

LANGERHANS' CELLS, VEILED CELLS, AND
INTERDIGITATING CELLS IN THE MOUSE RECOGNIZED
BY A MONOCLONAL ANTIBODY

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Langerhans' cells (LC)¹, veiled cells (VC), interdigitating cells (IDC), and dendritic cells (DC) form a group of nonlymphoid dendritic cells (NLDC) postulated to act primarily as antigen-presenting or accessory cells, in particular as inducers of T-dependent responses (1-5). The cells originate from bone marrow (3, 6, 7), but although it has been suggested that they belong to the mononuclear phagocyte system (8, 9), a direct relationship with monocyte/macrophage populations has not yet been proven. In contrast to typical macrophages, these DC show little phagocytic activity (10-12), whereas high levels of Ia antigens are constitutively expressed (13-16).

LC are described as the primary immunocompetent cell in the skin, playing a role in the presentation of antigen to T cells and the induction of contact sensitivity (2, 4, 17). They can migrate from the skin into the afferent lymph (18, 19) as VC, characterized by their long, actively moving processes (19, 20). During their travel via the afferent lymph, VC can carry antigen, which may be presented to lymphocytes in the draining lymph node (21). Based on morphological criteria and localization patterns after cell transfer, it has been hypothesized that the IDC of the T cell-dependent area in lymph nodes are derived from immigrating VC (22, 23).

An accessory role of the IDC in the immune response is assumed in studies with in vitro isolated DC. The in vitro isolated DC was first described by Steinman (3, 24) and although it has been suggested that IDC represent the DC (25, 26), it cannot be excluded that the in vitro DC belong to a functionally different subpopulation. The development of specific markers will give us more insight into the functions and relationships of these populations of nonlymphoid dendritic cells. In this study we describe an mAb that reacts with mouse LC, VC, and IDC.

Materials and Methods

Animals. BALB/c mice and Wistar rats were obtained from CPB-TNO (Zeist, The Netherlands). AKR mice were obtained from Nederlands Kankerinstituut, Amsterdam.

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¹ *Abbreviations used in this paper:* DC, dendritic cell(s); FCA, Freund's complete adjuvant; FDC, follicular dendritic cell(s); IDC, interdigitating cell(s); LC, Langerhans' cell(s); NLDC, nonlymphoid dendritic cell(s); PALS, periarteriolar lymphocyte sheath; SPIT, solid phase immunosolution technique; VC, veiled cell(s).

The Netherlands. C₃D₂F₁ mice were obtained from Bomholtgard Ltd., Rye, Denmark. Nude mice (B10) were a gift of Dr. H. A. Brouwer (Department of Experimental Medicine, Free University, Amsterdam). Animals were kept under routine laboratory conditions.

mAb Production. Wistar rats were immunized intraperitoneally with mouse lymph node stroma emulsified in Freund's complete adjuvant (FCA). This was repeated three times with 4-wk intervals. The lymph node stroma was obtained from lymph nodes (axillary and brachial) that had been stimulated by skin-painting with oxazolone (4-ethoxymethylene-2-phenyloxazol-5-one; Sigma Chemical Co., St. Louis, MO) 4 d previously. This resulted in enlarged, well-developed, T cell-dependent areas in the nodes. The organs were cut in smaller fragments on nylon gauze and rinsed gently with PBS to remove the majority of lymphocytes. The remaining stroma was scraped off the gauze and emulsified in FCA. Fusion of the rat spleen cells and the SP2/0 myeloma cell line was performed 4 d after the last booster injection. Screening of the supernatants from developing clones was performed on tissue sections using a two-step immunoperoxidase staining (see below). Clones of interest were subcloned by limiting dilution.

Immuno- and Enzyme Histochemistry. Cryostat sections of 8–12 μ m were picked up on slides and air-dried. Sections were fixed in acetone for 10 min and air-dried for at least 30 min. Sections were then incubated for 60 min with culture supernatant. After washing in 0.01 M PBS (pH 7.4), slides were covered with a 1:200 dilution of rabbit anti-rat Ig peroxidase (Dako, Glostrup, Denmark) in PBS with 0.2% BSA and 1% normal mouse serum for 60 min. After washing in PBS, the slides were stained for peroxidase activity with 3,3'-diaminobenzidine-tetrahydrochloride (DAB; Sigma Chemical Co.) (0.5 mg/ml Tris-HCl buffer, pH 7.6, containing 0.01% H₂O₂). Control slides were incubated in the same way, but we omitted the first step.

Acid phosphatase activity was shown according to Burstone (27), with naphthol AS-BI phosphate (Sigma Chemical Co.) as substrate and hexazotized pararosaniline as diazonium salt. This procedure was done on the cryostat sections following the immunoperoxidase procedure.

Double Staining by Immunohistochemistry. Lymph node sections or cytospin preparations of AKR (H-2^k) mice were stained with NLDC-145 antibody and incubated after washing with peroxidase-conjugated rabbit anti-rat Ig (Dako). Thereafter the staining was visualized by treating the sections with 3-amino-9-ethylcarbazole (AEC; Sigma Chemical Co.) containing 0.01% H₂O₂ for 10 min. The sections were then incubated with biotinylated anti-Ia^k (clone 11-52) (28). After washing, incubation was performed with alkaline-phosphatase-conjugated avidin (29, 30), and the enzyme was visualized with Fast Blue BB salt (No. F0250; Sigma Chemical Co.) and naphthol-AS-MX phosphate as substrate (27); 0.25% Levamisole (Sigma Chemical Co.) was added to reduce background staining. Slides were mounted in glycerin-gelatin.

Immunofluorescence. Double staining with NLDC-145 and anti-Ia was performed on epidermal sheets as described elsewhere (29) using directly FITC labeled anti-Ia (clone B 21-2). Briefly, the sheets were incubated with NLDC-145 supernatant (rat anti-mouse antibody) followed by incubation with a mouse antiserum against rat Ig. After washing, the tissue was incubated with rhodaminated goat anti-mouse Ig to which rat Ig was added, followed by FITC anti-Ia (rat anti-mouse clone B 21-2).

Cell Suspensions. Blood was collected by cardiac puncture and decoagulated with heparin. Red cells were lysed by ammonium-chloride treatment. Bone marrow suspensions were prepared by flushing femora with cell suspending medium (Earle's balanced salt solution). Suspensions of VC were prepared from lymph nodes (axillary and brachial) according to the method of Knight et al. (31), using collagenase treatment and density centrifugation with metrizamide (Nyegaard & Co., Oslo, Norway). Peritoneal macrophages were collected after intraperitoneal injection of 5 ml of medium. Peritoneal exudate macrophages were harvested 4 d after intraperitoneal injection of 2 ml of thioglycollate. Cytocentrifuge slides of the various cell suspensions were used for immuno- and enzyme histochemistry.

Isotype Determination. The isotype of the mAb was determined in a three step immunoperoxidase method on sections using rabbit anti-rat isotype-specific antisera (Nordic

Laboratories, Tilburg, The Netherlands) in combination with a peroxidase-conjugated anti-rabbit antiserum (Dako).

Molecular Weight Determination. Cell suspensions enriched for cells positive for the NLDC-145 antibody were prepared from peripheral lymph nodes by collagenase treatment and density centrifugation using a metrizamide gradient (Nyegaard) according to the method of Knight et al. (31).

Cells were iodinated using iodogen (Pierce Chemical Co., Rockford, IL) (32). A film of iodogen was plated onto the wall of 15 ml-Corex centrifuge tubes by adding 100 μ g iodogen in 500 μ l of chloroform, and evaporating the solvent with nitrogen gas. Cells (10^6) were added in 500 μ l of cold PBS, together with 1 mCi or carrier free 125 I (Amersham Corp., Arlington Heights, IL). Iodination was continued for 20 min at 0°C with gentle agitation. The cells were washed three times with PBS, 5 mM NaI, and lysed in 500 μ l lysis buffer (NP-40, 0.5% PMSF (Sigma Chemical Co.) in 1 mM PBS, pH 7.4) for 20 min at 0°C with gentle agitation. The labeled NLDC-145 antigen determinant was isolated by the solid-phase immunosolution technique (SPIT) (33). Briefly, in the indirect SPIT, 100 μ l multiple-step, affinity-purified-specific RAaRa IgG, at a concentration of 1 mg/ml, was added in 0.1 M carbonate buffer, pH 9.5, to each well of a U bottom polyvinyl chloride microtiter plate (Nunc, Roskilde, Denmark) and allowed to incubate overnight at 4°C. The well was washed three times with 200 μ l PBS, 0.05% Tween 20. Then 100 μ l of ammonium sulfate-precipitated α -NLDC-145, in PBS with 0.05% Tween, was added to the wells and incubated for 2.5 h at 4°C. As antibody control, the isotype matched mAb 11B5 (rat anti-mouse IgM) was used. The wells were washed three times in PBS with 0.05% Tween. A 100- μ l aliquot of cell lysate was added to each well and allowed to incubate at 4°C overnight. The lysate was then removed and the well was washed three times with PBS. For SDS-PAGE, 100 μ l of sample buffer containing 5% SDS, 20% glycerol, and 0.01% bromophenol blue in 25 mM Tris (pH 6.8) was then added and allowed to incubate for 5 min. The sample buffer was removed and the samples were heated for 3–5 min in a boiling waterbath. Samples were run on SDS-polyacrylamide gels as described by Laemmli (34). To increase the signal, several wells were coated with antibody and the antigen recovered in a minimal volume by sequentially eluting the wells with the same 100- μ l sample buffer (four wells per sample). Reduction was carried out with DTT (Sigma Chemical Co.), 0.2 M final concentration, in sample buffer. For autoradiography, X-Omat XAR-5 films (Eastman Kodak Co., Rochester, NY) were used and exposure was carried out with intensifying screen.

Results

The NLDC-145 antibody reacts with large nonlymphoid cells in the T cell-dependent areas of peripheral lymphoid organs. The cells have slender processes, giving them a characteristic dendritic appearance. When the immunoperoxidase staining was combined with acid phosphatase enzyme histochemistry, the cells showed weak acid phosphatase activity, often concentrated in a distinct area near the cytocenter. Double staining with NLDC-145 and anti-Ia antibody using immunohistochemistry clearly showed that cells positive for the NLDC-145 antibody were also positive for Ia antigens. From the morphology, enzyme pattern, and Ia staining we concluded that the NLDC-145 antibody reacts with the IDC characteristic for the T cell areas of lymphoid organs.

Tissue Distribution in Lymphoid Organs. In lymph nodes, the localization of cells reacting with the NLDC-145 antibody is restricted to the paracortical areas; no positive cells can be observed in the follicular B cell areas (Fig. 1). In the medulla of lymph nodes, positive cells can be detected occasionally, but the staining pattern is clearly different from macrophage staining as determined with Mac-1 (Fig. 2). When immunized lymph nodes were examined after skin-painting with oxazolone, many positive cells could be detected in the subcapsular sinus.

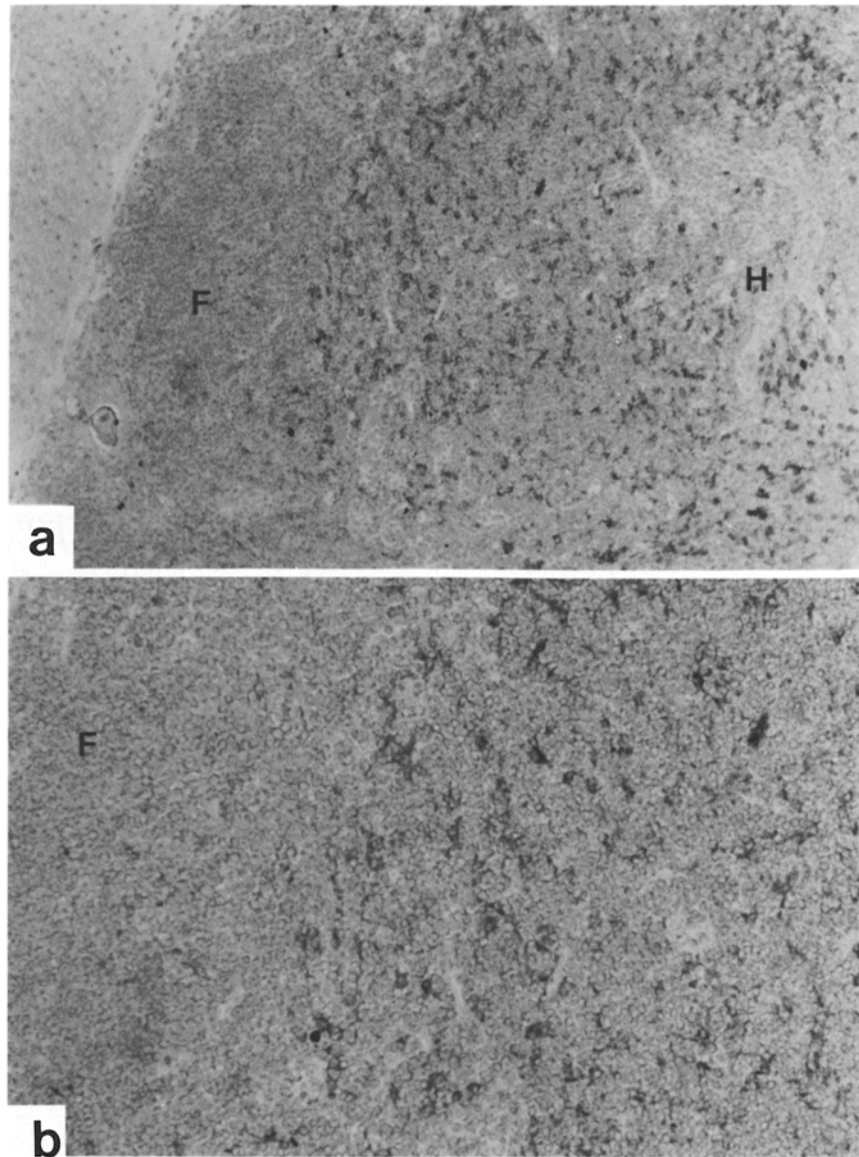


FIGURE 1. Interdigitating cells in the lymph node. (a) Section of an unstimulated axillary lymph node. Many NLDC-145⁺ cells can be found in the paracortical area, but no positive cells are present in the follicle (F). H, high endothelial venule ($\times 20$); (b) Detail of a showing follicle (F) and paracortical area.

These cells were large, with irregular outline, and resembled VC in morphology and localization (Fig. 3a). That NLDC-145 reacts with VC was confirmed by isolating VC from auxiliary lymph nodes using the method of Knight et al. (31). All cells with the morphological features of VC were positive for the antibody.

In the spleen, positive cells were predominantly found in the inner periarteriolar lymphocyte sheath (PALS) around the central arteriole (Fig. 4). No

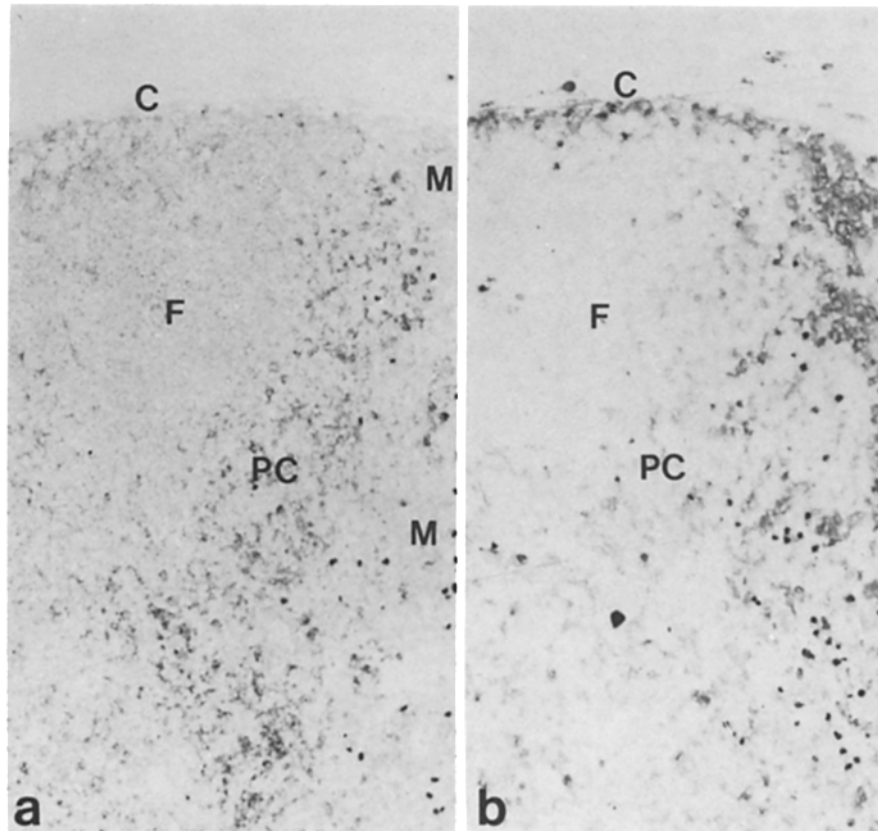


FIGURE 2. NLDC-145 and Mac-1 staining of lymph node. (a) Staining with NLDC-145. Many NLDC-145⁺ cells are present in the paracortex (PC) whereas these cells are absent from follicle (F) and medullary region (M) as well as from the subcapsular sinus (C); (b) Staining with Mac-1 (clone M1/70 [52]). Note the many positive cells in the subcapsular sinus and medulla.

staining was observed in follicles or marginal zone, and sometimes few positive cells were localized in the red pulp of the spleen. Similar staining profiles were observed in spleens (and lymph nodes) of nude mice. In contrast to the staining of IDC in other lymphoid organs, staining in the spleen is usually less well defined. The staining is often weaker and cell boundaries are harder to discriminate.

In Peyer's patches, positive cells with the characteristic morphology of IDC were localized in the small interfollicular T cell areas. Positive cells were also present in the lamina propria of the adjacent small intestines. In addition, positive staining of the epithelial cells of the villi could be observed (Fig. 5).

In the thymus the situation was more complicated. In addition to a staining of nonlymphoid cells with dendritic appearance in the medulla and corticomedullary region typical for IDC, an intense staining of cortical elements was observed. This staining was more intense than that on IDC, and seems to be associated with the epithelial components of the thymic cortex (Fig. 6).

Bone marrow was studied using cytocentrifuge preparations. No positive cells

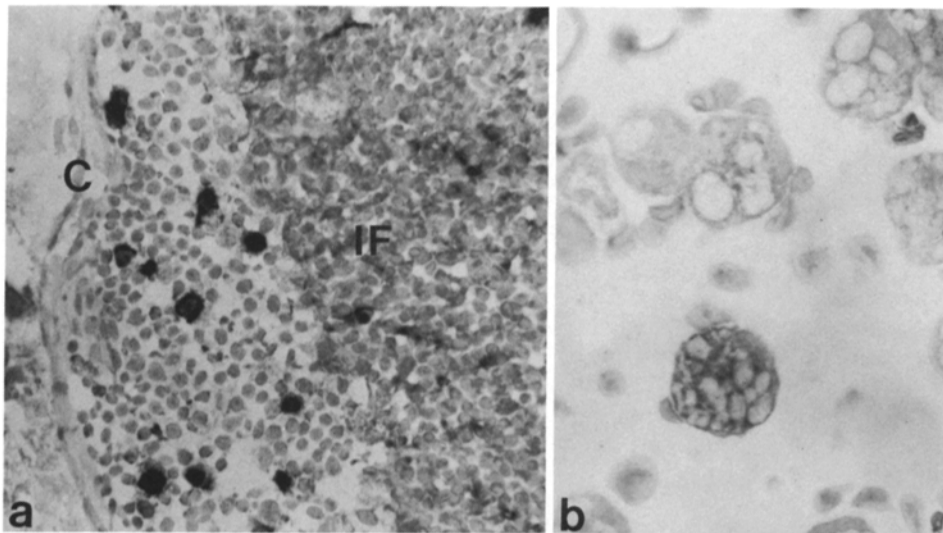


FIGURE 3. Veiled cells in subcapsular sinus of stimulated lymph node. (a) Section of an axillary lymph node 4 d after skin painting with oxazolone. Many NLDC-145⁺ cells can be found in the subcapsular sinus and also in the interfollicular areas (*IF*). *C*, capsule ($\times 40$); (b) Peritoneal exudate. Cytospin preparation of peritoneal exudate cells 4 d after thioglycollate administration. Large NLDC-145⁺ cells can be identified.

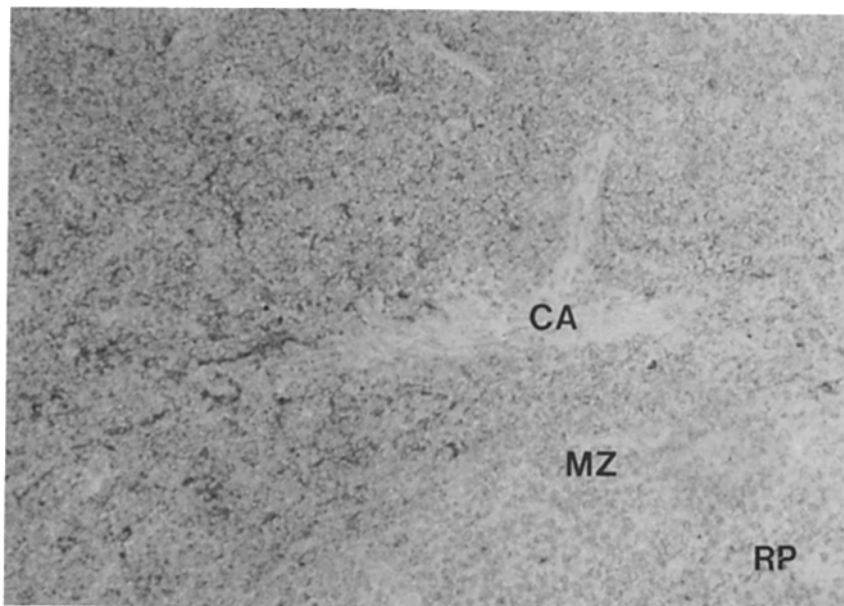
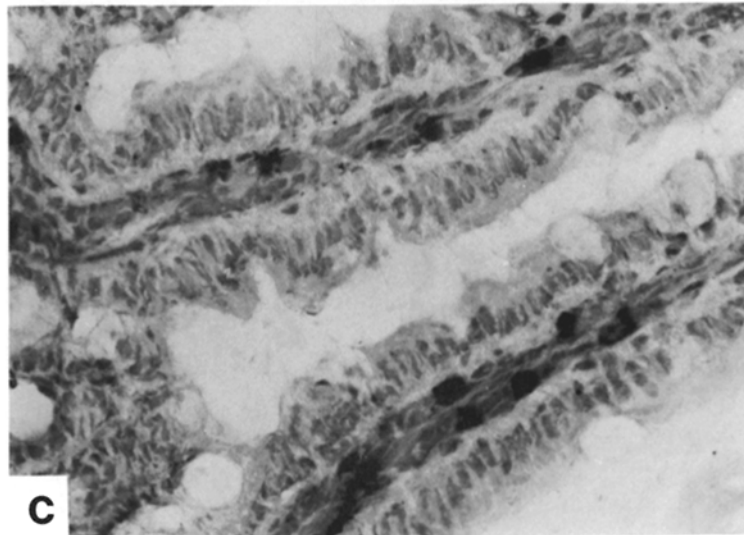
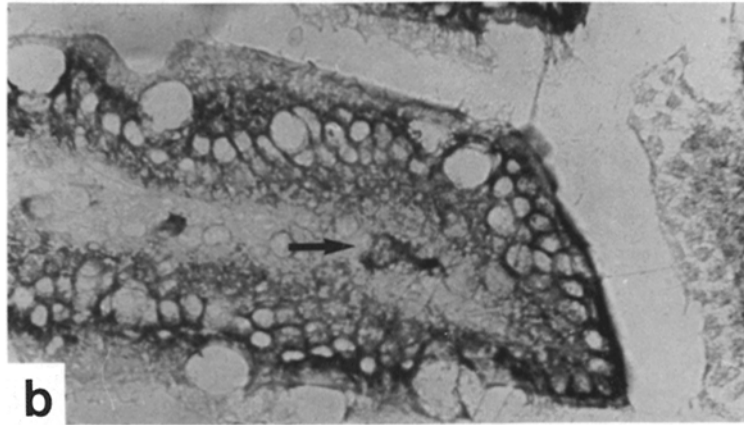
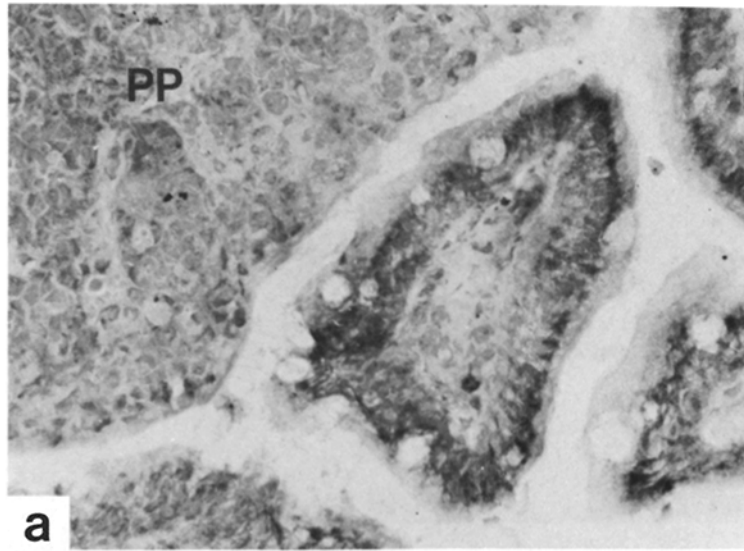


FIGURE 4. Interdigitating cells in the spleen. IDC can be found in the periarteriolar lymphocyte sheath as determined by NLDC-145 staining. *CA*, central arteriole; *MZ*, marginal zone; *RP*, red pulp ($\times 20$).

FIGURE 5. Villi of the small intestines. (a) The epithelial cells of the intestinal villi stain with NLDC-145. Note the absence of staining from the lymphoepithelium covering the Peyer's patch (*PP*) ($\times 20$); (b) Occasionally in the lamina propria large irregular-shaped NLDC-145⁺ cells can be found (arrow) ($\times 40$); (c) When stained with Mac-1 an abundant number of macrophages can be found throughout the lamina propria of the villi ($\times 40$).



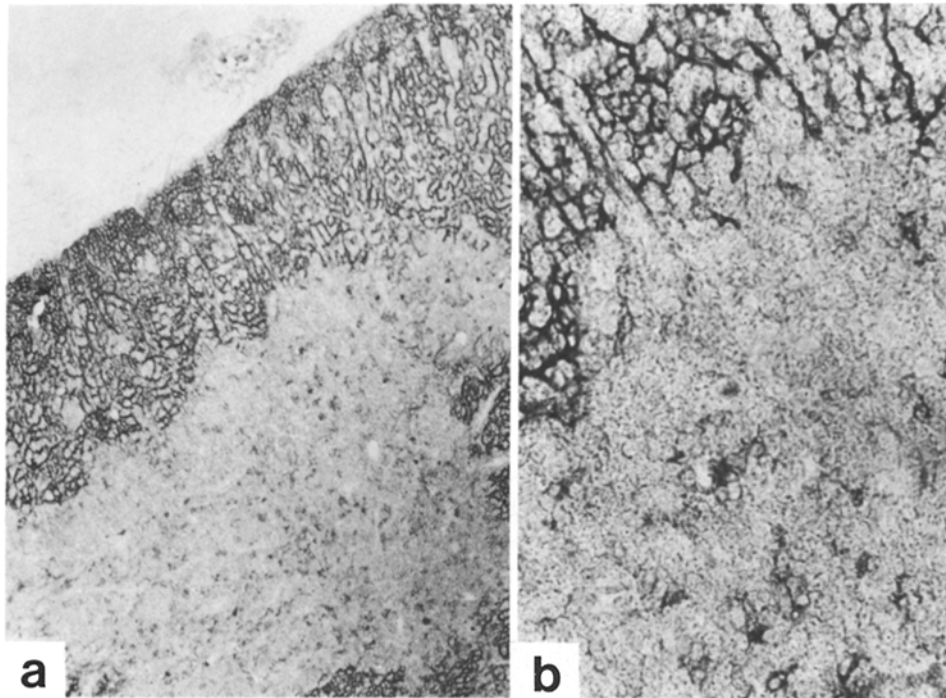


FIGURE 6. Thymus. In the thymus epithelial cells in the cortex stain intensely with NLDC-145. In addition many Interdigitating cells can be found in the medulla. (a) overview with cortex and medulla ($\times 10$); (b) detail medulla with IDC ($\times 25$).

could be detected in these preparations. Using cytocentrifuge slides of peripheral blood, no activity could be found on blood monocytes or any other blood cell.

When peritoneal macrophages of unstimulated mice were tested, no cells could be detected that reacted with NLDC-145. However, when peritoneal macrophages were harvested from mice that had been stimulated 4 d previously with thioglycollate, a distinct population of the collected cells was clearly positive for NLDC-145 (Fig. 3 b). Double immunohistochemistry for Ia-antigens on these cells showed that these cells were also positive for Ia. Combination of staining with either antibody and acid phosphatase histochemistry revealed that the positive cells in the peritoneal exudate showed weak enzyme activity, often concentrated in spots.

Radioresistance of NLDC-145⁺ cells was determined using lethal irradiation (900 rad; C₃D₂F₁ mice). Spleen and lymph nodes of irradiated animals were examined up to 7 d after irradiation. Positive cells were present at all time points tested, and especially in the spleen the staining looked more intense. This is in contrast to control animals where staining of NLDC-145⁺ cells is often weak (Fig. 7). Due to the loss of lymphocytes after irradiation, the cells were positioned more closely together and seemed to have lost some of their processes.

Tissue Distribution in Nonlymphoid Organs. Activity of the NLDC-145 antibody in other than lymphoid tissue was investigated using skin, kidney, liver, and brain. Only in the skin could activity be found. This activity was restricted to

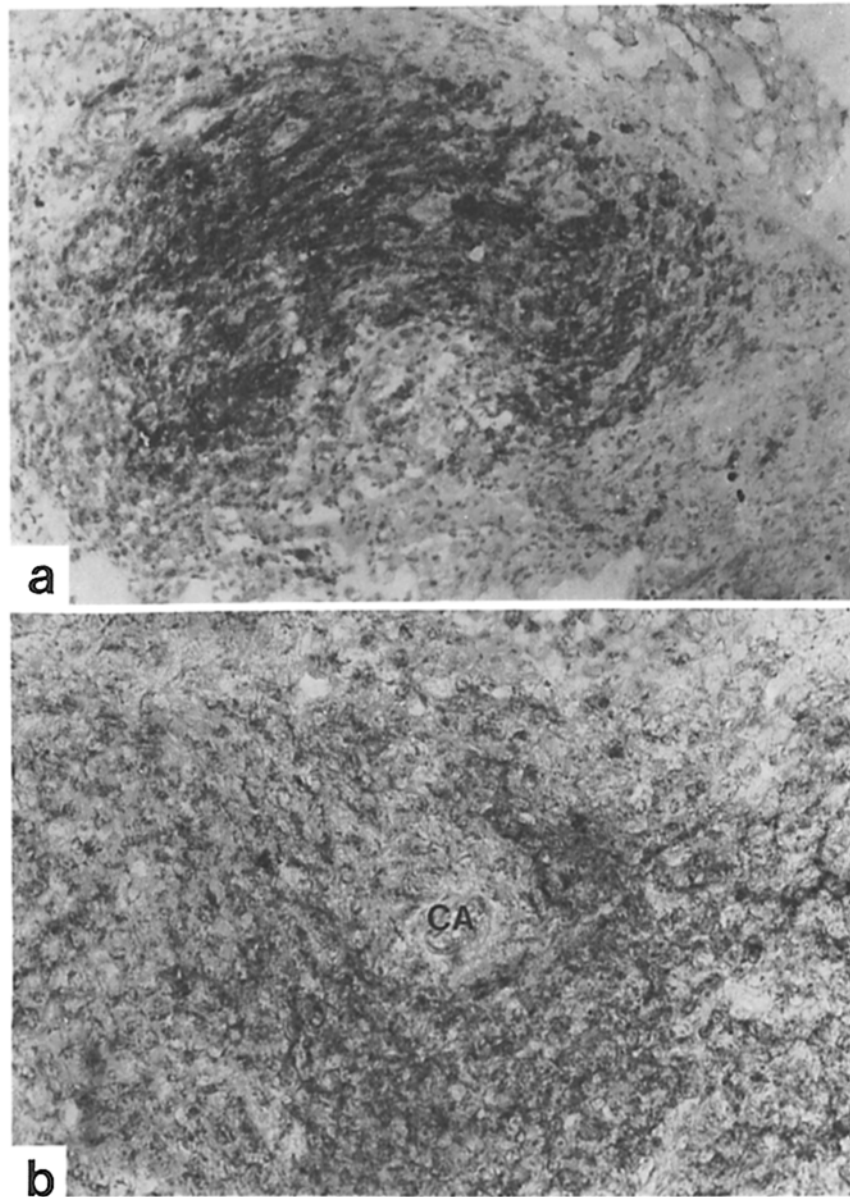


FIGURE 7. NLDC-staining after irradiation. Sections of lymph node (a) and spleen (b) 4 d after lethal irradiation. Lymphocytes are absent and many nonlymphoid cells stain intensely in the collapsed tissue of paracortex (a) and PALS (b). CA, central arteriole.

large irregular cells in the basal layers of the epidermis and scattered cells in the dermis (Fig. 8). Combining NLDC-145 with anti-Ia on dermal sheets, using double immunofluorescence, showed a complete correlation of the two stainings (Fig. 9). Therefore, NLDC-145 specifically reacts with the LC in the skin.

Isotype and Molecular Weight Determination. Isotype determination using immunohistochemistry showed that the NLDC-145 antibody is a rat IgG2a. Lymph

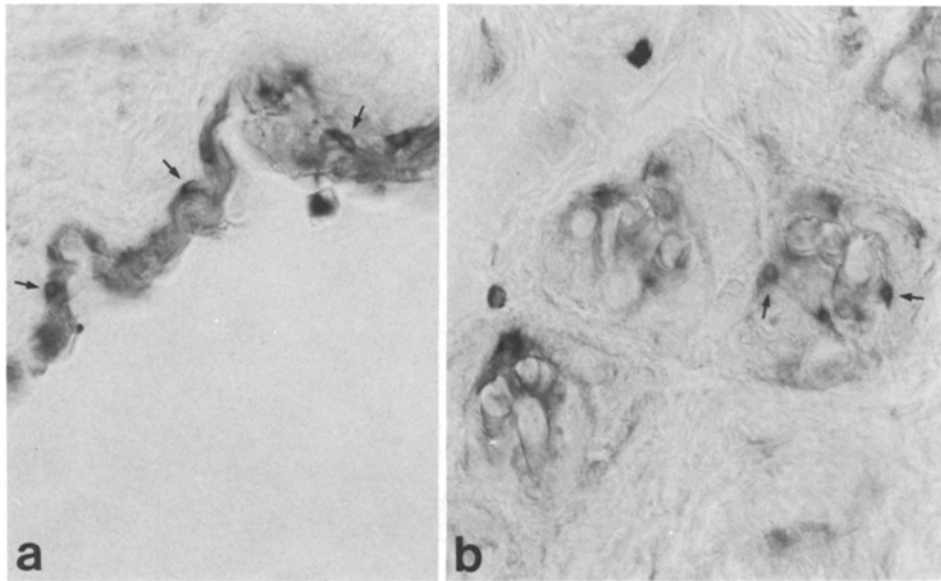


FIGURE 8. Langerhans cells in the skin. Section of the skin from the ventral wall. (a) Longitudinal section with Langerhans cells (arrows) present in the basal layer of the thin epidermis ($\times 20$); (b) Tangential section through skin papillae. Langerhans cells are indicated by arrows ($\times 20$).

node fractions enriched for NLDC-145⁺ cells by gradient centrifugation (31) were used to isolate iodinated cell surface antigens. The iodinated proteins were immunoprecipitated by the antibody and analyzed by SDS-PAGE followed by autoradiography. Using the density gradient enrichment, many B cell blasts are copurified. Therefore, an isotype matched anti-IgM (mAb; clone 11B5) was used as control. A single band with an approximate molecular weight of 145,000 was found under nonreducing as well as reducing conditions (Fig. 10, Lane B).

That NLDC-145 reacts with surface antigens was showed using unfixed suspensions of lymph nodes enriched for VC using metrizamide. A two-step immunofluorescence staining of these unfixed, live cells clearly showed membrane staining. From the staining on tissue sections and fixed cytopsin preparations it is obvious that the antigen is also present in the cytoplasm.

Discussion

In this study an mAb, NLDC-145, is described that reacts with LC in the skin, and with IDC and VC in lymphoid tissues (Table I). The specificity of the antibody that is inferred from the localization, enzyme pattern, and morphology of the cells, and the fact that these cells are also strongly stained by anti-Ia antibodies, clearly emphasizes the relationship between these cell types. LC and IDC have been described as the predominant Ia⁺ population in skin and lymph node paracortex, respectively (13–16). Our description of VC is based on their morphology in suspensions and position in the subcapsular sinus, as well as their increasing number after skin-painting with oxazolone. Final proof should come from the demonstration of Birbeck granules (23) in these cells by electron

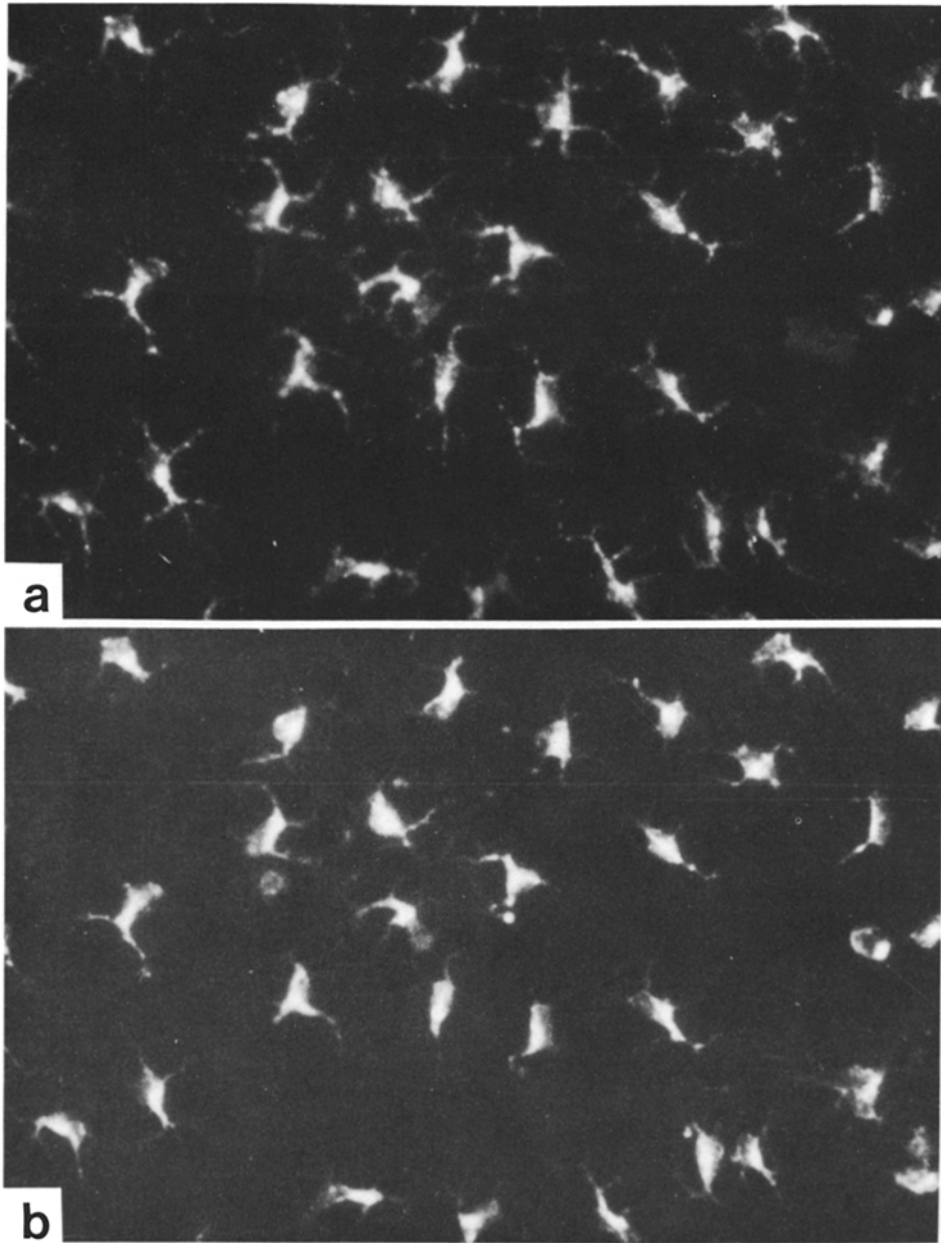


FIGURE 9. Langerhans cells in epidermal sheets. Simultaneous immunofluorescence staining of epidermal sheets with (a) rhodamin-detected NLDC-145 and (b) fluorescein-detected anti-Ia. Note the complete overlap between the two antibodies ($\times 40$).

microscopy, showing that they are truly derived from LC via the afferent lymph (35, 36).

LC have been described to act as APC in various experimental systems, and VC direct evidence for an accessory role in the immune response has been given

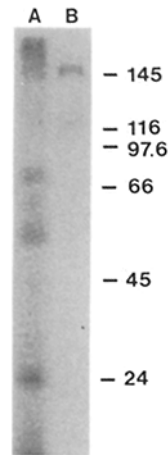


FIGURE 10. Immunoprecipitation and SDS-PAGE of detergent-solubilized membrane from lymph node suspensions enriched for NLDC-145 positive cells and labeled with ^{125}I . Immunoprecipitation was performed in microtiter plates using the SPIT methods. Gels were run under reducing conditions. Lane A, immunoprecipitation performed with the isotype matched control aIgM (clone 11B5); lane B, immunoprecipitation with NLDC-145.

TABLE I
Staining Pattern of NLDC-145 on Sections of Various Organs and on Cytospin Preparations of In Vitro Isolated Cells

Organ	NLDC-145 staining	Cell type and localization
Spleen	+	IDC in inner PALS
Lymph node	+	IDC in paracortex VC in subcapsular sinus
Peyer's patch	+	IDC in interfollicular T cell areas Villus epithelium, isolated cells in submucosa (VC)
Thymus	+	IDC in medulla Cortical epithelium
Skin	+	LC
Brain	-	
Kidney	-	
Liver	-	
Heart	-	
<i>In vitro isolated cells</i>		
Blood	-	
Bone marrow	-	
Peritoneal cells	-	
Peritoneal exudate cells*	+	Some positive cells (VC?)

All stainings were performed using a two-step immunoperoxidase technique on organs and cells of both BALB/c and $\text{C}_3\text{D}_2\text{F}_1$ mice with identical results.

* Peritoneal exudate cells were harvested 4 d after intraperitoneal thioglycollate injection.

for VC as well (17-20). The role of the IDC in the immune response is less clear. Originally described to play a role in the differentiation and maturation of T cells in peripheral lymphoid organs (1, 8), IDC have also recently been induced

in the group of APC. This is largely based on morphological similarities between IDC and in vitro DC (9, 25). The Ia^+ DC is an extremely potent stimulatory cell in T-dependent immune responses. Much of the experimental work has been done with DC that had been isolated from the spleen, but DC have also been found in other tissues, including thymus, lymph nodes, afferent lymph, and blood (12, 16, 37, 38), as well as in interstitial connective tissue (39, 40). Considering the many data that include the DC together with LC, VC, and IDC in one group of accessory cells, it will be important to screen in vitro isolated cell types to further establish the specificity of this antibody. So far the antigen has been detected on some spleen DC in vitro, and is clearly present on LC in culture (Schuler, G., M. Witmer, and R. Steinman, personal communication). The latter have been shown to resemble DC in in vitro stimulatory capacity when cultured for 2–3 d (29).

These data may imply that DC as characterized in vitro are the most differentiated cell type within the group of accessory cells. In this respect it is also pertinent that the antibody 33D1, which is cytotoxic for DC, does not react with LC and IDC (41, 42).

If we assume that this group of nonlymphoid DC belongs to the mononuclear phagocyte system, the NLDC-145 antibody makes these cells clearly stand out from the other members of this system; no precursors in bone marrow or blood, nor differentiated tissue macrophages are recognized by the antibody. F4/80, however, which is considered to be a general marker for murine macrophages, has been shown on LC but not on IDC (43). The data that show that the IDC population in lymph nodes is, at least partly, derived from LC; imply that the F4/80 antigen is lost during further differentiation. Dijkstra et al. (44) have reported that subpopulations of tissue macrophages are also $F4/80^-$.

Also, the demonstration of NLDC-145 activity on an Ia^+ subpopulation of peritoneal exudate cells is more likely to represent VC immigrating into the peritoneal cavity than differentiating macrophages, considering the weak acid phosphatase activity of these cells.

The cellular distribution and the molecular weight of the NLDC-145 antigen is different from Ia and from any other marker described for nonlymphoid dendritic cells in man and rodents. Yet, the concomitant presence of Ia and NLDC-145 on the various cell types is striking. Also the cross reactivity of NLDC-145 in thymus and intestinal epithelium closely resembles the staining observed with anti-Ia. Both antibodies react with cortical epithelial elements of the thymus (45, 46) but in contrast to the confluent medullary staining of anti-Ia, NLDC-145 only recognizes IDC in the thymus medulla. In this respect NLDC-145 staining is comparable to that of ER-TR4, a recently described monoclonal antibody that selectively reacts with Ia^+ thymus cortex epithelial cells (47). The cross-reactivity of NLDC-145 with epithelial cells in the thymus cortex and IDC and LC is interesting in view of the many implications for an important role of these cells in T cell differentiation and activation. The cross-reactivity of intestinal and thymus epithelium cannot be explained at present but it is remarkable that both epithelia are of entodermal origin. Ia expressed on villus epithelial cells is not acquired from bone marrow-derived cells as has been demonstrated with chimeric animals (48). It is not known whether this is also true for NLDC-

145 nor is it known at present whether the antigen on thymus- and intestinal epithelium is the same as found on the nonlymphoid DC. So far we have not been able to show an allotypic distribution of the NLDC-145 antigen using various mouse strains including all H-2 haplotypes (results and mouse strains not shown).

The absence of the NLDC-145 antigen on follicular dendritic cells (FDC) in B cell follicles agrees with recent findings that this cell type is probably not bone marrow derived and is more likely to be a differentiated reticulum cell involved in immune complex trapping (49-51).

Further use of surface markers may help us to delineate the precise relationships of the various nonlymphoid cell types and their functions in the immune response.

Summary

An mAb, NLDC-145, is described that specifically reacts with a group of nonlymphoid dendritic cells including Langerhans cells (LC), veiled cells (VC), and interdigitating cells (IDC). The antibody does not react with precursor cells in bone marrow and blood. Macrophages are not stained by the antibody, but a subpopulation of Ia⁺ peritoneal exudate cells is recognized. Possible relationships of the various nonlymphoid dendritic cell (NLDC) types are discussed.

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