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In situ immunological characterization of Langerhans cells with monoclonal antibodies: comparison with other dendritic cells in skin and lymph nodes

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Summary. The antigenic properties of epidermal Langerhans cells (LC) were determined and compared with those of non-lymphoid dendritic dermal cells (DDC), interdigitating reticulum cells (IRC), dendritic reticulum cells (DRC), and histiocytic reticulum cells (HRC) by examination of serial and double immunoenzymatic and -fluorescence stained frozen sections of skin and lymph node biopsies.

All of these cell types expressed leucocyte common antigen. LC, DDC, and IRC demonstrated similar antigenic phenotypes (HLA-DR$^+$, Leu3$^+$, OKT6$^{+/-}$, anti-C3 receptor$^-$, R4/23$^-$, Ig-complex$^-$, MO2$^-$), whereas the antigenic properties on DRC (HLA-DR$^-$, Leu3$^-$, OKT6$^-$, anti-C3 receptor$^+$, R4/23$^+$, Ig-complex$^+$, MO2$^-$) and HRC (HLA-DR$^{+/-}$, Leu3$^-$, OKT6$^-$, anti-C3 receptor$^+$ R4/23$^-$, Ig-complex$^+$, MO2$^+$) were markedly different.

These data suggest that LC, DDC, and IRC are closely interrelated cell types, and support the concept that DRC and HRC are unique cell types which do not appear to be related to LC, DDC, or IRC. The lack of labelling of LC with monoclonal anti-C3b receptor antibody, and polyclonal antiserum recognizing C3b, C3bi, and C3d receptors strongly indicate that the EAC-rosetting of LC previously described is not due to the presence of C3 receptors on these cells. Alternatively, LC may express C3 receptor molecules different from those previously identified (C3b, C3bi, and C3d).

Key words: Dendritic cells – Immunohistochemical staining – Monoclonal antibodies – Langerhans cells

Langerhans cells (LC) are T-cell interacting, dendritic cells which localize in the epidermis and take up and transport antigens to T-cell dependent paracortical areas in regional lymph nodes (Silberberg-Sinakin et al. 1976; Stingl et al. 1978). LC possess specific cytoplasmic granules (the Birbeck

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granules). Otherwise the ultrastructure of LC is similar to that of interdigitating reticulum cells (IRC) in lymph nodes, and to that of subsets of HLA-DR antigen positive dendritic dermal cells (DDC) (the so-called "indeterminate cells") (Rausch et al. 1977; Rowden et al. 1979). The concept has been proposed that these cell types form a reticulo-endothelial trap for epicutaneously applied antigens (Shelly and Juhlin 1976). However, at present the exact interrelationship of these cell types is debated, and recently it has been suggested that DDC comprise different cell types (Murphy et al. 1983). Furthermore, the relationship of LC, DDC and IRC to other types of dendritic and phagocytic marcrophages (e.g. dendritic reticulum cells (DRC) in B-cell follicles, histiocytic reticulum cells (HRC) in the sinuses of lymph nodes) is uncertain (Thornbecke et al. 1980).

Until recently, the above mentioned cell types could only be reliably identified by ultrastructural examination. However, within the last few years, a series of monoclonal antibodies recognizing some macrophage subsets (Fithian et al. 1981; Lampert et al. 1980; Murphy et al. 1981; Naiem et al. 1983; Todd et al. 1981) have become available, and it is now possible to identify these cell types at the light microscopic level by immunohistochemical staining techniques.

In this study the antigenic properties of LC, DDC, IRC, DRC, and HRC have been determined and compared by examination of serial and double immunoenzymatic and -fluorescence stained frozen section of skin and lymph node biopsies with a panel of recently developed antibodies.

Material and methods

Patients

4 mm skin punch biopsies were obtained from 6 adult, healthy volunteers, and from 10 patients with positive patch test followed in the Department of Dermatology, the Finsen Institute. Lymph nodes biopsies with benign follicular hyperplasia were obtained from 15 patients referred to the Department of Medicine, the Finsen Institute, because of local or generalized lymph node enlargement.

Processing of fresh biopsy material

Immediately following removal, biopsies were quickly frozen in 2-methyl-butane and dry ice. Biopsies were stored airtight at $-80°$ C until sectioning. 6 μm cryostat sections were lyophilized at $-20°$ C for 2–4 h, fixed in acetone for 10 min and subsequently processed for staining (Stein et al. 1980).

Antibodies

The monoclonal antibodies employed in this study are summarized in Table 1. A polyclonal rabbit antiserum directed against C3b, C3bi, and C3d receptors was prepared as described elsewhere (Gerdes et al. 1982b).

Immunoenzymatic staining of frozen sections

Single staining. Sections were incubated with monoclonal antibody (30 min), washed in Tris-buffered saline, and labelled with peroxidase-conjugated rabbit anti-mouse immunoglobulin

Table 1. Monoclonal antibodies

Antibody	Specificity	Ig-subclass	Reference (and/or source)[a]
PD7/26	Leucocyte common	IgG1	Warnke et al. 1983 (DAKO)
CR3/43	HLA-DR	IgG1	Mason and Stein unpublished
Anti-HLA-DR	HLA-DR	IgG2a	(BD)
To15	Pan-B-cell	IgG2b	(DAKO)
To5	C3b receptor	IgG1	Gerdes et al. 1982a (DAKO)
Nlo/2/27	Lambda	IgG1	(DAKO)
anti-kappa	Kappa	IgG1	(BD)
R4/23	Dendritic reticulum cells	IgM	Naiem et al. 1983 (DAKO)
OKT6	Langerhans cells cortical thymocytes	IgG1	Fithian et al. 1981 Reinherz et al. 1980 (Ortho)
Lyt3	E-rosette receptor	IgG2b	Kamoun et al. 1981 (NEN)
Leu4	Peripheral T-cells	IgG1	(BD)
Leu3	T-helper/inducer cells	IgG1	Ledbetter et al. 1981 (BD)
MO2	Monocytes	IgM	Todd et al. 1981 (CC)
Ap-7/6/7	Alkaline phosphatase	IgG1	Cordell et al. in press (DAKO)

[a] DAKOPATTS (DAKO), Becton-Dickinson (BD), Ortho Diagnostics (Ortho), New England Nuclear (NEN), Coulter Clone (CC)

(DAKOPATTS) diluted 1:10 with phosphate buffered saline (PBS) containing 33% (v/v) normal human serum (30 min). To further minimize unspecific binding of anti-mouse immunoglobulin to human proteins, the peroxidase conjugate was absorbed with insolubilized human serum prior to use. The peroxidase reaction was stained brown using 3-ethyl-9-aminocarbazole and H_2O_2 (10 min) as substrate. Sections were thereafter washed, counterstained with Harris' haematoxylin, and mounted in Aquamont®. The polyclonal anti-C3 receptor antibody was stained as described previously (Stein et al. 1980).

Coexpression of antigens was estimated by examination of serial sections.

Double staining was performed with the following combinations of antibodies. To15/R4/23, To15/To5, To15/anti-immunoglobulin, To15/CR3/43, To15/PD7/26, Leu4/Leu3, Leu3/R4/23, R4/23/Leu3 (lymph node biopsies), and CR3/43/OKT6 (skin biopsies). The basic principle is detailed in Fig. 1. After completion of the peroxidase reaction, sections were washed and incubated with the following reagents: monoclonal antibody (30 min), alkaline phosphatase conjugated rabbit anti-mouse immunoglobin diluted 1:10 with PBS containing 33% (v/v) normal human serum (30 min), and mouse monoclonal anti-alkaline phosphatase:alkaline phosphatase complexes (1 h) (Cordell et al. in press, Mason et al. 1981). The alkaline phosphatase was developed with naphthol AS-MX and fast blue BB salt. Immediately before staining 1 mM levamisole was added to the substrate solution to inhibit tissue endogeneous alkaline phosphatase activity (Ponder and Wilkinson 1981). This staining of alkaline phosphatase yields a blue colour contrasting with the brown colour of the peroxidase reaction.

Immunofluorescence staining of frozen sections

Sections were double stained with the following combinations of fluorescein (FITC)/phycoerythrin (PE) conjugated monoclonal antibodies: FITC-Leu4/PE-Leu3, FITC-OKT6/PE-Leu3, FITC-OKT6/PE-anti-HLA-DR, and FITC-anti-HLA-DR/PE-Leu3. Sections were incubated with a mixture containing equal amounts of 2 antibodies for 1 h, washed in Tris-buffered saline, mounted in glycerol phosphate buffer, and examined on the day of staining in a Zeiss epi-illumination fluorescence microscope fitted with fluorescein- and rhodamine-selective filters, as well as phase contrast optics.

Immunological control preparations

Negative controls were performed by omitting the specific anti-human antibodies. Positive controls were performed by staining of normal thymus, hyperplastic tonsils, and/or B- and T-cell malignant lymphomas with known antigenic phenotypes. The specificity of labelling in the double staining procedures was estimated by comparison between cellular reactivities in double and single stained serial sections, and by cross-comparison between sections doubled stained with various combinations of antibodies.

Results

The antigenic properties of LC, DDC, IRC, DRC, and HRC are summarized in Table 2.

LC were recognized as OKT6-positive, dendritic, epidermal cells in normal and diseased skin (Fig. 2). Examination of serial sections demonstrated that the OKT6-positive epidermal LC also expressed leucocyte common, and HLA-DR antigens. Furthermore, epidermal LC were cross-reactive with anti-T-helper/inducer antibody Leu3. The expression of HLA-DR, and Leu3 antigens on LC was confirmed by double immunofluorescence labelling with FITC-OKT6/PE-anti-HLA-DR, and FITC-OKT6/PE-Leu3. In both of these double staining procedures, the dendritic epidermal cells demonstrated a mixed colour of fluorescence (yellow) indicating coexpression of HLA-DR and Leu3 on OKT6-positive, epidermal LC. These cells did not react with anti-T-cell antibodies Leu4 or Lyt3, excluding the possibility that we might be detecting exocytic T-cells.

The double immunofluorescence staining of skin biopsies with FITC-OKT6/PE-anti-HLA-DR described above indicated that all OKT6-positive epidermal LC coexpressed HLA-DR antigen. This was confirmed by a more sensitive double immunoenzymatic staining technique in which sections were stained with anti-HLA-DR using an immunoperoxidase procedure, followed by staining with OKT6 with an alkaline phosphatase technique. In these

Table 2. Expression of antigens by dendritic and histiocytic cells in skin and lymph nodes

Tissue	Number of samples	Cell type[a]	Antigens								
			leucocyte common	HLA-DR	T6	T-helper associated	DRC-associated	C3 receptor	kappa	lambda	monocyte associated
Skin	16	LC	+	+	+	+	−	−	−	−	−
		DDC	+	+	+/−	+	−	−	−	−	−
Lymph node	15	IRC	+	+	+/−	+	−	−	−	−	−
		DRC	+	−	−	−	+	+	+	+	−
		HRC	+	+/−	−	−	−	+	+	+	+

[a] LC, Langerhans cells; DDC, Dendritic dermal cells; IRD, Interdigitating reticulum cells; DRC, Dendritic reticulum cells; HRC, Histiocytic reticulum cells

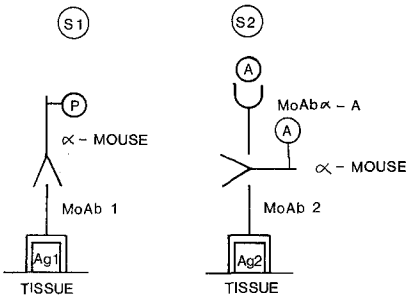

Fig. 1. Double immunoenzymatic staining of frozen sections: the first antigen (*Ag1*) is demonstrated with an indirect immunoperoxidase method using monoclonal anti-Ag1 (*MoAb1*), and peroxidase conjugated anti-mouse immunoglobulin (*P-α-mouse*). The peroxidase is stained with a substrate (*S1*) which yields a brown colour. The second antigen (*Ag2*) is demonstrated with an alkaline phosphatase technique using monoclonal anti-Ag2 (*MoAb2*), alkaline phosphatase conjugated anti-mouse immunoglobulin (*A-α-mouse*), and mouse monoclonal anti-alkaline phosphatase: alkaline phosphatase complexes (*MoAb-α-A : A*). The alkaline phosphatase is stained with a substrate (*S2*) which yields a blue colour. A cell expressing only *Ag2* demonstrates a blue colour, and a cell expressing only Agl a brown colour. A cell expressing both antigens also demonstrates a brown colour, since the immunoperoxidase reaction product appears to inhibit the binding of anti-*Ag2*. The method is therefore ideal for the identification of antigens on non-overlapping (Ag^+, $Ag2^-/Ag1^-$, $Ag2^+$) or partly overlapping ($Ag1^+$, $Ag2^+/Ag1^-$, $Ag2^+$) populations of cells

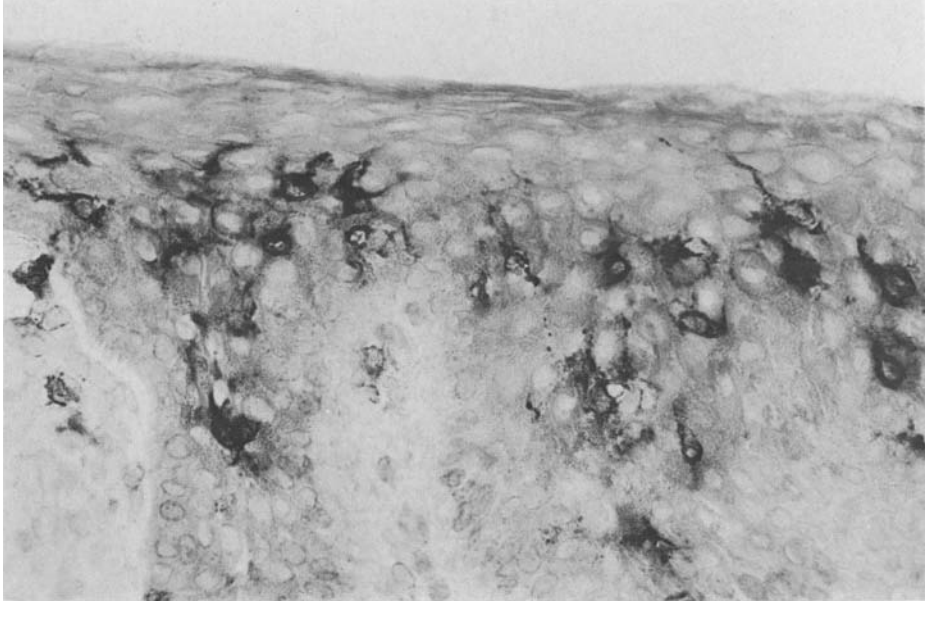

Fig. 2. OKT6 reactive, epidermal Langerhans cells (× 400)

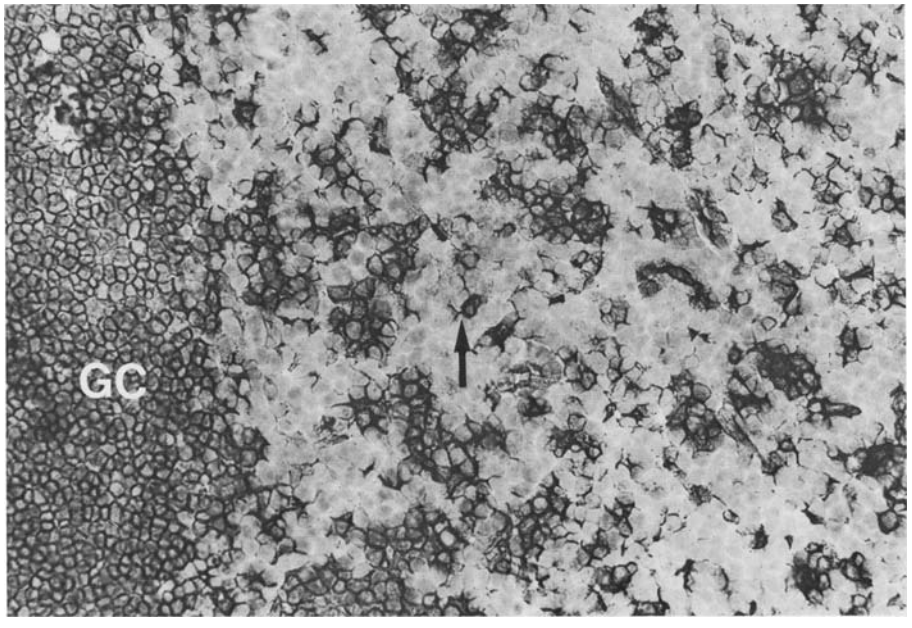

Fig. 3. HLA-DR-antigen positive germinal center cells (*GC*), and clusters of HLA-DR antigen positive cells in the paracortical region of lymph node biopsy (× 200). The latter population of cells demonstrates a dendritic outline (arrow), and thereby resembles interdigitating reticulum cells

sections, all epidermal dendritic cells stained brown. This confirmed that $OKT6^+$, $HLA\text{-}DR^-$ epidermal LC were not present in the examined series of skin biopsies, since such cells would have demonstrated a blue colour (see Fig. 1).

HLA-DR antigen positive DDC expressed a similar phenotype. However, only subsets of these cells expressed OKT6. In contrast they were all cross-reactive with anti-T-helper/inducer Leu3, as evidenced from examination of serial sections stained with anti-HLA-DR, anti-T-helper/inducer Leu3 and anti-T-cell antibodies Leu4 and Lyt3. In these preparations, anti-HLA-DR and anti-T-helper/inducer Leu3 appeared to stain non-overlapping populations of cells including activated T-cells ($Leu4^+$, $Lyt3^+$, $HLA\text{-}DR^+$) and non-lymphoid ($Leu4^-$, $Lyt3^-$, $HLA\text{-}DR^+$) dendritic cells. Double fluorescence labelling with FITC-Leu4/PE-Leu3, FITC-anti-HLA-DR/PE-Leu3, FITC-OKT6/PE-Leu3, and FITC-OKT6/PE-anti-HLA-DR confirmed the presence of a population of Leu3-reactive non-lymphoid ($Leu4^-$, $OKT6^{+/-}$, $HLA\text{-}DR^+$) dendritic cells in the dermis of all the skin biopsies examined.

IRC were identified as HLA-DR antigen positive dendritic cells in the paracortex of lymph nodes (Fig. 3). The phenotype of these cells was similar to that of DDC as evidenced from examination of serial sections stained with anti-HLA-DR, OKT6, anti-T-helper/inducer Leu3, and anti T-cell antibodies Leu4 and Lyt3. Coexpression of Leu3 and OKT6 on HLA-DR anti-

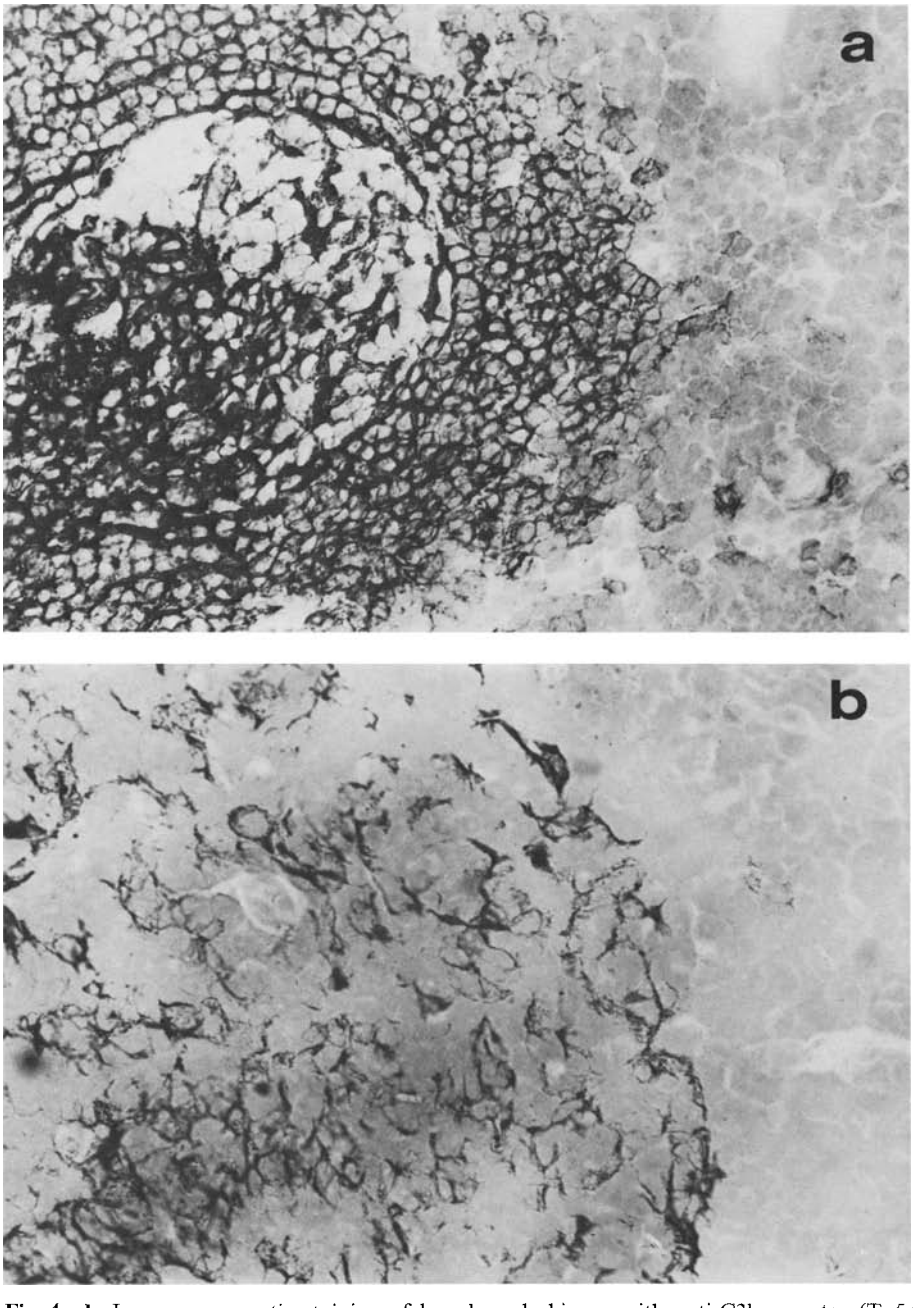

Fig. 4a–b. Immunoenzymatic staining of lymph node biopsy with anti-C3b receptor (To5 × 400). Application of undiluted antibody (**a**) labels follicular B-cells, and the network of dendritic reticulum cells. At a dilution of 1:40 (**b**) a selective staining of the latter cell types is obtained

gen positive IRC was confirmed by double fluorescence staining techniques using the same combination of antibodies described above.

LC, DDC, and IRC did not react with other antibodies employed in this study. In particular these cell types were consistently unreactive both with monoclonal anti-C3b receptor To5, and with a polyclonal antiserum recognizing C3b, C3bi, and C3d receptors.

DRC were identified as immunoglobulin-complex positive (kappa$^+$, lambda$^+$) dendritic cells in B-cell follicles. These cells reacted strongly with anti-C3 receptor antibodies. Labelling with undiluted anti-C3b receptor also stained the adjacent B-cells. However, application of anti-C3b receptor in dilutions of 1:40–1:80 permitted an almost selective labelling of DRC (Fig. 4), indicating a higher density of C3b receptors on DRC than on follicular B-cells. Staining with R4/23 also selectively labelled DRC. The expression of HLA-DR antigen by DRC was estimated with a double immunoenzymatic labelling technique in which sections were stained with anti-B-cell (To15) using an immunoperoxidase method followed by staining for HLA-DR with an alkaline phosphatase technique (see Fig. 1). In these sections DRC did not appear to express HLA-DR. In this context it should be emphasized that similar double immunoenzymatic staining of lymph nodes sections with To15/anti-C3b receptor, To15/R4/23, To15/PD7/26 and To15/anti-Ig demonstrated that the To15: immunoperoxidase reaction product on B-cells did not inhibit the binding of anti-C3 receptor, PD7/26, R4/23 and anti-Ig antibodies to DRC. DRC did not react with other antibodies used in this study.

HRC in the sinuses of lymph nodes were distinguished from the other cell types by their labelling with anti-monocyte antibody MO2. HRC also reacted with anti-kappa, -lambda, -leucocyte common, and -C3b receptor antibodies, whereas only subsets of HRC expressed HLA-DR antigen. HRC were unreactive with other antibodies used in this study.

Discussion

In this study, antigenic properties of epidermal LC have been determined and compared with those of other types of macrophages (DDC, IRC, DRC, and HRC) in skin and lymph nodes by immunohistochemical staining with a panel of recently developed antibodies. The cell types were distinguished on the basis of their tissue distribution, morphology and reactivity with one or several antibodies (OKT6, anti-HLA-DR, anti-immunoglobulin, anti-C3 receptor) whose labelling of macrophage subsets have been established previously (Gerdes and Stein 1982; Klaus et al. 1980; Lampert et al. 1980; Murphy et al. 1981; Rowden 1977; Rowden et al. 1979). Coexpression of other antigens was estimated by examination of serial and double stained sections using immunoenzymatic and -fluorescence labelling techniques. These staining techniques also served to distinguish macrophages from B- and T-lymphocytes; and the pattern of expression of B- and T-cell subset restricted antigens by the lymphocytes in the present material has been

described in detail elsewhere (Ralfkiær et al. 1984; Ralfkiær and Lange Wantzin 1984).

The data demonstrated that the antigenic phenotype of OKT6 reactive epidermal LC was identical with that of subsets of HLA-DR antigen positive DDC, and IRD; and in this study, the existence of HLA-DR antigen negative LC subsets (Harrist et al. 1983) was not confirmed. Furthermore, the dermis and the paracortex of lymph nodes contained another major population of non-lymphoid dendritic cells which were mutually indistinguishable, and which only differed from epidermal LC by their lack of reactivity with OKT6. These data strongly indicate that LC, DDC, and IRC are closely interrelated cell types. This is supported by experimental, ultrastructural, enzymecytochemical, and immunological data obtained in other studies: 1) LC are migratory cells which take up and transport antigens from skin to lymph nodes and localize in close apposition to IRC in paracortical areas (Rausch et al. 1977; Silberberg-Sinakin et al. 1976). 2) LC possess Birbeck granules, but are otherwise indistinguishable from IRC and HLA-DR antigen positive DDC (so-called "indeterminate cells") on ultrastructural examination (Rausch et al. 1977; Rowden 1977; Rowden et al. 1979). 3) The enzymecytochemical characteristics of LC (weak acid phosphatase and strong ATPase reaction) are similar to those of IRC, and differ markedly from those of histiocytic macrophages (Lennert et al. 1978). 4) LC and IRC both express the S-100 protein, whereas DRC and HRC are devoid of this protein (Nakajima et al. 1982; Takahashi et al. 1981). The identification that LC (Wood et al. 1983; this study), DDC, and IRC (this study) are all cross-reactive with anti-T-helper/inducer antibodies is further evidence that these cell types are closely interrelated.

In a recent study it has been proposed that HLA-DR antigen positive DDC are not homogeneous, but comprise "indeterminate cells" ($OKT6^+$, $HLA\text{-}DR^+$) and histiocytic macrophages ($OKT6^-$, $HLA\text{-}DR^+$) (Murphy et al. 1983). This suggestion was made on the basis of immunoelectronmicroscopic examination with a limited number of antibodies. Our data are not consistent with this suggestion, since the latter population of OKT6 negative, HLA-DR antigen positive DDC differ markedly from HRC with respect to their antigenic properties. Further studies of DDC by immunoelectronmicroscopy with more extensive panels of antibodies, and/or by in vitro functional assays are clearly necessary before the possible heterogeneity of these cells can be fully understood. Such studies may also further elucidate the concept that DDC and IRC are T-cell interacting, accessory cells (Rausch et al. 1977; Silberberg-Sinakin et al. 1976; Thornbecke et al. 1980), and at present this concept has only been directly confirmed for epidermal LC which have been isolated and analysed in suspension (Stingl et al. 1978).

The antigenic properties on DRC observed in this study correspond to those described in other studies using immunostaining techniques on tissue sections (Gerdes and Stein 1982). Recently, however, examination of DRC in imprint preparations has revealed a weak expression of HLA-DR molecules by DRC (Gerdes et al. 1983). The significance of this finding is uncertain and the mechanisms of macrophage/B-cell interactions are pre-

sently very poorly understood (Gerrard and Fauci 1983). Nevertheless, the data from this study clearly support that DRC are unique cell types (Naiem et al. 1983) which do not appear to be related to either LC, DDC, IRC or HRC. In particular, DRC were selectively labelled with R4/23, and expressed readily detectable C3b receptors, whereas LC, DDC, and IRC did not demonstrate any reactivity with R4/23, monoclonal anti-C3b receptor antibody or polyclonal antiserum recognizing C3b, C3bi, and C3d receptors. Similar results have been obtained in other studies of lymphoid organs (Gerdes and Stein 1982; Gerdes et al. 1982a and b, Naiem et al. 1983), and recently it has been demonstrated that epidermal LC are unreactive with monoclonal antibody recognizing the C3bi receptor (Haines et al. 1983). Our data clearly extend the latter finding and strongly indicate that the rosetting of LC in suspension with complement coated erythrocytes (EAC-rosettes) described previously (Berman and Gigli 1980; Burke and Gigli 1980; Stingl et al. 1977) is not due to the presence of C3 receptors on LC. In this context it should be emphasized that the anti-C3 receptor antibodies used in this study reliably identify C3 receptors not only on DRC, but on several other cell types (e.g. erythrocytes, neutrophils, B-cells, glomerular cells (Gerdes et al. 1982a and b; Stein et al. 1981). The possibility that LC may express C3 receptor molecules different from C3b, C3bi, and C3d requires further studies and should encourage research on the isolation and characterization of the LC EAC-rosetting receptor.

The possibility of specific labelling of macrophage subsets facilitates their identification in sections, and may be valuable for isolation and characterization of their cellular antigens, and in vitro functional properties.

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