CHARACTERIZATION OF SHEEP AFFERENT LYMPH DENDRITIC CELLS AND THEIR ROLE IN ANTIGEN CARRIAGE

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The functions of accessory cells in T-dependent responses to antigen appear to be at least twofold. First, to present antigen in association with MHC molecules for recognition by clonotypic antigen receptors on T cells; second, to promote activation and subsequent proliferation of antigen-specific T cells. Dendritic cells, including mature Langerhans' cells, afferent lymph veiled cells, and lymph node interdigitating cells appear to be unique accessory cells with respect to T cell activation (1–4). While a wide variety of MHC class II–positive cells induce proliferation of previously activated T cells, only dendritic cells are capable of clustering with and stimulating proliferation of normal resting T cells (5).

It is suggested these dendritic cells represent different stages along a migratory pathway that is related to their function. Langerhans' cells are believed to migrate from the dermis through afferent lymphatic vessels in the form of veiled cells that enter lymph nodes as interdigitating cells (6–8). The functional significance of this migration is the carriage of antigen from the dermis to the paracortical areas of the lymph node for presentation to T cells. Several observations support this scheme. Langerhans' cells, veiled cells, and interdigitating cells in the mouse are recognized by the mAb NLDC-145, suggesting a common lineage for these cells (9); cells with Birbeck granules, a marker of Langerhans' cells in some species, have been found in afferent lymph and T-dependent areas of lymph nodes following skin sensitization (6, 10); afferent lymph veiled cells and lymph node dendritic cells have been isolated with antigen on their cell surface following skin painting with dinitrofluorobenzene (DNFB)[1] (11) and FITC (12), respectively.

The low number of dendritic cells in tissues and the lengthy fractionation procedures required for their isolation have hampered studies on these cells. In the case of afferent lymph dendritic cells, these problems may be overcome by the cannulation of pseudo-afferent lymphatic vessels in relatively large experimental animals (13, 14). These lymphatics arise after lymphadenectomy when true afferent lymphatic vessels reanastomose with the former efferent duct (15). Lymph collected from

[1] *Abbreviations used in this paper:* CD, cluster of differentiation; DNFB, dinitrofluorobenzene; LFA-3, lymphocyte functional antigen.

these lymphatic vessels has a cellular composition similar to that of true afferent lymph and includes 1–10% nonlymphoid MHC class II–positive dendritic cells (13, 16, 17). In this report, we describe the properties of dendritic cells collected from sheep pseudo-afferent lymphatic vessels. In characterizing these cells, we have developed a mAb that reacts with sheep CD1 and identifies dendritic cells in afferent lymph as well as skin and lymph node tissue. The expression of MHC class II and CD1 by afferent dendritic cells suggests they are related to skin Langerhans' cells. This is supported by our observation that afferent dendritic cells migrating from a site of intradermal antigen challenge are able to present the injected antigen to specific T cells in vitro. In an accompanying paper (18) we show the changes in MHC Class II and CD1 expression by afferent dendritic cells as a consequence of in vivo antigen challenge.

Materials and Methods

Antigens

Ovalbumin was obtained from Sigma Chemical Co. Ltd (cat. no. A5503, Grade V; Poole, UK). Purified protein derivative of bovine tuberculin (PPD, batch 291) was kindly supplied by the Central Veterinary Laboratory, Weybridge, UK.

Immunization and Secondary In Vivo Challenge of Animals

1–3-yr-old Finnish Landrace sheep of either sex were purchased from the Moredun Research Institute, Edinburgh, Scotland. Sheep were immunized with 1 mg of ovalbumin injected over two sites intramuscularly in a 1-ml mixture of PBS and CFA containing H37Ra *Mycobacterium tuberculosis*. Sheep were also immunized with five human doses of Bacillus Calmette-Guerin (BCG; Glaxo Laboratories Ltd., Greenford, UK). Secondary in vivo antigen challenge of primed animals was by intradermal injection of 50 μg of antigen in 1 ml of PBS into several sites around the drainage area of the cannulated pseudo-afferent lymphatic vessel.

Medium

RPMI 1640 (cat. no. 074-1800; Gibco Biocult, Uxbridge, UK) was supplemented with 2 mM L-glutamine, 100 U/ml benzyl penicillin and 100 U/ml streptomycin, 5 × 10^{-5} M 2-ME, and 2 g/liter sodium b rbonate. Culture medium consisted of RPMI 1640 with supplements and 10% FCS (Flc Laboratories, Hertfordshire, UK). Wash medium consisted of RPMI with supplements and 1% FCS.

Collection and Fractionation of Lymph Cells

Surgical techniques for the cannulation of the popliteal efferent lymphatic vessels were as described by Hall et al. (19). Pseudo-afferent lymphatic cannulations were as those described for the prefemoral lymphatic except that the node had been excised at least 6 wk previously (13). Cannulations were allowed at least 3 d to stabilize after operative procedures. Lymph was collected from fully conscious animals into sterile siliconized bottles containing 200 U heparin and was centrifuged at 800 g for 10 min to separate cells from lymph fluid. Mononuclear lymph cells were harvested by centrifugation over Lymphoprep (Nyegaard, Oslo, Denmark).

Afferent Dendritic Cells.   Afferent mononuclear cells were fractionated by centrifugation over a discontinuous gradient of 14.5% (wt/vol) Metrizamide (Nyegaard) in RPMI 1640 plus 10% FCS (20). Cells and density medium were cooled on ice then centrifuged for 30 min at 4°C with 800 g applied to the interface of cells and metrizamide. Purified afferent lymph dendritic cells were collected from the top of the metrizamide gradient after centrifugation and washed twice.

Efferent T Cells.   Resting T cells were prepared from efferent lymphocytes after removal of plastic adherent cells by incubation at 37°C for 1 h. Cells were then frationated by nylon

fiber column according to Julius et al. (21). The nylon fiber nonadherent cells were routinely >95% CD5⁺ cells.

Peripheral Blood Mononuclear Cells.   PBMC were prepared by defibrination of venous blood followed by centrifugation over Lymphoprep (22).

Generation of Antigen-specific Cell Lines

Antigen-specific cell lines were generated and recovered as described previously (23). Briefly, PBMC, prepared from antigen-primed sheep, were cultured with antigen for 5 d. Viable blast cells were expanded by culture for 14 d with fresh additions of 120 pM human rIL-2 (Biogen SA, Geneva, Switzerland) every 3–4 d. Viable cells were harvested over Lymphoprep and restimulated with antigen in the presence of X-ray irradiated autologus PBMC and the resultant blast cells were again expanded with rIL-2. Viable resting cells collected after this second cycle of antigen stimulation and IL-2 expansion were stored under liquid nitrogen.

T Cell Proliferation

Resting T Cells.   T cells were cultured in 96-well round-bottomed microculture plates in a final volume of 200 µl. Triplicate cultures were stimulated with various numbers of X-ray irradiated afferent or efferent mononuclear cells or fractionated dendritic cells.

Antigen-specific T Cell Lines.   Antigen-specific T cells were recovered from storage under liquid nitrogen as described elsewhere (23). Cells were cultured in 96-well flat-bottomed microculture plates in a final volume of 200 µl. Triplicate cultures were stimulated with the indicated number of X-ray irradiated autologous afferent dendritic cells that were collected either before or at different times after in vivo antigen pulsing. Appropriate exogenous soluble protein was added to the cultures of T cells and dendritic cells at the indicated concentrations.

Culture plates were incubated at 37°C for 5 d in a humidified atmosphere of 5% CO_2/95% air. Cells were pulsed with 1 µCi [³H]thymidine (specific activity, 2Ci/mmol; Amersham Corp., UK) over the last 5 h of culture then collected onto glass filter paper using a semiautomated harvester. [³H]Thymidine incorporation was assessed by liquid scintillation counting. The data are expressed as the geometric mean for the triplicate cultures.

Dendritic-T Cell Clustering

A feature of cocultures between T cells and dendritic cells is the formation of discrete clusters involving both cell types. To demonstrate these clusters resting T cells were cultured at 2 × 10⁶/ml with irradiated unfractionated lymph cells or purified dendritic cells in 1 ml of culture medium in 24-well plates. Cultures were incubated for 3 d at 37°C in a humidified atmosphere of 5% CO_2/95% air. Clusters were photographed within the wells using an inverted Diavert microscope at ×40 magnification.

X-Ray Irradiation

Cells were resuspended in culture medium to a concentration of 2 × 10⁶/ml and irradiated with 2,500 rad using a cobalt-cesium source.

Production of VPM5

BALB/c mice were immunized by intramuscular injection with 10⁶ afferent lymph dendritic cells emulsified in 250 µl CFA, followed 3 wk later by the same inoculation given intraperitoneally, then intravenously with cells in PBS 4 d before fusion. Spleen cells from immunized mice were fused with the NSO cell line at a ratio of 1:5, respectively (24). The products of these fusions were seeded at 5 × 10⁴ NSO cells/well in 96-well flat-bottomed plates. After HAT selection, supernatants were screened on ELISA plates coated with 1% paraformaldehyde-fixed afferent lymph cells and hybrids from positive wells were cloned in soft agar. VPM5 was picked for further study.

Immunoprecipitation of VPM5 Antigen

Sheep thymocytes were treated with methyl-*p*-hydroxylbenzimidate HCl as described by Wall and Fitch (25) then iodinated with ¹²⁵I catalyzed by lactoperoxidase and glucose oxi-

dase. Cells were washed three times with PBS plus 0.1% sodium azide, resuspended at 5 × 10^7/ml, and aliquots of ~2.5 × 10^7 cpm were mixed with 10 μl of VPM5 ascites fluid at 37°C for 60 min. Cells were then lysed in 0.5 ml of 0.5% (vol/vol) Triton X-100 in 150 mM NaCl, 20 mM Tris-HCl, pH 8, and 0.2 mM PMSF at room temperature for 10 min. Cell debris was removed by microfuging for 5 min at 16,000 g and the supernatant was incubated for 16 h at 4°C. Immune complexes were isolated by incubation for 1 h at 4°C with 50 μl sheep anti-mouse Ig Affigel 10 (10 mg affinity-purified antibody per milliliter affigel), (Bio-Rad Laboratories, Watford, UK). The beads were washed four times in lysis buffer and the complexes were eluted by boiling in 50 μl SDS-elution buffer containing 5% 2-ME. Eluted material was separated by 5-20% linear gradient SDS-PAGE (26) followed by autoradiography using Kodak XS-1 film.

Monoclonal Antibodies

SBU-1, SBU-T4, SBU-T6, and SBU-T8 are mouse mAbs that recognize the sheep cell surface glycoproteins MHC class I, CD4, CD1, and CD8, respectively (27-29). These were a generous gift from Dr. M. Brandon (Melbourne, Australia). L/180/1 and I35-A are mouse mAbs that recognize sheep LFA-3 (30) and CD2 (31), respectively, and were a generous gift from Dr. T. Hunig, Max-Plank Institute, Munich, FRG. VPM8 is a mouse monoclonal antibody generated in our laboratory that has specificity for sheep Ig light chain (Jones, P., and I. McConnell, unpublished observation). SW73.2 and VPM16 are rat mAbs and mouse mAbs, respectively, that react with sheep MHC class II (Dutia, B., et al., manuscript in preparation).

Immunofluorescence and Immunohistology

Cells were washed twice with ice-cold PBS containing 1% BSA and 0.1% sodium azide (PBS-BA). 1-2 × 10^6 cells/tube were treated with the appropriate dilution of mAb for 30 min on ice, washed twice with PBS-BA, and reacted with sheep anti-mouse Ig-FITC for 30 min on ice. Cells were washed three times with PBS-BA and fixed with 1% paraformaldehyde in PBS then stored at 4°C. Cells were analyzed by FACS using either a Becton Dickinson & Co. (Mountain View, CA) FACS IV or a FACScan system. 10^4 cells were analyzed per sample with dead cells excluded on the basis of forward light scatter. The tissue distribution of the VPM5 antigen was by standard immunoperoxidase methods (32) using peroxidase-labeled antiglobulin for tissue sections and gold-labeled antiglobulin (Jannsen Biochemica, Beerse, Belgium) for smears.

Histochemical Staining

Staining for alkaline phosphatase, membrane ATPase, nonspecific esterase, and Sudan Black was carried out by routine histochemical techniques (32).

Results

We have collected afferent lymph dendritic cells in order to study their role in antigen carriage and presentation. A major problem associated with functional studies on afferent lymph cells is that single afferent lymphatics are small and are difficult to cannulate. These lymphatics have a low output of cells and lymph fluid. To overcome this problem we have ablated peripheral lymph nodes in sheep and subsequently cannulated the pseudo-afferent lymphatic vessel that arises as a consequence of afferent lymphatic vessels reanastomosing with the former efferent duct. The pseudo-afferent lymphatic vessel is easier to cannulate and routinely provides a greater output of cells than does the cannulation of true afferent lymphatic vessels. In our characterization of afferent dendritic cells we have isolated a mAb that reacts not only with these cells in afferent lymph but also dendritic cells in sheep peripheral lymphoid organs. Functionl studies show that afferent dendritic cells are capable of stimulating primary resting T cells and are involved in antigen carriage from the skin to draining lymph nodes.

Characterization and Distribution of VPM5 Antigen.   VPM5 (formerly A1E1) is a mAb isolated from mice immunized with purified afferent lymph dendritic cells. The monoclonal reacts in tissue sections with large nonlymphoid dendritic cells as described in Table I. In lymph node tissue, positive cells are localized to the paracortical T cell areas. The monoclonal does not stain all of the dendritic cells as VPM5⁻ MHC class II–positive dendritic cells can be identified in this area. VPM5⁺ cells within this site might represent recently arrived migrants that subsequently lose VPM5 staining. Cells staining with VPM5 in the spleen are found predominately in the inner periarteriolar lymphocyte sheath (PALS) around the central arteriole In the thymus, nonlymphoid dendritic cells of the medulla and the corticomedullar regions stained with VPM5 as did thymic cortical lymphocytes. Cells in the skin that stained with VPM5 were found within the dermis and at the junction of the dermis and epidermis. Cells were isolated from afferent and efferent lymph as well as peripheral blood. Positive cells were found only in afferent lymph and these were typical dendritic or veiled cells as described in afferent lymph of other species (33–35).

VPM5 staining is similar to that described for SBU-T6, a mAb that recognizes sheep CD1 (27) except that, unlike SBU-T6, VPM5 does not stain peripheral blood B cells or B cell follicles. SBU-T6, however, reacts strongly in an ELISA with immunopurified VPM5 antigen (data not shown). The staining pattern of VPM5 is also similar to that described for two anti–bovine CD1 mAbs, CC13 and CC14, which crossreact with sheep tissue (36).

Immunoprecipitation of radiolabeled sheep thymocytes with VPM5 followed by SDS-PAGE and autoradiography shows that VPM5 specifically precipitates polypeptides of 48 and 12 kD (Fig. 1). The tissue distribution together with the molecular mass of the antigen suggest that it recognizes sheep CD1. Furthermore, our data suggest that it recognizes a subgroup of the antigen recognized by SBU-T6.

TABLE I

Staining Pattern of VPM5 on Sections of Various Organs and on Cytospin Preparations of In Vitro Isolated Cells

Organ	VPM5 staining	Cell type and localization
Spleen	+	Dendritic cells in PALS
Lymph node	+	Dendritic cells in paracortex
Thymus	+	Cortical thymocytes Medullary dendritic cells
Skin	+	Dendritic cells within the dermis and at the junction of the dermis and epidermis
Peyer's patch	–	
Brain	–	
Heart	–	
Kidney	–	
Liver	–	
In vitro isolated cells		
PBMC	–	
Afferent lymph	+	Dendritic cells
Efferent lymph	–	

Staining of tissue sections and cytocentrifuge smears was carried out as described in Materials and Methods.
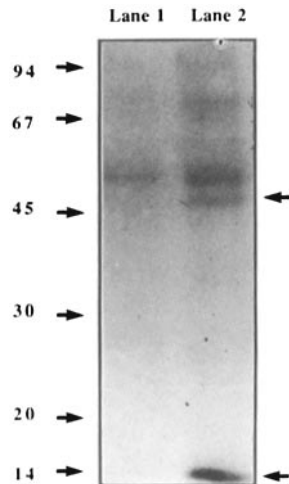
Figure 1. Autoradiograph of the material immunoprecipitated from ^{125}I-labeled sheep thymocytes using control (lane *1*) and VPM5 (lane *2*) ascites.

Isolation and Characterization of Afferent Dendritic Cells. Lymph collected from a pseudo-afferent cannulation contains cells at a concentration of 1–5 × 10^6/ml. The cellular composition of afferent lymph cells (Table II) contains 1–10% VPM5$^+$ cells that are large nonlymphoid dendritic cells. Enriched populations of dendritic cells were prepared by centrifugation of afferent mononuclear cells on a single density band of 14.5% metrizamide, according to the method of Knight et al. (20). After this, a population of cells that are routinely >75% dendritic cells, as seen by light microscopy, may be recovered from the top of the density gradient. Approximately 10^6–10^7 dendritic cells could be harvested by this technique after fractionating a 16-h collection of afferent cells. Afferent lymphocytes and some of the dendritic cells pass through this concentration of metrizamide. Purified dendritic cells were stained with a panel of mouse anti–sheep leukocyte mAbs and analyzed by flow cytometry (Table III). Contaminating lymphocytes that were mainly T cells and <5% B cells were excluded from the analysis on the basis of their lower forward and side scatter compared with dendritic cells. All of the dendritic cells expressed high levels of MHC class I and class II molecules. The majority of dendritic cells reacted with VPM5 mAb, confirming the expression of CD1 by these cells (27). Afferent dendritic cells were stained by the mAb 135/A, which identifies sheep CD2, a ligand of LFA-3 (30). VPM8, a monoclonal that reacts with sheep Ig light chain, stained the majority of afferent dendritic

TABLE II

Staining of VPM5 in Lymph Compartments of the Sheep

Source of cells	Percentage positive cells			
	CD5	sIg	VPM5	Class II
Efferent	67.2 (7.0)	22.8 (7.0)	<0.5 (0.06)	29.4 (7.1)
Afferent	83.4 (2.2)	11.0 (8.1)	8.2 (10.8)	57.0 (5.3)

Afferent and efferent lymph mononuclear cells were stained with mouse mAbs followed by rabbit anti–mouse Ig-FITC. Cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry. The data shown are the mean of four experiments with the standard deviation in parentheses.

TABLE III

Phenotype of Afferent Lymph Dendritic Cells

Name	Antigen	Reactivity with dendritic cells
SBU-T4	CD4	−
SBU-T6	CD1	+
SBU-T8	CD8	−
VPM5	CD1	+ +
L/180/1	LFA-3	+ + +
I35/A	CD2	+
VPM8	sIg	+
SBU-1	Class I	+ + +
SW73.2	Class II	+ + +

Afferent lymph dendritic cells were isolated as described in Materials and Methods then stained with mouse mAbs followed by (Fab)$_2$ rabbit anti–mouse Ig-FITC. Cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry. These results were seen in three of three experiments.

cells. The sIg$^+$ cells in afferent lymph, therefore, include some of the dendritic cells, in contrast to efferent lymph where the sIg$^+$ cells are all small lymphocytes (Table II). A representative sample of fluorescence staining of the dendritic cells analyzed by a Becton Dickinson & Co.. FACScan is shown in Fig. 2. The results show afferent dendritic cells displaying unimodal marginal positivity for CD4 expression. When analyzed by a Becton Dickinson & Co. FACS IV (Table III), these cells show no reactivity with anti-CD4 mAbs. The data also show afferent dendritic cells have a uniform expression of LFA-3 and MHC class II. The expression of LFA-3 is of equivalent intensity as shown by afferent lymphocytes while the level of MHC class II expression by afferent dendritic cells is 3–10 greater than resting B cells.

Although we cannot exclude the possibility that the purified population of dendritic cells includes macrophage cells, histochemical staining of fractionated dendritic cells showed no staining for the macrophage markers nonspecific esterase or Sudan Black. Positive staining was seen for membrane ATPase and alkaline phosphatase. We have also found that sheep macrophages collected by bronchiolar lavage do not express VPM5 or CD2.
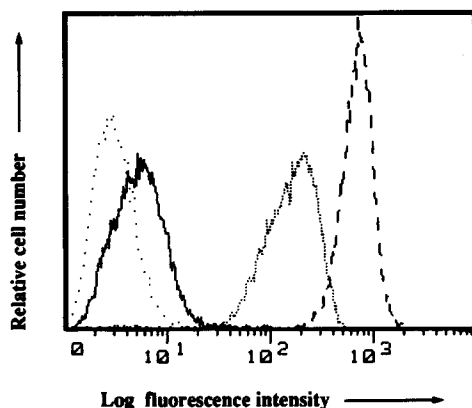

FIGURE 2. Immunofluorescence profile of afferent dendritic cells stained with various mAbs. Afferent lymph dendritic cells were isolated as described in Materials and Methods then stained with anti-CD4, anti-LFA-3, or anti-MHC class II mAbs followed by (Fab)$_2$ rabbit anti–mouse Ig-FITC. Cells were fixed with 1% paraformaldehyde and analyzed by FACScan flow cytometry. These results were seen in three of three experiments. (· · ·) Control, (—) CD4, (····) LFA-3, (---) MHC class II.

The results shown here, in particular the expression of CD1 and MHC class II by afferent dendritic cells, together with the results of Hein et al. (17) and Mackay et al. (28), suggest that afferent dendritic cells are lymph-borne Langerhans' cells or a migratory intermediatory between Langerhans' cells and interdigitating cells (9).

Accessory Functions of Afferent Lymph Dendritic Cells.   Both afferent and efferent lymph compartments contain MHC class II–positive cells and therefore the potential to act as accessory cells for T lymphocyte responses to antigen. This potential has been analyzed in this study by measuring the primary T cell response to alloantigen. Fig. 3 shows cocultures of allogeneic afferent or efferent lymph cells with resting T cells. Clustering does not occur when autologous (Fig. 3 *A*) or allogeneic (Fig. 3 *B*) efferent lymph cells are mixed with resting T cells. In contrast, when unfractionated allogeneic afferent mononuclear cells are cultured with resting T cells, small and infrequent clusters develop (Fig. 3 *C*). With purified allogeneic afferent dendritic cells as stimulators, larger and more frequent clusters are seen compared with cultures with unfractionated afferent cells (Fig. 3 *D*). Within these clusters one or more dendritic cells could be seen surrounded by numerous lymphocytes.

Afferent and efferent lymph cells differ in their ability to stimulate the proliferation of resting T cells. Fig. 4 *A* shows that unfractionated allogeneic afferent lymph cells stimulate proliferation of resting T cells but allogeneic efferent cells do not. This proliferative response can be stimulated by purified afferent dendritic cells, as shown in Fig. 4 *B*. At high numbers of stimulator cells/well, autologous dendritic cells stimulate a response of similar magnitude to that stimulated by allogeneic den-
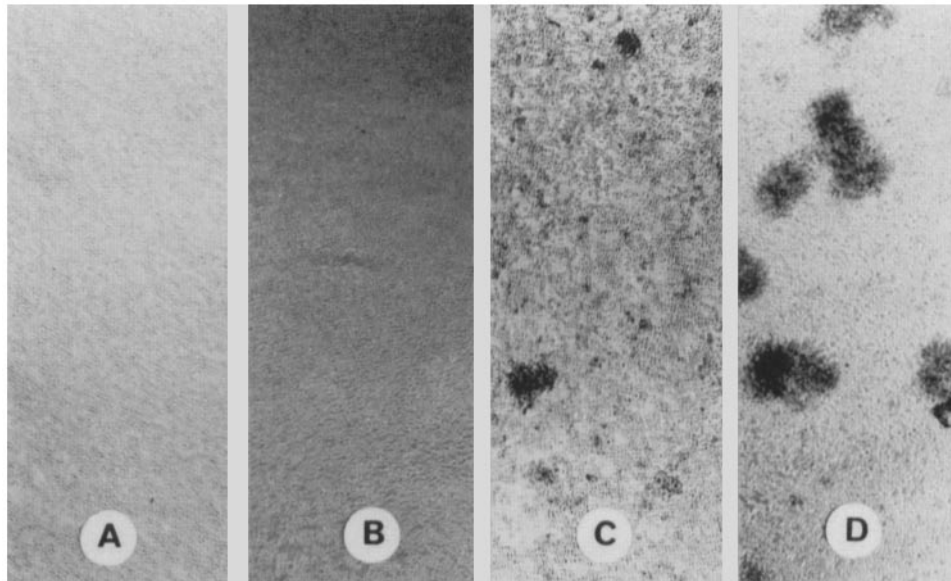

FIGURE 3. 10^5/well nylon fiber nonadherent resting efferent lymph T cells were cultured with irradiated lymph cells. (*A*) 10^5/well autologous efferent T cells. (*B*) 10^5/well allogeneic efferent cells. (*C*) 10^5/well allogeneic afferent cells. (*D*) 5×10^4/well allogeneic fractionated afferent dendritic cells. Cultures were incubated for 3 d in 24-well plates. Cells were photographed at ×40 magnification.
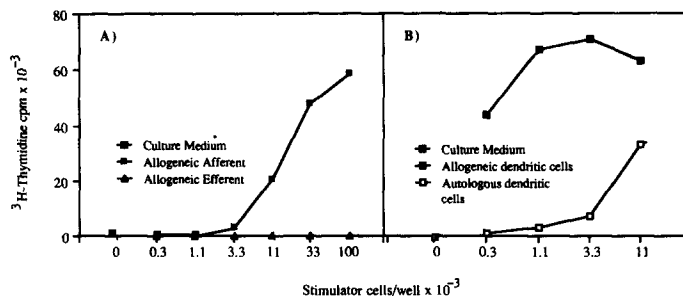
FIGURE 4. T cell proliferation stimulated by afferent dendritic cells. 10^5/Well nylon fiber nonadherent resting efferent lymph T cells were cultured with irradiated lymph cells in round-bottomed microculture plates with either (A) irradiated unfractionated afferent or efferent lymph cells or (B) irradiated fractionated autologous or allogeneic dendritic cells. Cultures were incubated for 3 d and proliferation was measured by the uptake of [^{3}H]thymidine over the last 5 h of culture. The data is expressed as the geometric mean of counts per minute from triplicate cultures.

dritic cells. As the number of stimulator cells/well is reduced then only allogeneic dendritic cells stimulate a significant response by the T cells. Although we have not demonstrated that proliferating cells arise from within these clusters, significant [^{3}H]thymidine uptake did not occur in the absence of cluster formation.

Both afferent and efferent lymph contains MHC class II–positive cells, yet only afferent lymph cells are capable of clustering and activating resting T cells. These functions can be carried out by enriched populations of afferent dendritic cells showing the similarity between these and other isolated dendritic cells in stimulating primary responses by T cells to antigen (5).

Afferent Dendritic Cells Are Involved in Antigen Carriage. We have investigated the hypothesis that dendritic cells are involved in antigen carriage from the skin to draining lymph nodes. Dendritic cells were collected 24 h after an intradermal injection of soluble protein antigen into the drainage area of a pseudo-afferent cannulation then cultured in the absence of exogenous antigen with antigen-specific cells prepared from the same animal.

Table IV shows the proliferative response of PBMC, from primed animals, stimu-

TABLE IV

The Response of PBMC to Autologous In Vivo Pulsed Dendritic Cells

Antigen pulse	Dendritic cells	Collection of dendritic cells (h)	
		Pre-pulse	24 h after antigen challenge
Ovalbumin (50 μg)	–	6,330	4,443
	+	5,078	10,777
PPD (20 μg)	–	2,143	2,132
	+	3,189	8,734

10^5 PBMC/well were cultured with 5 × 10^4/well irradiated fractionated afferent dendritic cells collected either before or at 24 h after an intradermal injection of antigen into the drainage area of the pseudo-afferent cannulation area. Cultures were incubated for 3 d and proliferation measured by the uptake of [^{3}H]thymidine over the last 5 h of culture. The data are expressed as the geometric mean of counts per minute from triplicated cultures.

lated by autologous in vivo antigen-pulsed dendritic cells. Dendritic cells collected after in vivo antigen challenge stimulate a significant response above that seen with pre-challenge dendritic cells. This response is seen when either ovalbumin or PPD is used to antigen pulse dendritic cells in vivo. These data show that dendritic cells may at least pick up antigen in vivo and either present antigen directly to T cells or pass the material to other accessory cells within the PBM population. To distinguish between these, the experiment was repeated using antigen-specific T cell lines that do not respond to antigen in the absence of added accessory cells (23). Table V shows dendritic cells, collected after in vivo antigen challenge with ovalbumin, stimulate a significant proliferative response by ovalbumin-specific T cells compared with that induced by pre-challenge dendritic cells. This effect is antigen specific as no response is seen by PPD-specific T cells. In Exp. 2, dendritic cells collected after in vivo antigen challenge with PPD stimulate a significant proliferative response by the PPD-specific cell line. No response is seen by the ovalbumin-specific T cells to the PPD-pulsed dendritic cells. The proliferation of antigen-specific T cells is dependent upon the number of in vivo pulsed dendritic cells added to the cultures. Fig. 5 shows the response stimulated by antigen-specific T cells to various numbers of ovalbumin-pulsed dendritic cells collected after in vivo antigen challenge. A significant response is seen by the ovalbumin-specific cell line down to 1.9×10^3 dendritic cells/well. In contrast, the PPD-specific cell line shows a background level of proliferation to all concentrations of added dendritic cells. The experiments described in Table V and Fig. 5 were set up at the same time. As in vivo antigen pulsed afferent dendritic cells were the only accessory cells added to these cultures these data show that these cells can directly stimulate T cells in an antigen-specific manner and do so with antigen acquired in vivo.

It may be argued that the failure to see proliferation by the PPD-specific cell line with ovalbumin-pulsed dendritic cells, or the ovalumin-specific cell line with PPD-pulsed dendritic cells, is due to the cell lines failing to survive liquid nitrogen storage. To show this was not the case, antigen-specific T cells were cultured with exogenous specific antigen in the presence of dendritic cells pulsed with nonspecific antigen. The results are shown in Table VI. On each day of recovery from liquid nitrogen

TABLE V

The Response of Cell Lines to Autologous In Vivo Pulsed Dendritic Cells

		Collection of dendritic cells	
Antigen pulse	Cell line	Pre-pulse	24 h after antigen challenge
Ovalbumin (50 µg)	Ovalbumin	5,704	169,588
	PPD	2,709	3,978
PPD (20 µg)	Ovalbumin	1,032	3,654
	PPD	1,212	20,040

5×10^4 ovalbumin-specific or PPD-specific T cells/well were cultured with 5×10^4/well irradiated fractionated afferent dendritic cells collected either before or at 24 h after an intradermal injection of antigen into the drainage area of the pseudo-afferent cannulation drainage area. Cultures were incubated for 3 d and proliferation measured by the uptake of [³H]thymidine over the last 5 h of culture. The data are expressed as the geometric mean counts per minute from triplicate cultures.
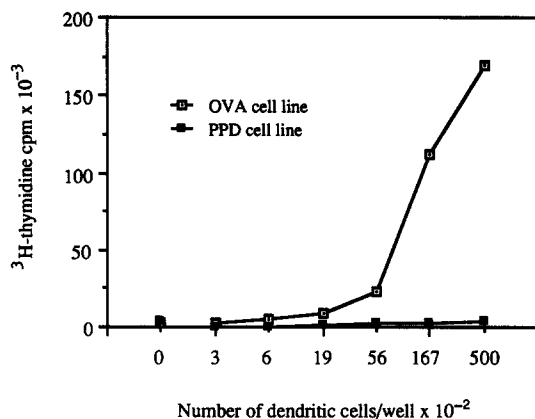
FIGURE 5. Response of cell lines to various numbers of dendritic cells pulsed in vivo with ovalbumin. 5×10^4 ovalbumin-specific or PPD-specific T cells/well were cultured with various numbers of irradiated fractionated afferent dendritic cells collected 24 h after in vivo antigen pulsing with 50 μg ovalbumin. Cultures were incubated for 3 d and proliferation measured by the uptake of [^{3}H]thymidine over the last 5 h of culture. The data are expressed as the geometric mean of counts per minute from triplicate cultures.

TABLE VI

The Response of Cell Lines to Specific Antigen with In Vivo Pulsed Dendritic Cells

Antigen pulse	Cell line	Antigen (27 μg/ml)	Collection of dendritic cells	
			Pre-pulse	24 h after antigen challenge
Ovalbumin	PPD	–	5,704	3,282
		+	136,593	122,980
PPD	Ovalbumin	–	1,325	4,822
		+	110,584	150,706

5×10^4 ovalbumin-specific or PPD-specific T cells/well were cultured with 5×10^4/well irradiated fractionated afferent dendritic cells collected either before or at 24 h after an intradermal injection of antigen into the drainage area of the pseudo-afferent cannulation area. T cells were cultured with dendritic cells in the absence or presence of 27 μg/ml soluble protein antigen. Cultures were incubated for 3 d and proliferation measured by the uptake of [^{3}H]thymidine over the last 5 h of culture. The data are expressed as the geometric mean of counts per minute from triplicate cultures.

the PPD-specific cell line, while showing no response to ovalbumin-pulsed dendritic cells alone, clearly responded to PPD antigen presented by the same dendritic cells. Similarly, the ovalbumin cell line responded to ovalbumin in the presence of PPD-pulsed dendritic cells. These data confirm the viability of cells recovered from liquid nitrogen.

Collectively, the data described in Tables V and VI show the ability of afferent dendritic cells to acquire soluble protein antigen either in vivo or in vitro for presentation to autologous T cells. As these experiments were set up at the same time they also show that as a population, the dendritic cells are able to present more than one antigen at a time.

Discussion

Dendritic cells first isolated from tissue sites by Steinman have been shown to be potent accessory cells for T cell responses to antigen (reviewed in 37). The in vivo

correlates of isolated dendritic cells include epidermal Langerhans' cells, afferent lymph veiled cells, and lymph node interdigitating cells. Furthermore, it is postulated that these dendritic cells represent intermediates along a pathway of migration involved in the carriage of antigen to lymph nodes (8). While information has been described for Langerhans' cells and lymph node interdigitating cells in this scheme, little data are available concerning the role of afferent lymph dendritic cells. Here we report on the isolation and characterization of dendritic cells from sheep afferent lymph. These cells are obtained by the cannulation of pseudo-afferent lymphatic vessels that arise after lymphadenectomy of peripheral nodes. A 16-h collection of lymph from these cannulations may contain between 10^6 and 10^7 dendritic cells. The bulk of these may be harvested at a purity of >75% following a single density-gradient centrifugation step. The ability to collect afferent dendritic cells in large numbers, and in high purity, provides an opportunity to characterize and investigate the role of these cells in antigen carriage and presentation of soluble protein antigen to T cells.

We have found that sheep afferent dendritic cells, like human and mouse Langerhans' cells, express CD1. This family of molecules is best described in the human system where three subgroups are defined, CD1a, CD1b, and CD1c, that have glycosylated heavy chains of 49, 45, and 43 kD, respectively, and that can be differentiated on the basis of their tissue distribution (38). All the anti–human CD1 mAbs stain cortical thymocytes, but while anti-CD1a monoclonals stain both dermal and epidermal dendritic cells, CD1b monoclonals stain only dermal dendritic cells. Anti-CD1c mAbs can be differentiated by their ability to stain B cells (39). The tissue distribution of VPM5 antigen is similar to that of the human CD1b antigen. VPM5 tissue staining resembles that of the anti–bovine CD1 monoclonals CC13 and CC14 (36), but differs from that of the sheep anti-CD1 marker SBU-T6 in that VPM5 does not stain B cells (27). SBU-T6, however, reacts strongly with VPM5 antigen. These data show that in the sheep the CD1 antigen consists of more than one product and demonstrate that there are reagents that can differentiate these products. Sheep afferent dendritic cells express high levels of surface molecules involved in antigen recognition by T cells. The expression of MHC class I and class II molecules allows afferent dendritic cells the potential to present antigen to $CD8^+$ and $CD4^+$ T cells (40). The level of MHC class II expression by afferent dendritic cells is approximately three times that seen on resting B cells (41). A feature of some dendritic cells is the ability to form clusters with resting T cells and is likely to involve an interaction between cellular adhesion molecules (42). LFA-3 was found on afferent dendritic cells at levels approximately equal to those on afferent lymphocytes. A human lymphoblastoid cell line has been described with two forms of cell surface LFA-3 (43). One of these is linked to the membrane by a hydrophobic transmembrane region followed by an intracytoplasmic tail. The other lacks the intracytoplasmic tail and is linked to the membrane by a phosphatidylinositol glycan moiety attached to the COOH terminus of the LFA-3 molecule. This second type of linkage has been identified on membrane proteins involved in transmembrane signaling, interleukin release, and intercellular adhesion (44). If both forms of LFA-3 are present on afferent dendritic cells then a dichotomy of function might exist for this molecule. One form may act as an anchor for clustering with T cells while the other may be involved in signal transduction and cytokine release. It is reported that perturbation of LFA-3 on human macrophages by anti-LFA-3 induces IL-1 release (45). We are currently

investigating the linkage and function of LFA-3 on afferent dendritic cells. Afferent dendritic cells were found to express CD2, a ligand for LFA-3 (46), on their cell surface. We do not know at the present whether this is due to expression of a CD2 gene or adsorption of the molecule (47) as a consequence of the high level of LFA-3 shown by dendritic cells.

Dendritic cells are potent accessory cells for T cell responses but significant differences exist in the accessory potential displayed by different dendritic cells. In vitro cultured mouse epidermal Langerhans' cells, like spleen dendritic cells, form clusters with resting T cells, whereas fresh Langerhans' cells do not (48). Fresh Langerhans' cells have similar amounts of MHC class II molecules to splenic dendritic cells and although levels increase during in vitro culture, it does not appear to be the sole determinant of this increased accessory potential. A maturation step for mouse Langerhans' cells has been demonstrated in vitro that can be mediated by recombinant granulocyte/macrophage colony-stimulating factor (GM-CSF) (49). As suggested by others (49), Langerhans' cells may differentiate in the epidermis under the influence of cytokines from keratinocytes and macrophages that produce GM-CSF and IL-1. Both of these cytokines increase the accessory function of dendritic cells and may allow Langerhans' cells to mature into competent accessory cells as they pass into afferent lymph.

A prediction of the hypothesis that afferent dendritic cells are involved in antigen carriage from the dermis to lymph nodes is that antigen deposited in the dermis should, at some stage, be associated with these cells. We have demonstrated that this is the case by a functional assay. Afferent dendritic cells collected from the site of an intradermal challenge with soluble protein antigen were able to induce proliferation of autologous antigen-specific T cell lines, in the absence of exogenous antigen or other accessory cells. Previous studies that have investigated this have shown that in vivo antigen-pulsed dendritic cells stimulate T cell proliferation but fail to distinguish between an antigen-specific response and that which occurs as a result of increased nonspecific accessory function (12, 50). The data presented here, together with the studies showing the localization of antigen on afferent veiled cells or lymph node dendritic cells after antigen challenge at the skin (11, 12), provide strong evidence for a direct role by dendritic cells in the carriage of antigen to lymph nodes. It is not clear how dendritic cells acquire and transport soluble protein antigen in vivo. It has been customary to assume that antigen becomes cell associated as a result of nonspecific pinocytosis and/or phagocytosis. However, Langerhans' cells and lymphoid dendritic cells fail to accumulate the pinocytotic tracer horseradish peroxidase and are regarded as poorly phagocytic (48). Recent studies by Lanzavecchia have shown that efficient uptake and presentation of antigen occurs only when antigen interacts in a specific manner with the presenting cell. This is demonstrated by antigen-specific EBV-transformed B cells that can present specific antigen at much lower concentrations than do nonspecific cells (51). Second, MHC class II–positive T cell clones only present antigen that binds with high affinity to their cell membrane such as recombinant HIV gp120 protein interacting with CD4 (52). Our observation that afferent dendritic cells have Ig on their cell surface reinforces the findings by Miller and Adams (53) that dendritic cells display membrane-associated IgM. This may reflect natural antibody passively acquired by the dendritic cells that functions as a receptor for antigen. The subsequent interaction of antigen with this anti-

body would allow foreign material to enter an antigen processing pathway that would exclude self material. A separation of foreign and self material is unlikely to occur if the process of uptake occurs by a nonspecific mechanism. By using antigen–antibody complexes, dendritic cells in vivo may efficiently concentrate and process small amounts of antigen. This may be particularly important during a primary response to soluble antigen for which dendritic cells appear to be the principal antigen-presenting cell.

It has become clear that dendritic cells play a major role in the pathogenesis of persistent viral infections by members of the lentivirus family. HIV infects CD4+ cells (54) and part of the immunosuppression caused by this virus is due to an alteration of CD4+ accessory cell function. Visna virus, the sheep lentivirus, also infects accessory cells including macrophages (55). The data reported here and in the accompanying paper (18), which describes the alterations of afferent dendritic cell MHC class II and CD1 expression as a consequence of in vivo antigen challenge, provide parameters with which to assess the role of infected dendritic cells in lentivirus infection.

Summary

We have ablated peripheral lymph nodes in sheep and subsequently cannulated the pseudo-afferent lymphatic vessel that arises as a consequence of afferent lymphatic vessels reanastomosing with the former efferent duct. This technique allows the collection of lymph with a cellular composition that resembles true afferent fluid, and in particular, containing 1–10% dendritic cells. A 16-h collection of this lymph may contain between 10^6 and 10^7 dendritic cells. This dendritic cell population may be enriched to >75% by a single-density gradient centrifugation step. We have generated a mAb that recognizes sheep CD1. This monoclonal not only reacts with afferent dendritic cells, but with dendritic cells in the skin and paracortical T cell areas of lymph nodes. The expression of CD1 suggests afferent dendritic cells are related to skin Langerhans' cells and other dendritic cells that act as accessory cells for T cell responses. Consistent with this is the high level of expression by dendritic cells of molecules involved in antigen recognition by T cells, including MHC class I and class II. Afferent dendritic cells express high levels of the cellular adhesion molecule LFA-3, and at the same time express a ligand for this molecule, namely CD2. The accessory functions of afferent dendritic cells resemble those displayed by mature Langerhans' cells and by lymph node interdigitating cells. These include clustering with resting T cells and stimulating their proliferation in a primary response to antigen. Afferent dendritic cells are capable of acquiring soluble protein antigen in vivo or in vitro and presenting the material directly to autologous T cells in an antigen-specific manner. We conclude that afferent dendritic cells represent a lymph-borne Langerhans' cell involved in antigen carriage to the lymph node.

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