

Dendritic cells (DC) are highly specialized to initiate primary immune responses.[1] They exhibit the necessary properties for this task at different stages of their lifespan. In an *immature state*, while resident in the tissues, they are very efficient in processing native protein antigens and thus generating MHC class II/peptide ligands for the antigen-specific T cell receptors. After undergoing a *maturation process* they acquire the adhesion molecules and costimulatory molecules necessary for the powerful stimulation of resting T cells[2-4] and they greatly reduce the capacity to process native proteins.[5-9] At the cellular level the decrease of processing activity in maturing DC was correlated with the disappearance of acidic, endosomal organelles and with the loss of invariant chain expression (reviewed in[1]). Furthermore it was shown that biosynthesis of MHC class II and invariant chain molecules is massive at a time when processing activity is high (i.e. in freshly isolated Langerhans cells); upon culture biosynthesis of both molecules is largely shut down (reviewed in[1]). These cellular and molecular observations may explain the down-regulation of processing.

Data that seemed to contradict the described concept were originally published by Hauser and Katz,[10] Shimada et al.[11] and, more recently, by Aiba and Katz.[12] These experiments clearly indicated that populations of cultured LC had substantial processing activity. De Bruijn et al.[13] and Liu and MacPherson[14] reported similar data of splenic and lymph-borne DC, respectively. These studies prompted us to take a second look at the relative processing abilities of immature and mature DC. We decided to undertake a detailled side-by-side comparison of immature and mature DC concentrating on murine LC as a model. Processing activity was measured with *T cell hybridomas* (kind gifts of Dr. S.I.Katz, Bethesda, MD) of defined peptide specificities.[6] We used hybridomas restricted by 5 different MHC-class II molecules: I-A^b, I-A^d, I-E^d, I-A^k, I-E^k.

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RESULTS

Populations of immature dendritic cells are consistently superior to mature dendritic cells in processing native protein antigens. When freshly isolated LC and cultured LC were compared *side by side* it became apparent that fresh LC stimulated the hybridomas always better than cultured LC. This was less pronounced at high doses of antigen presenting cells and/or at high concentrations of antigen (see Figure). The same phenomenon was observed with spleen DC: Populations of low density adherent cells (containing fresh, immature spleen DC) pulsed overnight with native protein antigen elicited vigorous hybridoma responses. Cells cultured overnight in parallel without antigen and subsequently tested with the hybridoma in the presence of native protein induced only poor responses. We were unable to correlate the degree of processing by mature DC populations with MHC haplotype; the decrease of processing capacity during culture was observed with all strains of mice and with all antigens tested.

Dendritic cell populations are heterogeneous. In populations of mature DC (here cultured LC) stained by immunoperoxidase with mAb's against the invariant chain it became apparent that most LC lacked this molecule as described.[6] When samples were more thoroughly evaluated, however, one could consistently observe a subpopulation of LC (5-10%) that expressed invariant chain intracellularly. It was also evident with overnight cultured spleen DC. Heterogeneity was observed with dendritic cells of all three MHC class II haplotypes.

DISCUSSION

Here we describe that populations of mature dendritic cells invariably contain a subpopulation of "stragglers", i.e. cells that lag behind in their maturation process. As a consequence, these few cells still possess the machinery for processing as shown by their expression of the invariant chain, a molecule critically involved in antigen processing. The experiments with fresh LC emphasize both the outstanding capacity of these cells to process native protein antigens and the sensitivity of T cell hybridomas: As few as 10-30 fresh LC can elicit a substantial antigen-specific hybridoma response. Therefore, it is most likely that the small numbers of "contaminating" immature DC are responsible for the processing activity observed in populations of cultured LC or spleen DC.

Our data confirm the concept of dendritic cell maturation, that is the upregulation of T cell sensitizing function and the down-regulation of antigen processing capacity during short term culture.[1,2,5] The data do not rule out MHC-haplotype-dependent differential processing abilities as discussed by Aiba and Katz.[12] We do also not see a contradiction to the data of De Bruijn et al.[13] and Liu and MacPherson[14] who reported that spleen or gut-derived lymph-borne mature DC can process native protein antigens very efficiently. Neither paper contains a direct comparison with an immature precursor of the DC tested. Fresh, immature spleen DC or fresh, immature gut-derived DC might have turned out to be even more potent when tested side-by-side. For obvious reasons these experiments could not be done by the authors.

In conclusion, processing activity observed in populations of mature DC may be explained by the heterogeneity of these populations: small minorities of not (yet?) mature DC are probably responsible for it. This may be way to reconcile the seemingly contradictory reports on "processing by dendritic cells".[5,6,8,9] vs. [5,12-14] The observations of Liu and MacPherson,[14] who showed processing capacity of DC populations which had matured *in vivo* (on their way from the gut into the lymphatic vessels), would suggest that heterogeneity of DC populations is not an in vitro artefact but may rather be of relevance in vivo.

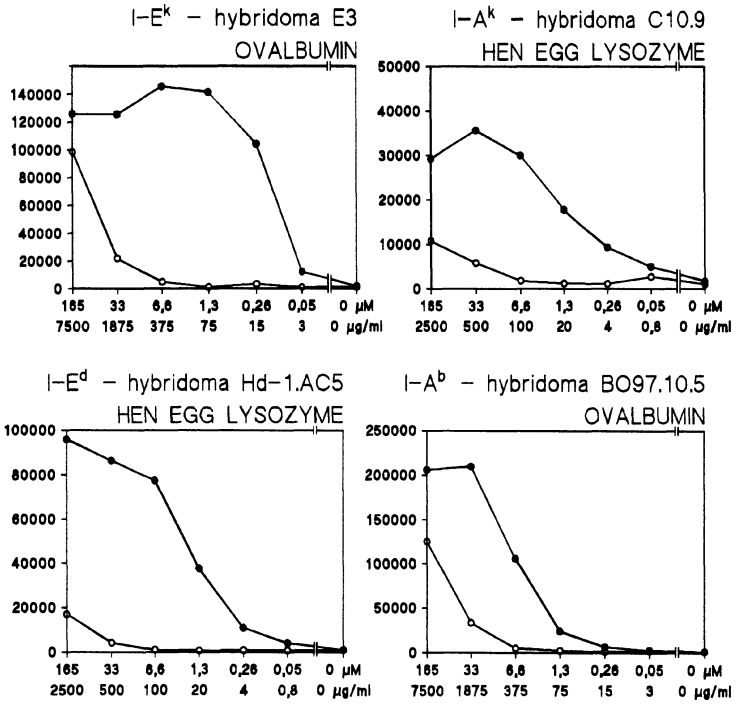

Figure 1. Processing of different native proteins by LC of different MHC class II haplotypes (1.000 LC per microwell). Note that freshly isolated LC (closed circles) are consistently superior to cultured LC (open circles) over a wide range of antigen concentrations (x-axis). Proliferation of CTLL indicator cells (cpm) is plotted on the y-axis. Note different scales on each panel!

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TNFα INTERRUPTS ANTIGEN-PRESENTING FUNCTION OF LANGERHANS CELLS BY TWO MECHANISMS: LOSS OF IMMUNOGENIC PEPTIDES AND IMPAIRMENT OF ANTIGEN-INDEPENDENT T CELL CLUSTERING

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INTRODUCTION

In vitro and in vivo experiments suggest that TNFα disturbs the antigen presenting function of epidermal Langerhans cells (LC), and contributes to the well known immunosuppressive effect of low-dose UVB irradiation. Presenting function of LC is impaired by low dose UVB.[1,2] The UVB effect was shown to be mediated - at least in part - by TNFα. Simon et al.[3] have shown that UVB irradiation converts LC to cells which can induce antigen-specific anergy in TH1 clones. Grabbe et al. demonstrated that LC cultured in the presence of TNFα were less effective in conveying protection from tumor in an adoptive transfer model.[4] Our own data indicate that TNFα distorts the maturation process of LC in culture.[5] Antigen processing appears not to be affected by TNFα but the capacity to stimulate resting T cells in the mixed leukocyte reaction is greatly impaired.[6] We have, therefore, studied the effect of TNFα on murine LC in more detail by examining antigen processing and retention as well as on T cell binding.

To this end *epidermal LC* of BALB/c mice were highly enriched to 90-95% from ear skin by a panning method.[7] Panned cells were cultured for 3d in the presence of recombinant murine TNFα (125U/ml; gift of Dr. G.R. Adolf, Bender, Vienna, A) or recombinant murine GM-CSF (100U/ml; gift of Dr. S. Gillis, Immunex, Seattle, WA). Processing activity was measured by means of *T cell hybridomas* of defined peptide specificities. The procedures outlined by Inaba et al.[8] were followed in order to study the antigen-*in*dependent T cell clustering ability of LC.

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RESULTS

As shown previously[6] highly enriched LC cultured in TNFα survived a 3d culture well; they were incapable, however, of stimulating resting T cells in an oxidative mitogenesis assay (see Table). Tissue DC, including LC, fulfill their function of generating immunity in vivo in that they capture and process antigens in the tissues and migrate to the draining lymphoid organs where they sensitize resting T cells with the immunogenic peptides they have "imported" from the various tissues. For this purpose LC must be able to retain immunogenic peptides for some time. When fresh LC were pulsed with a protein antigen for 12h, washed, and cultured for another 48h in the presence of GM-CSF they had retained enough immunogenic MHC/peptide complexes on their surfaces to effectively stimulate the peptide-specific T cell hybridoma (see Table). If, however, a parallel sample of the same antigen-pulsed LC was cultured in TNFα the hybridoma responded only poorly (Table). The addition of synthetic peptide to the TNFα-cultured LC lead to a vigorous hybridoma cell activation (Table). This ruled out the possibility to that TNFα had grossly altered the MHC class II molecules in qualitative or quantitative terms. The same phenomenon was observed when freshly isolated, purified LC were pulsed with synthetic peptide instead of native protein (not shown).

Table: Retention of immunogenic MHC-peptide fragments: TNF α induces loss of peptides generated by Langerhans cells.

Antigen during 12h pulse	Culture without antigen from 12 to 72h in	Antigen during hybridoma assay	Number of Langerhans cells per well 3000	1000	300	100	30	10	0
									HYBRIDOMA ASSAY
			Proliferation of CTLL-2 cells in CPMx10 $^{-3}$						
Myoglobin	GM-CSF	none	104.4	41.0	10.7	8.5	3.3	3.6	2.6
Myoglobin	**TNFα**	**none**	**4.2**	**2.9**	**3.1**	**4.1**	**3.5**	-	
Myoglobin	TNFα	peptide	94.1	26.6	7.3	1.7	2.0	1.1	
									OXIDATIVE MITOGENESIS ASSAY
			Proliferation of resting T cells in CPMx10 $^{-3}$						
None	GM-CSF			76.3	27.9	12.8	7.2		1.3
None	**TNFα**			**11.1**	**5.1**	**2.1**	**1.5**		

Cytofluorographic comparisons between LC cultured in GM-CSF versus LC cultured in TNFα showed that pronounced MHC class II upregulation occurred under both culture conditions. In our further studies we employed clustering assays. We observed that TNFα also impaired the ability of LC to cluster T cells in an antigen-independent way [8] (not shown).

DISCUSSION

We found that TNFα did neither inhibit nor enhance antigen processing, the first step of an immune response. It did, however, *influence the maturation process of LC* at least in two ways:

TNFα impairs the ability of mature dendritic cells to bind/cluster T cells in an antigen-independent way. This phenomenon is probably also responsible for the reduced

ability of TNFα-LC to stimulate resting T cells. The molecule responsible for antigen-independent clustering is still not known.

TNFα induces the loss of immunogenic peptides from MHC class II molecules. This conclusion is based on the fact that the MHC molecules of TNFα-LC are apparently intact: Exogenously added synthetic peptide induced strong responses by the hybridomas indicating that the presentation mechanism is not affected. Moreover, the reduced T cell binding capacity cannot sufficiently explain the low or absent responses of peptide-specific T cell hybridomas (i.e. T cells with altered/reduced activation requirements) to LC cultured in TNFα. Experiments to adress this question are in progress. It will be attempted to directly prove or disprove the disappearance of peptides from the MHC class II binding grooves, e.g. by using radioactively-labeled[9] peptides.

To what extent these *in vitro* observations will be relevant *in vivo* remains to be determined. One must keep in mind that our data were obtained in an experimental system where LC were highly enriched and cultured in medium containing only TNFα. Other investigators, in particular Sallusto & Lanzavecchia most recently[10], had studied the effects of TNFα in a cell culture environment, where other cytokines (GM-CSF, IL-4) were also present. It may be the proportions of various cytokines that finally determines the response of dendritic cells.

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THE ROLE OF CIS-UROCANIC ACID IN UVB-INDUCED IMMUNOSUPPRESSION

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INTRODUCTION

UV radiation causes many alterations in the skin, including loss of Langerhans' cells (LC) and results also in the accumulation of dendritic cells (DC) in the lymph nodes draining the site of irradiation.[1] One epidermal mediator which may be involved in the induction of UVB effects on LC is cis-urocanic acid (cis-UCA), formed from the naturally occurring trans-isomer following UV-exposure. It has been proposed that UCA acts as a photoreceptor for UV-induced immunosuppression. The action spectrum of UV-induced suppression of contact hypersensitivity is similar to the absorption spectrum of trans-UCA and mice deficient in UCA are resistant to UV-induced immunosuppression. Cis-UCA also has the ability to mimic some of the effects of UV-B such as suppression of delayed-type hypersensitivity responses to herpes simplex virus in mice and depletion of LC from the epidermis.[2] A murine monoclonal antibody, with specificity for cis-UCA has been developed[3] and was used in the present study to elucidate further the role of cis-UCA in modulating immune responses in the skin.

EXPERIMENTAL OVERVIEW

Female C3H-HeN mice received intraperitoneal (i.p.) injections of anti-cis-UCA two hours prior to irradiation with sub-erythemal doses (960 and 1440 J/m^2) of broadband UVB or ear painting with cis-UCA. Control mice were injected i.p. with an isotype matched (mouse IgG1) monoclonal antibody of irrelevant specificity, or with phosphate buffered saline (PBS) alone. Twenty four hours later, LC numbers in the ears were measured by counting ATPase^{+ve} cells in dorsal epidermal sheets (Figure 1). Forty eight hours later, DC numbers in the draining auricular lymph nodes were measured by counting DC microscopically after purification on metrizamide gradients (Figure 2).

RESULTS

Previous evidence has shown that exposure to UVB or ear painting with cis-UCA significantly reduces the density of ATPase^{+ve} LC in the epidermis.[2] In the present study it was found that the depletion of LC induced by either treatment was abrogated by prior i.p.

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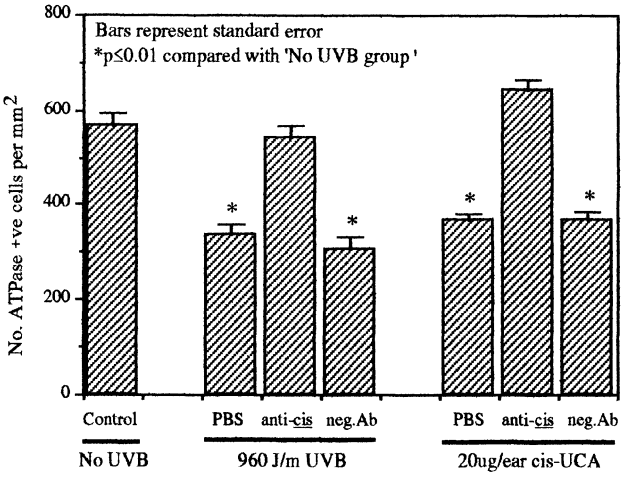

Figure 1. Groups of mice (n=4), received an i.p. injection of PBS, anti-cis-UCA or an irrelevant isotype matched antibody. Two hours later the mice were irradiated with a single dose (960 J/m^2) of broadband UVB or were ear painted with cis-UCA. Control mice received anti-cis UCA but were not irradiated. Twenty four hours later the mice were killed and the ears removed. Dorsal epidermal sheets were prepared and stained for ATPase activity. For each group the number of ATPase^{+ve} cells in 40 fields of view (10 random fields per dorsal epidermal sheet) were counted.

injection of anti-cis-UCA antibody (Figure 1). Injection of an irrelevant isotype matched control antibody or vehicle alone, did not affect the depletion of ATPase^{+ve} cells induced by either treatment. The ability of anti-cis-UCA to block the depletion of ATPase^{+ve} cells in the epidermis was dose dependent (data not shown).

As has been demonstrated previously, exposing the ears to sub-erythemal doses of UVB resulted in a significant increase in numbers of DC found within draining lymph nodes 48 hours later (Figure 2a). Although ear painting with cis-UCA caused a decrease in the number of ATPase^{+ve} cells in the epidermis, it did not induce a subsequent increase in the numbers of DC in the auricular lymph nodes (Figure 2a). In addition, prior injection of the anti-cis-UCA monoclonal antibody did not influence UV-B induced accumulation of DC in the auricular nodes (Figure 2b).

CONCLUSIONS

Following UVB irradiation, cis-UCA is an important mediator in reducing LC numbers in the epidermis. However, cis-UCA is not involved in the UV-induced migration of DC to the draining lymph node. The mechanism by which cis-UCA causes depletion of ATPase^{+ve} cells is unknown but published work has indicated that cis-UCA may act through histamine-like receptors.[2] Other mediators produced in the skin after UVB irradiation may act to induce the migration of DC to the draining lymph nodes. There is evidence that tumour necrosis factor-α(TNFα) production is induced in the epidermis after UVB exposure and that this cytokine may promote migration of DC to the lymph nodes.[4,5] It is possible that TNFα and cis-UCA may modulate immune responses by different mechanisms and the possible interactions between the two mediators are being investigated currently.

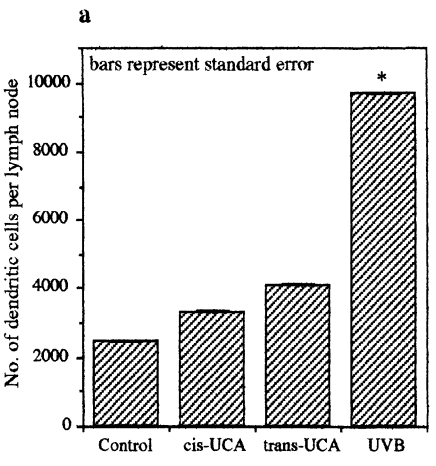

Figure 2a. Groups of mice (n≥6) were painted with 100 µg of cis or trans-UCA on both ears or received a single dose (1440 J/m^2) of UVB. Control mice were ear painted with the vehicle alone but were not irradiated.

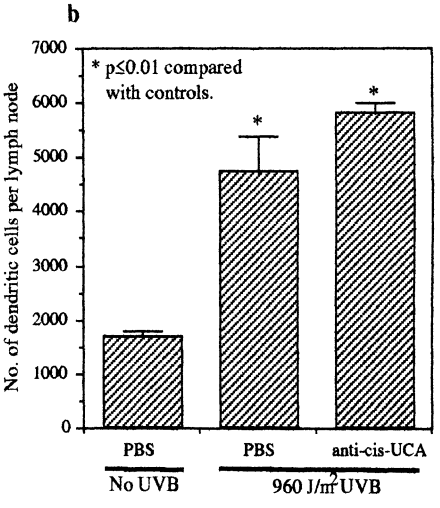

Figure 2b. Groups of mice (n=4) were injected i.p. with PBS or anti-cis-UCA (1:500 in PBS). Two hours later mice received a single dose (960 J/m^2) of UVB. Control mice received anti- cis-UCA but were not irradiated.

Forty eight hours later all mice were killed and the auricular lymph nodes were excised and pooled for each group. Single cell suspensions were prepared by disaggregation through a 70 µm nylon mesh. Dendritic cells were enriched by density gradient centrifugation and counted by direct morphological examination using light microscopy. For each group, five counts were made and the mean number of dendritic cells per lymph node calculated.

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ANTIGEN PROCESSING BY RAT LYMPH-BORNE DENDRITIC CELLS

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INTRODUCTION

The central function of dendritic cells (DC) is to present antigen to T lymphocytes (Reviewed in [1]). Several groups have shown that DC in peripheral tissues can capture antigens and can present them to sensitized T cells "in vitro" or to naive T cells "in vivo" [2-4]. We have shown that antigens injected into the small intestine or given by gavage are captured by DC in the intestinal wall and transported in lymph, and that such DC can prime naive T cells [5,6]. Studies of murine LC show that cells freshly isolated from epidermal sheets are very weak stimulators of resting T cells in the MLR, but are active antigen-processing cells and can present antigens to T cell lines. After culture "in vitro" however, they dramatically increase their ability to stimulate a MLR but lose the ability to process native antigen although their ability to present peptides is maintained [7,8]. A hypothesis which accommodates these observations is that peripheral DC acquire antigen locally but are unable to activate resting T cells in their vicinity, and travel to secondary lymphoid organs. Here, as they are unable to process fresh antigen, they retain an "image" of the antigens they acquired in the periphery and can present them to resting T cells.

In the model we have developed in the rat, DC derived from the small intestine are purified from pseudo-afferent lymph [9]. Although these cells have only just left the intestine and would normally arrive at the draining node in a matter of seconds, they are both powerful stimulators of the MLR and are able to process and present exogenous proteins efficiently [5,9]. As these DC represent a physiologically relevant population, we determined if such cells lose the ability to process antigens in a manner similar to LC. Our results show that cultured lymph-borne DC retain the ability to process exogenous antigens for at least 72h in culture. Thus, the hypothesis proposed for LC does not apply to rat lymph-borne dendritic cells.

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Cultured L-DC Are Able To Present Native OVA And G50-Filtered OVA To OVA-Sensitized Spleen T Cells

Highly enriched L-DC (85-95%) were cultured in the presence of murine recombinant GM-CSF (50ng/ml) for 0 (fresh), 20 or 48 hours. These L-DC were then pulsed with OVA for 3 hours, washed three times and added to OVA-sensitized spleen cells in an antigen presentation assay. The results show that both fresh and cultured OVA-pulsed L-DC could stimulate significant proliferation of OVA-spleen cells (Table 1).

To exclude the possibility that the OVA we used contained peptides which might directly bind to MHC molecules in or on cultured L-DC, OVA was passed through a Sephadex G50 column and the pure protein was used as antigen. Using G50-filtered OVA in an antigen presentation assay, the results were very similar to those obtained with the whole preparation. L-DC cultured for 24, 48 and 72 h presented G50-OVA efficiently to spleen T cells sensitized with filtered OVA (Table 1).

Controls in which antigen was omitted showed that the syngeneic MLR was relatively small and was similar whether fresh and cultured L-DC were used (data not shown). In parallel, a MLR was carried out using the same fresh and cultured L-DC. The results show that all populations of L-DC could stimulate a strong MLR, and that culture had no significant effect on their stimulatory capacity.

Table 1. Cultured L-DC can present native OVA to sensitized T cells (Gross CPM)

	OVA	MLR	G-OVA	MLR
Fresh	30555	105590	20400	91985
24h	30000	131643	20068	100121
48h	30347	134573	31504	95621
72h			29408	102211

L-DC were purified and cultured with murine recombinant GM-CSF (50ng/ml) for 0h (fresh), 24h, 48h or 72h They were then pulsed with OVA (1mg/ml) or Sephadex G50 filtered OVA (1mg/ml) for 2-3h and washed three times An antigen presentation assay was set up by culturing OVA-primed spleen cells with OVA-pulsed L-DC for 4 days and proliferation measured by tritiated thymidine incorporation and expressed as gross CPM A MLR was carried out with OVA- or G-OVA-pulsed fresh or cultured L-DC as stimulators

Cultured L-DC Are Able To Prime Naive Rats

As a central function of L-DC is to activate T cells in a primary response, it was of interest to know whether OVA-pulsed cultured L-DC can also prime naive T cells. To test this, fresh or cultured L-DC pulsed with filtered or normal OVA were injected into the footpads of naive rats. 10 days later, popliteal lymph node cells (PLNC) were cultured in the presence of OVA and proliferation was measured. The results show that both OVA-pulsed fresh L-DC and cultured L-DC are able to prime naive T cells efficiently. Table 2 shows the antigen-specific recall responses of PLNC primed with OVA-pulsed fresh or cultured L-DC.

Chloroquine inhibits the presentation of OVA by cultured L-DC

To show that the presentation of OVA to T cells requires antigen processing by L-DC we used chloroquine as an inhibitor. The results show that chloroquine inhibits the presentation of OVA by both fresh and cultured L-DC (data not shown).

Table 2. OVA pulsed cultured L-DC can prime naive T cells (Gross CPM)

	G-Ovalbumin			BSA	No Ag
Priming with	50µg/well	25µg/well	12 5µg/well	50µg/well	
0h G-OVA-L-DC	23600	19311	-	1676	2220
24h G OVA-L-DC	22566	16896	11151	767	181
24h N-OVA-L-DC	16871	10289	4812	358	290
48h G-OVA-L-DC	32403	20860	10633	3109	979
48h N OVA-L-DC	20868	23833	11620	952	1841

G50 filtered OVA or normal OVA-pulsed L DC were injected into the footpads of naive PVG rats 10-11 days later, popliteal lymph node cells were prepared and cultured in the presence of G50 OVA or BSA for 5 days Proliferation was measured by tritiated thymidine incorporation and expressed as gross CPM G OVA L DC G50 filtered OVA pulsed L DC N OVA L DC normal OVA pulsed L DC

Our results show clearly that lymph-borne DC do not lose the ability to process antigen for up to 72h in culture under a variety of conditions Fresh and cultured DC show similar capacities to stimulate sensitized T cells, and more importantly, are similarly effective in sensitizing naive T cells "in vivo" It is unlikely that cultured DC were exchanging MHC-associated peptide for free antigen-derived peptide since active antigen processing was necessary as shown in chloroquine inhibition assay Thus we conclude that the shut-down of processing seen in cultured LC is not common to all DC, and that shut down does not occur in at least one population of DC actively engaged in antigen transport to the draining node

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EVIDENCE THAT LANGERHANS CELLS MIGRATE TO REGIONAL LYMPH NODES DURING EXPERIMENTAL CONTACT SENSITIZATION IN DOGS

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INTRODUCTION

Langerhans cells are epidermal dendritic cells which take up antigens and transport them to paracortical areas in regional draining lymph nodes, where they induce T-lymphocyte activation. According to Moll [1], LC's play a key role in the capture, transport and presentation of leishmania to T cells, and in the initiation of the specific immune response in the mouse version of leishmaniasis. In order to establish a canine model of this disease, we first examined the ability of canine LC's to migrate to regional lymph nodes in response to cutaneous stimulation.

MATERIALS AND METHODS

Sensitization *in vivo*

Three healthy six-years-old female beagles were obtained from the Lyon National Veterinary School experimental kennels.

Fluorescein isothiocyanate (FITC, isomer 1, Sigma) was dissolved in a 50/50 v/v acetone/dibutylphtalate (Sigma) mixture. At day 0, the dogs were skin-painted on a shaved distal part of the legs (10 cm^2) either with 500 µl 2% FITC (left hind limb) or, as a control, 500 µl relevant vehicle alone (right hind limb). At day, the procedure was repeated.

Electron microscopy - Cell suspensions - Frozen sections -

The DLN's were removed on days 2, 4 and 7. A thin piece of each was fixed using 2% glutaraldehyde in cacodylate buffer at pH 7.3, processed for standard transmission electron-microscopy and embedded in epoxy resin. Ultrathin sections were examined, after post-staining, with a Hitachi electron microscope. A part of each lymph node was mechanically disaggregated through wire mesh to obtain single cell suspensions. Lymph node cells (LNC) were then centrifuged. Cytospins were observed with a fluorescence microscope, either directly to look for green fluoresceing FITC positive cells, or after indirect immunolabeling for red fluoresceing rhodamine positive cells.

The remaining tissue was frozen in liquid nitrogen-cooled isopentan, then cryosectioned (6 µm thin sections) and mounted on slides, which were examined after indirect immunolabeling.

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Indirect immunolabeling

Cytospun cells from the DLN's were analyzed with monoclonal antibodies (MAb) specific for canine MHCII, CD8, CD11a, CD11c, CD4, CD5, CD21, CD45, CD45R surface antigens (Ag), obtained from the first Canine Leukocyte Antigen Workshop (CLAW)[2] (respectively CLAW # 113, 97, 102, 103, 96, 28, 105, 35, 37, 110), and for canine CD1c kindly provided by P.F. Moore (Department of Veterinary Pathology, University of Davis, USA), and canine CD18 (clone MHM 23, a cross -reactive human specific MAb from DAKO). Frozen sections were labeled with MAb specific for canine CD5, CD21 (CLAW #28, 10) and CLAW cluster A to highlight T or B areas on lymph nodesin the rhodamine-conjugated mouse anti-rat IgG $F(ab)'_2$ (The Binding Site) or rabbit anti-mouse IgG (DAKO) were used as secondary antibodies.

RESULTS

The direct observation of cytospins from FITC-painted limb displayed that on day 2 some LNC's showed a moderate to strong, diffuse, spotted, or diffuse and spotted green fluorescence. Such cells were less frequently observed on days 4 and 7. With May-Grünwald-Giemsa staining, these FITC-positive cells appeared large, with abundant basophilic cytoplasm and an eccentric nucleus containing unique nucleolus. These cells were usually clustered with small lymphocytes. No FITC-positive cells were observed for vehicle-painted limb.

Indirect immunolabeling showed that FITC-positive cells were CD1c, CD18, MHCII, CD45, CD11a, CD11c, CD8 + and CD4, CD45R, CD5, Thy1, CD21 -.

On frozen sections, these cells were never located in ca CD21 or CLAW cluster A positive zones (B-cell areas), but always in ca CD5 positive zones (T-cell areas).

At the electron microscopic level, we observed in day 4 removed draining lymph nodes from FITC-painted limb large cells with folded nuclei, clear cytoplasm and round to oval dense bodies of variable size, from 150 to 950 nm, the majority being between 600 and 800 nm. These bodies were covered by a unit-membrane and contained an amorphous, fairly electron-dense substance. Often, they had a light halo between this substance and the limiting membrane. Some of them showed many small (40 nm) or a few large (120 nm) highly electron-dense spherical structures. Some of the cells contained BG : the rod was composed of two parallel layers of limiting membranes 10 nm thick and one central lamella. The average thicknesses of the rod and central lamella were respectively about 50 nm and 10 nm. The lamella had a periodicity of 20 nm, with the appearance of a linear and/or spotted pattern.

DISCUSSION

This study demonstrated the existence of fluorescent cells in regional draining lymph nodes two days after epicutaneous sensitization with FITC. The percentage of FITC-positive cells decreased on days 4 and 7. No fluorescent cells were observed in controlateral lymph nodes (CLN). The information which exists on immunophenotypes of antigen-bearing cells in the DLN's of contact-sensitized animals is limited and inconsistent. Cumberbatch [3] mentions in mice an Ia+, F4180-, Thy1- phenotype, while Kripke [4] demonstrated an Ia+, F4/80+ (for 75% of FITC-positive cells), Thy1-, Mac1-, Mac2+, Mac3+ phenotype,which, for him, is consistent with that of LC's. In our experiment, FITC-positive DLN's cells expressed the same surface antigens as canine LC's [5] (manuscipt in preparation). Not all these antigen-bearing cells were ca CD8-positive, in fact 37% were negative on day 2. This loss of antigen might be related to a maturation during lymph node vessels course.

The origin of these antigen-bearing cells is still open to discussion. Liddington suggested that, following skin painting, free FITC could travel directly to lymphoid tissue and to associate *in situ* with Ia+ cells in mice model [6]. This kind of binding would involve many more cells, and not just the small percentage of large CD1c + cells as we found in our study. Kripke [4] used the presence of BG to prove that the majority of the antigen-bearing cells that migrate from the skin to the DLN's after topical sensitization are LC's. The BG-containing cell

we found on day 4 DLN's have the same morphology as the "newly-arrived macrophages", *i.e.* LC's or veiled cells, as observed by Kamperdijk [7] in the paracortex of rat lymph nodes as a primary response to paratyphoid vaccine. Compared with resident IDC's, these cells were smaller, showed less interdigitation, had a higher nucleus/cytoplasmic ratio, contained many irregularly-shaped phagolysosomes and, in some cases displayed BG's. According to Kamperdijk [8], only dendritic cells isolated from LN's in the induction phase of the immune response displayed BG's. So we postulate that the Birbeck granules-containing cells we observed represent FITC-positive LC's newly-arrived in DLN's and not resident IDC's. Furthermore, the cytoplasm of these cells contains dense bodies of roughly the same size (800 nm) as the fluorescent spots observed in FITC-positive cytospin cells. Silberberg-Sinakin [9], using intradermal injection of ferritin in Guinea-pigs, demonstrated the presence of ferritin not only on the surface of LC's from skin DLN's, but also in the cytoplasm of such cells, in the form of membrane-bound aggregates. Arkema [10] and Kleijmeer [11] have demonstrated that DC's and LC's contain MHC II compartments with lysosomal characteristics. Therefore, we hypothesize that the above mentioned dense bodies are MHC compartments containing FITC.

Further work is needed to investigate the functional capacity of these FITC-positive cells *in vitro* by culturing with naive T cells and observing the resulting T-cell proliferation.

CONCLUSION

This study demonstrates, in canine model, that following the topical application of allergens, antigen-bearing epidermal cells migrate to draining lymph nodes. These cells display an immunophenotype and ultrastructural features (BG) typical of canine LC's. We are now in a position to investigate the migration of LC's in experimentally induced canine cutaneous leishmaniasis.

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ANALYSIS OF INVARIANT CHAIN PROCESSING IN 3 DAY CULTURED RAT LANGERHANS CELLS

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INTRODUCTION

MHC class II molecules, critical peptide binding elements involved in the presentation of exogenous antigen to T helper cells, are expressed constitutively by Langerhans cells (LC) within their epidermal microenvironment. Several studies in mouse and man demonstrated, that short term in vitro culture of LC entails remarkable functional and penotypic alterations, including a profound increase of class II elements exposed at the LC's surface[1]. Biosynthetic analysis revealed a downregulation of class II synthesis during the culture period[2,3]. In recent work on rat LC we described the uncoupling of the coordinately regulated biosynthesis of class II and invariant chain proteins in the course of culture[4]. Thus, LC undergoing 3 days of in vitro culture synthesize very high levels of γ-chains in the almost complete absence of class II synthesis. We extended these studies to explore invariant chain synthesis and processing in 3 day cultured LC more precisely.

RESULTS AND DISCUSSION

Invariant chain fragments in fresh and cultured LC

Invariant proteins accompany class II molecules on their biosynthetic route, thereby targeting them to an acidic intracellular processing compartment where selective proteolytic γ-chain cleavage allows peptide loading of the class II dimer[5]. Biosynthetic labeling of fresh LC combined with sequential immunoprecipitation analysis employing class II- and invariant chain specific-mAbs was performed. The specific immunoprecipitate obtained with the rat γ-chain-reactive mAb RG11[6] contained invariant chain proteins γ and p40 including their sialylated derivatives p35 and p45 respectively (Fig. 1A). In addition low molecular weight γ-species referred to as p28, p20 and p10 were observed.

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Detection of terminally glycosylated proteins p35 and p45 in the mAb RG11-precipitate derived from 3d cultured LC implicated Golgi-passage of the invariant chains, that notably occured in the absence of class II synthesis (Fig. 1B). Furthermore in 3d cultured LC a complex pattern of invariant chain fragments was observed, whose signal intensity was even stronger when compared to γ-chain fragments of fresh LC. Moreover, a staircase of acidic spots (see bracket in Fig. 1) was observed exclusively with cultured LC.

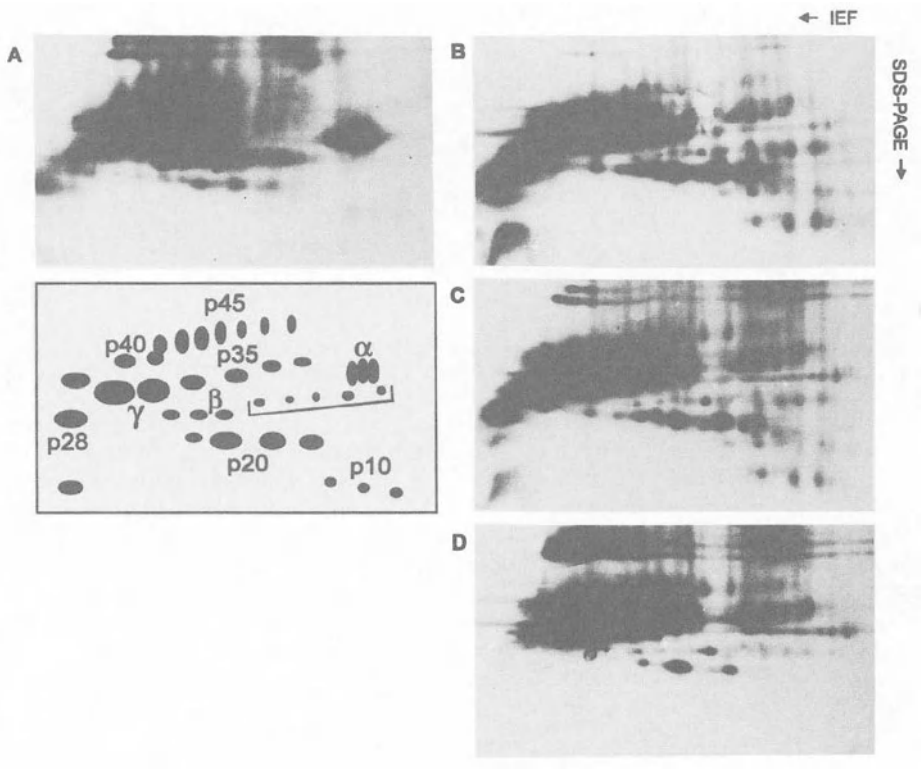

Figure 1. Identification of proteolytic invariant chain fragments by precipitation with γ-chain specific mAb RG11. Comparable numbers of fresh (A) and 3 day cultured LC (B,C,D) were metabolically labeled for 4 hours in the absence (A,B) or presence (C,D) of a protease inhibitor mixture (20 μg/ml Leupeptin; 20 μg/ml Pepstatin A, 8 μM Cbz-Phe-Ala-CHN_2) and were solubilized with detergent. In (D) the NP-40 buffer used was supplemented with protease inhibitors. Sequential immunoprecipitation was performed employing class II-specific mAbs (not shown) followed by mAb RG11-precipitation. Immunoprecipitates were resolved by two dimensional gel electrophoresis. Cell preparations, labeling procedure and immunoprecipitation were performed essentially as outlined in[4]. Note that polymorphic α- and β-chains are detectable exclusively in the mAb RG11-precipitate obtained from fresh LC.

Evidence for generation of γ-chain fragments by internal proteases

To investigate the role of intracellular proteases in γ-chain fragmentation in 3d cultured LC, experiments were performed in the presence of protease inhibitors, including inhibitors for cathepsin B (Cbz-Phe-Ala-CHN_2; Leupeptin) and cathepsin D (Pepstatin A)

respectively. These reagents were added at different steps of the biochemical analysis. Addition of protease inhibitors during the labeling period resulted in diminished amounts of γ-chain fragments p20 and p10 including the group of staircase-like spots (see bracket Fig. 1C). This result indicates extensive intracellular proteolytic cleavage of invariant proteins in 3d cultured LC and points to the participation of cathepsin B and D. If the inhibitors were added during cell labeling as well as during cell solubilization inhibiton of proteases was more effective (Fig. 1D). In this case only fragment p20 was visible, whose generation was reported to occur during or shortly after invariant chain synthesis[7]. The more efficient inhibition of γ-chain degradation observed when protease inhibitors were present during the whole experiment, demonstrates the release of cathepsin B and D from intracellular membrane systems, that were not accessible to the inhibitors on intact cells but became exposed by the solubilization procedure. Further evidence for proteolytic generation of γ-chain fragments within 3d cultured LC was obtained by in vitro treatment of mAb RG11-precipitates with cathepsin B, leading to a whole array of fragments including those described above (data not shown).

3d cultured LC were reported to show a decrease in antigen processing capacity[1]. This was described to be paralleled by disappearence of acidic processing organelles[8]. Combined with the downregulation of class II synthesis these data indicate, that antigenic peptide generation and peptide loading onto newly synthesized class II are shut off in 3 day cultured LC. The previous finding of an upregulation of invariant chain synthesis in 3d cultured LC[4] and results of this study showing a higher representation of invariant chain fragments in 3d cultured LC as compared to fresh LC demonstrate that intracellular invariant chain processing and antigen processing appear to be independently regulated in 3d cultured LC. In addition our data suggest that cathepsin B or a closely related SH-protease appear to be crucially involved in the processing of invariant chain and that this (these) enzyme(s) play only a marginal role in the processing of nominal antigen.

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FUNCTIONAL AND ULTRASTRUCTURAL ASPECTS OF ANTIGEN PROCESSING BY DENDRITIC CELLS

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INTRODUCTION

Major histocompatibility (MHC) class II molecules present peptides derived from exogenous antigens to $CD4^+$ T lymphocytes (reviewed [1,2,3]). The MHC class II molecule is a heterodimer of two transmembrane subunits, an α chain (33 kDa) and ß chain (29 kDa), both encoded in the MHC region[4]. The ends of the peptide-binding groove of MHC class II molecules are open so peptides can extend out. As a result, MHC class II-associated peptides have a length varying between 12-24 amino acids residues[5]. MHC class II molecules are primarily expressed on antigen presenting cells (APC), such as B cells, macrophages and dendritic cells (DC).

Since MHC class II molecules are evolved for the surface display of antigenic fragments derived from extracellular antigen, it is currently believed that the biosynthetic route of MHC class II molecules intersects the endocytic route to encounter internalized and degraded antigens[6]. Several studies have begun to identify the site of peptide loading of MHC class II molecules and the intracellular transport of MHC class II towards the cell surface. These studies have been performed with EBV-transformed B cells, B cell lymphomas, melanoma cells and activated peritoneal macrophages. In our study we determined the intracellular localization of MHC class II molecules in mouse spleen DC and human blood derived DC[7,8]. DC are specialized antigen presenting cells which are uniquely capable of inducing primary T cell immune responses (reviewed[9,10]). It is therefore of interest to study the intracellular class II distribution in DC in relation to the capacity to endocytose antigens. This manuscript shows data of the intracellular class II distribution in DC compared to the published intracellular MHC class II distribution in other types of MHC class II positive cells, such as B cells and macrophages.

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ASSEMBLY AND INTRACELLULAR TRANSPORT OF MHC CLASS II MOLECULES

The MHC class II α and ß chains associate in the endoplasmic reticulum (ER) with the 31-33 kDa invariant (I)-chain. Association of the I-chain with MHC class II α/ß dimer prevents the binding of endogenous peptides present in the lumen of the ER. Furthermore, binding of the I-chain induces efficient transport of α/ß dimer out of the ER. Cross-linking studies suggested that a nonameric complex of α, ß and I-chain is transported. After passage through the Golgi apparatus and the TGR where they are sorted to the endocytic route, the majority of the MHC class II α,ß/I-chain trimer are directed to a specialized endocytic compartment (reviewed[1,2]). As demonstrated by Lamb[11], the I-chain targets class II molecules to acidic endosomes containing influenza virus particles. Furthermore, formation of MHC class II-enriched compartments could only be established by transient transfection of class II together with I chain in COS cells[12]. Just before arriving at or in this compartment, the I-chain is dissociated from the α/ß dimer by proteolytic cleavage thereby restoring the peptide binding capacity of the MHC class II molecules. Once loaded with peptide, MHC class II molecules must find their way to the cell surface. It is unknown via which pathway this occurs.

The endocytic compartment exists as a complex network of tubulo-vesicular structures, including early endosomes, late endosomes, and dense lysosomes. In immunocytochemical studies, MHC class II molecules are found in post-Golgi, endosomal and lysosomal-related compartments. Peters[13] have shown compartments within the endosomal/lysosomal system in human EBV-transformed B cells, which are enriched for MHC class II molecules. These MHC class II-enriched compartments (MIIC) were characterized as mildly acidic and containing the typical lysosome-associated membrane proteins lamp-1 and CD 63 and ß-hexosaminidase.

Subcellular fractionation of B lymphoma cells, human melanoma cells and mouse activated peritoneal macrophages showed that fractions could be isolated which contained MHC class II molecules and the lysosomal markers Lamp-1[14,15,16] and ß-hexosaminidase[15,17] and were not positive for the endosomal marker CD-MPR[14,16]. The MHC class II enriched compartments were morphologically described as multivesicular structures with internal membranes and/or a dense content[14,16,17,18,20]. Intersection of this MHC class II enriched compartment with the endocytic route was visualized by the presence of an endocytic tracer[14,16,20]. Peptide loading of MHC class II molecules resulting in stable complexes can biochemically be visualized by SDS resistance[19]. In some studies stable MHC class II/peptide complexes resistant to denaturation by SDS could be demonstrated in these compartments[15,16,20]. Presence of peptide-MHC class II complexes in MIIC was also inferred from functional T cell responses[14,20]. Although internalization of MHC class Ii/peptide complexes from the plasma membrane has been reported and can not be excluded, the majority of peptide/MHC class II complexes are formed from newly synthesized MHC class II molecules[21].

INTRACELLULAR DISTRIBUTION OF MHC CLASS II MOLECULES IN DC

Mouse spleen dendritic cells were isolated using the standard method described by Steinman[22], with slight modifications. Our studies using immunoelectron microscopy showed that MIIC had either internal vesicles and/or a dense content with internal membranes. Using immunogold labeling on ultrathin cryosections of activated (cultured) mouse spleen DC, we found that MHC class II molecules were abundantly expressed on the plasma membrane. Compared to B cells, mouse spleen DC express eight times the amount of MHC class II (Ossevoort et al., unpublished results). Mouse spleen DC contained two times the number

of MIIC in comparison to B cells (Ossevoort et al., unpublished result). MIIC in mouse spleen DC have abundant expression of the lysosomal enzyme cathepsin D, the membrane protein lamp-1. Furthermore, these MIIC lacked the endosome-associated CD-MPR. In addition, we showed that the majority of the MIIC could be reached by the endocytic tracer BSA-conjugated to colloidal gold (BSA-gold)[7].

Human DC were isolated from peripheral blood mononuclear cells (PBMC) by negative selection for T cells, B cells, monocytes, NK cells and granulocytes[8]. Freshly isolated human DC (f-DC) with an 80 - 85 % purity could be obtained. Our study showed that f-DC displayed short cytoplasmic processes by immunoelectron microscopy. Isolation of human DC from PBMC with an additional culturing step (c-DC) resulted in a display of long cytoplasmic processes. Immunogold labeling on ultrathin cryosections demonstrated that c-DC displayed a denser MHC class II expression on the plasma membrane as well as intracellularly compared to f-DC. Intracellularly, the MHC class II labeling in both cell types was concentrated in typical MIIC's with either internal vesicles and/or membrane sheets. Further characterization of MIIC demonstrated that MIIC in f-DC and c-DC have abundant expression of the lysosomal enzymes lamp-1 and CD63 and were mildly acidic. In addition, the MIIC could be reached by the endocytic marker BSA-gold[8].

These data demonstrated that MIIC in mouse spleen DC and human peripheral blood derived f-DC and c-DC are late endocytic prelysosomal compartments as shown in other APC, such as human EBV-transformed B cells and peritoneal activated macrophages.

ANTIGEN PROCESSING AND PRESENTATION BY DC

In agreement with the competent internalization of the endocytic tracer BSA-gold[7] and rhodamine dextran[23], it was shown in a previous study that mouse spleen DC can efficiently process intact HEL protein and present the antigenic peptide to induce a specific T cell response[7,24]. The kinetics of antigen processing and presentation were measured by the arrival of a significantly amount of peptide/MHC class II complexes on the cell surface capable of inducing a specific T cell response. A specific T cell response induced by protein-pulsed DC could be measured 120 min after adding the protein. LPS activated B cell blasts showed similar kinetics of antigen processing and presentation, although more LPS activated B cell blasts were needed to obtain an optimal response[7,24]. Already after 30 min of HEL uptake, presentation of HEL protein by mouse activated peritoneal macrophages was demonstrable[14]. This can largely be contributed to the fact that macrophages have a higher endocytotic capacity than DC. Although HEL protein reaches the MIIC after 60 min, it is under current investigation if peptide/MHC class II complexes are derived from MIIC and reach the cell surface after a lag period.

CONCLUDING REMARK

Comparing the morphology and the characteristics of MHC class II-enriched compartments in different MHC class II positive cells, such as DC and macrophages and B cells, it seems that MIIC are very similar in the different cell types, although different technics are used to identify MIIC. Apart from variation in number of MIIC present in different cell types, additional qualitative differences can not be excluded.

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PROCESSING AND PRESENTATION OF PROTEIN AND PARASITE-DERIVED ANTIGENS BY 4F7$^+$ DENDRITIC CELLS

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INTRODUCTION

The dendritic cell (DC), a trace component of splenocytes is the principal cell type required for a primary mixed lymphocyte reaction.[1] Splenic DC are described as antigen presenting cells (APC) capable to generate immunogenic fragments of intact protein antigens for presentation to MHC class II restricted T cells,[2] in contrast to former findings.[3] Langerhans cells (LC) as potent APC are members of the dendritic cell lineage forming a system of potent APC, first identified by Steinman and Cohn.[4] Importanly Schuler and Steinman have subsequently presented experimental evidence suggesting that LC represent immature DC.[3] During infectious diseases caused by parasites of the genus *Leishmania* LC of skin capture, process, and transport antigen to the regional lymph nodes for subsequent presentation to specific T cells.[5] LC also play a critical role as APC in promoting contact hypersensitivity reactions. In order to obtain monoclonal antibodies (mAb) for characterization of DC involved in the induction and elicication of allergic contact dermatitis DC-specific mAb were selected. Rats were immunized with Ia$^+$ epidermal cells from BALB/c mice epicutanously treated with the contact sensitizer 2,4-dinitrofluorbenzene. The mAb 4F7 was generated and was found to recognize an epitope expressed on DC from various tissues.[6,7,8]

In this study we have investigated the capacity of highly purified freshly 4F7$^+$ DC, isolated from the spleen of BALB/c mice to present ovalbumin (OVA) and *Leishmania major* (*L. major*)-derived antigens. 4F7$^+$ DC were found to activate OVA/I-A^d restricted T cells in an antigen-dependent manner. This presentation is based on the uptake and proteolytic cleavage of the native antigen within the cell. In contrast to B cells DC present antigens derived from inactivated *L. major* promastigotes as described for macrophages (Mph).

RESULTS AND DISCUSSION

DC were prepared preventing overnight culture by using moAb 4F7 as described previously.[8,9] Spleen cell suspensions were depleted of B cells and Mph using magnetic beads specific for mouse-immunoglobuline (Ig) and rat-Ig loaded with mAb F4/80 respectively. The resulting cell suspension was positively selected for DC with sheep anti-rat-Ig beads bound to mAb 4F7. Preparations showed expression of MHC class II, 33D1, and HSA molecules. No

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staining of molecules located on B cells, granulocytes, Mph or T cells could be detected. To exclude contamination with T- or B cells the lipopolysaccharide- and concanavalinA-reactivity of the enriched 4F7$^+$ DC was investigated.[9]

We determined the number of highly enriched splenic 4F7$^+$ DC and spleen derived B cells as well as Mph required for optimal activation of the I-A^d-restricted 3DO-54.8 T cell line using OVA or OVA-peptide as antigen. DC presented both antigens as efficient as Mph and better than B cells (Fig. 1). As the amount of 4F7$^+$ DC within this preparation was above 93% it seems unlikely that contaminating Mph or B cells did account for the antigen presentation observed. Low numbers of DC were found to be sufficient for presentation. Titration of antigen revealed that the capacity of 4F7$^+$ DC to process OVA was comparable to Mph but more effective than B cells. No presentation of native proteins was obtained by fixed DC indicating that intracellular processing is required for presentation.[9]

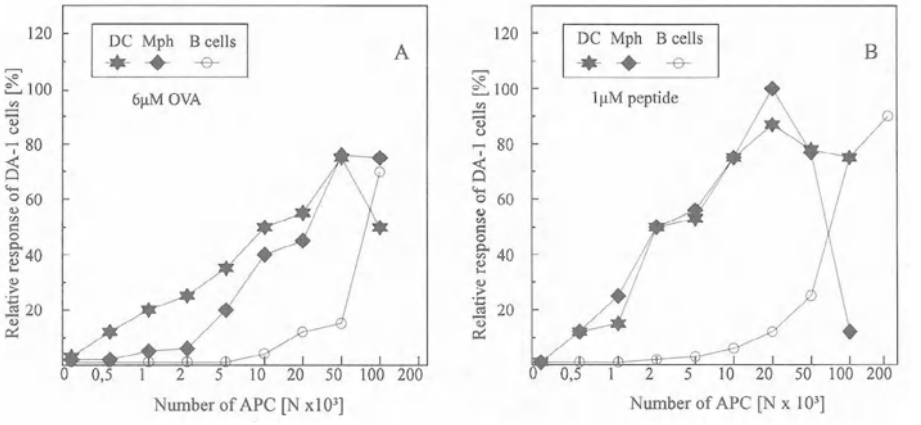

Figure 1. Titration of different APC for presentation of OVA and its antigenic peptide. Splenic 4F7$^+$ DC, B cells or Mph were cocultured with T hybridoma cell line 3DO-54.8 (2x10^4/well) in the presence of OVA (**A**) or the OVA-peptide (**B**) for 48h. 3DO-54.8 is specific for I-A^d and OVA-peptide corresponding to aa323-339. Cultures were assayed for their IL3-content using the indicator cell line DA-1 (4x10^3/well). Maximal response of DA-1 cells: 12 373 cpm; Medium control: 211 cpm.

After pulsing of 4F7$^+$ DC with native OVA in the presence of the lysosomotrophic drugs chloroquine or cystamine no activation of T cells was observed (Fig. 2A). Therefore intracellular degradation in acidic compartments like endo- or lysosomes must be involved. This capacity of 4F7$^+$ DC is also influenced by culture of DC. Freshly isolated cells induced optimal lymphokine production in CD4$^+$ T cells but culture of cells led to continuous lost of OVA-processing, while the presentation of processing-independent, and MHC II-binding OVA-peptide is not reduced.[9]

We further investigated the ability of 4F7$^+$ DC to process an antigen derived from *L. major* parasites. 4F7$^+$ DC were as Mph excellent stimulators of *L. major* specific Th2-type clone L1/1.[10] In contrast B cells failed to induce lymphokine production of T cells using complete parasites.Therefore it seems likely that splenic DC of BALB/c mice are potent APC for *L. major*-derived antigens (Fig.2B).

The processing capacity of DC had been discussed for a long time. Recent data show conflicting results which may be based on the heterogenity of DC populations. Our experiments show that 4F7$^+$ DC are in principle able to process antigens that depends on their physiological state which is influenced by culture. Nevertheless 4F7$^+$ DC used *ex vivo* did not only act as potent stimulator cells for allogenic[8] and syngenic stimulation of naive T cells (A.P. unpublished), but also process exogenous protein antigen, irrespective of their processing requirements. This feature of 4F7$^+$ DC decline upon culture, possibly reflecting maturation of cells.[3]

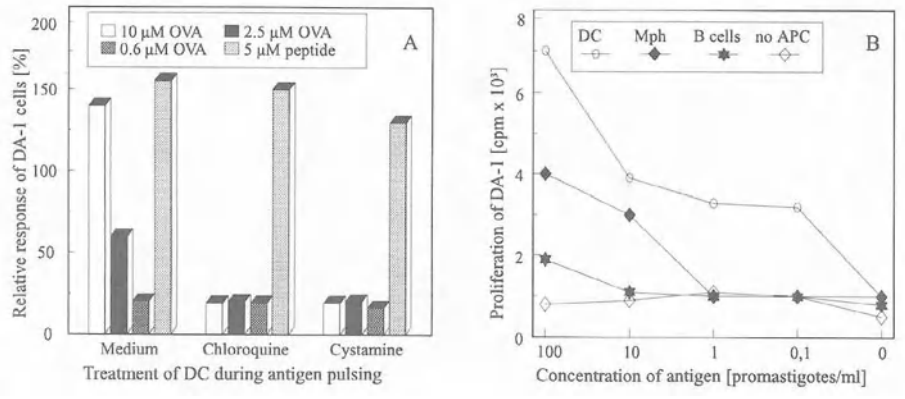

Figure 2. Intracellular processing is involved in presentation of OVA by DC. APC ($5x10^5$/well) were pulsed for 12h with antigen in the presence or absence of cloroquine (150µM) or cystamine (1mM). In control cultures OVA-derived peptide was added to inhibitor-treated or untreated DC. After incubation, cells were fixed with 0,05% glutaraldehyde for 40s. 10^5 cells/culture were used to stimulate OVA-specific 3DO-54.8 T cells ($2x10^4$/well). Supernatants were tested for their IL3-content. Maximal proliferation of DA-1 cells: 13 519 cpm; without growth factors: 125 cpm (**A**). $4F7^+$ DC stimulate *L. major* specific Th2-cells in an antigen specific manner like Mph. APC ($1x10^4$/well) were cocultured with T cell clone L1/1 ($2x10^4$/well) in the presense of different concentrations of inactivated *L. major* promastigotes. Supernatants of cultures were harvested after 24h assayed for IL3 (**B**).

ACKNOWLEDGEMENTS

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DENDRITIC CELLS MIGRATING FROM CARCINOGEN-TREATED SKIN HAVE REDUCED ANTIGEN-PRESENTING FUNCTION

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INTRODUCTION

Chemical carcinogens have a recognised ability to reduce the status of cutaneous immunity. Antigen presentation through carcinogen-treated skin results in immune suppression rather than activation of effector mechanisms (reviewed by [1]). Topical application of the complete chemical carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) leads not only to the development of skin tumours but also induces a rapid depletion of Langerhans cells (LC) from the epidermis - up to 50% of the LC are depleted from the epidermis in the 3-4 days following DMBA application [2].

To define the mechanism of this LC depletion, sheep prefemoral pseudoafferent lymphatic vessels draining a defined area of skin were cannulated to allow direct sampling and enumeration of LC migration. These experiments demonstrated that LC depletion a result of topical application of DMBA is due to a massive increase in the migration rate of LC from the skin towards the lymph node [3].

Since dendritic cells (DC), which include the LC, continue to migrate from the carcinogen-treated skin despite a reduction in the status of cutaneous immune status, the functional capacity of these migrating DC requires definition. The aim of this study was to analyse the antigen-presenting ability of DC migrating from carcinogen-treated sheep skin.

MATERIALS AND METHODS

Animals

Prefemoral pseudoafferent lymphatic vessels were generated and cannulated in outbred Polwarth/Comeback sheep as described previously [3,4]. Briefly, prefemoral lymph nodes were removed and the sheep rested for 6-8 weeks to allow the afferent lymphatics to reanastamose with the remaining efferent duct. During this period, animals were immunised to the antigen ovalbumin (OVA; Sigma) by subcutaneous intrascapular injection of an alum precipitate. Lymphography dye was injected into the prefemoral drainage area and pseudoafferent lymphatics carrying the dye progressively ligated and cannulated. Afferent lymph was collected from fully conscious animals.

Application of Carcinogen

Sheep were treated with a single 700 µl topical application of chemical carcinogen DMBA (1% w/v in lanolin/paraffin vehicle; Sigma, USA) to an area of approximately 100 cm^2 of closely shaved flank skin drained by the cannulated pseudoafferent lymphatic vessel. Control animals were treated with the lanoline/paraffin vehicle only. This dose of carcinogen is the same as that used in

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our previous studies [2,3]. The effect of DMBA on DC function was assessed in 3 separate experiments.

Antigen-Presentation Assay

DC were enriched from afferent lymph by density gradient centrifugation over 14.5% (w/v) metrizamide (Nyegaard, Oslo) [5] in RPMI-1640 (Hyclone, Logan, USA) and pulsed for 1 hour with OVA (1mg/ml in RPMI). Control DC were incubated in RPMI only. Antigen-pulsed and control DC were washed twice with PBS and treated with mitomycin C (Kyowa, Tokyo, Japan; 25 μg/ml) for 20 minutes at 37°C. DC were subsequently washed twice in PBS and resuspended in complete medium (RPMI+5% foetal calf serum+2mM L-glutamine+$5x10^{-5}$M 2-mercaptoethanol+ 100U/ml gentamicin) at 1 x 10^6 /ml. DC were co-cultured with autologous peripheral blood mononuclear cells (PBMC) at various DC:PBMC ratios for 5 days at 37°C / 5% CO_2. Cultures were pulsed with 0.5μCi [^{3}H]thymidine (Amersham Life Sciences, Australia) over the last 16 hours of culture and collected onto glass filter paper using a semi-automated harvester. [^{3}H]thymidine incorporation was assessed by liquid scintillation counting. The data are expressed as the mean of the triplicate cultures with standard deviation.

RESULTS

Antigen-Specific T-Cell Proliferation Induced by Afferent Lymph DC

The ability of afferent lymph DC to present antigen to and stimulate proliferation of peripheral blood T cells was investigated (Figure 1). *In vitro* pulsing of DC with the antigen OVA prior to culturing with PBMC resulted in a significant and substantial increase in T cell proliferation compared to control experiments. Maximal stimulation occurred when antigen-pulsed DC and PBMC were co-cultured at a ratio of 1:8 respectively, as determined by the stimulation index (SI)

Effect of DMBA on Antigen Presentation by Afferent Lymph Dendritic Cells.

The application of DMBA to the skin in the drainage area resulted in an immediate decrease in presentation of OVA to T cells as evidenced by the substantial decrease in the SI of DC collected 12 hours post-treatment (Figure 2). However, this initial abrogation of DC function was only temporary as DC collected at subsequent time-points exhibited increasing stimulatory capacity with a return to pre-treatment SI by 36 hours post-treatment. A second reduction in DC functional capacity was observed 72 hours after topical DMBA application. The SI continued to decrease until, by 144 hours post-treatment, there was no detectable antigen-induced proliferation of T cells by afferent lymph DC. This total loss of the accessory function of DC migrating from DMBA-treated skin was long-term and sustained. It was not until 8 weeks post-treatment that DC migrating from the treated skin were able to stimulate proliferation of peripheral blood T cells, with a return to pre-treatment functional capacity not occurring until 10 weeks.

Effect of Carcinogen Vehicle on DC Function

To ensure that the second, sustained loss of DC antigen-presenting function was due to the effects of the chemical carcinogen DMBA and not a result of reduced peripheral T cell immunity to OVA or an effect of the lanoline/paraffin vehicle, an experiment was performed in which both the left and right prefemoral pseudoafferent lymphatics of a sheep were successfully cannulated. The right side drainage area was treated with DMBA whereas the left side drainage area was simultaneously treated with the carcinogen vehicle. The results for the DMBA-treated side confirmed the earlier experiment. DC migrating from contralateral vehicle-treated sheep skin did not exhibit any reduction in their functional capacity to present antigen to T cells during the time course of the experiment (Figure 3).

DISCUSSION AND CONCLUSIONS

The ability of metrizamide-enriched, OVA-pulsed afferent lymph DC to stimulate primed T cell proliferation upon co-culture with autologous PBMC has been utilised in this study as a measure of DC antigen-presenting function. Utilising this system we have assessed the functional capacity of DC that have migrated from carcinogen-treated sheep skin.

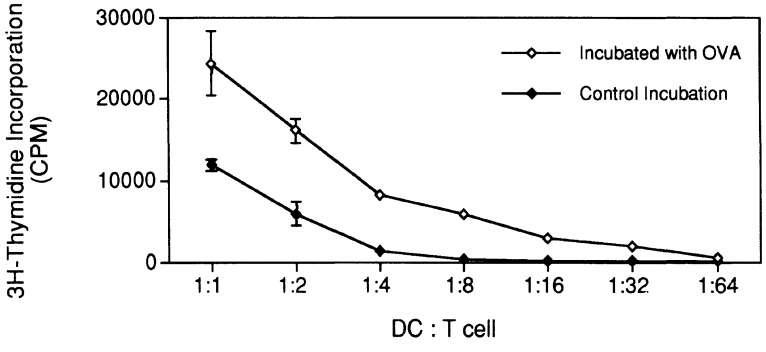

Figure 1 . *In vitro* pulsing of afferent lymph DC with the antigen OVA significantly increases T cell proliferation.

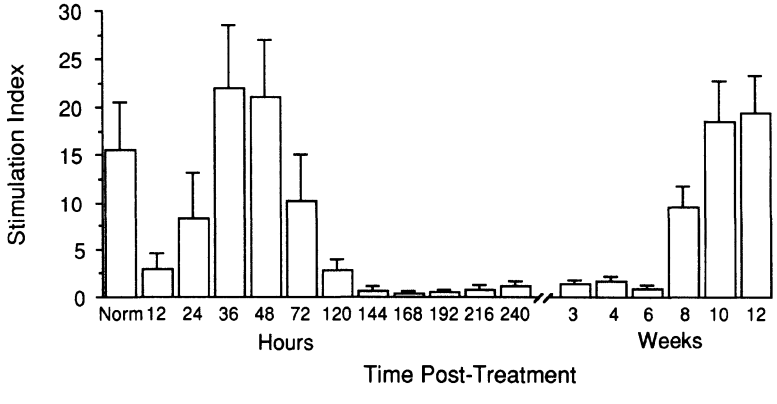

Figure 2. Afferent lymph DC function is abrogated by topical application of the carcinogen DMBA. Stimulation indices were calculated at the 1:8 DC:T cell ratio. Norm = pre-treatment control.

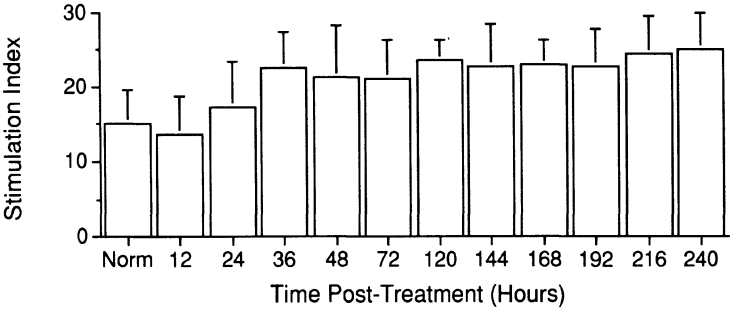

Figure 3. The lanoline/paraffin vehicle does not affect afferent lymph DC function Stimulation indices were calculated at a 1:8 DC:T cell ratio. Norm = pre-treatment control.

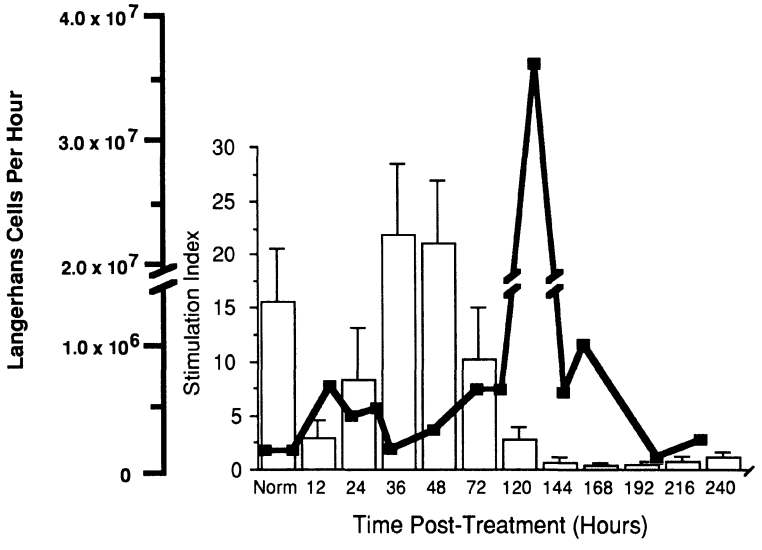

Figure 4. Correlation of LC migration and afferent lymph DC function after topical application of the carcinogen DMBA. LC migrating via pseudoafferent lymphatics were enumerated using indirect immunofluorescence and flow cytometry. Norm = pre-treatment control.

We propose that the initial failure of DC to present OVA to T cells may be a result of antigenic competition[6] since DC migrating from the skin in the first 24 hours post-treatment may be carrying carcinogen as antigen and therefore will not bind, process and present OVA. As a contact hypersensitivity response can be generated to cutaneously applied DMBA[7] this compound obviously has antigenic properties and it is well documented that DC perform the critical step of antigen presentation during the induction of hypersensitivity responses [8,9].

The timing and duration of the second, sustained loss of DC function following topical DMBA application makes it highly improbable that this is due to antigenic competition. Suggested mechanisms operating during this period may include downregulation of DC adhesion molecule expression, inhibition of IL-1 production or the synthesis of immunosuppressive cytokines either by DC or keratinocytes in the DMBA-treated skin.

The lanoline/paraffin vehicle does not affect DC function, a finding in line with previous experiments that have found that this vehicle does not alter LC density [2], the migration of LC from the skin [3] or the status of cutaneous immunity [10]. Therefore the reductions in DC function are a consequence of DMBA treatment and are not due to diminished peripheral T cell immunity to OVA. Additionally, this experiment has demonstrated that the impairment in DC antigen presentation is localised and confined to cells in the DMBA-treated area.

Correlation of the changes in LC migration and afferent lymph DC function that result from cutaneous application of DMBA are depicted in Figure 4. The immediate decrease in DC function parallels the primary moderate increase in LC migration, a finding which lends support to the proposal that these initial changes are related to the antigenic properties of DMBA. The massive increase in LC migration at 120 hours was accompanied by a sustained abrogation of DC function, a combination that has adverse implications for immuno-surveillance against emerging cutaneous neoplasms.

In conclusion, the complete chemical carcinogen DMBA reduces the status of cutaneous immunity by impairing the functional capacity of epidermal DC. In DMBA-treated skin, decreased numbers of functionally impaired DC are present during tumour formation, a situation which is indicative of severely compromised cutaneous immunity.

ACKNOWLEDGMENTS

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INHIBITORY EFFECTS OF ULTRAVIOLET B ON HUMAN LANGERHANS CELL ANTIGEN PRESENTING FUNCTION

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INTRODUCTION

Ultraviolet B (UVB) radiations are known to suppress induction of cutaneous immune responses. Since these radiations are almost completely absorbed within epidermis, epidermal cells were considered as potential targets for their various effects. Among epidermal cells, interest was focused on Langerhans cells (LC) because these cells are the antigen presenting cell of epidermis and play a key role in contact hypersensitivity reactions. In the murine models, UVB-induced immunosuppression has been described to both direct effect on LC function[1] and/or to indirect effect through the liberation of keratinocyte-derived factors[2]. In human being, only few data are available concerning the mechanisms involved in this process. Here, we analysed the effects of narrow-band of UVB radiation (312 nm) on human LC antigen presenting function by using the mixed epidermal cell lymphocyte reaction (MELR).

RESULTS

UVB Radiation Acts Directly on Human Langerhans Cell Antigen Presenting Function

We first asked whether UVB radiation inhibits allostimulatory function of epidermal cells suspensions by acting directly on LC. To this end, we compared the capacity of irradiated epidermal cells suspensions, containing few LC or almost completely composed of LC, to induced allogeneic T cell proliferation.
Epidermal cell suspensions were either partly enriched (eLC: 5-10% LC) or purified (pLC: 70-90% LC) for LC by successive density gradient centrifugations. Both type of suspensions were exposed to a single dose of UVB radiation (12 to 200 J/m^2) and then increasing numbers of viable LC were added to allogeneic T cells. T cell proliferation was assessed by [^{3}H] thymidine incorporation during the last 18 hr of culture. As shown in Figure 1, *in vitro* exposure to UVB radiation inhibits the allostimulatory property of both eLC and pLC (about 50% and 90% inhibition with 100 and 200 J/m^2 respectively), and inhibition of MELR was not more pronounced using irradiated eLC, as compared to irradiated pLC. A comparable pattern of inhibition was obtained in mitogen or recall antigen induced T cell responses. These results suggest that *in vitro* UVB radiation acts primarily on LC and not on keratinocytes to inhibit human LC antigen presenting function.

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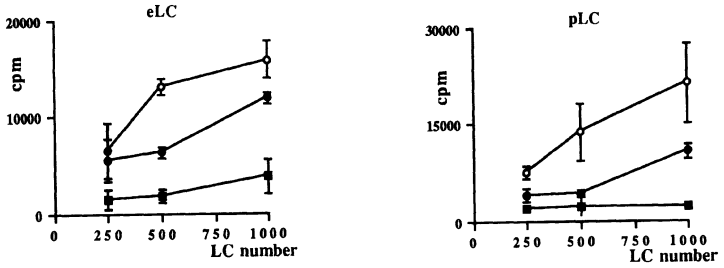

Figure 1. UVB radiation inhibits the allostimulatory function of both eLC and pLC. eLC and pLC from the same donor were exposed to a single dose of UVB radiation (312 nm). After washes, cells were numerated and increasing numbers of viable LC (250 to 1000) were co-cultured with 10^5 allogeneic T cells. T cell proliferation was assessed by [^{3}H] thymidine incorporation for the final 18 hr of culture. Results are expressed as the mean cpm ± SD of triplicate culture. Unirradiated cells (open circle), cells exposed to UVB radiation at 100 J/m^2 (closed circles), 200 J/m^2 (closed squares).

It may be possible that, upon irradiation, keratinocytes or LC released soluble factors that could be, at least in part, responsible for *in vitro* inhibition of T cell proliferation. We showed, however, that supernatants (SN) from UVB-irradiated eLC, that have been cultured for 18h or 48h, did not suppress T cell proliferation to untreated pLC (data not shown). Furthermore, addition of 200 J/m^2-irradiated eLC to MELR cannot inhibit the allogeneic T cell response to untreated pLC (Fig 2). These results provide evidence that *in vitro* UVB-induced immunosuppression was not mediated by suppressive soluble factors and that UVB radiation acts directly on LC to inhibit their allostimulatory function.

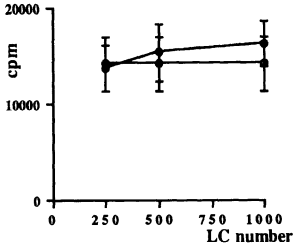

Figure 2. UVB induced immunosuppression in MELR is not mediated through soluble inhibitory factors. 10^3 pLC were co-cultured with 10^5 allogeneic T cells, in the presence (closed circle) or absence (open circle) of graded number of UVB-irradiated eLC from the same donor. T cell proliferation was assessed by [^{3}H] thymidine incorporation for the final 18 hr of culture. Results are expressed as the mean cpm ± SD of triplicate culture.

In additional experiments, we have tested SN from irradiated long-term keratinocyte cultures: undifferenciated keratinocyte monolayer or pluristratified epithelium. No inhibition was observed with any SN (data not shown), suggesting that upon UVB radiation human keratinocyte cultures were quite unable to release immunosuppressive factors.

UVB Radiation Does Not Prevent Up-Regulation of HLA-DR Expression on Human LC After a Short *In Vitro* Culture

We analysed the level of HLA-DR expression on human LC immediately after UVB exposure or 18 hr later. We first found that prior exposure to UVB radiation neither affects the percentage of HLA-DR positive cells, nor the level of HLA-DR expression on freshly isolated eLC suspensions. As reported earlier[3], we observed a strong up-regulation of HLA class II antigens on human LC after culture in medium alone. Exposure to UVB did not prevent this up-regulation although the number of highly positive cells was slightly lower

than that observed on sham-irradiated eLC. Furthermore it did not alter the percentage of HLA-DR positive cells recovered after the 18 hr incubation (data not shown). It is unlikely, therefore, that the decrease of UVB-irradiated LC allostimulatory function only reflected a decreased expression of HLA-DR expression at the human LC surface.

Two-Day Cultured human LC Are Less Sensitive to UVB Exposure Than Freshly Isolated LC

Cultured human LC have been reported to display higher levels of accessory molecules such as ICAM-1 and LFA/3 that might explain their enhanced capacity to promote T cell responses[3,4]. We have compared the effects of UVB exposure on the allostimulatory function of both freshly isolated and 2-day cultured LC. eLC suspensions were incubated for 2 days in medium alone and viable LC were then recovered on Lymphoprep before exposure to UVB radiation. As reported above, exposure of freshly isolated LC to UVB radiation at 100 J/m^2 caused significant inhibition of allogeneic T cell response whereas exposure at 200 J/m^2 almost completly abrogated the proliferative response. By contrast, 2-day cultured LC appeared less sensitive to UVB deleterious effects than freshly prepared LC. As shown in Figure 3, irradiation at 100 J/m^2 did not affect the allostimulatory property of 2-day cultured LC and exposure at 200 J/m^2 still allowed substancial T cell proliferation. This suggests that deleterious effect of UVB on human LC allostimulatory function may be related, at least in part, to impaired development of their accessory function.

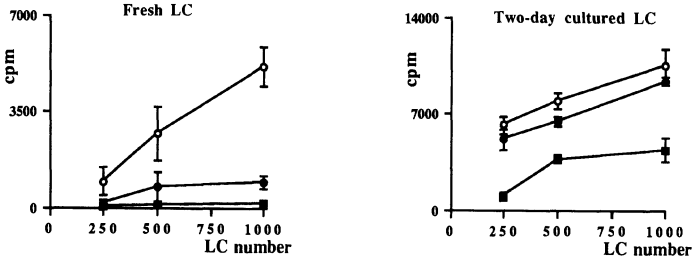

Figure 3. Two-days cultured human LC are less sensitive to UVB radiation than fresh LC. eLC were UVB-irradiated either immediately after isolation from the epidermis or after a 2-day culture in medium. Increasing numbers of viable LC were assayed in MELR and T cell proliferation was assessed 6 days later. Unirradiated cells (open circle), cells exposed to UVB radiation at 100 J/m^2 (closed circles), 200 J/m^2 (closed squares).

CONCLUSIONS

Taken together these results demonstrated that *in vitro* exposure to narrow-band of UVB radiation directly inhibits antigen presenting function of human LC. Furthermore, the deleterious effect is independent of the action of soluble suppressive factors and seems more likely related to impaired development of LC accessory function.

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PHENOTYPE OF CELLS MIGRATED FROM HUMAN SKIN EXPLANTS

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INTRODUCTION

Dendritic cells (DC) are very efficient antigen presenting cells, distributed in many tissues and organs (1). In the skin, they are characterized by CD1a expression and the most extensively studied cell among them is the epidermal Langerhans cell. However, the dermis contains also a population of CD1a+ cells with potent antigen presenting capacities (2, 3). It was described by Larsen et al. (4) that Ia+ cells migrated "spontaneously out of murine ear skin into the medium during culture.

In previous experiments (5), we were able to obtain human skin dendritic cells making use of their migratory capacities (skin explant culture model). Besides the CD1a+ DC, we observed that the migrated cell population consists of non-adherent macrophages and T cells that often cluster with the DC. In this report, we studied the immuno-phenotype of the migrated T cells and the T cells that clustered with DC.

MATERIALS AND METHODS

Normal human split skin (0.3-0.4mm), was obtained from six women undergoing corrective plastic surgery. The skin was floated on DMEM supplemented with 10 % FSC and cultured for three consecutive days in plastic petri-dishes. After each 24 h of culture, the medium containing the migrated cells was collected and the skin was replaced into fresh medium. The cells were spun down, counted and the viability was always > 95%, as determined by trypan blue exclusion. Thereafter, cyto-centrifuge preparations were made to study the immuno-phenotype of the cells. The indirect APAAP method described by Cordell et al. (6) was used to visualize the different antigens present. In some experiments, cell clusters were separated from single cells by 1-g sedimentation.

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Table 1. Percentages of positive cells in the migrated cell population

	Day 1 cells	Day 2 cells	Day 3 cells
CD3	17-40	19-45	23-48
CD1a	60-75	50-65	40-55
CD14	5-9	5-10	4-10
clusters	10-15	20-30	25-35

Table 2. Percentage of migrated T cells positive for the specific antigen.

	Specificity	Day 1	Day 2	Day 3
CD4	Helper T cells	12-30	14-35	16-37
CD8	Cytotoxic T cells	4-10	5-10	5-12
CD25	Il-2 receptor	12-14	13-14	9-13
CD45RO	Memory T cells	50-70	75-85	58-75
Heca 452	Cutaneous lymphocytes	44-49	48-52	44-50

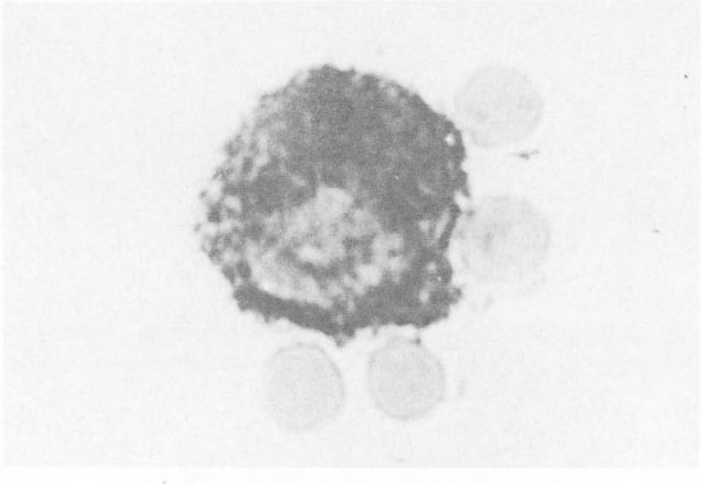

Figure 1: CD1a+ cell isolated on day 3, with clustered T cells.

RESULTS

Table 1 shows the changes in the cellular composition of the migrated cell population during the three different days of culture. The number of CD1a+ cells that had clustered 2-5 T cells (fig. 1) is increasing. The clustered cells were isolated from the non-clustered, it was not possible to dissociate the clusters with EDTA and vigorous pipetting. The isolated clusters were placed into culture for 6 days. However, they did not proliferate as determined by measuring 3H-Thymidine incorporation. A high response was measured when allogeneic T cells were added.

Table 2 summarizes the immuno-phenotype of the migrated T cells. The phenotype of the T cells that had clustered with CD1a positive DC was similar to the other migrated T cells. The DC were also positive for CD25 (80%) and sometimes expressed HECA 452 (2-7%).

DISCUSSION

In normal human skin in situ, many T cells are present, with an CD4/CD8 ratio of 1:1 (7). Our results show that predominantly CD4 positive T cells migrate out of human skin during culture (CD4/CD8; 3:1). The immunologic relevance of this observation is not yet clear. It appeared that the distribution of phenotype of the migrated T cells positive for the Heca 452 antigen is similar as described for T cells observed in normal skin in situ, not cultured (8). Most T cells are memory cells as shown by the expression of CD 45RO. Some of the T cells are in an activated state, i.e. they express the Il-2 receptor (CD25). It has been shown that DC exhibit a very strong capacity to cluster with T cells in vitro (9). This also occurs in our human skin explant culture model.

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We wish to thank Mr. S. Paniry for preparing the photograph. This works was financially supported by the Dutch Burns Foundation.

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UPTAKE OF BEAD-ADSORBED VERSUS SOLUBLE ANTIGEN BY BONE MARROW DERIVED DENDRITIC CELLS TRIGGERS THEIR ACTIVATION AND INCREASES THEIR ANTIGEN PRESENTATION CAPACITY

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INTRODUCTION

The property to internalize particles has for long time been ascribed primarily to macrophages. DC in contrast were considered generally as phagocytosis negative. Fully mature DC which can be isolated from various tissues of the body do indeed not take up particulate material; however immature DC which arise in differentiating bone marrow cultures do exhibit phagocytic capacity[1]. Consistent with their immature phenotype epidermal Langerhans cells were also described to possess phagocytic potential[2].

In this study we focused on the antigen presentation capacity of the phagocytically active progenitor DC. Dendritic cells were prepared from GM-CSF supplemented mouse bone marrow cultures[3] and their capacity to engulf particulate material was examined. Antigen was administered in microparticle-adsorbed and in soluble form and antigen specific T cell stimulation was assessed. It was found that antigen-pulsed Dendritic cells were far more stimulatory to T clone cells when the antigen used for pulsing was particle-adsorbed instead of being applied in soluble form.

RESULTS AND CONCLUSION

Preparation of antigen loaded microparticles for antigen presentation

FITC-labeled polystyrene beads which are in use as calibration beads for FACscan were employed as antigen carrier. Conalbumin (CA) was used as nominal antigen. One mg of the protein was incubated with 4.5×10^8 FITC-labeled polystyrene microparticles for 2h at 4° C. Unbound CA was removed by 5 cycles of washing using MEM supplemented with 5% FCS at ambient temperature. The amount of bead-adsorbed CA was estimated by photometric analysis. It was found that one particle had adsorbed roughly 0.4 picogram CA. Microscopic

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analysis revealed that phagocytic DC internalize on the average of 3 to 5 polystyrene particles per cell.

Phagocytosis enhances antigen presentation capacity

A series of antigen pulse-chase experiments was performed to test the potential of phagocytosis positive bone marrow derived Dendritic cells to process and present bead-adsorbed CA. The result was compared with that obtained by conventional antigen presentation testing namely pulsing with soluble CA. Thus day 6 bone marrow cultures were pulsed for 6 h with either 10 CA-loaded particles per cell corresponding to 2.5 µg CA/ml or 50µg soluble CA. Excess of antigen was removed by washing and the cells were placed back into culture without antigen for the chase times 6h, 12h, and 18h. Finally total bone marrow cells were harvested and depleted of macrophages by F4/80-mediated immunomagnetic bead selection. The F4/80 negative population containing most of the antigen pulsed dendritic cells was irradiated and cocultured with conalbumin specific T clone cells D10G4.1[4]. The uptake of ^{3}H-TdR by specifically stimulated T clone cells was estimated. The results are summarized in Figure 1.

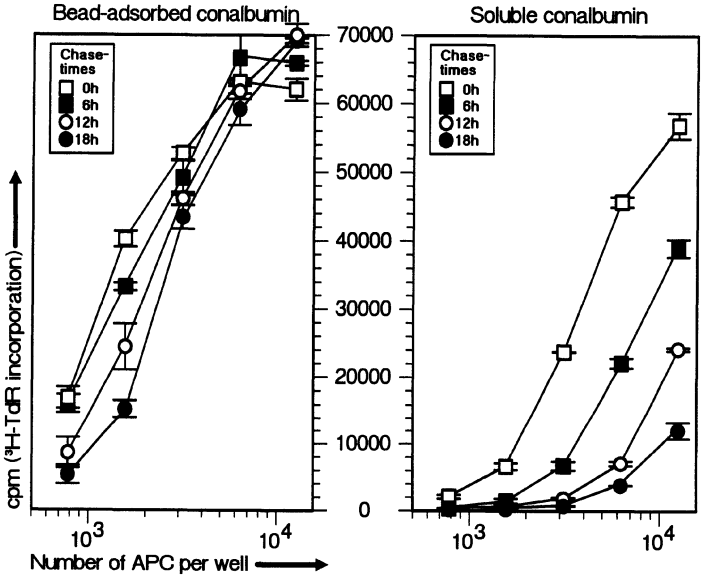

Figure 1. Comparison of the T cell stimulatory capacity of Dendritic cells pulsed with conalbumin in bead-adsorbed or soluble form following various periods of chase.

It is clearly evident, that progenitor DC pulsed in vitro with bead-adsorbed CA are far more stimulatory than DC pulsed with soluble antigen. Considering the fact that 20 times less CA was added to the cultures when bead-adsorbed CA was given, this finding is remarkable. Moreover progenitor DC pulsed with bead-adsorbed CA retain their stimulatory capacity much longer than DC given soluble CA. From these data it can be concluded that the physical state of the antigen given to DC either in particle-adsorbed or soluble form determines the route of antigen acquisition by the DC (i.e. phagocytosis or pinocytosis) and thereby greatly influences the outcome of the immune response. Uptake of particle-adsorbed antigen by phagocytosing DC triggers a number of activation events like upregulation of IL1α (not shown) which entail superior and prolonged presentation capacity of the DC.

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ECTROMELIA VIRUS ESTABLISHES A PERSISTENT INFECTION IN SPLEEN DENDRITIC CELLS AND MACROPHAGES OF BALB/c MICE FOLLOWING THE ACUTE DISEASE

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INTRODUCTION

The EV initially replicates in the skin and regional lymph nodes of mice. Next, it reaches the spleen and liver what might lead to death of mice due to irreversible damage of these organs[1,2]. However, some mice can survive acute disease (at peak between 5 and 10 days p.i.), and harbour EV for several months. To understand how EV avoid immune surveillance and persist, we have studied spleen dendritic cells (DC), macrophages (Mø), T and B lymphocytes of the BALB/c mice up to 60 days p.i. The number of cells harbouring EV from each subpopulation was determined by an infectious center assay.

MATERIALS AND METHODS

Mice, virus and cell preparation

BALB/c (H-2^d) mice, 4 - 6 weeks old, were injected via footpad with Moscow strain of EV (kindly received from Dr. R. Mark L. Buller, NIAID, NIH, Bethesda, MD, USA; present address: Saint Louis University Health Sciences Center, St. Louis, MO, USA) and the spleens were removed at 5, 10, 15, 20, 30, and 60 days p.i.

Splenocytes obtained from infected mice and naive controls were treated with ammonium chloride Tris buffer to deplete red blood cells. Next, the cells were separated into metrizamide-gradient derived DC, plastic-adherent Mø, immunoglobulin (Ig)-positive cells (B lymphocytes) and Ig-negative cells (T lymphocytes) using rabbit a-mouse Ig (Cedarlane Lab., Hornby, Ontario, Canada)-coated plates[3] (Figure 1). DC were identified by their distinctive morphology and ATP-ase staining and were more than 65 % pure.

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Infectious center assay (ICA)

The number of splenocytes harbouring EV from each cell subpopulation was determined by an ICA[4]. Briefly, permissive Vero cells were added to the DC, Mo, T and B cells, and co-cultivated for 5 days in a 5 % CO_2-95 % air at 37°C (Figure 1). After 5 days the monolayers were fixed in 10 % formaldehyde in PBS and stained with 1 % toluidine blue.

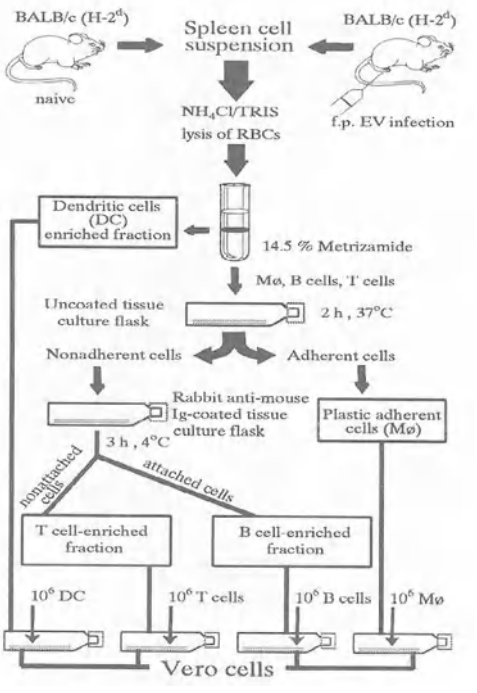

Figure 1. Schematic diagram illustrating the separation of splenic DC, Mø, T and B cells, and ICA.

Fluorescence analysis and transmission electron microscopy (TEM)

The fluorescence of splenocytes obtained after separating process was studied with anti-EV conjugate. Cytospin slides were examined in a Vanox Research Photomicrographic Microscope System AHBT3 (kindly accessible by Mr. K.Szukalski, Olympus Microscopes, Warsaw). For TEM the spleens were fixed for 2 h in 2.5 % glutaraldehyde in sodium cacodylate buffer (pH 7.4), postfixed in 1 % osmium tetraoxide in the same buffer, dehydrated in graded ethanol solutions and in propylene oxide, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate. Spleens sections were observed in a Jeol 100C TEM (Japan) operated at 80 kV.

RESULTS

In contrast to T and B cells, EV could be recovered from the metrizamide-gradient enriched DC and from the plastic-adherent Mø at least up to 60 days p.i. As shown in Figure 2, the number of infectious centers formed by DC and Mø increased in the first two

weeks p.i., peaking at day 15, and decreasing thereafter to 10 infectious centers/10^6 cells, a level that was maintained throughout the 60 days investigated. EV-specific immunofluorescence confirmed the presence of EV protein(s) in antigen presenting cells (APC) (DC and/or Mø) during the period examined with decreasing of fluorescence intensity after 20 days p.i. (Table 1 and Figure 3). We found that only between 5 and 10 days after the inoculation of mice, could EV be recovered from the T and B cells enriched fraction of splenic cells. Currently we are continuing these studies by examining the spleens ultrathin sections and different subpopulations of splenocytes in TEM (Figure 4).

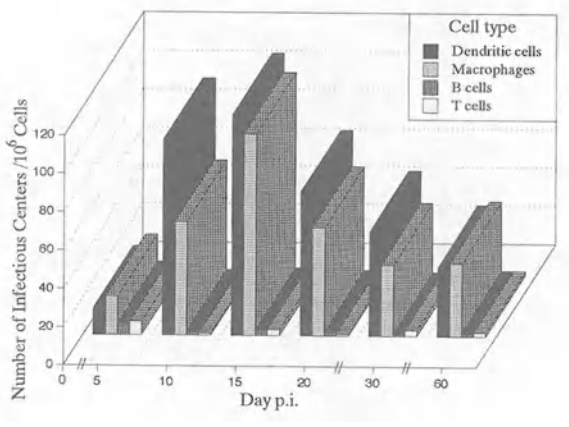

Figure 2. Kinetics of infectious centers formed in Vero cells by DC, Mø, T and B lymphocytes.

Table 1. Detection of EV Ag in various types of cells by direct IF at different times after infection

Type of cells	Day post-infection					
	5	10	15	20	30	60
DC	++*	++++	++++	+++ +	+++	++
Mø	++	++++	++++	+++ +	++	+++
T cells	++	+/-	+/-	-	-	-
B cells	++	+/-	+/-	-	-	-

*Fluorescence intensity: ++++ 80 - 100 % bright cells; +++ 50 - 80%; ++ 30 - 50%; + to 30%; - no reactivity.

DISCUSSION

It is well known that virus-infected cells of the host immune system may be lysed by the virus or became target cells for virus-specific immune response or can manifest impaired biological function(s) as a result of viral persistence. The role of lymphocytes and/or Mø

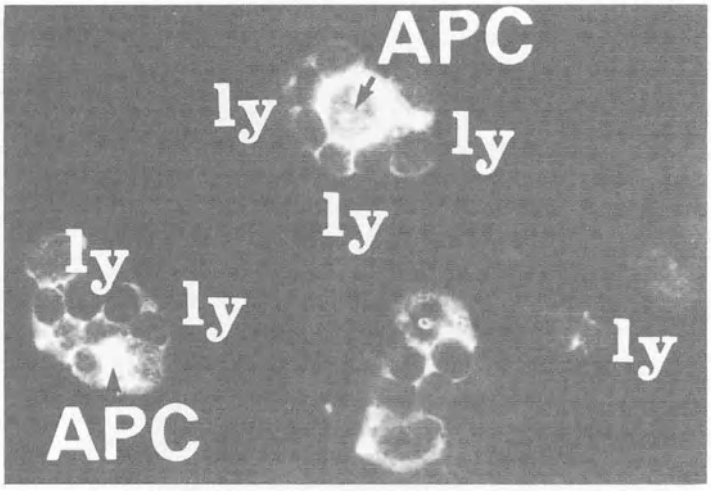

Figure 3. The immunofluorescence photomicrograph of splenic T lymphocytes (Ly) clustering with antigen presenting cells (APC) during EV Ag presentation. Original magnification = 600 x.

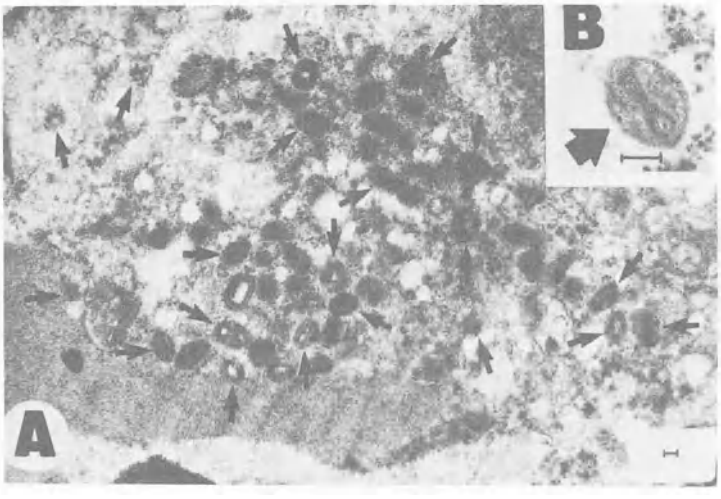

Figure 4. A transmission electron photomicrograph of EV particles in the spleen of BALB/c mouse experimentally infected 15 days earlier: (A) inclusion bodies formed by about 50 particles of EV, and (B) single EV particle. Calibration bar = 100 nm.

in viral persistence was highlighted by a number of studies[5]. In a natural infection(s), some viruses can persist in the immune cells in a latent form, i.e. HIV in T cells and monocytes, human cytomegalovirus in lymphocytes and monocyte/macrophages, and murine gammaherpesvirus 68 in B cells[4]. However, the poxvirus persistence is still poorly understood in many aspects of the immunobiologically-related functions.

In this paper, we show by ICA that DC and Mø, the main cells that form a system of APC, could actively produce EV up to 60 days p.i. peaking at 15 days. This suggest that particular cells of the immune system may harbour the latent EV for a long time after acute disease. Why and by what mechanism a selective decrease in the number of infectious centers formed by T and B cells occurs remain unknown. It is possible, that mousepox virus replication within DC and Mø is more effective than in the T and B cells and/or the accumulation of viral DNA sequences over time is more toxic to or lytic in T and B cells than DC and Mø. These two possibilities were considered by Borrow et al.[6] for lymphocytic choriomeningitis virus (LCMV) persistence.

In conclusion, we have demonstrated that DC and Mø are equivalently infected during the 60 day period examined and could serve as a important reservoir for the EV dissemination in vivo. The availability of transgenic mice with selective DC and/or Mø deficiency could be a useful tool for better understanding of the immunobiology of these cells during viral persistence.

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FUNCTIONAL EXPRESSION OF ICAM-3 ON HUMAN EPIDERMAL LANGERHANS CELLS

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INTRODUCTION

Optimal activation of T lymphocytes by APC requires two different sets of signals: MHC-peptide complexes that interact with the TCR and costimulatory signals. These are especially important for activation of resting and naive T cells, which in the absence of costimulation, may undergo to long lasting functional impairment[1]. Dendritic cells, including epidermal Langerhans cells (LC), are the most efficient activators of naive T cells and the most potent inducers of primary T cell mediated immune responses[2], a property that has been attributed, at least in part, to the constitutive expression of high levels of MHC and various accessory molecules. Human freshly isolated LC (fLC) do not display significant numbers of membrane costimulatory molecules. Following short-term culture, LC (cLC) upregulate the intercellular adhesion molecule (ICAM)-1 (CD54), the leucocyte function-associated antigen (LFA)-3 (CD58) and B7-1 (CD80) and markedly increase their antigen presenting capacity[3,4]. The LFA-1 (CD11a/CD18) adhesion molecule plays a pivotal role in cellular interactions between APC and T lymphocytes. At least three counter-receptors able to bind to LFA-1 have been described, namely ICAM-1, ICAM-2 (CD102) and ICAM-3 (CD50), all belonging to the Ig gene superfamily[5]. Recently, ICAM-3, but not ICAM-2, has been detected in LC in sections of human epidermis[6,7]. In this study, we examined the expression and function of ICAM-2 and ICAM-3 in human LC, and their possible regulation during culture.

ICAM-3 IS CONSTITUTIVELY EXPRESSED BY LC AND IS NOT SUBJECTED TO REGULATION DURING CULTURE

Double immunofluorescence on frozen skin sections confirmed that epidermal dendritic $CD1a^+$ cells in situ lack ICAM-2 and express abundant ICAM-3 (not shown).

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Two-color flow cytometry analysis of LC-enriched EC suspensions revealed that fLC ($CD1a^+$ cells) express ICAM-3 (Fig. 1), but not ICAM-2 (not shown). 72-hr cLC ($HLA\text{-}DR^+$ cells) displayed similar levels of membrane ICAM-3 (Fig. 1) and did not express ICAM-2. This finding is in agreement with data reported by de Fougerolles et al. showing that, in contrast to ICAM-1, ICAM-3 expression on T lymphocytes is only marginally dependent on the state of cell activation[5].

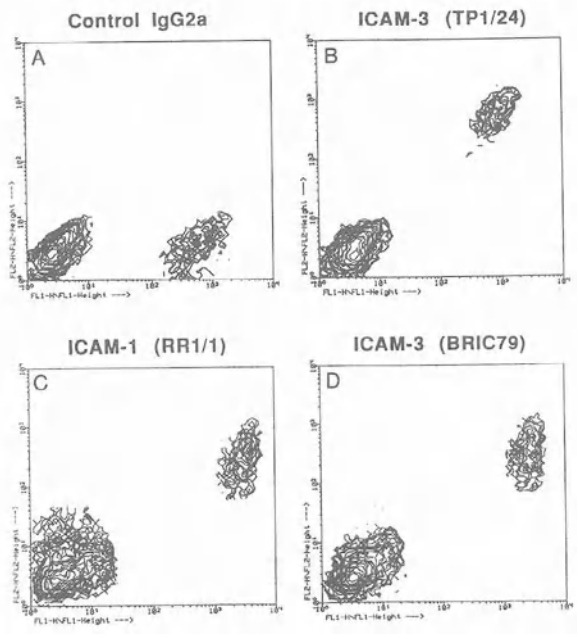

Figure 1. Expression of ICAM-3 but not of ICAM-2 by human LC. Freshly isolated epidermal cell suspensions enriched for LC were stained with matched isotype (A), or anti-ICAM-3 mAb TP1/24 (1:800) (B), followed by PE-conjugated anti-mouse Ig and FITC-conjugated anti-CD1a. Panels C and D show cLC stained for ICAM-1 (RR1/1, 1:100) or ICAM-3 (BRIC79, 1:400), respectively, and FITC-conjugated anti-HLA-DR. The anti-ICAMs mAb were obtained from the 5th International Workshop on Human Leukocyte Differentiation Antigens (Boston, MA, November 3-7, 1993).

ICAM-3 IS A RELEVANT COSTIMULATORY MOLECULE FOR BOTH fLC AND cLC

In order to determine the relevance of ICAM-3 to the APC function of LC, we studied the effects of adding mAb against ICAM-3 on the ability of both fLC and cLC to stimulate allogeneic T cells in a primary MLR assay. As shown in Fig. 2, mAb against ICAM-3 inhibited T cell proliferation induced by both fLC and cLC. In contrast, anti-ICAM-1 mAb had no effect on T cell proliferation promoted by fLC. The degree of inhibition by anti-ICAM-3 mAb ranged from 25 to 75% and varied according to the mAb used, with BRIC79 resulting the most potent. When cLC were used as stimulators, anti-ICAM-1 and anti-ICAM-3 mAb reduced T cell response to a similar extent. In addition, incubation of cLC with both anti-ICAM-1 and anti-ICAM-3 mAb had an additive effect (92% inhibition), suggesting that ICAM-1 and ICAM-3 on cLC cooperate in stimulating T cell activation.

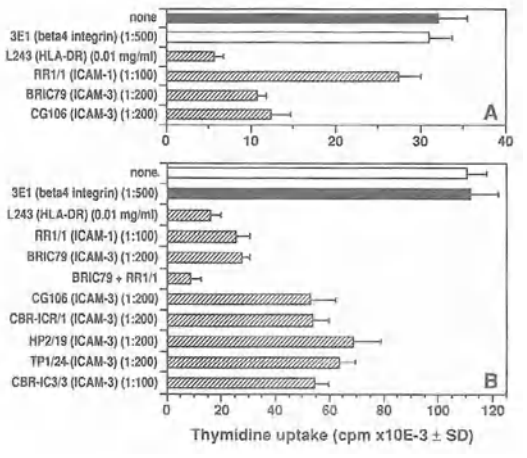

Figure 2. Inhibition of the alloantigen presenting function of fLC (A) and cLC (B) by anti-ICAM-3 mAb. Epidermal cells enriched for LC (3000 LC/well) were incubated with the mAb indicated (45 min, 4°C) at saturating concentrations. Thereafter, allogeneic PBMC deprived of adherent monocytes (150 x 10^3/well) were added and cocultured in flat-bottomed microwells for 5 days. Background proliferation of T cells and epidermal cells alone were 1520 ± 596 and 960 ± 380, respectively.

CONCLUSIONS

The results indicate that ICAM-3 is constitutively expressed by LC and serves as a relevant costimulatory molecule for both fLC and cLC. We postulate that ICAM-3 on fLC may play a very important role in the early contact between LC and T lymphocytes and be critical for initial T cell activation before expression of other costimulatory molecules on LC is upregulated.

Acknowledgments

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MULTIPLE LINES OF EVIDENCE FAVORING A BONE MARROW DERIVATION OF FOLLICULAR DENDRITIC CELLS (FDCs)

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INTRODUCTION

In early studies, FDCs were identified as a reticular cell type[1,2] primarily on account of the similarities between reticular cell and FDC nuclei. Although, the nuclei of FDCs tend to be more pleomorphic and to exhibit a greater variety of nuclear shapes, cells identified by electron microscopic antigen localization as FDCs were occasionally also shown to be closely associated with reticular fibers.[1,3]

According to the current theory on FDC origin, FDCs are probably derived locally from fibroblastic or primitive reticular cells[3-9] or from mesenchymal cells (pericytes around capillaries) found locally in follicles.[10] Humphrey's[8] report that FDCs in bone marrow chimeras were of the host phenotype further strengthened the belief that FDCs are of local origin.

Our report on antigen transport in the lymph node[11] suggested that antigen transporting cells (ATCs) were pre-FDCs, originating outside of the lymph node. We thus began to question the FDC local origin theory. In addition to the descriptive antigen transport data showing that ATCs mature to form FDCs, we recently obtained additional support from ATC/FDC phenotyping, the identification of FDC-M1 reactive cells in the blood and bone marrow, and data from experiments conducted with bone marrow chimeras[12]. In this chapter, we will give an account of our studies supporting the contention that FDCs are derived from tissues outside of the lymph node. A diagram summarizing our working model is presented in the section on *Antigen transport by ATCs* (Fig.1 below).

RESULTS and DISCUSSION

Antigen transport by ATCs

Antigen transport to lymph node follicles was first reported in 1983[11] and subsequently described in detail in several reviews.[13,14] Antigen transport is a cell-mediated mechanism and involves the migration of ATCs to follicles during the first 24-36 hrs after antigenic

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challenge.[11] Antigen transport was studied in C3H and C57BL/6 mice and can be recognized by light microscopy by the path of immune complex-coated ATCs as early as 1 min after antigen injection of passively immunized mice. This path fans out from a narrow section of the subcapsular sinus toward individual follicles in the cortex and appears in tissue sections as a triangular peroxidase positive area when using HRP as the antigen.[11] The triangular shape of the path in section is due to the distribution of ATCs carrying the immune complexes on their surfaces (see Figure 1., just above left follicle). Control mice, lacking the antigen-specific antibodies do not show any cell-mediated antigen transport after antigen injection. Consequently, this mechanism is antibody dependent and primarily functional during the late phases of the primary and the secondary antibody responses.

It should be pointed out that the immune complexes transported in this model were formed *in vivo* under physiological conditions. In contrast, attempts to induce antigen transport by others with *in vitro* (pre-) formed immune complexes were unsuccessful.[15,16] We found that we could correlate the difficulty of detection of this cell-mediated antigen transport by changing the ratio of antibody and antigen in the immune complexes formed *in vivo*. This was accomplished by varying the amount of antibody used for passive immunization and the dose of the challenge antigen (Kapasi *et al.* unpublished).

During antigen transport, the first cells observed with immune complexes on their surfaces are found in the afferent lymph of the subcapsular sinus.[11] These cells have a monocyte-like morphology, an indented relatively euchromatic nucleus and small, peroxidase positive cytoplasmic granules (primary lysosomes) associated with the Golgi apparatus.[11] On these cells, surface area is increased by numerous veils which bear a relatively large immune complex load. These nonphagocytic cells are quite distinct from the typical phagosome-rich sinusoidal macrophages that phagocytize the majority of immune complexes.[11] There is always a distinct spacial separation between these veiled and dendritic ATCs and the antigen laden macrophages in the subcapsular sinus.

ATCs penetrate the cortex through pores in the subcapsular sinus floor and this passage through the pores can be convincingly followed by electron microscopy.[11] As these cells move below the sinus floor, their nuclei increase in size and number of lobes. At this time, ATCs begin to develop long dendritic processes, which interdigitate with processes of other ATCs in the antigen transport pathway. Thus, these ATCs form a chain -- a reticulum -- which expands toward the nearby follicle in the cortex with the arrival of new ATCs. ATCs become increasingly more dendritic along this antigen transport chain and as the cells enter the follicles they take on the morphology of mature FDCs. By 24 hrs after antigen injection, the FDC antigen retaining reticulum is established at most sites through this mechanism.

In our original paper on antigen transport, we proposed three hypothetical mechanisms of antigen transport. According to one mechanism, ATCs may be cells strictly concerned with the transport and transfer of antigen to FDCs. According to the second hypothetical mechanism, ATCs may be pre-FDCs that mature to become FDCs (as described above) and would not involve a transfer of the transported antigen. A third alternative may be the combination of these two mechanisms. We reasoned that if ATCs were pre-FDCs then their antigenic phenotype should be either very similar or identical to that of mature FDCs. To determine this, we phenotyped ATCs *in situ* between 1-15 minutes after antigen injection in parallel with mature FDCs at 3 days after antigen injection. The results showed that ATCs and FDCs were of the same phenotype (for results of this phenotyping see Table I in the accompanying paper by Haley *et al.*). From this identity of the phenotypes and the observed maturation of ATCs into FDCs in the transport chain, as indicated by morphology, we concluded that ATCs are pre-FDCs. Furthermore, since the first monocyte-like, veiled ATCs were seen in the afferent lymph of the subcapsular sinus, we proposed that FDCs are derived from monocyte-like, veiled cells, originating outside of the lymph node.

The recognition of this cell-mediated, antibody dependent mechanism of antigen

transport to lymph node follicles prompted some pertinent questions. For a discussions of these questions and answers the reader is referred to reference 17.

To summarize this section, it appears that the cell mediated antigen transport mechanism can account for the transport of sufficient amount of antigen to follicles. Concomitantly, this antigen transport mechanism also provides pre-FDCs (ATCs) of an identical phenotype with FDCs. The pre-FDCs mature in transit from the afferent lymph to the follicles and ultimately form the FDC antigen retaining reticulum. By working in concert with the mechanisms of iccosome formation and dispersion[14] that result in partial or complete dissipation of FDC networks, a relatively steady state in the size of FDC networks may be achieved. Antigen transport, as it exists in the lymph node, supports the extranodal FDC derivation theory.

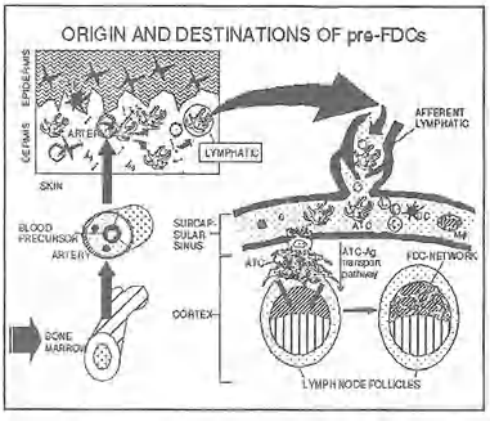

Figure 1. A working model of FDC origin depicting the path of migration from the bone marrow via the circulation to connective tissues and via lymphatics to the draining lymph node.

Studies of SCID mouse/bone marrow chimeras

Humphrey *et al.*[8] concluded, based on studies with mouse radiation chimeras, that FDC precursors were not derived from the bone marrow. In this study, chimeras maintained for over a year continued to express H-2 antigens of host phenotype on the FDCs. We have confirmed Humphrey's observation (Kosco and Burton *et al.*, unpublished observations). In our studies; however, an essential control failed. Due to the radio-resistance of FDCs [i.e., 1600 rads (Burton; unpublished); 18.5 Gy[18]] they were not eliminated from the host. Thus, it may be argued that because of the radioresistant nature of FDCs, host FDC were not eliminated and their presence may have inhibited donor FDC from developing normally. On account of this critical observation, we were prompted to use severe combined immunodeficient (SCID) mice for the construction of chimeras to circumvent this FDC radioresistance problem. SCID mice lack functional B and T cells[19] and as we have demonstrated, they also lack FDCs.[20] The absence of FDCs was shown by the lack of antigen localization on FDCs in lymph node and splenic follicles, and by the lack of labeling with the monoclonal antibody FDC-M1.[20] We reasoned that the SCID mouse would provide a useful model to study the origin of FDCs since the problem of eliminating radioresistant FDCs could be bypassed. However, since reconstitution studies with syngeneic B and T cells resulted in the development of FDC-networks, these studies clearly showed that SCID mice have FDC precursors.[20] In ontogeny, FDCs do not appear until about three weeks after birth[3,21,22]. Therefore, we reasoned that transferring bone marrow or fetal liver cells to newborn SCID mice would be advantageous and donor pre-FDCs would not have to compete with host-origin FDC precursors for location or development into mature FDCs. Since studies from other laboratories have demonstrated that SCID mice accept xenogeneic transplants in addition to F_1(Balb/c x C57BL) bone marrow, we also reconstituted SCID mice with rat bone marrow or rat fetal liver cells. We then evaluated the recipients for rat donor phenotype FDCs, 6 to 8 weeks after rat bone marrow or rat fetal liver cell transfers, using the mouse-anti-rat FDC specific monoclonal antibody, ED5. Recipients of F_1 mouse bone marrow were evaluated 6 months after cell transfer for donor-phenotype FDCs, using monoclonal antibodies against

C57BL/6 class I antigens.[12] According to the results of phenotyping, of 7 rat bone marrow reconstituted SCID mice, 3 mice clearly showed the presence of ED5+ FDC-networks in lymph nodes and spleens. Similarly, 1 out of 5 recipients of rat fetal liver also had rat FDC in their lymphoid organs. We also noted that a given follicle tended to have either donor or host FDC predominating, although there were follicles with both cell types. It should be noted that FDC-M1+ FDC were not reactive with ED5 indicating that the monoclonal antibody ED5, as reported,[23] does not cross-react with mouse FDC. Similarly, the ED5+ cells did not cross-react with the monoclonal antibody FDC-M1. The presence of host FDCs in our recipients is not surprising, considering that SCID mice have FDC precursors. Nevertheless, the presence of FDC-networks of donor and donor-host phenotypes were the major findings.[12] The results showed that bone marrow and fetal liver both contained FDC precursors. Chimeras constructed with F_1 bone marrow confirmed these observations. These observations also give credence to the pre-FDC nature of ATCs and to the extranodal origin of FDCs.

Further confirmation of the presence of FDC precursors in the bone marrow was obtained using the *lacZ* mouse model.[24] Rosa-26 mice transfected with the *lacZ* gene express the gene product, β-galactosidase, in all cells. Through the action of this gene product, the fluoresceinated substrate, fluorescein-di-β-galactopyranoside (FDG)[25,26] is cleaved and the fluorescein is released into the cytoplasm. As a result, the cell becomes fluorescent and detectable by flow cytometry. Using the same protocol for the construction of chimeras as above, newborn (3 day old) SCID mice received bone marrow cell transfers from Rosa-26 mice. We reasoned that if the SCID mice were repopulated by Rosa-26 bone marrow FDC precursors, then the developing FDCs will be identifiable by the presence of the *lacZ* gene product. For flow cytometry, control FDCs were isolated according to protocol[27] from Balb/c mice. This enriched preparation contained 23% FDCs. By FACScan after being reacted with biotinylated FDC-M1 and streptavidin-phycoerythrin, 99% of the FDC-M1+ population was shown to be negative for the *lacZ* product. When a similarly enriched preparation (25%) of FDCs from *lacZ* mouse bone marrow recipient SCID mice were tested, the results showed a ratio of 67% host to 33% donor (*lacZ*+) phenotype FDCs for the SCID-Rosa-26 chimeras. These results supported the results of our previous SCID mouse-rat and SCID mouse-F_1 chimeras and allowed us to conclude that FDC precursors *can* come from the bone marrow.

Putative FDC precursors in the blood

If FDC precursors are indeed come from the bone marrow, than it would be reasonable to expect that FDC precursors are also present in the blood. In fact, Parwaresch *et al.*,[28,29] using the monoclonal antibody KiM4, specific for human FDCs, identified KiM4+ cells in the human blood. We have also found similar size (14-16μm diameter) cells in mouse blood with the aid of the mouse FDC specific mAb, FDC-M1. For illustrations see the accompanying paper by Haley *et al.* in this volume. Further characterization of these blood-borne precursors is presently in progress. The finding of this potential FDC precursor in the blood made the connection between the bone marrow, a source of the precursors, and the tissues drained by the lymph nodes, and provided additional support for the idea of the bone marrow derivation of FDC precursors.

Identity of FDC-M1+ cells in the bone marrow

The main issue regarding the bone marrow derivation of FDC precursors is *whether these precursors are derived from hemopoietic cells or stromal cells.* We have approached this problem also with the use of the monoclonal antibody, FDC-M1. Preliminary studies identified stromal cells and megakaryocytes labeled with FDC-M1. Among the stromal cells,

we have tentatively identified, with light and electron microscopy, FDC-M1+ macrophage-like cells with prominent phagosomes, cells with multiple, elongated cell processes of a supporting reticular cell type, some of which appeared to contain multi-lobed nuclei or were multinucleated. However, we feel that the FDC bone marrow precursor has not yet been identified and a hemopoietic or a stromal origin of the FDC precursor are both possibilities. For illustration of these cells see accompanying paper by Haley *et al.*, in this volume.

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ADHESION AND COSTIMULATORY MOLECULES ON HUMAN FDC IN VITRO

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INTRODUCTION

In the previous study[1], we reported a unique accessory function of follicular dendritic cells(FDC), emperipolesis of germinal center lymphoid cells, which was maintained in FDC after long-term culture. The first intercellular phenomenon for emperipolesis is considered the adhesion between lymphoid cells and FDC. This suggests that some specific adhesion molecules are expressed on the surface of FDC in vivo and in vitro[2]. In this study, we attempted to demonstrate the adhesion and costimulatory factors in FDC after long-term culture using FACS analysis to evaluating their properties of emperipolesis.

METHODS

Surgically-removed, fresh palatine tonsils were used. The method used for enucleation and subsequent digestion of the lymph follicles, and purification of FDC clusters has been descrived in detail elsewhere[3]. After several days, FDC clusters dedifferentiated into flat plastic-adherent FDC, losing all engulfed lymphocytes. The dividing small FDC were differentiated from those flat non-proliferating FDC after approximately 14 days in culture. To determine the effect of cytokines on expression of surface molecules, we added γ-IFN, IL-4, and GM-CSF to the medium of target cells, and maintained this culture for 4 more days.

Cultured FDC on various days and control fibroblast cell lines (KD, TIG-7)(JCRB, Japan) were harvested. Those were incubated with primary MoAb for 1 h on ice, washed and reacted for 30 min with FITC-labeled Fab' goat immunoglobulin to mouse γ-chain. After washing,

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the fluoresence intensity of 1000-2000 FDC in each sample was analyzed with a FACScan(Becton-Dickinson).

To examine true emperipolesis[1], FDC(10^4/ml), at various days of culture with or without cytokine addition, were combined with tonsilar lymphocytes($5x10^5$/ml), and cultured in Terasaki plates. After 6 h, the incidence of FDC-lymphocyte spherical complexes was counted.

RESULTS

At 7 days of culture, the flat FDC did not express distinct VCAM-1 on their surfaces. CD54 was markedly expressed and VLA-4 and CD40 showed a similar positivity. After 14 and 35 days, FDC maintained the same pattern of expression of these molecules. With the addition of γ-IFN , however, MHC-classII were dramatically induced and more CD54, CD40 and VCAM-1 molecules were induced on the FDC after 14 days of culture. The same levels of induction were observed with the addition of IL-4. There was no distinct effect on those expressions in FDC with GM-CSF. On the dividing small FDC, CD54 was expressed, although other molecules were not detected. In contrast, addition of IL-4 and γ-IFN induced expression of CD40, CD54, VCAM-1 and MHC-classII on their surfaces. The control fibroblasts, KD and TIG-7 showed expression of CD54, but no significant expression of CD40. In TIG-7, there were no changes with the addition of cytokines.

At 14 to 16 days of culture, approximately 8% to 40% of the flat non-proliferating FDC formed spherical complexes with fresh lymphocytes. Those FDC, previously activated with IL-4 and γ-IFN, showed 2 to 4 times increased incidence of emperipolesis and a tendency to form larger FDC complexes. In contrast, the dividing FDC showed no significant emperipolesis. The cytokines could not enhance the incidence of emperipolesis.

DISCUSSION

We clearly showed that the adhesion and costimulatory molecules: CD40, CD54, VCAM-1 and MHC-classII are reliable surface markers of FDC in vitro. The expression of those molecules on the surface of FDC were independent of IL-4 and γ-IFN. This suggests that the functional state of FDC in situ is undercontrolled by cytokines and biological active factors[4].

The dividing small FDC differentiated from the large-flat FDC after 14 days of culture. The expression of the adhesion and costimulatory molecules was also influenced by cytokines, although they did not showed any emperipolesis to lymphocytes. These results strongly suggested that the adhesion and costimulatory factors that we examined in FDC are not essential factors for proceeding to emperipolesis. In a further study, we could culture two populations of FDC: emperipolesis positive and negative. This may demonstrate functional heterogenity in FDC.

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ARCHITECTURAL AND ANTIGENIC FEATURES OF FOLLICULAR DENDRITIC CELLS AS A CLUE TO THE HISTOGENESIS OF PRIMARY CUTANEOUS B-CELL LYMPHOMA*

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INTRODUCTION

Primary cutaneous B-cell lymphoma (CBCL) are B-cell non-Hodgkin's lymphoma primarily presenting in the skin without any detectable extracutaneous lesion despite careful and complete staging procedures.[1-4] Previous studies of our group indicate that CBCL should be considered as a unique type of a low grade lymphoma.[4] In fact, CBCL have: 1. non aggressive clinical behavior (mostly loco-regional extension, good response to local treatments, low tendency to spread, and excellent prognosis);[1-4] 2. uniform immunophenotypic (CD5-, CD10-)[2,4] and genotypic features of neoplastic B-cells (lack of bcl-2 gene rearrangement),[2,5] and 3. histologic evidence of centrocyte-like (monocytoid, parafollicular) cells in their whole morphologic spectrum, plasma cells, reactive lymphoid follicles and lymphoepithelial lesions.[4] These typical features are strikingly similar to those described in so-called MALT (Mucosa-Associated Lymphoid Tissue) lymphoma,[6,7] suggesting the interpretation of CBCL as the cutaneous counterpart of MALT lymphoma, i.e., Skin-Associated Lymphoid Tissue (SALT)-related B-cell lymphoma.[4,8,9] The close clinical, histologic, immunologic, and genotypic similarities of CBCL and MALT-lymphoma with monocytoid/parafollicular lymphoma[6] suggest the hypothesis of a common marginal cell origin for these neoplasms.[9]

The aim of our study was to investigate whether the architectural and antigenic features of follicular dendritic cells (FDC) may be: i) a useful additional tool for the differentiation between neoplastic and reactive lymphoid follicles, and ii) a possible clue to the marginal cell origin of CBCL.

PATIENTS AND METHODS

We studied 113 CBCL patients. Crucial criteria for the selection of cases were: 1. absence of any detectable extracutaneous lesion within 6 months from diagnosis; 2. expression of B-cell restricted antigens by neoplastic cells; 3. light chain monoclonal restricted or SIg negative staining by neoplastic B-cells. More that 350 biopsies - taken from lesions of different size, age and growth rate - were routinely processed for light

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List of abbreviations. CBCL: cutaneous B-cell lymphoma. CD: cluster of differentiation. MALT: mucosa-associated lymphoid tissue. SALT: skin-associated lymphoid tissue. FDC: follicular dendritic cells. NGFr: nerve growth factor receptor.

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microscopy and investigated with immunohistochemistry on both frozen and embedded material. We used a large panel of monoclonal antibodies specified elsewhere.[4]

RESULTS

Follicular DC were found in a large percentage of lesions, arranged in 2 main patterns. The corresponding figures are schematically shown in Table I.

Pattern 1

Follicular DC were found associated with CD5-/CD10- monoclonal B-cells to form follicle-like clusters. In fact, we have found DRC-1+, NGFr (Nerve Growth Factor receptor)+, CD14-, CD21-/+, and CD35+ FDC showing an aberrant "centrifugal" pattern. Indeed, they are loosely arranged in ill-defined meshworks with blurred and radiating contours, and they skip the nodule centers (Fig.1a). The B-cell FDC clusters are mainly found in the superficial dermal infiltrate of recently developed lesions and are irregularly surrounded by reactive T-cells (Fig. 1b), thus delineating a quite evident compartmentalization of the infiltrate not identifiable on histologic grounds alone .

Pattern 2

In the second pattern, typically found in slowly grown, long-standing lesions, FDC are located in true reactive follicles, mostly showing a morpho-immunologically typical organization. In fact, we can observe polyclonal, CD5-/CD10+ B-cells associated in the germinal center with FDC showing a CD14+/-, CD21+ phenotype, and polyclonal, CD5+/CD10- or CD5-/CD10- B-cells associated in the mantle zone with FDC showing a CD14-, CD21+/- phenotype. These polyclonal clusters are surrounded by broad strands of neoplastic B-cells, in a fashion reminiscent of the parafollicular and interfollicular pattern typically observed in parafollicular-monocytoid (marginal cell) lymphoma.[6,10]

Table 1. Architectural and antigenic features of B-cells and FDC in CBCL.

	Follicle-like neoplastic clusters	True reactive follicles
B-cell phenotype	CD5-/CD10-	CD5-/CD10+ (center), CD5+/CD10- or CD5-/CD10- (mantle).
FDC architecture	aberrant "centrifugal" pattern	typical (dense network in the center, looser in the mantle).
FDC phenotype	CD14-, CD21+/-, NGFr+	CD14+/-, CD21+, NGFr+ (center); CD14-, CD21+/-, NGFr+ (mantle).

CONCLUSION

In CBCL, the architectural and antigenic features of FDC are: i) a useful, additional tool for the differentation between neoplastic, follicle-like nodules and true reactive follicles, due to the differential expression of specific antigens; ii) an interesting clue to the marginal cell origin of this type of lymphoma. In fact, their NGFr+, CD14- phenotype is a further argument against the previously proposed follicular center cell origin of CBCL.[10] Indeed, the phenotype of FDC is NGFr-, CD14+/- in nodal lymphomas of follicular center cell

origin.[11] These characteristics, along with the CD5-/CD10- phenotype of neoplastic cells and their lacking rearrangement for the bcl-2 gene, speak in favor of a marginal cell origin.

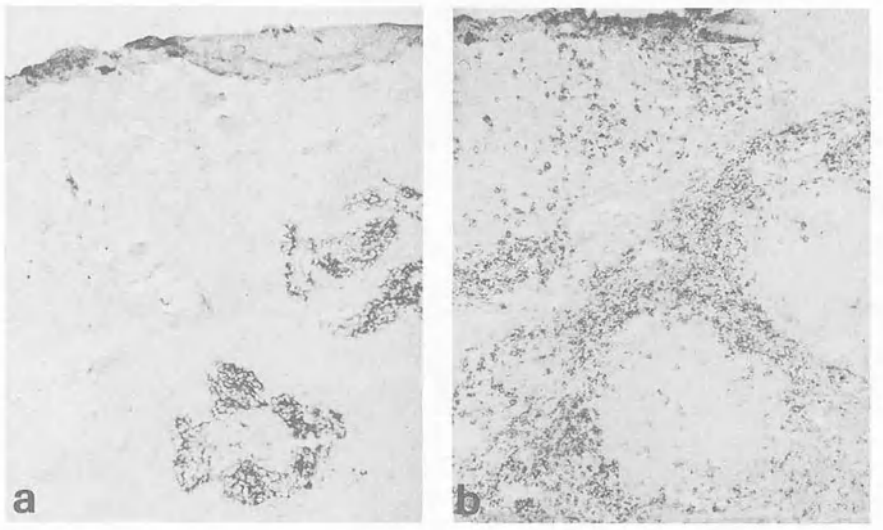

Figure 1a. CD35+ FDC show an aberrant "centrifugal" pattern and characteristically have a CD14- phenotype. **Figure 1b.** CD3+ T-cells irregularly surround the FDC/B-cell clusters. Immunohistochemistry (APAAP) on frozen section (original magnification x 250).

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AN IMMUNOHISTOCHEMICAL STUDY ON ISOTYPES OF THE IMMUNE COMPLEXES TRAPPED BY FOLLICULAR DENDRITIC CELLS (FDC) IN VARIOUS HUMAN LYMPHOID TISSUES

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INTRODUCTION

Follicular dendritic cells (FDC) are well known to trap and retain immune complexes (IC) containing various isotypes of immunoglobulins[1,2,3]. In addition, it has been reported that FDC retained IC with peculiar isotypes of immunoglobulins in certain diseases (IgA1 in IgA nephropathy[4,5], IgE in Kimura's disease[6] etc). These observations imply the particular relationships between isotype of the immunoglobulin constituting IC retained by FDC and immune responses or pathogenesis of certain immunopathological conditions. In the present study, distribution of each isotype of immunoglobulins was systemically examined in human various lymphoid tissues using immunohistochemical techniques with special reference to the reactivity within germinal centers (GC).

MATERIALS & METHODS

Specimens. The following tissues were examined: tonsils from patients with chronic tonsillitis (n=10), tonsils from patients with IgA nephropathy (n=10), superficial lymph nodes (LNs) including inguinal, submandibular and axillar LNs (n=9), intra-abdominal LNs including perigastric and mesenteric LNs (n=10), mucosal associated lymphoid tissues (MALT) including appendix, gastric mucosa and Peyer's patches (n=9) and LNs from Kimura's disease (n=3). These tissues were fixed in 10% formalin solution, processed routinely and embedded in paraffin. In addition, some cases of tonsils from both patients with chronic tonsillitis or with IgA nephropathy were freshly frozen or fixed in periodate-lysine-paraformaldehyde (PLP) solution for sensitive immunohistochemical examination or immunoelectron microscopy. These tissues revealed non-specific reactive appearance but not specific or neoplastic changes on their histology.

Antibodies. Rabbit polyclonal antisera and mouse monoclonal antibodies specific for each isotype or subclass of human immunoglobulins were employed as primary antibodies for the immunohistochemical labeling as described below.

Immunohistochemical procedures. The paraffin sections were pretreated with 0.1 % trypsin sufficiently (over 90 min at 37°c) to unmask the antigenecity[5] and then immunostained by PAP methods[6] using rabbit polyclonal antisera, the bridging antibody (swine anti rabbit antibody) and PAP (peroxidase- rabbit antiperoxidase complex) or by indirect immunoperoxidase staining using mouse monoclonal antibodies and peroxidase

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conjugated goat anti-mouse antibody. The cryostat sections of frozen tissues or PLP-fixed tissues were also labeled by the later .

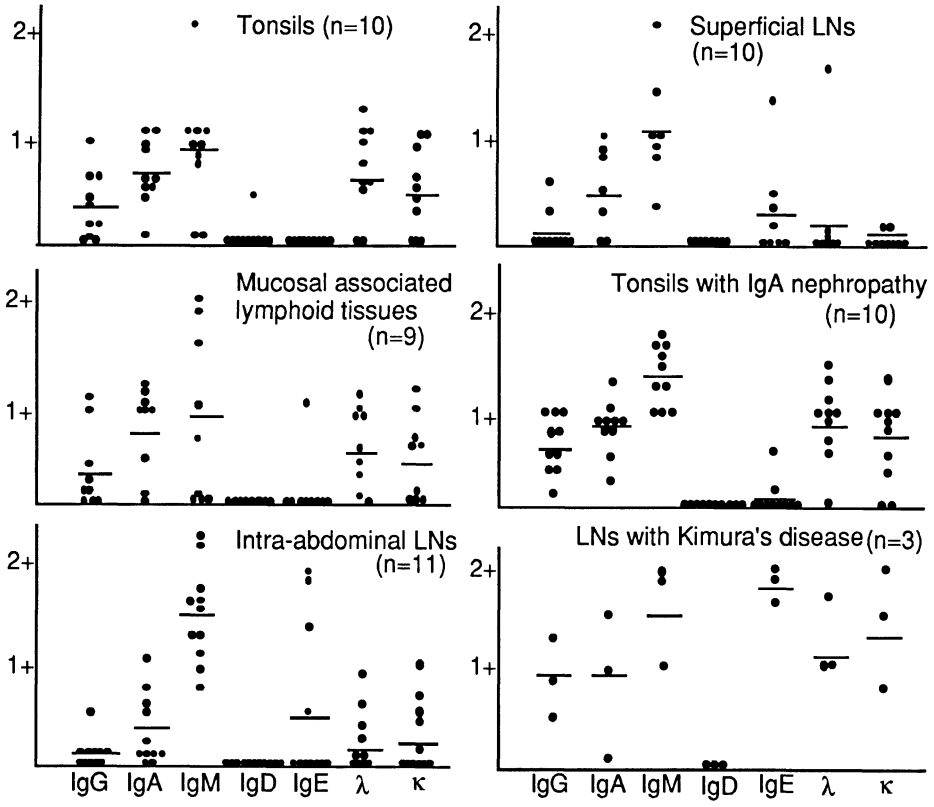

Fig. 1 Distribution of each isotype of immunoglobulins in various human lymphoid tissues.

RESULTS & DISCUSSIONS

Reticular pattern of distribution of immunoglobulins were recognized clearly within most of GC, especially in the light zone, in the examined tissues, even if they were fixed in formalin and embedded in paraffin routinely. These immunoglobulins were regarded as a constituent of IC trapped and retained by FDC. Indeed, our electron microscopic observations confirmed that the immunoglobulins located on the surface of the entangled cytoplasmic processes of FDC (data not shown). To compare quantitatively the distribution of isotypes of immunoglobulins within GC in the different tissues, intensity of the reactivity was scored as following: – or 0; no reactivity, 1+; the same intensity as cytoplasm of positive plasma cells, 2+; more intense reactivity than positive plasma cells and 3+; more prominent reactivity than positive plasma cells in wide area of GC. The average score of 30 randomly selected GC in each case was calculated and compared as shown in Fig.1. These results indicated that relatively intense reactivity on IgM was recognized in most of GC in the examined tissues. In contrast, IgD was constitutively negative. Our results were consistent with previous reports concerning the immunoglobulins present on FDC in human[1,2] and in mouse[3] and they suggested that IgM-IC might play general roles in the immune responses within GC. Previously Heinen et al[8] reported the controversial results in which experimentally administrated IgM-IC could not be efficiently retained by mouse FDC. Their IC, however, were produced in vitro by mixing of hapten (TNP)-carrier protein with only a clone of specific monoclonal

antibody. They might be somewhat different from physiological IC because IC were generally supposed to be polyclonal in vivo. In addition, the gold particles conjugated the IC might influence their physiological activity. Further experimental approaches are expected to elucidate particular immunological roles of IgM-IC.

The distribution of IgG and IgA within GC depended on the anatomical site. It was likely that both of the isotypes distributed more prominently in tonsils and MALT than other tissues (Fig.1). These observations suggested that more amount of IgG- or IgA-IC might be retained by FDC in more active GC because these lymphoid tissues were generally hyperstimulated with exogenous antigens. In particular, it was easy to image the IgA-IC might play important roles to provoke and maintain mucosal immune responses because IgA was well known to a major isotype of antibody in mucosal defence system. Among subclasses of IgA, IgA1 was constitutively detected in germinal centers and IgA2 was completely negative (Table 2). The same observations were confirmed even in the tonsils from IgA nephropathy. Among subclasses of IgG, IgG2 and IgG3 were recognized generally in GC whereas IgG1 and IgG4 were occasionally observed (Table 2). The observations were limited to tonsils in the present study and more extended examinations will be needed to establish the distribution of subclass of IgG. These uneven distribution of subclasses of immunoglobulins in GC might be related to the physiological amount in vivo or their ability to fix or activate complement system..

The peculiar isotypes were confirmed in tonsils from IgA nephropathy patients and in lymph nodes with Kimura's disease. These observations were consistent with previous reports[2,4,5,6] and the pathological or immunopathological roles of IC with particular isotypes are suggestive to elucidate pathogenesis of these diseases .

Table 1 Distribution of subclasses of IgG and IgA in tonsils.

	IgG				IgA	
	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2
reticular reactivity in GC	–/±	+/+++	±/+++	–/+	+/++	–
positive plasma cells	+	++	++	+	++	+

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THE INTERDEPENDENCE OF LYMPHOCYTE, STROMAL CELL, AND FOLLICULAR DENDRITIC CELL MATURATION

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INTRODUCTION

If asked to pick a cell type as a biological metaphor for an independent free-spirited entity, many biologists might pick the lymphocyte. Unlike most other cell types in the body, lymphocytes can and must be able to move about the body, a dynamic circulating surveillance system. The fast-moving lymphocyte- on the go- has been an obvious center of attention for decades now; where lymphocytes go, what they do, what they need in order to live and function are major issues for immunologists. By contrast, attached, relatively sessile cells seem to have fewer options, be less dynamic, less flexible, less interesting. One widely held assumption has been that those cells coming in contact with lymphocytes, by-in-large play nurturing or minor roles, hence the term "accessory" cell, accessory being defined in one dictionary as "aiding or contributing in a secondary way". They are viewed as "aides", even valets or butlers, for "executive" lymphocytes such as CD4+ T cells which have the really important jobs.

Until recently this lymphocentric view greatly influenced even the scientists who work on "accessory" cells such as dendritic cells (DC) -defined by their ability to stimulate autologous T cells- or follicular dendritic cells (FDC)- studied principally for how they affect B cells. Yet the concept that T cells in particular are the pivotal cells of the immune system- stimulated here, leading the band there- reflects the anthropomorphisms of certain immunologists more than reality. In fact, the immune system is relatively egalitarian. No cell in the body, probably even a tumor cell, is truly independent from the effects of and its effect on certain other cells. As outlined here, the development of the immune system reflects interdependent dialogues[1] between several cell types, each affecting each other, including T and B lymphocytes, DC, FDC, monocytic lineage cells, and stromal cells.

INTERDEPENDENCE OF LYMPHOID AND DC/FDC MATURATION

The accessory cell "aides" are affected by lymphocyte "executives" just as the executives are affected by the aides. A clear example of this is the fact that *scid* mice, which have few or no detectable functional FDC, if reconstituted with normal B cells, T cells or bone marrow then develop FDC which can bind immune complexes[2]. Similarly, mice depleted of B cells do not develop FDC[3]. A combination of B cells and T cells was most effective at inducing FDC development[2], suggesting T cells also play a key role in FDC maturation. The actually trapping of immune complexes on germinal center FDC requires lymphocytes, probably mature IgM+D- B cells[4]. Conversely, of course, memory B cell development requires germinal center formation and FDC[5,6]. The fact that CD40

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ligand (CD40L) -defective patients with hyper-IgM syndrome do not form germinal centers[7] again indicates that CD40L+ T cells are required for GC formation to crosslink CD40 on B cells, DC or FDC (see below). T cell/DC interactions reflect another example of reciprocal dialogues in the immune system: T cell-derived cytokines like GM-CSF and IL-4 promote DC maturation[8,9] and signaling of DC from T cell CD40L via CD40 may promote their maturation into more effective APC[9,10].

RELATIONSHIP OF FDC TO FIBROBLASTS AND STROMAL CELLS

Most studies of FDC function in vitro have been flawed by the fact that it is extremely difficult to isolate pure FDC with reasonable yields. For example, in a very detailed and controlled study, Kosco-Vilbois and coworkers[11] reported that FDC help resting B cells to become effective APC; however, the FDC preparations used contained only 25-40% FDC, some B cells and also about 40-50% fibroblast-like cells, which most likely would not be in the non-FDC control populations the authors prepared by mechanical disruption. Since fibroblasts, like FDC, can stimulate B cell proliferation[12] and differentiation[13], it is not clear to what extent fibroblasts and/or FDC were responsible for the reported effects on APC[11]. This study is mentioned not so much to single it out, but because it is one of the better papers in the field, e.g., the authors clearly state the purity of their preparations. I return to the fibroblast and FDC relationship below.

Recently, human FDC-like cells have been isolated, but use of these cells, as discussed below, has its own set of problems. Tsunoda and coworkers[14] first isolated and maintained human FDC in long-term culture, but these FDC did not divide in culture. Later we were able to establish an FDC line, designated FDC-1 with many properties of FDC and which grows in continuous culture without exogenous cytokines[15]. Two other groups have also developed FDC lines[16,17] including EBV-transformed FDC lines[16], and we have extended our initial characterization of FDC-1 cells[13]. These FDC-like lines have a number of common properties: They are very large with a fibroblast-like morphology; they bind B cells but not resting T cells; they do not express markers such as CD3, CD16, CD18, CD20, CD45 or HLA-DR but do express CD14, CD40 and CD54 (Table 1). The EBV-transformed FDC lines, unlike the untransformed lines, do not express CD106/VCAM-1 or VLA-4 (CD49d); also, unlike the non-transformed lines[15,17], they inhibit B cell proliferation rather than stimulating proliferation. Interestingly, these lines initially express the FDC marker, DRC-1, but then lose it in culture within a few weeks. This suggests that some FDC markers may be inducible and/or require a certain environment in vivo, such as contact with lymphocytes (see below) to maintain expression.

Table 1 summarizes some similarities and differences between FDC and fibroblasts and bone marrow stromal cells (BMSC). "Stromal cell", as far as I can tell, is a term often used by immunologists for adherent cells when it is hard to pinpoint the origin of the adherent cells or because we don't want to use distasteful words like "fibroblast". The first point of this Table is that the three cell groups are all CD45-, they can produce IL-6, and some of them express CD40 and vimentin. A second and major point is that FDC[6], fibroblasts[18], and BMSC[19] are all heterogeneous populations. Given this major caveat, some clear differences are evident: human BMSC do differ from classic fibroblasts in their expression of VCAM-1[20], which is noteworthy, since, like FDC[15,21], human BMSC bind to B lineage cells via VCAM-1 to VLA-4 (CD49d) interactions[20]. FDC and BMSC also differ from fibroblasts in that they can express CD73, smooth muscle actin, and a recently described stromal marker, Bst-1[22]. As discussed elsewhere[15], our current model is that, while FDC, fibroblasts and BMSC are related, FDC and BMSC have very similar properties because they both are in hematopoietic cell compartments and both function to stimulate the proliferation and maturation of surface μ+ B lineage cells. BMSC can be derived from pluripotent CD34+ HLA-DR- stem cells in bone marrow[23] suggests that FDC may have such a bone marrow origin (see also Szakal et al., this volume).

It may be difficult to make generalizations about the precise relationship between FDC and these other cell types, particularly if they change their phenotypes depending on the microenvironment in which they find themselves. The FDC marker, DRC-1, in all four studies of FDC lines in culture[14-17] was rapidly lost (Table 1). This suggests DRC-1 expression and perhaps FDC maturation may depend on a particular microenvironment. In their original characterization of DRC-1, Naiem et al.[24] found DRC-1+ FDC in foci of

follicular lymphoma metastasis in kidney, and a number of extranodal MALT-type lymphomas have extensive clusters of FDC[25,26] Thus, either certain extranodal lymphomas arise when both lymphoma cells and FDC migrate to the same site- a possibility consistent with the findings of Szakal et al (this volume) suggesting FDC precursors may migrate from the bone marrow- or certain lymphoma cells induce stromal cells already present at extranodal sites to mature into the FDC lineage- a possibility we are now testing in vitro Could the same cell in one context become a fibroblast, and in another, become an FDC or BMSC?

Table 1. Comparison of properties of FDC, fibroblasts, and BMSC

Property	FDC		Fibroblasts	Bone marrow
	Lines	In situ		stromal cells
Expression of				
CD45	-	-	-	-
CD40	+	+	some +	some +
vimentin	+	+	+	+
CD106 (VCAM-1)	+ or -	+	-	+
CD73	+	+	-	some +
α- muscle actin	+	+	-	+
Bst-1	+	-	- or weak	+ to ++
CD14	+		-	some +
DRC-1	lost	+	-	-
CD54 (ICAM-1)	+	++ or +	inducible	inducible
Production of				
IL-3	-	-		-
IL-6	+		+	+
IL-7	-	-		+
Effect on				
B cell proliferation	+ or -		+	NE
B cell maturation	+		+	weak?
T cell proliferation	NE		+	

DIFFERENTIATION OF THE IMMUNE SYSTEM: A SERIES OF INTERDEPENDENT RECIPROCAL DIALOGUES

Both the development of the immune system and the generation of immune responses[1] proceed by a series of interdependent interwoven reciprocal dialogues These processes cannot be fully understood without understanding the effects of developing or activated lymphocytes on stromal cells, endothelial cells, dendritic cells, and FDC Shifting experimental emphasis from studies of lymphocytes or accessory cells alone to studies of interdependent dialogues in context will deepen our understanding of how the immune system really works

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THE MONOCLONAL ANTIBODY FDC-M1 RECOGNIZES POSSIBLE FOLLICULAR DENDRITIC CELL PRECURSORS IN THE BLOOD AND BONE MARROW

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INTRODUCTION

Antigen transport cells (ATC) [1] transport immune complexes to the follicles of draining lymph nodes for longterm retention by the follicular dendritic cell (FDC) antigen-retaining reticulum (ARR). The purpose of this study was to determine the antigenic phenotype of ATC to further explore the relationship of ATC to FDC. For more detail on antigen transport refer to the accompanying paper by Szakal *et al.*, in this volume.

ATC have been identified in the afferent lymph [1]. This suggest that ATC migrate to draining lymph nodes from peripheral tissues. During their migration ATC become morphologically identical with FDC. A similarity or identity in antigenic phenotype would confirm the idea that ATC are pre-FDC. Since ATC and FDC both react with the monoclonal antibody against murine FDC, FDC-M1, we reasoned that precursors of ATC/FDC may also react with this antibody. Therefore, we reacted FDC-M1 with the mononuclear fraction of fresh mouse blood, and with whole bone marrow. Cells reactive with FDC-M1 were identified in the blood and bone marrow of immune mice.

MATERIALS AND METHODS

Animals

Six to 8 wk old female C3H/HenMTV-mice purchased from Taconic Farms, Germantown, NY., were housed in shoebox cages and were given food and water *ad libitum*. For antigen localization horseradish peroxidase (HRP; type VI, Sigma Chemical Co., St. Louis, MO. #P-8375 lot 121H9540) and HSA (Sigma, #A-3782 lot 127F-9310) were used. Mice were passively immunized with rabbit anti-HRP (Sigma #P-7899 lot 062H-8975) or rabbit anti-HSA (locally produced) i.p. 24 hours before footpad injection of 5 μg of the appropriate antigen (HRP or HSA).

Preparation of cells and tissues for immunofluorescence and immunohistochemistry

Axillary, brachial and popliteal lymph nodes were obtained 1-15 minutes after antigenic challenge of mice for localization of HRP transport sites as described [1] and 3 days after challenge for localization of FDC-antigen-retaining reticula. Bone marrow was obtained from mice 24 hours after challenge by flushing the femurs and tibias with media. The lymph nodes and bone marrow were sectioned at 7 μm on a cryostat. FDC were isolated from lymph nodes by enzymatic digestion as previously reported [2]. Fresh blood mononuclear cells were obtained by density centrifugation of whole mouse blood over a ficoll gradient. Cells were cytobucketed onto slides prior to staining.

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Antigenic phenotyping

Before application of the primary antibody to tissue sections or cytobucketed cell preparations, 10% normal mouse serum (NMS) was applied to block nonspecific reactivity. Alternate sections were incubated in primary antibody at optimal dilutions overnight at 4°C, washed, and incubated with biotinylated mouse anti-rat $F(ab')_2$ fragment of IgG overnight, followed by Streptavidin (SA) -FITC.

The localization of antigen at the sites of antigen transport (at 1-15 min) and at sites of fully developed FDC-reticula (at 3 days) were accomplished by incubating alternate sections with the appropriate biotinylated antigen (i.e. B-HSA or B-HRP) followed by SA-FITC.

Controls for nonspecific reactivity included sections incubated without the primary antibody and sections in which a primary antibody was substituted with an antibody of inappropriate specificity but the same isotype as the original antibody. All controls were negative.

RESULTS

Phenotypes are listed in table I. Note the identity of ATC and FDC phenotypes.

Table I.

The antigenic Phenotype of ATC and FDC				
Antibody	ATC (1-15 min)	FDC (24 hours)	Reactivity	Previously reported results
FDC-M1	+++	+++	FDC	FDC +++, [3]
MK-1	+++	+++	ICAM-1	ATC and FDC +++, [4]
2.4G2	+++	+++	FcγRII	FDC +++, [5], [6]
8C12	+++	+++	CR1	FDC +++, [6]
F4/80	+	+	Mϕ	FDC -, [5], [6]
Mac-2	-	-	subset of Mϕ	FDC -, [5], [6]
MOMA-2	+	+	Mo, Mϕ, LDC±	NPR for FDC or ATC
MIDC-8	+	+	LDC, Mϕ, VC	NPR for FDC or ATC
NLDC-145	+	+	LDC, Mϕ, VC	NPR for FDC or ATC
anti-CD45 clone M1/9.3	++	++	All 3 isoforms of CD45	FDC+, [5]
anti CD45 clone 30F11.1	-	-	Only isoforms present on B and T cells	FDC -, [6]

In the mononuclear fraction of blood, FDC-M1 reacted with a large (14-16 μm in diameter) cell with a characteristic knobby appearance (Fig. 1). This cell had an occurrence of approximately 1 in 100,000 cells. Lymphocytes showed no reactivity with FDC-M1. Cells were also found in sections of murine bone marrow that reacted with FDC-M1. These cells were also rare, contained phagosomes, and morphologically resembled bone marrow stromal cells.

DISCUSSION

This is the first phenotype study of the ATC. The phenotype of FDC has been studied in the human and murine systems. Unique to this report is the reactivity of murine ATC/FDC with two macrophage and two dendritic cell (DC) markers, although at a lower level than macrophages or DC present in the same tissue section (Fig. 1). Several factors may account for these results. Our fixation is very mild, and we have used lower concentrations of antibody and 24-48 hour incubations. In

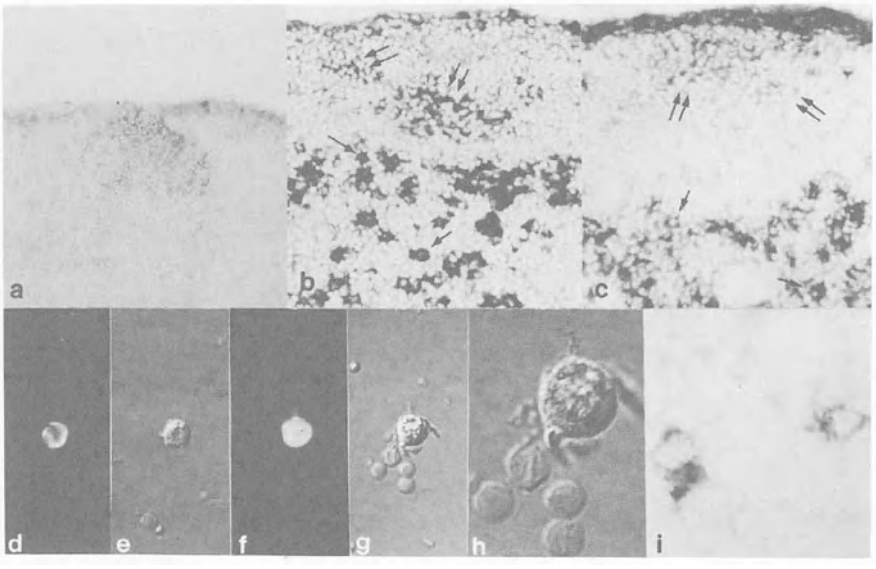

Fig. 1. First row: Antigen transport sites (1-15min)· a, 8C12 (CR1)+; b, NLDC-145+(double arrows), IDC (single arrows); c, MIDC-8+ (double arrows), IDC (single arrows); **Second row:** a large mononuclear blood cell: d, FDC-M1+; f, 2.4G2+; e, g, and h (high mag) same cells by Nomarski optics; and e, FDC-M1+ bone marrow (phagocytic/stromal?) cells Note lymphocytes are negative except for slight reactivity with in f with 2.4G2.

addition we have utilized a biotinylated secondary antibody together with a streptavidin conjugated marker to achieve higher sensitivity

The expression of CD45 (leukocyte common antigen) by FDC is controversial. We found FDC not to react with anti-CD45 clone 30F11.1, but FDC did react with anti-CD45 clone M1/9.3. The reactivity of clone 30F11.1 is restricted to the 220 KD chain expressed on B cells and the 200 KD chain expressed on T cells. However, clone M1/9 3 reacts with an element common to the B cell and T cell forms as well as the 205 KD chain found on macrophages. Taken together this suggest that ATC and FDC express the macroph age form of LCA.

This study found no disparity in ATC and FDC antigenic phenotype. Furthermore, ATC and FDC react with monoclonal antibodies specific for DC and macrophages. These results, taken with the reactivity of FDC-M1 with cells in the peripheral blood and bone marrow, further indicate that FDC are of bone marrow origin.

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EXPRESSION OF COMPLEMENT REGULATING PROTEINS ON FDC

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Introduction

In the generation of the secondary immune response FDC play a pivotal role as antigen retaining cells. Regularly, the antigens on the surface of FDC are complexed with complement and antibodies, thus forming immune complexes[1]. The antigen binds to FDC via Fc-receptors and complement receptors. The extracellular localisation of the antigen on the plasma membrane has been shown for many antigens including HIV-1[2,3]. Immune complexes on cell surfaces can activate the complement cascade, which eventually leads to lysis of the cells. This activation is regulated by membrane proteins, which protect host cells from the lytic action of autologous complement[4]. The membrane proteins, decay accelerating factor (DAF; CD55), complement receptor 1 (CR1; CD35), and membrane cofactor protein (MCP, CD46) induce the dissociation of the C3 convertase, thus preventing further activation of complement. In addition, CD35 and CD46 act as cofactors for C3b cleavage by the serum factor I. Protectin (CD59) is a membrane protein that prevents the assembly of the terminal complement complex (TCC). To elucidate how FDC as antigen retaining cells escape lysis by complement, we investigated whether the complement regulating proteins are expressed on FDC, using either isolated FDC or tonsil sections.

Materials and Methods

Palatine tonsils from ten children aged 2 - 10 years were obtained by standard surgical procedures. One part of the material was prepared for cryosections and for

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routine histological investigations. The remaining material was used for enrichment of FDC, as described elsewhere[5]. Briefly, single cell suspension were generated by mechanical and enzymatic disruption and an enriched FDC-fraction was obtained after discontinuous density gradient centrifugation. From these FDC fractions cytospin slides were prepared. The tonsil sections and cytospin slides were investigated by staining with the APAAP method (single staining). Furthermore, the cytospin slides were double stained, using primary mouse IgG (mAb directed to complement regulating proteins) and IgM (mAb directed to FDC: DRC1) antibodies and anti mouse IgG and IgM secondary antibodies coupled to peroxidase or alkaline phosphatase. Table I contains a list of the antibodies to the complement regulating proteins used in this study.

Table 1: Antibodies to complement regulating proteins used for this study

mAb specific for/ antigen recognized	origin	
MAC-inhibitor	CD59	Monosan (clone MEM 43)
MAC-inhibitor	CD59	IBGRL (clone Bric-229)
MAC-inhibitor	CD59	gift: Morgan, Cardiff (clone A35)
DAF	CD55	Serotec (clone MCA 914)
DAF	CD55	Serotec (clone MCA 737)
DAF	CD55	Wako Pure (clone WDR 7843)
MCP	CD46	Dianova (clone IOL 46)
CR1	CD35	Dianova (clone IOT 17)
TCC		Würzner, Innsbruck (clone WU 7-2)
anti factor B	--	Serotec (clone AHC 019)

Results

The tonsil sections as well as the isolated FDC on the cytospin slides showed expression of CD46, CD55 and CD59 on FDC. In comparison to the strong expression of CD55 (all clones used), an intermediate expression of CD46 and CD59 (clone Bric-229 and MEM-43) was observed on FDC. Furthermore, T-lymphocytes and endothelia showed a strong expression after staining with the mAbs to CD59 (clone Bric-229 and MEM-43). The clone A35, which is directed to CD59 as well, showed only a weak reaction on T-lymphocytes, but a strong reaction on FDC and endothelia. The highest concentration of the complement regulating proteins was observed in the apical light zone of the germinal centers. In addition, this area demonstrated a strong reaction with CD23 and with an antibody to the terminal complement complex (TCC). A weak expression of factor B was observed in macrophages, while FDC did not react.

Discussion

In earlier studies it has been demonstrated that FDC express complement receptors (CR1, CR2 and CR3) on their surface[6]. In addition to these molecules we are now able to show that FDC express complement regulating proteins on their surface as well. In contrast to Lampert et al.[7], who could not find expression of CD59 on FDC, we could detect expression of CD59, using three different mAbs. Factor B, which is involved in activation of the alternative pathway, was only weakly expressed on macrophages, but FDC were negative.

Our results suggest that FDC as antigen retaining cells escape the lytic activity of the complement activation in germinal centers by the expression of complement regulating proteins. This activation was clearly demonstrated with a mAb to the terminal complement complex (TCC; C5b-9), as published elsewhere[8].

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DISTRIBUTION AND CHARACTERIZATION OF FOLLICULAR DENDRITIC CELLS (FDCs) IN NON-HODGKIN'S LYMPHOMAS

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INTRODUCTION

Follicular dendritic cells (FDCs) are present in primary follicles and germinal centres of secondary follicles, forming a reticular spherical network by their elongated dendritic processes[1]. FDCs play an important role for immune response, trapping antigen and retaining immune complexes[1], and, in recent years, they are also considered to have a relation with apoptosis of B lymphocytes[2,3]. FDCs are also present in B cell lymphomas (follicular and diffuse lymphomas), considering that they may have a correlation with the differentiation of neoplastic B cells[4,5]. However, there are a few reports on the relationship between FDCs and extranodal B cell lymphomas and/or T cell lymphomas. It was attempted in this study to clarify the distribution of FDCs in these lymphomas and the relationship between FDCs and tumor cells, 156 cases with non-Hodgkin's lymphomas were examined immunohistochemically.

MATERIALS AND METHODS

Biopsy materials obtained from 156 cases with non-Hodgkin's lymphomas were used. Non-Hodgkin's lymphomas, except immunoblastic lymphadenopathy-like T cell lymphoma (IBL-like T) and/or AILD-like T cell lymphomas, were classified according to the criteria of the Working Formulation[6]. IBL like-T cell lymphoma was diagnosed on the basis of the histologic criteria described by Shimoyama et al.[7]. Histological subtypes are shown in Table 1.

All tissues were fixed in periodate-lysine-paraformaldehyde (PLP) fixative for 4 hours and snap-frozen at -70°C. Immunohistochemical staining was performed using Streptavidin biotin complex (StreptABC) method. Monoclonal antibodies used in this study are the following; R4/23 (DRC-1, DAKOPATTS(DA), Denmark), B2 (CD21, Coulter Clone (CC), USA), H107 (CD23, Nichirei, Japan), VCAM-1 (British Bio-technology, UK), and Ber-MAC-DRC (CD35, DA).

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RESULTS AND DISCUSSION

A summary of the distribution and characterization of FDCs in non-Hodgkin's lymphomas is shown in Table 1. All cases were classified into two types by immunostaining pattern of DRC-1. The 1st type showed FDCs were distributed with a dense and small nodular network pattern, corresponding to FDC-distribution pattern in non-neoplastic follicles This type was found in 39 of 108 cases with B cell lymphomas and 25 of 48 cases with T cell lymphomas. The reactivity of CD21, CD23, VCAM-1 and CD35 to FDCs in these cases was similar to that of DRC-1 The 2nd type showed FDCs were distributed with an irregular and well-developed network pattern (Fig.1a). This type was found in 17 cases of B cell lymphomas and 6 cases of T cell lymphomas The reactivity of CD21, VCAM-1 and CD35 to FDCs in these cases was similar to that of DRC-1, but the reactivtiy of CD23 was somewhat different from that of these markers. The staining pattern of CD23 was not a well-developed irregular network pattern, but a sparse and loose pattern (Fig.1b). Many FDCs in the neoplastic lesions of extranodal B cell lymphomas and nodal T cell lymphomas were negative for CD23, but were positive for CD23 in nodal B cell lymphomas.

Many investigators have reported that FDCs were found in neoplastic follicles of follicular lymphomas and in diffuse lymphomas. They also reported that the network pattern of FDCs in diffuse lymphomas was almost atrophic or destroyed by the proliferation of tumor cells, but large network pattern of FDCs was found in some cytological types, such as diffuse small cleaved cell type. Our data including extranodal lymphomas and T cell lymphomas were similar to their data. Fellbaum et al.[8] reported on the distribution pattern of FDCs of extra-

Table 1. Distribution pattern and characterization of FDCs in non-Hodgkin's lymphomas.

Distribution pattern of FDCs	Histology	No. of positive cases		Characterization of FDCs			
		Nodal	Extranodal	CD21	CD23	VCAM-1	CD35
irregular and well-developed network	B lymphoma						
	small cleaved cell	8/20	9/25	+	+/-[1]	+	+
	T lymphoma						
	small and large	2/9		+	+/-[2]	+	+
	large	2/19		+	+/-[2]	+	+
	IBL-T	2/8		+	+/-[2]	+	+
dense and small nodular network	B lymphoma						
	small lymphocytic	4/4	2/2	+	+	+	+
	small and large cell	4/7	2/9	+	+	+	+
	large cell	3/11	5/30	+	+	+	+
	small cleaved cell	7/20	12/25	+	+	+	+
	T lymphoma						
	small cleaved cell	2/4		+	+	+	+
	small and large cell	4/9		+	+	+	+
	large cell	10/19		+	+	+	+
	lymphoblastic	1/2	2/2	+	+	+	+
	IBL-T	6/8		+	+	+	+
no network	T lymphoma						
	polymorphous type	0/1					
	large cell		0/1				
	lymphoblastic		0/2				

+; The reactivity of these antibodies were similar to that of DRC-1. [1]Positive areas of CD23 were smaller than that of DRC-1 in 3 cases of 8 cases with nodal lymphomas and 8 cases of 9 cases with extranodal lymphomas. [2]Positive areas of CD23 were smaller than that of DRC-1 in each one case of small and large cell type, large cell type and IBL-T.

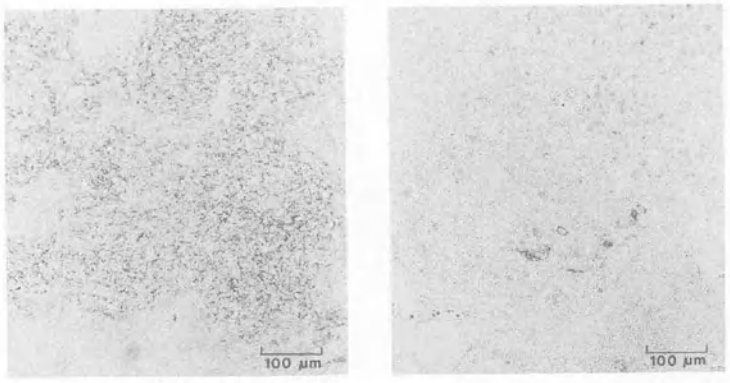

Figure 1. The distribution pattern of FDCs in extranodal B cell lymphoma, diffuse, small cleaved cell type. FDCs (DRC-1 positive) were distributed with an irregular and well-developed network pattern (1a), but the staining pattern of CD23 was a sparse and loose pattern (1b).

nodal lymphomas and Patsouris et al.[9] reported on the distribution pattern of FDCs of AILD type lymphomas. However, they didn't described the reactivity of various moAb to FDCs or the relationship between FDCs and tumor cells. Our data suggest that the tumor cells showing some cytologic subtypes of diffuse B cell lymphomas and of T cell lymphomas seems to have a capacity to induce FDCs. However, FDCs of extranodal B cell lymphomas and nodal T cell lymphomas are somewhat different in the expression of CD23 in comparison to those of nodal B cell lymphomas. A further study will be necessary to clarify the role of FDCs and/or CD23 in the neoplastic condition.

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FOLLICULAR DENDRITIC CELLS INDUCE B CELL ACTIVATION

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INTRODUCTION

Germinal centers are sites of B cell activation in secondary lymphoid tissues. B cell memory clones and plasma cell precursors are generated within these dynamic microenvironments[1,2]. It is not clear how germinal center reactions are initiated but the following scenario is quite plausible. Following immune complex deposition on follicular dendritic cells (FDC), the formation of iccosomes occurs and appears to at least partially trigger the response[3]. Circulating antigen specific resting B cells then come into contact with the FDC and iccosomes, becoming activated to a state such that they effectively process and present antigen[4]. This event results in the genesis of T cell factors necessary for invoking the germinal center reaction.

In an attempt to study the initial events leading to germinal center formation, we employed two different models to assess the antigen presenting cell function of a potential germinal center B cell population. The first model involved the *in vitro* activation of resting B cells. FDC loaded with immune complexes were incubated with small resting B cells obtained from immunoglobulin transgenic mice. Their ability to present antigen was compared to that of activated B cells and monitored using an antigen specific T cell clone. The second experimental system evaluated the ability to form germinal centers *in vivo*. Utilizing a different source of transgenic mice expressing the secreted form of a CTLA-4 fusion protein, that effectively blocked the co-stimulatory effect between B7 and CD28/CTLA-4, the need for this early B-T cell signal was assessed. While the results of these two studies have been presented elsewhere[5,6], a brief account of the data and an integrated discussion of the relevance of these findings will be presented here.

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IN VITRO ACTIVATION OF RESTING B CELLS BY FDC

In order to evaluate the activation capacity of FDC on small resting B cells, single cell suspensions were prepared from the spleens of SP6 mice (H-2^d) transgenic for dinitrophenol (DNP) specific immunoglobulin molecules[7]. The various populations were obtained as described elsewhere[5]. Briefly, small resting B cells were purified using discontinuous density gradients (1.079-1.085 g/cc band) and magnetic bead separation (anti-CD4, anti-CD8 and anti-Thy 1 coupled to Dynabeads). As a positive control for antigen presentation, activated B cells which are of a more buoyant density (1.063-1.070 g/cc) were also isolated from the transgenic mice. FDC and non-FDC populations which included macrophages and dendritic cells were obtained from C57BL/6 mice (H-2^b) immune to DNP-ovalbumin (OVA). Finally, the T cell clones used were restricted to H-2^d and specific for OVA.

Within 24 hrs of coculturing with FDC, the resting B cells showed an increase in both forward and side scatter profiles as well as levels of MHC Class II. By 48 hrs, these B cells also showed significant de novo expression of the T cell costimulatory molecule, B7-2. Neither dendritic cells, macrophages nor supernatants from FDC cultures provoked a similar response. These observations revealed that stimuli associated with FDC membranes induced changes in B cells that resulted in an antigen presenting cell phenotype.

To examine if these results reflected a change in their functional state, cultures containing resting B cells with and without soluble DNP-OVA, FDC carrying DNP-OVA or non-FDC (i.e. macrophages and dendritic cells) were prepared, irradiated and the OVA-specific T cell clones added[5]. As a positive control, low density activated B cells were also evaluated. After 48 hrs, ^{3}H-thymidine was introduced and incorporation by the T cell clones was measured over the next 24 hrs. The results in figure 1 show that resting B cells acquire the ability to function as antigen presenting cells only after coculturing with FDC (36,049 cpm). In contrast, B cells previously activated *in vivo* can present antigen obtained from FDC (34,130 cpm) and also the exogenously added soluble antigen (30,827 cpm). As the number of FDC per well was lowered, so did the stimulation capacity (36,049, 20,707, 9,955, 3,124, 624, 1,070, 660 cpm). This observation again suggested that FDC-B cell contact was necessary for the effect. Non-FDC populations such as macrophages and dendritic cells could not induce this antigen presenting capacity by the resting B cells (397 cpm).

Co-incubation with FDC clearly augmented the resting B cell's ability to function as an antigen presenting cell. These experiments support the concept that *in vivo*, small recirculating B cells can encounter antigen on FDC and become stimulated such that they can elicit the necessary T cell help leading to germinal center formation. The induction of higher levels of MHC Class II and the co-stimulatory molecule, B7, on potential germinal center B cells may be the earliest events required for producing a germinal center reaction.

IMPAIRMENT OF GERMINAL CENTER FORMATION IN CTLA-4 TRANSGENIC MICE

Interactions between the B7 family of molecules, expressed on dendritic cells, macrophages and activated B cells, and their ligands, CD28 and CTLA-4, expressed on T cells, are essential for optimal T cell activation[8,9]. To investigate an early role of B7-ligand interactions for generating germinal center responses, an *in vivo* model was examined.

Table 1. Effect of FDC on the Antigen Presentation Capacity of Resting B cells

	Thymidine Incorporation (cpm)[1]	
	Resting B cells	Activated B cells
B cells[2] alone	433	467
B cells + Ag[3]	365	645
B cells + T cells	453	1,036
B cells + T cells + Ag	544	30,827
B cells + FDC	758	729
B cells + T cells + FDC[2]	36,049	34,130
B cells + T cells + FDC (1/2)	20,707	19,928
B cells + T cells + FDC (1/4)	9,955	15,145
B cells + T cells + FDC (1/8)	3,124	6,898
B cells + T cells + FDC (1/16)	624	4,469
B cells + T cells + FDC (1/32)	1,070	2,427
B cells + T cells + FDC (1/64)	660	2,028
Additional Controls:		
B cells + T cells + non-FDC[2,4] + Ag	397	30,591
T cells + FDC (no B cells)	1,066	

1- Resting B cells were compared to activated 'control' B cells for their capacity to present antigen to T cell clones. Antigen was either added to the wells at 10 µg/ml or in the form of immune complexes on FDC. The concentration of cells per well was as follows: B cells, 3 x10^5; T cells 2 x 10^4; FDC starting concentration was 5 x 10^4 and then diluted 2 fold (e.g. 1/2, 1/4, etc.) The uptake of 3H-thymidine (by the T cell clones) was measured during the last 24 hrs of a 72 hr culture. All values represent the average of triplicate wells and the standard deviation was <5%.

2- irradiated with 1000 rads;

3- Ag = DNP-OVA;

4- Non-FDC refers to a population of macrophages and dendritic cells that were isolated from the same immune mice but specifically deleted for FDC.

Using the higher affinity ligand for B7, a chimeric immunoglobulin construct (human IgG1) expressing the extracellular portions of the mouse CTLA-4 gene was introduced into fertilized egg pronuclei[6]. The resulting transgenic mice maintained per ml of serum 10-30 µg of the soluble CTLA-4-human IgG1 molecule. This concentration was sufficient to saturate the B7 molecules expressed by activated B cells *in vitro*.

When CD4+ T cell priming was assessed in these transgenic mice, no impairment was observed[10]. However, in response to a challenge with T dependent antigens, antibody responses were significantly affected[6]. While antigen specific IgM responses were comparable to wildtype control littermates, all IgG isotypes were decreased by at least 10 fold. Following a primary, secondary or tertiary injection of antigen, germinal centers remained absent. Using the antigen, 4-hydroxy-3-nitrophenyl-acetyl chicken gamma globulin (NP-CγG), a reduction in cells undergoing somatic mutation and selection was documented. These observations of minimal somatic mutation and selection in conjunction with impaired class switching correlated functionally with the lack of germinal centers.

DISCUSSION

The data from the above *in vitro* model documenting the de novo induction of B7 expression on B cells by FDC combined with these *in vivo* observations strongly supports a role for B7 co-stimulatory signals for initiating germinal center reactions. As such, we would argue that FDC "prime" resting B cells for antigen-specific, cognate interactions with a T cell[5]. In order to generate a germinal center response, antigen specific B cells entering a primary follicle must be induced to express appropriate peptide/MHC molecules as well as function as antigen presenting cells. If the B cell fails in either aspect, no T cell help is elicited and no germinal center reaction can be provoked. In this manner, it appears that this specialized microenvironment significantly influences the humoral response. In other words, it insures that high affinity antibodies of isotypes that have specialized functions (for example, IgG2b versus IgE) are generated only after several criteria have been fulfilled. The complete understanding of these various steps will lead to better strategies for generating vaccines as well as therapies for reversing pathological conditions.

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GERMINAL CENTER T CELLS : ANALYSIS OF THEIR PROLIFERATIVE CAPACITY

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INTRODUCTION

Several immunohistochemical studies have revealed the existence of T cells expressing the CD57 antigen in the germinal center[1]. A few are also found in the interfollicular zones and the mantle zone[2]. Phenotypically they are $CD4^+$, $CD8^-$ cells. They do not express Leu8, CD16 or CD11b[3,4,5]. These cells are not fully activated being $CD25^-$,$CD71^-$ and HLA-DR^- cells[6].

There is no data as to the function of these germinal center T cells. Here, we report on the prolifertive capacity of germinal center T cells : after isolation from human tonsils, we stimulated them with mitogens alone or in coculture systems with B cells, fibroblasts or follicular dendritic cells. These results were compared to those obtained with $CD4^+CD57^-$ T cells (classical T helper cells) which are mainly located in the interfollicular areas but are also present in the germinal centers.

MATERIAL AND METHODS

The T lymphocytes were isolated from tonsils by rosetting with sheep red blood cells (SRBC) or by running the lymphocyte suspensions through nylon wool columns. The recovered T cells were labelled with anti-CD8 and anti-CD19 mAbs and dynabeads to discard the contaminant B and $CD8^+$ cells. An anti-CD57 mAb and a magnetic cell sorter were used to sort the $CD4^+$T cells into $CD4^+CD57^+$ and $CD4^+CD57^-$ cells. B cells, fibroblasts and follicular dendritic cells (FDC) were also isolated from tonsils (see Bosseloir et al., this volume).

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Cultures were grown in RPMI 1640 supplemented with 10% FCS. 1.10^5 T cells were cultured in 96-well microtiter plates in a final volume of 200µl per well. Different polyclonal T cell activators (PHA: 2µg/ml; ConA: 10µg/ml; IL-2: 10U/ml) were added to the cultures. For cocultures 3.10^5 B cells, 10.10^4 fibroblasts or 5.10^3 follicular dendritic cells were added to 1.10^5 T cells per well in a final volume of 200µl. B cells, fibroblasts and follicular dendritic cells were pretreated with mitomycin (50µg/ml) to prevent their proliferation. The cultures were incubated for 48h and the cell proliferation was tested by pulsing the cells with ^{3}[H]-thymidine during 16-hours of incubation.

RESULTS

The $CD4^+CD57^+$ cells (germinal center T cells) or $CD4^+CD57^-$ cells were cultured with PHA or ConA additioned or not with IL-2 for 2 days and then checked for their capacity to incorporate tritiated thymidine. In absence of mitogens, no proliferation was found. The proliferation of the $CD57^-$ cells was markedly increased in the presence of each of the different activators used especially when IL-2 was present in the medium. The $CD57^+$ cells did exhibit no or a clearly less proliferative activity than $CD57^-$ cells. Only ConA additioned with IL-2 induced some multiplication of these cells (Fig 1).

When PHA + IL-2 stimulated T cells were cocultured with mitomycin-treated B cells or fibroblasts, a clear improvement of the proliferative activity was observed in the $CD57^-$ cells but weak or no proliferative activity was found in the $CD57^+$ cells even in contact with accessory cells (Fig2).

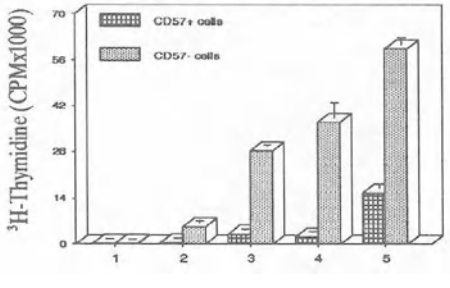

Figure 1. Proliferative test of $CD57^+$ or $CD57^-$ T cells stimulated with PHA (2µg/ml), ConA (10µg/ml) in presence or not of IL-2 (10µg/ml). The level of tritiated thymidine uptake is expressed in CPM.
1 : No mitogen; 2 : PHA; 3 : PHA + IL-2; 4 : ConA; 5 : ConA + IL-2.

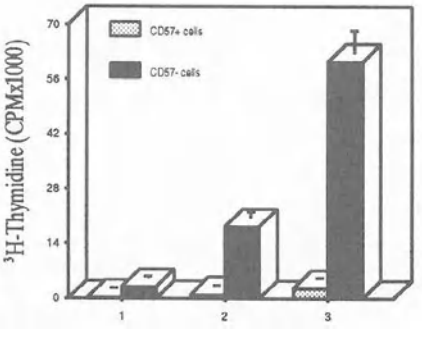

Figure 2. Proliferation test of $CD57^+$ or $CD57^-$ T cells stimulated with PHA + IL-2 (1) and cocultured with mitomycin C-treated B cells (2) or fibroblasts (3). Only weak level of tritiated thymidine incorporation was observed in mitomycin C-treated cells.

Similar results were obtained when. The CD57$^+$ or CD57 T cells were cocultured with mitomycin C-treated follicular dendritic cells (Fig3).

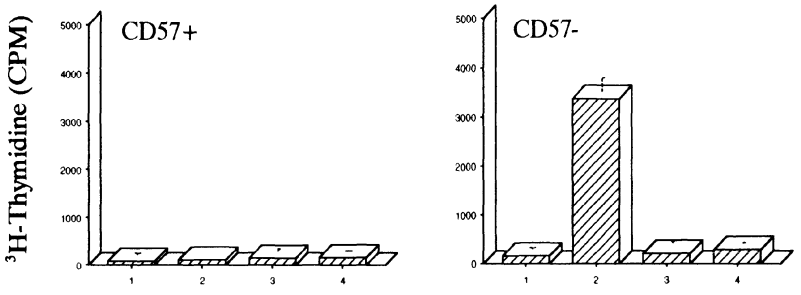

Figure 3 : Proliferation test of CD57$^+$ or CD57 cells cultured alone (1) or cocultured (2) or not (3) with mitomycin C treated follicular dendritic cells. Only a weak proliferation was measured in the FDC population (4).

DISCUSSION

We show here that the germinal center CD4$^+$CD57$^+$ T cells respond only weakely to mitogens even when cocultured in contact with accessory cells (B cells, fibroblasts, follicular dendritic cells). On the contrary, the CD4$^+$CD57 cells (classical T helper cells) are sensitive to mitogens used and proliferate even more intensively in cocultures. These CD57$^+$ T cells appear to be not fully activated[6] and contrary to the CD57 cells, they do not provide any help to B cell proliferation or differentiation in Ig-secreting cells (Bouzahzah et al. to be published). The capacity of these cells to secrete cytokines is much debated : Bowen et al.[6] found that the isolated CD4$^+$CD57$^+$ cells do not secrete cytokines whereas Butch et al.[7] showed that they consistently contained mRNA for IL-4. Germinal center CD57$^+$ T cells constitute thus a peculiar cell population. Their precise function remains unknown.

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ICCOSOMES AND INDUCTION OF SPECIFIC ANTIBODY PRODUCTION IN VITRO

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INTRODUCTION

Follicular dendritic cells (FDCs) are immune accessory cells, located in lymphoid follicles[1]. FDCs can trap and retain antigen antibody complexes on their long slender dendrites for a long periods of time[2] and it appears that antigen retained on FDCs can induce primed B cells to make specific antibody[3]. FDCs can form liposome-like immune complexes coating bodies (iccosomes)[4]. These iccosomes may be released and then engulfed by adjacent B lymphocytes. The engulfed iccosomes appeared to disintegrate in the B cells close to Golgi complex. We believed the iccosomal Ag is being processed and Ag fragments are being delivered to the B cell surface for presentation to T cells[5]. Antibody forming cells (AFC) emerge in germinal centers 3 to 4 days after antigen challenge[6]. The coincidence of iccosome release and AFC emergence led to the hypothesis that antigen bearing iccosomes might be able to induce B cells to differentiate into AFC[7]. In this study, we sought to test the hypothesis that iccosomal Ag is involved in the induction of specific Ab production. Iccosomes were prepared from FDCs 3 days after OVA challenge by sonicating FDCs or shaking them in the upper chamber of a transwell apparatus with a 3.0 μm pore diameter filter and collecting icccosomes from the lower chamber of the apparatus. Both iccosome preparations were able to induce OVA primed lymphocytes to produce anti-OVA. However intact FDCs were critical for optimal Ab production.

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MATERIALS AND METHODS

BALB/c mice were primed with the OVA precipitated by aluminum potassium sulfate containing heat killed Bordetella pertusis. Antigen bearing FDCs were isolated from draining lymph nodes[8] of OVA immunized mice 3 days after OVA challenge. Non-OVA bearing FDCs ($FDCs^-$) were isolated from non-immunized mice. OVA primed lymphocytes were isolated from draining lymph nodes 30 days after OVA challenge. Iccosomes were prepared by sonicating FDCs (s-FDCs) and then pelleting it by ultracentrifugation or by shaking FDCs over 3 μm pore size transwell and collecting the iccosome rich filtrate from the lower chamber of the transwell. Non-OVA bearing iccosomes (I^- vs I^+ for OVA bearing iccosomes) were also prepared from $FDCs^-$ by transwell shaking. Anti-OVA total IgG was monitored in the supernatant using an ELISA .

RESULTS & DISCUSSION

The essential results are recorded in table 1. Iccosomes, prepared by sonicating and transwell shaking, were cultured together with lymphocytes and induced a modest anti-OVA production (groups 1 and 2). However addition of $FDCs^-$ markedly improve the response (group 3). FDCs + lymphocytes represents the positive control (group 4). Negative controls (groups 5, 6, 7, 8, 9, 10, 11, 12) made no measurable anti-OVA.

Table 1. In vitro anti-OVA total IgG production

Groups	anti-OVA IgG	Groups	anti-OVA IgG
1) I^+ +LCs	4.2±0.3 (ng/ml)	7) I^- + LCs	non measurable
2) s-FDCs + LCs	10±4.04 (ng/ml)	8) I^0 + LCs	non measurable
3) I^++ $FDCs^-$ +LCs	180±58 (ng/ml)	9) s-FDCs alone	non measurable
4) FDCs + LCs	1300±404 (ng/ml)	10) Ag + LCs	non measurable
5) LCs alone	non measurable	11) Ag-Ab+LCs	non measurable
6) I^+ alone	non measurable	12) $FDCs^-$+LCs	non measurable

LCs = memory lymphocytes; I^+ = OVA bearing iccosome preparation; I^- = non-OVA bearing iccosome preparation; I^0 = filtrate from 0.1 μm pore size transwell; s-FDCs = sonicated FDCs bearing OVA; $FDCs^-$ = non-OVA bearing FDCs; Ag = free OVA; Ag-Ab = OVA-anti-OVA complex.

The ability of s-FDCs bearing OVA or transwell filtrate from shaking OVA-bearing FDCs to stimulate lymphocytes to produce anti-OVA IgG in vitro implies that iccosomes or iccosome-like entities are capable of eliciting specific antibody responses. Since iccosomes are liposome-like particles with average size of 0.3 to 0.7 μm[5], they should be spun down during ultracentrifuge and should not pass 0.1 μm pores. The results in this study are consistent with these properties. Similar amount of free antigen or immune complex failed to elicit anti-OVA production, indicating that iccosomes are very potent stimulators. It was also noticed that non-OVA bearing FDCs can enhance iccosome elicited anti-OVA production by providing costimulatory signals. All these results support the hypothesis that antigen bearing iccosomes released in vivo stimulate B cells to differentiate into AFC and make specific antibody[7].

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LOCALIZATIONS OF REGULATORY PROTEINS OF COMPLEMENT COMPONENTS ON RA SYNOVIAL TISSUES– ESPECIALLY GERMINAL CENTERS

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INTRODUCTION

We reported that lymphoid follicles of the rheumatoid arthritis(RA) synovial tissues ranged among the most important sites for local immunoreactions[1,2]. They are thought to promote the production of immunoglobulins during the acute inflammatory phase. Recently regulatory proteins(RPs) have been found which control the complement(C) activation cascade[3,4]. The aim of the present study is to evaluate the localization of RPs of C components on RA synovial tissues,especially germinal centers.

MATERIALS & METHODS

Synovial tissue samples were obtained from 21 RA patients. The samples were fixed in 10% formalin and 2% periodate-lysine-paraformaldehyde. The former were embedded in paraffin and the latter in OCT compound. The samples were cut into 4-6 micrometer slices,placed on albumin-coated glass slides,and immunostained using a direct or an indirect immunoperoxidase method (Table1:1st antibodies for thisstudy),and finally examined by light and electron microscopy.

RESULTS and CONCLUSION

Immunohistochemical results are summerize in Table 2. RPs,C4 binding protein(C4bp), decay accelerating factor(DAF),comlement receptor(CR1),CR2,S-protein/vitronectin(VTN) were expresed as a lacy network by light microscopy similar to that of a monoclonal antibodies,R4/23, specific for follicular dendritic cells(FDCs). By electron microscopy,positive immunoreactions for DAF and VTN were found on the surface of GC cells and were especially marked on the labyrinth-like cytoplasmic extensions of FDCs.

Our results suggest that cells in the GCs were pretected from membranolysis by some of the RPs.

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Table 1

Antibodies	Immmunized animal	Source
Factor B	Rabbit	Haechst
C5b-9(membrane attack complex)	Mouse	Dakopatts
C4 binding Protein(C4bp)	Sheep	Serotec
Decay accelerating factor(DAF:CD55)	Mouse	Wako
Factor H	Goat	Cappel
Properdin	Rabbit	Miles
Complement receptor 1(CR1 CD35)	Mouse	Dakopatts
Complement receptor 2(CR2:CD21)	Mouse	Becton Dickinson
S-protein(Vitronectin:VTN)	Mouse	Boehringer
R4/23	Mouse	Dakopatts

Table 2

	RA synovial tissues	
Antibodies	GC	lining cell layer
Factor B	+	+
C5b-9(membrane attack complex)	+	+
C4 binding Protein(C4bp)	+	+
Decay accelerating factor(DAF·CD55)	+	+
Factor H	+	+
Properdin	+	+
Complement receptor 1(CR1:CD35)	+	-
Complement receptor 2(CR2:CD21)	+	-
S-protein(Vitronectin·VTN)	+	+
R4/23	+	-

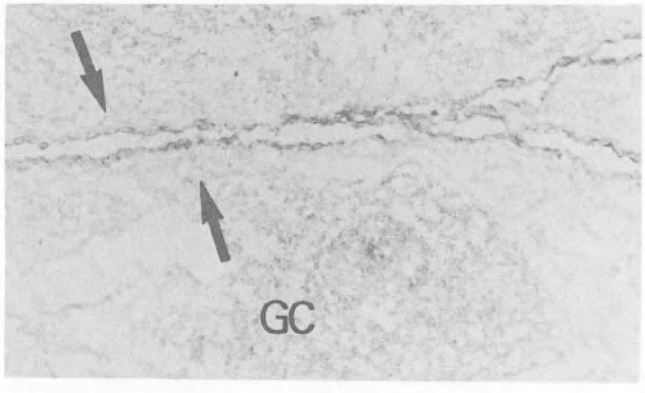

Figure1. Immunostaining for DAF in the synovial tissue with RA by serial section technique (**Figure1&2**) The germinal centers(GCs) and lining cell layers(arrows) show positive reactions.

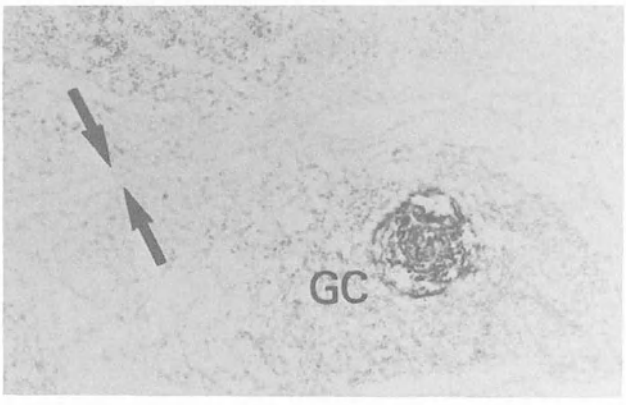

Figure2. Immunostaining for CR1.The GCs shows positive reactions but lining cell layers(arrows) negative reactions.

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IN VITRO IMMUNE COMPLEX BINDING ASSAY TO EXAMINE THE MECHANISM OF IMMUNE COMPLEX TRAPPING BY HUMAN FOLLICULAR DENDRITIC CELLS (FDC)

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INTRODUCTION

The trapping and long term retention of exogenous or autogenous antigens in the form of immune complex (IC) is one of major cardinal features of follicular dendritic cells (FDC). This tapping are thought to be mediated mainly by complement receptors (C'Rs), which are distributed abundantly on the surface of FDC[1,2]. The precise molecular mechanisms of this phenomenon, however, are still obscure. For example, controversial ideas have been reported as to the contribution of Fc-receptors (FcRs) to the trapping[3,4,5]. Especially the investigations are very limited in human system because it is difficult to establish the experimental approaches *in vivo*.

In the present study, an *in vitro* assay system to assess the IC-trapping ability of FDC in human tissues has been developed. This assay uses heterogeneous peroxidase-antiperoxidase complex (PAP), fresh human serum as complement source and unfixed cryostat sections from reactive tonsils or lymph nodes. This system will be expected to provide the further informations on the mechanisms of IC-trapping by human FDC in physiological or pathological conditions.

MATERIALS & METHODS

The assay was performed as follow: The mixture of heterogeneous PAP and fresh human serum were incubated on the unfixed cryostat sections of human tonsils for 30 min at 37°C. After washing, some sections were fixed in glutaraldehyde and then developed in 3-3'-diaminobenzidine solution to detect the peroxidase activity which indicated the localization of the IC. Simultaneously the other incubated sections were fixed in absolute acetone or periodate-lysine-paraformaldehyde solution and followed by incubation with suitable maker (FITC, alkaline phosphatase etc) conjugated anti-heterogeneous immunoglobulin antibody to confirm that the immunoglobulins constituting the PAP also revealed the same distribution as the antigen (peroxidase). Heterogeneous PAP employed in the present study were listed in Table 1.

Inhibition experiments were performed by addition of a panel of monoclonal antibodies against human C'Rs, FcRs and other related molecules or heat aggregated (63°C, 30 min) human immunoglobulins to the PAP-serum mixture.

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Table 1. Binding of PAP from different species to GC on cryostat sections of human tonsils.

species of PAP	working concentration	complement source	results
Mouse (polyclonal) PAP	0.5 mg/ml	fresh human serum	+/–
Mouse (monoclonal IgG1) PAP	0.106 mg/ml	fresh human serum	–
Rat PAP	0.5 mg/ml	fresh human serum	+/–
Guinea pig PAP	0.5 mg/ml	fresh human serum	–
Rabbit PAP	unknown (1:5 ~ 1:50)	fresh human serum	+/– or +
Goat PAP	0.5 mg/ml	fresh human serum	++
Sheep PAP	0.5 mg/ml	fresh human serum	++

Table 2. Binding of PAP to GC of tonsils with inactivated serum or overdiluted serum.

species of PAP	concentration of PAP	treatment of human serum	results
rabbit PAP	1:5 dilution	Non-treated (fresh serum)	+
rabbit PAP	1:5 dilution	heat inactivated	–
rabbit PAP	1:5 dilution	overdiluted (1:1000) serum	–
Goat PAP	0.5 mg/ml	Non-treated (fresh serum)	++
Goat PAP	0.5 mg/ml	heat inactivated	–
Goat PAP	0.5 mg/ml	overdiluted (1:1000) serum	–

Table 3. Inhibition of PAP-binding to GC by a panel of monoclonal antibodies.

monoclonal antibody [manufacture]	concentration or dilution	inhibition
1F8 (CR2/CD21) [DAKO]	27 – 54 mg/ml	+/–
BU32 (CR2/CD21) [BindingSite]	10 – 50 mg/ml	–
BU35 (CR2/CD21) [BindingSite]	10 – 50 mg/ml	–
HB-5 (CR2/CD21) [Becton-Dickinson (B-D)]	1:10 (100 tests)	–
To5 (CR1/CD35) [DAKO]	27 mg/ml	+/–
Ber-Mac-DRC (CR1/CD35)[DAKO]	21 mg/ml	–
Mixture of 1F8 + To5		+
Mo1 (CR3/CD11b) [Coulter]	1:10 (100 tests)	–
Mac-1 (CR3/CD11b) [Serotec]	1:10 (100 tests)	–
Leu11b (FcγRIII/CD16) [B-D]	1:5 – 1:10 (100 tests)	–
3G8 (FcγRIII/CD16) [Immunotech]	40 mg/ml	–
GRM1 (FcγRIII/CD16) [Clonab]	1:5 (100 tests)	–
2E1 (FcγRII/CD32) [Immunotech]	40 mg/ml	–
10.1 ((FcγRI/CD64) [Serotec]	1:5 (200 tests)	–
mixture of all monoclonal antibodies to Fcγ receptors		–
BU38(FcεRII/CD23)[BindingSite]	10 – 50 mg/ml	–
H107 (FcεRII/CD23) [Nichirei]	1:10 (100 tests)	–
HLA-DR [DAKO] + [Becton-Dickinson]	mixture	–
R4/23 [DAKO]	30 - 70 mg/ml	–
DF-DRC [Sera-Lab]	1:10	–
BU-10 [BindingSite]	10 - 50 mg/ml	–
Ki-M4 [Serotec]	20 mg/ml	–
VIIIA7 (DAF/CD55) [Dr. Kinoshita, Osaka]	1:10	–
inappropriate mouse IgG1 (control)	40 mg/ml	–
inappropriate mouse IgG2 (control)	40 mg/ml	–
normal mouse serum (control)	1:200	–

RESULTS AND DISCUSSIONS

The PAP derived from several different species were used as heterogeneous IC (Table 1). When goat and sheep PAP were incubated on sections, the complexes bound

very efficiently to germinal centers (GC), especially their light zone, with reticular pattern. Such binding pattern of PAP suggested that they might be trapped *in vitro* by FDC. In contrast, PAP from rodent animals such as mouse, rat and guinea pig did not bind to GC significantly(Table 1). Such differences by species of PAP are quite interesting to consider the molecular mechanism of IC-trapping by FDC.

The binding of PAP in our system is complement-dependent because PAP did not bind to GC when heat inactivated serum or overdiluted serum were used instead of fresh serum (Table 2). In addition, an mixture of anti-CR1 and anti-CR2 could inhibit the binding (Table 3). These indicated that C'Rs (CR-1 and CR-2) constitutively involve to the binding in our system as well as *in vivo*. On the other hands, the contribution of FcRs could not be confirmed in our experiments because none of monoclonal antibodies against human FcRs could inhibit the binding (Table 3). Nevertheless, the possibility that certain FcRs might participate to the binding could not be eliminated because heat aggregated IgG could efficiently inhibit it (Table 4). Indeed, controversial observations have been reported[3,4,5,6]as to the expression of FcRs on FDC and their contribution to the IC-trapping. Further analysis using our system may provide us a clue to solve these disputes.

Dijkstra et al[7] and Yoshida et al[5] reported the similar system in rat or mouse, respectively. The advantages of our system are as follow: 1) unfixed sections were used to get more physiological condition,.2) many tools such as numbers of monoclonal antibodies against surface molecules and biomedical reagents are available, 3) both of antigen and antibody constituting the IC can be detected easily and 4) it can be applied in pathological situation. In addition, this system provide us one of principle methods to identify FDC in section or in suspensions of human tissues.

Table 4. Competitive inhibition of PAP-binding to GC by heat aggregated human Igs.

human aggregated immunoglobulins	concentration	PAP	inhibition
heat aggregated IgG	1.0 mg/ml	goat PAP	++
heat aggregated IgM	1.0 mg/ml	goat PAP	+/–
heat aggregated IgA	1.0 mg/ml	goat PAP	+/–

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SIV INFECTION OF FOLLICULAR DENDRITIC CELLS (FDC) AND OTHER SPLEEN CELL SUBSETS IN EXPERIMENTALLY INFECTED CYNOMOLGUS MONKEYS

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Introduction

Follicular dendritic cells (FDC) are localized in primary and secondary lymphoid follicles (germinal center) and are instrumental as antigen presenting cells for the humoral response, particular for the development and maintenance of B-cell memory[1]. During HIV-1 infection immunohistochemical studies of lymphnode sections revealed that continous destruction of FDC by yet unknown mechanisms was a hallmark of progression to AIDS[2,3].

We recently could demonstrate that highly purified normal human tonsil FDC were susceptible to HIV-1 infection *in vitro* in a CD4-independent way[4]. These findings therefore raised the important question as to the possible *in vivo* permissivnes of FDC to HIV as an explanation for their destruction during HIV-1 infection. However, although EM studies demonstrated virus budding on FDC membranes in hyperplastic lymph node follicles of HIV-1 infected individuals[5], documented histological observations are at present controversial[6]. Obviously this controversy is related to methodological difficuilties of such studies given that the FDC only represent approx. 2% of total germinal center cells[7], therefore requiring extensive screening of tissue sections. To overcome this sampling problem we have enriched FDC from spleens of SIVsmm3-infected monkeys (*Macaca fascicularis*), which have been shown to develop a simian immunodeficiency syndrome with clinical, immunological and pathological hallmarks of human AIDS, including lymphadenopathy and progressive involution of germinal centers[8]. Spleen FDC from such animals were immunoaffinity enriched and their *in vivo* permissivnes for viral infection was compared with that of spleen B/T lymphocytes and macrophages (MΦ).

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Material and Methods 14 cynomolgus monkeys experimentally infected with SIVsmm3 and displaying different disease stages as described elsewhere[9] were entered in this study. At necropsy the spleen was immediatly dissected and cell suspensions obtained by enzymatic digestion (16 µg/ml Collagenase VII, 20µg/ml DNAse I; Sigma) were further fractionated. FDC were enriched using the FDC-specific antibody KIM4 (Behring-Werke, Marburg, Germany) and immunomagnetic separation (MACS) as recently described for human tonsil FDC[10]. The macrophage fraction was enriched by use of MACS prior to the separation of FDC and the B/T cell fraction, which was depleted of macrophages and FDC. Cytospins of the enriched cell fractions were characterized by immunophenotyping using mAbs to CD2 (Leu 5b; Becton-Dickinson), CD20 (L26; Dakopatts) and CD68 (KIM6; Behring-Werke) and by labelling of cell surface markers and *in situ* hybridization for viral RNA or double spliced *tat/rev* mRNA. For the detection of DNA or mRNA by nested PCR, limiting dilutions of cell subsets from seven spleen specimen were prepared prior to lysis of the cells. cDNA was prepared by use of random hexamer priming, subsequently followed by reverse transcritption. *In vitro* co-culture experiments were performed to quantify virus RT acivity associated with cell subsets.

Results

Phenotypic Analysis of Fractionated Spleen Cell Subsets Cytospin preparations immunostained for FDC (KIM4), MΦ (CD68) and B/T lymphocytes (CD20, CD2) revealed that the FDC fractions prepared from early stage monkeys contained 10%-20% KIM4+ FDC and approx. 80% B lymphocytes which were trapped by FDC-dendrites forming FDC-B-cell clusters[11]. The number of contaminating MΦ and T-cells was <5%. The MΦ fraction contained 30%-50% macrophages, 40%-50% B cells and 5%-10% T-lymphocytes. This was confirmed by RT-PCR analysis for rearranged TCR-cβ mRNA (table 1). The B/T lymphocyte fraction contained 60%-70% B-cells, 20%-30% T-cells and 5% MΦ.

Analysis of Spleen Cell Subsets for Latent and Productive Virus Infection Latent or productive SIV infection of the fractionated cells was assayed by PCR and RT-PCR analysis respectively on limiting dilutions of cell subsets (10^5-10^1 cells) prepared prior to cell lysis in order to prevent dilution of target copies. A PCR detection limit (DL) was defined by the minimum number of cells needed for a positive PCR result. Considerable differences were found in the DL's of the cell subsets from early compared to late stage infection (table 1).Thus, in early stage the DL for proviral DNA (pol) varied between 10^3 for MΦ to 10^5 for B/T lymphocytes. However. the DL for spliced *env* mRNA was clearly different in the various cell subsets and did not correlate with that for SIVpol . Thus, the amount of

amplifiable *env* mRNA target was 100-fold higher in the FDC fraction when compared to MΦ and B/T lymphocyte subsets (table 1). Parallel amplification of TCR-cβ chain mRNA demonstrated a DL of 10^5 in all FDC fractions, indicating that T-cell contamination could not account for the results obtained neither contamination with MΦ since the*env* DL was 10^5 for the MΦ fraction. In late stage animal little variation was seen in both latent and active infection, where the DL for SIVpol was 10^5 in both FDC and B/T fractions but undetectable in 10^5 MΦ (table 1).

***In situ* Hybridization (ISH) for Viral RNA and Spliced *tat/rev* mRNA**
The spleen fractions were studied simultaneously for surface markers by immunohistochemistry and viral RNA or double spliced *tat/rev* mRNA by ISH. Whereas ISH signals were almost absent in the MΦ and B/T lymphocyte fractions, KIM4-labelled FDC were found to express both, viral RNA as well as spliced mRNA, confirming the RT-PCR results. Furthermore, as shown in Fig. 1, two different hybridization patterns were evident . While ISH detection for viral RNA resulted in a diffuse labeling of FDC-B cell clusters indicative for virus immunecomplexes trapped by FDC (Fig. 1A) a more focal signal accumulation on individual FDC was seen for *tat/rev* mRNA (Fig. 1B, C).

Demonstration of RT activity in Spleen Cell Subsets The spleen cell subsets were cultured *in vitro* in the presence of normal human monocyte-derived macrophages. Results of such a experiment revealed that the FDC fraction produced a 8- to 15-fold higher RT-activity compared to the other cell subsets from the same animal (table 2).

Discussion The results described above indicate that spleen FDC in SIV infected monkeys become infected *in vivo* particulary at early stage of infection. This results agree with ultrastructural studies performed on PGL lymphnode biospies of asymptomatic HIV-1 patients showing virus budding on FDC-dendrites in hyperplastic germinal centers[12], and also confirm previous reports on the*in vivo* permissivness of human FDC for HIV-1 infection[13,14]. The low frequency of SIVpol positive cells in the FDC fraction may reflect a differential permissivnes among the different maturation stages described for FDC in a germinal center[15]. The observed decrease in the number of KIM4+ FDC recovered from late stage animals and the fact that these late stage FDC exhibited little virus replication could suggest that virus replicating FDC are eliminated during the course of disease. Similarily It was experimentally demonstrated that virus-infected FDC in the animal model of LCMV-infected mice are eliminated by cytotoxic CD8+ cells[16]. Also during HIV/SIV infection CD8+ cells were demonstrated to infiltrate germinal centers[17,18]. In summary our findings and that of other groups[5,10-12] provide compelling evidence that FDC may serve as at target cell for virus infection *in vivo* . Thus, a subsequent destruction of such cells may indeed constitute a major pathway of HIV-1 pathogenesis as recently proposed[19].

Table 1. Detection limit (DL) for latent (pol) and productive (env) SIV infection as well as for TCR-cβ mRNA in spleen cell subsets of early and late stage animals, expressed as the lowest amount of PCR-positive cells for the indicated product.

	DNA				mRNA					
PCR-Product	β - globin		SIVpol		β - actin		SIVenv		TCR-cβ	
stage of animal[1]	*early**	*late¤*	*early*	*late*	*early*	*late*	*early*	*late*	*early*	*late*
B/T	10^1	10^1	10^5	10^5	10^2	10^2	10^5	10^5	10^3	10^3
MΦ	10^1	10^1	10^3	$>10^5$.	10^2	10^2	10^5	$>10^5$	10^4	10^5
FDC	10^1	10^1	10^4	10^5	10^2	10^2	10^3	10^5	10^5	10^5

[1] = *asymptomatic or ¤symptomatic animal with clinical manifestations indicative for simian AIDS[8].

Table 2. RT-activity after 12 days of co-cultivation of spleen cell subsets with human monocytes-derived macrophages

spleen cell subset[1]	RT counts in cpm/10^6 cells
FDC	64.000
MΦ	4.300
B/T*	1.500
B/T4¤	8.000
T8+	0

[1] all subsets but T8+ cells were PCR-positive for SIVpol before onset of culture; RT = reverse transcriptase; * = B/T lymphocytes not depleted of CD8+ cells; ¤B/T lymphocytes depleted of CD8+ cells by use of mAb Leu2a and MACS.

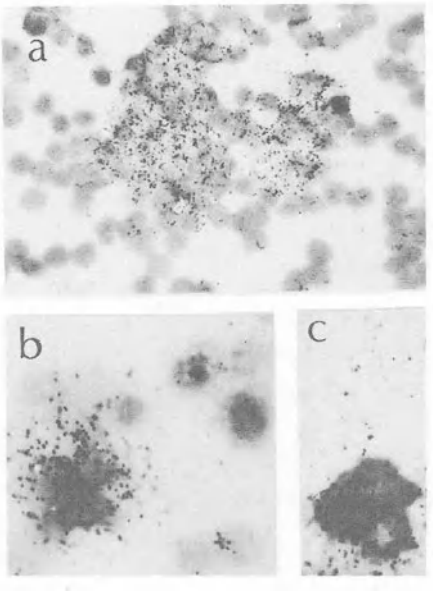

Fig.1 ISH demonstrating association of SIV RNA or mRNA with FDC; A) RNA localized on a FDC-B-cell cluster; B) *tat/rev* mRNA detection on a small FDC-B-cell cluster, and C) *tat/rev* mRNA detection on a single KIM4+ FDC

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MORPHO-FUNCTIONAL CHANGES OF FOLLICULAR DENDRITIC CELLS (FDC) AND LYMPH NODE STRUCTURE IN SIMIAN IMMUNODEFICIENCY VIRUS (SIV) INFECTION

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Introduction

The onset of acquired immunodeficiency syndrome (AIDS) has been reported to coincide with destruction the network of follicular dendritic cells (FDC) in germinal centers of lymph nodes (LNs)[1]. These observations have led to the theory in which one of underlying mechanisms of HIV pathogenesis may be loss of FDC in LNs[2]. This process can be investigated in rhesus macaque infected with simian immunodeficiency virus (SIV), developing an immunodeficient disease closely resembling AIDS[3]. Although the specific role of FDC has been recognized in both HIV- and SIV-induced diseases[1,4], up to now little is known about morpho-functional changes of these cells during follicular hyperplasia and follicular involution in LNs. Investigating LNs of monkeys infected with SIV we found that morpho-functional peculiarities of FDC were closely related to virus distribution, local cytokine production and structure of lymphoid tissue.

Materials and Methods

Seven monkeys (Macaca mulatta) were inoculated i.v., as previously described[5], with 4-400 median macaque infectious doses of SIVmac 251. Three animals were sacrificed 13, 27 and 29 months respectively after inoculation when they became moribund and showed signs typical for AIDS. Four other macaques were euthanized while still asymptomatic 28-34 months after inoculation. Specimens from three groups of LNs (inguinal, mesenteric, axilary) were collected at the time of postmortem examination. Human LNs which did not show activated germinal centers or paracortical hyperplasia were obtained during postmortem examination and used as tissue controls. For each specimen a part was processed for conventional histology, another was snap-frozen in liquid nitrogen for immunohistochemistry and a small one was prepared for transmission electron microscopy. Immunohistochemistry was performed on 6 μ cryostat sections to detect SIV*gag* p27 antigen, macrophages, B, CD4+, CD8+ lymphocytes, FDC, proliferating Ki67 positive cells, expression of CD23, vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), endothelial cell adhesion molecule 1 (ELAM-1), tumor necrosis factor α (TNF α), interleukins 1 α (IL-1) and 6 (IL-6).

Results and Discussion

Four patterns of morphological alterations (follicular hyperplasia - 19%, follicular

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involution - 54%, diffuse follicular and paracortical depletion - 24% , and granulomatous lymphadenitis - 5%) were found in LNs of rhesus monkeys infected with SIVmac 251. In hyperplastic follicles the network of FDC was expanded and these cells formed numerous short processes (Fig.1). A fine reticular staining for SIV*gag* p27 antigen was predominantly associated with outer part of FDC network and viral particles were localized between processes of FDC (Fig.1). In involuted follicles, reduced germinal centers were permeated by low number of FDC and viral antigen and particles were associated with isolated FDC processes or enclosed in paracortically located macrophages.

A strict zonal division of germinal centers into light zone permeated by CD23 positive FDC and highly enlarged dark zone occupied by Ki67+ proliferating centroblasts was preserved in some hyperplastic germinal centers. However, the other follicles demonstrating striking hyperplasia displayed Ki67+ cells in both CD23+ and CD23- zones of FDC network. In involuted follicles, the number of Ki67+ centroblasts was significantly reduced and they were absent in CD23+ part of FDC. Strong CD23 presentation on FDC appears to be unique for LNs of HIV-infected individuals[6] or as reported here in those of SIV-infected monkeys. Since the CD23 labeling was found to correspond the SIV antigen distribution, it is possible that presence of SIV may up-regulate CD23 expression.

It has been shown that adhesion of B lymphocytes to FDC is mediated by interaction of very late antigen 4 with VCAM-1[7]. Although the enhanced expression of VCAM-1 has been recently reported in reactive human LNs[8], there is no information about VCAM presentation in LNs in HIV- or SIV-induced lymphadenopathy. We found a interdependence between the morphological peculiarities of follicles (follicular hyperplasia or follicular involution) and intensity of expression of VCAM-1 on FDC (Fig.2) in whole light zone and a part of a dark one. A gradient of VCAM presentation appeared to exist between subcapsular-intermediary sinuses (no expression), mantle (low membrane labeling) and FDC network (strong presentation). Such a pattern of VCAM distribution diplayed in this part of LN though which the lymphocytes coming from afferent lymphatic vessels may influence the capture of cells expressing appropriate ligands. Degenerative changes and decrease of surface of FDC processes together with diminished expression of VCAM-1 may be responsible for inefficient trapping of infected cells and virus in germinal centers in SIV-induced disease. Presentation of other adhesion molecules was very weak (ICAM-1) or absent (ELAM-1) on FDC in both SIV-infected and control LNs.

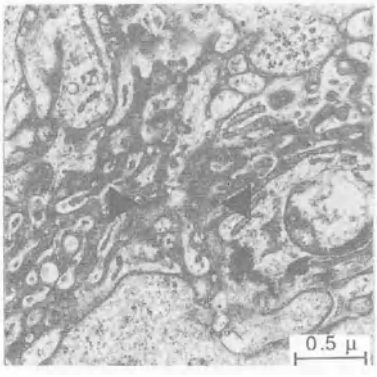

Figure 1. SIV particles (arrowhead) between FDC processes (bar 0.5µ).

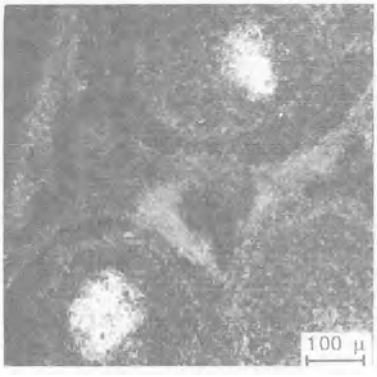

Figure 2. Strong expression of VCAM-1 on FDC network in hyperplastic follicles (bar 100µ).

Ultrastructural markers of interferon alpha production (confronting cisternae and tubulo-reticular structures) were found in cytoplasm of sinusoidal macrophages (Fig.3), lymphocytes and FDC in LNs of SIV-infected monkeys.

Numerous IL-1 and IL-6 positive lymphocytes were detected in mantle, interfollicular area and medulla in hyperplastic LNs (Fig.4) as previously reported for HIV-associated lymphoadenopathy[9]. Additionally, many cells in germinal centers presented IL-6 membrane staining. During follicular involution IL-1 and IL-6 containing lymphocytes were detected in medullar cords. IL-1 and IL-6 positive macrophages were found in sinuses or medullar cords in LNs with follicular hyperplasia, follicular involution and granulomatous

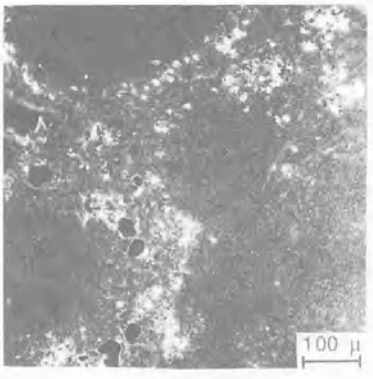

Figure 3. Confronting cisternae (arrowhead) in macrophage cytoplasm (bar 1µ).

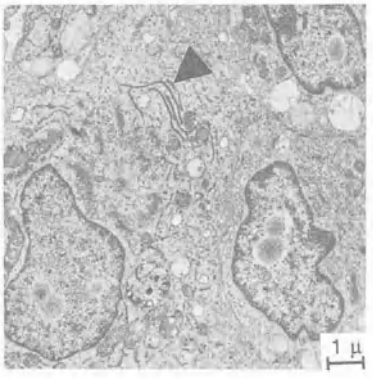

Figure 4. IL-6 positive lymphocytes and macrophages in mantle and paracotex (bar 100µ).

lymphadenitis. The number of positive cells was constantly lower in LNs with follicular involution than in ones with follicular hyperplasia. IL-1 and IL-6 producing cells were rather rare in both control LNs and LNs with diffuse follicular and paracortical depletion. Few cells having morphology of FDC or macrophages were stained positively for TNFα in LNs with follicular hyperplasia, follicular involution and granulomatous lymphadenitis.

Increased local production of cytokines (IL-1, IL-6, TNFα) may be the sign of SIV replication in macrophages and can facilitate the infection of susceptible cells[10]. Augmented cytokine synthesis may be responsible for up-regulated expression of VCAM-1 on FDC in hyperplastic follicles. Since IL-6 has been described as an important factor for B lymphocyte differentiation[10] its local expression in the cells of germinal centers may play a role in polyclonal B cell activation and follicular hyperplasia in HIV and SIV diseases. Our results indicate the close interrelationship between morpho-functional changes of FDC, virus distribution, local cytokine production and LN structure in SIV-induced lymphadenopathy.

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MODULATION OF B LYMPHOCYTE PROLIFERATION INSIDE THE GERMINAL CENTER

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INTRODUCTION

Germinal centers of stimulated lymphoid follicules comprise different microenvironments where B cells proliferate or differentiate into memory B cells or precursors of Ig-producing cells [1]. Follicular dendritic cells (FDC), unique non lymphoid cells, mainly compose these microenvironments. FDC are closely associated with B cells and can be distinguished from other accessory cells in secondary lymphoid tissues by a number of features, as lack of phagocytic activity and a characteristic set of cell surface markers.
Murine and human B cell proliferation in vitro has been reported to be enhanced by poorly FDC enriched preparations (2 to 10% among lymphocytes) [2-3]. Recently, Freedman et al.[4] showed that highly purified FDC (more than 80%) inhibit SAC activated B cell proliferation which contradicts results of Burton et al.[5] showing that murine FDC enhance through either a sIg-dependent or -independent pathway murine B cells stimulation obtained by polyclonal activators.
At view of these conflicting results, we analysed the possibility that the action of FDC on B cell proliferation depends on the mitogen used. We tested the proliferation of B cells stimulated or not with activators which mimiked situations encountered in germinal centres in adding anti-immunoglobulin, anti-CD40 antibodies or even Staphylococcus areus strain Cowan I and we also analysed the proliferation of IgD+ (mantle zone) and IgD- (interfollicular and GC) B cell subsets activated by anti-CD40 antibodies in the presence or not of FDC.

MATERIAL AND METHODS

FDC isolation and enrichment

FDC were prepared according to Marcoty et al. [6], with several modifications. The remaining cells were treated with mitomycin C (50µg/ml) for 45 min at 37°C. These preparations, containing 20 to 50% pure FDC (DRC-1+, CD35+, CD54+), were then plated at 4.10^4 to

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5.10^4 cells/well in 96-well microtitre plates and cultured for 16 to 24 hours in RPMI 1640 containing 10 % FCS. Thereafter, the micro-wells were cautiously washed with culture medium. During this procedure, the FDC remained attached to the substrate and most contaminating lymphoid cells were removed (non-adherent cells).

B cell preparation

B cells were isolated as described by Lagresle et al. [7]. The purity of the B cell populations obtained by this procedure was routinely superior to 95 %. IgD+ and IgD- B cells were isolated using the MACS procedure. The purity obtained were routinely superior to 95% for IgD+ B cells and to 80% for IgD- B cells.

B-cell cultures

Purified tonsillar B cells and B cell subsets were plated at 1.10^5 cells/well in 96-well microtitre plates and cultured alone or in the presence of either purified FDC (5.10^3/well) or non-adherent cdlls (5.10^4 cells/well). The culture medium was RPMI 1640 supplemented with 10 % FCS with or without activators (rabbit anti-Ig coupled to polyacrylamide beads were used at a final dilution of 1/600, Staphylococcus aureus strain Cowan I (SAC) were used at a final concentration of 1:10,000 and the anti-CD40mAb G28-5 used in functional assays was kindly provided by Dr E.A. Clark (University of Washington, Seattle, WA) and used at a final concentration of 100ng/ml in a final culture volume of 0.1ml. Proliferation was assessed by adding 1µCi of [^{3}H]-thymidine during the last 16 hours.

RESULTS

In the absence of any activator, only basal-level incorporation was recorded (data not shown). When anti-CD40 mAb and anti-Ig were used as activators, B cells in contact with FDC intensely incorporated tritiated thymidine (Fig. 1). After 5 days in culture, the B cells in all experimental sets exhibited low proliferative activity (data not show). In the case of CD40 antibodies, the Fc receptors of FDC don not play any role in CD40-antibody interaction. We preincubated the FDC for 1hr at 37°C with 100 µg/ml of human gammaglobulin, aggregated or not, before being cocultured with B cells. This did not significantly alter the results (data not shown). B cells cultured alone or with non-adherent cells in the presence of SAC intensely incorporated tritiated thymidine. In the presence of this stimulant, however, coculturing with FDC significantly reduced B-cell proliferation (by about 50%) (Fig. 1). The same inhibitory effect was observed with supernatants from 16-hour cultures of highly purified FDC (data not shown).
In culture, FDC favour, in the presence of anti-CD40 antibodies, the proliferation of both B cells subsets (Fig2.).

DISCUSSION

Our results demonstrate that FDC improve the proliferation of B cells stimulated with anti-CD40, anti-Ig but not that of SAC-activated B cells. This is the first reported observation of FDC affecting B cells differently according to the mitogen used.

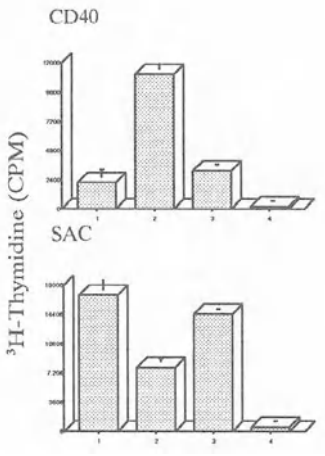

Fig. 1: Tritiated thymidine incorporation in B cells (1); in FDC plus B cells (2); in B cells plus non-adherent cells (3); in FDC (4).

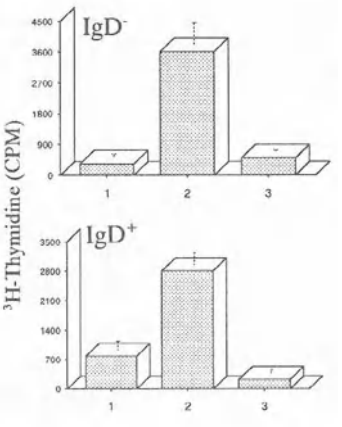

Fig. 2: Tritiated thymidine incorporation in B cells subsets (1); in FDC plus B cell subsets (2); in FDC (3).

Previous work on FDC has generated conflicting data. Most authors have found FDC to stimulate B cell proliferation [2-3,5] but Freedman et al.[4] found an inhibitory effect. Our results may somewhat explain these contradictory findings. We also showed that the proliferation of B cells activated with anti-CD40 antibodies is due to the effect of FDC on both IgD+ and IgD- B cells.

Our results may reflect the in-vivo situation: centroblasts actively divide in the dark zone while centrocytes seldom do; it is generally accepted that centroblasts are B cells activated by native Ag and Ag-specific T cells in the T-dependent zone, whereas centrocytes are intermediates in the evolution of centroblasts to B memory lymphocytes [8]. FDC would thus appear, according to their location and to the stimulatory conditions, either to favour or reduce B cell proliferation.

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HUMAN FOLLICULAR DENDRITIC CELLS PROMOTE BOTH PROLIFERATION AND DIFFERENTIATION OF CD40 ACTIVATED B CELLS

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INTRODUCTION

Follicular dendritic cells (FDCs) are unique stromal cells within the germinal centers, where intensive B cell proliferation was identified. The main feature of these cells is their capacity to trap antigen-antibody immune complexes for long periods of time (1). FDCs have been shown to have a stimulatory effect on B cell proliferation in both human and mouse systems by many groups (2, 3, 4, 5). In contrast, Freedman *et al* (6) have shown that FDCs inhibit SAC dependent B cell proliferations.

CD40-Ligand on activated T cells has been shown to play important roles in B cell proliferation and rescueing germinal center B cell from apoptosis (7). Since germinal center T cells in human have been shown to express CD40-Ligand constitutively *in situ* (8), we wondered if FDCs could promote B cell activation induced by anti-CD40 antibody and cytokines. We show that FDC clusters promote both proliferation and differentiation of cytokine and CD40 activated B cells.

MATERIALS AND METHODS

Isolation of FDC-lymphocyte clusters:

a) Small pieces of tonsil were digested by DNAse and collagenase.

b) The cell suspension was centrifuged through Ficoll to remove dead, red and epithelial cells.

c) The cells were centrifuged through a 1.5% BSA gradient for 10 minutes at 10g.The pellet contains the FDC-lymphocyte clusters.

Isolation of B cells:

The cells in suspension, following the BSA gradient, were used to purify B cells by sheep red blood cells rosetting and magnetic depletion of residual T cells.

RESULTS

We first determined the effect of FDCs on the spontaneous proliferation of autologous B cells. FDC clusters were shown to induce moderate and short term B cell proliferation (data not shown). The limited FDC dependent B cell proliferation suggested the involvement of other mechanisms for the intense B cell

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proliferation in germinal center. In this context, a wide range of cytokines was tested for their capacity to enhance FDC dependent B cell proliferation. IL-2 was the only cytokine that consistently potentiated FDC dependent B cell proliferation (data not shown) but this was still a moderate proliferation.

To further improve the FDC dependent B cell proliferation, we investigated the effect of CD40 triggering in this culture system. We used soluble anti-CD40 antibody, G 28.5 (9), rather than the more efficient CD40 system (10) to better appreciate the FDCs contribution. Figure 1 shows that anti-CD40 antibody in combination with IL-2, IL-3, IL-4, IL-10 or IL-13 significantly potentiated FDC dependent B cell proliferation. Maximal proliferation was observed with the combination of IL-2+IL-10 or IL-4+IL-10. These strong proliferations were maintained up to 5 days with a peak time at day three (data not shown).

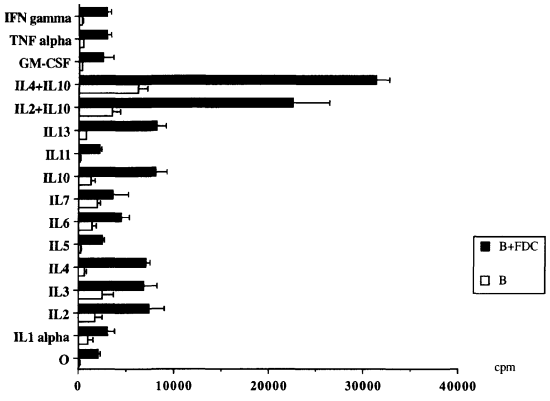

Figure1: FDCs promote the growth of B cells activated by cytokines and anti-CD40

5.10^4 B cells were cultured with 2000 irradiated (20 Gy) FDC clusters with anti-CD40 antibody (1 µg/ml). IL-1α was used at 500 pg/ml, IL-2 at 10 U/ml, IL-3 at 10ng/ml, IL-4 at 50 U/ml, IL-5 at 500 ng/ml, IL-6 at 250 ng/ml, IL-7 at 10 ng/ml, IL-10 at 100 ng/ml, IL-11 at 250 ng/ml, IL-13 at 50 ng/ml, GM-CSF at 100 ng/ml, TNFα at 2,5 ng/ml and IFNγ at 100 U/ml.

It has been recently shown that together with CD40 triggering, IL-2+IL-10 induce B cells to produce large amount of Ig (11). FDCs were added into the above culture to see if they can modulate B cell differentiation. The Ig levels of culture supernatants were analysed at day 10 by ELISA. Table 1 shows that together with anti-CD40 triggering, IL-2+IL-10 induce B cells to produce significant amount of IgM (130-960 ng/ml), IgG (380-720 ng/ml) and IgA (50-580 ng/ml). FDCs further potentiate the Ig production by B cells in all three Ig subclasses. This results in 6 to 40 fold increase in IgM, 8 to 37 fold increase in IgG and 11 to 16 fold increase in IgA.

Table 1: FDCs enhance Ig secretion of B cells activated by anti-CD40 and IL-2+IL-10

		B	B+FDC	FDC
Exp 1	IgM	130±30	5850±500	90±5
	IgG	720±240	28000±3200	1330±90
	IgA	580±50	6800±1600	250±70
Exp 2	IgM	960±80	6500±500	900±150
	IgG	380±80	3600±280	400±50
	IgA	50±20	850±80	20±10

DISCUSSION

The present study demonstrates that FDCs enhanced cytokine-dependent growth and differentiation of CD40 activated B lymphocytes. These results support and further extend previous studies showing that FDCs have a stimulatory effect on mouse and human B cell proliferation (2, 3, 4, 5). When anti-CD40 antibody was added into the cultures, IL-2, IL-3, IL-4, IL-10 and IL-13 had comparable enhancing effects on FDC-dependent B cell proliferation. Maximal B cell proliferation was observed only when IL-2+IL-10 + anti-CD40 antibody or IL-4+IL-10 + anti-CD40 antibody were provided. Our results are consistent with studies using FDC-like cell lines that are able to promote CD40 activated B cell proliferation (12). In line with this, Kosco *et al* showed the importance of T cell signals for FDC-dependent B cell proliferation. Indeed depletion of T cells abolished this proliferation (4) that could not restore by adding soluble cytokines, possibly due to lack of CD40-Ligand. The detection of CD40-Ligand expressing T cells in human tonsillar germinal centers (8) demonstrates the *in vivo* relevance of the present *in vitro* observations. The key role of CD40-CD40-Ligand interaction has been further demonstrated by the lack of germinal center in secondary lymphoid tissues of patients with hyper-IgM syndrome, who carry mutations in their CD40-Ligand gene (13).

Previous experiments, suggested that FDCs promote B cell proliferation but inhibit Ig production (2). These experiments were carried out by culturing FDCs (1-2%) with lymphocytes (25% T cells and 75 % B cells) activated by pokeweed mitogen. Our present results show that FDCs strongly potentiate the differentiation of B cells induced by IL-2+IL-10 and CD40 triggering. This is consistent with the in situ observation that there are many plasma cells within the germinal centers of human tonsils (14, 15)

Our results strongly suggest that the cognate interaction between B cells, T cells and FDCs is required for germinal center reaction.

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DENDRITIC CELLS AS STIMULATOR CELLS OF MHC CLASS I-RESTRICTED IMMUNE RESPONSES

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INTRODUCTION

Members of the dendritic cell (DC) family are potent stimulators of syngeneic and allogeneic, naive $CD4^+$ T cells[1]. This functional capacity is linked to their expression of MHC class II moieties[2,3] and of certain costimulatory molecules of T cell activation, such as ICAM-1[4,5], LFA-3[6], as well as B7-1 and B7-2[7-10].

Some evidence exists that DC and, perhaps, also other accessory cells can stimulate $CD8^+$ T cells and generate MHC class I-restricted CTL responses against alloantigens and viral antigens[11-16]. However, the mechanism(s) by which DC accomplish this task is(are) still a matter of debate. While certain studies emphasize the need for MHC class II-restricted T helper activity in this process[17-20], the observation that CD4-deficient mice can mount effective antiviral CTL responses[21-24] implies that the activation of MHC class I-restricted $CD8^+$ T cells can occur in the absence of T cell help. The fact that the accessory cells used for most of the in vitro studies on the activation of $CD8^+$ T cells express both MHC class I and class II antigens opened the possibility that minute numbers of $CD4^+$ T cells, contaminating the "purified" $CD8^+$ T cell populations employed, may have contributed to the generation of MHC class I-restricted CTL activity.

In the past two years, we have established cell lines with a phenotypic profile which allowed us to address this question.

GENERATION AND FUNCTIONAL ANALYSIS OF CYTOKINE-DEPENDENT DC LINES FROM MURINE FETAL SKIN

In the course of studying the ontogeny of murine dendritic epidermal T cells[25], we have established three long-term, growth factor-dependent cell lines {80/1, 86/2 (IL-2 + Con A; derived from C3H mice) and 18 (GM-CSF; derived from BALB/c mice)} from mouse fetal

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skin[26]. Phenotypically, they are $CD3^-$, $CD45^+$, $CD18^+$, $CD44^+$, $CD32^+$, HSA^+, MHC class I^+, MHC class II^-, asialo $GM1^+$, exhibit pronounced dendritic configurations and, thus, somewhat resemble fetal Langerhans cells (LC)[27,28]. We therefore tested whether these fetal skin-derived DC lines are capable of functioning as accessory cells for T cell activation. Using the primary MLR, we found that all three lines {80/1 > 18 > 86/2} induce a vigorous proliferation of allogeneic, but not syngeneic lymph node T cells. Flow cytometric analyses of cells 3 days after initiation of the fetal skin cell line (FSCL)-induced allogeneic MLR revealed that all blastoid cells belong to the TCR $\alpha\beta^+$, $CD8^+$, $CD4^-$ T cell subset[26]. In further studies, we cultured memory cell-depleted, i.e., naive single $CD4^+$ and $CD8^+$ T cells from selected mouse strains ($H\text{-}2^b$, $H\text{-}2^d$, $H\text{-}2^k$) with x-irradiated 80/1 cells ($H\text{-}2^k$), and for control purposes, with LC ($H\text{-}2^k$) from 72 hour epidermal cell cultures (=cLC). Results showed that 80/1 cells and cLC induce massive proliferative responses of equal magnitude in allogeneic, but not syngeneic, $CD8^+$ T cells. In sharp contrast to the exclusive $CD8^+$ T cell-activating property of 80/1 cells, cLC also induced strong proliferative responses in allogeneic, and, to a lesser extent, in syngeneic $CD4^+$ T cells. Our contention that the 80/1 cell-induced activation of $CD8^+$ T cells occurs indeed in the absence of T cell help was substantiated by (i) the lack of FACS-dectectable $CD4^+$ T cells in the purified $CD8^+$ T cell population; (ii) the lack of reactivity of purified $CD4^+$ T cells to MHC class I-disparate fetal skin cell lines; (iii) the inhibition of the MLR by anti-CD8/MHC class I but not by anti-CD4/MHC class II mAb; (iv) the finding that the addition of graded numbers of $CD4^+$ T cells to fixed numbers of FSCL and $CD8^+$ T cells had essentially no effect on the magnitude of the proliferative response; and finally, (v) the lack of inducibility of MHC class II expression on FSCL by either IFN-γ or GM-CSF. To determine the role of soluble mediators in the 80/1-driven T cell proliferative response, x-irradiated 80/1 cells were cocultured with allogeneic $CD8^+$ T cells for 3 days in culture wells that allowed or prevented physical contact (transwells). Vigorous proliferation of $CD8^+$ T cells was measurable only when stimulator and responder cells were cultured in the same well suggesting that for the direct activation of $CD8^+$ lymphocytes by dendritic accessory cells, physical cell - cell contact is necessary and that soluble mediators do not suffice to trigger $CD8^+$ T cell proliferation[26].

Due to the fact that CD8 molecules act as coreceptors for MHC class I molecules, we studied the cytolytic activity of 80/1 cell ($H\text{-}2^k$)-induced $CD8^+$ lymphoblasts ($H\text{-}2^b$) against syngeneic and allogeneic target cells. We observed that 80/1-primed $CD8^+$ lymphoblasts significantly lyse $H\text{-}2^k$, but not $H\text{-}2^d$ and $H\text{-}2^b$ targets and that the lysis of $H\text{-}2^k$ targets was independent of their MHC class II expression. These findings indicate that the interaction between 80/1 cells and $CD8^+$ T cells is mediated and restricted by MHC class I antigens[26]. However, the exact mode of alloantigen recognition ("empty" vs. peptide-laden MHC class I molecules[29,30]) by $CD8^+$ T cells remains to be elucidated.

To determine whether the strong allostimulatory capacity of the FSCL 80/1 is an intrinsic property of all MHC class I^+ cells, provided these antigens are expressed at high density, we tested a variety of MHC class I^+ cells (Ia^-/$Thy\text{-}1^-$ spleen cells, peritoneal cells, the fibroblast cell line L929 and the T cell hybridoma BW5147) for their capacity to initiate proliferation of allogeneic $CD8^+$ T cells. We found that all of these cells were substantially less efficient stimulators of allogeneic $CD8^+$ T cells than 80/1 cells. The only other cells that proved to be equally potent in their stimulatory capacity for $CD8^+$ T cells were cLC. Subsequent immunolabeling studies showed similar levels of class I antigens on most of the stimulator cells/cell lines tested arguing against the possibility that differences in class I expression could account for differences in their MLR-stimulatory potency and rather suggesting that the superior allostimulatory capacity of 80/1 cells and cLC is due to the delivery of one or more

costimulatory signals[26]. Although a number of candidate molecules have been identified as being capable of delivering costimulatory signals, accumulating evidence assigns greatest relevance to interactions between T cell surface antigens CD28/CTLA-4 and B7 molecules (B7-1 and B7-2) expressed on antigen presenting cells[9,10,31]. Indeed, it has been shown that transfection of keratinocytes with B7-1 confers effective antigen presenting function on this nonlymphoid cell[32]. We, therefore, directly assessed the cell surface expression of the B7-1 antigen on cell lines tested previously for their allostimulatory capacities and found that the level of costimulatory activity of each cell population correlated closely with the level of B7-1 expression. The decisive role of B7-1 in the 80/1 cell-driven T cell proliferation was further evidenced by functional assays conducted in the presence of either anti-B7-1 mAb or Ab against other costimulatory molecules. We found that FSCL-induced $CD8^+$ T cell responses could be almost completely inhibited by an anti-B7-1 mAb but only modestly or not at all by Ab against other costimulatory molecules such as ICAM-1, LFA-1, IL-1 and IL-6[26]. In contrast to the observations made with FSCL, we found that cLC-induced $CD8^+$ T cell responses were only partially inhibited with an anti-B7-1 mAb. This lack of complete inhibition is most likely due to the fact that cLC, in contrast to the FSCL 80/1, express B7-2 (A. Elbe, unpublished observation) which is another molecule capable of transmitting costimulatory signals[7-10]. The finding that FSCL-primed lymphoblasts can lyse B7-negative targets with equal efficiency as B7-positive ones, suggests that the expression of B7 by target cells is not essential for the effector function of CTL, thus, confirming and extending previous results showing that CD28/B7 interactions are required for the generation, but not for the effector function of CTL[33-35].

SUMMARY

We have shown that growth factor-dependent, MHC class I^+/II^- dendritic cell lines established from mouse fetal skin, can stimulate naive, allogeneic but not syngeneic $CD8^+$ T cells in the absence of $CD4^+$ T cells and that this T cell response is restricted by MHC class I molecules. We further showed that the FSCL-induced activation of naive $CD8^+$ T cells is critically dependent on the physical contact between stimulator and responder cells and the expression of the costimulatory molecule B7 on FSCL. An important question that remains to be addressed concerns the derivation of FSCL. One could argue that they are members of the LC/DC family because they (i) exhibit certain features of fetal murine LC (i.e., $CD45^+$, $CD44^+$, $CD32^+$, MHC class I^+, MHC class II^-, asialo $GM1^+$, TCR^-)[27,28] including membrane-bound ADPase activity (A. Elbe, unpublished observation) and (ii) exhibit a pronounced dendritic configuration when cultured. If these cells are indeed derived from fetal LC, they should undergo the same phenotypic changes (MHC class $II^- \rightarrow$ MHC class II^+) under in vitro culture conditions as do fetal LC in situ[28]. However, our FSCL are phenotypically stable, and attempts to induce MHC class II expression with cytokine cocktails were unsuccessful. One explanation for this phenomenon could be that stimulatory signals provided by fetal keratinocytes or other skin cells are responsible for LC maturation in vivo and that, due to the early demise of these "stromal" cells in fetal skin cell cultures, the maturation process would not have been completed. Alternatively, the attractive possibility exists that we have generated a population of "mature" MHC class I^+/II^- cells, which, perhaps because of their paucity, have so far escaped in vivo detection by conventional immunolabeling procedures. Evidence for the latter hypothesis comes from studies by Sprent et al.[16] and Holt et al. (3^{rd} International Symposium on Dendritic Cells in Fundamental and Clinical Immunology), who described the existence of MHC class II^- antigen presenting cells

in the spleen, bone marrow, and fetal liver by functional criteria, and of MHC class I^+/II^- DC in situ in the conducting airways, respectively.

While in this study FSCL were used for allo-sensitizing purposes, it is not unreasonable to assume that they can also function as potent vehicles for the induction of primary responses against nominal antigens. As such, they may be ideally suited to study (i) the MHC class I-associated immunogenicity of selected (tumor, viral) peptide antigens and (ii) to generate $CD8^+$ immune responses, in vitro and in vivo, against such antigens.

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DENDRITIC CELLS REGULATE DEVELOPMENT OF ALLOANTIGENIC AND MITOGENIC T_H1 VERSUS T_H2 RESPONSES

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INTRODUCTION

Previous studies indicate that different T helper cells may regulate humoral and cell-mediated immunity to foreign antigens. This regulation may be controlled in part by cytokines produced by different helper T cell (T_H) populations, i.e., T_H1 cells that produce IL-2 and IFN-γ, and T_H2 cells that produce IL-4, IL-5, IL-6, and IL-10.[1,2] However, the induction and regulation of these distinct T_H subsets and their derived cytokines *in vivo* is still under investigation. It remains to be elucidated whether a given antigen presenting cell may induce similar or divergent cytokine production profiles or patterns in its regulation of cell-mediated versus humoral immunity. The studies presented here have addressed the possibility that DC regulate these divergent immune responses through induction of divergent T cell-derived cytokine production profiles.

Historically, DC have been isolated from different tissues using dissimilar separation and isolation techniques. This practice imposed certain limitations with regard to interpreting reported differences or similarities in the features and functions of these distinct DC populations as was previously addressed.[3] Therefore, we combined novel techniques with previously published methods to develop a unified approach for the isolation of DC from spleen and Peyer's patch, and DC isolated under these conditions were used in our previous work.[3] This isolation scheme permitted a meaningful comparison of the function of DC from different tissues.

Recent experiments in our laboratory have suggested that DC from different tissues possess divergent functions in their capacity to stimulate B cells, also from various tissues, to secrete IgA. Specifically, we showed that Peyer's patch DC were able to preferentially induce spleen B cells, Peyer's patch B cells, or a pre-B cell line, in the presence of either spleen or Peyer's patch T cells, to make IgA.[4,5] These data suggested that DC from Peyer's patch may function as specific coordinators of IgA production and mucosal responses. In further studies along these lines, we determined that IL-6 preferentially induced surface IgA-bearing B cells to secrete high levels of IgA.[6] Taken together, these data suggest that Peyer's patch DC may

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be able to specifically induce T cells to produce high levels of IL-6, especially when compared with those levels induced by spleen DC. These increased levels of IL-6 may then go on to induce B cells to specifically secrete high levels of IgA.

To address these questions, we determined whether spleen DC versus Peyer's patch DC induced similar levels of T cell-derived cytokines such as IFN-γ, IL-2, and IL-6. Using identical procedures to harvest spleen DC and Peyer's patch DC, we mixed these DC populations with allogeneic spleen T cells. We then tested 72-h culture supernatants for production of cytokines.

METHODS

Cells, Cell Enrichment, Culture Conditions, and Cytokine Determinations

Spleen and Peyer's patch cells were derived from 8-20 week old C3H/HeN and BALB/c mice (Frederick Cancer Research Facility, Frederick, MD).

We had previously isolated DC from spleen and Peyer's patch tissues using the unified approach of clustering buoyant accessory cells with periodate-modified dense thymocytes.[3] This isolation procedure yielded cell populations enriched to approximately 60-80% DC as determined by morphological characteristics. Since that time however, hamster anti-mouse DC mAb raised against the CD11c molecule (DC restricted in mice) (N418 from American Type Culture Collection, Bethesda, MD) has been shown to efficiently stain murine spleen DC[7]. Therefore, we mixed spleen and Peyer's patch buoyant cells with anti-FcR Ab 2.4G2 (ATCC) to block Fc receptor-mediated binding of N418, and followed this by staining with N418 antibodies and sorting for $N418^+$ cells. These $N418^+$ DC were shown to be >96% positive for class II MHC (Ia) antigens by flow cytometry. DC were washed, counted, and placed in culture in graded numbers with responder T cells as indicated below.

Cell-free supernatants were derived from cultures of 2.5×10^5 spleen or Peyer's patch DC plus enzyme-dissociated, anti-Ia^k- and complement-treated, allogeneic spleen T cells cultured at 5×10^6 cells/mL in complete medium (RPMI 1640, 50 μg/ml gentamicin, 2 mM L-glutamine, 5% heat-inactivated fetal calf serum, 50 μM 2-ME, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μg/ml indomethacin) as previously described.[8] Culture supernatants were harvested from 24-well, flat-bottomed tissue culture wells after 72-h of culture, filter-sterilized, and stored at 4°C until assayed for cytokine content.

Cytokine levels were determined using the cytokine-sensitive cell lines described below by comparison of supernatant dilutions with recombinant cytokine dose-response curves as previously described.[8] IL-2/-4, IFN-γ, and IL-6 levels were determined using HT-2, WEHI-279, and 7TD1 cells as previously cited.[3,8]

RESULTS AND DISCUSSION

Oxidative mitogenesis reactions using periodate as the mitogen have been demonstrated to elicit T cell activation by predominantly, and perhaps uniquely, stimulatory DC.[9] Therefore, previous studies have used periodate-modified cells and periodate-modified cell mixtures as models for determining the potential of DC from spleen and Peyer's patch to induce cytokine production by T cells derived from these tissues.[3] Those data suggested that spleen DC induce spleen or Peyer's patch T cells to produce predominantly T_H1 cytokines, while Peyer's patch DC induce spleen or Peyer's patch T cells to produce prodominantly T_H2 cytokines.

We wanted to extend the mitogenic data to address a similar question in the alloantigenic system. Therefore, culture supernatants were generated using spleen T cells as a model for induction of cytokines in an alloantigenic system using allogeneic spleen or Peyer's patch DC as inducers of allogeneic mixed leukocyte reactions. The trend of these results paralleled those derived from oxidative mitogenesis reactions;[3] however, in the current study, only IL-6 levels

were statistically significantly different in spleen T cell-derived cytokine levels induced by DC from spleen versus Peyer's patch (Table 1). These results support our previous data using mitogenic stimulation and suggest that the DC tissue of origin is critical in determining the type of cytokine profile elaborated from a given T cell population. Moreover, these data support the notion that DC coordinate immune responses to at least some degree by controlling the cytokines produced in a given tissue or milieu.

Table 1. IL-2 and IL-6 levels in allogeneic mixed leukocyte reactions[1]

Cells added to culture	IL-2 [U/ml]/(n value)	IL-6 [U/ml]/(n value)
spleen T cells only	11.30 ± 3.78 (2)	1.11 ± 0.18 (2)
spleen DC only	5.58 ± 1.40 (6)	2.58 ± 0.02 (2)
Peyer's patch DC only	6.01 ± 2.46 (6)	2.00 ± 0.32 (2)
spleen T cells + spleen DC	328.8 ± 93.60 (8)	23.58 ± 6.34 $(10)^2$
spleen T cells + Peyer's patch DC	296.4 ± 20.14 (8)	28.44 ± 3.88 $(10)^2$

[1]Representative of 3 different allogeneic mixed leukocyte reactions using BALB/c spleen T cells with or without stimulatory C3H/HeN DC to generate 72-h supernatants.
[2]IL-6 values are significantly different at $p < 0.05$ using a one-tailed *t* test.

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NEW MOLECULES ON DENDRITIC CELLS AND THEIR INTERACTIONS WITH T LYMPHOCYTES

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Dendritic cells (DC) are a subpopulation of leucocytes with an important role as specialist antigen presenting cells (APC), being the only APC capable of initiating a strong primary T lymphocyte response Using this functional property (ie their ability to stimulate a primary mixed leucocyte reaction) to detect DC we have identified a small population of potential DC precursors within human bone marrow (BM) (1) Further fractionation of this minor population of BM mononuclear cells (BMMC) using mAb with immunomagnetic separation and flow cytometry suggests this population has a close relationship with myeloid precursors and that even CD34 positive BMMC initiate an allogeneic MLR (2) It is possible to maintain these mAb mix-negative, lineage negative, BMMC in culture but it is not yet clear which cytokines optimize their growth and differentiation The CD1a antigen has not proven to be a marker restricted to human DC in BM In our hands, GM-CSF and TNFα induce CD1a expression on myeloid cells, but also distort myeloid differentiation, altering phenotypic appearances without selectively encouraging DC stimulatory function (3)

The application of similar immunoselection techniques to T lymphocyte depleted peripheral blood mononuclear cells (PBMC) enabled us to isolate a mononuclear cell population with strong allostimulatory activity (4) More recently we have exploited a 16 hr period of tissue culture and a Nycodenz gradient to isolate blood DC in high purity (McLellan et al, submitted) These latter cells are clearly more activated and express the activation antigens CMRF-44 (5) and HB15 (CD83) (6), whereas unactivated blood DC, prepared by the former method, do not Blood DC probably act as precursors for interstitial DC including Langerhans cells (LC), which migrate after exposure to antigen to the T cell dependent area of lymph nodes Thus tonsil DC, as described previously (7), probably represent the natural in vivo differentiated and activated DC

The molecular interactions which take place enabling the DC to stimulate a primary T lymphocyte response have been studied in our laboratory Recognition of the MHC molecule antigenic peptide complex provides the essential and specific signal 1 to the responding T lymphocyte A series of accessory molecules facilitate the interaction In broad terms this accessory activity may be due to

i molecules with predominant adhesion function which maintain DC-T lymphocyte membrane proximity These may induce some degree of T lymphocyte activation

ii molecules with predominant "costimulatory" activity providing an essential second signal for T lymphocyte activation The costimulatory molecules currently under extensive investigation are ligands for CD28/CTLA-4 on the T lymphocyte The

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absence of this latter signal duriong exposure to signal 1 may lead to anergy and a failure to respond effectively to repeat antigenic challenge.

Several adhesion molecules are present on tonsil DC of which LFA-3 (CD58) provides a highly relevant interaction with CD2 on T lymphocytes (8) The ability of DC to maintain a close physical relationship with T lymphocytes also depends on DC intercellular adhesion molecules (ICAMs) binding to T lymphocyte LFA-1 (8,9) Both ICAM-1 and ICAM-2 are present on DC but blocking studies suggested that another ligand was likely to be involved Another molecule, ICAM-3, with five Ig-like domains homologous to ICAM-1 was recently identified by several other groups (10,11,12) and shown to be the antigen recognised by CD50 mAb However, no CD50 mAb with specificity for the LFA-1 binding site, ie functional blocking antibodies were detected in the Vth Leucocyte Differentiation Antigen Workshop (LDAW)

An analysis of the expression of ICAM-1, 2 and 3 on blood DC was undertaken Blood DC prepared by immunodepletion (4) and therefore subjected to minimal activation had low/moderate levels of ICAM-1 and ICAM-2 staining In contrast, ICAM-3 was present in relatively high density and showed little increase, despite exposing DC to IFNγ (500 u/ml) in culture IFNγ, however, considerably increased their expression of ICAM-1 Recombinant ICAM-3-Fc was shown to provide a stimulatory signal via LFA-1 to T lymphocytes similar to ICAM-1-Fc and ICAM-2-Fc Finally, evidence that ICAM-3 was a ligand on DC for LFA-1 mediated adhesion and signalling of T lymphocytes was sought In the absence of blocking CD50 mAb, a polyclonal rabbit antiserum was raised which immunoprecipitated ICAM-3 and bound specifically to ICAM-3 without cross-reactivity to ICAMs 1 and 2 This antiserum produced substantial inhibition in the MLR compared to either control antisera (or to blocking mAb to anti-ICAM-1 and 2) Thus, ICAM-3 appears to be the most important ligand present on DC for T lymphocyte LFA-1, which participates in primary immune responses ICAM-3 probably contributes to initial DC-T lymphocyte adhesion but activation of DC during its interaction with T lymphocytes may lead to rapid upregulation of ICAM-1 and other adhesion molecules on DC, concomitant with an increase in LFA-1 avidity, thereby strengthening DC-T lymphocyte adhesion

The costimulatory molecules, which act as ligands for CD28/CTLA4 on T lymphocytes, have also been studied using blood DC and tonsil DC as examples of DC at different activation/differentiation stages The B7 1 molecule (CD80) was not present on unactivated blood DC either as surface protein or mRNA (detected using RT-PCR) However both mRNA and surface staining for CD80 was readily detectable on tonsil DC Reasoning that this was induced by DC activation, we showed that blood DC induced expression of CD80 after a period of tissue culture in the presence of cytokines (13) The soluble fusion protein CTLA-4-Ig binds to CD80 and was recently shown to bind other homologous co-stimulator molecules Thus, the cDNA sequence of B7 2 (14) or B70 (15), now identified as CD86, confirms its homology to CD80 Yet a further ligand(s) is predicted by binding of the mAb BB-1 and CTLA-4 Ig which is not accounted for by CD80 or CD86

Our recent studies have investigated CD80 (Vth LDAW panel), CD86 (FUN-1 from Vth LDAW) and CTLA-4 Ig (provided by Dr P Linsley, Bristol-Myer Squib, Seattle) binding to DC exposed to different activating conditions RT-PCR was also used to assess CD80 and CD86 mRNA expression Blood DC prepared by immunodepletion (4) did not bind CTLA-4 Ig or stain with the CD80 or CD86 reagents indicating a lack of CTLA-4 Ig ligands on minimally stimulated DC DC isolated by this method lacked CD80 mRNA but contained some message for CD86 A period of culture induced surface expression of both CD80 and CD86 These are important ligands for DC co-stimulation as indicated by the fact that CTLA-4 Ig profoundly inhibited the DC-induced MLR The failure of the mAb BB-1 (CD80 plus additional non-CD86 specificity) to inhibit the MLR significantly suggests that CD86 acts as the predominant functional CTLA-4 Ig binding ligand The presence of preformed RNA for CD86 within DC may well allow for rapid induction of this molecule on the DC surface

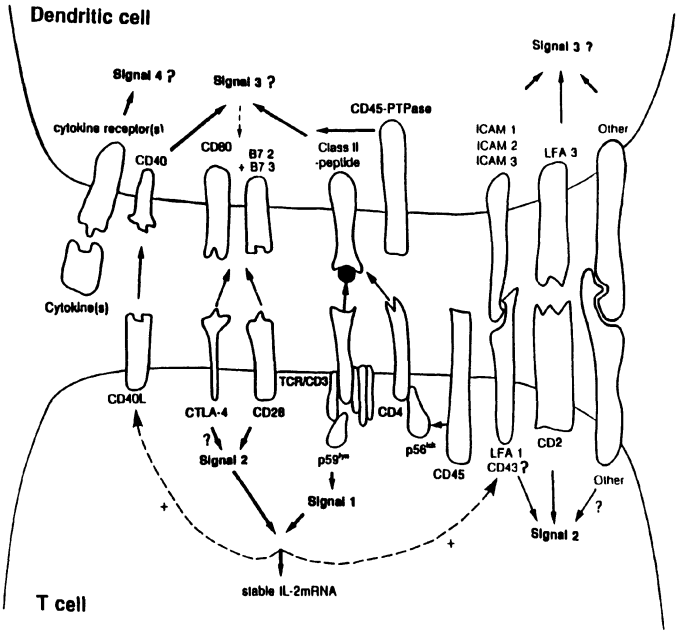

FIGURE 1 Schematic representation of the major DC/T lymphocyte interactions presently known to be important in T lymphocyte activation

Subsequent RT-PCR analysis of the DC stimulated MLR revealed that, as predicted, CD80 and CD86 mRNA was induced rapidly during these conditions

These experiments provide initial confirmatory evidence for our original hypothesis that DC co-stimulatory activity is not constitutive but is under regulatory control and dependent on the state of DC activation (1,13,16)

We suggest (see Figure 1) that initial adhesion events (probably ICAM-3 mediated) facilitate specific antigenic (signal 1) triggering of the T lymphocyte - possibly supplemented by additional DC LFA-3/CD2 triggering of T lymphocytes The resulting partial activation is postulated to increase T lymphocyte LFA-1 avidity and perhaps increased production of other membrane molecules (eg CD40L) and a variety of cytokines These reciprocal signals (signal 4 & 5) from the partially activated T lymphocyte provide the potential for further activation of the DC resulting in full co-stimulatory molecule (signal 2) upregulation The DC may also receive an additional upregulatory stimulus (signal 3) as a result of MHC class crosslinking This model allows for considerable control of DC co-stimulatory activity This might enable thymic DC to deliver a tolerogenic signal, ie signal 1 in the absence of full co-stimulatory activity and may conceivably contribute to maintenance of peripheral tolerance by resident unactivated tissue DC Evidence that in certain circumstances the expression of CD80 and CD86 costimulatory molecules are regulated (perhaps even downregulated) on DC is found in our recent discovery that DC isolated from chronic inflammatory joints lack CD80 and CD86 antigens (17)

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PHENOTYPE AND ALLOACTIVATING CAPACITY OF DENDRITIC CELLS GENERATED UNDER DIFFERENT CULTURE CONDITIONS FROM HUMAN PERIPHERAL BLOOD

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INTRODUCTION

Dendritic cells (DCs) are a family of specialized cells distributed in various tissues with stimulatory capacities for primary and secondary immune responses especially by antigen presentation to T-lymphocytes. The isolation of skin associated DCs from skin biopsies is hampered by the limited numbers of DCs and tedious isolation procedures. Recently, several investigators described procedures for the in vitro generation of cells with the feature of DCs/Langerhans cells (LCs) from human peripheral blood to have sufficient numbers of cells to investigate the characteristics of the cells (Caux et al., 1992; Thomas et al., 1993; Sallusto and Lanzavecchia, 1994).

In order to generate in vitro a LC like cell type from peripheral blood which is characterized by the expression of CD1a and HLA-DR antigens and the ability to act as antigen presenting cell we analyzed the influence of different media conditions to morphology, the phenotype and the stimulatory capacities towards T-lymphocytes of in vitro generated DCs.

MATERIAL AND METHODS

Reagents: McCoy's 5A medium and human AB serum were purchased from Sigma. SFM-medium without serum, fetal calf serum (FCS), buffers and medium supplements were purchased from Gibco, BRL.

Recombinant human GM-CSF and TNF-α were purchased from Genzyme. Ficoll-paque and Percoll were purchased from Pharmacia. All monoclonal antibodies (HLA-DR, ICAM 1, CD11a, CD64) were purchased from Dianova except for CD1a (Coulter), ^{3}H-thymidine was purchased from Amersham.

Cells: Blood cells from healthy human donors were isolated according to Zwadlo et al. Briefly, the monocytic cell fraction was isolated from heparinized blood by sequential density centrifugations on Ficoll-paque and Percoll density gradients. The cells of the

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monocytic cell fraction were incubated in teflon bags at $1x10^6$/ml in various media for 3-9 days. The expression of CD1a antigen on cells was stimulated by the addition of 100U. GM-CSF and 50U. TNF-α/ml medium. Mixed lymphocyte reaction was performed according to Schopf et al. 1986.

Cytofluorometric analysis: For cytofluorometric analysis cells were harvested by incubation on ice for 30 min, washed with buffer (phosphate buffered saline with 0.1% bovine serum albumin, PBSB) and incubation for 5 min with PBSB with 1mM EDTA at 37°C. The cells were washed with PBSB and incubated for 30 min at 4°C with PBSB with 2μg/ml human IgG. After washing with PBSB cells were incubated with direct labeled (FITC or phycoerythrin) monoclonal antibodies for 45 min at 4°C. The cells were washed two times with PBSB and analyzed in a Profile II flowcytometer (Coulter) for fluorescence.

For indirect immunostaining the cells were incubated after preincubation with human IgG for 45 min at 4°C with monoclonal antibodies, washed three times with PBSB, and incubated for 45 min with FITC-conjugated goat-anti-mouse IgG F $(ab)_2$-fragment. After washing two times with PBSB the cells were analyzed in a Profile II flowcytometer.

RESULTS

After stimulation with GM-CSF and TNF-α in serumfree medium the cells developed a high grade of DC/LC like shape with long dendritic branches connecting the cells to a complete network. In media supplemented with sera and cytokines fewer cells developed shorter branches without building up a network. The highest frequencies of $CD1a^+/HLA\text{-}DR^+$ DCs were obtained with the FCS-supplemented medium in the presence of GM-CSF and TNF-α as shown in Tab. 1. The CD1a expression increased until day 6 of culture and decreased on day 9 of culture. The same pattern of increasing CD1a expression until day 6 and a decrease until day 9 could be seen if the cells were incubated in serumfree medium. No CD1a expression could be measured if the cells were grown in media without cytokine.The addition of human serum inhibited the expression of CD1a even in the presence of GM-CSF and TNF-α. The expression of HLA-DR, ICAM 1 and CD11a on cells was similar in all media supplemented with cytokines. In medium without serum there was no expression of CD64 on the cells whereas by incubation in medium with FCS or human serum an expression of CD64 could be observed.

Table 1: CD1a cells [%] cultivated in different culture media supplemented with GM-CSF and TNF-α

culture period (d)	medium with 10% FCS	medium with 5% human AB serum	medium without serum
3	25	0	15
6	70	0	60
9	45	0	35

In parallel to the CD1a expression the allo-activating capacity of cells grown in different media towards allogeneic T-lymphocytes was tested.

As shown in Tab. 2 the DCs generated in presence of cytokines under serumfree or FCS supplemented conditions - with a high percentage of $CD1a^+$ cells - revealed a higher allogeneic stimulation potential than the CD1a negative cell population generated in human AB serum.

Table 2: Allo-stimulation potential of cells after incubation in different culture media supplemented with GM-CSF and TNF-α

culture period (d)	medium with 10% FCS	medium with 5% human AB serum	medium without serum
3	3,0*	1,8	3,0
6	2,8	2,1	3,1
9	2,6	1,8	3,1

* Incorporation of 10^4 cpm ^{3}H-thymidine into $1x10^5$ lymphocytes after stimulation on $1,25x10^4$ cultivated cells

From the data shown in Table 2 and from phenotype and morphology the cells grown for 6 days in serumfree medium supplemented with cytokines exhibited the most LC like shape. These cells were used to test their capacity to stimulate autologous T-lymphocytes in the presence of a mitogen. The cytokine stimulated serumfree cultured cells, enriched in $CD1a^+$ cells, showed a significant capacity to stimulate autologous T-lymphocytes to proliferate in the presence of mitogen..

By in vitro incubation of the monocytic cell fraction of the peripheral blood from normal human volunteers under serumfree conditions it was possible to generate a cell population which resembles from phenotype and stimulatory capacities towards T-lymphocytes that of LCs.

In summary these in vitro generated cells could be used as LCs equivalents circumventing the necessity to isolate LCs by tedious isolation procedures from human skin.

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INHIBITORY EFFECT OF IL-10 ON HUMAN LANGERHANS CELL ANTIGEN PRESENTING FUNCTION

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INTRODUCTION

IL-10 has been previously described by Mossman and coworkers as a product of murine Th2 clones that inhibits the production of cytokines by Th1 clones (1). Accumulated evidence now indicate that this cytokine is secreted by other cell types including monocytes and B cells and that it displays pleiotropic activities (2). Apart its stimulating and differenciating effects on B cells, IL-10 was shown to inhibit antigen presentation to T cells by acting on the antigen presenting monocytes. The recent finding that keratinocytes might be a potent source of IL-10 (3) prompted us to analyse its effect on human Langerhans cells (LC), the antigen-presenting cells from epidermis.

RESULTS

IL-10 Inhibits Human Langerhans Cell Antigen Presenting Function

We first determined the effect of IL-10 on the primary allogeneic T cell response induced by human LC. To this end, LC were purified from epidermal cell suspensions and added to allogeneic T cells in a mixed skin cell lymphocyte reaction (MSLR). T cell proliferation was assessed 6 days later by incorporation of 3H-thymidine during the last 18 hrs of culture. As shown in Figure 1, addition of IL-10 at the beginning of MSLR inhibits the proliferative T cell response in a dose-dependent way, with maximal inhibition at concentrations as low as 5 to 10 ng/ml. This inhibitory effect can be reversed by a neutralizing anti-IL-10 antibody (not shown), therefore demonstrating the specificity of IL-10 inhibitory effect.

We determined at which step IL-10 was required to inhibit allogeneic T cell response by adding the cytokine at different times after the initiation of MSLR. We found (data not shown) that IL-10 was most effective when added at the onset of culture. Significant inhibitory effect was still detected when the cytokine was added 24 hrs afer the initiation of culture, but it disappeared completely if IL-10 was added one day later. These results indicate that IL-10 interferes with an early step of T cell activation. Furthermore they eliminate the possibility of a direct cytotoxic effect of IL-10 on the T cells.

During the MELR, $CD4^+$ and, to a lesser extent, $CD8^+$ T cells are induced to proliferate. Enriched populations of these two cell subsets were prepared using a negative panning method and used in the MSLR. We observed that IL-10 inhibited allogeneic T cell responses

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to both CD4+ and CD8+ T cells in a similar dose-dependent way and that the extent of inhibition was similar to that observed with unseparated T cells (data not shown).

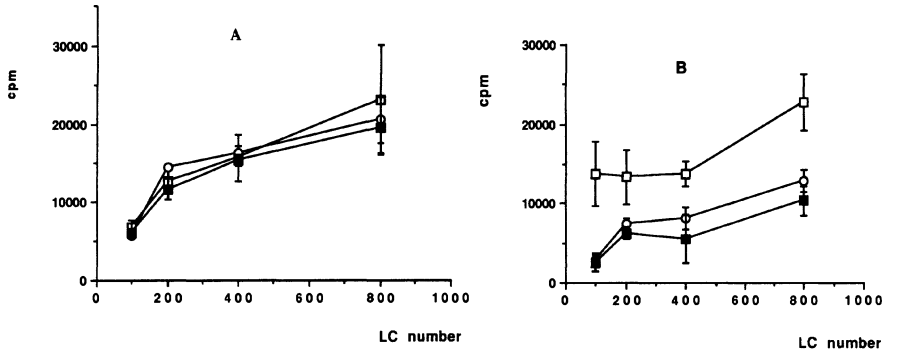

Figure 1. Inhibitory effects of IL-10 in MSLR. T cells (10^5 cells) were cultured for 6 days with purified allogeneic LC (10^3 cells) and increasing concentrations of IL-10 were added at the onset of culture. T cell proliferation was quantified by 3H-thymidine incorporation during the last 18 hrs of culture. Results are the mean cpm±SD of triplicate experiment.

IL-10 Inhibits the MSLR by Acting on Human LC Allostimulatory Function

We asked whether IL-10 inhibits the MSLR by acting on the T cells, or by affecting human LC function. Purified LC, or T cells, were therefore incubated for 18 hrs in the presence or not of the cytokine, then extensively washed and tested in MSLR. Since many LC died after the 18 hr incubation with or without the cytokine, viable LC were recovered on Lymphoprep and enumerated before carrying out the functional assays. As shown in Figure 2, IL-10 treatment of T cells did not impair their proliferative capacity. By contrast, we found that IL-10 incubated LC induced lower allogeneic T cell response than LC incubated in medium alone, therefore indicating a direct effect of the cytokine on human LC function. This inhibitory effect was not mediated by soluble suppressive factors since addition of IL-10 treated LC did not impair T cell response to non-treated LC (not shown).

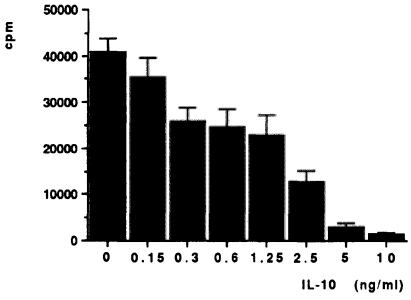

Figure 2. IL-10 acts directly on human LC to inhibit MSLR. Purified T cells (A) or LC (B) were incubated for 18 hrs in the absence (open squares) or presence of IL-10 at 1.25 (open circles) or 2.5 ng/ml (closed squares). After washing, the cells were assayed in MSLR and T cell proliferation was assessed 6 days later.

IL-10 did not Prevent Upregulation of HLA-DR Antigen during LC Culture

IL-10 has been reported to inhibit monocyte antigen-presenting function by downregulating HLA class II antigen expression at the cell surface (2). We therefore analyzed HLA-DR expression on human LC before and after a 18 hr incubation in the presence or not of the cytokine. As reported earlier (4), we observed a strong upregulation of HLA-DR antigen on human LC after incubation in medium alone. LC treatment with IL-10 did not prevent this upregulation although the number of highly positive cells was slightly

lower than that observed in the absence of the cytokine. Moreover, we found that IL-10 was quite able to inhibit T cell allogeneic proliferation induced by cultured LC which already display high level of MHC class II antigens. It seems unlikely, therefore, that inhibitory effects of IL-10 on LC allostimulatory function was merely related to a decreased expression of class II antigens at the cell surface.

IL-10 Inhibitory Effect on MSLR Can Be Partly Overcome by Addition of Exogeneous IL-1

IL-1 is secreted by LC and we (4) and others have reported a costimulatory function of this cytokine on the primary allogeneic T cell response induced by human LC. We found that addition of IL-1 (IL-1α or IL-1β) at the beginning of MSLR could reverse, at least partly, the IL-10 inhibitory effect (Fig 3). Many other cytokines are known to be released during the MSLR, such as IFN-γ, GM-CSF, IL-6 or TNF-α. By contrast with IL-1, however, none of these cytokines could reverse the IL-10 inhibitory effect (Fig 3). It is likely, therefore, that suppressive effect of IL-10 on human LC allostimulatory function is linked, at least partly, to a suppression of IL-1 activity during the primary allogeneic T cell response.

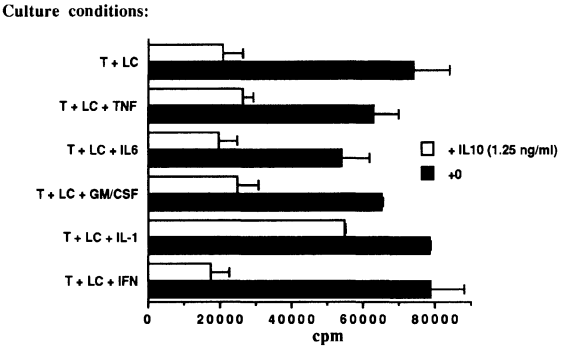

Figure 3. Addition of IL-1 during MSLR partly reversed IL-10 inhibitory effects. MSLR was performed in the presence, or not, of IL-10 and other cytokines were added at the onset of culture at the concentration of 50 U/ml. T cell proliferation was assessed 6 days later. Results are the mean cpm ± SD of triplicate experiment.

CONCLUSIONS

In conclusion, we demonstrated here an inhibitory effect of IL-10 on human LC allostimulatory function and this effect has been confirmed when human LC were used as accessory or antigen-presenting cells in mitogen or recall antigen-induced T cell proliferative assays. These findings may contribute to the down-regulating effects of the cytokine on contact hypersensitivity reactions in vivo (5) and may provide new insights into the potential clinical use of the cytokine.

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IL-10 INHIBITS THE PRIMARY ALLOGENEIC T CELL RESPONSE TO HUMAN PERIPHERAL BLOOD DENDRITIC CELLS

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INTRODUCTION

Most of the immunosuppressive properties of IL-10 are related to its action on antigen presenting cells (APC). Until now, several mechanisms have been described by which IL-10 inhibits the ability of monocytes/macrophages to induce T cell activation. Indeed, IL-10 down-regulates their expression of MHC class II molecules[1] as well as of ICAM-1 and B7-1 accessory molecules[2,3]. Moreover, the inhibition by IL-10 of IL-12 and IL-1 synthesis by monocytes was shown to be responsible for the inhibition of IFN-γ production by peripheral blood mononuclear cells (PBMC)[4] As far as the effects of IL-10 on dendritic cells are concerned, IL-10 was shown to inhibit the ability of mouse splenic dendritic cells to induce interferon (IFN)-γ production by antigen-specific TH1 clones or alloreactive splenic T cells[5]. In addition, human epidermal Langerhans cells preincubated with IL-10 were found to be deficient in the triggering of a mixed lymphocyte reaction[6] As a first approach to study the effects of IL-10 on human peripheral blood dendritic cells (PBDC), we analyzed the influence of IL-10 on a primary allogeneic T cell response induced by those cells

MATERIALS AND METHODS

Peripheral blood dendritic cells were purified according to a protocol adapted from Steinman et al.[7] Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors by density centrifugation on lymphoprep (Nycomed, Oslo, Norway).T cell-depleted PBMC obtained by rosetting with AET-treated sheep red blood cells were cultured two times ($4x10^6$/ml) at 37°C on tissue culture dishes coated with human immunoglobulins (Fluka, Switzerland). The nonadherent cells were further incubated with

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anti-CD19, anti-CD14, anti-CD56, anti-CD3 mAbs (Becton Dickinson) for 30 min at 4°C, then washed and incubated with goat anti-mouse IgG-coated magnetic beads (Dynabeads M450; Dynal, Oslo, Norway). After 1h incubation at 4°C, uncoated cells were removed using a magnet. The resulting fraction was treated with an anti-CD45RA mAb (Becton Dickinson) for 30 min at 4°C. After washing, cells were incubated for 30 min at 37°C on a 24-well-plate coated with goat anti-mouse IgG (Immunotech, Marseille, France). The nonadherent cells correspond to the dendritic-cell-enriched fraction. T lymphocytes were purified as previously described[8]. The medium used in all experiments was RPMI 1640 supplemented with 2 mM L-glutamine, gentamicin and 10 % fetal calf serum (Myoclone, Gibco, Paisley, Scotland). Mixed lymphocyte reactions (MLR) were carried out in round-bottom microtiter plates by adding T cells ($2x10^5$/ml) to a varying number of allogeneic dendritic cells in the presence or absence of 500 ng/ml recombinant human IL-10 (rIL-10)[8]. After 5 days of incubation, proliferation was measured by ^{3}H-thymidine incorporation.

RESULTS AND DISCUSSION

As shown in fig 1, the addition of 500ng/ml of rIL-10 at the beginning of the MLR induced inhibition of the proliferative response of alloreactive T cells as measured by ^{3}H-thymidine uptake. Since recent data indicate that IL-10 might directly affect T cells[8], we performed additional experiments to determine whether rIL-10 directly impairs the allostimulatory function of dendritic cells. For this purpose, dendritic cells were pretreated with rIL-10 and then washed before to be included as stimulators in the MLR. Our results indicate that, as in the case of Langerhans cells[6], IL-10-pretreated PBDC are defective in the activation of alloreactive T cells (data not shown). In order to elucidate the mechanism(s) of the inhibitory action of IL-10 on human PBDC, we are currently investigating the influence of rIL-10 on the expression of MHC class II as well as of ICAM-1, B7-1 and B7-2 molecules at the surface of these cells (manuscript in preparation).

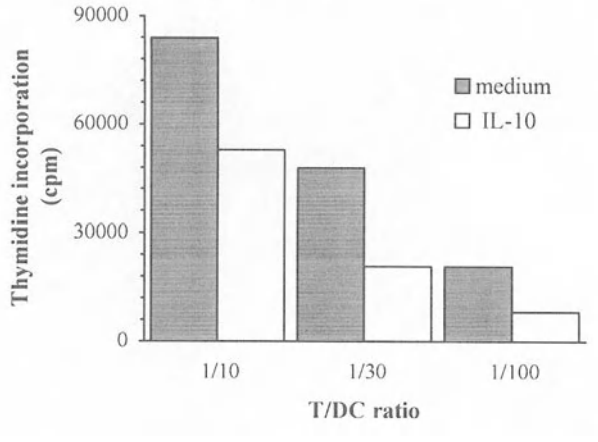

Figure 1. T cells ($2x10^5$ cells/well) were cultured for 5 days with PBDC at the indicated T cell/PBDC ratios in presence or absence of 500 ng/ml rIL-10. T cell proliferation was assessed by ^{3}H-thymidine incorporation during the last 16h of culture. Data are shown as mean cpm of triplicate cultures.

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A MOLECULAR STRATEGY TO IDENTIFY MOLECULES THAT ARE SPECIFIC FOR DENDRITIC CELLS AND/OR CRITICAL TO THEIR UNIQUE IMMUNOSTIMULATORY FUNCTION

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INTRODUCTION

The molecular basis for the unique capacity of dendritic cells (DC) to stimulate resting helper T-cells[1] is still unknown. Attempts to produce antibodies against functionally relevant molecules of DC have so far been unsuccessful. We have therefore chosen molecular strategies as a new approach to identify such molecules.

METHODS

When we started this project methods to grow DC from proliferating precursors were not yet available. One of our major concerns was therefore to choose methods that would keep the number of cells needed within a reasonable limit. A solution to this problem was to start by establishing a cDNA library. After a series of pilot experiments we chose the following cDNA subtraction strategy: From a DC cDNA library a pool of plasmids was grown and the inserts released by EcoRI digest. Similarly, from a cDNA library of the macrophage cell line J774 we released inserts by pstI/HindIII digest and biotinylated the DNA by photocrosslinking to photoactivatable biotin (PAB, Clontech). After hybridizing a 10-fold excess of biotinylated J774 cDNA to DC cDNA in solution, biotinylated DNA and hybrids were removed by phenolextraction, leaving behind DC cDNA that had not found homologous structures in the J774 fraction[2,3]. These inserts were ligated into phage arms and packaged to create a subtracted DC cDNA library.

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For a screening 1200 clones of this library were isolated and tested as follows: First we released the inserts by EcoRI digestion and performed Southern blots using as probes the inserts which were visible on ethidiumbromide stained agarose gels. By doing so we crosshybridized the clones to each other in order to eliminate multiple clones. Next we discarded all clones with inserts smaller than 100 bp as they would not give good signals in the next step, i.e. hybridizing lifts of the original DC cDNA library. This was done to check the frequency of the clones as a relation to their level of expression in DC's. Rare clones were discarded, more frequent ones were further characterized by partial sequencing and homology studies as a hint to their identity. Northern blots were then performed with RNA's from different tissues and cell types to check the expression pattern and specificity of the isolated clones.

RESULTS

Currently we have several clones under investigation of which we will present two: The first clone is fully sequenced and has very little homologies with known sequences except for a short stretch on the 3' end that has high homology to ubiquitin, a molecule that marks protein in the cell for proteolysis. We do not yet know whether this limited homology to ubiquitin points to a related function of the novel molecule identified.
Northern blot analysis shows strong expression in DC, none in the other leukocytes or several hematopoietic cell lines tested so far, but varying levels of expression in RNA's derived from different organs. Experiments in order to define the exact location and nature of cells expressing the molecule are in progress.
The second clone under investigation has strong homologies to signal transducer and activator of transcription (STAT)-proteins, and might represent a new member of that family. Several organs and cell lines express the respective mRNA.. This might be due to the strong homologies between different members of STAT's or to a ubiquitous expression of this molecule.

DISCUSSION

We have been able to establish a method which allows us to search for molecules expressed in DC's, that might be related to their unique immunostimulatory capacity. There are, however, a series of problems that will have to be addressed in the future. For example it is important to find a more efficient strategy to check for the specific expression of the molecules tested.
The combination of the novel molecular approach described here with emerging new techniques to obtain dendritic cells in large quantities[4,5] by strikingly simple methods has recently proven very successful.

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ACTIVATION OF PRIMARY ALLOGENEIC CD8+ T CELLS BY DENDRITIC CELLS GENERATED IN VITRO FROM CD34+ CORD BLOOD PROGENITOR CELLS

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INTRODUCTION

Dendritic cells (DC) have been shown to be the most potent antigen presenting cells (APC). Due to the difficulty to obtain large numbers of mature dendritic cells, several groups have developed ways to generate DC *in vitro* from culture of hematopoietic progenitors in presence of cytokines (1-5). We have described the generation *in vitro* of dendritic/Langerhans cells from culture of CD34+ cord blood hematopoietic progenitor cells (HPC) in presence of GM-CSF and TNFα. After 12 days of culture in presence of cytokines, up to 80% of the cells express CD1a and are very potent in stimulation of allogeneic T cells (4). In the present study, we have compared the activation and proliferation of alloreactive CD4+ and CD8+ T cells after stimulation with allogeneic DLC as well as their cytolytic activity.

RESULTS

Primary stimulation of CD8+ T cells by DLC

DLC derived from a 12 days culture in presence of GM-CSF and TNFα were used as stimulator cells for allogeneic adult CD8+ and CD4+ T cells isolated from the same donor. Fig.1 shows that DLC induce a strong stimulation of CD4+ T cells (Fig.1A) whereas the proliferation of CD8+ T cells is lower (Fig.1B). Ten times more DLC were required to induce significant proliferation of CD8+ T cells. However, when exogeneous IL-2 is added to the culture, lower numbers of cells are needed. Other cytokines such as IL-4, and IL-7 could also enhance CD8+ T cell proliferation but to a lesser extent (data not shown).

Since the proliferation of CD8+ T cells is relatively limited when compared to CD4+ T cells, we characterize the cytokines produced during a primary stimulation with DLC. Fig.2 shows that coculture of CD4+ T cells and DLC yielded measurable amounts of IL-2, TNF and INFgamma whereas supernatants from coculture of DLC and CD8+ T cells contained no detactable amounts of IL-2 but concentrations of IFNgamma and TNF comparable to those produced by CD4+ T cells stimulated with DLC. This suggests that DLC can strongly activate allogeneic primary CD8+ T cells as they produce cytokines although their proliferation is limited possibly because of the low IL-2 production.

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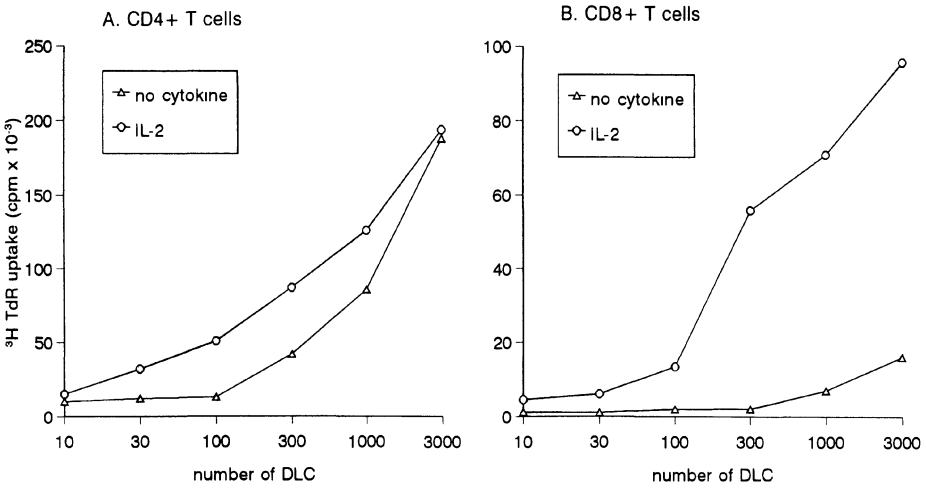

Figure 1. 10^4 CD4+ and CD8+ T cells were cocultured with various doses of DLC in absence or presence of rh-IL-2 (10 U/ml).

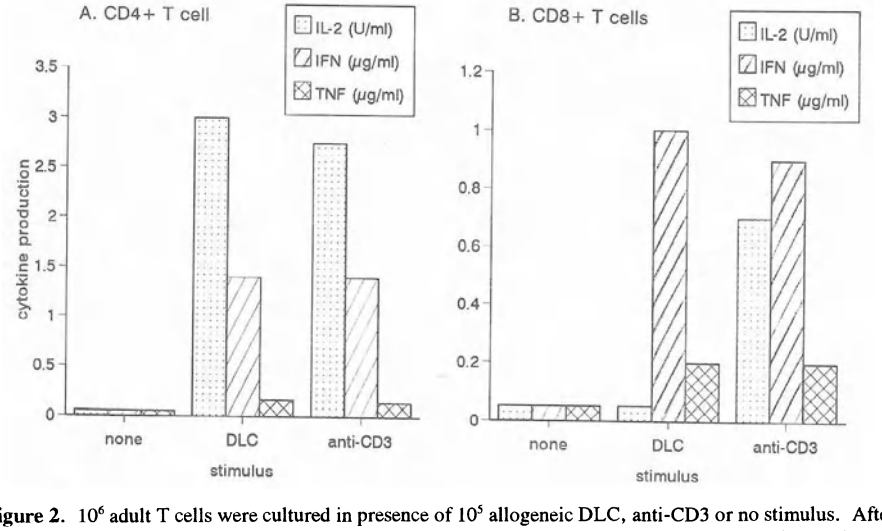

Figure 2. 10^6 adult T cells were cultured in presence of 10^5 allogeneic DLC, anti-CD3 or no stimulus. After 24 hr of culture supernatant was harvested for testing IL-2 production and after 72 hr for testing of IFNgamma and TNF secretion. Concentrations are reported in units derived from a standard curve with graded doses of purified cytokines.

Recruitment and expansion of allospecific CD8+ T cells

In order to determine whether DLC could allow recruitment and expansion of allospecific T cells, CD4+ and CD8+ T cells isolated from peripheral blood and cultured on DLC samples. After 7 days of culture, T cells were expanded in presence of IL-2 (10 U/ml) or IL-4 (500 U/ml) over another 5 day period. At that time, another 7 day stimulation with DLC from the same origin was performed and IL-2 or IL-4 was added 5 days later. Finally, non specific expansion on feeder cells (allogeneic PBMC and EBV transformed B cells), PHA and IL-2 was performed. Viable cells were enumerated at each time point as shown in Fig.3. Although the expansion of CD8+ T cells is not as strong as the expansion of CD4+ T cells, it is significant and the addition of exogeneous cytokines during the generation of CD8+ T cell lines enhances the proliferation of the cells.

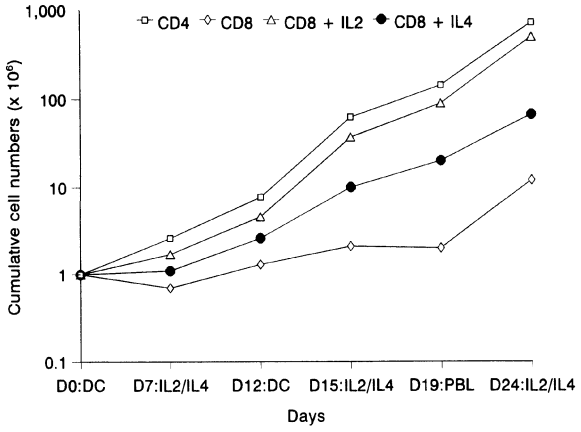

Figure 3. CD4+ and CD8+ T cells were cultured with DLC at a ratio 10/1 and expanded as described in the text. The proliferation was revealed by viable cell numeration at the different time points indicated.

Cytotoxic activity of allogeneic T cells

The cytolytic activity of allogenic CD8+ T cells was tested after 7 days of culture of total T cells in presence of DLC. CD8+ T cells were purified and tested for cytolytic activity against an allogeneic target (EBV transformed B cell) and the NK sensitive cell line Daudi in a standard 4hr ^{51}Cr-release assay. As shown in Fig.4A, T cells generated after stimulation with DLC specifically lyse the allogeneic EBV-B target cell line at E/T ratios as low as 1 but not the unrelated EBV-B cell line. Some NK lysis is observed (~10% lysis of Daudi at an E/T ratio of 1) is not surprising since 11% of the population is CD56+ as determined by flow cytometry (data not shown).

Interestingly, the specific cytolytic activity is increased during repeated cocultures of the cells with allogeneic DLC as shown in the same figure.

To demonstrate that the lytic activity of the total T cell population was due to CD8+ T cells, CD4+ and CD8+ T cells were purified from total T cells and tested for cytolytic activity. Fig.4B clearly shows that the specific lytic activity of the total population (85% at an E/T ratio=0.3) is due to CD8+ T cells (53% lysis) and not to the CD4+ T cells (6% lysis).

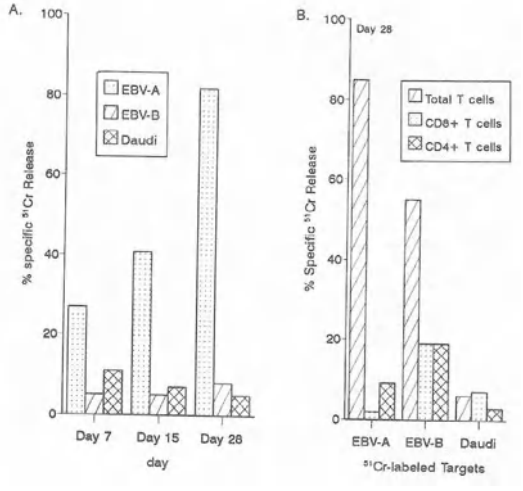

Figure 4. Allogeneic total T cells generated by successive stimulations with DLC were tested for their cytotoxic activity at day 7, 15 and 28 (Fig.4A). (E:T ratio = 1) At day 28, CD4+ and CD8+ were purified and tested for cytotoxic activity (Fig.4B). (E:T ratio = 0.3)

CONCLUSION

We have shown that *in vitro* generated DLC induce proliferation of CD8+ T cells although at a lower magnitude than that of CD4+ T cells in the same conditions probably because of the limited amounts of IL-2 produced by CD8+ T cells. The activation of CD8+ T cells could however be demonstrated by TNF and IFNgamma. Consistent with the low amounts of IL-2 produced by CD8+ T cells stimulated by DLC, addition of IL-2 greatly enhanced T cell proliferation.

Repeated cocultures of T cells and DLC followed by non specific stimulation of the cells allow the expansion of T cell lines which were found to be specific for the alloantigen. Specific cytotoxicity as well as NK lysis is observed after 7 days of culture but the specificity of the cell lines increases with time. These results suggest that DLC are important for the recruitment of allospecific CD8+ T cells but once they have been activated, their expansion is dependent on exogeneous IL-2.

As a result of their unique properties of *in vitro* and *in vivo* priming of naive T cells, DLC are an efficient tool to study and manipulate immune responses and develop vaccines against, for example, tumor associated antigens (TAA). We have also shown that a single injection of a small number of syngeneic DLC pulsed with TAA results in a long lasting resistance to subsequent injection of tumor cells. The *in vitro* generation of DLC from hematopoietic progenitors should overcome this difficulty to obtain large numbers of mature DC and open new ways of immunotherapy. DLC could be used for active immunotherapy of tumors when the TAA is known by injection of antigen or peptide pulsed DLC in patients, or for the generation of large numbers of specific anti-tumor CTL *in vitro* which could be then used for passive immunotherapy.

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STIMULATION OF HUMAN ANTI-VIRAL CD8+ CYTOLYTIC T LYMPHOCYTES BY DENDRITIC CELLS

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INTRODUCTION

Antigen-specific CD8+ cytolytic T lymphocytes [CTLs] are considered to be important mediators of resistance in several human infections [e.g. influenza (1), HIV-1 (2), cytomegalovirus (3), malaria (4)] and malignant diseases [e.g. melanoma (5)]. Vaccines or immunotherapies that preferentially prime this arm of the immune response could be essential for effective host immunity. However, the cellular requirements for eliciting specific and potent CTLs from human lymphocytes are not well defined, even in tissue culture where repetitive stimulation with antigen and exogenous cytokines are often required.

Evidence has emerged primarily from studies of killer cells to transplantation antigens, that an effective way to generate human CTLs, is to present antigens on dendritic cells (6). Here we describe the use of dendritic cells to stimulate virus-specific CD8+ CTLs from human blood. Influenza virus was chosen as a model since improved mechanisms for prophylaxis and therapy are needed in influenza, and the appearance of influenza virus-specific CTLs is associated with a more rapid clearance of virus from nasal washings (1). We find that influenza A virus establishes a nontoxic infection of human dendritic cells, and that these infected dendritic cells induce the development of strong virus-specific CTLs within 7 days.

INFLUENZA VIRUS UPTAKE AND INFECTION IN HUMAN DENDRITIC CELLS

Murine splenic dendritic cells [20% or so] can be infected by influenza virus and elicit potent CTL responses (7,8). The responses are dependent upon the synthesis of endogenous viral proteins (7). Human blood dendritic cells seem to be more efficient at supporting viral protein synthesis (9). When pulsed with live influenza virus [PR8; 1000 HAU/ml or MOI of 2-4], and cultured for 16 hours, > 90% of human dendritic cells expressed viral nucleoprotein [NP] and hemagglutinin [HA]. The viral proteins were detected by immunohistochemistry with anti-HA and anti-NP mAbs. Furthermore, no change in viability was detected for up to two days after infection. In sharp contrast, freshly isolated monocytes or week old cultured macrophages were infected to a lesser extent [<70%] but died within 16 hours of infection, a result of apoptosis (10,11). B cells and T cells were not susceptible to infection as assessed by staining with anti-HA and NP mAbs.

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DENDRITIC CELLS ARE POTENT STIMULATORS FOR THE INDUCTION OF INFLUENZA SPECIFIC KILLER CELL RESPONSES

When tested for their ability to induce human influenza virus-specific CTL responses, dendritic cells were found to be 50-100 fold better than macrophages. The experimental system involved coculturing APCs with highly purified syngeneic T cells for 7 days [time of peak CTL responses]. CTL activity was then measured on chromium labeled uninfected and infected macrophage targets (9). The distinguishing features of this system are [1] only a few dendritic cells [0.5-1% suffice] are required to generate highly potent CTL; [2] only partially purified dendritic cells [T cell and monocyte depleted] need be used to generate CTLs; [3] depletion of monocytes is necessary to remove potential inhibitory and toxic effects on the effector cells; [4] while monocytes do not induce CTLs effectively, they serve as efficient targets in short term chromium release assays [5 hours] when a majority express viral proteins as demonstrated by immunohistochemistry. Most donors, > 90%, could be stimulated to form CTLs with influenza virus-infected dendritic cells, indicating that the majority of our donor pool had been previously exposed to influenza. Since CTL activity was measured on influenza A PR8-infected targets, and the prevalent strains are different, the CTLs generated appear to be crossreactive, thereby confirming other studies of human influenza-specific CTL (12).

CD8+ T CELLS ARE THE PRINICIPAL CTLS INDUCED WITH INFECTED DENDRITIC CELLS

The potent CTLs generated by influenza virus-pulsed dendritic cells were found to be CD8+. This was demonstrated by stimulating bulk T cells with infected dendritic cells for 7 days, staining with CD4-FITC and CD8-PE mAbs and sorting the T cells on a FACSTAR into >98% pure CD4+ and CD8+ populations. Lytic activity was detected only in the CD8+ population as well as the unsorted bulk cells. The CD8+ CTLs generated were also shown to be class I restricted, in that they kill class I matched macrophage targets but not mismatched targets.

Dendritic cells also have the capacity to induce *purified* CD8+ T cells to proliferate and develop influenza virus-specific cytolytic activity [Fig. 1]. As in bulk cultures of T cells, CTL activity can be generated with relatively few dendritic cells. Dendritic cells also directly induce human and murine CD8+ T cells to develop cytotoxic activity in the MLR (6,13). In contrast to these findings, Nonacs et al (7) found that mouse dendritic cells could not induce influenza virus-specific CTL activity in purified CD8+ T cells, unless a source of CD4+ T cells or helper lymphokines was available. Key differences may be the number of antigen-specific IL-2 producers in the murine primed CD8+ population and that far fewer murine dendritic cells synthesize viral proteins than human blood dendritic cells.

INFLUENZA VIRUS-SPECIFIC CD4+ CTLS ARE ALSO GENERATED BY DENDRITIC CELLS

When separated from CD8+ T cells by cell sorting, purified CD4+ cells could also be induced by blood dendritic cells to proliferate and develop cytolytic activity [Fig. 1]. However, it was critical to remove the CD8+ T cells to observe both the blastogenesis and CTL responses. This could be due to killing of CD4+ T cells by CD8+ CTLs in bulk cultures. Alternatively, there might be selective inhibition of exogenous antigen presentation via the class II pathway, as previously described (14). This possibility seemed less likely since influenza-virus-infected APCs could present PPD to M.tuberculosis-reactive CD4+ T cell clones as well as uninfected APCs. Influenza-specific CD4+ CTL have been described in both human and mouse systems (15-17), but their role in disease resistance has yet to be defined.

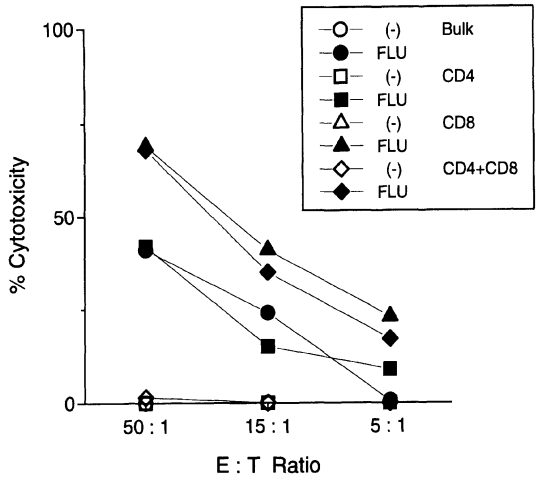

Figure 1. Dendritic cells stimulate the development of both CD4+ and CD8+ influenza virus-specific CTL.
Bulk T cells, sorted purified CD4+ or CD8+ cells were stimulated with uninfected [open symbols] or infected [closed symbols] partially enriched dendritic cells for 7 days. The T:APC ratio was 3:1. Cytolytic activity was measured on uninfected [not shown] and infected syngeneic monocytes. CD4+ and CD8+ cells were also added together at a 2:1 ratio prior to the CTL assay. Lysis of uninfected target cells was <5% at all E:T ratios used.

ANTIGEN PRESENTING CELL REQUIREMENTS FOR THE GENERATION OF INFLUENZA VIRUS-SPECIFIC CTLS

Several features may account for the observed differences between dendritic cells and monocytes in the induction of influenza CTLs. First, dendritic cells retain viability for >24 hours after infection, while macrophages die. Second dendritic cells are specialized APCs for the presentation of several other viruses to T cells in both the mouse (18,19) and human systems (20). Third, dendritic cells deliver signals to the TCR-CD3 complex on T cells far more efficiently than B cells or monocytes (21). This is due, in part, to the fact that dendritic cells express and upregulate many accessory molecules that are critical during the initiation of T cell immune responses [e.g. MLR (22), superantigens (21)]. They include B7/BB1 [CD80], ICAM-1 [CD54] and LFA-3 [CD58], ligands for CD28, CD11a and CD2, respectively. There is evidence that interaction of CD28 with its ligand is a critical element in the activation of cytotoxic CD8+ T cells (23). The CD28-B7/BB1 interaction provides a critical costimulatory mechanism for IL-2 gene expression (24-26). This would explain the CD4 helper independence of CTL induction by dendritic cells i.e. their ability to present antigen together with costimulatory molecules like B7/BB1 that enhance the production of IL-2.

POTENTIAL USES OF CD8+ CTLS IN IMMUNOTHERAPY AND PROPHYLAXIS

The ability of dendritic cells to directly induce strong CD8+ CTL responses, as shown here

for influenza virus, implies a critical role for these cells in the design of vaccines where CTL generation is the desired endpoint. Targeting antigen to dendritic cells has several advantages; one can maximize the efficiency of T cell activation (27), and avoid the use of adjuvants (28). Mouse dendritic cells pulsed with class I restricted peptides of NP (7), HIV peptides (29), or given antigen via pH-sensitive liposomes (30) into the cytoplasm can induce CTL responses. To elicit CTL responses in humans one might consider pulsing dendritic cells directly with NP peptides or with attenuated influenza virus, and delivering these APCs in vivo. Current vaccines for influenza boost antibody responses to antigens that undergo antigenic shift and drift (31), but do not enhance CTL responses which play a role in recovery from infection (12). Influenza viruses engineered to express CTL epitopes to other infectious organisms are now available (32). Thus influenza virus could be used as a vector for targeting desired antigens to dendritic cells. Dendritic cells could also be used for generating large numbers of CD8+ CTL, for adoptive transfer to immunosuppressed individuals who are unable to mount normal immune responses. Immunotherapy with CD8+ CTL has been shown to amplify the immune response. Bone marrow transplant recipients given CMV-specific CTL by adoptive transfer, do not develop disease or viremia (3). These novel approaches for immunoprophylaxis and therapy should be applicable to several situations where CD8+ CTLs are believed to play a therapeutic role e.g. HIV infection (2,33,34), malaria (4) and malignancies such as melanoma (5,35).

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T-LYMPHOCYTE PROLIFERATION STIMULATED BY αβTCR/CD2 BISPECIFIC ANTIBODY IS DEPENDENT ON LFA-1/ICAM-1 RECOGNITION OF ACCESSORY CELLS

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INTRODUCTION

Resting lymphocytes preferentially recirculate via the blood and lymphatics, migrating to the lymph nodes and spleen for efficient antigen encounter. Following activation, lymphocytes display altered migration pathways in that they preferentially return ("home") to their site of activation. Activated lymphocytes also migrate to non-lymphoid organs and sites of inflammation, ensuring efficient antigen elimination. The receptor mechanisms that regulate migration are, as yet, unclear but appear to be altered upon lymphocyte activation and by the action of cytokines on endothelial cells. In order to study the role of activated T lymphocytes in lymphocyte recirculation and migration into inflammatory sites, a reproducible means of generating large numbers of lymphoblasts is required. We have investigated the use of a bispecific antibody (BsAb) constructed by Tutt et al.,[1] consisting of chemically crosslinked mouse Fab' fragments from R73 (anti-rat αß T cell receptor) and MRC-OX54 (anti-rat CD2) to stimulate the proliferation of rat T cells.

MATERIALS AND METHODS

Lymphocyte suspensions were prepared from peripheral lymph nodes from (AOxDA)F_1 hybrid or AO rats (8-20 weeks old) in PBS containing Ca^{2+} and Mg^{2+} salts, antibiotics and 1% heat inactivated foetal calf serum (FCS). Cells were resuspended at the required cell density in RPMI-1640 medium containing 2mM L-glutamine, antibiotics and 5% FCS (RPMI-5). An enriched population of T lymphocytes was prepared from lymph node cells (LNC) by depletion of B cells and macrophages using mouse monoclonal antibodies (mAbs) against surface immunoglobulin and MHC Class II molecules, unwanted cells being removed using goat anti-mouse IgG-coated magnetic particles. Lymphocyte proliferation was assessed using [^{3}H] thymidine incorporation (10μCi/ml) and liquid scintillation counting. Antibody blocking experiments were carried out by pre-incubating the lymphocytes (4°C; 30min) with mAbs against CD11a, CD18, ICAM-1, VLA-4, CD4, CD8, MHC Class II and CD45. Flow cytometric analysis was used to identify the cell populations.

RESULTS

BsAb stimulated incorporation of ^{3}H-thymidine by LNC in a dose-dependent manner which peaked at 100ng/ml BsAb (Figure 1). The number of lymphoblasts increased with time and plateaued between 72 and 96h (Figure 2).

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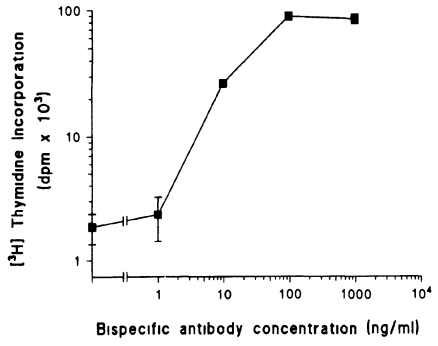

Figure 1. LNC were incubated with a range of concentrations of BsAb for 72 hours in the proliferation assay Optimal LNC proliferation was induced with 100ng/ml BsAb, with maximum [^{3}H] thymidine incorporation after 72 hours in culture

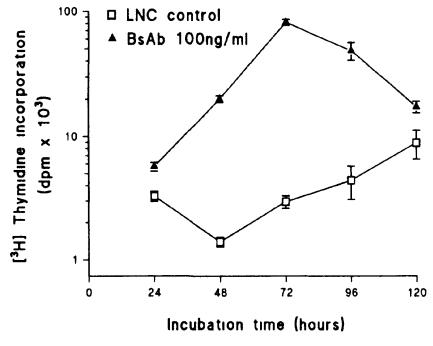

Figure 2. LNC were incubated in the proliferation assay for 24, 48, 72, 96 and 120 hours with 100ng/ml BsAb The number of lymphoblasts increased with time and plateaued between 72 and 96 hours

[^{3}H] thymidine incorporation by T lymphocytes purified from LNC by depletion of sIg$^+$ and Class II$^+$ cells was <10% of that by LNC (Figure 3). BsAb stimulation was accompanied by the formation of large cell clusters, which were not seen using purified T lymphocytes, suggesting a role for adhesion molecules in BsAb-induced proliferation.

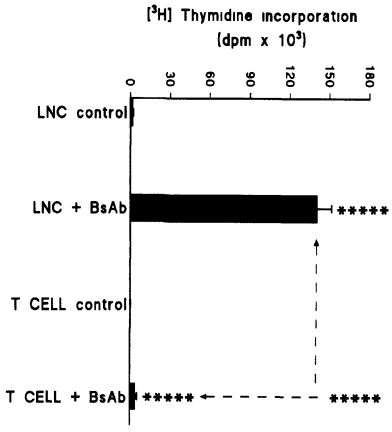

Figure 3. In BsAb-treated cells (100ng/ml, 72h), [^{3}H] thymidine incorporation by T lymphocytes purified from LNC by depletion of sIg$^+$ and Class II$^+$ cells was <10% of that by LNC (*****p<0 0005)

Table 1. Flow cytometric analysis of BsAb activated lymph node cells.

Marker	Antibody	Starting Population	Lymphoblasts
CD4	W3/25	46	30
CD8	OX8	25	58
IL2-R	OX39	3	76
CD5	OX19	69	75
VLA-4	HP2/1	85	92
CD45	OX1	99	94

Flow cytometric analysis of BsAb-stimulated LNC showed a population of large cells which were not present in the starting population. Analysis of this population demonstrated 30% CD4$^+$, 58% CD8$^+$, 75% CD5$^+$ and 76% IL2-R$^+$ cells (Table 1).

Thymidine incorporation by LNC was inhibited by 90% by the inclusion of antibodies to LFA-1 (CD11a, CD18) or ICAM-1; antibodies to VLA-4 had no effect (Figure 4). Antibodies against other cell surface molecules such as CD4, CD8, MHC Class II and CD45 were also without effect.

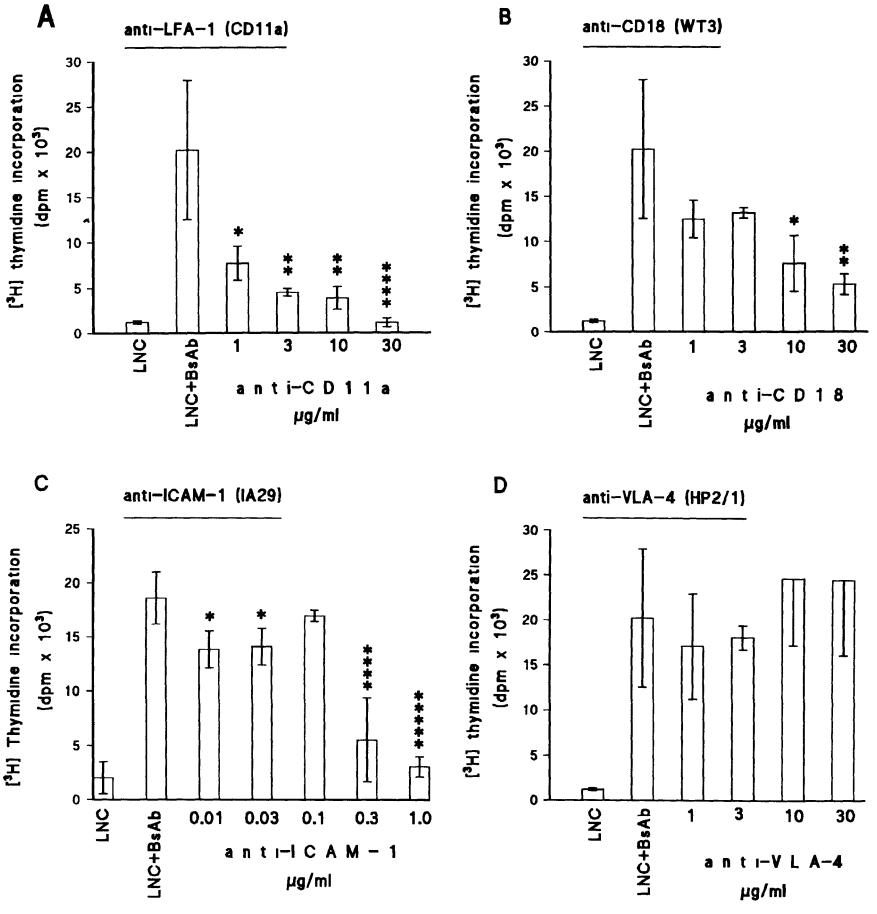

Figure 4. LNC were pre-treated with a range of concentrations of antibodies against (A) CD11a (WT1), (B) CD18 (WT3), (C) ICAM-1 (IA29), and (D) VLA-4 (HP2/1) prior to their inclusion in the proliferation assay Antibodies against CD11a (A), CD18 (B), and ICAM-1 (C), inhibit BsAb-induced LNC proliferation in a dose-dependent manner, by up to 90% Antibody against VLA-4 (D) showed no significant blocking effect (*$p<0.05$, **p,0 025, ***$p<0.005$, ****$p<0.0005$) Antibodies against CD4 (W3/25), CD8 (OX8), MHC Class II (OX6) and a control mAb against CD45 (OX1) were also without effect

CONCLUSION

The $\alpha\beta$TCR/CD2 BsAb has proved to be a reproducible means of generating T lymphoblasts *in vitro* in an Fc-independent manner. Proliferation-blocking assays have revealed that, in addition to the BsAb, LFA-1/ICAM-1 dependent interactions between T cells and accessory cells are also required for T cell activation. Experiments are underway to identify which cells in lymphoid organs provide this accessory activity for the BsAb.

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LOW REACTIVITY OF PERIPHERAL BLOOD DENDRITIC CELLS RESPOND TO IL-1 AND GM-CSF IN SLE PATIENTS

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INTRODUCTION

We reported previously that peripheral blood dendritic cells (PBDCs) have strong accessory cell (AC) function and are more potent ACs than macrophages. Further, IL-1 and GM-CSF were found to act on PBDCs but not on T cells, as a result, they enhance the concanavalin A (Con A)-stimulated T cell proliferative response[1]. Also, the Con A-stimulated T cell proliferation was reduced significantly in SLE patients compared to normal subjects[2]. Therefore, we investigated whether the PBDC function in patients with SLE can be restored by IL-1 and GM-CSF.

Materials and Methods

The subjects were 11 patients who were diagnosed with SLE according to American Rheumatism Association's criteria. The control subjects were 11 age- and sex- matched healthy volunteers.

Cell separation was performed by a previously reported method[1].

5.0×10^3 PBDCs and 1×10^5 T cells were cultured in 0.2ml of RPMI 1640 medium supplemented with 10% fetal calf serum and 1 μ g/ml Con A with or without IL-1 (100U/ml) or GM-CSF (1000U/ml) in 96-well microplates at 37℃ for 96 hours in a 5% CO_2 incubator. T cell proliferation was assessed by ^{3}H-TdR incorporation during the last seven hours of culture.

RESULT

IL-1 and GM-CSF enhanced significantly PBDC-mediated Con A response in normal subjects, however no such enhancement was found in SLE patients (Fig. 1 and Fig. 2, $P<0.01$).

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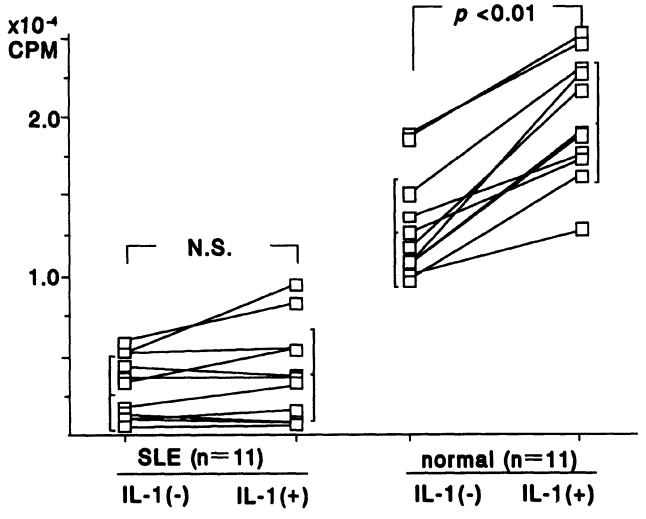

Fig.1 Effect of IL-1 on Con A-induced T cell responses in patients with SLE and normal subjects

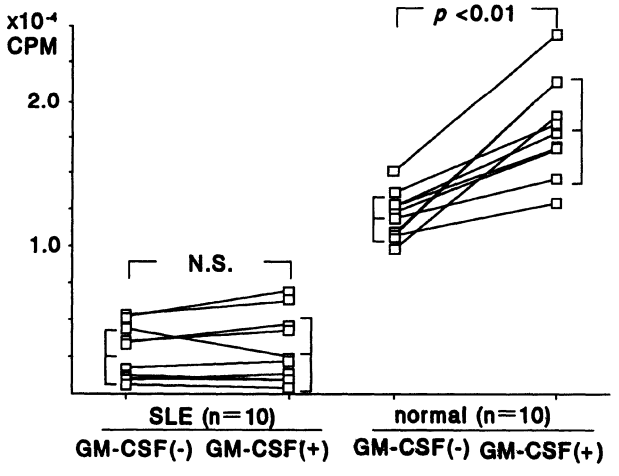

Fig. 2 Effect of GM-CSF on Con A-induced T cell responses in patients with SLE and normal subjects

CONCLUSION

PBDCs in SLE patients respond poorly to IL-1 and GM-CSF.

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DEFECT OF THE CTLA4-Ig LIGANDS ON TUMOR-INFILTRATING DENDRITIC CELLS

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The main paradox of tumor immunity is that cancer grows and ultimately kills its host, even though tumor cells express tumor-associated antigen(s) (TAA). One possible reason why immunogenic tumors can escape host immunity is that tumor-reactive T-cells receive inadequate co-stimulation from cells presenting TAA. Little is known concerning the way TAA are presented to the immune system, but tumor antigens, like other antigenic peptides, have to be presented to $CD4^+$ T-cells by accessory cells bearing MHC-II molecules[1]. Among the antigen-presenting cells (APC), there is evidence that dendritic cells (DC) play a critical role in activating resting T-cells much more efficiently than other APC[2].

In addition to presenting the processed antigen, APC have to provide a second or co-stimulatory signal for leading to T-cell growth and effector function. Recent studies have shown that engagement of CD28, a molecule constitutively expressed on resting T-cells, by either its two distinct counter-receptors on APC, B7.1 (CD80) and B7.2 (CD86), is sufficient to provide T-cell co-stimulation[3, 4]. CTLA4 is expressed on the surface of activated T-cells and is a high affinity receptor for B7.1 and B7.2. In the absence of co-stimulation, TCR engagement is ineffective in inducing T-cell activation and can instead induce T-cell anergy[5, 6].

PROb and REGb cells are cellular variants issued from a colon cancer chemically-induced in an inbred rat[7]. PROb tumors were rejected in previously immunized animals[8] and PROb cells expressed TAA evoking a proliferative response of tumor-immune $CD4^+$ T-cells in the presence of splenic APC[9]. However, PROb cells induce a specific immune tolerance and give rise to progressive, often metastatic and always lethal tumors when injected into syngeneic hosts[8]. Experimental data suggest that tumor-progression was made possible by an anergy restricted to the T-lymphocytes able to respond to the individual TAA[9]. In contrast, REGb cells are immunogenic and are rapidly rejected after injection into naive immunocompetent hosts[7].

PROb and REGb cells do not express MHC-II molecules *in vivo* and *in vitro*. Thus, they require MHC-II-bearing APC for TAA presentation to $CD4^+$ T-cells. PROb tumors contain a dense network of highly MHC-II positive cells identified as DC. PROb-associated DC were isolated, purified and characterized[10]. Using CTLA4-Ig, a fusion protein which blocks interactions between CD28, CTLA4 and their ligands B7.1 and B7.2, we showed that most of PROb tumor-infiltrating DC were severely deficient in the CTLA4-ligands, in marked contrast with splenic DC or peritoneal macrophages (Fig. 1).

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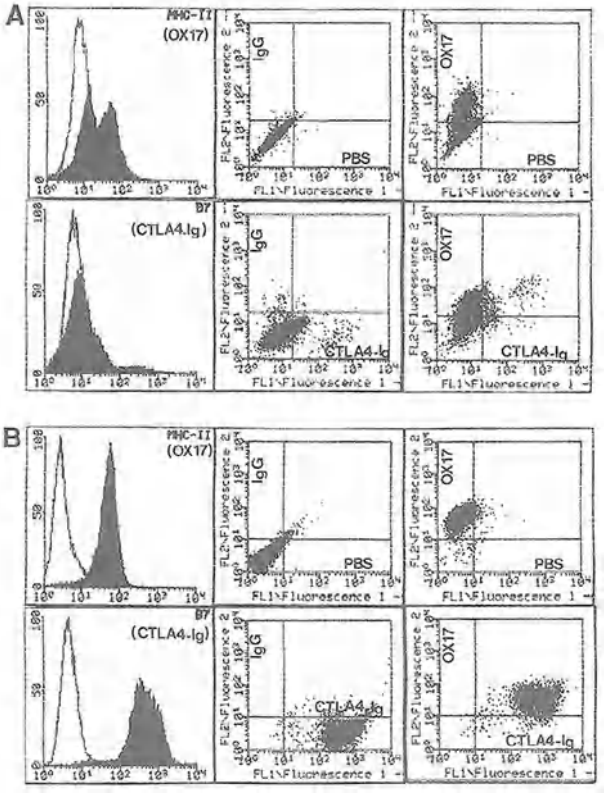

Figure 1. Most TIDC do not express B7. (a) TIDC-enriched populations were prepared from PROb tumors and labeled with the CTLA4-Ig fusion protein followed by FITC-conjugated goat anti-human IgG. Cells were double-labeled using OX17 (a mouse anti-rat RT1-D molecule monoclonal antibody) as a second stage reagent followed by PE-conjugated rabbit anti-mouse IgG. For comparison, splenic DC (b) were also studied. Control unstained or single-stained cells are shown in the dot-plots.

A weak primary allogeneic T-cell proliferative response and a secondary allogeneic-specific hyporesponsiveness could be induced in T-cells by exposure to histoincompatible tumor-associated DC (Fig. 2).

To investigate the role of the B7/CD28 co-stimulation pathway in REGb tumor regression, we used the CTLA4-Ig fusion protein that specifically inhibits B7 binding to its receptors on T cells. Studies have demonstrated the induction of an immunosuppression state following the administration of CTLA4-Ig[11]. Blocking the B7/CD28 interaction with a single dose of 0.5 mg CTLA4-Ig two days after REGb injection resulted in a dramatic decrease of tumor-infiltrating T-lymphocytes (not shown) and in an enhanced and prolonged growth of REGb tumors in CTLA4-Ig treated animals when compared to control rats (Fig. 3).

We suggest that the CTLA4-ligands deficiency could play a role in the specific anergy of $CD4^+$ T-cells to TAA and in the immune tolerance to the tumor.

Acknowledgments

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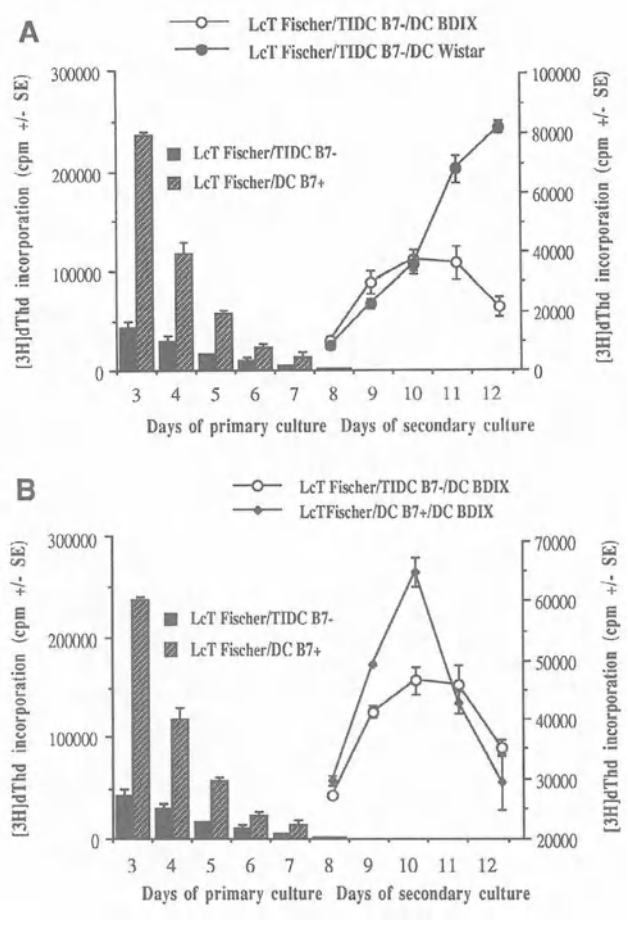

Figure 2. Comparison of the primary allogeneic T-cell proliferative responses to BD-IX alloantigens elicited by splenic versus intratumoral DC and secondary allogeneic MLR. Unprimed Fisher splenic T-cells were stimulated with BD-IX splenic (▨) or intratumoral (■)DC. Splenic DC are much more efficient compared to tumor-associated DC in activating allogeneic resting T-lymphocytes. Addition of CTLA4-Ig (5 μg/ml) inhibited the proliferation (not shown). Secondary allogeneic MLR. Seven days primary MLR contained freshly isolated allogeneic Fisher responder T-cells that had been cultured with BD-IX mitomycin-C treated splenic (DC $B7^+$) (▨) or tumor-associated (TIDC $B7^-$) (■) dendritic cells. Secondary stimulators were BD-IX or third party donor (Wistar) mitomycin C-treated splenic dendritic cells, used at a T:APC ratio of 10:1. Cells primed with $B7^-$ tumor-associated dendritic cells showed a weak response when rechallenged with splenic dendritic cells from the original BD-IX donor strain (○). On the contrary, when stimulated with splenic dendritic cells from a third donor, the primed cells showed a typical primary response (●). Cells primed with BD-IX $B7^+$ splenic dendritic cells showed a typical secondary response (◆)when rechallenged with BD-IX splenic dendritic cells.

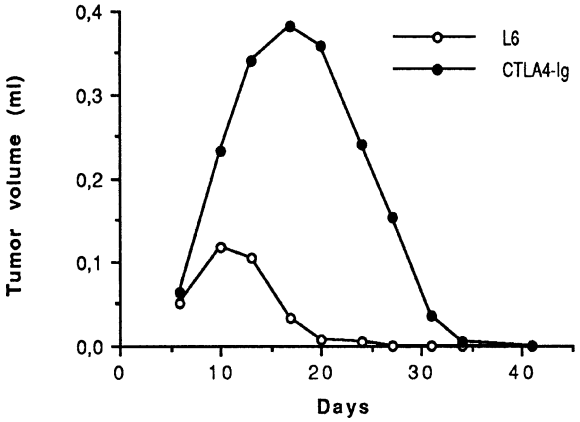

Figure 3. Effect of the CTLA4-Ig fusion protein on the growth of REGb tumors. Animals received $1x10^6$ REGb cells s.c on day 0 plus 0.5 mg of the control L6 (○) or 0.5 mg of CTLA4-Ig (●) fusion protein on day 2. From the 10th day to the 32nd day after tumor cells inoculation, tumor volumes of CTLA4-Ig-treated animals were significanlty higher than those of L6-treated control group.

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MIXED EPIDERMAL CELL LYMPHOCYTE REACTION: HLA-DR$^+$ CELLS EXHIBIT A GREATER IMMUNOSTIMULATORY ACTIVITY THAN CD1a$^+$ CELLS

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INTRODUCTION

Induction and expression of immunity depends upon processing and presentation of antigens to T-cells by bone marrow derived HLA-DR$^+$ antigen presenting cells (APC). In the induction of immunity to cutaneous antigens, the initial stages of this process begin within the skin itself, and both epidermis and dermis contain bone-marrow derived cells that have the capability of processing and presenting antigens. In normal epidermis, HLA-DR expression is believed to be confined to Langerhans cells with Birbeck granules and indeterminate cells without Birbeck granules. In diseased skin, particularly the inflammatory dermatoses, HLA-DR is commonly expressed by keratinocytes and has been related to the presence of intra-epidermal IFN-γ producing lymphocytes. Fresh and cultured Langerhans cells display disparate functional programs, based on their capacities to acitvate autologous and allogeneic T cells. Recent studies have revealed that the capacity of freshly prepared Langerhans cells to activate T cells is relatively poor. However, the antigen presenting capabilities can be considerably enhanced if the Langerhans cells are placed in culture in presence of GM-CSF, a cytokine produced by keratinocytes. On the other hand fresh Langerhans cells are at least tenfold more efficient at processing antigens than cultured cells.[1] These issues are relevant to psoriasis, a cutaneous disease in which antigen-presenting cell functions of epidermal cells have found to be abnormal.[2] We have studied the importance of epidermal Langerhans cells and other epidermal APC subsets for the stimulation of T-cells in patients with psoriasis and healthy individuals.

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METHODS

Mononuclear leukocytes were isolated from the peripheral blood of 7 healthy individuals and 6 otherwise healthy patients with psoriasis vulagris by density gradient centrifugation. None of the patients had received any systemic treatment, including irradiation, during the previous three months. T-lymphocytes were enriched to 96 % by passage over nylon wool columns. Epidermal cells from healthy skin, lesional and non-lesional psoriatic skin were isolated from the roofs of suction blisters by trypsinization and depletion of $CD1a^+$ and $HLA\text{-}DR^+$ cells by immunobeads. Cell enrichment and depletion were ascertained by flow cytometry. $1x10^5$ enriched T-cells were incubated for 6 days in coculture with $1x10^4$ allogeneic or autologous epidermal cells. Cell proliferation was measured semiquantitatively by ^3H-TdR uptake. The proliferative response was expressed as stimulation index (degree of change compared to the control T-lymphocytes incubated alone). Statistical significance was assessed using the two-tailed Wilcoxon signed rank test.

RESULTS

The T-cells of all psoriasis patients and healthy individuals showed a clear proliferative response to *allogeneic* normal epidermal cells. The level of the responses showed considerable inter-individual variability. To determine which epidermal APC subset was involved in the activation of T-lymphocytes, epidermal cells of healthy individuals were depleted of $HLA\text{-}DR^+$ and $CD1a^+$ cells. When the CD1a-depleted cell fraction was cocultured with allogeneic psoriatic and normal T-cells, a minimal inhibition of the mixed epidermal cell lymphocyte reaction was observed, a value to low to reach any statistical significance. However depletion of $HLA\text{-}DR^+$ cells in normal epidermis resulted in a strong inhibition of the mixed epidermal lymphocyte reaction. These results indicate that epidermal $CD1a^-/HLA\text{-}DR^+$ APC mainly stimulate allogeneic T-lymphocytes, whereas $CD1a^+/HLA\text{-}DR^+$ APC did so weakly or not. This was rather unexpected as in normal human skin $CD1a^+/HLA\text{-}DR^+$ Langerhans cell are thought to be the only $HLA\text{-}DR^+$ cells.

Table 1. Allogeneic mixed skin lymphocyte reaction: stimulation index (Mean ± SEM).

	Epidermal cells	$CD1a^+$ Depletion	$HLA\text{-}DR^+$ Depletion
Healthy T-cells (n=6)	11,8 ± 4,1	8,9 ± 4,1	4,1 ± 1,3 (p = 0,0313)
Psoritaic T-cells (n=6)	33,9 ± 16,1	20,9 ± 8,9	2,7 ± 1,0 (p = 0,0313)

Our observation that $CD1a^-/HLA\text{-}DR^+$ epidermal cells from normal skin stimulated allogeneic T-cells raised the question of whether $CD1a^-/HLA\text{-}DR^+$ epidermal cells would be able to stimulate *autolgous* healthy and psoriatic lymphocytes. This was studied in the autologous mixed epidermal cell lymphocyte reaction. Studies with unfractioned epidermal cells could only be performed with non-lesional psoriatic skin owning to insufficient number of epidermal cells. Removal of $CD1a^+$ cells did not exert any significant influence on the blastogenesis of T-lymphocytes. Compared to $CD1a^+$ cells, depletion of $HLA\text{-}DR^+$ epidermal cells diminished the proliferation level of autologous T-cells significantly. Concerning psoriatic skin this observation was expected as

in lesional psoriatic skin beside Langerhans cells other cell types also express HLA-DR (i.e. keratinocytes and lymphocytes). On the contrary, mixed epidermal lymphocyte reactions conducted either with non-lesional or healthy skin presented unexpectedly the stimulatory activity of $CD1a^-/HLA\text{-}DR^+$ cells.

Table 2. Autologous mixed skin lymphocyte reaction: stimulation index (Mean ± SEM)

	Epidermal cells	$CD1a^+$ Depletion	$HLA\text{-}DR^+$ Depletion
Healthy T-cells (n=7)		20,6 ±16,7	12,7 ± 9,8 (p = 0,0781)
Healthy skin			
Psoriatic T-cells (n=6)	7,3 ± 4,6	9,3 ± 4,0	2,9 ± 0,8 (p = 0,0625)
Non-lesional skin			
Psoriatic T-cells (n=6)		11,4 ± 5,4	3,0 ± 1,0 (p = 0,0625)
Lesional skin			

DISCUSSION

The existence of $HLA\text{-}DR^+/CD1a^-$ cells in epidermal cell suspensions may well explain the different degrees of reduction in mixed skin lymphocyte reactions conducted with either $CD1a^+$ depleted or $HLA\text{-}DR^+$ depleted epidermal cell suspensions. Concerning psoriatic epidermis the findings in this study are complementary to earlier findings. Activated lymphocytes in psoriatic lesions which themselves are $HLA\text{-}DR^+$ may lead to the expression of HLA-DR on keratinocytes by means of IFN-γ production.[3] These cells may then be able to stimulate the blastogenesis of lymphocytes.[4] The new aspect in this study is the stimulatory activity of $CD1a^-/HLA\text{-}DR^+$ cells in normal human skin. Our findings may be due to the fact that Langerhans cells display disparate funcitonal properties. Recently it was reported that cultured Langerhans cells lost the expression of CD1a and cytoplasmatic Birbeck granules in the presence of GM-CSF produced by keratinocytes, while concurrently the expression of MHC class II was upregulated and powerful accessory functions were acquired.[5,6,7] "Fresh" and "cultured" Langerhans cells may be considered as in-vitro representatives of different phenotypes of epidermal Langerhans cells. Indeed, epidermal $CD1a^+/HLA\text{-}DR^+$ Langerhans cells may undergo a functional process and mature into $CD1a^-/HLA\text{-}DR^+$ Langerhans cells, which acquire the capacity to activate autologous and allogeneic T-cells. One may speculate that these cells correspond to the so-called indeterminate cells.

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HUMAN DENDRITIC CELLS ENHANCE GROWTH AND DIFFERENTIATION OF CD40 ACTIVATED B CELLS

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INTRODUCTION

During an immune response, dendritic cells (DC) capture the antigen at site of injury, and migrate through the afferent lymph stream to the lymph-nodes where they efficiently activate naive T cells. This T cell activation is followed by B cell recruitment, which occurs in the extrafollicular area, where DC home[1]. Accordingly, we wondered herein whether DC might directly interact with B cells, using DC generated *in vitro* from $CD34^+$ progenitors, called Dendritic-Langerhans cells (D-Lc). As both DCs[2] and B cells[3] express functionnal CD40, we used CD40-ligand transfected L cells[3] as activated T cells surrogate, to study the effect of DCs on B cell activation. We show that D-Lc enhanced both CD40-L dependent B cell proliferation (3-6 fold) and Ig productions (10-200 fold). In presence of exogenous cytokine such as IL-10, D-Lc further increased Ig production and allowed low numbers of B cells to produce detectable amounts of Igs.

MATERIALS AND METHODS

Umbilical cord blood samples were obtained according to institutional guidelines. Cells bearing CD34 antigen were isolated from non adherent mononuclear fractions through positive selection, using anti CD34 Monoclonal Antibody (MAb) Immu-133.3 (Immunotech, Marseille, France) and a Magnetic Cell Sorter (Mini Macs, Miltenyi Biotech). Cultures were established in the presence of GM-CSF (100 ng/ml) and TNF α (2,5 ng/ml) in complete medium (10% FCS). After 12 days, cultures contained 50 to 90% $CD1a^+$ D-Lc[4]. Total B cells were purified from tonsil through Ficoll-Rosetting and depletion with MAbs directed against non B cells (T cells, monocytes and NK cells). The purity was routinely more than 98% ($CD19^+$ cells).

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B cell proliferation was measured after 6 days of culture and incubation with [3H]TdR for the last 16 hours. Igs productions were determined by standard indirect ELISAs.

RESULTS

Tonsillar B cells (10^4 per well) were cultured over irradiated CD40-L L cells ($2{,}5.10^3$ per well, 7500 rad) in presence or absence of $CD34^+$ progenitors derived D-Lc (3000 rad). Although, D-Lc had no effect on B cell proliferation in the absence of any activator (data not shown), D-Lc enhanced the proliferation of CD40-L activated total B cells ($sIgD^+$ and $sIgD^-$) in a dose dependent manner (3-6 fold). In the experiment shown in figure 1A, with two different D-Lc, DNA synthesis of B cells, cultured over CD40-L transfected L cells during 6 days, was enhanced 3 times when 10^4 D-Lc were added to the culture (30.9±2.1 to 102.8±14.4 10^3 cpm for D-Lc 1). 50% of this effect is maintained with 10^3 D-Lc.

Most notably, in the absence of exogenous cytokine, the addition of D-Lc dramatically increased the production of IgG, IgA and, to a lesser extent IgM, by CD40-L activated B cells. Figure 1B shows that addition of 10^4 D-Lc increased the productions of IgG by 80 fold, IgA (by 20 fold) and IgM (by 10 fold) over the background (≤ 300 ng/ml).

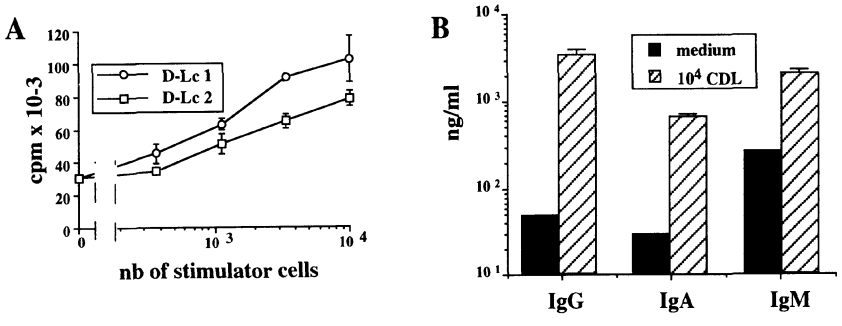

Figure 1: $CD34^+$ progenitors derived D-Lc were used, after irradiation (3000 rad) as stimulators of CD40-L dependent B cell proliferation and differentiation. Total tonsillar B cells (10^4 per well) were cultured over irradiated CD40-L transfected L cells (7500 rad, $2{,}5.10^3$ per well) in the presence of A) 2 different D-Lc (from $3{,}7.10^2$ to 10^4 cells per well) B) 10^4 D-Lc. **A)** B cell proliferation at day 6. **B)** production of IgG, IgA and IgM measured at day 14.

Interestingly, in the presence of IL-10, D-Lc further increased Ig production and allowed low numbers of B cells to produce significant amounts of Ig (data not shown). Limiting dilution experiments showed that addition of D-Lc lowered by two orders of magnitude the detection threshold of B cell proliferation and differentiation. Although D-Lc did not induce by themselves isotype switch, they strongly enhanced IL-10 dependent

isotype switch[5]. Furthermore, contacts between D-Lc and B lymphocytes in these cultures were observed by MGG staining and immunostainings (data not shown).

DISCUSSION

We have shown that D-Lc generated *in vitro* enhanced both proliferation and differentiation of B lymphocytes activated through their CD40, in absence of exogenous cytokines and T cells. Characterization of D-Lc sub-populations is currently under investigation[6]: D-Lc are composed of LC and other DCs related to Interdigitating Dendritic Cells of the extrafollicular area of secondary lymphoid organs. The latter cells are particulary important in the initiation of primary or secondary humoral response. Thus in addition to activate T cells in this area, DCs could directly interact with CD40 activated B cells, during primary and/or secondary humoral response. In this context, it would be interesting to determine whether the effects of D-Lc are restricted or not to certain B cell sub-population and/or DC sub-population.

Many authors have underlined the critical role of DCs in humoral response[7-9]. The direct modulation of B cell responses together with T cell priming may explain the primordial role of DCs in this process.

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HUMAN DENDRITIC CELLS CAN DRIVE CD40-ACTIVATED sIgD+ B CELLS TO MOUNT MUCOSAL-TYPE HUMORAL RESPONSE

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INTRODUCTION

Following tissue injury, invading antigens are captured by dendritic cells (DC) which migrate to secondary lymphoid organs where they initiate immune responses that involve triads composed of T cells, B cells and DC. As both DC[1] and B cells[2] express functional CD40, we studied the effects of *in vitro* generated DC on B cells responses in a culture system where CD40-Ligand-transfected L cells are used as surrogate membranes of activated T cells.

MATERIALS AND METHODS

Hematopoietic progenitors were obtained from umbilical cord blood through positive selection by anti-CD34 monoclonal antibody (Mab) (Immu 133.3, Immunotech) and magneting-sorting with MiniMacs (Miltenyi Biotech). After 12 days of culture in the presence of 100 ng/ml GM-CSF (Schering-Plough) and 2,5 ng/ml TNFα (Genzyme) in complete medium (10% FCS), 50 to 90% cells are $CD1a^+$ DC[3].
B cells were purified from tonsils after rosetting-Ficoll and negative depletions with Mabs against T cells and monocytes. B cells were incubated with an anti-IgD Mab (Sigma Chemicals) and $sIgD^+$ B cells are sorted in magnetic field with Macs (Miltenyi Biotech).
For Ig productions, 10^4 DC and 10^4 $sIgD^+$ B cells were cultured with $2x10^3$ irradiated (7500 rads) CD40-ligand-transfected L cells (CD40L-system)[2] during 12 days and Ig levels were measured by ELISA. IL-2 and IL-10 (Schering-Plough) were used at 20 U/ml and 200 ng/ml respectively.

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RESULTS

1- DC enhance the IL-10-induced Ig secretion of CD-activated sIgD$^+$ B cells

In the absence of any exogenous cytokine, DC induced a 3-fold enhancement of DNA synthesis by CD40-activated sIgD$^+$ B cells and further enhanced IL-10-induced proliferation in a similar fashion (data not shown). However, addition of DC to cultures of CD-activated B cells did not result in increased Ig secretion (figure). In response to IL-10, CD40-activated sIgD$^+$ B cells were shown to produce essentially IgM, IgG$_1$ and IgG$_3$ and to a lesser extent IgA[4,5]. Interestingly, DC amplified IL-10-induced B cell differentiation. IgM production increased from 35 to 55 µg/ml, IgG production from 3 to 9 µg/ml and IgA production from 0,8 to 15 µg/ml (figure). Even if IgG$_1$ and IgG$_3$ levels were higher, addition of DC did not modify qualitatively the IgG subclasses produced (data not shown). However, when DC were added, CD40-activated sIgD$^+$ B cells secreted more IgA than IgG, thus reversing the IgG:IgA ratio. This indicate that DC may play a major role in induction of IgA secretion.

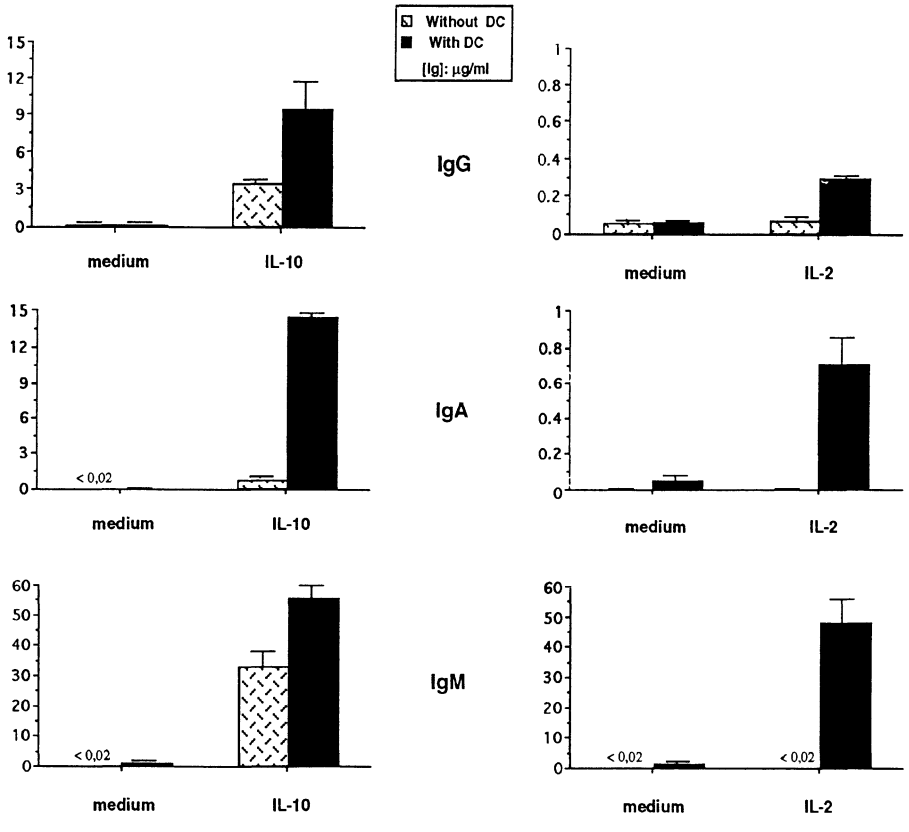

Figure: DC enhance Ig production by sIgD$^+$ B cells in response to IL-2 and IL-10. 10^4 DC, 10^4 B cells and $2x10^3$ irradiated CD40-Ligand-transfected L cells were co-cultured with 20 U/ml IL-2 or 200 ng/ml IL-10. Supernatants were harvested after 12 days and Ig levels were measured by ELISA.

2- DC induce CD40-activated sIgD$^+$ B cells to secrete IgM in response to IL-2

CD40-activated B cells do not mount increased proliferation in response to IL-2. However, further addition of DC results in enhanced B cell proliferation (data not shown) and most stikingly in high IgM secretion. Levels IgM obtained in response to IL-2 were comparable to those obtained with IL-10. Low but significant and reproducible levels of IgA (0,7 µg/ml) and IgG (0,3 µg/ml) were obtained under these co-cultures conditions.

DISCUSSION

Addition of DC to CD40-activated sIgD$^+$ B cells cultured in the presence of IL-2 or IL-10 resulted in the production of higher levels of IgA than IgG. This is reminiscent of responses occuring in mucosal-associated tissues. Our data confirm previous studies in mice which demonstrated that DC could induce a switch of B cells towards IgA secretion in the presence of activated T cells[6]. In current models, antigen presenting cells initiate immune response by activating T cells which stimulate B cells to control humoral response. However, the replacement of T cells by CD40-Ligand transfected L cells and cytokines show that direct interactions may occur between human DC and B lymphocytes which result in proliferation and Ig production by B cells[7, this report].
Taken together, these results suggest a major role of DC in the cytokine-induced B cell response which occurs *in vivo* during the primary immune reaction within extra-follicular areas of secondary lymphoid organs.

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FACTORS AFFECTING SPONTANEOUS DENDRITIC CELL-LYMPHOCYTE CLUSTERING IN SKIN AFFERENT LYMPH

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INTRODUCTION

Dendritic cells (DC) are essential accessory cells for the growth of T lymphocytes and there are evidences that DC start to migrate to regional lymph nodes after contact with antigen. Skin afferent lymph freshly drawn from lymphatics contains DC and lymphocytes (LY), and 3-6% of DC form clusters with LY [1]. Direct contact between these cells seems to be an integral part of their interaction in vivo. Clustering is the first phase of antigen presentation to LY and modulation of lymph cell cooperation may prove useful in mitigating skin immune response. In this study we demonstrate factors affecting the "spontaneous" binding of DC with autologous LY in their own environment, that is the lymph.

MATERIAL AND METHODS

Dogs

Outbred dogs with chronic lymphedema after surgical interruption of hind limb afferent lymphatics served as lymph donors [2].

Collection of lymph

Lymph was obtained by direct percutaneous puncture of dilated lymphatic and collected into tubes with heparin solution (10 U/ml). The percent of cells with DC morphology was 6.5 ± 2.6 and of lymphocytes 82.7 ± 9.2 ($CD4^+$ 58.4 ± 2.5% and $CD8^+$ 8.7 ± 4.6%). Cell concentration was $4.4 \pm 3.7 \times 10^6$/ml.

DC-LY binding assay

Binding of cells was quantitated in 0.2 ml samples after 4h incubation in lymph

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mixed 1:1 with 0.15 M NaCl (control) or with appriopriate reagents at 37°C. The number of DC with two or more lymphocytes attached per 100 of DC was counted under light microscope.

Reagent solutions

Recombinant human IL1 α and IL1 ß (Genzyme) were prepared in 0.15 M NaCl and used in final concentration 1000 pg/ml and 100 pg/ml, respectively. Polyclonal rabbit anti-IL1 ß antibody (Genzyme) was used in concentration 100 μg/ml. Recombinant human IL1ra (receptor antagonist) and anti-IL1ra neutralizing antibody (R&D Systems) were used in concentration 400 ng/ml and 10 μg/ml, respectively. The following mouse anti-human (cross-reactive with dog) antibodies were used: anti-CD1a, -CD18, -DR (Dakopatts); anti-CD54, -CD49 d,e,f (Immunotech) and anti- CD58 (Becton Dickinson). The following polyclonal rabbit antibodies (Dakopatts) were used: anti-fibronectin (FBN) and anti-S100 protein. Antibodies were incubated with lymph cell pellets for 30 min at 4°C and then for 4h at 37°C in presence of 50% lymph. The doses of reagents were chosen depending on the peak of their effect. Monoclonal antibodies against canine CD4 and CD8 were rat IgG (kind gift of dr S.Cobbold, Cambridge,UK).

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

RESULTS

We observed the lower lymph cell binding in the presence of 100% lymph as compared to lymph diluted 1:1 with saline (Table 1). When exogenous IL1 ß was added the enhanced cell clustering was seen, and that effect was abrogated by treatment with anti-IL1 ß antibodies or IL1ra. Exogenous IL1 α alone as well as IL1ra had no effect, however IL1ra neutralizing antibody showed enhancing effect on cell binding.

Table 1. The modulatory effect of IL1 on cell cluster formation (mean±SD, n=8). * $p<0.05$ vs control, # $p<0.05$ vs IL1ß

Reagents	Clusters (%)
Experiment 1	
control	29.6± 5.8
+ IL1ß	41.7± 9.4 *
+ anti-IL1ß	22.5± 4.4 *
+ anti-IL1ra	42.8± 9.0 *
Experiment 2	
control	19.5± 7.5
+ IL1α	22.5± 2.0
+ IL1ra	21.2± 6.2
+ IL1ß	30.2± 8.1 *
+ IL1ß + IL1ra	22.3± 6.2 #
100 % lymph	13.9± 5.2 *

Table 2. The effect of Abs on lymph cell clustering (mean±SD, n=8). * $p<0.05$ vs control

Abs against	Clusters (%)
control	22.6±5.2
CD54	17.3±6.6 *
CD58	11.1±2.9 *
CD1a	23.2±4.7
HLA DR	25.3±8.4
CD18	30.0±3.7 *
CD49d	30.5±3.1 *
CD49e	33.2±8.6 *
CD49f	29.7±4.7 *
FBN	14.5±1.9 *
S-100 protein	21.7±4.6

Spontaneous cell attachment in afferent lymph was reduced by monoclonal antibodies against CD54 and CD58 (Table 2). In contrast, antibodies against CD18 and CD49 (d,e,f) had the proaggregatory properties. Other control antibodies against CD1a, DR and S-100 protein had no effect on cluster formation. Rabbit anti- fibronectin antibodies had inhibitory activity (Table 2).

DISCUSSION

A network of cytokines mediates communication between cells of the immune system. These cytokines are active locally in the microenvironment of cells in tissues. In skin cytokines produced by keratinocytes and Langerhans cells play a dominant role. In previous studies we identified IL1 in normal human skin afferent lymph as well as IL1 inhibitor[3]. Koide et al.[4] observed a more effective DC clustering with allogenic lymphocytes in the presence of IL1 in MLR. Our present results indicate that IL1 ß rather than IL1 α is a candidate for such amplifying role. Imbalance in IL1 and IL1 inhibitor (IL1 receptor antagonist) levels in afferent lymph may be involved in regulating spontaneous cell clustering. It raises the possibility of regulation of skin immune responses with exogenous IL1ra.

Both CD54 and CD58 adhesion molecules were directly effective in our system in spontaneous cell binding suggesting that both CD2-LFA3 and ICAM1-LFA1 pathways were involved in cell attachment. Antibodies against CD18 and CD49 were found proaggregatory for cells. Accelerated attachment of T cells with DC after using of anti- CD18, -CD44, or -CD45 was observed by others[5,6]. In agreement with studies of Klingemann et al., [7] fibronectin was found another factor involved in cluster formation of the canine lymph DC with lymphocytes. Further studies concerning the effect of soluble IL1 receptor and other cytokines present in normal afferent lymph on cell clustering are carried out.

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CHARACTERISTICS OF ANTIGEN-INDEPENDENT AND ANTIGEN-DEPENDENT INTERACTION OF DENDRITIC CELLS WITH CD4+ T CELLS

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Dendritic cells (DC) are the main antigen-presenting cells for the initiation of primary T cell-mediated immune responses. In the first stage of activation, T cells bind to DC in an antigen-independent manner. We analysed the contribution of adhesion molecules to interactions between resting and alloantigen-activated CD4+ T cells and dendritic cells. Human dendritic/Langerhans cells were generated by 12 or 13 days of culture of CD34+ haematopoietic progenitors from bone marrow or cord blood in the presence of GM-CSF and TNF-α, according to the technique of Caux et al.[1]. These cells had the morphology, phenotype and functions of dendritic cells. They were 70% pure (CD1a, CD40+, HLA class II+, CD14-), had no detectable phagocytic activity and had a strong stimulatory effect on allogeneic CD4+ T cells. Interactions of CD4+ T lymphocytes to DC were assessed in adhesion assays by means of fluorescence microscopy and flow cytometry and primary mixed leukocyte reactions.

We found that at least four pathways (LFA-1/ICAM-1,3, ICAM-3/LFA-1, CD2/LFA-3 and CD28/CD80) were involved in the adhesion between dendritic cells

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and CD4+ T lymphocytes in antigen-independent and alloantigen-dependent situations, as shown by blocking experiments using monoclonal antibodies, preincubated with each population. The antibodies also blocked a primary mixed lymphocyte reaction (MLR) in which DC were used as stimulatory cells. The LFA-1 ligands involved in adhesion of resting CD4+ T cells to DC and alloreactive CD4+ T cells to specific DC differed in part, since ICAM-3 on resting CD4+ T lymphocytes and ICAM-1 on alloreactive T lymphocytes preferentially bound LFA-1. On dendritic cells, ICAMs 1, 2 and 3 interacted indiscriminately with LFA-1 on alloreactive T cells, whereas only ICAM-1 and ICAM-3 were involved in the adhesion to resting CD4+ T lymphocytes. De Fougerolles et al.[2] have demonstrated that binding of resting CD4+ T cells to purified LFA-1 is almost completely ICAM-3 dependent, whereas after mitogenic activation with PHA, adhesion occurs chiefly through ICAM-1 (surface expression of which increases) and to a lesser degree, through ICAM-3. Our data also show functional differences in the three ICAMs as regards the adhesion of resting and activated T lymphocytes to LFA-1 expressed on dendritic cells.

Adhesion of resting CD4+ T cells and alloreactive CD4+ T lymphocytes to antigen-presenting DC was transient, but maximal adhesion between alloreactive T cells and DC occured earlier (after 20 minutes whereas 5 minutes for resting CD4+ T cells) and was stronger, pointing to the involvement of an antigen-specific process upregulating adhesion.

Since interaction between transiently expressed CD40L on activated CD4+ T cells and CD40 on DC has been shown to play a role in mutual signaling leading to upregulation of B7 molecule expression and increased LFA-1-mediated adhesion[3,4], we assessed the role of this receptor/ligand interaction in the adhesion of PMA/ionomycin-activated T cells to DC. Reagents interacting with CD40L and CD40 partially blocked this adhesion. Since the inhibitory effects were additive with blockade of the main adhesion pathways, these results point to an adhesion mechanism mediated by CD4OL/CD40, although they do not rule out an additional regulatory mechanism. The role of the CD40/CD40 ligand activation pathway remains to be determined in more physiological conditions of cell activation.

We also found that anti-CD4 antibodies inhibited the adhesion of dendritic cells to resting, alloreactive and PMA-activated T cells. Inhibition occurred regardless of which cell type was preincubated with the antibody, and could be explained either by blockade of an adhesion force mediated by CD4/HLA class II interaction, or rather by a CD4-mediated regulatory inhibition of adhesion, since resting CD4+ T cells do not express MHC class II molecules[5]. Inhibitory activity of HLA class II-specific antibodies was detected when they were added to DC but not to resting, PMA-activated or alloreactive T cells, even though HLA class II molecules were expressed on the alloreactive CD4+ T cells. CD4 expressed on dendritic cells, by binding to HLA class II

molecules or anti-CD4 mAb, might thus induce negative regulation of adhesion between dendritic cells and resting, alloreactive or PMA-activated T cells. It has been reported that adhesion of resting and activated T cells to B cells can be negatively regulated by CD4, since the kinetics of CD4+ T cell binding to HLA class II+ cells was transient, whereas binding to HLA class II- cells was stable, and since putative ligands of CD4 inhibited this binding[5] . The transient nature of dendritic cell adhesion to T cells could also in part be accounted for by a negative regulatory role of CD4. Other studies have also provided evidence for a role of CD4 in conjugate dissociation of both resting and activated T cells[6,7]. Interaction between CD4 and its ligands can have negative effects on T cell adhesion and, probably, T cell activation[8,9] provided that CD4 is not functionally associated with the TcR/CD3 complex. In contrast, in the context of antigenic peptide-MHC class II-specific recognition, CD4 induces a positive signal[10,11]. Our results suggest that, in addition to negative regulation of low-affinity antigen-independent adhesion of resting T cells to DC, CD4/HLA class II interactions may also partially down-regulate high-affinity T cell adhesion in the antigen-dependent context. Anti-CD4 antibodies could inhibit up-regulation of adhesion by dissociating the TcR/CD3/CD4 complex.

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PRIMING OF T CELLS WITH DENDRITIC, MACROPHAGE AND B CELL LINES IN VIVO REQUIRES MORE THAN SURFACE EXPRESSION OF MHC II AND B7 MOLECULES - POSSIBLE ROLE OF CD44 AND INTEGRINS

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INTRODUCTION

Dendritic cells (DC) are the most effective cell type in presenting peptides associated with MHC I and II molecules to T cells (1). It was shown that freshly isolated DC and activated B cells are able to prime T cells in vitro and in vivo, in contrast to resting B cells and macrophages (2,3,9). Flamand et al. (4) injected DC's pulsed with tumor antigen in mice and achieved protection from tumor growth. However, experiments with freshly isolated DC's are restricted to the large amounts of tissue necessary to obtain sufficient numbers of cells in high purity.

In our laboratory a method was established to immortalize specifically cells from the phagocyte/dendritic cell lineage. This was enabled by the MIBΨ2-N11 retroviral vector introducing an env^{AKR}-myc^{MH2} fusion gene into the infected cells (5). We previously showed that one of the immortalized cell clones, named CB1, display the phenotypic and functional properties of an immature dendritic cell (6). In the course of studying the use of immortalized or transformed cell lines in antigen presentation, we tried to prime T cells for MHC II restricted antigens in vivo. Three cell lines all derived from BALB/c mice were used: the MIBΨ2-N11-immortalized D2SC/1 dendritic cell clone (7), that exhibit a more mature phenotype than CB1, the J774 macrophage (ATCC TIB 67, spontaneous tumor) and the A20 B cell lymphoma (ATCC TIB 208, spontaneous reticulum cell neoplasm). The A20 and J774 were chosen for our in vivo priming experiments because of their constitutively high expression of MHC II and B7 molecules. Therefore the cells have the prerequisites for

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optimal T cell activation as they can provide both the antigen specific signal (MHC/peptide complex) and the accessory signal (B7 molecules) (for review see 8).

We compared the surface phenotype of the three different cell lines and their ability to perform MHC class II restricted antigen priming in vivo. The additional requirement of receptors for homing of antigen presenting cells to the lymph nodes is discussed.

RESULTS

The D2SC/1, J774 and A20 cell lines were loaded with chicken ovalbumine protein over night, injected subcutaneously and the draining lymph nodes were removed after 9 days. Antigen specific, dose-dependent proliferation of lymph node cells was observed with the D2SC/1 and J774 cells, but not with the A20 (Fig. 1). For the proliferation assay, culture medium without FCS but supplemented with 1% normal mouse serum (NMS) was used, to exclude priming against FCS proteins which often occurs with DC. Moreover, the use of NMS in the proliferation assay did not allow the growth in vitro of the injected cells. All cell lines, in fact, showed little proliferation without FCS.

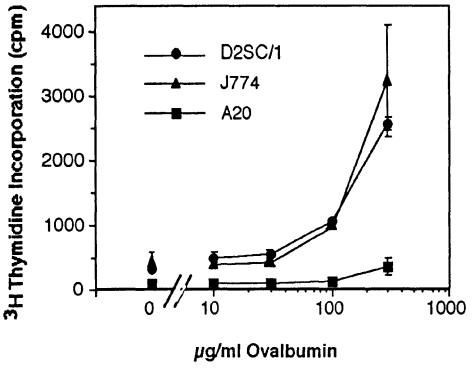

Figure 1 ***In vivo priming***

D2SC/1, J774 and A20 cells were cultured 16 h with 10µg/ml Ovalbumine in suspension culture dishes to detach them after incubation just by pipetting. Cells ($2,5x10^5$cells/50µl PBS) were injected into each hind footpads subcutaneously. Two mice per group were used. After 9 days the popliteal lymph nodes were removed, and from the single cell suspensions $1x10^5$ were cells plated into a 96-well flat bottom culture plate. Culture medium was IMDM (SIGMA) with 1% normal mouse serum. At day 4 the cells were pulsed with 1 µCi/well [^{3}H]-Thymidine (Amersham, 2Ci/mmol) for 16 h.

In order to understand their different priming capacities, the cell lines were examined for their surface expression of several markers by flowcytometry (Fig. 2). The expression of MHC class II molecules was magnitudes higher on the A20 B cell line (mean fluorescence intensity (MFI): 2123 for I-A, 939 for I-E) than on the J774 macrophage (MFI: 56 for I-A, 33 for I-E) or the dendritic D2SC/1 (MFI: 32 for I-A, 34 for I-E). Furthermore, all cell lines express the costimulatory receptor B7, as assessed by binding of CTLA4-Ig (A20: MFI 47.7, J774: 65.7 and D2SC/1: 78.6; background substracted). The expression of all other markers tested is approximately the same for the D2SC/1 and J774 cells, whereas the A20 cell line is lacking the F4/80, CD44, CD11b and CD11c molecules.

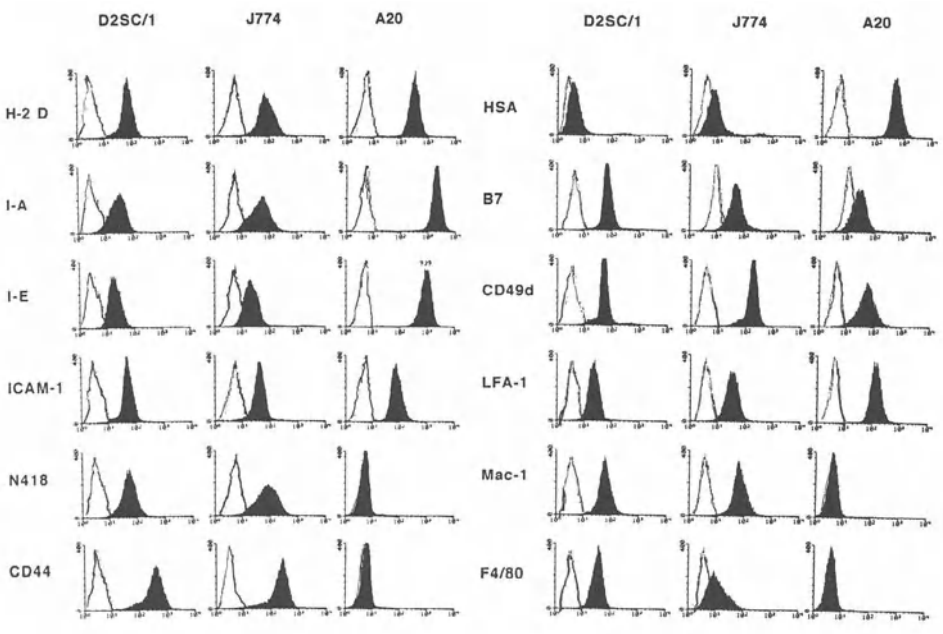

Figure 2 ***Surface Phenotype***

Y-axis represent the cell number, x-axis the relative fluorescence intensity. Open histograms show staining without primary antibody, closed histograms staining for the indicated markers.
For immunostaining biotinylated monoclonal antibodies against the following surface makers were used: H-2 D^d, CD54 (ICAM-1), CD11b (Mac-1), CD44, CD11a (LFA-1), B220, all purchased biotinylated from Pharmingen), HSA (J11D, supernatant), CD49d (α4-Integrin chain, Southern Biotechnology), B7 (CTLA4-Ig fusion protein, P. Lane, Basel), F4/80 (Serotec), I-E and I-A (14-4-4S resp. MKD6, R. MacDonald, Epalinges), CD11c (N418, J.C. Guèry, Milano),. Second step reagents were: Streptavidin/ Phycoerythrin (SIGMA), Goat-anti-rat IgM/FITC (Pharmingen), Goat-anti-human IgG/FITC (Janssen).
Surface staining of the cells followed standard procedures. Briefly $5x10^5$ cells were incubated with 50μl 2.4G2 supernatant (B.A. Kyewski, Heidelberg) to block unspecific binding to Fc-receptors. The cells were then stained with primary antibodies, washed and incubated with the secondary reagents (all steps 30 min on ice).

DISCUSSION

In this study, we compared three different antigen presenting cell lines in their capacity to induce in vivo priming and in their surface phenotype. The retrovirally immortalized dendritic D2SC/1 and the spontaneous macrophage tumor cell J774 could sensitize T cells specifically for ovalbumine. The priming capacity of the J774 cells was unexpected, because macrophages should not be able to prime T cells. We suspect that this tumor cell line underwent certain changes compared to freshly isolated macrophages that enables it to present antigen as efficiently as a DC but the surface phenotype of D2SC/1 and J774 cells is similar. We do not know yet the nature of these changes.

In spite of the higher expression of MHC II molecules and similar amounts of B7, the B cell lymphoma A20 was not able to prime the mice. This is in agreement with data from Ronchese and Hausmann (9), that reported B lymphocytes to fail to prime T cells in vivo. However, Croft et al. (3) had shown that freshly isolated and activated B cells can prime naive T cells in vitro. Phenotipically, A20 cells represents a fairly activated B cell. Furthermore, they can present hen egg lysozyme (HEL) to a specific T hybridoma with high efficiancy, but could not prime T cells for HEL in vivo (data not shown). The priming capacity in vitro however needs to be tested.

One explanation for the different priming capacities between dendritic, macrophage and B cells could reiside in the distinct homing receptor profile. The phenotypic differences that were detectable with our panel of antibodies were the lack of CD44, CD11c, CD11b and the F4/80 antigen on the A20 cells. Because of the involvement of CD44 and integrins in cell homing, the lack of these molecules could render A20 cells to adequately home to the lymph nodes. All three cell lines could reach the draining lymph node, as detected by outgrowth of the cells after culturing lymph node cells in medium with 5% FCS, but the A20 cells might be trapped in B cell areas and not reach the appropriate T cell compartements in the lymph node. This issue is under active investigation.

In summary, our data indicate that cell lines can be used to study some aspects of antigen presentation in vivo. T cell priming in vivo by APC requires appropriate expression of homing receptors for T cell areas of lymphoid organ, other than MHC and B7 molecules. Candidates for such molecules could be certain isoforms or glycoforms of CD44 and/or certain integrin molecules like CD11b and CD11c.

ACKNOWLEDGEMENTS

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IDENTIFICATION OF NOVEL cDNAS DERIVED FROM HUMAN LUNG DENDRITIC CELLS USING A SUBTRACTIVE CLONING APPROACH

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INTRODUCTION

Dendritic cell populations in lung (LDCs) play an important role in the development of primary immune responses to inhaled antigen[1]. Dendritic cells process incoming antigen, migrate to T cell areas of associated lymphoid tissue where they present antigen to T cells, and provide all the known costimulatory signals necessary for T cell activation[2]. In order to identify novel proteins which may be important for dendritic cell function, we prepared a cDNA library from human lung dendritic cells. The library was screened by differential hybridization to three different probes: an LDC cDNA probe; an LDC cDNA probe subtracted with pulmonary alveolar macrophage (PAM) mRNA and a PAM cDNA probe. Large scale partial sequencing of clones which hybridized to the subtracted probe was used to identify cDNAs which exhibited interesting sequence homologies on database searching with both nucleotide and protein translations. A number of cDNAs were then examined further by Northern blot analysis and *in situ* hybridization to human lung tissue and cytospins of lung dendritic cells. Our preliminary findings are described in this report.

METHODS

Preparation of a human lung dendritic cell and pulmonary alveolar macrophage cDNA libraries

Lung dendritic cells were isolated from resected human lung parenchyma as described previously[3]. Total RNA was isolated from approximately 3.7 x 10^5 FACS purified dendritic cells and used to prepare an oligodT-primed cDNA library in the plasmid vector pcDNA1 (Invitrogen). The resultant library contained approximately 10^5 independent clones with insert sizes ranging between 0.2- >2.5 kb. Total RNA was isolated from PAMs purified from BAL fluid. Starting from 50 μg of total RNA, the resultant oligo dT-primed cDNA library in pCDNA1 contained 5 x 10^5 independent clones with insert sizes ranging between 0.2 - >3.0 kb.

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cDNA library screening

Approximately 20000 clones from the LDC library were plated in duplicate on nitrocellulose filters (approximately 2000 colonies/filter) and screened sequentially with the following probes: (a) [32P]- labelled PAM cDNA probe derived from of 24 μg of total RNA using AMV reverse transcriptase (Life Sciences) (b) [32P]-labelled LDC cDNA probe derived by reverse transcription of total RNA from 2.5 x 10^5 FACS purified LDCs and (c) LDC cDNA subtracted with biotinylated macrophage mRNA generated from the PAM cDNA library by *in vitro* transcription, with SP6 and T7 RNA polymerases (Promega) using the method of Swaroop et al.[4]. For all screenings, probes were used at a specific activity of 10^5 cpm/ml. Only colonies which hybridised to both DC probes (subtracted and non-subtracted) or which were strongly positive with DC probes compared to macrophage probe were selected. The resultant positive colonies were then rescreened with a new macrophage cDNA probe to eliminate false positives.

Analysis of LDC specific clones

PCR analysis using SP6 and T7 primers was used to determine the insert size of selected clones and to verify that each pick contained only a single clone, prior to sequencing. Magic mini-prep DNA (Promega) was isolated from single clones which contained inserts greater than 0.4 kb in size and was sequenced bidirectionally with SP6 and T7 sequencing primers using Sequenase™ (USB). Sequences obtained were analysed using the FASTA program to search the GenBank/EMBL/DDBJ databases for homologous sequences. Following sequence analysis, a number of clones were further studied by Northern blot analysis and *in situ* hybridization.

RESULTS AND DISCUSSION

In an attempt to identify novel dendritic cell clones which may be important in DC function, we have constructed a human lung dendritic cell DNA library from FACS purified dendritic cells. Due to the limiting amount of material available, we used oligodT-primed total RNA for cDNA synthesis. The resultant library contained 100000 independent clones. Primary screening of approximately 20000 LDC clones by differential hybridization yielded 518 individual clones which were positive with both the subtracted and non-subtracted LDC cDNA probe and negative with the macrophage probe (+/+/- clones). On rescreening of (+/+/-) clones with a second macrophage cDNA probe (prepared from new total RNA) 326/518 were positive. The remaining macrophage probe negative clones (192) were then subjected to direct PCR analysis using SP6 and T7 vector primers prior to partial sequencing analysis, in order to eliminate clones which contained insert sizes less than 400 bp and thus minimise the sequencing of cDNAs which may correspond to 3 ' untranslated regions. Of 36 clones sequenced to date, 17 sequences (47%) were unwanted and include rRNAs, *Alu*I-like repeats, E.coli and yeast genes (subcloning artefacts) and vector sequences. The presence of rRNA clones probably

Table 1. Northern blot analysis of novel LDC clones

Cell type	9A11	9D11	7A5	8D6	8D4
A549 cells	+	-	-	-	-
A549 + TNFα	+	-	-	-	-
lung fibroblasts	+	-	-	-	-
total lung	+	-	-	-	+
lung lymphocytes	+	-	-	-	+
lung tissue macrophage	+	-	-	-	+
EOL-3 cell line	-	-	-	-	-
PMNs	-	-	N.D.	N.D.	N.D.
PBMCs	+	-	-	-	+
PB T cells	+	-	N.D.	N.D.	N.D.
endothelial cells	N.D.	-	-	-	-
Raji (B cell line)	N.D.	-	N.D.	N.D.	N.D.
HL-60 cells	+	-	-	-	+

Table 2. *In situ* hybridisation of novel LDC clone riboprobes to FACS sorted LDCs.

riboprobe	9A11	9D11	7A5	8D6	8D4
antisense	+	+	-	-	+
sense	-	-	-	-	-

reflects the use of total RNA as starting material for the library preparation. Ten sequences (28%) showed >70% sequence identity or were identical to previously cloned genes and interestingly included human cathepsin D, which is probably associated with antigen processing; mouse TCTEX-1, human myoblast cell surface antigen and human activin β - A subunit (also known as erythroid differentiation factor). The significance of the latter three genes is unknown. In addition we have identified 9 sequences (25%) which showed no significant homology with any primate or rodent gene sequence in the database; they presumably represent unknown genes. We studied the expression patterns of five of these cDNAs designated 9A11, 9D11, 7A5, 8A6 and 8D4 by Northern blot analysis, and by *in situ* hybridisation to cytospins of FACS purified LDCs, using SP6 and T7 RNA polymerase-generated riboprobes. Results are summarised in tables 1 and 2 respectively.

Clone 8D4 detected an approximately 2.6 kb cDNA in lung tissue, lung macrophages and lung lymphocytes in addition to PBMCs, and was also positive in DCs on cytospins. Clone 9A11 detected a 2.2 kb mRNA in most of the cell types tested, including dendritic cells. These two clones do not appear to be dendritic cell specific. This may be due to the presence of contaminating cell types (lymphocyte, monocyte, macrophage) in the dendritic cell population used to prepare the cDNA library and probes. We were unable to detect a signal on northern blots using 9D11, 7A5 and 8D6 as probes, suggesting that the corresponding mRNAs may be of low abundance. However, 9D11 mRNA was detected in DCs on cytospins suggesting that this may indeed be a dendritic cell specific gene. Further experiments on 9D11 will include complete sequence determination, analysis of tissue distribution and demonstration of function using antibodies raised to the expressed protein.

In conclusion, such a differential screening process should be useful in the identification of dendritic cell specific mRNAs, when combined with Northern blot analysis and *in situ* hybridisation. However in future experiments, it will be necessary to use rigorously purified dendritic cells in both the library construction and screening stages to avoid false positives. To this end, we have now developed a FACS procedure for the isolation of dendritic cells from human lung at purities of up to 95% (see J-P. Aubry et al., this volume).

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MECHANISMS OF RETROVIRALLY INDUCED IMMUNOSUPPRESSION ACTING VIA DENDRITIC CELLS

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INTRODUCTION

We have proposed that a major mechanism producing immunological abnormalities in HIV-1 infection is the infection and dysfunction of dendritic cells (DC). This article reviews evidence for this from our laboratory and discusses possible mechanisms by which infection of DC may influence the development of immunological abnormalities.

The susceptibility of human DC to infection with human immunodeficiency virus type 1 (HIV-1) was first described from studies of *in vitro* infection of peripheral blood DC (1) and of skin Langerhans' cells (2). Since that time a number of papers have demonstrated that HIV infection of DC also occurs *in vivo* (3 for references). Productive infection of DC was only seen in cells of a mature phenotype which represent only a small proportion of peripheral blood DC (4) and differences in the numbers of mature DC obtained during different isolation procedures may account for the failure of some authors to see significant infection of peripheral blood DC (5). Observations that human DC can also become infected with HTLV-1 (6) and that mouse DC are targets for *in vivo* and *in vitro* infection with Rauscher leukaemia virus (RLV) (7) suggest that DC infection is a common feature of retroviral infections.

Dendritic Cell Infection with HIV-1 and T Cell Function

In HIV seropositive individuals who are asymptomatic our studies have shown an abnormality in a function of DC in the presence of normally functioning T cells and macrophages (Table 1; 8,9). However, DC were fuelling antibody production in cultures contaning B and T cells (10).

Two ways in which infection of DC could influence the proliferation of T cells were identified (8). Firstly, DC infected with HIV-1 were cultured with T cells *in vitro* and infection of some T cells in cultures could be observed. This indicated

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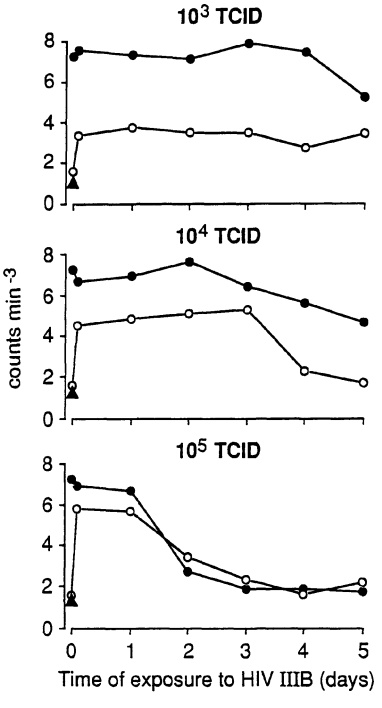

Figure 1.

Dendritic cells from normal human blood were exposed to increasing doses of HIV-1 *in vitro* for between two hours and 5 days before being used to stimulate T cell proliferation in 20 µl hanging drop cultures (12). Stimulation of normal allogeneic cells (filled circles) in a mixed leukocyte reaction or of autologous T cells (open circles) to show the stimulation of response to the HIV-1 itself are shown.

that transfer of virus to the T cells and subsequent damage to the infected responder cells could be occurring during the clustering and activation of T cells. Secondly, in the absence of T cell infection infected DC also failed to stimulate T cell activity. This suggested that a second mechanism preventing T cell activation could be operating via DC.

These two different possibilities may best be discussed in relation to the effects of different doses of HIV-1 on T cell stimulation shown in Figure 1. DC exposed to low doses of HIV-1 *in vitro* or for short periods of time were able to stimulate normal allogeneic mixed leukocyte reactions. They also stimulated a primary proliferative response to the virus itself. Under these circumstances where there are large numbers of activated T cells infection of T cells in cultures is liable to be a predominant feature. High levels of T cell infection in cultures of this type have been described (5). However, exposure to higher doses of virus or to longer exposure times resulted in a loss of the capacity of DC to stimulate either allogeneic mixed leukocyte reactions or autologous T cell stimulation. Under these circumstances in the presence of low levels of T cell activity there was only a low number of T cells infected with the virus during the culture (8). The important question is the extent to which these two possibilities contribute to *in vivo* effects of HIV-1. In extensive studies on the function of DC taken from patients, most individuals with asymptomatic infection showed some loss of stimulatory capacity of T cells by DC. The defect in T cell stimulation was shown to reflect the block in function of DC rather any effect of the responding T cells. This was demonstrated by the observation that T cells responded well to allogeneic DC in the mixed leukocyte reaction. Normal recall responses to influenza and to tetanus toxoid were also seen in T cells from these patients when macrophages were used to stimulate the responses. Thus this work indicated that *in vivo* there was a specific defect in antigen presentation by DC while T cell and macrophage function remain normal in early HIV infection (Table 1).

Table 1.

CELLULAR FUNCTION
HIV+/Asymptomatic

Cells	Test	Function
'Naive' T	MLR	Normal (9)
'Memory' T	Recall Responses (FLU/TETANUS)	Normal (9)
Macrophages		Normal
Dendritic	MLR/AMLR	Low (9,15)
	Recall Responses	Low (9)
	Antibody to HIV-1	High (10)

It thus appears that lack of recruitment of T cells by DC may be a major feature in HIV-1 infection (9). More recent studies using the highly immunosuppressive retrovirus Rauscher leukaemia virus in mice has also indicated that similar mechanisms operate; infected DC can cause infection of T cells or can show defective stimulation of T cells in the absence of T cell infection.

Mechanism of the Dendritic Cell Defect in Retroviral Infection

Recent studies on Rauscher leukaemia virus in mice have suggested that specific modulation of certain cell surface receptors may underlie some of the defects in DC function during infection (7. Down-regulation of surface expression of ICAM-1 and PGP-1 have been described on the DC taken from mice infected with Rauscher leukaemia virus. Additionally, down-regulation of class II molecules on the surface of DC has been observed. Low expression of class II MHC molecules was reported on skin Langerhans' cells in patients with HIV-1 infection (13) and a loss of class II bearing low density cells reported in peripheral blood (14). Thus a loss in this molecule essential to the antigen presenting function of DC could be important as a mechanism in the block of DC function.

Conclusions

Work from our laboratory has demonstrated that DC are susceptible to infection with a number of retroviruses including HIV-1 and HTLV-1 in humans and Rauscher leukaemia virus in mice. Infected DC have been shown to cause infection of activated T cells which may provide one mechanism for T cell loss in HIV-1 infection. However, a major effect seen in both HIV-1 and Rauscher leukaemia virus infection may be a loss in the capacity of DC to recruit T cells into immune responses. Evidence suggests that a loss in MHC Class II molecules from the surface of DC during infection could underlie this block in T cell activation.

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PREFERENTIAL ENTRY AND PRODUCTIVE INFECTION OF CD4 EXPRESSING LYMPHOID DENDRITIC CELLS BY MACROPHAGE-TROPIC HIV-1

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Introduction

Although cells of the dendritic cell [DC] lineage have been found in most tissues, only Langerhans cells and blood DCs have been examined for HIV-1 infection *in vivo* and *in vitro*. Reported levels of infection of blood DCs *in vivo* have ranged from high level infection with up to 20% of DCs infected[1] to virtually undetectable in patients at similar stages of the disease[2, 3]. A wide range of infectibility has also been found for blood DCs *in vitro* [see Cameron et al[4] for review]. The differences may be at least partially attributed to the different isolation methods used and resultant phenotypic differences in the DC populations studied. Some studies have used DC-enriched populations that contain only 30-75% DCs. In our hands, isolation methods that do not include specific depletion and cell sorting produce populations with unacceptable contamination with other cells including T cells. Dendritic cells are potent APCs and contamination with small numbers of T cells may assume a much greater significance than with less efficient APCs. DCs may induce the expansion of residual T cells and may be able to signal memory T cells to become productively infected without antigen recognition or overt stimulation and detectable proliferation[5]. For studies of DCs it is therefore critical to remove contaminating cells that may interact with the DCs, particularly when long term culture and p24 assays are used.

Differences in the phenotype of the DCs may also determine if HIV-1 will bind and enter the DCs and whether virus that does enter the DCs will induce a productive infection. Cultured blood DCs, as used in most of the studies of *in vitro* infection of DCs, express little if any CD4[6, 7]. Fresh DCs have been shown to express high levels of CD4[7] and DCs from other sites may also express moderate to high levels of CD4 *in vivo* and after isolation. Freshly isolated thymic DCs were recently shown to express high levels of CD4[8, 9] and tonsil DCs express CD4 after culture for 1-2 days in FCS-containing medium[10]. Constitutive high level expression of CD4 on these cells may render them susceptible to HIV-1 entry. We also considered that the thymic DCs might allow entry by T-cell-line-tropic isolates such as IIIb since recent data from the mouse suggest the thymic DCs arise within the thymus from a precursor common to both DCs and T cells[11].

HIV-1 infection of CD4 expressing lymphoid DCs

The methods we used for isolation of thymic DCs were as previously reported[8]. After removal of the capsule thymic tissue was disrupted by repeated aspiration through a transfer pipette during collagenase digestion. Low density cells were isolated over an isotonic metrizamide column [d=1.065] and the contaminating cells labelled with antibodies to CD14, CD19, CD20, CD2, CD3, and CD7 and removed by 2 rounds of depletion with magnetic beads. The DCs were

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labelled with directly conjugated HLA-DR. Thymic DCs that express high levels of HLA-DR and a second population with intermediate expression of HLA-DR were collected . This second population was composed predominantly of less mature DCs that, after culture, assumed a dendritic morphology and a similar immunophenotype to the mature, strongly HLA-DR$^+$ cells. Both populations were found to have similar levels and pattern of viral susceptibility.

Tonsil DCs were isolated using a similar enrichment and sorting strategy. Fresh tonsils were cut into small fragments after stripping off the epithelial layer and the tissue disrupted by repeated aspiration during collagenase digestion. Viable cells were isolated from a ficoll hypaque column and the cells cultured for 2 days in medium containing human serum. Low density cells were isolated over a metrizamide column [d=1.065], depleted of CD3, CD11b, CD16, CD3 and CD19 by panning and DCs sorted as large, cocktail negative, strongly HLA-DR$^+$ cells.

In contrast to blood DCs that rapidly lose CD4 expression during culture after T cell depletion, CD4 expression was maintained on tonsil DC after 48hrs and thymic DCs also continued to express CD4 after 24 hrs of *in vitro* culture. Cells were pulsed in microtitre plates with the IIIb and Ba-L isolates for 2 hours at 37°C. Virus stocks were treated with DNase I and filtered [0.2um]. Controls included cells permissive for the virus isolates. Macrophages cultured for 5-7 days before infection were used as targets permissive for the macrophage-tropic isolate Ba-L and the cell line CEM as the targets permissive for IIIb. After pulsing with virus the cells were washed and cultured in T-cell-conditioned medium for 24-36 hours. Cells were harvested and lysed as described [12]. Cell lysates from 25,000-50000 cells were amplified for the DQA genomic sequence and for gag in a semi-quantitative PCR[13]. Results of PCR on the thymic DCs were as shown in figure 1. The entry of Ba-L into the cultured macrophages was similar to the entry of this isolate into the thymic DCs but the entry of IIIb into the thymic DCs was much less than the entry of this virus into the permissive cell line CEM.

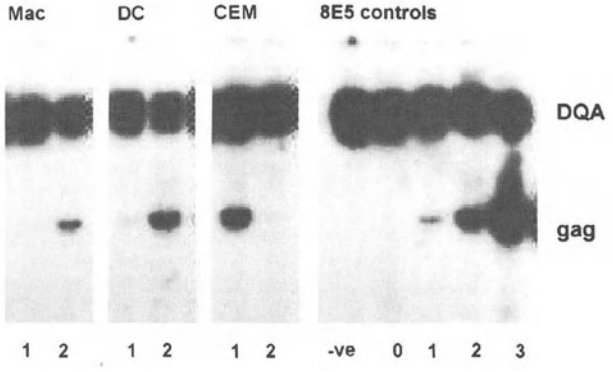

Figure 1 Entry of macrophage-tropic HIV-1 into thymic DCs is more efficient than entry of T-cell-line-tropic HIV-1. Cultured macrophages [Mac], thymic DCs [DC], and the T cell line CEM [CEM] were pulsed for 2 hours with Ba-L [lane 1] and IIIb [lane 2] and cultured for 36 hrs before harvesting DNA for PCR. DNA lysates corresponding to 25,000 cells were amplified for genomic sequence [DQA] and for HIV-1 gag [SK38, SK39 primers]. Controls were a dilution series of the 8E5 cell line that contains a single integrated copy of HIV-1 diluted in uninfected T cells. For the controls 3 = 1000, 2 = 100, 1 = 10, 0 = 1 copies of 8E5 per sample.

Productive infection of CD4$^+$ lymphoid DCs *in vitro*

DCs were examined for expression of viral antigens to determine if the virus that was able to enter the DCs and undergo reverse transcription was able to go on to produce viral antigens. DC-enriched populations were pulsed with virus and cultured for 5 days in T-cell-conditioned medium. Cytocentrifuge preparations of the cells were then fixed in methanol/acetone and examined for expression of HIV-1 p24 by immunoperoxidase labelling. Dendritic cells were found to express p24 in a cytoplasmic distribution in the cultures that had been pulsed with the macrophage-tropic isolate Ba-L [Figure 2] but not with the IIIb isolate.

Virus carriage and transfer from CD4$^+$ DCS to T cells

Virus pulsed DCs were added to allogeneic CD4$^+$ T cells to determine if the lymphoid DCs infected by HIV-1 could act as a reservoir of virus and infect T cells during cluster formation and expansion of antigen specific T cells . DCs were pulsed and washed in the same way as for PCR assays, and added to T cells. Virus reverse transcriptase [RT] was monitored in supernatants.

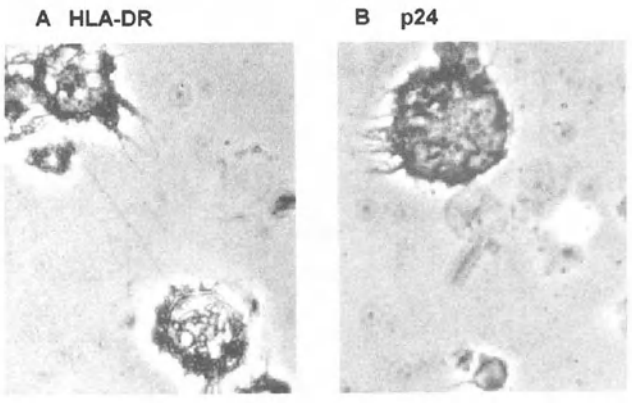

Figure 2 Ba-L infected thymic DCs are productively infected. By immunhistochemistry, 5 days after pulsing with Ba-L a small proportion [2-5%] of the thymic DCs expressed p24 antigen in a cytoplasmic distribution. Characteristic morphology and high level expression of HLA-DR was found on most of the cells [left panel]. The cells expresssing p24 [right panel] had a similar morphology. IIIb pulsed DCs did not express p24.

The CD4 expressing DCs were efficient in the transfer of virus to the expanding T cell population [Figure 3]. If the addition of the T cells to the virus pulsed DCs was delayed until the DCs had been cultured for 24 hours there was a reduction in the RT production but some virus transmission persisted. In 3 experiments we estimated the mean half life for virus associated with the DCs in these co-cultures to be 28.4 hrs for the macrophage-tropic isolate Ba-L and 14.5 hrs for the T-Cell-tropic isolated IIIb. The longer half life, reflecting prolonged carriage of the macrophage-tropic isolate that is able to enter and productively infect the DCs, does suggest that the infected DCs can act as a reservoir of virus for long term transmission to T cells. The rapid decline in virus carriage in these short term transfer experiments, however, argues that passive virus carriage, as we have shown for the $CD4^-$ blood DCs[14, 4], may be quantitatively more important than virus derived from replication in the DCs even when the virus is a macrophage-tropic virus and is able to infect the DCs.

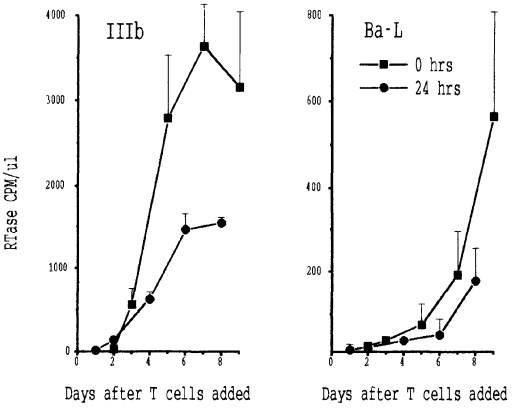

Figure 3 Both T-cell-line-tropic and macrophage-tropic HIV-1 is transmitted to $CD4^+$ T cells from virus pulsed DCs during an allogeneic response. Transmission occured when T cells were added directly after virus pulse [0 hrs] or if virus pulsed DCs were washed and cultured for 24 hours before adding T cells [24hrs]. Virus production was lower and slower for the macrophage-tropic virus [Ba-L] than for the T-Cell-tropic virus [IIIb]. Reduced transmission after 24hrs was found with both isolates but transmission from the 24 hr DCs pulsed with Ba-L was proportionately higher than for IIIb.

HIV-1 infection of DCs and the evolution and pathogenesis of AIDS

There seems little doubt that viral entry into dendritic cells including blood DCs can occur after *in vitro* exposure and that some DCs are infected *in vivo*. What has been contentious however is the extent of this infection and its significance for AIDS pathogenesis. Our previous study[2] supported the view that infection of DCs *in vivo* is much less frequent than the infection of $CD4^+$ T cells and is insufficient to account for DC dysfunction and loss of CD4 cells *in vivo*. We consider DCs integral to the pathogenesis of AIDS by two main mechanisms. DCs create a permissive environment for viral replication in antigen specific T cells by clustering T cells and inducing viral suceptibility in antigen specific T cells. Secondly DCs are able to efficiently take up virus and for a short time are able to re-present virions to the T cells entering the cluster.

Viral carriage and productive infection in DCs. We have previously shown that blood DCs isolated as pure populations by cell sorting are not readily susceptible to HIV-1 infection[12]. These cells express little if any CD4 but have been used at varying levels of purity to show that viral entry can occur[15-20] and that they are more susceptible to macrophage-tropic virus[20]. Methods of detecting infection have included viral p24 in supernatants of long term cultures. Residual T cells contaminating the preparations however may contribute to the infection. Infection in these DCs has rarely been quantitated relative to permissive cells such as mitogen-activated T cells, cultured macrophages or a permissive T cell line. It is therefore not clear how permissive the $CD4^-$ cultured DCs alone are for the different viral isolates.

In this study we have shown that entry of the T-cell-line-tropic isolate IIIb into $CD4^+$ DCs is inefficient and quantitatively similiar to the entry into cultured macrophages. We have also shown that virus pulsed DCs have an ability to transfer infection to T cells during the clustering of an immune response. This transmission is independent of the tropism of the virus isolate used. More prolonged or higher levels of carriage may occur with macrophage-tropic virus because of viral entry and replication in the DCs but the bulk of the virus carried by the DC is infectious virions. We made similar observations with IIIb and cultured blood DCs[14].

Several implications arise from a virus carriage model for the interaction of DCs and T cells in the presence of HIV-1. If DC carriage of virions in a trypsin resistant intracellular compartment[4] is the predominant source of the virus infecting T cells during an immune response then the viral load *in vivo* in the T cells will always be greater than in the DCs. T Cells will be both target and source of virus and the DCs will participate in the viral dissemination mainly by clustering and activating antigen specific T cells. The dissemination and cell-cell transmission of virus within clusters will not exhibit selectivity for either T-cell-line-tropic/ syncytia-inducing [SI] or macrophage-tropic/ non-syncytia-inducing [NSI] virus. The observation that AIDS and immune deficiency can occur with either NSI or SI isolates [21, 22] does suggest that the loss of antigen specific T cells can occur in the absence of virus able to efficiently infect either DCs or macrophages.

Discrepancies between observations made with sorted blood DCs and the work of Knight, Langhoff and others could be attributed to differences in the susceptibility of DCs when they are cultured as pure populations. DCs may only become susceptible to HIV-1 when they have been appropriately activated during isolation or culture. Several phenotypic markers on DCs have been found to change during culture with T cells[23], and virus production from DC enriched populations of epidermal cells is greatest in the conjugates of DCs and T cells and little if at all from the isolated T cells or DCs [5]. Our results using lymphoid DCs however indicate that sorted DCs are as susceptible as cultured macrophages to viral entry by Ba-L and do not require any interaction with T cells for efficient viral entry. Although NSI and SI may differ in requirements for activation it seems most likely that the increased viral replication in DC-T cell conjugates is due to changes in permissiveness of the T cells rather than the DCs in the clusters.

Viral tropism for DCs and selective infection by NSI . We have shown preferential entry into CD4 expressing DCs of the macrophage-tropic isolate Ba-L. This is similar to observations from another group[20] but the DCs used did not express CD4 by flow cytometry or RT-PCR. A non-CD4 dependent pathway for uptake was suggested by the lack of blocking with antibodies to CD4. Entry into $CD4^+$ lymphoid DCs is blocked by anti-CD4 antibodies or by sCD4. It is likely that the molecular mechanisms restricting the range of virus able to infect macrophages are also present on the DCs. Although the ontogeny of thymic DCs in the mouse suggests a more direct relationship to T cells the thymic DCs showed no greater susceptiblity to T-Cell-tropic virus than did the tonsil DCs.

It has been shown that the initial isolates of HIV-1, found during the viraemia of acute infection and early in the course of the disease have as part of their biological phenotype the ability to enter and productively infect macrophages[24]. Either a range of quasi-species are transmitted and there is subsequent preferential expansion of virus with the NSI biological phenotype or preferential passage of NSI virus occurs at the site of entry. Dendritic cells are present at the sites of viral entry where they take up antigen and actively migrate to the draining lymph node or splenic white pulp and interact with responding T cells. Few macrophages compared to DCs migrate from skin explants and it is likely that the ability to infect DCs rather than macrophages is important in restricting the virus crossing mucosa or skin. Our demonstration of restricted HIV-1 entry into DCs and the transient but variable carriage of all virus strains points to mechanisms that may mediate this restriction of viral phenotype. SI isolates infect DCs poorly and the DCs pulsed with such isolates lose their ability to infect T cells at a faster rate than DCs pulsed with macrophage-tropic virus. *In vivo* any virus taken up by the APC but unable to infect is likely to enter the acidic compartment and be degraded before the DC has migrated to the paracortical region of the lymph node and stimulated an immune response. An NSI virus that is able to infect the APC will be carried to the node without loss. NSI viruses may

be biologically important because they exhibit dendritic cell tropism rather than because of their macrophage tropism.

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EXPRESSION OF CYTOKINE mRNA BY HUMAN PERIPHERAL BLOOD DENDRITIC CELLS STIMULATED WITH HUMAN IMMUNODEFICIENCY VIRUS AND HERPES SIMPLEX VIRUS

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INTRODUCTION

Dendritic cells (DC) have been implicated in antigen presentation to naive and memory T lymphocytes and in pathogenesis of human immunodeficiency virus type 1 (HIV-1) infection. In order to define the mechanisms underlying the immunostimulatory capacities of DC in HIV-1 infection, it is critical to explore their ability to deliver the signals needed for T cell activation. We have demonstrated by using a bioassay that DC purified from human blood are the major producers of interferon-α (IFN-α) in response to HIV-1 and herpes simplex virus (HSV).[1] The present studies were undertaken to determine the ability of DC to express mRNA for IFN-α and other cytokines potentially involved in host immunity.

METHODS

$2x10^4$ to 10^5 DC were purified from blood by flow cytometric sorting[1] and stimulated with HIV-1 IIIb ($10^{7.5}$ $TCID_{50}$) or HSV-1 (10^5PFU) by incubation at 37^0C for optimal time periods. Total cellular RNA was isolated by using RNAzol (Biotecx, Houston, TX, USA). The RNA was reverse transcribed using M-MLV reverse transcriptase, oligo-dT (15 mer, Promega, Madison, WI, USA) and dNTPs in presence of RNAase inhibitor. The cDNA was amplified using cytokine-specific primers (Clontech, Palo Alto, CA, USA). The specificity of the products was tested by hybridization using internal oligonucleotide probes (Clontech).

In order to quantitate the amount of cytokines in DC stimulated with HIV-1, two methods, viz. cytokine-specific PCR mimics (Clontech) and internal standard RNA, were used. The mimic is a nonhomologous DNA fragment derived from V-erb β gene to which primer templates have been added. RNA equivalents of IFN-α and interleukin 6 (IL6) in DC stimulated with HIV-1 were quantitated using PCR mimics as specified by Clontech. After hybridization of the PCR products with ^{32}P labeled 5' primer, the ratio of radioactivity in the

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target band (782 bp) and the mimic band (596 bp) was calculated by measuring the intensity using ImageQuant software (Phosphorimager SF; Molecular Dynamics, Sunnyvale, CA, USA) and the concentration of the sample cDNA was calculated as suggested by Clontech.

Internal standard RNA for IFN-α was synthesized *in vitro* after deletion of PCR product by nested PCR using modified 3' primer and cloning into plasmid vector PDK101 (provided by David Rowe, Univ of Pittsburgh). A known concentration of this RNA was mixed with known cell equivalents of sample RNA before starting the RT reaction. PCR was performed on serial three fold dilutions of cDNA using the standard primer pair. After hybridization of the PCR products, the intensities of the two bands (782 bp and 600 bp) in the same lane were measured using the Phosphorimager and the concentration of sample RNA was extrapolated

RESULTS

PCR amplification and hybridization data for IFN-α confirmed our earlier observation that DC are potent producers of IFN-α in response to HIV-1 (data not shown) DC were able to synthesize mRNA for IL1-α, IL1-β, IL6, and tumor necrosis factor-α (TNF-α), when stimulated with HIV-1 (Fig 1, lane 2). DC also synthesized mRNA for IL4 and IL10 in response to HIV-1 as compared to mock stimulated DC (data not shown).

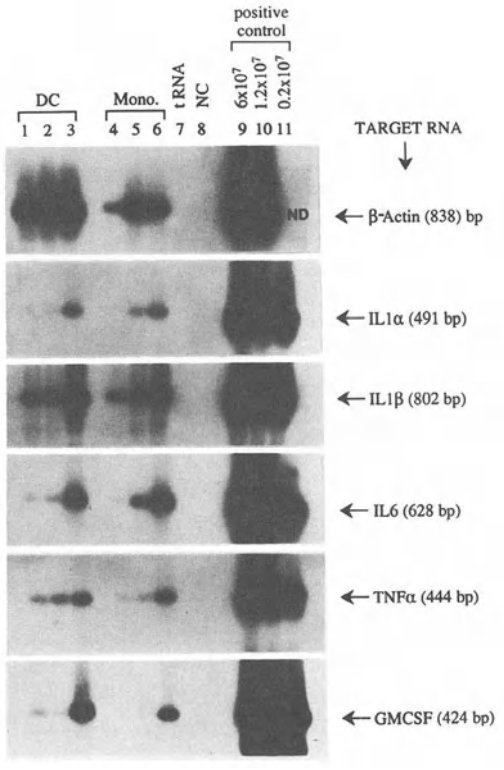

Figure 1. Synthesis of cytokine mRNA by normal human blood DC ($3\ 3x10^3$) and monocytes ($2\ 5x10^3$) stimulated for 4 hours, lanes 1 & 4 mock stimulated, lanes 2 & 5 HIV-1 stimulated, lanes 3 & 6 HSV-1 stimulated "ND" not done

DC synthesized RNA for granulocyte/monocyte colony stimulating factor (GMCSF) in addition to IL1-α, IL1-β, IL6 and tumor necrosis factor (TNF-α) in response to HSV (Fig.1, lane 3). Although the signals for all cytokines varied in their intensities, HSV was a better inducer of these cytokines in DC than was HIV-1. Despite endotoxin-free conditions, mock-stimulated DC occasionally showed the presence of mRNA for IL1-β, IL6, TNF-α, GMCSF and IL10. DC were not able to synthesize mRNA for IL2 when stimulated with PHA (data not shown). β-actin was used as an endogenous RNA control for each RNA sample.

Quantitation of IL6 in $1.3x10^3$ cell equivalents of DC RNA by PCR mimics could detect 10^5 RNA equivalents/10^6 DC stimulated with HIV-1. Quantitation of IFN-α in $5x10^3$ cell equivalents of DC using PCR mimics could detect 10^6 RNA equivalents/10^6 DC stimulated with HIV-1. As measured by using internal standard deleted RNA, however, $1.2x10^5$ molecules of IFN-α RNA/cell were detected in the same population of DC stimulated with HIV-1.

DISCUSSION

It is well established that cytokines play a major role as accessory molecules in T cell activation. These proteins may be crucial to the superior immunostimulatory function of DC in the antigen presentation pathway. In the present study using the RT-PCR technique we confirmed the presence of IFN-α mRNA in HIV-1 stimulated human blood DC. In addition, we now demonstrate that DC (>96% purity) were capable of synthesizing mRNA for IL1-α, IL1-β, IL4, IL6, IL10, TNF-α and GMCSF in response to stimulation with HIV-1 and/or HSV. It was observed that HSV was a better inducer of all these cytokines as compared to HIV-1.

Quantitation of IFN-α using DNA mimics underestimated the molecules of IFN-α mRNA as compared to that using internal standard deleted RNA, i.e., 1 RNA equivalent/DC as compared to $1.2x10^5$ molecules of IFN-α RNA/DC, respectively. The quantitation of various cytokines using internal standard RNA would facilitate studies of DC function.

The ability of DC to produce cytokines such as GMCSF may be important in autocrine regulation of their growth. Moreover, IFN-α may inhibit virus replication in DC, although the replication of viruses in DC is still ambiguous. Upregulation of IFN-γ by IFN-α produced by DC could be important in T helper-1 regulation of cytotoxic mechanisms against viruses and parasites.

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SPLENIC INTERDIGITATING DENDRITIC CELLS IN HUMANS: CHARACTERIZATION AND HIV INFECTION FREQUENCY *IN VIVO*

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In humans, dendritic cells have mainly been characterized from the blood and, as Langerhans cells, from skin samples [1]. Langerhans cells express the CD4 receptor and were found infected by HIV *in vivo* in several reports [2-4]. Peripheral blood dendritic cells express CD4 only as freshly isolated precursors and not as mature cells [5]. Reports on their infection by HIV *in vivo* and *in vitro* are controversial, probably because of the difficulty of obtaining pure populations [6-9]. However, as could be predicted from their major role in antigen presentation, it was shown *in vitro* that they could transmit the virus very efficiently to T lymphocytes during the course of a superantigen presentation [7]. Since antigen presentation to T cells occurs in secondary lymphoid organs, and since the highest numbers of HIV-infected cells in patients can be found in these organs [10-12], isolation methods derived from the published methods used for peripheral blood dendritic cells were adapted to the spleen.

ISOLATION AND CHARACTERIZATION OF HUMAN SPLENIC DENDRITIC CELLS

The experiment presented here is representative of several experiments performed with spleens from HIV-infected patients [13]. These spleens were obtained after splenectomy undertaken as a therapy for idiopathic thrombopenic purpura. After mechanical dissociation, mononuclear cells were separated by centrifugation over Diatrizoate-Ficoll. Results from a representative patient are shown in Table 1.

For direct isolation of the dendritic cells, mononuclear cells were successively depleted of T cells by erythrocyte rosetting, Fc receptor-positive cells by panning, and CD3, CD19, CD56-positive cells by immunomagnetic separation (adapted from ref. 5). Dendritic cells were analysed by flow cytometry. They expressed HLA-DR, but not other surface molecules specific for monocytes/ macrophages (CD14) which represented the major contaminating population, B lymphocytes (CD22), T lymphocytes ($TCR_{\alpha\beta}$) or NK cells (CD16). They were enriched to 8% in the resulting population (Table 1, 9% on average in a total of five spleens[13]). All dendritic cells expressed CD4, as shown by triple labelling.

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For the isolation of mature dendritic cells, a 36 hour culture step was performed (adapted from ref. 14). Non-adherent cultured cells were further depleted of adherent cells by two rounds of culture on plastic, and of Fc receptor-positive cells by panning. Low-density cells were isolated after centrifugation over metrizamide. The resulting population was enriched to 2% for mature dendritic cells as shown by morphology (not shown) and flow cytometry ($CD19^-$, $HLA\text{-}DQ^+$ medium-size cells, Table 1, 11% on average in a total of 5 spleens [13]). The main contaminating cells were B lymphocytes ($CD19^+$ $HLA\ DQ^+$). Moreover, with this isolation procedure, contaminating $CD4^+$ T lymphocytes were often found (2% in the isolation shown in Table 1).

INFECTION FREQUENCY OF SPLENIC DENDRITIC CELLS IN HIV^+ PATIENTS

Dendritic cells were further purified by fluorescence-activated cell sorting. They were diluted into non-infected Daudi cells, and each dilution was divided into ten replicates. The presence of HIV-1 envelope DNA was tested in each replicate by nested PCR using primers specific for conserved regions of the V1 and V2 loops. The method [13] was able to detect one infected cell in 10000 non-infected cells (data not shown). The proportion of infected cells in each purified cell population was determined using the Poisson law. Directly isolated dendritic cells were infected at a low frequency of 1/ 18000. Surprisingly, the mature dendritic cell population, which did not express CD4, seemed to be significantly more infected (1/5020). This apparent infection however could be related to the presence of contaminating $CD4^+$ T lymphocytes in the enriched population used for sorting, as well as in the control $CD19^+HLADQ^+$ population. Indeed, these control cells also seemed infected (1/17000), whereas $CD19^+$ B lymphocytes isolated directly from the same patient using immunomagnetic beads were clearly not infected (10/10 negative replicates at 100000 cells/replicate). $CD4^+$ T lymphocytes, isolated by immunomagnetic separation from the mononuclear splenocytes, were by far the most infected population (infection frequency: 1/67). Therefore, in this patient, splenic dendritic cells were infected at a frequency of 1/18000 and $CD4^+$ T lymphocytes were more infected than dendritic cells by a factor of 269. This experiment is representative of several experiments performed with spleens from HIV-infected patients [13], where dendritic cells were less infected than $CD4^+$T lymphocytes by a factor of 10 to 100 or more.

CONCLUSION

Dendritic cells enriched from human splenocytes have similar features to dendritic cells enriched from human blood [5, 14], in terms of their morphology and expression of many surface molecules. In the spleen of the HIV-infected patient presented here as well as in several other HIV^+ spleens [13], dendritic cells were less infected than $CD4^+$T lymphocytes by a factor of 10 to 100 or more. Therefore, dendritic cells from lymphoid organs do not seem to be a major source of HIV infection.

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Table 1. Infection frequency of splenic CD4 T lymphocytes and dendritic cells in an HIV$^+$ patient.

Cell population	Direct isolation		Isolation after culture	
	Phenotype	Infection frequency	Phenotype	Infection frequency
CD4+ T lymphocytes	CD4$^+$, low FSC, SSC	1/67	/	/
Sorted Dendritic cells	cocktail$^-$ [1],HLA-DR$^+$	1/18000 [2]	CD19$^-$HLA-DQ$^+$	1/5020 [3]
Control sorted cells	cocktail$^+$ [1], HLA-DR$^+$	1/132000 [2]	CD19$^+$HLA-DQ$^+$	1/17000 [3]

1: cocktail = mixture of FITC-labeled antibodies directed against CD14, 16, 22, TCR$\alpha\beta$
2: % T lymphocytes in the enriched population before sorting: 0.8%
3: 5% T lymphocytes, 2% CD4 T lymphocytes in the enriched population before sorting.

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HIV INFECTION OF BLOOD DENDRITIC CELLS IN VITRO AND IN VIVO

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INTRODUCTION

The question of whether blood dendritic cells (DC) can be infected with HIV is controversial (1,2,3). In further studies aimed at resolving this problem DC were shown to express CD4 and cells with the distinctive morphology of veiled DC were found to support productive virus growth. In vitro infection was also demonstrated by PCR on highly purified preparations of DC. Finally, PCR was used to show that highly purified DC from HIV-infected individuals contained HIV provirus DNA.

METHODS

Cells

Blood mononuclear cells were prepared by Ficoll separation and then cultured overnight on plastic to remove adherent cells. Low density cells (LDC), containing 20-40% DC, were obtained by centrifuging the mononuclear cells over a 13.7% metrizamide gradient. To look for CD4 expression on DC LDC were labelled with a cocktail of PE-conjugated antibodies directed against T (anti-CD3) B (CD19), NK (CD56) and monocytic (CD14) cells, with an APC-conjugated anti-DR antibody and with an anti-CD4 FITC-conjugated antibody. Labelled LDC were analysed by FACS and DC identified by the expression of DR and the absence of markers for other cell types detected by PE-labelling. LDC preparations were also labelled with isotyped matched control antibodies.

In vitro infection of DC with HIV

LDC were infected with the IIIB strain of HIV and after 5d examined by electron microscopy. Highly purified DC were prepared from LDC by removing non-DC by panning with a cocktail of antibodies directed against CD3, CD14, CD19 and CD56.In some experiments inoculum virus was treated with DNAse I (10U/μl for 10 min at 37°C) prior to infection and the cells washed to remove unadsorbed virus after 1h at 37°C. After 5-7d the amount of HIV provirus in infected cultures was estimated by nested PCR using gag or polymerase primers on limiting dilutions of DNA. The effect of infection in the presence of anti-CD4 blocking antibody was also examined.

In vivo infection of DC

Highly purified DC were prepared from the blood of HIV-infected individuals. This was achieved by panning LDC preparations with a cocktail of antibodies directed against non-DC as described above. The provirus load was then estimated by nested PCR on limiting dilutions of DNA.

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RESULTS

DC were identified in LDC preparations by FACS by the absence of markers for other cell types and the expression of high levels of MHC class II DR. Analysis of this population indicated that 30-80% of the cells expressed a low level of CD4.

LDC were infected *in vitro* with HIV in 12 separate experiments and then examined by electron microscopy. In 7 experiments infection of DC was observed. One of the 7 preparations contained DC with the highly distinctive morphology of veiled DC which are more commonly found in afferent lymph. Mature and budding virus was seen on these cells. Highly purified DC prepared by panning were shown by FACS to contain less than 2% contaminating CD3, CD14, CD19 and CD56 positive cells. After 5-7d infection HIV provirus was detected in the cultures. Up to 1 provirus copy per 5 cells was detected (Fig. 1). To ensure that this observation was not merely due to contaminating DNA in the input inoculum virus was treated with DNase I prior to infection. After 1h adsorption the cells were washed and then processed for PCR immediately or after 5-7d in culture. Provirus was detected after 5-7d in culture but not immediately after adsorption. In addition infectious virus was detected after 5d but not after 24h.

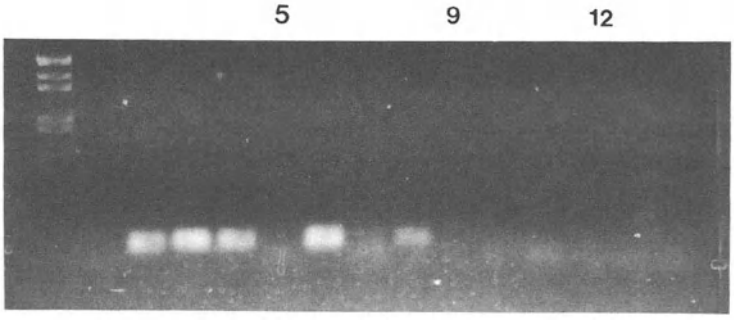

Figure 1. Detection of infected DC by nested PCR using polymerase primers on cells purified by panning. Cells analysed at 5d post infection. PCR performed in quadruplicate on doubling dilutions of DNA. Lanes 1-4 represent 5 cell equivalents of DNA, lanes 5-8 represent 2.5 cell equivalents of DNA, lanes 9-12 represent 1.25 cell equivalents of DNA.

The number of newly synthesised provirus copies was reduced up to 100-fold when the cells were infected in the presence of an anti-CD4 antibody that blocks the binding of gp120 to CD4. There was no reduction when cells were infected in the presence of a non-blocking
CD4 antibody.

The cytokine GM-CSF was reported to help maintain the viability of DC in culture (4). In one experiment when DC were infected with HIV and then maintained in GM-CSF for 7d the number provirus copies exceeded the number of cell genome copies as estimated by PCR for the single copy human gene b-globin.

HIV provirus was found in purified DC obtained from healthy HIV-infected asymptomatic individuals and also from patients with AIDS. For 2 asymptomatic patients there was 1 provirus copy per 120 and 1,100 cells. The number of cells per provirus copy for 3 AIDS patients was 80, 300 and 1,800. The second AIDS patient was receiving DDI and the third AZT. Provirus was also detected in the cells depleted from the LDC preparations by panning. However, the number of provirus copies in the DC fraction could not be accounted for by contamination with other infected cell types. Thus DC from infected individuals were clearly infected.

DISCUSSION

The data presented show that blood DC express the HIV receptor, CD4. Three lines of evidence, electron microscopy, PCR and assays for virus growth indicate that DC are susceptible to HIV infection. Recent studies have shown that expression of CD4 on DC is reduced when the cells are cultured (5, 6). Differences in time between removal of blood and infection in vitro may thus account for the conflicting reports on the susceptibility of these cells to infection. Although DC can bind, process and present a vast array of antigens by a pathway that is not generally considered to be CD4 dependent, entry of HIV appeared to require CD4.

The *in vitro* susceptibility to HIV infection was mirrored by the *in vivo* situation since provirus was detected in highly purified DC from asymptomatic and AIDS patients. These findings suggest that infection and the possible consequential impaired ability to present antigens may contribute to the immunosuppression seen in this disease. Future studies in this area will hopefully provide a better understanding of the pathogenesis of AIDS and may lead to the development of new therapies.

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IN VIVO AND IN VITRO INFECTION OF HUMAN LANGERHANS CELLS BY HIV-1

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INTRODUCTION

The skin and mucosa are the first line of defense of the organism against external agents, not only as a physical barrier between the body and the environment but also as the site of initiation of immune reactions[1]. The immunocompetent cells which act as antigen-presenting cells are Langerhans cells (LC). Originated from the bone marrow, LC migrate to the peripheral epithelia (skin, mucous membranes) where they play a primordial role in the induction of an immune response and are especially active in stimulating naive T lymphocytes in the primary response through a specific cooperation with CD4-positive lymphocytes after migration to proximal lymph nodes[2]. Apart from many plasma membrane determinants, LC also express CD4 molecules which make them susceptible targets and reservoirs for human immunodeficiency virus type 1 (HIV-1)[3]. Once infected, these cells due to their localization in areas at risk (skin, mucous membranes), their capacity to migrate from the epidermal compartment to lymph nodes and their ability to support viral replication without major cytopathic effects, could play a role of vector in the dissemination of virus from the site of inoculation to the lymph nodes and thereby to contribute to the infection of T lymphocytes.

IN VIVO HIV-1 INFECTION OF HUMAN LANGERHANS CELLS

In 1984, Belsito[4] reported that the absolute number of epidermal LC in the epidermis or AIDS/ARC patients was significantly reduced. Since 1987, different groups[5-7] have published electron microscope studies on skin from HIV-positive patients, in whom HIV particles have been observed in the close proximity of LC and buds of retroviral particles were observed from LC membrane. At the same time, the expression of CD4 antigens by LC has been shown to be up-regulated in certain clinical conditions (ARC)[7]. In a transgenic mouse model, carrying the HIV-1 promoter (long terminal repeat; LTR)- reporter genes, the HIV-1 LTR gene is preferentially expressed in epithelial dendritic cells that is LC and

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activated by ultraviolet light[8, 9] or skin-sensitizing chemicals[10]. The HIV-1 proviral DNA sequences) has been detected in the epidermis of seropositive patients[11-15]. Conflicting results have been published on this subject. The method used for the dermoepidermal dissociation (chemical/enzymatic procedures[12, 15], or suction blister[11, 14]) or for the purification LC suspensions[13] could account for the discrepancies. Recently, epidermal LC from seropositive patients were shown to actively transcribe HIV-1 proviral DNA[16] and be able to infect peripheral blood mononuclear cells from healthy donors in vitro[17] although the frequency of infected LC is very low (from 107 to 3, 645 HIV-1 DNA copies/10^5 LC)[16].

IN VITRO HIV-1 INFECTION OF HUMAN LANGERHANS CELLS

Between 1989 and 1991, we were able to demonstrate the *in vitro* capacity of normal LC to bind and to internalize HIV-1 gp120 or gp160 recombinant proteins[18]. This internalization occurs by receptor-mediated endocytosis and by Birbeck granules progressively induced from the plasma membrane[19]. In suspensions of epidermal LC obtained through trypsinization of dermo-epidermal sheets, the binding of viral glycoproteins occur *via* membrane receptor(s) which are different from CD4 molecules since these latter molecules are cleaved and released during the preparation of epidermal LC[18]. Furthermore, receptors resistant to trypsin which are involved in the binding of viral glycoproteins are up-regulated within 24h following isolation, whilst the cleaved CD4 are internalized from LC membranes and are not resynthesized. *In vitro* gp120 internalization may be the result of complex interactions occuring between viral envelope proteins and cellular factors in addition to the gp120/CD4 interactions[19]. In parallel to these results, we were able to demonstrate that LC are also capable of binding and internalizing viral particles by receptor-mediated endocytosis[15, 20]. Such an observation was made within the three first hours of co-culture of suspensions of LC and chronically infected promonocytic cells (U937 cells infected by the HIV-IIIB isolate). The route of entry of virus was shown to be identical to that of envelope proteins; nevertheless, the mechanism of fusion cannot be excluded since this latter is extremely rapid and, consequently, difficult to be observed by electron microscopy. By prolonging the time of co-culture of LC with the infected promonocytic cells, the LC show signs of permissivity to replication. After four days of co-culture, HIV-1 was clearly seen budding and being released from epidermal LC[21]. Recently we experimentally infected epidermal LC by HIV-1 provided by a cell-free infection system[22]. Viral particles were made cell-free by a low speed centrifugation followed by a 0.45 μm filtration. Proviral DNA (*gag* gene sequences) was found in LC-enriched epidermal cellular DNA from day 4 of post-infection with the isolate HIV-IIIB and from day 7, with isolate RF. Although the reverse transcriptase activity did not reach a significantly high level, viral RNA could be determined in supernatant of LC-enriched epidermal cell cultures at the time when proviral DNA was detected. These last findings indicate that the cell-free virus infection model leads to a later-occuring infection and a lower HIV-expression as compared to the cell-to-cell transmission. Nevertheless, this model allowed us to confirm the CD4-independent infection of isolated trypsin-treated LC and thus may be attractive for *in vitro* study of this type of infection. The fact that LC seem to support replication of HIV suggests that these cells may play an important part in viral pathogenesis. Taken together these results emphasize the point that LC, in genital mucosa, might play a role in the initial viral inoculation. The persistence of HIV in LC may favour a systemic dissemination of the virus from the areas of inoculation to regional lymph nodes where infected LC could serve as a constant source of infection for CD4+ T lymphocytes. Furthermore, it is easy to hypothesize a putative role of LC as a vector for dissemination although the consequences resulting from such an infection on their antigen-presenting cell functions are not still fully explored.

CONTRIBUTION OF LANGERHANS CELLS TO THE FIV MODEL IN MUCOSAL TRANSMISSION

Despite the fact that the heterosexual contact is the predominant route of transmission, the biology of the heterosexual transmission of HIV remains poorly understood. Mucosal transmission of feline immunodeficiency virus (FIV) in cats under experimental conditions would constitue a valuable model for the heterosexual transmission of HIV. In 1987, Pedersen[23] isolated in cats a new retrovirus very similar to the HIV, the FIV. This virus was detected in saliva, and it is now assumed that bites are the major route of FIV transmission in nature[24]. Furthermore, it has been proved that FIV transmission may occur through the oral, vaginal and rectal mucous membranes[25]. We tried to identify the equivalent of human LC in skin and mucous membranes and to find out whether these cells could be a target for FIV in order to demonstrate the contribution of feline LC as an experimental model for the transmission of virus through the mucous membranes. CD18-positive dendritic epithelial cells were found in all epidermal locations and in the mucous membranes (oral, vaginal, rectal and œsophageal membranes)[26]. Furthermore, these cells presented morphological and ultrastructural characteristics (Birbeck granules) which favour the hypothesis that these CD18-positive cells in cat stratified epithelia are the equivalent of human LC. This observation makes the feline LC a good candidate for an FIV model for exploring the infection of human LC located in the mucous membranes in the initial viral inoculation process.

COMMENTS

Human LC can be considered as targets for HIV infection. HIV-1 proviral DNA is detected in LC from AIDS patients and epidermal LC isolated from normal skin can be infected *in vitro* with HIV-1. Such experimental infections represent powerful approachs to understand the regulation of HIV infection of LC. The sites of LC infection are not been clearly established. Such an infection may occur either in LC precursors (found in bone marrow, peripheral blood and in dermis) or in mature LC in squamous epithelia. HIV transmission by sexual contact would lead to a direct infection of LC in mucosa. In addition infected LC may constitue a source of HIV infection and transmit virus to T lymphocytes in dermis as well as in peripheral lymph nodes where LC migrate following antigenic stimulation.

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IN VITRO INFECTION OF HUMAN EPIDERMAL LANGERHANS CELLS WITH HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

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INTRODUCTION

Members of the dendritic cell (DC) lineage, including Langerhans cells (LC) of squamous epithelia and peripheral blood (PB) DC, have been suggested to play an important role in the pathogenesis of HIV-1 infection[1]. PB DC from HIV-1 infected patients contain significant amounts of HIV-1 proviral DNA and can show viral budding[1]. Human epidermal LC express CD4 molecules on their membrane and are thus a likely target of HIV infection. We have demonstrated that purified preparations of epidermal LC, but not other epidermal cell (EC) types, harbor HIV-1 proviral DNA and RNA, indicating that LC are productively infected and can serve as a reservoir of the virus[2,3]. Moreover, the HIV DNA copy number in LC of AIDS patients is comparable to that found in PB $CD4^+$ T cells of patients at the same disease stage[3]. PB DC from healthy seronegative donors have been infected *in vitro* with HIV and could transmit the infection to other cells of myeloid origin[4]. Few studies have addressed the *in vitro* infection of epidermal LC. LC possess membrane gp120-binding sites[5]; in addition, Berger and co-workers have been able to infect *in vitro* both LC histiocytosis cells and LC-enriched EC, but the nature of the EC type infected could not be determined[6].

In the present study we investigated whether normal epidermal LC could be infected *in vitro* with HIV-1 and could transmit the infection to lymphoid cells.

LC CAN BE INFECTED *IN VITRO* WITH HIV-1IIIB

To avoid trypsin treatment, which removes membrane CD4 molecule, LC were allowed to migrate spontaneously out of epidermal sheets into the culture medium. Flow

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cytometry analysis confirmed that EC collected in the medium after 24-48 hrs of culture and subjected to gradient centrifugation contained 10-25% $CD1a^+/CD4^+$ cells, i.e. LC (not shown). EC were first deprived of passenger T cells by immunomagnetic separation using a cocktail of mAb against specific T cell markers and then HIV-1IIIB was added to the culture for 24 hrs. To evaluate HIV infection of LC, highly purified LC and LC-depleted EC fractions were prepared by immunomagnetic method using anti-CD1a mAb. Both cell preparations were devoid of T cells as confirmed by RT-PCR analysis for T cell receptor β chain gene (data not shown)[3]. LC and LC-depleted EC were then examined for the presence of proviral HIV-1 DNA by PCR analysis (*gag*). The purified LC fractions showed the presence of HIV-1 DNA, thus indicating that LC can be infected *in vitro* with HIV-1. In contrast, no HIV DNA could be detected in purified LC exposed to heat-inactivated virus or in LC-depleted EC (Fig. 1).

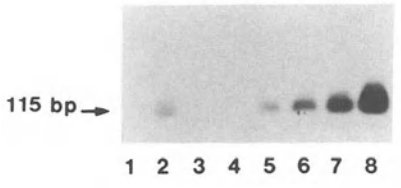

Figure 1. Analysis of HIV-1 proviral DNA by PCR amplification of *gag* region (primers SK 38/39) in purified LC and LC-depleted epidermal cells harvested 24 hrs after HIV-1IIIB infection. Lane 1, LC-depleted epidermal cells; lane 2, purified LC; lanes 3, purified LC exposed to heat-inactivated virus; lane 4, negative control: no DNA; lanes 5-8, positive controls: 1 μg of DNA from uninfected peripheral blood mononuclear cells containing 5, 10, 50 and 100 8E5/LAV cells, respectively.

HIV-INFECTED LC CAN TRANSMIT INFECTION TO C8166 CELLS

C8166 cells are lymphoblastoid cells known to be highly susceptible to HIV infection *in vitro*. Co-cultures of C8166 cells with LC-enriched EC previously deprived of T cells and challenged with HIV showed a time-dependent increase in the amount of HIV proviral DNA. In contrast, no signal could be detected when C8166 cells were co-cultured with HIV-1IIIB-treated TF1 cells, a cell line not susceptible to HIV infection (Fig. 2). HIV DNA was also detected in co-cultures of C8166 cells and purified LC, but not in co-cultures of C8166 cells and LC-depleted EC (Fig. 2), indicating that LC are the EC type which transmits HIV infection. By light and electron microscopy, C8166 cells exhibited formation of giant, multinucleated syncytia cells, with numerous membrane budding figures and abundant viral particles with a characteristic cone-shaped core adjacent to the plasma membrane (not shown).

CONCLUSIONS

Infection of LC by HIV is relevant for several reasons. LC of mucosal epithelia may be among the first cells to be infected following mucosal HIV-1 exposure. Secondly, LC may serve as a reservoir for continued infection of $CD4^+$ T cells[7], especially in lymph nodes where epidermal LC migrate following antigenic activation. Thirdly, as suggested for peripheral blood DC, HIV-infected LC may have a reduced antigen presentation capacity and thus contribute to the depletion of memory T cells and the circulating T-cell pool. Here we have shown that LC can be efficiently infected *in vitro* with HIV and can transmit the infection to other susceptible target cells. This *in vitro* model may be helpful in studying the mechanisms and regulation of LC infection with HIV.

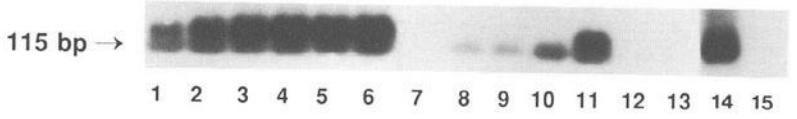

Figure 2. Analysis of HIV-1 proviral DNA by PCR amplification (*gag* region, primers SK 38/39) of C8166 cells co-cultured with LC-enriched epidermal cells, purified LC, LC-depleted epidermal cells or TF1 cells previously challenged with HIV-1IIIB. Lanes 1 and 2, LC-enriched epidermal cells + C8166 cells after 48 hrs of co-culture; lanes 3 and 4, LC-enriched epidermal cells + C8166 cells after 72 hrs of co-culture; lanes 5 and 6, LC-enriched epidermal cells + C8166 cells after 96 hrs of co-culture; lane 7, TF1 cells + C8166 cells after 96 hrs of co-culture; lanes 8 to 11, positive controls: 3, 5, 10 and 100 8E5/LAV cells, each cell containing a single copy of HIV-1 proviral DNA mixed with 1 μg of DNA from non-infected peripheral blood mononuclear cells; lane 12, negative control: all the reagents except DNA; lane 13, negative control: C8166 non-infected cells; lane 14, purified LC+ C8166 cells after 48 hrs of co-culture; lane 15, LC-depleted epidermal cells + C8166 cells after 48 hrs of co-culture.

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DENDRITIC CELL-T CELL CONJUGATES THAT MIGRATE FROM NORMAL HUMAN SKIN ARE AN EXPLOSIVE SITE OF INFECTION FOR HIV-1

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INTRODUCTION

Distinctive features of dendritic cells include their potent antigen presenting capabilities and their migratory properties. Stimulation of the skin using contact sensitzing agents[1,2], or following transplantation[3], "activates" dendritic cells to migrate into the afferent lymphatics and on to the draining lymph node. Movement of dendritic cells via the lymph[1,4-6] to the lymph node provides an explanation for the dependence on intact, cutaneous afferent lymphatics for effective primary immune responses to contact allergens[7] and transplants[8] in situ. Furthermore, injection of ex vivo antigen-pulsed dendritic cells back into mice results in migration of the dendritic cells to the draining lymphoid tissue[9], where $CD4^+$ T cells are primed[10-12].

We have studied the susceptibility of cutaneous dendritic cells to infection with HIV-1. Skin-derived dendritic cells are being used as a model for the dendritic cells located in the mucous membranes [skin-like epithelia] covering the organs involved in sexual transmission of HIV-1[13-15]. Cutaneous dendritic cells express detectable levels of the CD4 molecule[16], and may, therefore, be susceptible to direct infection with HIV-1 and be critical to subsequent transmission and dissemination of infection.

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ISOLATION OF CUTANEOUS DENDRITIC CELLS

Organ culture experiments carried out using explants of mouse skin[3] and more recently with human skin[17], revealed that dendritic cells selectively migrate into the surrounding medium. Based on this, we have established a technique for the isolation of normal cutaneous human dendritic cells[18]. In brief, pieces of split thickness skin removed during reductive plastic surgery, were placed into culture medium [dermal side down] and cultured for 2-4 days. After culture, the skin was removed and the cell suspensions incubated with Collagenase D. The cells were harvested and washed to provide the skin cell emigrants. Unlike the traditional methods of isolating cutaneous dendritic cells using enzymic digestion of the tissue[19,20], this procedure provided suspensions made up almost exclusively of dendritic cells and small T cells, with >95% viability, and few [if any] contaminating keratinocytes[18].

PHENOTYPIC CHARACTERIZATION OF SKIN CELL EMIGRANTS

Cytofluorography was used to characterize the dendritic cells and other cell types present in the skin cell emigrants. The latter were stained with a wide panel of monoclonal antibodies and FITC-goat anti-mouse Ig and counterstained with PE-anti-HLA-DR to identify the dendritic cells. Skin cell emigrants were all $CD45^+$ leukocytes, with only rare [if any] monocytes, B cells, and NK cells. Staining with anti-HLA-DR and anti-CD3, as well as cell sorting on the basis of size and CD3 expression, documented the presence of three cell subsets: $CD3^-$ $HLA\text{-}DR^+$ free dendritic cells, $CD3^+$ $HLA\text{-}DR^-$ free T cells, and $CD3^+$ $HLA\text{-}DR^+$ dendritic cell-T cell conjugates[18].

Strongly $HLA\text{-}DR^+$ cells had the typical mature dendritic cell phenotype[19-21], expressing high levels of Class II and Class I MHC products, many costimulatory and adhesion molecules[18]. The majority of the $HLA\text{-}DR^-$ cells expressed T cell markers including: CD2, CD3, CD5, CD7, and TcR$\alpha\beta$. Approximately 2/3 of the T cells were $CD4^+$ and 1/3 $CD8^+$. Most of the T cells were $CD45RA^{weak}$, $CD45RO^+$, and CD58/LFA-3^+, the phenotype characteristic of memory T cells[22]. Expression of T cell activation antigens was, at best, trace [CD25 and CD80/B7-1] or absent [HLA-DR].

Interestingly, $HLA\text{-}DR^+$ $CD3^+$ dendritic cell-T cell conjugates were a consistent feature of the emigrants. Immunohistochemical staining of cytospins confirmed the presence of such conjugates [i.e., small cells that were $CD3^+$ $HLA\text{-}DR^-$ attached to large cells that were $CD3^-$ $HLA\text{-}DR^+$[23]]. Both $CD4^+$ and $CD8^+$ T cells formed conjugates with the dendritic cells[18]. These antigen-independent conjugates were stable [the majority remaining intact during cytofluorography] and did not appear to be actively proliferating[18,23]. The susceptibility of the skin cell emigrants and the individual subsets to infection with HIV-1 was then investigated.

HIV-1 INFECTION OF CUTANEOUS DENDRITIC CELL-T CELL CONJUGATES

Bulk skin cell emigrants and the sorted cell fractions were exposed to various isolates of HIV-1. Virus entry was measured by the presence of HIV-1 gag specific DNA sequences, while infection and viral spread was assessed by the production of HIV-1 p24 antigen and reverse transcriptase activity[23]. DNA PCR analysis documented similar levels of virus entry into the skin cell emigrants as that seen in control permissive cell suspensions [macrophages, activated T blasts, or the CEM T cell line].

In the absence of any exogenous stimuli, bulk skin cell emigrants were permissive to productive infection with all seven HIV-1 isolates tested. Large multinuceated syncytia were the major site of virus production, as determined by immunohistochemical staining with a monoclonal antibody specific for HIV p24 antigen[24] and electron microscopy.

All these manifestations of HIV infection were dependent upon both dendritic cells and T cells. When dendritic cells and T cells were separated from each other by cell sorting, they were not productively infected. Immunophenotyping of the infected cultures revealed that the virus-producing syncytia were $CD1a^+$, $HLA\text{-}DR^+$, and $CD3^+$ and had formed as a result of dendritic cells and T cells fusing together.

CUTANEOUS DENDRITIC CELLS AND HIV-1 PATHOGENESIS

We report that both dendritic cells and T lymphocytes with a memory phenotype [$CD45RA^{low}$ $CD45RO^{high}$ $CD58/LFA\text{-}3^{high}$] migrate from normal human skin in organ culture. Some of these dendritic cells and T cells form tight, non-proliferating conjugates[18]. This migration system provides ready access to these cutaneous cell populations, which should be useful in the study of many cutaneous immune responses. Cutaneous cells may prove to be an accessible model for studying the mechanism of transmission of HIV-1 across the sexual mucosa. We have demonstrated that the cutaneous dendritic cell-T cell milieu, and not free dendritic cells or T cells, were highly permissive to productive infection with HIV-1[23].

Other reports are contradictory regarding the productive infection of skin dendritic cells in situ[25,26], although proviral DNA has been identified in dendritic cells isolated from AIDS patients at autopsy[27]. Furthermore, HIV nucleic acids have been detected in the skin-like mucosa of the cervix[28] and rectum[29] biopsied from HIV-infected individuals. Cervical and vaginal mucosa of SIV-infected rhesus macaques also contain SIV-infected cells[30,31]. Our findings suggest that the cutaneous dendritic cell-T cell microenvironment, but not the free dendritic cells, may be especially important for active productive infection with HIV-1 in the mucosa. It is possible that dendritic cells in the mucosa of the oral, anal, or genital tracts become activated following injury or infection and begin to migrate, as described in the explants of mouse[3] and human skin[17,18,23] and in in vivo mouse experiments[3,32]. During activation, the dendritic cells could associate with the memory T cells that normally reside in these tissues[18,33] and the draining afferent lymph[34]. Should this occur in the presence of HIV, the productive infection seen in these cultures might occur at the sites of sexual transmission. Even in the absence of cognate antigen, this could contribute to, or initiate, the progressive loss of memory $CD4^+$ T cells, that is the hallmark of HIV-1 infection[35,36].

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CELL-CELL INTERACTIONS REGULATE DENDRITIC CELL-DEPENDENT HIV-1 PRODUCTION IN CD4+ T LYMPHOCYTES

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SUMMARY

We investigated the role of blood dendritic cells (DC) in transmission of HIV-1 from infected to uninfected CD4+ T cells, and the accessory molecules involved. DC promoted transmission from infected to uninfected CD4+ cells, but blood DC themselves were not infectable. DC-mediated transmission was blocked by mAb to CD4 and MHC class II, but strongly increased by mAb to CD40 on DC or CD28 on T cells. The DC-dependent infection was inhibitable by anti-CD80 and a soluble fusion protein of the CD80 ligand, CTLA4; soluble CTLA4Ig also blocked infection augmented by crosslinking CD40. We also demonstrated that mAb to CD40 up-regulate the expression of CTLA4 ligands CD80 and B70/B7-2 (CD86) on DC. These data suggest that the dialog between CD40-CD40 ligand (CD40L) and CD28-CD80 counter-receptors on DC and T cells may be linked to HIV infection in vivo.

INTRODUCTION

Dendritic cells are extremely effective antigen-presenting cells because they express not only high levels of MHC class I and II products but also several accessory molecules including CD80 and B-cell-associated marker CD40 (1–4). The CD40-CD40L and CD28-CD80 receptor-ligand pairs appear to play a key role in the cognate T-B dialog (5).

In our previous studies we confirmed the finding of Cameron et al. (6) that after exposure to HIV-1, highly purified blood DC (negative for other cell-specific markers, CD40-positive and the most potent stimulators in allogeneic mixed leukocyte reaction) were not susceptible to HIV-1 infection, unlike stimulated CD4+ T lymphocytes or peripheral blood mononuclear cells (7). We also demonstrated that DC promote transmission of HIV-1 from infected to uninfected CD4+ T cells. Such transmission is strongly increased by anti-CD40 and anti-CD28 and blocked by CTLA4Ig and anti-CD80. CTLA4Ig blocked the anti-CD40-induced DC-dependent increase in HIV-1 DNA levels. We found that mAb to CD40 up-regulate the expression of CD80 and CTLA4 binding to DC (7).

The fact that CTLA4Ig binding was significantly greater than CD80 mAb (7) suggested that anti-CD40 increases expression of both CD80 and CD86 on DC, as has been reported for B cells (8,9).

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RESULTS AND DISCUSSION

MAb to CD40 Up-regulate the Expression of B70/B7-2 (CD86) on DC

To test whether anti-CD40 induces increases in DC-dependent HIV-1 replication via the regulation of B70/B7-2 on DC, we analyzed the expression of this ligand for CD28 and CTLA4 using cytofluorography. DC that were 40–60% pure were analyzed by FACS as described (7, 11) with some modifications. We analyzed a subset of the cells by gating on the cell population with large forward scatter that did not stain with a panel of phycoerythrin-conjugated cell-specific mAb. Having excluded nondendritic cells from the analysis, we analyzed DC using biotin-anti-CD86 (IT2.2, PharMingen), CTLA4Ig (kindly provided by Dr. Peter Linsley), or isotype-matched mAb followed by a streptoavidin fluorescein conjugate. DC expressed low levels of CD86 and CTLA4 family ligands immediately after isolation and after treatment with isotype control mAb throughout 4 days of culture. In contrast, DC treated with anti-CD40 had increased expression of B70/B7-2 and CTLA4 ligands were detectable by day 1 after treatment, peaked by day 2, and decreased by day 4 (Fig. 1).

These results suggest that CD40 is functionally active on DC and that activated T cells expressing CD40L may contribute to DC-dependent HIV-1 transmission. Thus, a CD80-CD28 and/or CD86-CD28 interaction appears to be required for anti-CD40-induced increases in HIV-1, perhaps by increasing levels of CD80 or CD86 (8) on DC. Activated $CD4^+$ T cells can up-regulate the expression of CD80 on B cells (9) and possibly on DC; CD80 and CD86 in turn can signal T cells via CD28 (5, 10). This reciprocal dialogue between the CD40L-CD40 and CD80-CD28 cell-cell interaction pairs (5) may be essential to promote HIV-1 expression and spread.

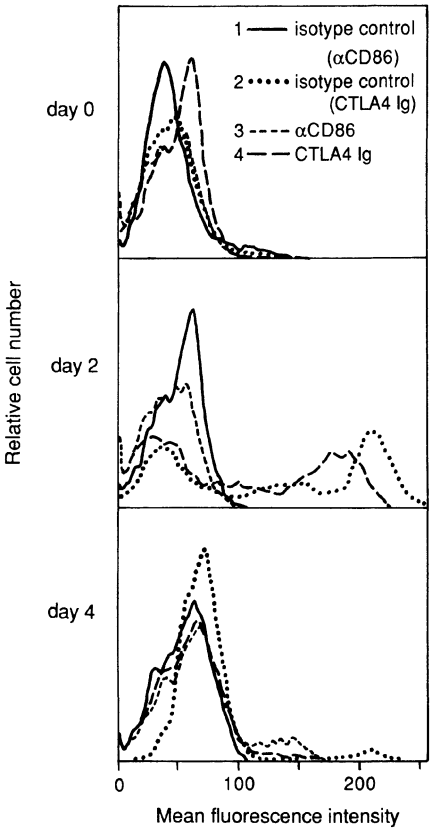

Figure 1. MAb to CD40 induce the expression of B70/B7-2 (CD86) and other CTLA4 ligands on DC. DC were assessed for expression of CD80 family members, using biotinylated anti-B70/B7-2, CTLA4Ig, or isotype-matched control antibodies followed by a streptoavidin-fluorescein conjugates. Isotype-matched mAb to (1) anti-CD86 (line); (2) human IgG control for CTLA4Ig (dotted line); (3) anti-CD86 (short broken line); (4) CTLA4Ig (long broken line).

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IN VITRO HIV-1 INFECTION OF ISOLATED EPIDERMAL LANGERHANS CELLS WITH A CELL-FREE SYSTEM

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INTRODUCTION

Langerhans cells (LC) belong to the antigen-presenting cells which are found in all lymphoid and non-lymphoid organs (1). Originating from the bone marrow the LC migrate to the peripheral epithelia (skin, mucous membranes) where they play a key role in the immune surveillance against foreign antigens then migrate to lymph nodes. LC are localized in areas at risk for inoculation by Human Immunodeficiency Virus (HIV). In seropositive patients, LC are susceptible to be targets for HIV (for review, see 2) that it had been shown by a large variety of methodologies such as electron microscopy, immunocytochemistry, polymerase chain reaction (PCR) amplification of virus-specific DNA, reverse transcription of HIV-1 spliced mRNA region of the *tat* gene (3). In a coculture with HIV-1-carrying U937 cells, we had demonstrated the infectiousness of LC isolated from human normal skin in vitro which supported replication (4). The aim of this study was to investigate whether LC can be infected in vitro by HIV-1 provided by a cell-free infection system.

MATERIALS AND METHODS

LC suspensions were prepared from normal human skin. Dermo-epidermal sheets were treated either with 0.25% trypsin (0.25% at 4°C / 18h) followed by gradient enrichment or with dispase (1.2 U/ml at 37°C / 1h) followed by passive migration (18h / 37°C) from epidermal (epidermal dendritic cells or EDC) and dermal (dermal dendritic cells or DDC) strips. Viral particles provided by chronically infected cell lines (H9 and U937 cells) were made cell-free by a low-speed centrifugation followed by a 0.45µm filtration. We used two lymphocytotropic isolates (HTLV-IIIB and RF). LC, DDC and control cell (U937) suspensions are infected with cell-free viral suspensions. After 3 days of culture, cell suspensions were washed and resuspended in fresh culture medium. The control of infection was made by measuring RT activity in the supernatant and by searching of specific sequences

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of proviral DNA by polymerase chain reaction (PCR) in *gag* gene, and specific sequence of viral RNA by RT-PCR in *gag* gene, in the supernatant.

RESULTS

Infection of Epidermal LC by HIV-1

Figure 1 shows the results of the proviral DNA analysis by PCR from DNA of LC after an infection by cell-free viral suspension of HTLV-IIIB isolate. A positive signal corresponding to the *gag* gene sequence (115 bp DNA) was found from day2 to day7 from DNA of U937 cells (lanes 4, 9, 11) and from DNA of LC (lanes 5, 8 and 10) that suggests epidermal LC were infected by HTLV-IIIB isolate.

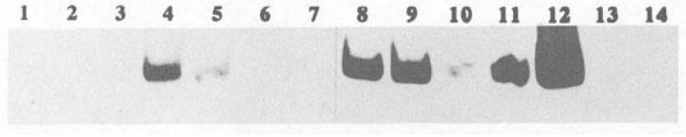

Figure 1. Analysis of PCR products cellular DNA of cells infected by HTLV-IIIB isolate. 1: non-infected LC; 2: non-infected U937 cells; 3 LC at day0; 4: U937 cells at day2; 5: LC at day2; 6: U937 at day0; 7, 14: molecular weight markers ; 8: LC at day4; 9: U937 cells at day4; 10: LC at day7; 11: U937 cells at day7; 12: HTLV-IIIB-infected U937 cells; 13: H2O.

With the RF isolate, a lower positive signal (115 bp DNA) was detectable at day4 and day7 for DNA of U937 cells (figure 2, lanes 4, 10) and only at day7 with DNA of epidermal LC (lane 9) which suggests a possible lower infectivity of the RF isolate than the HTLV-IIIB isolate in U937 cells and epidermal HIV-1-sensitive cells.

Figure 2. Analysis of PCR products cellular DNA of cells infected by RF isolate. 1: non-infected LC; 2: non-infected U937 cells; 3 LC at day0; 4: U937 at day0; 5: LC at day2; 6: U937 cells at day2; 7: LC at day4; 8: U937 cells at day4; 9: LC at day7; 10: U937 cells at day7; 11: RF-infected H9 cells; 12: H2O; 13: molecular weight markers .

The RT activity of the supernatant of epidermal LC was not high enough to assess a release of viral particles (data not shown). Nevertheless, with the more sensitive technique of RT-PCR from the supernatant of infected cells, we observed positive signals from day4 from the supernatant of U937 cells (figure 3, lanes 11, 15) and of epidermal LC (lanes 9, 13) which suggest a possible HIV-1 replication in epidermal LC with a release of viral particles.

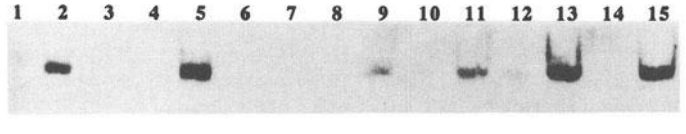

Figure 3. Analysis of RT-PCR products cellular RNA from supernatant of cells infected by RF isolate. 1: RF-infected H9 cells without RT; 2: RF-infected H9 with RT; 3 H2O; 4: LC at day3 without RT; 5: LC at day3 with RT; 6: U937 cells at day3 without RT; 7: U937 cells at day3 with RT; 8: LC at day4 without RT; 9: LC at day4 with RT; 10: U937 cells at day4 without RT; 11: U937 cells at day4 with RT; 12: LC at day7 without RT; 13: LC at day7 with RT; 14: U937 cells at day7 without RT; 15: U937 cells at day7 with RT.

Infection of Migrating LC by HIV-1

With the migrating cells (EDC and DDC), a positive signal was detectable from day4 after an infection with HTLV-IIIB isolate (figure 4, lanes 6, 7, 9 and 10). These data suggest migrating DDC and EDC were able to be infected by HIV-1.

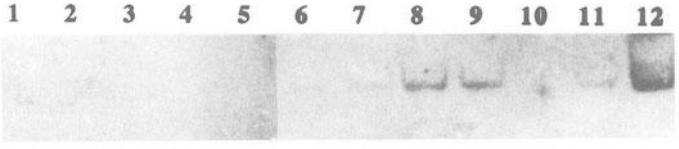

Figure 4. Analysis of PCR products cellular DNA of cells infected by HTLV-IIIB isolate. 1: non-infected LC; 2: non-infected U937 cells; 3 LC at day0; 4: U937 at day0; 5: molecular weight markers; 6: EDC at day4; 7: DDC at day4 ; 8: U937 cells at day4; 9: DDC at day8; 10: EDC at day8; 11: U937 cells at day8; 12: HTLV-IIIB-infected U937 cells.

With the RT assay, we observed a large increase at day9 (figure 5) in the supernatant of control cells (U937 cells) and migrating EDC but not in the supernatant of migrating DDC. So migrating LC could release a higher amount of viral particles than epidermal LC and migrating DDC.

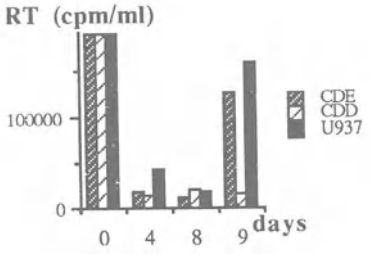

Figure 5. Reverse transcriptase (RT) activity detected in the supernatant after an HIV-1 infection

DISCUSSION

In this study, we showed that both isolated LC subpopulations (epidermal and migrating) could be infected by HIV-1 in a cell-free system. Indeed, proviral DNA was detected in the experimentally infected cells from day4 of postinfection. But the infection time course seemed to be related to the isolate since isolate RF appears less efficient and slower infectious than the HTLV-IIIB. With this cell-free system, LC which had been experimentally infected were susceptible to release viral particles. To compare these results with a previous study where we developped an in vitro cell-to-cell infection of epidermal LC (4), we conclude that this cell-free model of infection results in a latter-occuring infection and a lower HIV-1 expression since with a cell-to-cell infection HIV-replicative LC are found from day4 Nevertheless this cell-free system could be an excellent model for in vitro investigation of exploring the parameters of HIV infection on their way from epidermis to lymph nodes.

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DEFECTS IN THE FUNCTION OF DENDRITIC CELLS IN MURINE RETROVIRAL INFECTION

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INTRODUCTION

An inadequate immune response is one of the most important features of retroviral infections. Antigen presenting cells, in particular dendritic cells (DC), are a key element of the immune system, and assessment of their function in retroviral diseases may be very important in understanding of the pathogenesis of these infections. It has been established that DC are reservoir of HIV infection in the human body and can transmit the virus to T cells (1-3). However, the data about functional activity of DC in HIV infection are contradictory. Because of difficulties in obtaining purified DC from human lymph nodes we used in this study a murine retrovirus Rauscher leukemia virus (RLV) as an animal model of human retroviral diseases. This virus causes acute pathological effects (malignant erythrocytopoesis and splenomegaly associated with anaemia) which are rapidly lethal. Immunosuppressive effects *in vitro* and *in vivo* of RLV have been documented (4) earlier.

MATERIALS AND METHODS

Mice and viral inoculation. The suspension of RLV contained $1x10^5$ focus forming units (ffu)/ml. BALB/c mice were inoculated intravenously (tail vein) with 0.1 ml either of saline (controls) or of suspension containing RLV. Mice were sacrificed 3, 7, 14 and 21 days after inoculation and the infection was verified by the presence of virus activity in spleen cells

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(XC plaque "infectious centre" assay (5) with some modifications (6)). Inguinal,axillary,popliteal and brachial lymph nodes were taken.

Cell preparation. DC from single cell suspensions from lymph nodes were prepared by centrifuging of the cells onto metrizamide (Nygaard,Oslo).Pelleted cells from the lymph node suspension were passed through nylon wool columns to obtain >90% pure T cells. DC from the lymphocyte fraction of lymph node cells were removed using anti-DC antibody (33D1;(7)) and complement.

LN cell proliferation and mixed leucocyte reaction (MLR). Various numbers (25-100x10^3) of non-separated lymph node cells or DC depleted LN cells were cultured in 0.02ml hanging drops in inverted Terasaki plates (8). Cells were stimulated with either 0.005 mg/ml ConA (Sigma) or 0.01 mg/ml anti-CD3 antibody. For MLR DC (500 cells) were cultured with 25-100x10^3 enriched T cells from either C57BL/10 or BALB/c mice. After 3 days the cultures were pulsed for 2 hr with 0.001 ml ^{3}H thymidine (2 Ci/mmol; Amersham International, Amersham, Bucks, UK) to give 0.001mg thymidine/ml and harvested by blotting onto filter discs.

RESULTS AND DISCUSSION

Spontaneous proliferation of unseparated lymph node cells was not altered at any time after infection. However, depletion of DC from the lymph node cell suspension led to a decrease of ^{3}H uptake in control mice but to a considerable increase in RLV-infected mice in the week after infection (Fig.1). This effect was seen also in the second and third weeks of infection. The response, of both unseparated LN cells and DC depleted cells on stimulation by anti-CD3 antibody was significantly higher in infected mice than that in control mice (Fig.1). ConA stimulated proliferative response of LN cells was inhibited in RLV-infected mice by the end of second week of infection. There were no differences in proliferative response of LN cells after DC depletion at any time after infection. A suppression of the ability of DC to stimulate allogeneic T cells from non-infected mice was found even 3 days after infection (data not shown). This impairment became greater two and three weeks later. At three weeks following infection the T-cell response after stimulation by DC from infected mice was less than half of that of DC from control mice.

Thus, lymphocytes (after DC depletion) from infected mice showed a higher level of spontaneous proliferation than those from control animals. There was a parallel in our study between the level of RLV infection in lymphocytes and the level of proliferation (data not

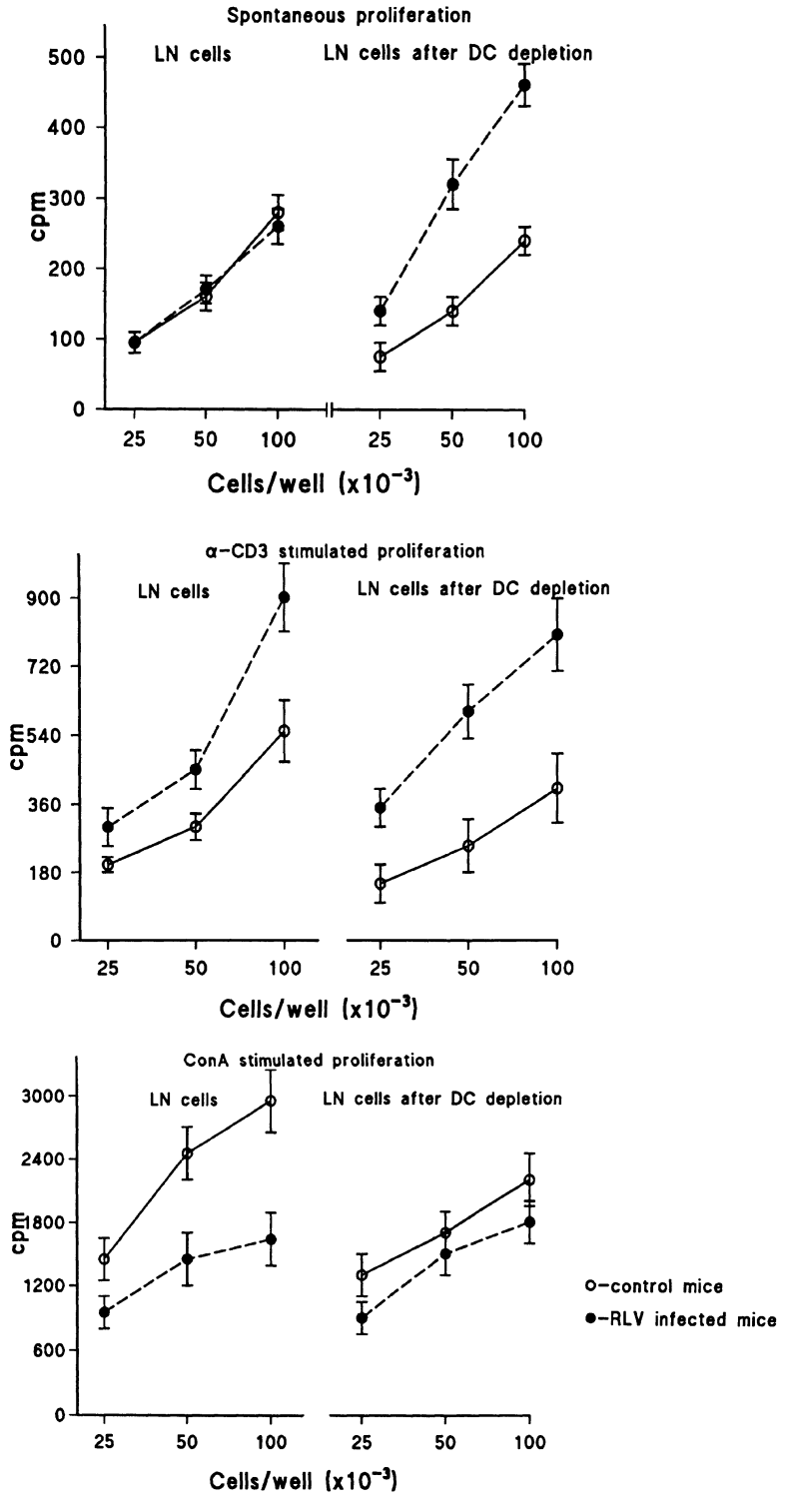

Figure 1. Effect of DC on proliferation of LN cells taken on 14 day of RLV infection.

shown). Infection of lymphocytes may induce lymphocyte proliferation directly or through the increased production of cytokines. However, a different effect was found when proliferation of non-separated LN cells was assessed. No difference in the level of spontaneous proliferation was obtained between control and infected animals at any time of infection. Anti-CD3 antibody stimulated T cell proliferation was increased in RLV infected mice regardless of presence of DC in LN cell fraction. However, significant decrease in ConA stimulated proliferation was found in presence of DC in LN cell fraction of RLV infected mice. Taking into account that anti-CD3 antibody acts directly to T cells, but ConA effect on T cells partly mediated by DC we can conclude that DC play an important role in producing the immunosuppressive action of RLV.

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HUMAN EPIDERMAL LANGERHANS CELLS EFFICIENTLY PRESENT HIV-1 ANTIGENS TO SPECIFIC T CELL LINES

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INTRODUCTION

Dendritic cells (DC) play a dominant role in T cell priming both in vitro and in vivo. We have shown that peripheral blood (PB) DC are the most efficient APC for presentation of the HIV-1 envelope glycoprotein gp120 to specific $CD4^+$ T cells and are required for in vitro generation of gp120-specific T cell lines from unprimed individuals[1]. Epidermal DC, i.e. Langerhans cells (LC), are the critical APC for the induction of T cell immunity against Ag that are present in or penetrate through squamous epithelia[2]. LC obtained from HIV-infected subjects contain HIV-1 proviral DNA and RNA[3], and can release HIV virions, thus indicating that LC are productively infected by the virus in vivo.

LC exhibit marked changes in both immunophenotype and functional properties during short-term culture. Compared to freshly isolated LC (fLC), cultured LC (cLC) display increased expression of membrane MHC and costimulatory molecules and enhanced alloantigen presenting function[4]. However, cLC are commonly believed to become rather inefficient in presenting intact proteins to T cells. In this study, we examined the ability of LC to present native HIV-1 protein Ag and immunogenic peptides to specific $CD4^+$ T cell lines generated from HIV-seronegative volunteers; and, secondly, we compared the Ag-specific accessory properties of fLC, cLC, and PB monocytes.

cLC ARE SUPERIOR TO fLC AND PB MONOCYTES IN PRESENTING HIV-1 ANTIGENS TO SPECIFIC $CD4^+$ T CELLS

Epidermal cell suspensions enriched for LC (5% $CD1a^+$ cells) were prepared from suction blister roofs and used either freshly isolated or after 48-hr culture. $CD4^+$ T cell lines

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specific for the envelope glycoprotein gp120 or reverse transcriptase (p66) were generated by repeated stimulation of PB T lymphocytes with Ag-pulsed and γ-irradiated PB monocytes followed by IL-2 expansion[5]. The p66-specific T cell line recognized two different immunodominant peptides (peptides 29 and 43). fLC could stimulate T cells at lower Ag (native protein and immunogenic peptides) concentrations (Fig. 1A) and were more efficient on a per cell basis (Fig. 1B) compared to unfractionated PBMC. In addition, cLC were more potent than fLC, PBMC or PB adherent monocytes both in terms of the amount of Ag required (Fig. 1C) and on a per cell basis (Fig. 1D). Both fLC and cLC utilized the exogenous pathway of Ag processing, as chloroquine or leupeptin inhibited presentation of intact p66, but not of peptide 43 (not shown).

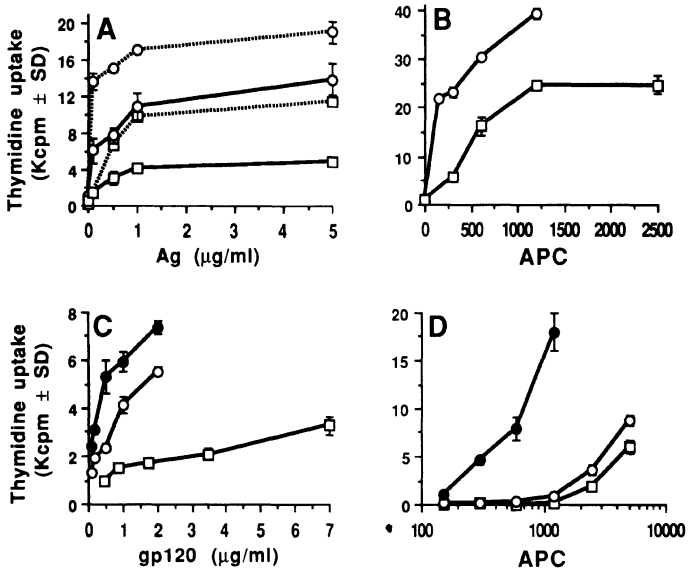

Figure 1. (A) Freshly isolated epidermal cells enriched for LC (600 LC/well; *white circles*) or PBMC (600 monocytes/well; *white squares*) were pulsed with 0.1-5 μg/ml of either intact p66 (*solid lines*) or peptide 29 (*broken lines*) and cocultured with 20 x 10^4 T cells for 3 days. (B) Increasing number of fLC or PBMC were pulsed with peptide 43 (2 μg/ml) and then cocultured with T cells. Epidermal cells enriched for LC either freshly procured, 48-hr cultured LC (*black circles*) or PB monocytes (500 APC/well) were pulsed with gp120 (1 μg/ml) (C) or p66 (1 μg/ml) (D) and then cocultured with T cells.

SEVERAL COSTIMULATORY MOLECULES ARE INVOLVED IN OPTIMAL ANTIGEN-SPECIFIC PRESENTATION BY cLC

Except for ICAM-3, fLC do not display significant numbers of costimulatory molecules. After short-term culture, LC express and then upregulate ICAM-1, LFA-3 and B7-1[4]. Although some studies have proved the importance of such membrane molecules in the LC-dependent activation of allogeneic T cells, only few of them have analyzed the role of costimulatory molecules on LC-driven Ag-specific T cell proliferation[5]. We therefore investigated the contribution of several accessory molecules in cLC-dependent stimulation of the T cell lines specific for gp120 or p66. Incubation of cLC with mAb against HLA-DR completely blocked T cell proliferation (95% inhibition) (Fig. 2). As expected, mAb against

MHC class I molecule had no effect mAb to ICAM-1, ICAM-3, LFA-3, B7-1 and β2-integrins reduced T cell proliferation between 50% and 90%

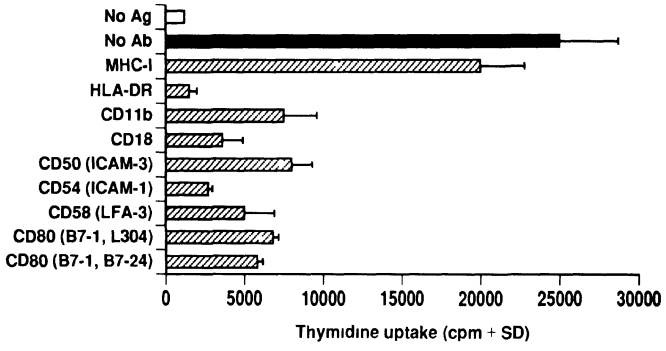

Figure 2 Costimulatory molecules involved in the presentation of gp120 by cLC to specific $CD4^+$ T cell lines Epidermal cells enriched for LC were cultured for 48 hr, incubated at 4°C for 45 min with saturating concentrations of dyalized mAb, pulsed with Ag and then cocultured with 20 x 10^4 specific T cells for 3 days Similar results were obtained using the p66 specific T cell line

CONCLUSIONS

Our study demonstrates that cLC fully retain the capacity to process intact protein Ag, as previously described by Cohen and Katz[2], and compared to fLC, cLC markedly augments their Ag presenting function to specific T cell lines Ag processing by cLC occurs through chloroquine- and leupeptin-sensitive mechanisms, similarly to fLC Optimal T cell activation by cLC could be diminished by mAb against different costimulatory molecules, confirming the complexity of membrane interactions involved in Ag presentation Finally, the high efficiency of LC to present HIV-1 Ag suggests that these cells have a very important role in the generation (mucosal LC) and maintenance of T cell immunity to HIV-1 infection

Acknowledgments

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INFECTION OF CULTURED IMMATURE DENDRITIC CELLS WITH HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

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ABSTRACT

An *in vitro* culture system was developed that facilitates detailed studies of the interaction of Human Immunodeficiency Virus (HIV) with dendritic cells (DC). Cultured immature DC were generated from adherent peripheral blood mononuclear cells in the presence of GM-CSF and IL-4. These cells were non-adherent, non-phagocytic and had a veiled surface appearance. They expressed high levels of MHC class I and II proteins, CD1a, B7/BB1 and low levels of CD4, and were known to possess a potent soluble antigen presenting capacity. Upon infection with the HIV-1 strains Lai (lymphocytotropic) and BaL (monocytotropic), the viral RNA was reverse transcribed to complete DNA provirus. However the infection was non-productive as judged from measuring the activity of the virus encoded reverse transcriptase in the culture supernatant. Thus HIV infection was restricted at a step post entry.

INTRODUCTION

The interaction of HIV with DC has been proposed to contribute significantly to the deterioration of the immune system in vivo[1,2]. However, due to the lack of specific cell surface marker for human DC and their minor proportion within the population of peripheral blood mononuclear cells (PBMC), detailed in vitro studies have been technically limited. Recently DC have been obtained by differentiation of CD34 positive precursor cells in the presence of GM-CSF and TNF-alpha[3,4] and from adherent cells of PBMC in the presence of GM-CSF and IL-4[5,6,7]. These methods guarantee a relatively easy access to human DC and allow more precise investigations about their interaction with HIV. The data presented here illustrate the initial findings on the characteristics of the HIV infection of cultured immature DC.

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RESULTS AND DISCUSSION

Based on surface antigen expression (table1), several morphological and cytochemical features, and the described capacity to efficiently present soluble antigens to T-lymphocytes[5], the DC generated from PBMC in the presence of GM-CSF and IL-4 were immature and resembled Langerhans cells (LC) of the skin.

Table 1. Surface antigens of various cell types

	Monocytes	Mø/M-CSF	DC(cultured immature)	LC(skin)[1]	DC(blood)[1]
CD1a	-	-	+	+	-
CD14	+	+	-	-	-
25F9	-	+	-	nd	nd
CD4	+	+	+	+	+
HLA-DR	+	+	+	+	+
B7	+	nd	+	nd	+

1 from: Caux et al., Nature 360, 259 (1992); nd= not done

The following mouse monoclonal antibodies conjugated with FITC or PE were used: CD1a (Ortho,OKT6, IgG1), CD14 (Dianova, RMO52, IOM2, IgG2a), HLA-DR (Dianova, B8.12.2, IOT2a, IgG2b), CD4 (Dianova, 13B8.2, IOT4a, IgG1), CD3 (Dianova, UCHT-1, IOT3a, IgG1), CD19 (Dianova, J4.119, IOB4a, IgG1), BB-1/B7 (Becton Dickinson, L307.4, IgG1), 25F9 (Boehringer, IgG1, indirectly stained with a second labeled goat antibody). The samples were analysed on a FACSort (Becton Dickinson).

Susceptibility of these cultured immature DC to HIV-1 infection was tested with the monocytotropic HIV-1 BaL and lymphocytotropic HIV-1 Lai strains (fig.1). Only the control infections of lymphocytes and macrophages respectively resulted in detectable virus production. However complete provirus synthesis was detected by PCR (fig.2). Treatment with azidothymidine (AZT) prior and during the infection did not result in an HIV-specific amplification product. Because AZT inhibits provirus synthesis within infected cells, the PCR data demonstrated that cultured immature DC were infectable by both HIV-1 strains and that HIV provirus synthesis was completed within the cells.

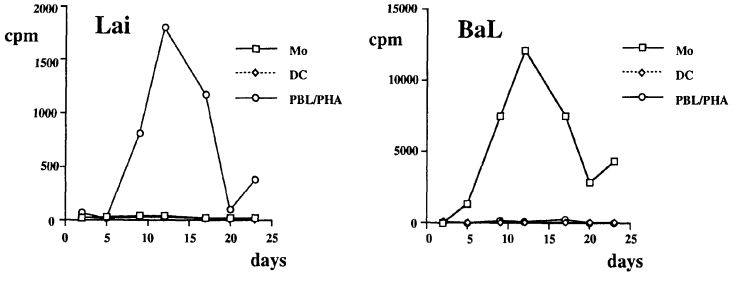

Figure 1. Kinetics of the HIV-1 infection of various cell types.
Cultured immature DC, macrophages and PHA stimulated peripheral blood lymphocytes (PBL) were infected for 2 hours with HIV-1 Lai (lymphocytotropic) and HIV-1 BaL (monocytotropic), respectively. Cells were subsequently washed and cultured in RPMI/10% FCS, L-glutamine and antibiotics, and GM-CSF/IL-4 (DC), human sera (Mø) or IL-2 (PBL). Reverse Transcriptase (RT) activity was measured from culture supernatants after [^{3}H]-thymidine incorporation for 1 hour at 37°C by Berthold-Inotech Trace-96 counter.

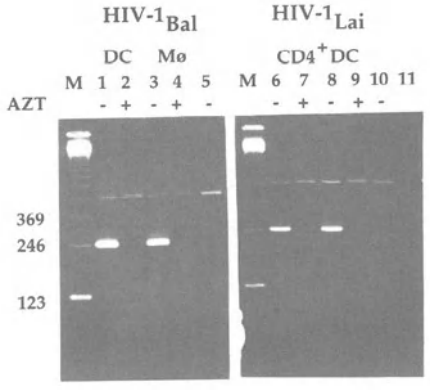

Figure 2. HIV-1 entry and provirus formation in cultured immature DC.
Cultured immature DC, macrophages and purified $CD4^+$ T-cells were infected with the HIV-1 Lai or BaL strains with and without pretreatment of the cells with AZT for 6 h at 200 μM. Cells were cultured for another 12 h, washed and used for PCR. To remove extracellular contaminating DNA, cells were incubated with DNase I (Boehringer- Mannheim) in the presence of 5 mM $MgCl_2$ for 30 min prior to lysis. PCR was carried out with HIV-1 specific primers that span the LTR/gag region.

The mechanism by which productive HIV-1 infection was restricted in immature DC is unknown. A reduced rate of provirus synthesis due to a low intracellular dNTP pool, as would be expected for nonproliferating cells, and the lack of appropriate transcription factors in this immature state of the DC, are likely possibilities. The subsequent work is concentrated on the restriction mechanism and the potential ways of HIV reactivation. The results should give a coherent view of the molecular characteristics of DC infections with HIV-1.

Acknowledgments

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DISCONTINUOUS DISTRIBUTION OF HIV-1 QUASISPECIES IN EPIDERMAL LANGERHANS CELLS OF AN AIDS PATIENT AND EVIDENCE FOR DOUBLE INFECTION

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In the present study HIV-1 quasispecies were analysed in Langerhans cells (LC) derived from eight split-thickness skin patches (B1-8) obtained from clinically normal skin taken soon after autopsy from an AIDS patient (coded RI). RI was an Italian intravenous drug user who had been HIV-1 Ab positive from 1986. Fifteen days before death, the blood $CD4^+$ and $CD8^+$ T-cell counts were 9 and 165/μl respectively, while the serum p24 titre was 73pg/ml. Purification of LC from the skin samples was carried out as previously described[1]. The LC preparations were devoid of T cells, as demonstrated by reverse transcription PCR analysis for the T-cell receptor β-chain gene constant region[2]. Immunostaining of cytospin preparations[2] failed to reveal any $CD14^+$ cells (monocytes/macrophage lineage) in purified LC fraction. Patches B1, B2, B3, B4 were disposed in a clockwise manner and derived from one thigh, while B5, B6, B7, B8 were similarly related yet from the other.

Between $1.5\text{-}2.0 \times 10^5$ LC were recovered from each sample and lysed. DNA corresponding to the hypervariable V1V2 regions of HIV-1 gp120 was amplified by nested PCR as previously described[3], the products were cloned and 20 recombinants sequenced for each of the eight skin patches.

An extraordinary complexity characterized each quasispecies. From 160 recombinant clones there were 91 unique amino acid sequences. Those present at least twice within, or between, samples are shown in Fig. 1.

Sequences A and E were the most widely distributed being present in 7 and 6 skin patches respectively (Table 1).

Beside these two, only sequences B, H and L were present in patches from both thighs. Sequence H was a remarkable outlier, differing from all other amino acid sequences in 28% of sites (Fig. 1). There was a sharp distinction between skin patches from the two thighs in terms of the number of unique sequences (44% v 70%), or the number of defective genomes (5% v 20%, identified as encoding substituted cysteine residues, stop codons and -1/+1 frameshifts), again emphasizing the fact that each skin patch harboured a unique collection of sequences. A statistical analysis allowing the comparison of two observed percentages[4] showed that the distribution of unique sequences or defective genomes between the two legs was significant ($p < 0.001$ and $p < 0.01$ respectively).

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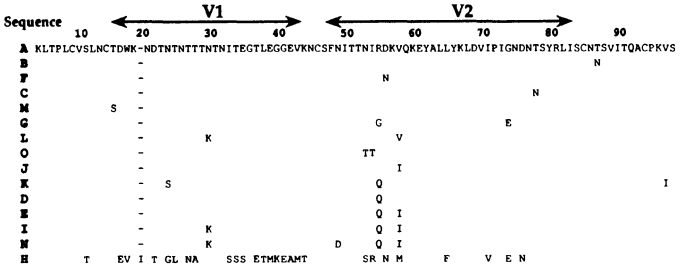
Patch	% sequence/sample															
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	UNIQUE SEQUENCES
B1	40	5	10	5	5											35
B2	15					10	10	5								60
B3	5			15	15	5			5	15						40
B4	5				30				5	5	10	5				40
B5						5			5				15	10		65
B6	20				5									10		65
B7	5														20	75
B8	5	5			10								5			75

Figure 1. LC derived HIV-1 protein sequences corresponding to the V1V2 regions of HIV-1 gp120. The sequences, in the one letter code, were aligned and gaps (-) introduced to maximise the homology. Only differences were scored, a dot indicating sequence identity. Of the 160 sequences, only those common to two or more skin patches are shown.

How can such a spatial distribution of HIV-1 sequences in Langerhans cells be understood? LC originate in the bone marrow and migrate to the epidermis via the capillaries terminating in the dermis. Were the LC precursors infected by HIV-1 in the bone marrow or during migration to the skin, a more homogenous distribution of sequences would have been expected between different skin patches, for there is no reason why an infected LC precursor cell should preferentially migrate to a given patch on one leg, or between adjacent skin patches.

Table 1. Frequency distribution of V1V2 sequences among the eight different patches B1-B8. Only sequences common to two or more skin patches are shown



The exquisite spatial partition of LC derived HIV-1 sequences might suggest that infection takes place in the skin, perhaps as the LC precursors passage through the dermis to the epidermis. Normal human dermis harbours T lymphocytes, a significant proportion of which are $CD4^+CD45RO$ memory T-cells bearing a specific skin homing receptor, the cutaneous lymphocyte-associated antigen[5]. Such T cells may be activated by antigen; those cells bearing HIV proviruses would consequently produce virions which might infect permissive LC precursors migrating to the epidermis. Alternatively, virus itself might migrate to the epidermis. Both HIV-1 proviral DNA and RNA have been detected by PCR in dermal samples from seropositive patients[6]. Thus, the spatial discontinuities in HIV-1 quasispecies might be reflecting local antigen dependent activation of HIV-1 from T cells in the dermis.

Hitherto, intrapatient sequence variation for the V1V2 regions was of the order of 5-17% [7, 8, 9]. However, among the 160 sequences, seven H-like sequences (1 in B2, 1 in B4, 2 in B5 and 3 in B6) were identified which differed from all others at 25-30% of sites. Such marked sequence divergence was indicative of two different co-circulating HIV-1 strains. If this was really the case then extensive sequence divergence should characterize all 5 hypervariable regions of the gp120 envelope sequence. Accordingly, the V3 region was amplified from B4 and B6 DNA, each sample belonging to a different thigh. Twenty recombinants per skin patch were sequenced (Fig. 2).

In both cases two distinct clusters of sequences were identified, their consensus sequences differing by 6 residues (17%). Such divergence is perfectly compatible with the notion that they were derived from a more distantly related HIV-1 strain, supporting the possibility of double infection.

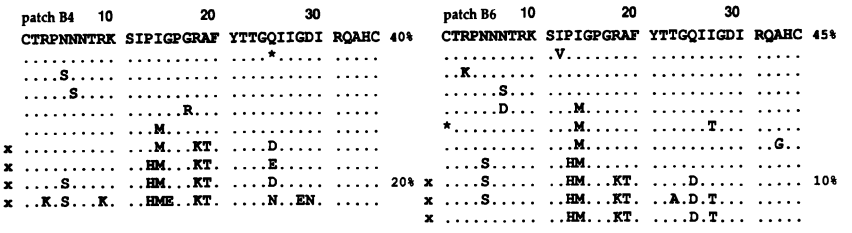


Figure 2. HIV-1 gp120 V3 loop quasispecies from purified Langerhans cells derived from the skin patches B4 and B6.

Certainly the ETMKEAMT (residues 36-43, Fig. 1) and the PIE (residues 71-73) motifs of the clone H are either non-existent or rare among V1V2 sequences, while the V3 GPGKT motif (residues 15-19, in sequences marked with an x, see Fig. 2) was totally absent from a collection of more than 410 V3 sequences from around the world[8]. Thus it is probable that the molecular clones bearing these motifs were derived from the same, or highly related proviruses, again supporting the notion of a double infection. It is not possible to formally conclude that patient RI was infected twice, although being an intravenous drug user, he had an obvious risk of multiple infection. RI could have been infected once by blood from a doubly infected individual. However double infection clearly must have occurred at some point. A number of independent experimental studies and phylogenetic analysis provides strong evidence for double infection[10, 11]. That detection is so infrequent might reflect the fact that a second infection most probably would occur in the face of a HIV-specific immune responses, so restricting viral replication[9].

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CONDITIONAL ABLATION OF DENDRITIC CELLS IN MICE: COMPARISON OF TWO ANIMAL MODELS

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INTRODUCTION

Dendritic cells (DC) knockout in mice should be a powerful mean to appreciate the role of these cells in physiological or pathological situations. In order to generate such an animal model, we used a stategy based on the DC-specific expression of a suicide gene in transgenic mice. We used the herpes simplex virus type 1-thymidine kinase (HSV1-TK) which allows conditional ablation of dividing HSV1-TK expressing cells by converting the non toxic ganciclovir (GCV) into a toxic metabolite[1,2]. DC expression of HSV1-TK in transgenic mice was attempted with the HIV-LTR promoter which had been previously shown to preferentially direct the expression of a CAT transgene in Langerhans cells[3]. We generated LTR-TK transgenic mice expressing the HSV1-TK gene under the control of the HIV-LTR promoter. We showed a low but preferential expression of the transgene in DC, leading to DC depletion in spleen and thymus following GCV administration[4]. This depletion was often associated with a thymic atrophy and a wasting syndrome. To rule out the possibility that a transgene expression leakiness in different tissues could be responsible for these pathological findings, we analysed the effects of GCV treatment in syngenic normal mice engrafted with transgenic bone marrow cells. In this situation, HSV1-TK expression is limited to hematopoietic cells.

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RESULTS AND DISCUSSION

Effects of GCV administration in LTR-TK transgenic mice

The LTR-TK transgenic mice of C57Bl/6-DBA/2 genetic background were treated with GCV for seven days. The different cell populations of the spleen were analysed with a panel of monoclonal antibodies. Whereas the proportion of monocytic and lymphoid cell populations were little modified, the DC population was severely affected since the percentage of DC dropped by 90%[4]. In the same time, the density of DC in the thymus and of skin Langerhans cells dramatically decreased as assessed by MHC class II staining on epidermal sheets. For immunological studies we backcrossed the LTR-TK transgenic mice with C57Bl/6 or DBA/2 mice. Compared to mice of C57Bl/6 genetic background, the DC depletion in mice of DBA/2 genetic background was more profond, and was associated with a thymic atrophy, the disappearence of $CD4^+CD8^+$ thymocytes, a downregulation of MHC class II expression, an enteritis and a wasting syndrome. Some of these biological and clinical perturbations ressemble those observed in stressed animals[5,6], and could be due to a GCV dependant destruction of non hematopoietic cells weakely expressing the transgene. This phenomenon was investigated with chimeric mice.

Effects of GCV administration in chimeric mice and comparison of the two animal models

In order to restrict the conditional toxin expression to hematopoietic cells, we generated chimeric mice. Normal irradiated mice were engrafted with transgenic bone marrow cells. After at least 45 days following the bone marrow transplantation, the mice were GCV treated for 7 days. This resulted in a splenic DC depletion comparable to the depletion observed in treated transgenic mice. However, the monocytic and lymphoid cell populations were unaffected, and the decrease of skin Langerhans cells density was less severe compared to GCV treated transgenic mice. Finally, thymic atrophy, downregulation of MHC class II expression, and wasting syndrome were never observed in treated chimeric mice (Table 1). Therefore, DC ablation per se was probably not responsible for the wasting syndrome and thymic modifications observed in transgenic mice.

Table 1. Biological and clinical pertubations in transgenic and chimeric mice.

	Transgenic	Chimeric
Clinical status	Wasting syndrome	Apparently healthy
Thymic atrophy	+++	+/0
Splenic atrophy	++	+
Splenic DC depletion	++++	++++
MHC class II downregulation	+++	0
Langerhans cells depletion	+++	+

These two animal models of conditional DC ablation should be useful in appreciating the functions of these cells in physiological and pathological situations. DC role in allograft rejection would be better explored with DC depleted organ or tissue obtained from transgenic mice (problem of a possible partial chimerism in bone marrow transplantated recipient mice). DC role in thymocytes selection, immune responses, lymphocyte homeostasis,... would be better explored in chimeric mice that survive GCV treatment.

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ACTIVATION OF AN HIV-LTR-CAT TRANSGENE IN MURINE MACROPHAGES BY INTERFERON-γ IN SYNERGISM WITH OTHER CYTOKINES OR ENDOTOXIN

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The initial infection with human immunodeficiency virus (HIV) and early viremia are usually caused by macrophage (MΦ)-tropic and non-syncytium- inducing HIV variants, while syncytium-inducing T cell infecting HIV arise later in the disease (1). Therefore, factors that influence the propagation of HIV within MΦs may be critical in determining the progression of AIDS. Among the cytokines frequently elevated in HIV^+ patients and capable of influencing MΦ functions are IFN-γ, IL-6, TNF-α and TGF-ß (2-5). Secondary infections with gram negative organisms common in HIV patients are probably sources of LPS. The purpose of the present investigation was to identify modulatory factors that suppress and/or activate the HIV genome within MΦs in a noninfectious animal model and to study their mechanism of action. Transgenic FVB/N mice homozygous for the LTR sequence (-453/+80) which contains the transcriptional regulatory elements of HIV linked to the bacterial gene encoding CAT as the reporter gene were used as an experimental model (6). Thus, alterations in HIV-LTR directed expression of CAT would be analogous to changes in viral expression in cells harboring integrated virus. Results from studies on the influence of biological response modifiers (BRMs) and UV-irradiation on HIV-LTR-CAT expression in MΦs and/or epidermis indicate the usefulness of this animal model (6-9), particularly when compared with those obtained with HIV-LTR (with or without Tat) transfected human MΦs (10).

Our findings indicate that IL-6 or LPS, when added alone, cause a moderate enhancement of HIV-LTR directed CAT expression (Fig. 1). The effect of IL-6 is not abolished by addition of polymyxin B, whereas LPS activity is blocked (data not shown). In contrast, when added alone, IFN-γ or TNF-α cause no significant enhancement of CAT expression. Despite this, IFN-γ acts synergistically with each of the other three BRMs to upregulate HIV-LTR directed CAT expression. Similar results are obtained with both fresh and precultured MΦs (not shown). IFN-γ primes MΦ for the subsequent induction of LTR directed CAT expression mediated by signals provided by exposure to LPS, IL-6 or TNF-α. The sequence of signals is important for maximum response, as the reverse sequence of addition of these BRMs is either much less (IL-6) or not at all (LPS, TNF-α) effective (Table 1). The signals initiated by IFN-γ within the cells are rapid ones, occurring in $< 1.5 - 3$ h. There is a striking similarity between the nature and the sequence of

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Table 1
PRIMING EFFECT OF rIFN-γ FOR CYTOKINE/LPS-INDUCED ACTIVATION OF LTR-CAT

Additions to Medium During*		% Acetylation**	p***
Priming	Activation		
IFN	None	0.32 ± 0.64[a]	NS
IFN	LPS	8.11 ± 3.06[a]	<0.0001
LPS	IFN	-1.07 ± 0.57	
IFN	IL-6	8.13 ± 3.07[b]	<0.002
IL-6	IFN	2.22 ± 0.26[b]	0.001
IFN	TNF-α	3.77 ± 0.95[c]	<0.0005
TNF-α	IFN	0.25 ± 0.67[c]	NS

* Macrophages were "primed" (3 h) in either AIM-V medium alone or with murine rIFN-gamma (300 U/ml), rIL-6 (100 U/ml), or rTNF-alpha (1000 U/ml). The cells were extensively rinsed with warm medium and incubated (48 h; 37°C) in AIM-V supplemented as indicated. LPS was used at 10 ng/ml.

** The data are expressed as the percentage of chloramphenicol acetylated by 0.1 mg of cell lysate protein in the experimental group minus the medium control (1.32 ± 0.51). Each value is the mean ± SD of at least two separate experiments each containing duplicate or triplicate samples/group.

*** P values were determined by Student's t-test for the comparisons with cells incubated in medium alone throughout (None, None). Values with the same footnote designation were compared: a) $p = < 0.0001$; b) $p = 0.022$; c) $p = 0.0009$.

Table 2
SUPPRESSION OF HIV-LTR ACTIVATION IN MACROPHAGES

Conditions During Incubation Periods			% Acetylation*	p vs
1.5 h Pre-Incubation	18 h Priming	48 h Activation	± SD	100% value**
Medium	IFN	Medium	-0.27 ± 0.50	
Medium	IFN	LPS	8.83 ± 2.62 (= 100%)	
Medium	IFN	LPS + TGF-β	7.95 ± 1.68	NS
Medium	IFN + TGF-β	LPS	7.55 ± 1.81	NS
TGF-β	IFN + TGF-β	LPS	2.33 ± 1.20	< 0.0001
TGF-β	IFN + TGF-β	LPS + TGF-β	2.49 ± 1.37	0.0001
Medium	IFN	LPS + NAC	1.91 ± 1.53	0.0005
Medium	IFN + NAC	LPS	2.01 ± 1.55[a]	0.0005
Medium	IFN + NAC	LPS + NAC	0.71 ± 0.53[a]	<0.0001

* Macrophages were incubated in AIM-V medium, supplemented as indicated (rIFN-gamma: 300 U/ml; NAC 30 mM; LPS 10 ng/ml; TGF-β_1 100 ng/ml). After "priming" (18 h, 37°C) the cells were extensively rinsed with warm medium and further incubated ("activation stage", 48 h, 37°C). A 1.5 h incubation between priming and activation with or without TGF-β made no difference to the results. The data are expressed as the amounts of chloramphenicol acetylated by 0.1 mg of cell lysate protein in experimental group minus the medium control. Each value is the mean ± SD of at least two separate experiments each comprising duplicate or triplicate samples/group.

** Comparisons between each group and cells in IFN and LPS (100% control group) were made employing the Student's t-test. Values with the same footnote designation were also compared: a) $p = 0.05$.

stimuli leading to activation of HIV-LTR directed CAT expression and to the induction of cytotoxic and microbicidal activity in MΦs.

Thus, the activation of HIV-LTR-CAT is minimally a two-step phenomenon, that of priming followed by activation. These steps act synergistically, but are independently controlled. TGF-ß inhibits IFN-γ + LPS induced transcription of LTR-directed CAT by suppressing the priming rather than the activation step. Fresh MΦs are sensitive to the suppressive effects of TGF-ß only if they are exposed to it prior to contact with IFN-γ. It is possible that this effect of TGF-ß may in part be due to its known ability to influence the expression of IFN-γ receptors. TGF-ß is without effect on the activation step (Table 2). In contrast to TGF-ß, N-acetyl-L-cysteine (NAC) appears to mediate its effect on the activation step. NAC (30 mM) inhibits CAT expression almost completely, whether present throughout or when added only during the LPS-mediated activation step. In a dose-responsive manner, NAC at 10 and 20mM is also significantly inhibitory (not shown). It is likely that NAC enters the cells and cannot be washed out. It therefore remains within the cells throughout the activation step even when added only during priming. However, its effect is

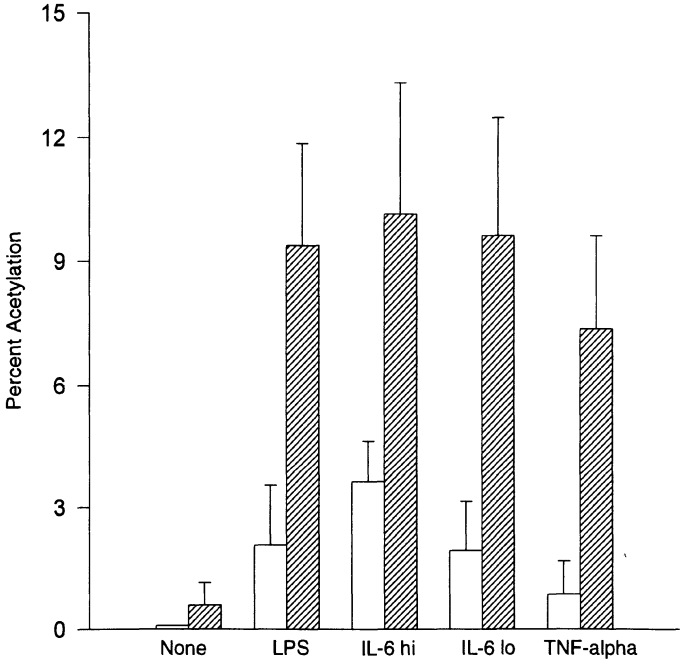

Fig.1. **Synergistic effects of rIFN-gamma and other biological response modifiers (BRMs).** Fresh macrophages were incubated (48 h; 37°C) in AIM-V medium ± 300 U of murine IFN/ml, supplemented as indicated (Hi IL-6 = 1000 U, Lo IL-6 100 U, and rTNF-alpha 1000 U/ml). The data are expressed as the percentages of chloramphenicol acetylated by 0.1 mg of cell lysate protein in the experimental group minus the medium control (1.65 ± 0.63). Each value is the mean ± SD of at least two separate experiments, each containing duplicate or triplicate samples/group. The statistical significance of the effect of BRMs alone, as determined by comparisons with macrophages cultured in medium only, was: LPS $p = 0.04$; IL-6 Hi $p < 0.001$; IL-6 Lo $p < 0.03$; IFN and TNF-alpha not significant (NS). The significance of the effect of added IFN, as determined by comparisons with macrophages cultured under similar conditions but without IFN, was: + LPS $p = 0.002$; + IL-6 Hi $p < 0.008$; + IL-6 Lo $p < 0.003$; + TNF-alpha $p < 0.002$.

significantly greater when added throughout than when added only during priming ($p = 0.05$), and it is also effective when added only during the activation stage.

The present results suggest that the regulation of HIV expression in MΦs is complex and that BRMs have various influences that depend on previous exposure of the cells to other BRMs. Obviously, neither of the viral regulatory proteins Tat or Rev are necessary to obtain a significant activation of HIV-LTR directed CAT transcription in murine MΦs.

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DENDRITIC CELLS, APOPTOSIS AND MURINE RETROVIRUS

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INTRODUCTION

Apoptosis is a universal form of "programmed" cell death that appears to have a key role in the development and regulation of the immune system[1]. Induction of the apoptotic pathway is a complex event, but in most instances requires an initial stimulus. Such a stimulus can include the removal of key growth factors, stimulation of immature T cells or inappropriate activation signals. Both activated and immature T lymphocytes appear to undergo apoptosis when stimulated through the T cell receptor, indicating that a combination of the stimulatory events and the state of maturation of the T cells is important in determining a cell's outcome.

Dendritic cells are potent stimulators of T lymphocytes. When infected with the Rauscher leukaemia virus (RLV), these cells have a reduced stimulatory capacity[2], which is similar to that observed for HIV infection of human dendritic cells. Consequently the immune changes associated with RLV infection provide a useful model for studying aspects of HIV infection. The observation that follicular dendritic cells protect germinal centre B lymphocytes from apoptotic death[3] and the concept that apoptosis of T cells has an important role in the immune system[4] suggests that an investigation into the role of dendritic cells in the apoptosis of T lymphocytes is warranted. In preliminary experiments we determined that T cells undergo apoptosis *in vitro* when cultured in the absence of any growth factors and that this apoptosis was reduced in the presence of allogeneic and syngeneic dendritic cells. To understand further the role of dendritic cells in apoptosis, and to classify in more detail the defects of retrovirus infected dendritic cells, we investigated the induction of apoptosis in normal and activated T cells in the presence of dendritic cells and analysed the ability of RLV infected dendritic cells to protect from (or induce) apoptosis of allogeneic and syngeneic T cells.

MATERIALS AND METHODS

Apoptosis identification and analysis

DC ($1x10^4$) from RLV infected or control BALB/c mice were co-cultured with either normal syngeneic (BALB/c) or normal allogeneic T cells (C57BL/10) or activated allogeneic T cells (C57BL/10) at a ratio of 1:100. T lymphocytes alone were set up in parallel. These cultures were incubated for 24 and 72 hours after which time cells were fixed sequentially in 1% formaldehyde in PBS followed by 100% ethanol. Quantitation of the number of cells in apoptosis was performed by detecting single strand breaks in DNA using an in situ nick translation technique[5]. The

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formaldehyde and ethanol fixed cells were washed with nick translation buffer (50mM Tris-HCl, pH 7.8, 5mM $MgCl_2$, 0.1M β-mercaptoethanol, 10 mg/ml BSA) and resuspended in 10 µl of nick translation buffer. To this suspension 1.3 µl dATP, dCTP, dGTP (each 0.2mM; Perkin-Elmer-Cetus, USA), 1.6 µl nick translation buffer containing 0.3 µl 1mM biotinylated-11-dUTP (Sigma) and 1 unit Escherichia coli DNA polymerase (Sigma) was added and incubated at 15°C for 90 minutes to repair the broken DNA strands. To detect these breaks, 40 µl avidin-FITC (2.5mg/ml) was added for 30 minutes at room temperature to allow the avidin-FITC to bind to the biotinylated-11-dUTP that had been incorporated into the cellular DNA. Immediately prior to analysis on a FACScan (Becton Dickinson) propidium iodide (PI) was added at a final concentration of 10mg/ml.

Apoptotic cells were identified as FITC and PI positive cells. The FITC detected cells with damaged DNA and the PI was required to exclude small apoptotic bodies with low levels of DNA.

Dendritic cell and T cell preparations

Lymph node cell suspensions were layered onto 2ml metrizamide (14.5g plus 100ml medium) (RPMI-1640, Dutch modification, Flow Laboratories, Irvine, Ayrshire, UK, with 100 IU/ml penicillin, 0.1 mg/ml streptomycin, $1x10^{-5}$M β-mercaptoethanol and 10% foetal calf serum) and centrifuged for 10 min at 600g. Dendritic cells at the interface were collected, washed once and resuspended in medium.

Pelleted cells from the lymph node suspensions were passed through nylon wool columns to obtain T cells.

Rauscher infection of dendritic cells

BALB/c mice were inoculated intravenously (tail vein) with 0.1 ml either of saline (controls) or of suspension containing RLV. Mice were sacrificed 21 days after inoculation and RLV infection of the dendritic cells was confirmed by polymerase chain reaction[6].

Separation of T cells from DC-T cell clusters

At the conclusion of the 72 hour culture the cell suspensions, in 1 ml, were very gently layered on top of 10 ml of a 20% FCS in medium solution (in a 30 ml tube) and allowed to settle for 30 minutes at 4°C. The top 2 ml was collected as the non-clustering T cells and the pellet (2 ml) was collected as the clustering T cells. The intermediate fraction was discarded.

Allogeneic activated T cells

BALB/c dendritic cells (10^5) were cocultured with C57BL/10 lymph node cells (10^7) in 10 ml culture medium in 25 cm^2 culture flasks for 5 days after which a second round of dendritic cell stimulation was performed by adding a further 10^5 BALB/c dendritic cells and supplemented with 10U/ml recombininant IL2. This culture was monitored for a minimum of 20 days with continuous IL2 maintenance.

RESULTS AND DISCUSSION

In the absence of growth factors, or stimulation, purified T lymphocytes were found to die by apoptosis; this apoptotic death was significantly reduced in the presence of allogeneic DC and slightly reduced in the presence of syngeneic DC (Table 1). Dendritic cells provide a powerful stimulus for T cell activation but when DC are infected with the Rauscher leukaemia virus they fail to induce significant proliferation[2]. An explanation for this lack of stimulation and proliferation could be that the infected dendritic cells activated the apoptotic pathway. An induction of apoptosis instead of proliferation, could also provide an explanation for the loss of $CD4^+$ cells in HIV patients. As shown in Table 1, RLV infected DC did not induce apoptosis in allogeneic or

Table 1. Percent apoptosis of T cells from allogeneic or syngeneic DC-T cell cultures

	Allogeneic T cells (BALB/c DC + C57B10 T cells)				Syngeneic T cells (BALB/c DC+ BALB/c T cells)			
	Exp I	Exp II	Exp III	Exp IV	Exp I	Exp II	Exp III	Exp IV
T cells alone	63	72	67	63	53	54	61	53
T cells + DC *	49	48	63	49	33	48	62	34
T cells + DC** (RLV infected)	43	39	60	43	38	39	51	38

Percent apoptosis of T cells following a 72 hour culture either alone (T cells alone); cultured with normal allogeneic or syngeneic dendritic cells (T cells + DC) or cultured with Rauscher leukaemia virus (RLV) infected allogeneic or syngeneic dendritic cells (T cells + DC-RLV infected)
* $p< 0.005$ T cells + allogeneic DC compared to T cells alone (paired t-test)
* $p=0.12$ (NS) T cells + syngeneic DC compared to T cells alone (paired t-test)
**$p< 0.005$ T cells + allogeneic RLV infected DC compared to T cells alone (paired t-test)
**$p< 0.005$ T cells + syngeneic RLV infected DC compared to T cells alone (paired t-test)

Table 2. Percent apoptosis of T cells isolated from DC-T cell clusters

	Allogeneic T cells (BALB/c DC + C57B10 T cells)		Syngeneic T cells (BALB/c DC+ BALB/c T cells)	
	Exp I	Exp II	Exp I	Exp II
T cells alone	68	64	61	63
Clustered T cells	26	31	44	23
Non-clustered T cells	28	35	42	27

Statistics were not performed due to the low number of experimental groups

Table 3. Percent apoptosis of allogeneic activated T cells following restimulation by DC

	24 hour culture*			72 hour culture**		
	Exp I	Exp II	Exp III	Exp I	Exp II	Exp III
activated T cells alone	4	2	29	13	7	25
activated T cells + allogeneic DC	4	5	34	20	20	30

There was no significant difference between activated T cells + allogeneic dendritic cells compared to activated T cells alone after 24 hours** ($p=0.2$) and 72 hours** ($p=0.07$).

syngeneic T cells. In fact, RLV infected DC were as effective as normal DC in protecting T cells from *in vitro* induced apoptosis. The protective effect was found to be greater with allogeneic DC than with syngeneic DC, which correlated to a greater stimulation. Upon further analysis it was revealed that, even though apoptosis of T cells was reduced in the presence of allogeneic and syngeneic DC, there was still a background level suggesting that some cells could have been induced to undergo apoptosis. By analysing the T cells isolated from DC-T cell clusters it couldn't be confirmed that a continuous contact between the DC and T cells was essential for the protection of apoptosis (Table 2). It is possible that T cells could have received a signal from the DC during cluster formation and that these T cells disaggregated from the cluster as single cells. Preliminary electron microscopic analysis of the DC-T cell clusters provided evidence that the majority of T cells within the clusters were not apoptotic. Occasionally an apoptotic cell was observed in a cluster (data not shown) and it is feasable that during the process of T cell activation some T cells became apoptotic. To evaluate this further, and, as it has been shown that secondary stimulation of activated T cells via the T cell receptor with e.g. phytohaemagglutinin, anti-CD3 antibodies[7], or superantigen[8,9] induced apoptosis, we investigated T cells which had previously been activated by allogeneic stimulation. When allogeneic activated T cells were cultured with DC a small, but significant increase in apoptosis was detected following 72 (but not 24) hours of culture (Table 3). This increase was much less than that previously reported for the above mentioned restimulation systems. It would therefore appear that DC primarily provide stimulatory signals, in the absence of apoptotic signals, and that RLV infected DC do not cause apoptosis.

CONCLUSIONS

Dendritic cells infected with RLV failed to induce apoptosis of T cells. An implication is that the loss of $CD4^+$ cells in HIV infected patients is unlikely to be due to inappropriate signals from the infected dendritic cells. In general, dendritic cells normally provide signals which protect T cells from apoptosis and it is more than likely that it is the nature of the T cell rather than the DC that determines the induction of apoptosis.

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EARLY EVENTS IN CONTACT SENSITIVITY

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For many years, contact sensitivity has served as a very useful model for defining constituents of the skin immune system and for understanding the dynamics of the afferent and efferent limbs of various forms of delayed-type hypersensitivity reactions. A precise understanding of the mechanisms involved in these types of reactions would provide a more sound basis for modulating certain types of immunological, inflammatory, infectious and neoplastic diseases.

Considerable research has indicated the critical role of Langerhans cells in the induction of contact sensitivity and other forms of delayed-type hypersensitivity reactions[1]. In non-sensitized mice, after interaction with antigen, Langerhans cells, the antigen presenting dendritic cells of the epidermis, migrate from skin to regional lymph nodes and present the antigen to naive T cells. For the past several years, we have been studying the molecular mechanisms involved in these early events in the induction of contact sensitivity. In this review, we will summarize findings that demonstrate that in the very early phase of contact sensitivity 1) Langerhans cells are activated after hapten painting, and 2) Langerhans cell-derived and certain keratinocyte-derived cytokine mRNAs are selectively enhanced after interaction with allergens. Furthermore, our most recent studies indicate that 3) in contact sensitivity, hapten-specific CD4+ T cells are triggered by hapten-peptide complexes that are formed either by direct interaction of the hapten with peptides anchored to MHC class II proteins or after processing of extracellular or membrane associated haptenated proteins.

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Activation of Langerhans cells after allergen painting

Within 24 hours of the application of allergens such as di- or tri-nitrochlorobenzene (DNCB and TNCB), epidermal Langerhans cells enlarge, become more dendritic and exhibit enhanced expression of MHC class II molecules[2]. These changes do not occur with irritants or with the vehicles alone (without hapten). The upregulation of MHC class II molecules is accompanied by more potent immunological function of Langerhans cells as evidenced by their induction of enhanced syngeneic, allogeneic and antigen-specific T cell responses compared to that induced by Langerhans cells obtained from control-treated mice[2]. These phenotypic and functional changes of Langerhans cells are consistent with those seen when Langerhans cells are cultured for short periods of time[3,4]. Taken together, these findings suggest that after hapten painting, Langerhans cells become activated and migrate to regional lymph nodes where they induce primary immune responses[5].

Allergen induction of Langerhans cell- and keratinocyte-derived cytokines

As keratinocytes, the major subpopulation of epidermal cells, are a rich source of cytokines and growth factors[6], we hypothesized that haptens may cause Langerhans cell activation through cytokine (or growth factor) induction. We used an RT-PCR technique in combination with liquid hybridization to quantitatively assess cytokine mRNA signals. Signal strength at various times after hapten painting was compared to the baseline of each individual mRNA.

RNA was extracted from the epidermis - the pattern of mRNA production was clearly different when signal strength (of a large group of cytokine and growth factor mRNAs) after allergen painting was compared to that after vehicle, irritant, or tolerogen painting. Whereas IFN-γ, TNF-α and GM-CSF were found to be upregulated even after nonspecific stimuli (including tolerogens and irritants), only contact sensitizers like TNCB and DNCB upregulated IL-1α, IL-1β, MIP-2, IP-10, or I-Aα mRNA[7]. Signal strength for IL-1β and TNF-α mRNA increased within 15 minutes and 30 minutes, respectively, of hapten painting and both reached their peak strength (more than 10-fold upregulation) at 2-4 hours after skin painting. The strength of IL-1α increased more than 10-fold at 2 hours when it reached its peak. In all of these experiments upregulation of I-Aα mRNA was used as the positive control since we assumed that this would precede detection of upregulation of Langerhans cell surface expression of class II molecules.

The source of the mRNA signals was determined by depleting epidermal cell populations of Langerhans cells, Thy 1+ dendritic epidermal cells, and infiltrating T cells. Langerhans cells were the primary source of the IL-1β and class II I-Aα mRNA, whereas keratinocytes were the primary source of the IL-1α, TNF-α, IP-10, and MIP-2 and infiltrating T cells were the source of the IFN-γ[7]. The findings indicate that haptens that are capable of inducing allergic contact sensitivity cause selective changes in the signal strength of the mRNAs of several cytokines. The earliest of these changes is seen in the IL-1β mRNA signal

strength that is increased as early as 15 minutes after hapten painting.

To determine whether the enhanced IL-1β production could be linked to the activation of Langerhans cells and to the induction of contact sensitization, we injected IL-1β into the ears of mice and extracted total epidermal RNA 4 hours later. IL-1β injection resulted in a 5- to 100-fold enhancement of mRNA signals for IL-1α, IL-1β, MIP-2, TNF-α and class II I-Aα as well as enhancement of Langerhans cell MHC class II expression, mimicking the changes induced by allergens[8]. Furthermore, Langerhans cells taken from IL-1β-injected skin were 2 to 3 times more potent accessory cells in an anti-CD3 proliferation assay than Langerhans cells from IL-1α or sham injected skin.

Further studies demonstrated the induction of keratinocyte-derived IL-10 mRNA and protein after hapten (as compared to irritant or vehicle) application to skin[9]. Since IL-10 has been shown to be immunosuppressive via inhibition of antigen presenting cell function, we determined the effect of IL-10 on the antigen presenting function of Langerhans cells and found that IL-10 markedly diminished the proliferation of Th1, but not Th2 clones, when Langerhans cells were used. The IL-10 suppressive effects were most pronounced when the Langerhans cells were preincubated with IL-10 for at least 24 hours. The inhibitory effects of IL-10 were independent of antigen processing by Langerhans cells since they were seen with both protein- and peptide-pulsed Langerhans cells[10]. More recent studies by Enk et al have shown that IL-10 is capable of inducing hapten-specific tolerance in vivo[11].

Taken together, our studies have demonstrated that early in the induction of contact sensitivity there is a rather specific pattern of cytokine production by both keratinocytes and by Langerhans cells. Langerhans cell-derived IL-1β appears critical in directing primary immune responses towards sensitization whereas keratinocyte-derived IL-10 inhibits the induction of contact sensitivity and may even induce specific immunological tolerance. Further studies should elucidate those factors involved in determining the ultimate outcome of this delicate balance. This information would certainly help in the development of strategies that may be used to modulate certain types of inflammatory, infectious and even neoplastic diseases.

Characterization of the epitopes generated after hapten painting

CD4+ T cell responses to protein antigens are dependent on T cell receptor antigen recognition of small peptides that bind the MHC class II groove. Little is however known about the precise epitopes recognized by hapten-specific CD4+ T cells. To better understand the nature of these epitopes, we assessed the ability of TNP-modified class II binding peptides to activate T cells obtained after in vivo induction of contact sensitivity. Using Langerhans cells as the antigen presenting cells and (haptenated or non-haptenated) 10mer synthetic peptides of hen egg lysozyme that were known to bind to I-A^k, we found that in vivo primed CD4+ T cells proliferated preferentially to peptides that were derivatized at certain amino acid positions[12]. Other derivatized peptides and other strains of mice were also utilized in these studies. The results indicate that hapten-modified MHC class II binding peptides are

recognized by hapten-specific CD4+ T cells and that precise positioning of hapten molecules on peptides binding MHC class II molecules is required for optimal CD4+ T cell recognition. The hapten-peptide complexes are probably formed either by direct interaction of the hapten molecule with peptides anchored to MHC class II proteins or after uptake and processing of extracellular or membrane-associated haptenated proteins. Knowledge of the precise position of the hapten molecule on the peptide may facilitate the production of blocking peptides that may be used to alter certain types of hapten-specific immunological responses.

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DENDRITIC CELLS CAN BE USED AS PHYSIOLOGICAL ADJUVANT IN VIVO

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The first step in the induction of an immune response is the presentation of the antigenic peptide in the MHC groove, a step which is required to activate MHC-restricted T helper cells. Although they represent a minor subset among the antigen presenting cells, the dendritic cells (DC) have the unique property to activate antigen-specific, naive T cells and seem to play an essential role in initiating immune responses[1].

ANTIGEN-PULSED DC CAN EFFICIENTLY INDUCE AN ANTIBODY RESPONSE IN VIVO

The primary immunization of mice is classically performed by injecting the antigen emulsified in external adjuvant. However, we and others have shown that it was possible to prime mice using elements of the immune system, and in particular DC. Inaba et al. have indeed reported that a single injection of DC, pulsed with antigen, into syngeneic mice activated antigen-specific, MHC-restricted T cells[2]. To induce an humoral response using the same experimental system, we injected a low dose of soluble antigen 5 days after the priming with antigen-pulsed DC, in order to activate B cells which recognize the unprocessed antigen. Our data show that syngeneic DC, which have been pulsed *in vitro* with the antigen, induce a strong antibody response in mice, whereas B cells do not prime the mice in the same conditions[3].

The injection of soluble antigen is not necessary for all antigens used: for example, the injection of human gamma globulins (HGG) pulsed DC alone results in high antibody

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response (unpublished results). By contrast, the immunization to other proteins, like BCL1 IgM, myoglobin or conalbumin, requires the injection of pulsed DC and an antigen boost 5 days later (data not shown). This observation could be due to the binding of HGG in a native form to FcR on the surface of the injected DC, whereas antigens which do not bind FcR may be present in a processed form only.

Of note, the injection of antigen-pulsed DC induces the synthesis of isotypes similar to the immunoglobulin classes detected after immunization with the same antigen emulsified in complete Freund's adjuvant; i.e.specific IgM, IgG1, IgG2a and IgE antibodies[3]. In particular, high amounts of IgG2a antibodies are produced, suggesting that interferon-γ producing T helper cells (Th1-type) have been activated *in vivo*. Recent data from our laboratory show indeed that an injection of antigen-pulsed DC into the rear footpads results in priming of the afferent lymph node cells which produce interferon-γ upon *in vitro* restimulation with the antigen (not shown).

To compare the priming of mice using the 3 major populations of APC, able to activate class II restricted $CD4^+$ cells, we injected HGG-pulsed DC, resting B cells or peritoneal macrophages, and 5 days later 5 μg of soluble HGG. Our data[4] (and G. De Becker et al., these proceedings) show that DC and macrophages, but not B cells, prime the mice. The humoral response, however, is characterized by distinct isotypic profiles: the synthesis of high levels of antigen-specific IgG1 and IgE, but very low levels of IgG2a in macrophage-injected mice, suggests that Th2-type cells are activated by peritoneal macrophages.

DENDRITIC CELLS PULSED WITH A TUMOR ANTIGEN INDUCE TUMOR RESISTANCE IN VIVO

The same immunization protocol was applied to a tumor model in mice. We used the BCL1 lymphoma expressing high levels of idiotype (IgM, λ) which can be considered to bear tumor-associated antigens. We have shown that the majority of mice, immunized with syngeneic dendritic cells pulsed *in vitro* with the IgM, displayed an antiidiotypic antibody response and were protected against a subsequent injection of a lethal dose of lymphoma cells[5]. These data demonstrate that effector cell(s) able to confer tumor protection can be activated in the animals and suggest that the lack of an efficient immune response in tumor-bearing animals could be due to inadequate antigen presentation. The mechanism of protection remains to be determined. Experiments are underway to characterize the effector cells ($CD4^+$ helper cells, cytotoxic $CD8^+$ cells or antibody-producing cells) able to prevent tumor growth, as well as to determine the conditions leading to protection to an established tumor, and in particular the optimal Th1/Th2 balance. The induction of tumor resistance *in vivo*, using elements of the immune system, should theoretically offer a non toxic form of therapy, specific for the tumor cells and characterized by an anamnestic response which would prevent cancer resurgence.

B7.1 AND B7.2 MOLECULES ARE UPREGULATED DURING MATURATION IN VITRO

The initiation of an immune response requires two signals from the antigen presenting cells: an antigenic signal, i.e. the presentation of the peptide in the groove of MHC molecules (signal 1), and a second signal which determines the outcome of T cell receptor occupancy

(costimulatory signal). Schwartz and his collaborators have shown that the presentation of an antigen to T cell clones in the absence of the second signal could induce a state of unresponsiveness to this antigen[6]. Therefore, the MHC/peptide complex is necessary but not sufficient to induce an immune reaction, and the amplitude of the response induced is dependent on the presence or absence of the costimulatory signal. Several reports have shown that dendritic cells constitutively expressed the best defined costimulatory molecules: B7-1 and B7-2[7,8]. The constitutive expression of costimulatory signal(s) required to optimally activate T cells would have explained why dendritic cells are the only APC able to trigger naive T cells. However, the analysis of purified dendritic cells may not reflect the reality *in vivo*, since dendritic cells present two distinct stages of maturation in culture: fresh DC actively process antigens but weakly activate resting T cells, whereas mature DC have lost the capacity of processing while acquiring the ability to activate naive T cells[2]. We therefore compared the expression of B7 molecules on fresh (day 0) versus cultured (day 1) dendritic cells. Our data in Table 1 show that the expression of B7-1 and mainly B7-2 was upregulated during culture and to a lesser extent the expression of class II and ICAM-1 molecules. A similar increase in B7 expression was observed on dendritic cells from athymic mice, showing that this phenomenon is independent of T cell activation (data not shown). Since there is evidence that surface expression of B7 on B cells and macrophages requires their activation[9,10], the distinct regulation of B7 on DC versus B cells and macrophages may explain why DC are the only APC able to sensitize resting T cells, which themselves could not induce costimulatory activity in B cells or macrophages. Thus, although fresh DC express low, but significant levels of B7, the costimulatory signal may be upregulated at least *in vitro,* in the absence of external stimuli.

Of note, the addition of neutralizing anti-GM-CSF antibodies in the overnight culture results in decreased B7 expression, as compared to dendritic cells cultured in the absence of antibodies, suggesting that GM-CSF is required for optimal dendritic cells maturation (Table 1).

Table 1. Upregulation of B7.1 and B7.2 expression on dendritic cells during maturation in culture.

	control	CTLA4-L	B7.1	B7.2	CD54	I-E^d
DC day 0	6	40	13	5	110	61
DC day 1 + -	20	1106	20	96	202	196
DC day 1 + a-GM-CSF	14	654	21	47	211	193

Two color immunofluorescence analysis: cells from DBA/2 mice [either low density spleen cells (DC day 0) or purified dendritic cells incubated during maturation with (DC day 1 + a-GMCSF) or without anti-GM-CSF (DC day 1 + -)] were doubled stained for red fluorescence with N418-biotin followed by phycoerythrin-conjugated streptavidin and for green fluorescence with either goat anti-human (control), CTLA4-Ig + goat anti-human (CTLA4-L), 1G10 (anti-B7-1), 2D10 (anti-B7-2), anti-CD54 (CD54) or 14.4.4 (I-E^d), coupled to fluorescein. The analysis was performed on N418 positive cells (gate on N418 positive cells) and the data are expressed as mean of fluorescence intensity.

MODULATION OF DC FUNCTION IN VIVO

Since DC pulsed extracorporeally with a protein antigen induce a strong humoral response when injected into syngeneic animals, we tried to target an antigen *in situ* on DC, using two approaches. First, mice received sequential injections of biotinylated anti-DC N418 mAb, streptavidin 20 h later and HGG coupled to biotin 4 h later. FACS analysis revealed the presence of N418 on DC 20 hours after injection (not shown). Our data[11] showed that these mice displayed a strong humoral response, although lower that the antibody response of mice primed with the antigen emulsified in complete Freund's adjuvant. More importantly, the immunotargeting *in situ* induced the secretion of high levels of IgG1 antibodies but very low levels of specific IgG2a antibodies, suggesting that mainly Th2-type cells were induced to differentiate in these animals. In a second approach, we injected HGG in Langerhans cells-rich tissues, i.e. subcutaneously[12]. This protocol induced a strong antibody response, of Th2-type. Based on the assumption that optimal activation of Th1 cells requires fully matured (cultured?) DC, we conclude from this experiment that the maturation of DC, if occurring *in vivo*, most probably requires the antigen and an additional signal. Table 2 summarizes various protocols of immunization to HGG and the type of immune response induced. Importantly, 3 types of immunization appear to induce a Th1-type response: the injection of the antigen emulsified in complete Freund adjuvant, the administration of HGG pulsed on DC during maturation in culture, or the inoculation of antigen in saline supplemented with 5 daily injections of IL-12 (Heynderickx et al., manuscript in preparation), which has been shown to promote Th1 development[13]. Therefore, we favor the hypothesis that only fully mature DC induce an optimal Th1 response, and that the maturation can be induced *in vivo* by elements of complete Freund's adjuvant. The factor(s) provided by DC which are required for sensitization of Th1-type cells could include IL-12, since this lymphokine alone with the antigen is sufficient to drive a strong Th1-type response.

Table 2. Summary of humoral responses induced by various modes of immunization.

Antigen	route	antibodies	IgG1	IgG2a
HGG-DC	i.v.	+++	+++	++
HGG-Mac	i.v.	+++	+++	-
HGG-Br	i.v.	-	-	-
sol. HGG	i.p.	+	+	-
	s.c.	++	++	-
HGG CFA	i.p.	+++	+++	++
	s.c.	+++	+++	++
N418-HGG	i.v.	++	++	-
HGG+IL12	i.p.	+++	+++	++

Groups of mice were immunized by injection of : 3 x 10^5 DC pulsed *in vitro* with antigen (HGG-DC), 3 x 10^5 peritoneal macrophages pulsed *in vitro* with HGG (HGG-Mac), 10^6 resting B cells pulsed *in vitro* with HGG (HGG-Br), 100µg of HGG in saline (sol HGG), 100µg of HGG emulsified in complete Freund's adjuvant (HGG-CFA), biotinylated N418 mAb followed 20h later with avidin and HGG coupled to biotin 4h later (N418-HGG), or 100 µg of soluble HGG on day 0 with daily injections on days 0 to 4 of 0.2 µg IL-12 (HGG+IL-12). The injections were administered intravenously (i.v.), intraperitoneally (i.p.) or subcutaneously (s.c.).

CONCLUSION

The potent "adjuvant" capacity of DC *in vitro* and *in vivo* correlates with the spontaneous upregulation of B7-1 and B7-2 molecules upon maturation in culture, in the absence of intentional stimulation. Although DC maturation *in vivo* is still a matter of speculation, some factors like complete Freund's adjuvant, GM-CSF, TNFα, IL-1, together with the antigen, could induce increased immunostimulatory properties of these cells, and in particular would confer to them the capacity to promote Th1 development. The identification of the factors inducing DC maturation *in vivo* would lead to optimalisation of immune responses and open new strategies in development of new vaccines

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DENDRITIC CELLS SECLUDE *LEISHMANIA* PARASITES THAT PERSIST IN CURED MICE - A ROLE IN THE MAINTENANCE OF T-CELL MEMORY?

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INTRODUCTION

In humans, infections with *Leishmania* parasites range from self-healing cutaneous to uncontrolled diffuse cutaneous disease and from subclinical to fatal visceral disease. The clinical symptoms depend not only on the parasite species but also on the efficiency of the host's immune response that is influenced by genetic factors or by infections with other pathogens. For example, patients with human immunodeficiency virus infection may display severe visceralization of *Leishmania* parasites which, in other subjects, show characteristics of low virulence and are confined to the skin[1]. The disease profiles seen in humans can be mimicked by experimental infection of mice with *Leishmania major*, one of the causative agents of cutaneous leishmaniasis in man. Mice of resistant inbred strains, such as C57BL/6, are able to control the disease with lesions healing spontaneously, whereas genetically susceptible BALB/c mice develop progressive ulcers followed by visceralization of the parasites and eventual death of the animals. The outcome of the infection depends on the quality of the T cell-mediated immune response to *Leishmania*, i.e. the cytokine production pattern elicited by $CD4^+$ T cells[2].

Leishmania parasites are transmitted by sandflies and the infection commences at the site of the vector's bite in the skin. In the mammalian host, the parasites are obligatory intracellular. Until recently, it was assumed that macrophages are the only cells with the ability to take up *Leishmania* and to present parasite antigen to specific T cells. However, it has now become evident that another type of cells, dendritic cells in the skin and in the lymph nodes draining the cutaneous lesion, play a critical role in cutaneous leishmaniasis[3].

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THE ROLE OF LANGERHANS CELLS IN THE EARLY PHASE OF CUTANEOUS LEISHMANIASIS

We have analyzed the function of Langerhans cells, the dendritic cells of the skin, in experimental cutaneous leishmaniasis with *L. major*. It was shown that freshly isolated Langerhans cells (representing the intracutaneous state of differentiation) but not cultured Langerhans cells (resembling lymphoid dendritic cells) can phagocytose the organisms in vitro and that *L. major*-infected Langerhans cells are present in vivo in the dermal infiltrate of lesional skin[4]. Interestingly, dendritic cells restrain intracellular parasite replication by an as yet unknown mechanism because the parasite burden of *L. major*-containing Langerhans cells was found to be consistently low. A distinguished feature of Langerhans cells was their capacity to transport the ingested *L. major* parasites from the site of infection in the skin to the T-cell areas of the draining lymph node[5]. This migration could be detected within 48 h after dermal infection and was never seen with infected macrophages. During the movement, Langerhans cells developed into lymphoid dendritic cells with the ability to activate resting T cells as well as primed T cells with specificity for *L. major*. Macrophages were unable to induce a primary response. Furthermore, macrophages were much less potent than Langerhans cells in stimulating a secondary T-cell response to *L. major*[6].

Taken together, the distinctive function of Langerhans cells in the initial phase of cutaneous leishmaniasis in both susceptible and resistant mice appears to be the uptake of parasites in the dermis and their transport to the regional lymph node. At this location, Langerhans cells present *L. major* antigen to naive T cells for initiation of the specific cellular immune response.

THE ROLE OF LYMPH NODE DENDRITIC CELLS IN MICE THAT HAVE RECOVERED FROM CUTANEOUS LEISHMANIASIS

After spontaneous recovery from cutaneous leishmaniasis, mice of resistant strains are completely protected from subsequent infections. Nevertheless, small numbers of parasites persist in the lymph nodes draining the site of the prior skin lesion[7,8]. Persistence of *Leishmania* parasites has also been documented in humans[9]. Analysis of the phenotype of long-term host cells in immune mice revealed that similar numbers of both macrophages and dendritic cells harbored viable *L. major* parasites that were fully virulent[10]. Strikingly, however, only lymph node dendritic cells were able to present endogenous antigen from persistent parasites to *L. major*-reactive T cells in vitro. Furthermore, they were extremely efficient because a small number of dendritic cells with a low parasite load was sufficient to stimulate maximal T-cell responses to *L. major*. In vivo tracking experiments suggested that the infected dendritic cells in the lymph nodes of recovered mice are derived from *L. major*-laden Langerhans cells that have emigrated from the former skin lesion.

PERSISTING *LEISHMANIA* PARASITES - FRIENDS OR FOES?

Our studies show that dendritic cells are important not only in the early phase of cutaneous leishmaniasis for transport of parasites from the infected skin to the lymph nodes and initiation of the cellular immune response, but also at later stages after cure of cutaneous lesions in resistant mice. Lymph node dendritic cells carry persistent parasites and, as opposed to macrophages, present endogenous antigen to *L. major*-specific T cells.

What are the consequences of parasite persistence? In *immunocompetent* hosts, an efficient T-cell response limits the replication of persisting organisms. It may well be

possible that survival of a few pathogens results in the sustained stimulation of memory T cells and allows the maintenance of protective immunity. This notion is supported by the suggestion that T-cell memory requires perpetual restimulation caused either by cross-reactions with related or environmental pathogens or by the continuous presence of antigen from the priming inoculum[11]. Lymph node dendritic cells may be particularly well equipped to serve as long-term host cells for continued presentation of persistent antigen. In addition to a potent T cell-stimulatory activity, they have the unique ability to retain antigen in an immunogenic form for long periods[12]. This is achieved by down-regulating the synthesis of MHC class II molecules during differentiation from Langerhans cells, while migrating from the skin to the draining lymph node. Consequently, the MHC class II/peptide complexes of dendritic cells have a very long half-life, whereas those of macrophages turn over and decrease quickly[13]. In *immunosuppressed* hosts, the situation turns into a health hazard. The reduced function of T cells results in reactivation of persistent parasites and relapsing disease. It remains to be shown whether recurrence of leishmaniasis involves both the organisms persisting in dendritic cells and those residing in macrophages and whether this would have an effect on the clinical manifestation, such as the tissue preferences of the reactivated parasites. Because persistence of pathogenic micro-organisms has been reported not only for leishmaniasis but also for other infectious diseases (e.g., tuberculosis and Lyme borreliosis), elaboration of the precise mechanisms will be important for the development of more effective treatments and vaccination strategies.

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EXPOSURE TO TYPE-I COLLAGEN INDUCES MATURATION OF MOUSE LIVER DENDRITIC CELL PROGENITORS

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INTRODUCTION

Liver interstitial dendritic cells (DC), are localized in the connective tissue comprising the portal triads, and have been characterized by immunohistochemical studies both in rodents (1,2) and humans (3). There are however, few published data on the in vitro properties of liver-derived DC (4) and no reports of attempts to propagate DC lineage cells from normal liver.

We report here on the immunophenotype and in vitro allostimulatory activity of DC progenitors propagated selectively from granulocyte macrophage colony stimulating factor (GM-CSF)-stimulated B10.BR mouse liver non parenchymal cells (NPC). Liver DC progenitors (MHC class II^{dim}) proliferated rapidly, but resisted conventional attempts to induce maturation, which uncluded the use of GM-CSF alone or in combination with either TNF-α or IFN-γ. These same cells (or Ia-depleted, GM-CSF propagated cells) could however, be induced to mature (in the continued presence of GM-CSF) after culture on plates coated with type-I collagen, an extracellular matrix protein spatially associated with DC in normal liver (5) (Table 1).

MATERIALS AND METHODS

Isolation of Non-Parenchymal Cells (NPC) from Liver

Liver NPC were isolated from B10.BR ($H\text{-}2^k$; $I\text{-}E^+$) mice as described elsewhere (6).

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Table 1. Distribution of type 1 collagen in normal liver.

Portal tracts	In stroma surrounding vessels and ducts; absent from basement membranes
Lobules	Discrete, small bundles in space of Disse; regularly found at branching points
Central vein	Discontinuous layer beneath endothelial basement membrane
Liver capsule	Near terminal lymphatic channels in capsule

Modified after A. Martinez-Hernandez, Lab Invest 51:57(1984)

Culture of NPC with GM-CSF

2-5x10^5 liver NPC or freshly-isolated spleen cells were placed in each well of a 24-well plate in 2 ml of RPMI-1640, supplemented with 10% FCS and 0.4 ng/ml r mouse GM-CSF (R&D Systems, Minneapolis, MN). The cultures were "fed" every other day by aspirating 50% of the supernatant after gentle swirling, and replenishing with an equivalent volume of fresh GM-CSF-supplemented medium. An objective of these washes was to remove non-adherent granulocytes, without dislodging clusters of developing DC that attached loosely to firmly-adherent macrophages. After d4, granulocytes were no longer significant contaminants of the cultures which were maintained routinely for 7-10d. In some experiments, in addition to GM-CSF, r mouse TNF-α (500U/ml; Genzyme, Cambridge MA) and/or r human IFN-γ (1000U/ml; Collaborative Research, Inc., Bedford, MA) was also incorporated in the culture medium.

Culture of Liver DC Progenitors on Collagen-Coated Plates

Each well of a 24-well plate was coated with collagen type-I (50μg/ml in 0.02 N acetic acid) purified from rat tail tendon (Collaborative Research Inc.), for 15 min at 37°C. The collagen solution was then decanted and the wells were allowed to air dry (15 min at room temperature; RT) before washing twice in RPMI-1640 containing 10% FBS. Cells released from growing clusters in 6 or 7d GM-CSF-stimulated cultures which were free of adherent cells and granulocytes, were transferred to the plates (7x10^5 cells/well) and maintained for an additional 3d in the presence of GM-CSF (0.4 ng/ml). In some experiments, prior to collagen exposure, the cells were pre-treated to deplete all MHC class II^+ cells .

Flow Cytometric Analysis

Liver or spleen cells (5x10^5/tube) in HBSS with 1% w/v bovine serum albumin (BSA; Sigma) and 0.1 % sodium azide (Sigma) were stained either by direct or indirect immunofluorescence, the details of which are described elsewhere (6). Flow cytometric analysis was performed in a FACSTAR® flow cytometer (Becton Dickinson, San Jose, CA).

Mixed Leukocyte Cultures

One-way mixed leukocyte cultures ($4x10^5$ cells in 200 μl per well in 96-well, round-bottom microculture plates) were performed with γ-irradiated (20 Gy) allogeneic (B10.BR) or syngeneic (B10) liver NPC or spleen cells as stimulators. B10 spleen T cells were used as responders. Cultures were maintained in RPMI-1640 complete medium supplemented with 10% heat-inactivated FCS for 72 h in 5% CO_2 in air; for the final 18 h, 10 μl [^{3}H]TdR (1μCi) was added to each well.

Immunocytochemistry

Cytocentrifuge preparations were stained using avidin-biotin-peroxidase complex (ABC) staining procedures as described previously (6).

RESULTS

Culture and Immunophenotypic Analysis of GM-CSF-Stimulated Liver and Spleen Cells

After 4 d culture of liver or spleen NPC in GM-CSF, during which time non-adherent granulocytes were removed by gentle washes, growth of cell "clusters" attached to a layer of adherent cells was evident; many dendritic-shaped cells appeared to have been released from the clusters and exhibited sheet-like cytoplasmic processes. With more prolonged culture in GM-CSF, these cells detached from the aggregates and many mononuclear cells with a typical dendritic shape were seen either loosely attached or floating in the culture medium. The floating or loosely-adherent putative DC were harvested by gentle aspiration for further phenotypic or functional analyses. By d 7 of culture, approximately 2.5 x 10^6 of these cells per mouse liver could be harvested from the cultures.

At the microscopic level, the cells released from proliferating aggregates of GM-CSF-stimulated liver or spleen cells exhibited typical morphological features of DC, including (in many but not all cells) irregular-shaped eccentric nuclei, numerous "veil"-like cytoplasmic dendrites, abundant mitochondria and few electron-dense granules. To ascertain the surface phenotype of cells released from proliferating aggregates, flow cytometric analysis was performed after 6-10 d or further periods of culture in GM-CSF. Staining for cells of lymphoid lineage, including NK cells was absent. The floating cells in liver-derived cultures strongly expressed surface antigens that are known to be associated with mouse DC. These included CD45 (leukocyte common antigen), heat stable antigen, ICAM-1, CD11b (MAC-1), and CD44 (non-polymeric determinant of Pgp.1 glycoprotein). In addition, staining of weak to moderate intensity was observed for the DC-restricted markers NLDC-145 (interdigitating cells), 33D1 and N418, F4/80 (Fig. 1) and for FcγRII. The intensity of expression of these markers on GM-CSF-stimulated spleen cells was similar, except that 33D1 and NLDC 145 were slightly more and less intense respectively, compared with the liver-derived cells. The liver-derived, GM-CSF-stimulated cells however expressed only a low level of MHC class II (I-E^k) surface antigen molecule when compared to GM-CSF-stimulated spleen cells propagated under the same conditions. The intensity of I-E^k expression could not be increased on either the liver or spleen-derived cells by increasing the concentration of GM-CSF (0.4-0.8ng/ml). Further efforts to induce MHC class II antigen expression included combination of GM-CSF with TNF-α (500U/ml) and/or IFN-γ (1000U/ml) for up to 5d. None of these

treatments affected significantly the expression of cell surface I-E^k on the putative "immature" liver DC.

Induction of MHC class II Expression on GM-CSF-Stimulated Liver-Derived Dendritic Cells Following Exposure to Type-I Collagen

Seven day GM-CSF-stimulated liver cells expressing low levels of MHC class II were transferred to culture plates pre-coated with type-I collagen and maintained for 3 additional days in the presence of GM-CSF. Cell proliferation was observed on the collagen-coated plates, accompanied by a relative increase in non-adherent cells as compared with control cultures (collagen-free). Immunophenotypic analysis of the non-adherent cells showed marked upregulation in the intensity of expression of the DC markers NLDC145, 33D1 and N418 (Fig. 1). Such upregulation of DC markers has been shown previously in GM-CSF-stimulated mouse bone marrow cultures (7). Of particular interest, however, was the marked upregulation of MHC class II expression observed on liver DC propagated for an additional 3d on collagen-coated

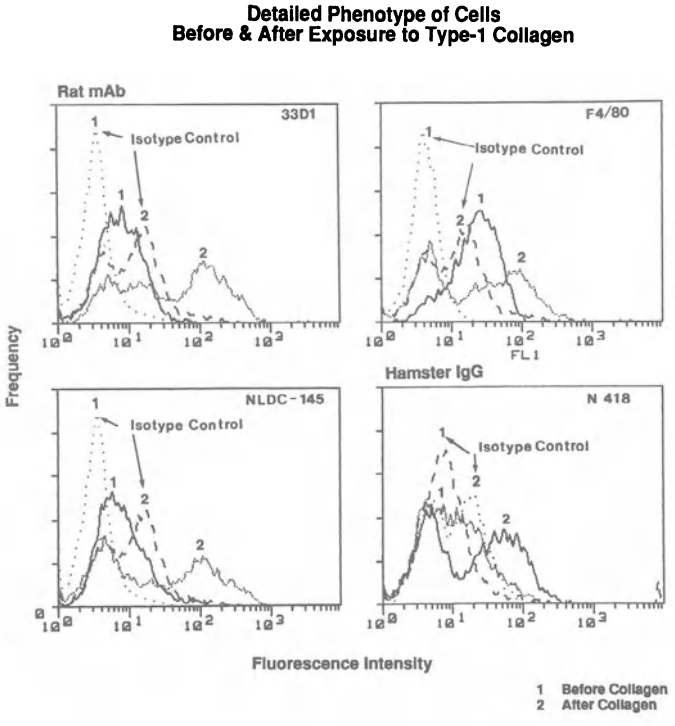

Figure 1. The expression of various DC-restricted markers and of F4/80 on GM-CSF-stimulated putative liver DC before and after the exposure of the cells to type-I collagen Seven-day cultures of liver-derived cells released from aggregates in GM-CSF- supplemented medium were exposed for a further 3 d to type-1 collagen or maintained without collagen in the continuous presence of GM-CSF (0.4ng/ml). An isotype-matched irrelevant antibody was used as a negative control. The results are representative of 3 separate experiments.

plates as compared to similar cells maintained in collagen-free cultures. This observation was confirmed by immunocytochemical staining of cytospins of these cells (data not shown). In order to address the possibility of enrichment of class II^+ cells, vs "upregulation" of class II in collagen and GM-CSF cultures, the Ia^+ population was depleted by anti-I-E^k mAb dependent complement-mediated lysis before exposure to collagen. Similar to the 7d GM-CSF propagated DC, the Ia^- population obtained from

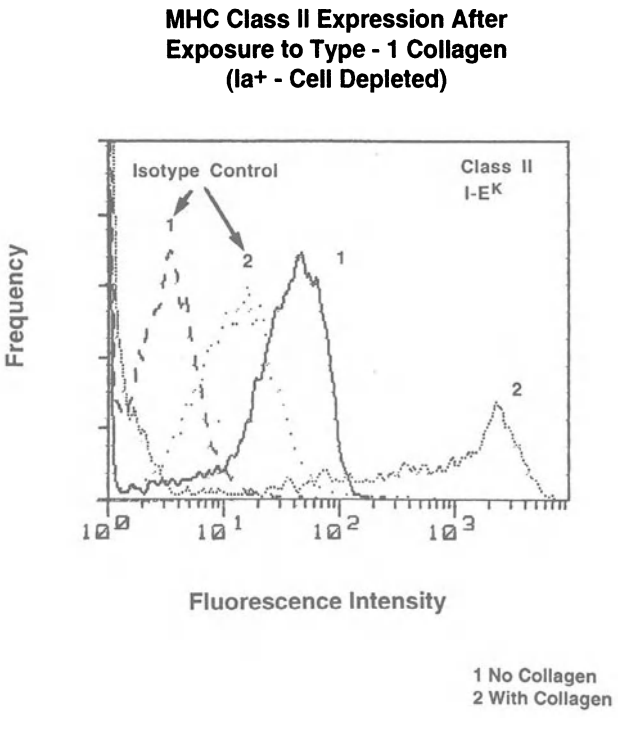

Figure 2. Flow cytometric analysis of the expression of MHC class II (I-E^k) on GM-CSF-stimulated putative liver DC with or without subsequent exposure to type-1 collagen. Ia^+ cells were depleted from 7d cultures of liver-derived cells released from aggregates in GM-CSF-supplemented medium by treatment with anti-Ia (I-E^k) mAb and complement; the cells were then exposed for a further 3 d to type-1 collagen or maintained without collagen in the continuous presence of GM-CSF (0.4ng/ml). An isotype-matched irrelevant antibody was used as a negative control. The results are representative of 3 separate experiments.

these cells also markedly upregulated its MHC class II expression when exposed to collagen for 3d in the continued presence of GM-CSF (Fig. 2). To control for possible trace contamination of the collagen with endotoxin and to rule out possible endotoxin-mediated upregulation of class II on the developing liver DC, 7-d cultures were exposed to LPS (50μg/ml) for a further 3 d period in the presence of GM-CSF. No increase in class II expression was observed.

Development of MLR Stimulatory Activity Following Exposure of Liver DC Progenitors to Type-1 Collagen

Following exposure to collagen, Ia-depleted liver DC became potent inducers of MLR, in marked contrast to Ia-depleted cells maintained in GM-CSF alone, which failed to elicit T cell proliferation (Fig. 3). These class II^{bright} liver-derived DC also proved much stronger MLR stimulators than freshly-isolated spleen cells although not as potent as GM-CSF stimulated splenocytes.

DISCUSSION

Unlike DC cultured from the spleen, which exhibited the morphologic, immunophenotypic and functional characteristics ascribed to DC (8), the majority of

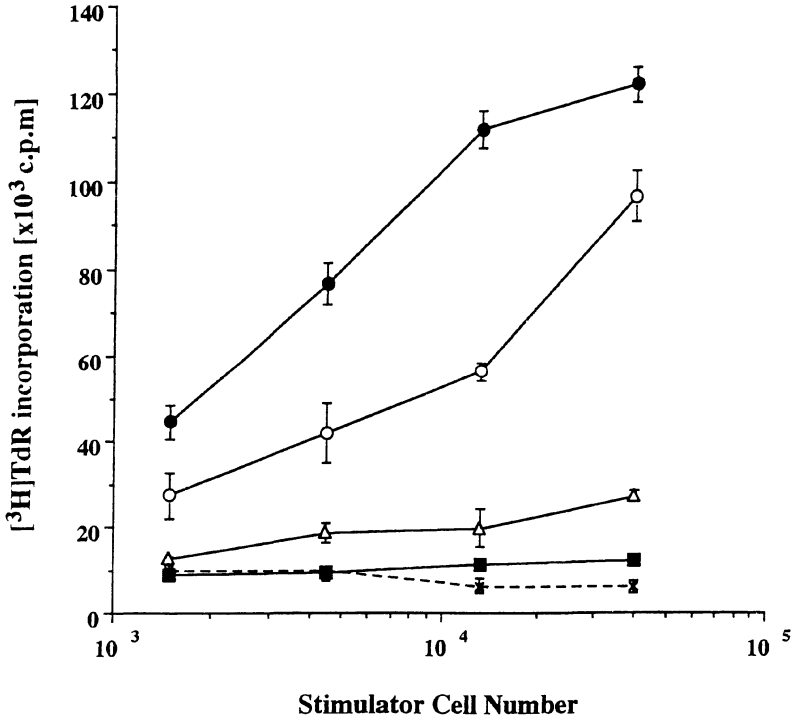

Figure 3. Allostimulatory activity of γ-irradiated, GM-CSF-stimulated B10.BR liver-derived DC from which all class II^+ cells were depleted (after 7d culture) with anti-Ia mAb and complement; the cells were then exposed to type 1-collagen for 3d. (▪), Ia-depleted stimulator cells maintained for 3d with GM-CSF in collagen-free wells; (○), similarly-derived and treated cells, except for exposure for 3d to type-1 collagen with continued presence of GM-CSF. There was no increase in the MLR-stimulatory activity of untreated or Ia- depleted liver-derived cells maintained in GM-CSF alone for extended periods beyond 7 d (up to 35 d; data not shown). The allostimulatory activity of freshly-isolated (△) and GM-CSF-stimulated (d10) B10.BR spleen-derived DC (●), and (X), syngeneic (B10) spleen cells is also shown. The results are expressed as mean counts per minute ± 1SD.

GM-CSF-stimulated NPC derived from the liver had weak or no surface expression of MHC class II and failed to stimulate naive B10 T-lymphocytes in MLR. Consequently, their identity as DC could not be established unequivocally. This dilemma was resolved by culturing the liver cells in the presence of type-I collagen, a strategy employed to mimic the microenvironment of the intact liver, where mature DC are found in areas that are rich in type-I collagen; - portal tracts, around central veins, and in Glisson's capsule (5; and Table 1). After 3d of exposure to type-I collagen in the presence of GM-CSF, the putative hepatic DC markedly upregulated the expression of MHC class II and acquired potent allostimulatory activity. These observations could reflect selective "enrichment" after exposure to collagen of the few Ia^{dim} cells, rather than the functional and phenotypic maturation of liver DC precursors. This argument however, was dismissed after our findings that Ia^{-} GM-CSF propagated liver DC, when exposed to collagen also matured into potent antigen-presenting cells expressingly equally high levels of MHC class II.

The tolerogenic implications of the rapid egress from transplanted whole organs (the liver above all) of postulated precursor or progenitor DC exhibiting low MHC class II expression and low T-cell stimulating activity are considerable. The existence of subpopulations of murine DC with a veto function has recently been proposed (9). Moreover, $HLA\text{-}DR^{dim}$ allogeneic donor bone marrow cells, shown to exhibit veto cell activity (inactivation of T-helper cells or cytotoxic T-cell precursors) have been postulated to be immature DC (10). Such immature DC might be expected to elicit a "deviant" (tolerogenic) local and systemic immune response shortly after their injection. A well studied example of an atypical or "deviant" cytokine-modulated immune response induced by bone marrow-derived APC has been provided by Streilein etal (11). The precise basis of the DC-T-cell interaction leading to tolerance induction is uncertain, but would clearly depend on the relative affinity or avidity (compared with effective APC) of the donor DC-TCR interactions and on the expression on the former cells, of adhesins and costimulatory molecules, such a B7/BB1. These aspects of the developing liver-derived DC are under further investigation in our laboratory.

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HUMAN KERATINOCYTE-DERIVED IL-12 AFFECTS LC-INDUCED ALLOGENEIC T-CELL RESPONSES

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INTRODUCTION

Our laboratory has recently defined the cytokines involved in the early induction phase of contact sensitivity that help LC to become more potent APC and that help to direct this classical Th1 immune response[1,2].

Lately the novel cytokine IL-12 has evolved as a major player in the regulation of T cell development towards a Th1 phenotype[3,4]. IL-12, is a heterodimeric molecule composed of two covalently linked chains, p40 and p35[5]. Whereas the p35 chain of IL-12 is expressed by numerous, including non-lymphoid, tissues, expression of p40 was thought to be restricted to the lymphoid cell system.

Goal of this study was to investigate whether skin cells are capable of producing IL-12 mRNA and protein in a Th1 type immune response such as contact sensitivity. We demonstrate herein that non-lymphoid cells like human keratinocytes release IL-12 following stimulation with allergen and that IL-12 seems to be involved in T cell responses induced by human LC. The data demonstrate that IL-12 might also have a role in directing contact sensitivity reactions in skin.

RESULTS

Induction of IL-12 mRNA in human epidermis

3% TNCB was applied to skin of human healthy volunteers. Skin punch biopsies were obtained after various time points (0-48h) following application of the allergen. Afterwards EC suspensions were prepared and total epidermal RNA was extracted immediately. RNA was quantitated and subjected to 25 cycles of RT-PCR followed by liquid hybridization. Signals for p35, p40 and ß-actin control were detected by autoradiography. Whereas signals for p35 chains were expressed constitutively in human epidermis, p40 chain signals were only found following treatment with allergen. Time course studies showed that p40

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was found within 4h of hapten application reaching peak strength after 6h and being downregulated after 24h. ß-actin control signals remained unchanged during treatment with allergen. These data indicate that epidermal cells following stimulation are capable of producing IL-12 p35 and p40 chain mRNA's.

Induction of IL-12 p40 chain is allergen-specific

3% TNCB (an allergen), 2% DNTB (a tolerogen), 20% SLS (an irritant), or nothing was applied to skin of healthy normal volunteers. 6h later punch biopsies were obtained and EC suspensions were prepared. Following RNA extraction RT-PCR was performed with 25 cycles and signals for p35, p40, and ß-actin control were sought by liquid hybridization. Whereas the p35 chain of IL-12 was again found to be expressed constitutively and did not change during activation, p40 chain mRNA signals could only be detected following stimulation with contact allergen, not following stimulation with tolerogen, or irritant. These data indicate that IL-12 p40 chain is selectively induced by contact allergen in our test system.

Cell depletion assays

EC suspensions were prepared and stimulated with TNBS (a water-soluble analogue of TNCB) as described. Afterwards EC suspensions were treated with either anti-HLA-DR mAb or CD4/CD8 mAb or an unrelated control mAb followed by treatment with complement to deplete LC or T cells respectively. Afterwards aliquots were stained again with the respective mAb and subjected to FACScan analysis to control for cell depletion. The remaining cells were lysed immediately by RNAzol B for RNA extraction and subjected to PCR analysis for expression of p35, p40 and ß-actin. The purity of our keratinocytes was 100% as controlled for by PCR. None of the lytic cocktails could deplete the cells responsible for the signals for p35 or p40 indicating that human keratinocytes are the major sources of this cytokine in skin.

To furthermore support this finding, the immortalized human keratinocyte cell line HaCat was cultured as described and treated with PDB (a phorbolester). Following RNA extraction and PCR analysis, p35, p40 and ß-actin signals were sought. Again p35 chain mRNA signals were expressed constitutively, whereas only PDB-treated HaCat cells expressed mRNA signals for p40. ß-actin controls remained unaffected. Our data indicate that human keratinocytes are capable of producing IL-12 mRNA.

Identification of keratinocyte-derived IL-12 protein

EC suspensions were prepared as described and either left untreated or stimulated with 0.2% SLS, or 1mM TNBS as described. After 24h of culture, supernatants were harvested and concentrated by centrifuge concentrators about 25x. These concentrates were then analysed by a sensitive capture bioassay for IL-12. Only concentrated supernatant from allergen-stimulated EC-suspensions, not control-treated concentrates contained measurable amounts of IL-12 protein. Our data indicate that human keratinocytes are capable of producing functionally active IL-12 protein following allergen stimulation.

Anti-IL-12 mAb inhibits proliferation of allogeneic T cells induced by haptenated LC

EC suspensions were prepared as described and either left untreated or haptenated with TNBS. Blood from healthy human volunteers was enriched for $CD4^+$ T cells by sequential treatment with Ficoll gradient centrifugation followed by positive enrichment

with paramagnetic beads as described in the methods section. Cells were >95% pure $CD4^+$ T cells. Afterwards cells were cocultured with haptenated or non-haptenated EC containing about 3% LC as APC for 4 days in the presence or absence of 25µg/ml anti-IL-12 mAb. ^{3}[H]-Thymidine at 1µCi/well was added for the last 12h of culture to measure proliferation. Addition of anti-IL-12 mAb to cocultures of T cells and haptenated EC inhibited proliferation by about 50%, whereas control mAb or treatment of non-haptenated EC only showed minimal inhibition. Our data indicate that IL-12 is of functional importance for the induction of human T cell proliferation by epidermal LC in the induction of primary immune responses and that the enhanced proliferation of allogeneic T cells towards haptenated EC may be due to release of IL-12 by keratinocytes following hapten application.

DISCUSSION

We demonstrate that epidermal cells are capable of producing IL-12. In cell depletion assays as well as by using immortalized keratinocyte cell lines, we show that keratinocytes are the major sources of this cytokine in the epidermis. We demonstrate by using an IL-12 capture assay that keratinocytes are able to release bioactive IL-12 protein following stimulation with contact allergen. In a LC-induced proliferation assay we demonstrate that IL-12 might also be involved in the induction of T cell proliferation by LC, as a monoclonal anti-IL-12 mAb inhibited T cell proliferation by 50% in an allogeneic proliferation system. In aggregate our data indicate that functional IL-12 is produced by epidermal cells. We furthermore provide evidence that this cytokine might be involved in balancing the intricate homeostasis of the early induction phase of contact sensitivity by helping direct T cell responses induced by LC.

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STUDIES ON LANGERHANS CELL PHENOTYPE IN HUMAN AFFERENT SKIN LYMPH FROM ALLERGIC CONTACT DERMATITIS

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It is generally accepted that the bone marrow-derived epidermal Langerhans cells (LC), together with keratinocytes, represent the most peripheral outpost of the immune system, and that they play a major role in antigen presentation[1-3]. Circulating in the blood, LC or their bone-marrow-derived precursors leave the dermal postcapillary venules and populate the epidermis. After taking up appropiate antigens, they migrate via the lymphatic vessels to the paracortex of the regional lymph nodes, where they present the processed antigens to T-cells. Investigations in animals[4-6] sustain these hypothesis on the role of LC in the skin immune system, but in humans the suggested migration of LC has still to be verified. In allergic skin disorders, the phenotype of LC migrating in the afferent lymph towards the regional lymph nodes is of considerable interest. Currently, the CD1a antigen is still accepted as a reliable cell surface marker for immunocytochemical identification of LC[1]. Ultrastructurally, the main marker for LC are the so called Birbeck granules (BG)[7]. Formed from the cytomembrane, BG may possess an endocytotic capacity, which enables the transport of extracellular solutes to the endosomal system[8]. While the origin and function of LC and the role of BG are increasingly understood, there is controversy concerning the sensitivity of BG as a LC marker, since in vitro and in vivo data indicate that the phenotype of LC essentially depends on the microenvironment in which they reside[1, 9, 10]. So far, LC were extensively investigated in human skin diseases, mainly through biopsies. In contrast, data on LC in the afferent human skin lymph are still scarce[11-13]. Today, afferent lymph exclusively derived from a selected skin area can be collected and analyzed[14]. Since the composition of the afferent lymph in all probability reflects processes in the drained skin area, investigation of lymph sampled after induction of specific skin lesions may provide new information on the pathogenesis of skin disorders.

Using the recently described system of lymph cannulation[14] we have investigated afferent lymph derived from an induction[13] as well as an elicitation (manuscript in

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preparation) phase of allergic contact dermatitis. Diphenylcyclopropenone (DPCP) was used as sensitizing agent. For electron microscopy and immunocytochemistry lymph cells from the late phase of an induction as well as an elicitation reaction were isolated and reacted with sheep anti-mouse IgG antibody-coated Dynabeads coupled with mouse anti-CD1a monoclonal antibodies. For comparison, freshly prepared epidermal cell suspensions were analysed in the same way. The ultrastructural analysis revealed that in lymph as well as in epidermal cell suspensions cells containing BG had formed rosettes after anti-CD1a/Dynabead treatment[13]. But, in contrast to the rosetted epidermal cells, which all exhibited sections through typical BG, the majority of cells binding anti-CD1a in the lymph had none. Moreover, only few anti-CD1a/Dynabead - rosetted lymph cells had BG in a number equivalent to freshly isolated epidermal LC, whereas a larger fraction, however, was found to have only one or two BG per crossection[13]. As indicated by the proliferation of some subcellular structures the CD1a-positive cells from the lymph appeared to be also more active than those from the epidermis. The immunocytochemical analysis of CD1a/Dynabead - rosetted cells showed that these cells also expressed protein S-100, and the surface antigens HLA-DR, ICAM-1 and in part LFA-3, but they did not express the monocyte surface markers CD36, CD68 and MAC 387[13]. Based on these results we speculate that most of the cells forming rosettes belong to the population of LC, and that this particular phenotype of anti-CD1a/Dynabead - rosetted cells might be due to the specific microenvironment in afferent skin lymph.

In conclusion, using antibody-coated Dynabeads as a simple but specific immunolabelling technique, suitable for both electron and light microscopy, we demonstrated CD1a-positive dendritic lymph cells, which did not express monocyte surface markers and contained no or markedly fewer BG than epidermal LC. These data suggest that during the late induction and elicitation phases of an allergic contact dermatitis in humans, LC partly devoid of BG migrate from the skin to the regional lymph nodes. The surface antigens detected on the majority of these cells such as HLA-DR, ICAM-1 and in part LFA-3 as well as the ultrastructural findings are similar to LC cultured in vitro[10]. Thus, these CD1a/Dynabead - rosetted cells correspond well to the dendritic cells formerly designated as "veiled"[1, 10].

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ROSETTES OF LANGERHANS CELLS AND ACTIVATED T CELLS IN HUMAN SKIN LYMPH DERIVED FROM IRRITANT CONTACT DERMATITIS

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In addition to its role as a mechanical and physicochemical barrier the skin acts as an immunological control and defence system. Thereby, immunological surveillance is principally performed by circulating cells in concert with soluble mediators. In this context the lymphatic system plays a major role, as its vessels allow the clearance of proteins and fluid from the tissues and provide the exit pathway for immunocompetent cells. Starting in the papillary dermis, the skin lymphatics drain the adjacent tissue in a one-way flow towards the regional lymph nodes. Thus, the composition of the afferent lymph reflects inflammatory processes in the drained tissue. Today, in human probands, it is possible to collect afferent lymph exclusively derived from a selected skin area[1]. By inducing specific skin lesions in the drained skin area, this system of lymph cannulation allows to get insight into the local pathomechanisms of skin disorders. In contrast to studies taking single skin biopsies of certain time-points of an experiment, the kinetics of skin processes can be assessed continuously.

Contact dermatitis is classified into irritant and allergic, the former resulting from toxic chemicals and the latter representing a delayed type hypersensitivity reaction. The pattern of imflammatory cells was reported to be essentially identical in both type of reactions, consisting of T-lymphocytes of the helper/inducer type mainly in association with both Langerhans cells (LC) and HLA-DR positive dermal macrophages[2-9]. Concerning the presence of LC in the skin in irritant versus allergic patch test reactions, there is still controversy with reports on increased, unchanged or even decreased numbers of LC[7]. Fluctuations in epidermal LC density, however, are not specific for contact dermatitis. They occur in other types of inflammatory skin reactions and even after simple occlusion of the skin with water[7, 9] Nevertheless, it is generally believed, but not yet demonstrated in humans, that the migration of LC from the skin to the regional lymph nodes is an essential feature of allergic contact dermatitis

Using lymph cannulation we have investigated human afferent lymph derived from a mild contact dermatitis induced by the application of 10 % sodium lauryl sulphate. In parallel to the skin reaction the lymph flow and the output of total cells increased and remained elevated for 2-3

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days even after the clinical signs of contact dermatitis had completely disappeared[1] Concerning the lymph cells, the main feature was an impressive absolute and relative increase of LC as well as of T and B cells during and after the irritative skin reaction[10-12] In addition activation markers (Il-2 receptors, MHC class ll antigens) were detected on various cells Immunocytochemically, high numbers of $CD3^+$ / $CD4^-$ / $CD8^-$ cells were counted, which might represent a characterisitc feature of T cells in lymph from irritant contact dermatitis Recently an increased number of $CD3^+$ / $CD4^-$ / $CD8^-$ / γδ T-cell receptor-positive cells was reported both in lesional epidermis and dermis of contact hypersensitivity reactions[13] A further surprising finding was the presence of cell rosettes[12], which were detected throughout the experiment, i e even before induction of the irritant skin reaction Immunocytochemically and ultrastructurally, these rosettes revealed a central LC surrounded by in part activated T cells Focal contacts between lymphoid cells and LC as well as expression of ICAM-1 on the LC and LFA-1 on the apposed T cells indicate a close cooperation of these cells

The large number of in part activated and interacting immunocompetent cells migrating from the skin to the regional lymph nodes, the increased functional capacities of the lymph cells[14] as well as the augmented output of cytokines[15] and complement proteins[16], all point to an activation of the skin immune system also in the course of so called irritant skin processes The formation of rosettes, for instance, occurring in all phases of the experiment, might well be a "normal" feature resulting from the continuous exposure of the skin to environmental agents In this context, keratinocytes could act as pro-inflammatory signal transducers, responding to nonspecific external injuries with the production of cytokines, further chemotactic factors and adhesion molecules[17] Thus, irritant contact dermatitis, previously considered as a simple toxic damage to the cutaneous cell system, presents a very complex reaction pattern, which most probably also includes immunoregulatory processes

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MIGRATION OF INTERLEUKIN-6 PRODUCING LANGERHANS CELLS TO DRAINING LYMPH NODES FOLLOWING SKIN SENSITIZATION

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INTRODUCTION

Epidermal Langerhans cells (LC) have potential to develop into active antigen presenting cells for T cell-dependent immune responses. It is likely that the accessory function of these cells is dependent not only on their ability to express certain costimulatory adhesion molecules such as B7[1], but also on their capacity to elaborate cytokines[2,3]. LC and/or the dendritic cells (DC) into which they mature, were considered originally not to produce cytokines. However, it is now known that cultured LC, which are in many ways analogous to the immunocompetent LC which have migrated to draining lymph nodes following skin sensitization, have the ability to synthesize and secrete interleukins 1β and 6 (IL-β and IL-6) *in vitro*[4]. The production by LC of IL-1β and IL-6 is of interest as both cytokines are known to be important accessory molecules for T cell activation[5] and may therefore participate in LC/T lymphocyte interactions during antigen presentation *in vivo*. Certainly, LC-derived IL-1β has been reported recently to be essential for the induction of primary immune responses following skin sensitization of BALB/c mice[6]. Evidence that IL-6 may also be important, comes from studies in mice where the induction phase of skin sensitization was found to be associated closely with the production by draining lymph node cells of IL-6[7]. Although T lymphocytes have the capacity to produce IL-6, proliferating T cells were found not to be the major source of this cytokine in allergen-activated lymph nodes. We chose to

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examine whether the DC which accumulate in draining lymph nodes following skin sensitization, and the epidermal LC from which they derive, express this cytokine.

EXPERIMENTAL

IL-6 is known to be produced *in vitro* by a number of different skin cells, including keratinocytes, endothelial cells and fibroblasts[8]. To investigate whether epidermal LC are a source of this cytokine in normal skin we have developed a sensitive biotin-streptavidin immunoperoxidase staining technique for the *in situ* immunocytochemical detection of IL-6. Using a monoclonal antibody directed against murine IL-6 (rat IgG1 isotype), it was found that epidermal LC, examined in frozen skin sections prepared from the ears of naive BALB/c mice, constitutively express IL-6. No IL-6 activity was evident in adjacent keratinocytes (Figure 1). Serial skin sections treated with an isotype-matched control antibody or an irrelevant anti-cytokine antibody (anti-murine IL-4; rat IgG1), in place of the primary antibody, exhibited no staining.

Given the central role of LC during the induction phase of skin sensitization and the potential costimulatory activity of IL-6 during T cell activation, we have examined whether DC accumulating in the lymph nodes of sensitized mice express IL-6. Preliminary studies were carried out on sections of lymph nodes, snap-frozen in isopentane cooled in liquid nitrogen, 24 hours following exposure on the dorsum of both ears to the contact allergen oxazolone (1%). A small number of cells resident within lymph nodes prepared from vehicle treated and naive mice were found to express IL-6 constitutively. However, following exposure to oxazolone, an increased frequency of IL-6 producing cells was detectable in allergen-activated lymph nodes compared with control lymph nodes. Importantly, all IL-6 staining was localized to the paracortex of lymph node sections, with no activity in lymphoid follicles. These observations are consistent with the production of IL-6 by DC already resident within resting lymph nodes and with the localization of IL-6$^+$ LC within the T cell-dependent area of draining lymph nodes following skin sensitization.

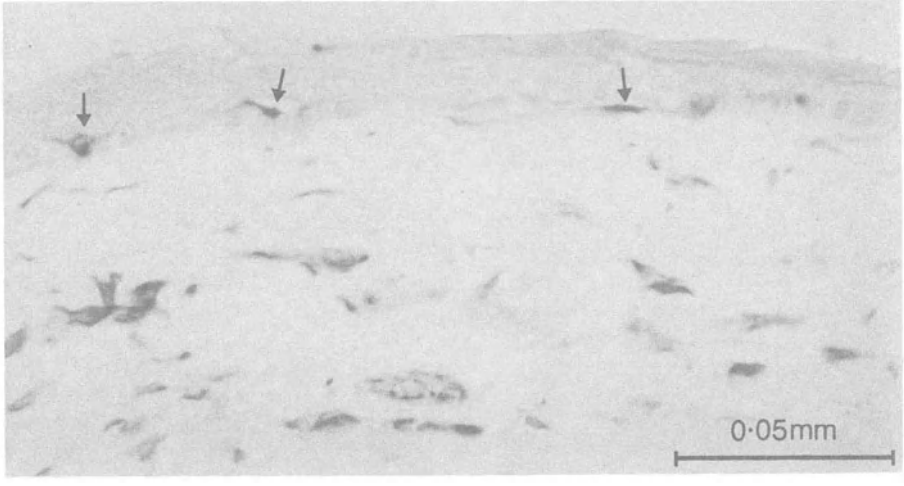

Figure 1. Detection of immunoreactive IL-6 protein in murine epidermal LC (↓) by immunocytochemistry

In an attempt to identify the cellular source of IL-6 within the lymph nodes of BALB/c mice, DC enriched populations of lymph node cell suspensions from both sensitized and non-sensitized mice were examined. Such cell populations, prepared 18-24 hours following exposure to allergen have been shown previously to contain greater than 80% DC[9]. Exposure to oxazolone resulted routinely in a 7-10 fold increase in lymph node DC numbers measured 18 hours later. Immunocytochemical analysis of cytospins revealed that the majority of DC, prepared from sensitized or non-sensitized animals, were positive for IL-6. Contaminating lymphoblasts (< 1%), macrophages (< 2%) and resting T and B lymphocytes were negative for IL-6, as were populations depleted of DC.

CONCLUSION

These results demonstrate that DC, and the epidermal LC from which they derive, represent an important source of IL-6. We suggest that LC induced to migrate to draining lymph nodes following skin sensitization have the capacity to elaborate IL-6 which, in addition to LC-derived IL-1β, may serve as an important cofactor for T lymphocyte activation during antigen presentation.

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THE ROLE OF CYTOKINES IN THE PATHOGENESIS OF PULMONARY LANGERHANS' CELL HISTIOCYTOSIS

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INTRODUCTION

Langerhans' cell histiocytosis (LCH) is characterized by an accumulation and/or proliferation of cells with a Langerhans' cell (LC) phenotype. Immunohistochemically, the cells express CD1a and S-100, and with electron microscopy Birbeck' granules can be identified in the cytoplasm of the LCH cells.

Most patients affected by the disease are children. The clinical manifestations of LCH may be variable: some patients present with skin involvement, whereas others present with solitary or multiple bone lesions. Lymph nodes may also be involved. In the most severe cases, the patients present with multi-organ involvement. Lung involvement, either in the context of multi-organ involvement or as solitary lesions, may occur.

Little is known of the pathogenesis and etiology of LCH. At present, an immunological dysregulation is thought to underlie the disease, although recent findings suggest that LCH is a clonal disorder.[1] In addition to characteristics of the epidermal LC, the LCH cells express cell-surface molecules of activated LCs. Also, due to abnormal expression of cellular adhesion molecules, the LCH cells may show abnormal migration.[2]

Cytokines are of particular interest in the pathogenesis of LCH. LCH cells have been found to produce interleukin (IL) 1, and immunohistochemically staining for interferon gamma (IFNg) and GM-CSF was found.[3-6] It is to be expected that additional cytokines will play a role in the pathogenesis of LCH. The LCH cells, as well as other cells, may be the producing cells of these cytokines. Especially cytokines produced by macrophages, eosinophilic granulocytes and T-lymphocytes usually are abundantly present in the lesions will be of interest. Cytokines, similar to the situation regarding normal Langerhans' cells, may influence activation and differentiation of LCH cells, as well as migration and distribution of these cells. Also, cytokines will be of particular interest to investigate in pulmonary LCH, since this form of the disease is characterized by distinct phenomena, such as intraluminal fibrosis and elastic fiber degradation. Cytokines are implicated in the development of pulmonary fibrosis. Therefore, in this study, we investigated specimen of a patient with

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pulmonary LCH that had to undergo a unilateral lung transplantation because of extensive interstitial fibrosis.

MATERIAL AND METHODS

Immunohistology was performed on frozen, acetone-fixed sections from tissue stored at -80 °C using a biotin-streptavidin peroxidase method. Biotin-labeled rabbit anti-mouse immunoglobulin (Dako, Glostrup, Denmark) was used as second step reagent, and peroxidase-labeled streptavidin (Dako) was used as third step reagent. The peroxidase label was visualised using 3-amino-9-ethylcarbazol (AEC, Aldrich, USA), together with H_2O_2. Slides were counterstained with hematoxylin. To prevent nonspecific background staining endogenous avidin-binding activity and endogeneous peroxidase activity were suppressed, and slides were pre-incubated with a 10% dilution of normal rabbit serum. Also, the second- and third step reagents dilutions contained 1% normal human AB serum.

Antibodies were used against CD1a (T6 and BL-Thy1, obtained from Dako and Monosan, Uden, The Netherlands, respectively) and the cytokines IL4, IL5, and granulocyte/macrophage-colony stimulating factor (GM-CSF) (all obtained from Genzyme, Cambridge, Massachusetts, USA), IL1a, granulocyte-colony stimulating factor (G-CSF), and basic fibroblast growth factor (bFGF) (all Oncogene, Cambridge, Massachusetts, USA). Replacement of the primary antibody by a non-relevant antibody of same immunoglobulin-isotype was used as negative control. To identify macrophages, staining for CD68 (Dako) was performed. Staining for eosinophil major basic protein (Monosan) was used to identify eosinophilic granulocytes.

A specimen of a lymph node affected by a dermatopathic reaction was used as a control of normal activated LCs.

RESULTS

The histopathological diagnosis of LCH was confirmed by means of staining for CD1a (Figure 1). Staining for CD68 revealed that numerous macrophages were present within the lesions. Also, many eosinophils were present as identified with staining for eosinophil major basophilic protein.

Using the anti-cytokine antibody panel, within the LCH lesions staining was found for IL1a and GM-CSF. The cells expressing these cytokines most probably were macrophages. GM-CSF was not found in the LCH cells, but was seen in a somewhat reticular staining pattern, possibly representing matrix bound GM-CSF (Figure 2). Some IL4 positive cells were found within LCH lesions, likely to be eosinophilic granulocytes. No staining was found for IL5. Expression of bFGF was found surrounding vessels and bronchioli. Staining for G-CSF was found abundantly in the lung tissue, but leaving the LCH regions unaffected.

In the lymph node affected by a dermatopathic reaction only sporadic expresion was found for the cytokines. The LCs present within the paracortical area of the lymph node did not stain for the cytokines investigated.

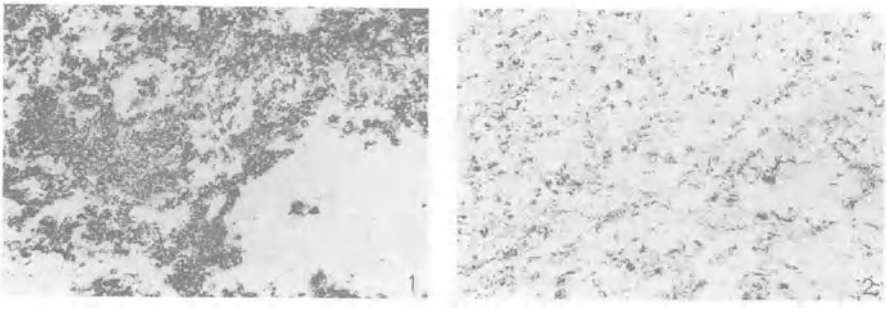

Figure 1 and 2. Staining in the pulmonary LCH lesions for CD1a and GM-CSF, respectively (x140)

DISCUSSION

LCH cells have been reported to stain for IL1, IFNg and GM-CSF. However, on the expression of IFNg data are not consistent. In addition to the immunohistochemical expression of these cytokines, production of IL1 by LCH cells has been demonstrated.

Staining for IL1a was found strong within the LCH lesion, although it seemed that not the LCH cells but macrophages within the lesions were the positive staining cells. The presence of IL1a within the lesions may have implications for the pathobiological behavior of the LCH cells. Continuous production of IL1a may affect the differentiation of the LCH cells, and may in addition up-regulate cellular adhesion molecules on as well the LCH cells as endothelium.

Only weak expression of bFGF was found within the lesion. The presence of bFGF may be expected within pulmonary LCH lesions, often characterized by pulmonary fibrosis. When considered the extensive pulmonary fibrosis in the LCH lesion investigated, the only limited expression we found may be related to the fact that in our patient a end-stage pulmonary fibrosis was present. Possibly that in early stages of the disease this cytokine is more abundantly present, or alternatively, that other cytokines not tested play a role in the development of pulmonary fibrosis. Interestingly, staining for bFGF was found surrounding vessels. This staining pattern may be explained by the ability of proteoglycans, components of the basal membrane, to bind bFGF.

In the pulmonary lesion of our patients with LCH, no GM-CSF could be detected in the LCH cells in contrast to earlier findings in LCH.[4] This may indicate that the production of cytokines by the LCH cells is variable, and may be related to the micro-environment in the lesion or the differentiation of the LCH cells. We did find a somewhat reticular staining pattern, possibly indicating extracellular matrix bound GM-CSF. GM-CSF has been found essential to viability and differentiation of normal LCs, and may have similar effect on LCH cells. Also, it has been suggested that GM-CSF is the principal regulator of distribution of normal LCs in lung. The presence of GM-CSF, not present in LCH cells but localized in the immediate micro-environment as demonstrated in our lesion, may in part explain the occurence of LCH lesions in lung. In addition GM-CSF may influence the biological behavior of the LCH cells contributing to the pathogenesis of LCH.

In conclusion, the cytokines present in the LCH micro-environment, whether or not produced by LCH cells themselves, may contribute to the pathogenesis of pulmonary LCH lesions and the clinical course of the disease.

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IMPORTANCE OF HLA-DR+ AND CD1a+ EPIDERMAL CELLS FOR CYTOKINE PRODUCTION IN PSORIASIS

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INTRODUCTION

Psoriasis is an inflammatory skin disease characterised by marked hyperproliferation of keratinocytes in association with vascular expansion, leukocyte infiltration and lymphocyte activation[1]. Cytokines are thought to play a pivotal role in the pathogenesis. It is speculated that a dysregulation of the cytokine network involving tumor-necrosis-factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8) might be the basic mechanism of the psoriatic inflammatory response[2]. TNF-α is the prototype of a proinflammatory peptide exerting immunomodulatory effects. IL-6 is a multifunctional cytokine with a broad range of biological functions in both acute and chronic inflammatory reactions. IL-8 is a potent chemotactic and activating factor for both neutrophils and lymphocytes. However, no information is available to which extent the production of these cytokines is (dys)regulated in psoriatic skin. Therefore, this study was meant to investigate the role of HLA-DR+ and CD1a+ epidermal cells in the production of TNF-α, IL-6 and IL-8 in the autologous mixed skin lymphocyte reaction.

METHODS

6 patients having plaque type psoriasis were included. Patients had not received any systemic treatment or UV irradiation for at least 3 months before. Mononuclear cells (MNC) were isolated by density gradient centrifugation from peripheral blood drawn by venipuncture[3]. By passing MNC over a nylon wool column suspensions were enriched for T-lymphocytes to >96 % as checked by flow cytometry[4]. Vacuum-induced suction blisters were raised over normal, psoriatic uninvolved and psoriatic involved abdominal skin[5]. CD1a+ and HLA-DR+ epidermal cells were depleted using immuno-magnetic beads (Dianova) exactly as described by the manufacturer. Flow cytometry was used to control depletion. 10^5 responder T-cells were incubated with 10^4 stimulator epidermal cells in

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RPMI 1640/10% AB-serum for 5 days (AMSLR)[6]. TNF-α, IL-6 and IL-8 concentrations were measured in cell culture supernatants using two specific monoclonal antibodies in a sandwich enzyme linked immunosorbent assay (ELISA). Results are shown as pg/ml (mean ± SEM). To evaluate statistical differences the two-tailed Wilcoxon signed-rank test was employed and *p*-values < 0.05 considered significant.

RESULTS

Lympho-epidermal interaction of T cells and psoriatic skin led to the secretion of markedly increased amounts of TNF-α in comparison with normal cells. Depleting HLA-DR+ epidermal cells in psoriatics but not in healthy controls resulted in a significantly diminished TNF-α production. Depleting CD1a+ cells did not affect TNF-α production in healthy human controls but significantly reduced TNF-α secretion in cultures with psoriatic skin (p = 0,0313). No statistical difference could be detected comparing the effects of HLA-DR and CD1a depletion neither in cultures with healthy nor with psoriatic skin.

Table 1. Cytokine concentrations pg/ml, mean ± SEM, n=6 (CD1a-: depletion of CD1a+ epidermal cells, MHC II-: depletion of HLA-DR+ epidermal cells)

	HEALTHY SKIN			UNINVOLVED SKIN			INVOLVED SKIN		
	TNF	IL-6	IL-8	TNF	IL-6	IL-8	TNF	IL-6	IL-8
AMSLR	256±59	991±78	317±113	593±57	948±93	1060±632	552±88	891±169	801±649
CD1a-	253±92	4905±2450	2380±441	236±55	1161±86	2427±753	242±57	1187±124	2324±641
MHC II-	161±57	1910±895	2583±529	205±101	1130±97	1959±684	175±42	1393±228	1696±554

Regarding IL-6 concentrations, no significant differences were measured in cultures with normal or psoriatic epidermal cells. Depleting HLA-DR+ cells did not change significantly IL-6 concentrations in cultures with healthy or uninvolved psoriatic skin but resulted in a clearly increased IL-6 release in cultures with involved psoriatic skin (p = 0,0313). Further analysis of the effects of CD1a+ cells showed that these cells had no significant influence on the production of IL-6 neither in healthy nor in psoriatic skin. Comparing the effects of HLA-DR and CD1a depletion a significant decrease in IL-6 release (p = 0,0156) could be measured only in healthy skin which is in agreement with a stimulatory activity of CD1a-/HLA-DR+ epidermal cells on IL-6 secretion in healthy skin.

In the AMLSR with psoriatic skin as stimulator cells markedly higher IL-8 concentrations were measured in respect to cultures with healthy epidermal cells. Depleting healthy CD1a+ or HLA-DR+ epidermal cells resulted in a similar and significantly (p = 0,0313) increased IL-8 release compared to the results after stimulation with unfractioned epidermis. In psoriasis, depletion of CD1a+ epidermal cells led to a markedly increased IL-8 production. IL-8 concentrations were significantly lower after depletion of HLA-DR+ cells of both uninvolved and involved psoriatic skin compared to IL-8 concentrations in cultures stimulated with CD1a depleted epidermal cells (p = 0,0313). This is in contrast to healthy controls, where no statistical difference was measured comparing the effects of CD1a vs. HLA-DR depletion.

DISCUSSION

Our data clearly demonstrate that neither CD1a+ nor HLA-DR+ epidermal cells of healthy individuals have an influence on the in-vitro release of TNF-α. However, CD1a+ cells of psoriatics are potent stimulators of the secretion of TNF-α as the concentrations of TNF-α decrease significantly after depletion. Further analysis comparing the effects of CD1a and HLA-DR depletion gives indirect information about the importance of CD1a-/HLA-DR+ cells[7]: infiltrated lymphocytes, activated keratinocytes or indeterminated cells of mononuclear or dendritic origin. Therefore, the cytokine concentration after the depletion of HLA-DR+ cells is the results of the synergistic and/or antagonistic effect of CD1a+/HLA-DR+ and CD1a-/HLA-DR+ epidermal cells.

Whereas no influence of CD1a-/HLA-DR+ cells could be demonstrated in respect to TNF-α release, IL-6 and IL-8 production is partly regulated by these cells. With regard to IL-6 secretion, CD1a+ and HLA-DR+ epidermal cells have gradually different, however principally inhibitory effects in psoriatics and healthy individuals. In contrast, CD1a-/HLA-DR+ cells stimulate IL-6 release only in healthy skin whereas in involved skin an inhibitory influence is exerted by these cells. Regarding IL-8 production, CD1a+ and HLA-DR+ epidermal cells clearly, partly even significantly inhibit IL-8 release. CD1a-/HLA-DR+ cells have only minor effects in healthy individuals whereas a significant stimulatory function can be seen in psoriatics.

The proliferative response of autologous T cells stimulated with epidermal cells has been reported[7]. It was concluded, that mainly CD1a-/HLA-DR+ epidermal cells from psoriatics are potent stimulators of autologous T cells in an MHC class II-restricted fashion. Whether this stimulating effect is the result of a direct lympho-epidermal interaction or the consequence of an indirect influence by alterations of the cytokine network is not known. Our results seem to support the hypothesis that beside direct effects, also indirect effects mediated by changes of cytokine production might play an important role. Interestingly, the existence of CD1a-/HLA-DR+ cells in healthy skin has not been reported before, although our results give only indirect evidence for the existence of these cells.

Our data suggest that HLA-DR+ epidermal cells have a crucial influence on production of TNF-α, IL-6 and IL-8. The effects are dependent in part on CD1a+/HLA-DR+ but also on CD1a-/HLA-DR+ epidermal cells. The different effect of HLA-DR+ epidermal cells in healthy human controls and psoriatics on the secretion pattern of these cytokines indicates a pathogenetic role of these mediators in psoriasis. A potential use of the AMSLR to measure these cytokines seems advantageous to characterise patients concerning their genetic risk, therapeutical progress or prognostic fate.

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ISOLATION OF DENDRITIC LEUKOCYTES FROM MOUSE LIVER

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INTRODUCTION

Dendritic cells (DC) are a minor population of large, bone marrow-derived leukocytes that are distributed ubiquitously throughout the body (1,2). DC resident in the interstitial connective tissue of non-lymphoid organs are believed to be important "passenger" leukocytes that migrate to T-dependent areas of host lymphoid tissue following organ transplantation (3). Recently, it has been suggested that the chimeric cells observed in various organs of recipients of liver or other allografts include, predominantly, cells of DC lineage. These cells may play an important role in modulation of the immunological interaction between host and donor (4,5). Since the liver is the most tolerogenic of all transplanted organs (6-8) and is accepted spontaneously across major histocompatibility complex (MHC) barriers in mice without the need for immunosuppressive therapy (8), studies on mouse liver DC may provide important clues to mechanisms underlying tolerance induction. We have isolated DC-enriched cell populations from normal mouse liver and describe herein their immunophenotype and in vitro allostimulatory activity compared with freshly-isolated liver non-parenchymal cells (NPC).

MATERIALS AND METHODS

Animals

Adult 8-12 week old male B10.BR ($H\text{-}2^k$;$I\text{-}E^+$) and C57BL/10SnJ (B10, $H\text{-}2^b$, $I\text{-}A^+$) mice were purchased from the Jackson Laboratory, Bar Harbor, ME and

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maintained in the specific pathogen-free facility of the University of Pittsburgh Medical Center.

Isolation of NPC from Mouse Liver

Mice were anesthetized with metofen. The liver was perfused for 3 min in situ via the inferior vena cava, using 30 ml Hank's BSS. Two ml collagenase solution (Sigma, St. Louis MO; type IV; 1mg/ml) was then injected; the liver was excised immediately, diced into small pieces and digested in collagenase solution (20ml/liver) for 30 min at 37°C with constant stirring. After removing debris and connective tissue, the cell suspension was washed twice in RPMI-1640 (Gibco, Grand Island, NY) then resuspended in 7 ml self-generating Percoll solution (Sigma; 1.079 relative density) and centrifuged at 4°C for 10 min at 39000g in an ultracentrifuge. The top layer of cells, containing intact hepatocytes and hepatocyte fragments was removed and discarded. The cell suspension between the upper and lower (erythrocyte) layer was collected and washed x 2 (400g; 5 min) in RPMI-1640. These cells constituted the freshly-isolated NPC population.

Enrichment for DC

NPC were placed in plastic tissue culture flasks and incubated overnight (18 hr) at 37°C in 5% CO_2 in air. Non-adherent cells were recovered and resuspended in 8 ml 10% RPMI-1640, then layered onto 2 ml columns of 14.5% w/v metrizamide (Sigma) in 15 ml conical tubes. The cells were sedimented at 600g for 15 min at room temperature. Both the interface population (low density, LD) referred to as "DC-enriched cells," and the cell pellet (high density, HD) were collected separately, washed twice, and resuspended in 10% FCS RPMI-1640 for further morphologic, phenotypic and functional studies.

Immunocyto- and Histochemistry

Cytocentrifuge preparations of isolated cells were stained using the avidin-biotin-peroxidase complex staining procedure. Controls included the use of isotype-matched irrelevant mAb.

Flow Cytometric Analysis

Liver NPC or DC-enriched cells were stained either by direct or indirect immunofluorescence using an extensive panel of monoclonal antibodies (9). Flow cytometric analysis was performed in a FACSTAR® flow cytometer (Becton Dickinson, San Jose, CA).

Mixed Leukocyte Cultures

One-way 3-day mixed leukocyte cultures were set up in 96-well plates with variable numbers of γ-irradiated allogeneic (B10.BR) or syngeneic (B10) liver NPC or splenocytes or DC-enriched populations as stimulators. B10 spleen T-cells were used as responders (2 x 10^5/well). [^{3}H]TdR (1μCi) was added to each well 18 hr before harvest.

RESULTS

Isolation and Immunophenotypic Analysis of Hepatic NPC

Approximately 10^7 NPC were isolated per liver, with less than 5% hepatocyte contamination on microscopic examination of Giemsa-stained cytocentrifuge preparations. These cells were strongly positive for the leukocyte common antigen (CD45), and were positive for MHC class II (I-E^k). They stained both for lymphoid markers (Thy 1.2, CD3, B220, and heat stable antigen; HSA) and the macrophage cell surface antigen F4/80; they also expressed the following receptors/adhesins: CD11b, CD11c, CD44 and CD32 (FcγRII). Expression of the DC-restricted marker NLDC145 was also detected, but that of 33D1 was low. Further details are shown in Table 1.

Enrichment for DC

The LD fraction of overnight cultured non-adherent NPC comprised 45-50% mononuclear cells with distinct DC morphology. Giemsa-stained cytocentrifuge preparations exhibited cells with irregular-shaped, eccentric nuclei, absence of prominent cytoplasmic granules and abundant cytoplasmic projections or "veils."

Immunophenotypic Analysis of DC-Enriched Populations

Immunocytochemical staining of cytospins for MHC class II (I-E^k) revealed that LD cells with distinct DC morphology were strongly class II positive (Fig. 1). More extensive immunophenotypic analysis of the DC-enriched cell population was

Table 1. Cell Surface Markers Expressed by Mouse Liver NPC and DC-enriched populations

MAb: Supplier/Clone Name (ATCC#)	Antigen	NPC	DC-enriched
M1/9.3.4 (TIB 122)	CD45	+++	+++
RA3-3A 1/6.1 (TIB 146)	CD45RA;B220	+	-
PharMingen; 53-2.1	Thy 1.2	+	-
PharMingen; 145-2C11	CD3-ϵ	+	-
PharMingen; RM-4-5	CD4	ND	-
PharMingen; 53-6.7	CD8α	ND	-
J11D (TIB 183)	Heat stable antigen	++	++
PharMingen; 14-4-4S (HB32)	Class II; I-$E^{k,d,p,r}$	+	++
33D1 (TIB 227)	Lymphoid DC	±	±
NLDC-145; Dr. R.M. Steinman	Interdigitating Cell	+	++
F4/80 (HB 198); Dr. R.M. Steinman	Macrophage	++	-
PharMingen; 2.4G2 (HB 197)	CD32, FcγRII	++	-
M1/70 (TIB 128)	CD11b, MAC-1α unit; C3biR	+	++
N418; Dr. R.M. Steinman	CD11c, p150/90	+	±
PharMingen; 2D2C (TIB 235)	CD44, Pgp-1	±	-

ND = not determined

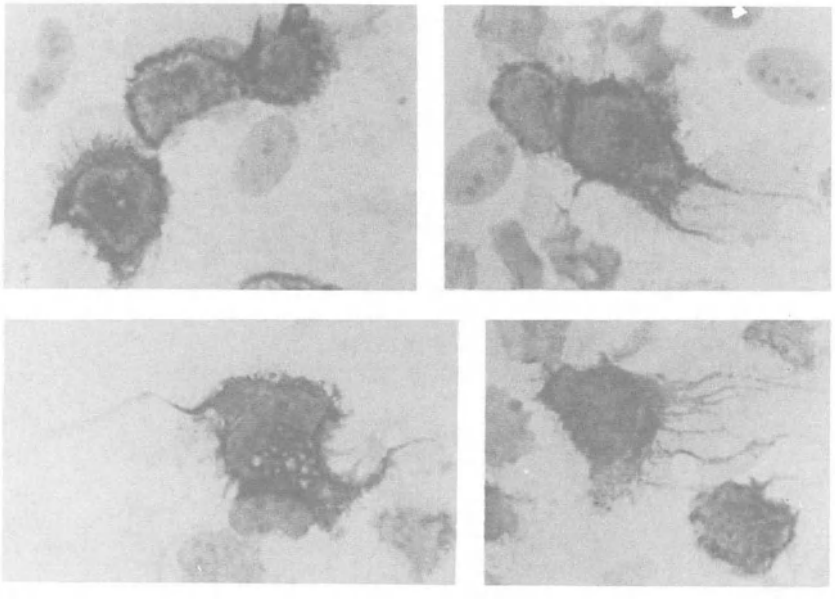

Figure 1. Strongly MHC class II^+ cells with distinct dendritic morphology present in the non-adherent, LD fraction recovered from overnight-cultured B10 BR mouse liver NPC Cells were stained using a mAb to $I\text{-}E^k$ and the avidin-biotin-peroxidase procedure Counterstained with hematoxylin x 1000

undertaken by flow cytometry. The cells were $CD45^{bright}$ and strongly MHC class II positive. They were Thy1.2, CD3, CD4, CD8, B220, HSA^+, CD32 ($Fc\gamma RII$), $CD11c^{/+}$, F4/80, CD44, and showed moderate expression of CD11b, and of the DC-restricted marker NLDC145 but were $33D1^{dim}$. The phenotypic differences between the freshly-isolated liver NPC and the DC-enriched population are summarized in Table 1.

Comparative Allostimulatory Activity of Overnight-Cultured NPC and DC-Enriched Populations

The freshly-isolated liver NPC induced proliferation of unprimed allogeneic T cells and were similar in potency to freshly-isolated allogeneic spleen cells. As shown in Fig 2, overnight cultured, transiently adherent, low buoyant density DC-enriched populations were highly effective in inducing proliferation of naive, alloreactive T cells. In contrast, the overnight-cultured HD liver NPCs or bulk NPC exhibited at least 9-27 fold less allostimulatory activity. Interestingly, as with freshly-isolated NPC, a progressive reduction in MLR was observed when an excess of bulk cells or HD cells over responder cells was used.

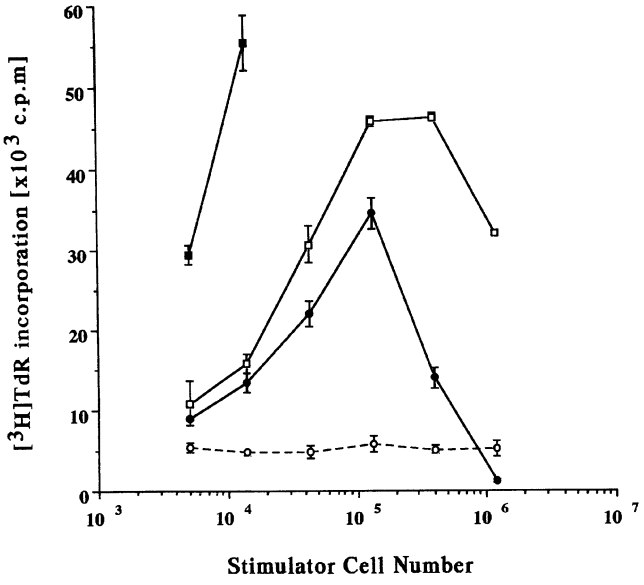

Figure 2. Allostimulatory activity for 2.10^5 naive B10 (H-2^b) mouse splenic T-cells of variable numbers of γ-irradiated, overnight-cultured NPC or DC-enriched stimulator cell suspensions prepared from normal B10.BR (H-2^k) mouse liver. Cells were cultured for 72 hr; [^{3}H]TdR was added to the cultures 18 hr before harvesting. Results are mean cpm ± 1SD and representative of 3 separate experiments. (▪), DC-enriched cell population (non-adherent LD NPC population after overnight culture); (□), HD NPC population after overnight culture; (▵), Bulk NPC; (●), fresh B10.BR spleen cells; (○), unstimulated splenic T-cells. Syngeneic (B10) liver NPC stimulators gave results almost identifical to those obtained with unstimulated T cells.

DISCUSSION

Liver-derived DC have previously been investigated using immunohistochemical procedures in both the rat and human (10,11). There are however, no published data on hepatic DC isolated from the mouse. Isolation of non-adherent, LD cells after overnight culture of liver NPC permitted the recovery of cells, many of which had the morphological features of DC. Approximately 50% of cells in these enriched populations were DC, with some contamination of lymphocytes and a small proportion of macrophages visible in cytospin preparations. This degree of enrichment is similar to that reported by Lautenschlager et al (12), for rat liver DC that induced accelerated cardiac allograft rejection. In keeping with findings on murine DC isolated from other sites, the liver DC-enriched population after overnight culture was strongly positive for CD45, and showed apparent upregulation of cell surface MHC class II expression, with concomitant downregulation of FcγRII and F4/80. These phenotypic changes are consistent with earlier observations regarding the in vitro maturation of murine Langerhans cells (13), and DC isolated from other mouse non-lymphoid organs (14).

Most splenic DC, which are considered representative of mature DC, express 33D1 but not NLDC145. Mouse liver DC share similar characteristics with freshly-isolated and cultured Langerhans cells which are 33D1$^-$, NLDC145$^+$. In addition to strong MHC class II expression, the liver-derived, DC enriched cells were highly active in stimulating unprimed allogeneic T cells. These findings are similar to reports that

freshly-isolated DC populations from other non-lymphoid tissues, i.e. the skin, heart, kidney and lung, acquire stimulatory function after a 24 hr period of culture (13, 15-17).

Freshly-isolated liver NPC were as good as fresh spleen cells in initiating proliferation of naive T-cells. These observations are similar to early reports (14) that freshly-isolated, rat liver DC have the capacity to stimulate naive T-cells, but only following removal of inhibitory macrophages. The latter may have accounted for the reduced MLR seen in this study when an excess of liver NPC stimulator cells over splenic responder cells was tested. Similar findings have been made for DC isolated from rat pulmonary tissues (16).

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ELECTRONMICROSCOPIC STUDY OF CANINE CUTANEOUS HISTIOCYTOMA : A BENIGN LANGERHANS CELL TUMOR

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INTRODUCTION

Canine cutaneous histiocytoma (CCH) is a benign dermal self-healing tumor of the young dog. CCH cells and Langerhans cells are thought to be related because they share immunologic markers as CD 1c, CD 18, CD 45, MHC II. In this study we investigated the ultrastructure of five CCH in order to confirm this relationship.

MATERIALS AND METHODS

Five CCH selected on clinical and cytological features were removed surgically. A part of each tumor was fixed by immersion in 10% buffered formalin for the histological diagnosis. An other part was fixed in 2% glutaraldehyde in cacodylate buffer at PH 7.3, embedded in epoxy-resin and processed for standard transmission electronmicroscopy. Ultrathin sections were examined under JEOL 1200 EX electron microscope.

RESULTS

The light-microscopic examination of sections, confirmed the clinical and cytological diagnosis. At the electron microscopic level, tumor cells usually clustered together with closely opposed plasma membranes, were irregular in shape and moderately large, with a diameter range from 10 to 14 µm. They lacked cellular junctions with adjacent cells and displayed central large (5 to 8 µm) indented nucleus containing clumps of clear heterochromatine opposed to the inner face of the nuclear envelope and a dark eccentric nucleolus. The cytoplasm was rather clear with a large number of vesicles limited by membranes, and some of them contained electron-dense material, moderate rough endoplasmic reticulum and small mitochondria. Microfilament accumulations were rare. Few to many small coated vesicles with a diameter ranging between 80 to 85 nm may be observed. Specially in one case, tumor cells tended to form profound invaginations of the plasma membrane up to 2 µm deep. Usually, the incomplete opposition of plasma membranes formed a bleb at the inner end of the invagination. A fairly electron-dense material partly filled the invaginations. However, tumor cells displayed few to many cytoplasmic pleiomorphic inclusions, regularly laminated bodies and paracrystallin structures. Pleiomorphic inclusions observed had two fusing components : one membranous and one electron-dense vesicular. The mixing of these two elements gave rise to various granule shapes : basket shape appearance, composed of membrane limited dense body partially surrounded by a double joining membrane forming handle, saddle shape appearance formed by dense bodies connected with a curved double membrane,

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signet ring shape composed by a wall of two limiting parallel membranes with dense material accumulation between them, or ring shape appearance formed by two concentric membranes. Some granules displayed a central linear density, bounded by two plasma membranes in part of the organelle, fusing with a membrane limited dense body. Such structures have also been observed within lysosome-like granules. Regularly laminated bodies contained parallel membranes concentrically arranged and separated by a lighter space of approximately 20 nm. Accumulation of numerous regularly disposed 20 nm diameter tubular arrays formed membrane limited paracrystalline or honey-comb-shaped structures.

DISCUSSION

Three authors have already investigated the CCH at ultrastructural level : Kelly (1970) [1], Glick (1976) [2], and Kelm (1982) [3]. We found similarities with them : the size of the tumor cells, the general aspect of the nuclei, the poverty in organelles of the cytoplasm, the presence of vesicles containing electron-lucent to electron-dense material were all similar.. Paracrystalline structures found in variable amount for 4 out of 5 tumors, were expected. In fact, Glick [2] in his study of five CCH, have already noticed in 2 tumors such large reticular aggregates. The author suggested a possible relationship between these structures and a viral induced tumor. However, the small diameter of each tubule (20 nm), their regular disposition in quinconx rather than in square, the presence of membrane limiting the aggregates and the lack of plasma membrane budding disageed with this hypothesis. More probably may these inclusions represent crystallization of accumulated and unused proteins. More exciting was the description by Schneider [4] in a case of human histiocytoma, of such tubular formation with quite similar dimension (30-50 nm in diameter) in the cytoplasm of a LC present in the overlaying epidermis.

However, the main dissimilarities were the presence in our experiment of coated vesicles, deep invaginations of the plasma membrane, regularly laminated bodies and pleiomorphic inclusions in the cytoplasm of tumor cells.

It was the first description of regularly laminated bodies in CCH cells. Such cytoplasmic markers were already described in congenital self-healing reticulo-histiocytosis (CSRH) [5]. The signification of such markers was disputed. Because of the CD1a positivity of parallel membranes, Schaumburg-Lever [6] described them as transformed Birbeck granules. Laugier [7] raised the hypothesis that they may represent damaged phagocyted red blood cells. Hashimoto [8] noticed their resemblance with myelin figures seen in the residual bodies of lysosomes. In man, the presence of regularly laminated bodies is not reserved to LC tumors but may also be observed in macrophagic tumors as generalized eruptive histiocytoma [9].

Similar pleiomorphic inclusions to those we described for the first time in CCH cells were already reported in connection with normal activated or neoplasic histiocytes. Young, studying the mouse vaginal epithelium, found, particularly at diestrus, LC's containing such ultrastructural markers [10]. Some years latter, Parr confirmed this observations [11]. Dezutter-Dambuyant noticed such inclusions in humans 12 days *in vitro*-maturated LC precursors from cord blood CD34+ cells (personnal communication). El-Labban observed in histiocytes from human lichen-planus lesions numerous inclusions with various shapes : basket handle, circle... [12]. Pleiomorphic inclusions were also described as well as in LC tumor : Kanitakis in congenital self-healing histiocytosis [13] as in macrophagic originated tumors : Caputo in papular histiocytoma [14], Degos in reticulo-histiocytoma [15], Piette in multiple reticulo-histiocytoma [16] or Sigal-Nahum in generalized eruptive histiocytoma [17]. The origin and signification of these inclusions was not clear, and several interpretations were put forward. According to Young [10], they were BG attached with electron-dense, membrane bound bodies, presumed to be lysosomes, given the whole a basket-shaped appearance. El-Labban interpreted them as lysosomes with slender projections in which enclosed material appeared as a central band similar to BG [12]. Caputo proposed two possible mode of formation of these granules : a peculiar kind of endocytosis or the expression of an exocytosis phenomenon [14]. We agree with Degos [15] that these inclusions may reflect a severe malfunction with an exaggerated endocytic activity of the plasma membrane and an overproduction of lysosomal material. According to the antigen-presenting cell function of histiocytes, this lysosomal material might be MHC II molecules. In fact, Arkema [18] studying human dendritic cells have obser-

ved in the cytoplasm of these cells class II containing vesicles in a juxta-nuclear position. Only ultrastructural immunolabelings of CCH cells with MHC II MAb will confirm the hypothesis that vesicular parts of pleiomorphic inclusions are class II compartments.

The presence in CCH cells of ultrastructural markers : regularly laminated bodies, pleiomorphic inclusions, sometimes with Birbeck-like granules, frequently observed in congenital self-healing histiocytosis, a human benign LC tumor, and of paracrystalline structures sometimes described in human LC's, plead in favor of a LC origin of this tumor.

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ISOLATION AND CHARACTERISTICS OF DENDRITIC CELL PROGENITORS FROM THE BONE MARROW OF THE HODGKIN'S DISEASE PATIENTS

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INTRODUCTION

It has been previously shown that GM-CSF promotes differentiation and survival of human peripheral blood dendritic cells (DC) in vitro[1] Human bone marrow progenitors of dendritic cells were identified in semi-solid cultures supplemented with conditioned media from PHA-stimulated leukocytes[2] GM-CSF and TNF-α have been shown to cooperate in the regulation of DC growth from $CD34^+$ progenitors in vitro[3,4,5]

Early impairment of cell-mediated immunity is characteristic for Hodgkin's disease That raises the question, if DC growth, differentiation and function as highly potent antigen presenting cells may be involved in pathogenesis of Hodgkin's disease

MATERIALS AND METHODS

Bone marrow was obtained from Hodgkin's disease patients following approval from the Ethical Committee Part of bone marrow aspirate sample obtained from the posterior iliac crest was mixed with preservative-free heparin Peripheral blood T lymphocytes were obtained from healthy volunteers

Bone marrow mononuclear cells (BMMC) were isolated by Ficoll-Uropoline gradient centrifugation Fresh or cultured BMMC were fractionated in principle according to procedures previously used for the isolation of human peripheral blood DC[1,6] BMMC were depleted of FcR-positive cells by a solid-phase absorption (panning) on human IgG-coated Petri dishes Low-density and high-density cells were separated by centrifugation on discontinuous Percoll or Nycoprep gradients BMMC were cultured in RPMI-1640 medium suplemented with 2 mM L-glutamine, 50 μg/ml gentamycin and 10% FCS (Gibco) or 10% human AB serum for MLR cultures (Sigma) Semi-solid cultures of unseparated BMMC or BMMC depleted of FcR-positive cells (FcR^-BMMC) were established at 0,5-2,0 x 10^5 cells/ml in 35 x 10mm Petri dishes in the presence of 20% Tsn or rGM-CSF (Genzyme) at 50 U/ml to 500 U/ml or rGM-CSF and rTNF-α (Genzyme) at 5 U/ml to 50 U/ml Semi-solid culture medium contained 1,66% methylcellulose (Sigma) and was supplemented

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with 20% FCS Pure and mixed colonies containing DC were scored on the inverted phase-contrast microscope after 9 to 14 d of incubation in 5% CO_2 in air Colonies containing differentiated DC were recognized and scored in situ by their distinctive dendritic appearance T cell supernatant (Tsn) was obtained from cultures of peripheral blood T lymphocytes pulsed 18 h with 5μg/ml PHA, washed four times and maintained at 37°C for additional 5 days Allogeneic MLRs were carried out for 6 days using 50 x 10^3 T cell responders obtained form peripheral blood of healthy donors and graded doses of fresh or pre-cultured BMMC or BMMC fractions obtained from patients with Hodgkin's disease BMMC were irradiated (20 Gy from a ^{60}Co source) before the onset of MLR cultures 0,5μCi per well [^{3}H]-TdR (spec act 5 0 Ci/mmol) was added to the wells 16 hrs before cell harvesting

RESULTS

To identify progenitors of dendritic cells in the bone marrow of untreated patients with Hodgkin's disease, bone marrow mononuclear cells were cultured in the presence of rGM-CSF or supernatant from PHA-activated T lymphocytes (Tsn) Characteristics of patients were as follows age (years) - median (range) 26 (15-58), sex - M/F 6/8, histology - Nodular sclerosis 8, Mixed cellularity 2, Lymphocyte depletion 1, Lymphocyte predominance 1, not done. 2, BM status - involved 3, uninvolved 9, not known 2 The number of DC containing colonies per 1 x 10^5 unseparated BMMC cultured in the presence of 20% Tsn ranged from 0,5 to 37,0 , median 5,3 DC progenitors capable to form colonies were recovered in BMMC fraction depleted of FcR^+ cells When FcR^-BMMC cultures were supplied with rGM-CSF and rTNF-α simultaneously, the number of DC containing colonies increased in comparison to the cultures supplied with rGM-CSF alone (Tab 1) The total number of generated GM-CFU colonies was reduced or not changed by addition of rTNF-α to the cultures supplied with rGM-CSF (data not shown) Differentiated DC were found mostly in the mixed type colonies containing also macrophages and/or granulocytes Pure, dispersed DC colonies were found rarely The appearances of differentiated bone marrow DC and previously described[1] peripheral blood DC were identical

Table 1. DC colony growth in semi-solid cultures of FcR^-BMMC - effect of cytokines compared in 8 patients

Tsn (20% v/v)	rGM-CSF (100 U/ml)	rGM-CSF (100 U/ml)+ + rTNF-α (50 U/ml)
12,8* (3,5 - 63,3)	7,4* (0,5 - 60,5)	28,0* (1,1 - 80,0)

* number of DC colonies per 1 x 10^5 FcR BMMC - median (range)

To isolate and characterize DC progenitors fresh and cultured BMMC were depleted of FcR^+ cells and fractionated on Percoll or Nycoprep density gradients This procedure resulted in a minor low-density, FcR^- cell fraction enriched in DC progenitors capable to proliferate and differentiate to cells with a distinctive dendritic appearance This fraction contained highly potent allostimulatory cells Bone marrow DC cultured in the absence of exogeneous GM-CSF, in contrast to peripheral blood DC, maintained their allostimulatory activity at least for 7 days of culture (Fig 1) and were able to differentiate, if supplied with GM-CSF By cytofluorographic analysis low-density, FcR^- cell fraction was depleted of $CD14^+$ cells and enriched in cells strongly staining for HLA-DR and simultaneously staining for HLA-DQ (Fig 2) Two-colour FACS analysis revealed, that cells stained positive with

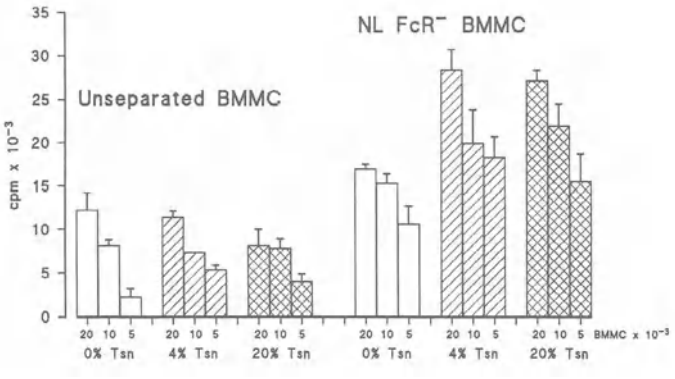

Figure 1. Allostimulatory activity of unseparated BMMC and Nycoprep low-density, FcR⁻ cell fractions (NL FcR⁻BMMC) enriched in DC and DC progenitors (mean cpm values ± SE). DC-enriched fractions were isolated after 7 days of pre-culture with or without Tsn. The incorporation of [^{3}H]-TdR by T cells cultured alone was <300 cpm.

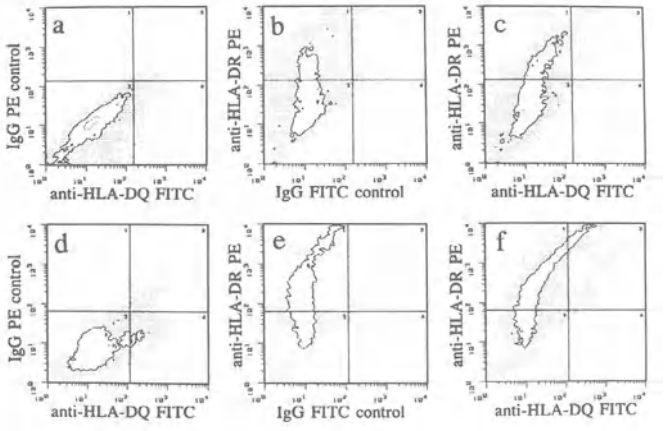

Figure 2. Analysis of HLA-DR and HLA-DQ expression on unseparated BMMC (a, b, c) and DC-enriched BMMC fraction (d, e, f). Cell separation and cytofluorographic analysis on FACStar (Becton Dickinson) were performed after 6 days of culture. DC-enriched, Percoll low-density, FcR⁻ cell fraction was negatively panned with the use of a combination of moAbs to hematopoietic cell lineages other than DC.

a combination of moAbs to the myeloid, lymphoid and erythroid lineages (anti-CD11b, anti-CD15, anti-CD14, anti-CD3, anti-CD19, anti-CD16 and anti-Glycophorin A), contaminating DC enriched fraction, were HLA-DR negative or weakly positive.

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DEFECTIVE DENDRITIC CELL (DC) FUNCTION IN A HLA-B27 TRANSGENIC RAT MODEL OF SPONDYLOARTHROPATHY (SpA)

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INTRODUCTION

Transgenic (Tg) rats carrying high copy numbers of both B27 and human β2m on either the Lewis or F344 genetic backgrounds spontaneously develop a multisystem inflammatory disease similar to human SpA with arthritis, gut and male GU inflammation and psoriasiform skin and nail changes[1]. Histological examination of the lesions suggested a disease with an immunological basis[1]. Cell transfer experiments[2] and the derivation of germ free animals[3] suggest a critical role for antigen presenting cells (APC), T cells and the gut flora in the joint and gut inflammation.

Given the importance of DC both in initiating responses to foreign antigen[4] and in shaping the T cell repertoire[5], we have compared the phenotype and function of these cells in Tg and non-Tg (NTg) rats. We describe a profound functional defect in the cells isolated from Tg animals.

MATERIALS AND METHODS

DC were isolated from the spleens of pre morbid 21-4H (Lewis) and 33-3 (F344) Tg rats and from NTg littermates by overnight culture of single cell suspensions and separation of low density cells on hypertonic metrizamide gradients[6].

The phenotype of isolated cells was analysed by staining with the following monoclonal antibodies: OX6 (rat MHC class II), OX18 (rat MHC class I), B1.23.2 and ME1 (B27), OX33 (B cells), R73 (T cells), 3.2.3 (NK cells), ED1 (macrophage subset). Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View CA.)

The function of DC was assessed by measuring their ability to activate allogeneic T cells in a mixed leukocyte reaction (MLR). Graded numbers of DC were irradiated (2000rad) and cultured with $4x10^5$ lymph node cells from PVG or DA rats for 4 days in flat bottomed microtitre plates (Costar Cambridge, MA). T cell proliferation was measured by ^{3}H-Thymidine incorporation. In some experiments the effect of the addition of monoclonal antibodies (5μg/ml) at the initiation of the cultures was studied.

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RESULTS

Enriched spleen DC preparations were successfully isolated from NTg rats of both genetic backgrounds and from pre morbid Tg animals. In dual colour flow cytometry DC were identified as large cells with low granularity expressing high levels of MHC class II antigens but negative for staining with antibodies to T and B cells, NK cells and macrophages. Similar levels of MHC class II antigens were found on the surface of both Tg and NTg DC (not shown).

DC from NTg , or low copy number 21-4L, rats were very potent stimulators of allogeneic MLR responses with fewer than 1% required to induce a powerful proliferative response. However DC from high copy number Tg (both 21-4H and 33-3) animals were only weakly stimulatory across a wide range of stimulator cell numbers. (See Fig.1 for example and note different scale for responses stimulated by NTg and Tg cells).

Furthermore, the small MLR response stimulated by Tg DC, but not the response stimulated by NTg DC, was almost completely inhibited by monoclonal antibodies to B27. Isotype matched controls had little or no effect. In contrast, the response stimulated by both Tg and NTg DC was completely inhibited by an antibody to rat MHC class II (Fig.1). Antibody to rat MHC class I antigens had little effect. The effect of anti-B27 on responses stimulated by Tg DC was observed even when purified CD4+ T cells were used as responders (not shown) suggesting an unusual mode of antigen recognition, or else a facilitatory effect of B27 on class II responses.

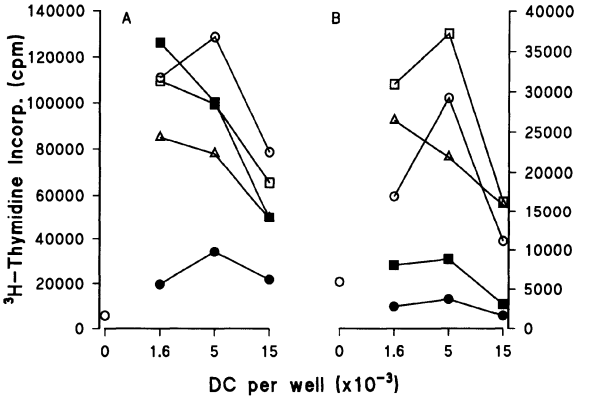

Figure 1. Stimulation of MLR by (A) NTg and (B) 21-4H Tg DC. Note different scale. Cultures contained no antibody (○), OX6 (●), B1.23.2 (■) or isotype matched controls (□, Δ).

Cell mixing experiments demonstrated that the failure of Tg DC to simulate a powerful MLR response was not due to the activation of suppressor T cells and pre-culture experiments failed to provide evidence for the induction of anergy by Tg DC.

Preliminary evidence suggested that *in vivo* exposure of Tg rats to antigen resulted in the generation of only weak proliferative and cytotoxic T cell responses suggesting that the DC defect may also occur *in vivo*.

DISCUSSION

The major finding of this study is that DC isolated from the spleens of transgenic rats expressing high copy numbers of B27 and human β2m display a striking functional defect *in vitro*. Such animals proceed to develop a severe inflammatory disease similar to human SpA and it is possible that a defect in antigen presentation by DC may be central to this process. It is possible, for instance, that inefficient presentation of self antigens by DC during thymic or extra-thymic education of T cells may lead to the escape of autoreactive clones into the periphery. Alternatively, poor presentation of antigens derived from the gut flora, which is essential for at least some manifestations of the disease in Tg animals, may in some way lead to an inappropriate immune response and the development of immunopathology. The observation that monoclonals to both B27 and rat MHC class II antigens inhibit the small response of allogeneic T cells to Tg DC suggests an unusual mode of antigen recognition but this requires further study particularly at the clonal level.

In the future, additional experiments will attempt to identify the molecular basis for the defect in DC function. Levels of MHC class II antigens appear similar on Tg and NTg DC but it remains to be determined whether there are differences in expression of other APC molecules involved in T cell activation such as co-stimulators or adhesins which may account for the functional differences. Alternatively, over-expression of the transgenes may disrupt the antigen processing machinery perhaps by the generation of large concentrations of peptides which compete with self and foreign antigens for occupation of rat MHC molecules. High copy-number transgenics with other human MHC molecules will give information about the specificity of any such effects but preliminary data suggest the disease has specificity for B27[7].

Such studies may provide important information about antigen processing by DC as well as the disease process in B27 Tg rats.

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IMPROVED ISOLATION OF DENDRITIC CELLS IN CHRONIC ARTHRITIC JOINTS REVEALS NO B7 (CD80) SURFACE EXPRESSION

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INTRODUCTION

The aetiology and pathogenesis of most chronic arthritic diseases is unknown Immunohistochemical studies on animal models of chronic arthritis (1,2) and on human rheumatoid synovium (3) suggest that dendritic cells (DC) are involved in the disease process DC have been isolated from the synovium (4), synovial fluid (SF) (5) and blood (6) of chronic arthritic human subjects These studies were limited by difficulties defining DC, but now with more experience, improvements in isolation and DC identification have encouraged us to re-examine the role of DC in this disease We have now developed an improved technique, using minimal cell manipulation, for the isolation of highly pure, fresh DC from joint aspirates of chronic arthritic subjects (manuscript submitted) Phenotypic analyses of these SF DC has provided new evidence regarding the potential role of costimulator molecules in this disease

PURIFICATION OF FRESH SF DC

SF was collected into EDTA following routine knee joint aspirations from chronic arthritic patients Mononuclear cells were isolated on a Ficoll-Hypaque density gradient and T lymphocytes depleted using standard rosetting techniques with neuraminidase-treated sheep red blood cells Non-T cells were stored overnight at 4°C in 10% human AB serum to avoid aberrant activation and the introduction of foreign antigens These conditions were maintained for the remainder of the preparation The next day, cells were labelled with mAb to T lymphocytes (CD3), B lymphocytes (CD19), NK cells (CD16,CD57), granulocytes (CD15) and monocytes (CD14), and were depleted on RAM Ig-coated plates Residual labelled cells were further removed using immunomagnetic beads The final lineage-negative (lin-) cells obtained constituted the SF DC population and comprised 0 4-3 5% of the starting mononuclear cell population

IDENTIFICATION OF SF DC

Using this purification method, we obtained cell preparations of which 63-90% were lin- (X=80%) They were the most potent stimulatory cells in SF, as demonstrated by the increasing ability of cells at each enrichment step to stimulate in an allogeneic mixed leukocyte reaction MGG-stained cytospins revealed 2 morphologically distinct populations of lin- cells The predominant population (57-80%) consisted of larger macrophage-sized cells with an irregular shaped nucleus, little cytoplasm and variable presence of dendrites The minor population comprised smaller lymphocyte-sized cells

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with a rounded nucleus, little cytoplasm and no dendrites Two similarly morphologically distinct lin- populations have been isolated from peripheral blood These smaller cells have been found to be less potent stimulators in the mixed lymphocyte reaction and are thought to represent naive DC or DC precursors (7) MHC class II molecules were expressed on both cell populations, although at a much higher density on the larger lin- cells

PHENOTYPE OF SF DC

Cell phenotype was determined by immunoperoxidase staining and flow cytometry The larger lin- cells were $CD4^{+}$, $CD14^{+/}$, $CD40^{++}$, whereas the smaller lin- cells were CD4-, CD14-, $CD40^{+}$ Both populations had strong MHC class I expression Of the new activation antigens described on DC, CD83 (8) was absent or very weakly expressed on SF DC whereas CMRF-44 (9) was expressed on the larger cells but was either weak or negative on the smaller cells The larger lin- cells had stronger expression of the CD54 (ICAM-1) and CD58 (LFA-3) molecules Both populations stained weakly for CD102 (ICAM-2) but strongly for CD50 (ICAM-3) In comparison, CD4 has been detected on blood DC (10) and little CD40 is present on fresh blood DC (11) but it is induced upon activation (12) Activated blood DC also express CD83 and CMRF-44 Tonsil DC have a similar phenotype to activated blood DC in that they both express CMRF-44 (9), CD40, CD54, CD58 (13), CD102 (14) These results indicate that the large lin- SF cells resemble activated blood DC

COSTIMULATOR MOLECULE EXPRESSION ON SYNOVIAL DC

The costimulator molecule B7 1 (CD80) was not detected on SF DC using 5 different CD80 mAb, or the CTLA4-Ig fusion protein, which has high avidity for B7 1 Likewise, we detected either no or limited amounts of CD80 mRNA in SF DC using reverse transcriptase polymerase chain reaction (RT-PCR) Interestingly, a recent immunohistochemical study demonstrated weak B7 expression on only a few cells, primarily macrophages, in the rheumatoid synovium (15) In comparison, fresh blood DC lack CD80 mRNA and surface protein, whereas activated blood DC and tonsil DC produce both CD80 mRNA and surface antigen (16) In further studies, we incubated SF DC with allogeneic T lymphocytes plus BB1 (B7 1,B7 3) or CTLA4-Ig (B7 1,B7 2,B7 3) and found minimal inhibition of the MLR with BB1, yet nearly complete inhibition with CTLA4-Ig Thus, despite the absence of B7 1 on fresh SF DC and the failure of BB1 to block the SF DC stimulated MLR, other CTLA4-Ig ligands on SF DC are important for costimulation Detection of CD80 mRNA in some SF DC implied that CD80 surface expression might be induced if they were removed from the chronically inflamed joint Preliminary results show that both B7 1 (CD80) and B7 2 (CD86) can be upregulated on SF cells and normal PB monocytes following culture in IL-12 (B7 2>B7 1), implying that the CTLA4-Ig ligands (B7 1, B7 2 and B7 3) may be downregulated on SF DC by an inhibitory factor present in the fluid (manuscript in preparation) In additional studies, we incubated L428 cells, which express both B7 1 and B7 2 molecules, in SF and observed downregulation of these molecules (manuscript in preparation)

CONCLUDING REMARKS

We have developed a reliable technique for the isolation of pure, fresh DC from SF of chronic arthritic patients Phenotypic analyses suggest that these cells are not, despite the inflammatory environment, in a fully activated state The absence of costimulator molecules on SF DC indicates that a factor down regulating B7 1 and other CTLA4-Ig ligands may be present in SF Induction of these molecules on SF DC following their removal from the chronic inflammatory environment, as well as inhibition of their expression by SF is suggestive evidence for a soluble regulator of costimulator molecules present in arthritic joint fluid These results are likely to have relevance to the immunopathogenesis of chronic arthritis

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VACCINE ADJUVANCY: A NEW POTENTIAL AREA OF DEVELOPMENT FOR GM-CSF

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INTRODUCTION

Granulocyte-macrophage colony stimulating factor (GM-CSF) is an hematopoietic growth factor that has been named after its ability to induce the proliferation and maturation of precursor cells into colonies of granulocytes and macrophages[1,2]. However, GM-CSF is not simply a proliferative stimulus, but also is a polyfunctional regulator that affects the growth and function of granulocytes, macrophages, eosinophils, basophils, keratinocytes, dendritic, endothelial cells and also mature, cloned T-lymphocytes[3]. It increases DNA synthesis in transformed cells, enhances neutrophil chemotaxis, and the phagocytic, cytotoxic and microbicidal action of neutrophils and macrophages[3].

Currently, non-glycosylated recombinant human GM-CSF (rhuGM-CSF, molgramostim) is licensed in Europe as LEUCOMAX® for the following indications:

- reduction in duration of neutropenia and incidence of febrile neutropenia after cytotoxic chemotherapy for non-myeloid malignancy;
- reduction of duration of neutropenia and clinical sequela after myeloablative therapy preceding bone marrow transplantation;
- treatment of ganciclovir-induced neutropenia in AIDS patients with cytomegalovirus retinitis.

It has been demonstrated[4] that administration of rhuGM-CSF to patients undergoing intensive chemotherapy reduced the time to recovery of neutrophils from 26 to 13 days, resulting in fewer febrile days, less antibiotic use, and, although not statistically significant, a shorter duration of hospital stay.

It is noteworthy that all current clinical indications of rhuGM-CSF exploit mainly its "anti-granulocytopenic" effect and they do not profit of the unique characteristic of rhuGM-CSF of acting on antigen presenting cells like macrophages and dendritic cells[5]. The dendritic cell system comprises cells distinct from the well-recognized lineages of granulocytes and macrophages[6]. These cells form an interconnected system of antigen- presenting cells which

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regulates the primary T-dependent immune responses. Since none of the other colony stimulating factors can activate the antigen-presenting and accessory functions of dendritic cells, it has been suggested that rhuGM-CSF may represent a novel strategy to increase the immune response to vaccines.

RATIONALE FOR USING GM-CSF AS VACCINE ADJUVANT

Vaccines represent one of the most effective options to control infectious diseases in humans and animals. They prevent outbreaks of infectious diseases, by inducing, without causing a disease, a state of immunity against a pathogen similar to that caused by actual infection with that pathogen. However, despite the success of vaccines against human diseases such as smallpox, poliomyelitis, tetanus and pertussis, there are still conditions for which effective vaccines are not yet available or, if a vaccine is available, it is less effective in those patients for which vaccine efficacy is most desirable, for example in elderly or in immuno-compromised patients. Therefore, there is a substantial effort in developing new vaccines or in improving the efficacy of the existing vaccines by administering them in conjunction with immunoadjuvants.

To date, the only registered vaccine adjuvants in humans are those based upon aluminum salts, discovered over 50 years ago[7]. Unfortunately aluminum salts show a great variation in their level of effectiveness, depending on the nature of vaccine with which they are formulated[8]. Generally, they perform poorly, and there are various attempts being made to develop better adjuvants, adjuvant formulations or, more recently, new immunomodulators. Immuno-modulators are substances, like interferons, interleukins or growth factors, that directly influence or modify one or more components of the immunoregulatory network to achieve an effect on a specific immuno function.

The mechanisms of the response generated after vaccination, leading to a long lasting immunity, involve different components of the immunoregulatory network[9]. The first step is the uptake of the antigen by an antigen presenting cell (APC), such as a macrophage or a dendritic cell. After the antigen has been processed within the APC, antigen fragments are shuttled back to the cell surface in a non-covalent association with class II major histo-compatibility complex (MHC) molecules, to activate T-cells. It is presumed that T-cell activation would require cross linking of the T-cell receptor as well as an association between the T-cell's CD4 molecules and the antigen presenting cell's MHC[10]. Once T-cells are activated, they differentiate and replicate to form regulator (T-helper and T-suppressor) and effector cells. Differentiation and replication of B-cells to become antibody secreting cells such plasma cells are induced by the activated T-helper cells.

In some cases and for different reasons, vaccine antigens may not be able to trigger the immune reactions depicted above. For example, subunit preparations are notoriously poor at generating a complete and rigorous immune response and this observation has stimulated the search for effective immunoadjuvants. GM-CSF has been shown to be able to influence and to activate the first critical steps of the immunoregulatory pathway:

- it cooperates in the generation of dendritic cells from $CD34^+$ hematopoietic progenitors[11];
- it activates APC, such as macrophages and dendritic cells[12];
- it increases the expression of the Class II MHC molecules in APC[13];
- it enhances the antigen presenting activity of APC[14];
- it augments the primary antibody response by enhancing the function of APC[15].

These observations suggest that GM-CSF, by increasing antigen presenting activity, may enhance activation of T and B cells, leading to generation of high yield memory cells after

vaccination, and, as a consequence, it may improve the protection provided by the vaccine.

Some of the vaccines for which rhuGM-CSF may be potentially used as an adjuvant include:

- influenza vaccine in the elderly;
- hepatitis B vaccine to reduce the number of doses required to provide adequate protection or to enhance immunogenicity in selected populations, for example hemodialyzed patients;
- to enhance immune response to vaccines in HIV patients;
- to increase antigenicity of tumour or HIV vaccines currently under development.

The concept of using rhuGM-CSF as vaccine adjuvant has initially been tested with the flu vaccine in the elderly because there is a large population receiving flu vaccine every year that can be easily monitored and followed up.

GM-CSF AS FLU VACCINE ADJUVANT

Despite the availability of influenza vaccines since the 1950s, annual influenza epidemics are still claiming a heavy toll both in terms of human lives and health care costs. In the United States alone there are more than 20,000 excess deaths and $5 billion expenses every year due to flu epidemics and flu-related complications[16]. Since the influenza vaccine has been shown to provide benefits in reducing both morbidity and mortality, the flu vaccine is strongly recommended in subjects at a high risk to develop flu-related complications, and substantial resources are expended annually in an effort to vaccinate high-risk subjects, mainly elderly people[17]. In fact, approximately 80 to 90 percent of the excess deaths and hospitalizations occur in persons 65 years of age or older, particularly those with underlying cardiac or pulmonary disease[16]. Unfortunately, among the elderly, the vaccine may only be 30 to 40 percent effective in preventing clinical illness, even though it may provide the benefit of lessening the severity of the disease[18,19]. The protection offered by the influenza vaccine correlates with the development of serum anti-hemoagglutinin (HAI) antibodies[20,21].

Recently, the use of rhuGM-CSF as vaccine adjuvant was tested in 60 healthy elderly patients receiving flu vaccine[22]. The study was designed as a double-blind, placebo-controlled, dose finding study. The subjects were healthy elderly people of both sexes and of at least 65 years of age with baseline HAI antibody titers $\leq$ 1:40 for the A-H1N1 and B influenza strains and $\leq$ 1:80 for the A-H3N2 influenza strain contained in the 1992-1993 French flu vaccine. The 60 subjects enrolled in the study were divided into five dose groups of 12 subjects each. In each dose group, 9 patients received a single dose of 0.25, 0.5, 1, 2.5 or 5 μg/kg of rhuGM-CSF and the other 3 patients were administered placebo. Immediately after the subcutaneous administration of rhuGM-CSF or placebo, all the subjects received intramuscolarly 0.5 mL of a licensed 1992-1993 trivalent subvirion vaccine that contained 15 μg each of hemo-agglutinin from A/Singapore/6/86 (H1N1), A/Beijing/353/89 (H3N2) and B/Yamagata/16/88 viruses. Blood serum and throat swabs for virological cultures were obtained from each subject before the start of the study and 1, 3, 6, 12 and 26 weeks after vaccination. Subjects were considered seroconverted if a four-fold increase of the HAI antibody titers over baseline occurred at Week 6.

Table 1 shows the number of patients who seroconverted to all three strains 6 weeks after the administration of rhuGM-CSF/placebo and flu vaccine. Results also are reported for each strain. None of the 15 subjects who received placebo with the flu vaccine showed simultaneous seroconversion to all three flu virus strains, compared with 5 of 9 (56%) and 3 of 9 (33%) of the subjects treated with 2.5 and 5 μg/kg of rhuGM-CSF respectively who were seroconverted to all three strains. In addition, when the results are examined separately for each flu strain,

seroconversion rates for 2.5 and 5 μg/kg of rhuGM-CSF are consistently higher (ranging from 44% to 67%) than those observed with placebo (ranging from 13% to 20%). Interestingly, seroconversion rates observed with the lowest dose (0.25 μg/kg) have been higher than those observed with 0.5 and 1 μg/kg. However, the small number of subjects for each group does not allow to draw a final conclusion about the dose response. Very few adverse events were reported and the administration of rhuGM-CSF was well tolerated.

The first relevant finding of the study is the confirmation of the poor immunogenicity of the flu vaccine in elderly subjects. Seroconversion to all the three flu strains contained in the vaccine was observed in none out of 15 subjects who received the vaccine with placebo. Similar findings were observed in 63 elderly subjects administered with the same vaccine (M. AYMARD, personal communication). Only 3 out of 63 subjects (5%) developed seroconversion to all three flu strains within 6 weeks from vaccination.

Therefore, considering the relatively low immunogenicity of the flu vaccine in the elderly, the results obtained with rhuGM-CSF are very promising. Immunoresponse to flu vaccine was higher in all rhuGM-CSF treated patients than in those treated with placebo, and with the highest doses (2.5 and 5 μg/kg), 56% and 33% of the subjects were seroconverted to all three flu strains. The findings of this preliminary study support the concept of using rhuGM-CSF as a vaccine adjuvant even though additional studies are warranted to further define its role in this indication.

Table 1. Results of seroconversion. Seroconversion is defined as a four-fold increase of the HAI antibody titers over baseline.

	Patients simultaneously seroconverted to all three strains	Number of seroconversion observed for the different flu strains		
		A/Beijing [H3N2]	A/Singapore [H1N1]	B/Yamagata
Placebo + flu vaccine	0/15 (0%)	3/15 (20%)	3/15 (20%)	2/15 (13%)
rhuGM-CSF + flu vaccine				
0.25 μg/kg	2/9 (22%)	4/9 (44%)	4/9 (44%)	3/9 (33%)
0.5 μg/kg	0/9 (0%)	2/9 (22%)	3/9 (33%)	1/9 (11%)
1 μg/kg	1/9 (11%)	2/9 (22%)	1/9 (11%)	4/9 (44%)
2.5 μg/kg	5/9 (56%)	6/9 (67%)	5/9 (56%)	5/9 (56%)
5 μg/kg	3/9 (33%)	4/9 (44%)	4/9 (44%)	5/9 (56%)

CONCLUSIONS

Recombinant human GM-CSF has attracted the interest of many scientists wishing to exploit its unique characteristics as an enhancer of the host immune system response. The most promising area for the continued clinical development of rhuGM-CSF is probably vaccine adjuvancy. To test the concept of using rhuGM-CSF as a vaccine adjuvant, a pilot double-blind, placebo-controlled, dose-escalation study was carried out to determine whether the immuneresponse to flu vaccine is enhanced by the administration of rhuGM-CSF. The results of this pilot study are promising suggesting that rhuGM-CSF may have a role as a flu vaccine adjuvant in the elderly. Further studies are warranted to define the optimal dosage in this indication, and to confirm the good safety profile observed in the pilot study. If these results are confirmed, some of the vaccines for which rhuGM-CSF could be used as adjuvant include

the influenza vaccine in the elderly, hepatitis B vaccine to reduce the number of doses required to provide adequate protection or to enhance immuneresponse in the hemodialyzed patient, and tumour or HIV vaccines currently under development.

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