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Some properties of dendritic macrophages from peripheral lymph

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SUMMARY

Peripheral lymph was collected from the skin and liver of sheep, and from the intestine of rats. The dendritic macrophages contained in it were isolated by centrifuging the lymph over a layer of 'Nycodenz'. Similar cells were produced by culturing mononuclear cells from venous blood, but the yields were very small. The numbers of dendritic cells in the lymph from the legs of sheep increased five-fold after xylene had been applied to the skin. Dendritic macrophages displayed abundant class II histocompatibility antigens on their surfaces, as well as immunoglobulins. Although the latter were probably acquired passively, they remained present for several days on cells cultured *in vitro*. When *in vitro*, dendritic cells could be shown to phagocytose marker particles, such as latex beads, but their performance was unimpressive compared to macrophages from the peritoneal cavities of rats. In contrast, their ability to phagocytose rapidly T4 phage or influenza viruses was unequivocal and striking.

INTRODUCTION

Early studies of peripheral lymph from dogs' legs (Haynes & Field, 1931) showed it to be different from intermediate and central lymph in that some of the few white cells it contained were monocytes and macrophages. The unusual, dendritic nature of these cells escaped notice until Horii, Tamaki & Terada (1950) applied vital stains to cells in the peripheral lymph of rabbits and guinea-pigs, but their observations attracted little attention. By using phase–contrast and electron microscopy, Morris (1968) defined the morphological phenotype of such cells and established their presence in peripheral lymph from many major organs of sheep (Smith, McIntosh & Morris, 1970). Later, apparently identical cells were found in the peripheral lymph of pigs (McFarlin & Balfour, 1973), man (Spry *et al.*, 1980) and cattle (Emery, MacHugh & Ellis, 1987). The introduction of techniques for collecting peripheral intestinal lymph (Hall, Hopkins & Orlans, 1977) allowed the collection of dendritic lymph cells from rats (Pugh, Macpherson & Steer, 1983), and it is reasonable to assume now that dendritic macrophages are a characteristic constituent of the tissue fluid of all mammals.

The increased numbers of such cells in lymph from allografts (Hall, 1967; Pederson & Morris, 1970) and sites of application of sensitizing chemicals (Hall & Smith, 1971; Hall, 1980), taken together with the intimate contacts that these cells make with lymphocytes (Morris, 1968), suggested that they play a part in the induction of cell-mediated immune responses (Hall, 1971), but initial attempts to test this in sheep gave equivocal results (Scollay, Hall & Orlans, 1976). The use of inbred rats, and purer preparations of cells, showed that dendritic lymph cells were indeed efficient in inducing T-dependent immunity to tumours (Gyure *et al.*, 1987). In spite of the fact that dendritic lymph cells are obviously phagocytic *in vivo* (Morris, 1968; Hall, 1979; Hall & Robertson, 1984), their status as phagocytes of the monocyte/macrophage lineage has remained controversial. A description of their phagocytic and other properties is set out below.

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MATERIALS AND METHODS

Animals

Cross-bred ewes and wethers, about 18 months old, were obtained from local livestock auctions.

Inbred rats of the Wistar (RT1u) strain were obtained from the National Institute for Medical Research, London, and used for most experiments. Occasionally, 'Hooded' CBH/Cbi (RTlc) and the related rnu/rnu 'Nude' rats, were taken from our own specific pathogen-free colony, as required.

Surgical techniques and the collection of peripheral lymph

These have been described elsewhere (Lascelles & Morris, 1961; Hall & Morris, 1962; Smith *et al.*, 1970; Hall *et al.*, 1977; Styles *et al.*, 1984). Heparin was used to prevent coagulation of the lymph. To provide control material, conventional macrophages were harvested from the peritoneal cavity of rats (Denham, Barfoot & Jackson, 1987).

Identification of dendritic cells

Dendritic cells were identified on the basis of their structural phenotype when viewed in the living state with phase–contrast

optics using a ×100 oil immersion objective. Under these conditions, the characteristic features of these cells are so striking and immediately apparent that their identification presents no problems. Some such cells have occasionally engulfed amorphous detritis, red cells and lymphocytes and start to take on the appearance of ordinary macrophages, while, at the other end of the spectrum, some are smaller and have many of the features of conventional blood monocytes.

Isolation of dendritic lymph cells

Whole lymph was centrifuged at approximately 1000 ***g*** for 15 min over a cushion of 'Nycodenz' (Nyegaard Diagnostics, Oslo, Norway), with a specific gravity of 1·063. The dendritic cells were aspirated from the interface, deposited by centrifugation and resuspended in a convenient volume of Dulbecco's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS). The cell suspension was inspected by phase-contrast microscopy using a ×100 oil immersion objective to verify viability and to count the percentage of dendritic cells. These usually accounted for at least 50% of the cells, the remainder being small lymphocytes and occasional immunoblasts. In the case of rat lymph some of the mononuclear cells were clearly conventional macrophages, and they were removed by incubating the suspension on plastic for 3 hr at 37° (Denham *et al.*, 1987). This step was never necessary with cells from sheep lymph.

Preparation of dendritic cells from peripheral blood mononuclear cells

Ten-millilitre volumes of heparinized blood from rats or sheep were layered over an equal volume of 'Lymphoprep' (Nyegaard) and centrifuged for 20 min at 1000 ***g***. The mononuclear cell fraction was aspirated from the interface, washed in RPMI medium and cultured at a modulus of 10^6 cells per ml in RPMI supplemented with 10% FCS at 38° in an atmosphere of 5% CO_2 in air. The cultures were sampled daily and the cells inspected using phase-contrast optics. Some dendritic cells appeared within 24–48 hr, but highest numbers were seen after 5 days of culture. Even at this time the dendritic cells accounted for less than 1% of the initial number of cells.

Electron microscopy

Cells were suspended in small (100 μl) volumes of medium to which 10 volumes of freshly made fixative were than added. The fixative comprised 2% formaldehyde and 0·2% glutaraldehyde, buffered between pH 7·5 and pH 7·8 with 0·1 M cacodylate. Fixation was allowed to proceed for 1–2 hr at room temperature, after which the cells were deposited by centrifugation and the supernatant fixative aspirated and replaced with phosphate-buffered saline (PBS). The pellets of fixed cells were treated with osmic acid, dehydrated with acetone and embedded in araldite, sectioned and stained with lead citrate or uranyl acetate prior to examination in the electron microscope, as described elsewhere (Hall *et al.*, 1978).

Antibodies for immunocytochemistry

Monoclonal mouse antibodies to rat $F(ab')_2$ (OX12), and rat MHC class II antigens (MRC OX6) were obtained from Serotec Ltd, Oxford. Mouse monoclonal antibodies to classes and subclasses of rat Igs were prepared here by standard techniques. These mouse antibodies were detected by specifically purified rabbit or sheep anti-mouse Ig antibodies that had been treated with NHS-LC-biotin (Pierce Chemical Co. Ltd, Luton, Beds) so that they would react with streptavidin-horseradish peroxidase (Sigma, Poole, Dorset), which could be developed to give a light and electron-dense reaction product with diamino-benzidine HCl (Hall *et al.*, 1978).

A mouse monoclonal antibody to the class II histocompatibility antigens of sheep was prepared at Melbourne, Australia, under the aegis of Dr M. R. Brandon (Puri, Mackay & Brandon, 1985). The binding of this mouse antibody to sheep cells was detected as above. Specifically purified antibodies to sheep IgG1, IgG2, IgA and IgM and $F(ab')_2$ were obtained from the sera of appropriately immunized rabbits, as described elsewhere (Hall *et al.*, 1977), and their binding to sheep cells was detected by specifically purified, biotinylated sheep anti-rabbit Ig using the above immunoperoxidase methods.

Immunocytology of dendritic cells

Although the surfaces of living dendritic cells can be stained with immunological reagents, the washing of such cells which necessarily involves repeated aggregation and resuspension is liable to damage them severely and, where small numbers of cells are involved, which is usually the case, the yield of intact cells is often too small to be either representative or easy to handle. Accordingly, the cytochemical and immunocytochemical techniques reported here were carried out on fixed cells.

For light microscopy, conventional cell films were prepared after resuspending pellets of cells that had been washed once with PBS in an equal volume of 7% bovine serum albumin (BSA) or, alternatively, 'cytocentrifuge' preparations were made. In either case the air-dried slides were fixed for 1–2 min in absolute methanol. Cells for electron microscopy were fixed in the aldehyde fixative described above.

After fixation the slides were washed in running tap water for 2 min, rinsed in PBS and exposed to an appropriate dilution of the primary antiserum for 10 min in a damp box. The antiserum was washed off in a stream of tap water, the slides rinsed as before and the next reagent applied, and so on.

Aldehyde-fixed cells for electron microscopy were first 'quenched' with Tris-buffered BSA and then treated similarly, but the washing had to be accomplished by serial centrifugations and resuspensions.

In order to control the adventitious interaction of the immunoperoxidase reagents with Fc receptors, etc., the procedures included a parallel series wherein the primary antibody was replaced with an Ig of identical origin and isotype, etc., but with irrelevant antigen-binding specificity. Similarly, all reagents were diluted in solutions containing either 7% BSA or 10% FCS to saturate non-specific protein binding sites.

Cytochemistry

ATPase, non-specific esterase and acid phosphatase were demonstrated in cell films and cytocentrifuge preparations by standard techniques (Lojda, Grossrau & Schiebler, 1979).

Marker particles for studies of phagocytosis

Fluorescein-labelled 'Floresbrite latex microspheres', 0·8μ diameter, were obtained from Polysciences Ltd, Moulton Park, Northampton.

After uptake by the cells under test, they were viewed in a Zeiss photomicroscope III using incident UV together with

transmitted white light and phase–contrast optics. In this way, both the living cells and the fluorescent beads they contained could be observed.

Influenza virus suspension was obtained as an inactivated, polyvalent 'Influvac' vaccine, containing 1420 IU per ml (Duphar Laboratories Ltd, Southampton, Hants).

A suspension of T4 phage containing 10^{10} particles per ml was prepared by Dr M. Tilby of this department.

RESULTS

General observations and quantitative aspects

Peripheral lymph was collected from 35 preparations in sheep and over 100 rats.

The output of dendritic cells in lymph from the subcuticulum of a single hind limb of a sheep averaged approximately 5×10^5 per hour. Lymph from the subcuticulum of the flank, which embraces a larger area and is kept warm by the proximity of the viscera, yielded up to 5×10^6 dendritic cells per hour, as did peripheral hepatic lymph. Roughly, throughout the body, peripheral lymph contains between 10^5 and 10^6 dendritic cells per ml; in sheep, the total amount of lymph formed is 2·5 l per day (Yoffey & Courtice, 1970); it can be calculated that approximately $1{\cdot}4 \times 10^8$ dendritic cells are needed each day to populate the peripheral lymph. This number is of the same order as the number of typical monocytes present in the peripheral blood at any one time. Of course, such calculations are extremely crude, and the derivation of dendritic lymph cells from blood monocytes has yet to be proved conclusively. However, evidence consistent with the latter proposition is shown in Fig. 1, where the effect of painting the skin with xylene on the flow and composition of peripheral lymphs is illustrated. Xylene induced immediately a mild, short-lived, cutaneous hyperaemia so that the flow of lymph was slightly increased. In some sheep a substantial neutrophil granulocytosis occurred in the lymph but in many the increased extravasation of blood cells was confined to lymphocytes and dendritic macrophages. The outputs of these cells increased several-fold and the output of dendritic cells reached a peak about 24–48 hr after the application. Interestingly, the increased numbers of dendritic cells were accompanied often by countable numbers of cells with

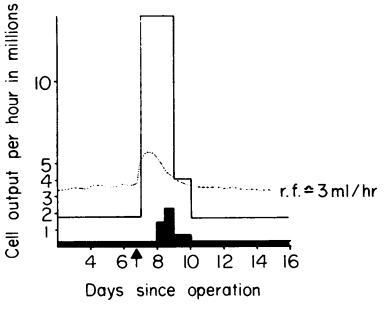

Figure 1. Graph to show the flow and composition of the peripheral lymph from the leg of a sheep before and after the application of xylene to the skin. The arrow shows the time at which the xylene was applied, and the blocked area of the histogram shows the proportion of the total white cells accounted for by dendritic macrophages. The broken line shows the flow rate of the lymph which was about 3 ml per hr under normal conditions.

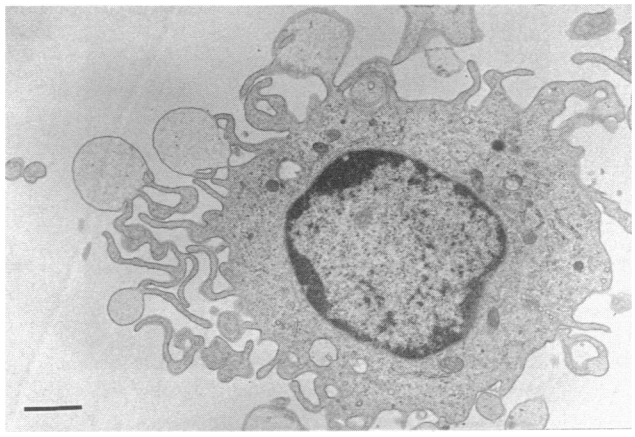

Figure 2. Electron micrograph of a dendritic macrophage isolated from sheep lymph to show the terminal blebs on the cytoplasmic processes which appeared a few hours after xylene had been applied to the skin from which the lymph came. The scale bar represents 1 μm.

morphology of blood monocytes, and, in addition, up to 3% of eosinophil granulocytes usually appeared. This manoeuvre could be repeated *ad infinitum*, though the gradual accretion of desquamated epithelium and the regrowth of wool or hair reduced its effect unless the skin was thoroughly cleaned and shaved beforehand. Many other organic solvents, including methyl salicylate, were tried but none reproduced the effect of xylene.

Figure 2 shows an electron micrograph of a dendritic cell isolated from peripheral lymph collected from the leg of a sheep 5 hr immediately after the application of xylene. The cytoplasmic processes show peculiar terminal blebs, suggesting that they had been exposed to some physical or chemical insult. This feature was not seen in cells collected at other times.

The outputs of dendritic cells in the thoracic duct lymph from previously mesenteric lymphadenectomized Wistar rats varied between individuals from 10^5 to 10^6 per hr. In any one individual the output was relatively constant and was maintained as long as the preparation functioned, irrespective of the dwindling numbers of recirculating lymphocytes. The outputs of dendritic lymph cells from athymic 'nude' rats were at least as high, and usually higher, than those recorded from euthymic rats. The maintenance of constant outputs of dendritic lymph cells from rats again suggests that such cells are end cells derived from the blood. Further evidence for their haematogenous origin is presented in Fig. 3, which shows typical dendritic cells isolated from cultures of peripheral blood cells from rats and sheep. We never saw such cells in fresh peripheral blood or bone marrow from either species.

Cytochemical and immunocytochemical characteristics

Dendritic cells from both rats and sheep stained intensely for ATPase, and strongly for non-specific esterase and acid phosphatase. Similarly, cells stained with anti-Ig reagents were so darkly stained that their internal features were sometimes obscured; electron microscope studies showed that the Ig was confined to the cytoplasmic membrane, and no intracytoplasmic Ig was observed (Fig. 4). In the case of sheep this surface Ig appeared to be mainly IgM; IgG1 and traces of IgG2 were

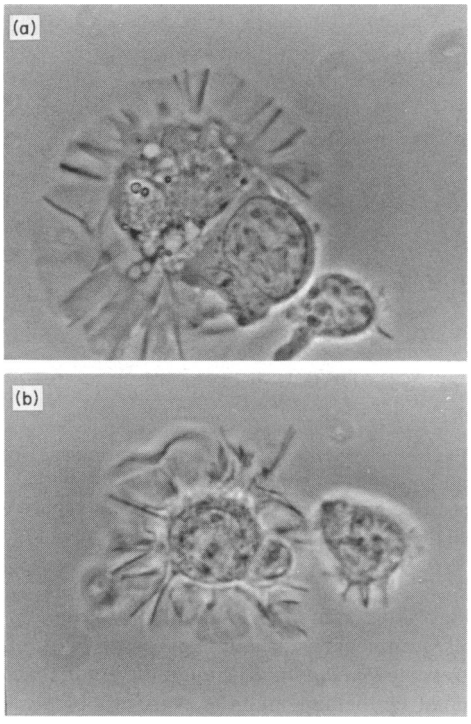

Figure 3. Phase-contrast photomicrographs, approximately × 1020, of dendritic macrophages produced by culturing the mononuclear cells of venous blood for 5 days. (a) Sheep; (b) rat.

present also but no IgA was found (cells from peripheral intestinal lymph of sheep were not examined). The surface Ig on dendritic cells from rat lymph comprised IgG and IgM. It is likely that this surface Ig was passively acquired and it seemed to turn over very slowly. Dendritic cells incubated *in vitro* for up to 4 days in media that lacked Ig did not lose their surface staining. The presence of this surface Ig naturally made testing for Fc receptors extremely difficult to interpret; none was detected on sheep cells but, using established methodology (Denham *et al.*, 1987) it was shown that rat dendritic cells were still able to bind

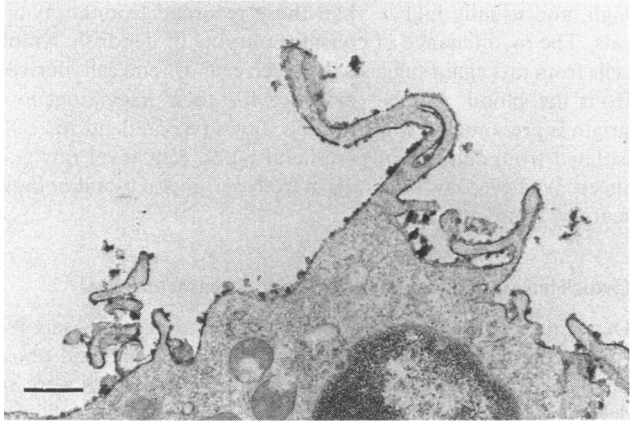

Figure 4. Electron micrograph of a dendritic macrophage from the peripheral lymph of sheep, to show the staining of surface Ig by the immunoperoxidase technique. The primary antibody was a specifically purified rabbit anti-sheep $F(ab')_2$, and the scale bar represents 500 nm.

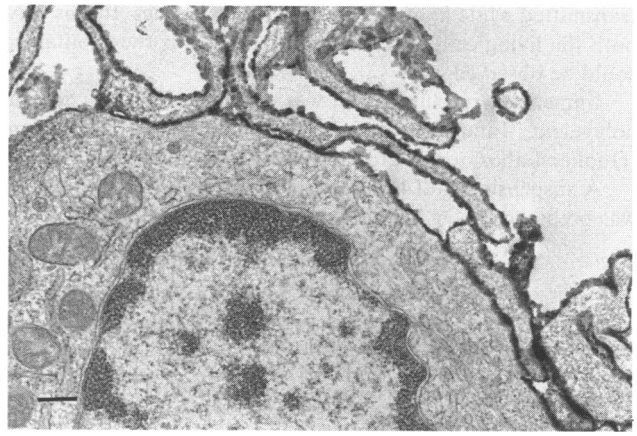

Figure 5. Electron micrograph of a dendritic macrophage from the peripheral lymph of a sheep, to show staining of the class II histocompatibility antigens on the surface by the immunoperoxidase technique. The primary reagent was a mouse monoclonal antibody against class II antigens of sheep, and the scale bar represents 200 nm.

some IgG2b. It is hard to prove that the acquisition of surface Ig was not an artefact produced by the conditions of collection. However, cells collected from sheep for short periods, using EDTA as anti-coagulant instead of the usual heparin, still showed staining for surface Ig.

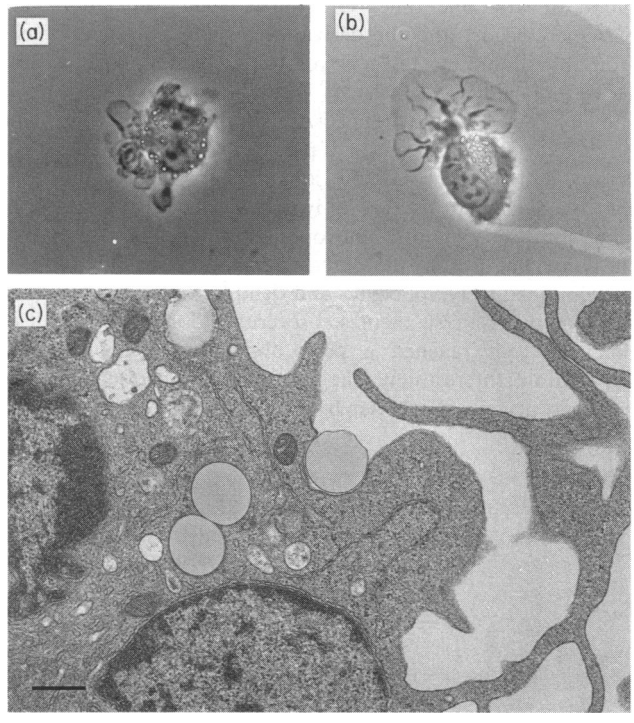

Figure 6. Phase-contrast and electron microphotographs to show the uptake *in vitro* of fluorescent plastic beads by dendritic macrophages from the peripheral lymph of sheep. The photographs were taken after the cells and beads had been cultured together for 18 hr. In the upper phase-contrast, photographs (a) and (b), the cells were illuminated also by incident UV light to highlight the plastic beads which otherwise would have been invisible. The accompanying electronmicrograph (c) shows that most of the beads were intracytoplasmic (scale bar = 500 nm).

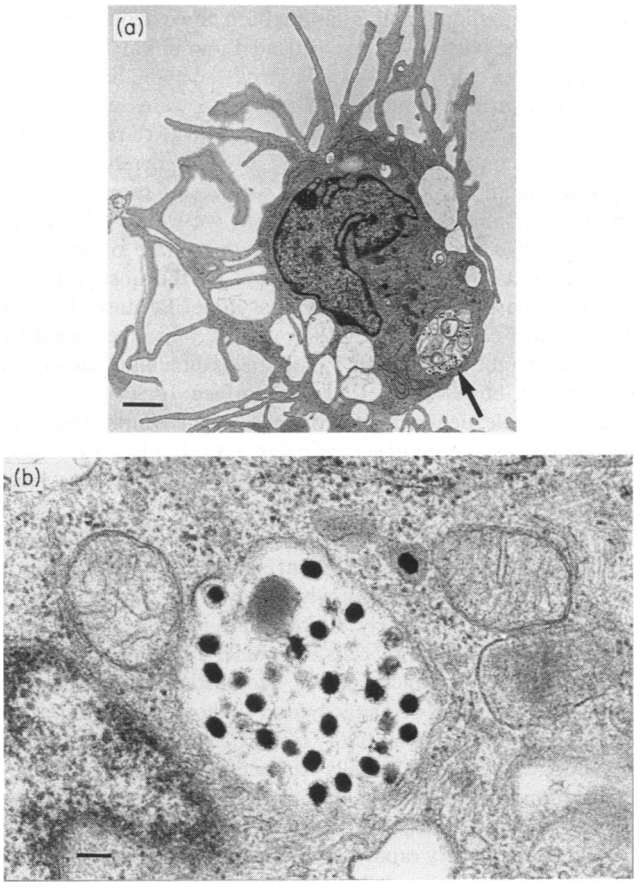

Figure 7. Phagocytosis of T4 phage virus particles by lymph-borne, dendritic macrophages of sheep after 40 min culture. (a) Low power electronmicrograph (scale bar = 1·25 μm) shows clearly the dendritic phenotype, and the arrow indicates a phagocytic vacuole in which the ingested virus can be seen as small black dots. (b) High power electronmicrograph (scale bar = 100 nm) of virus particles in phagocytic vacuole.

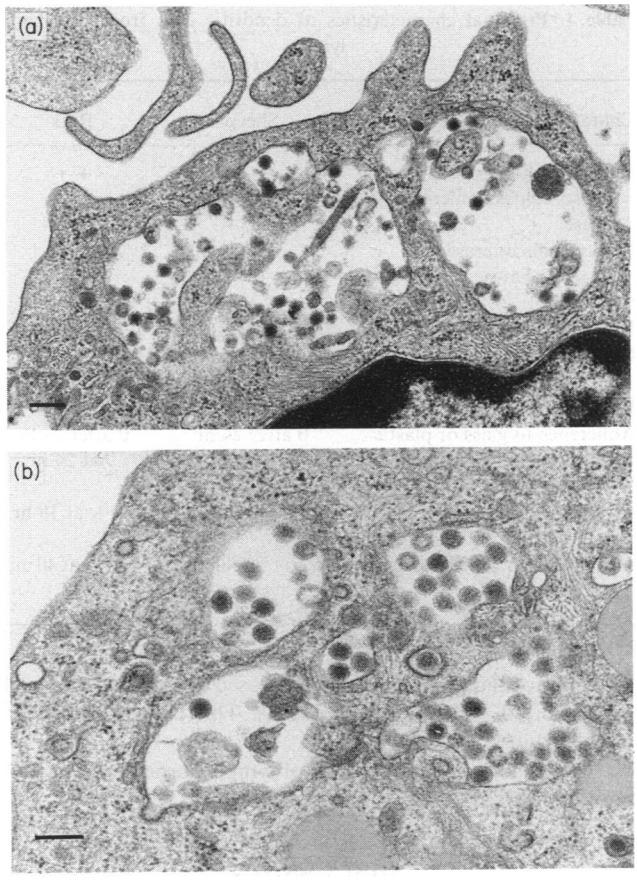

Figure 8. Phagocytosis of influenza virus particles by lymph-borne dendritic macrophages of rats and sheep after 40 min culture. (a) Sheep cell containing virus particles, scale bar = 200 nm. (b) Rat cell containing virus particles, scale bar = 150 nm.

Dendritic cells from both rats and sheep showed abundant class II histocompatibility antigens on their surfaces; a sheep cell stained for this antigen is shown in Fig. 5, electron micrographs of rat cells stained in this way have been published (Hall, 1985).

Phagocytosis by dendritic cells *in vitro*

Dendritic cells from rats and sheep were incubated under standard culture conditions with fluorescent latex beads at a modulus of approximately 20 beads per cell. Little unequivocal uptake of the beads was observed during the early hours of the culture, but by 18 hr examination of the cells by fluorescence microscopy showed that nearly half the cells had obvious fluorescent beads either in them or on them (Fig. 6). Parallel electron microscope studies showed that many of the beads were indeed intracytoplasmic and had been phagocytosed (Fig. 6).

Phagocytosis occurred much more rapidly and abundantly when the dendritic cells were exposed to viruses. The suspensions of either influenza or T4 phage viruses were added to suspensions of dendritic cells at a ratio of 1 : 10 by volume. After 40 min the cultures were stopped by the addition of aldehyde fixative and, after centrifugation, the cell pellets were examined in the electron microscope. Even in ultra thin sections the dendritic cells were crowded with virus particles. The phage particles were confined for the most part to obvious phagolysosomes, and seemed to have lost their outer coat during the process of phagocytosis. The influenza virus particles, too, were mainly in phagolysosomes but some were in isolated 'private' endocytotic vesicles, which might indicate the special intracellular routing of potential pathogens. These phenomena are illustrated in Figs 7 and 8.

Longer term culture of dendritic cells

Although dendritic cells survive well in culture they show little ability to undergo cell division. When co-cultured with isologous lymphocytes they apparently exert a trophic effect and provide mitogenic stimuli which could account for the 'autologous MLR' (Crow & Kunkel, 1984). Whether this was due to the presentation of previously ingested antigens or to the *de novo* production of lymphokines is unclear. Certainly, as the days passed, many dendritic cells gradually phagocytosed the dead cells and detritus which inevitably were present, and assumed the characteristics of conventional macrophages, i.e. they

Table 1. Principal characteristics of dendritic cells from peripheral lymph

Characteristic under test	Sheep	Rats
Dendritic phenotype as seen by phase-contrast microscopy	+++	++
ATPase	+++	+++
Non-specific esterase	++	+++
Acid phosphatase	++	+++
Surface Ig	+++	+++
Isotypes of surface Ig	IgM, IgG1 (IgG2)	IgM, IgG
Class II histocompatability antigens	+++	+++
Fc receptor	?	? IgG2b
Adherence to glass or plastic	0 after 24 hr	0 after 24 hr
In vitro phagocytosis of opsonized RBC or bacteria	0 at 24 hr	0 at 24 hr
In vitro phagocytosis of latex beads	++ at 18 hr	++ at 18 hr
In vitro phagocytosis of virus particles	+++ at 40 min	+++ at 40 min

became more adherent, and phagocytosed marker particles such as opsonized red blood cells (RBC). This change sometimes happened in as little as 3 days.

The principal characteristics of lymph-borne dendritic cells are summarized in Table 1.

DISCUSSION

In simple cytological terms the dendritic cells we obtained from the peripheral lymph of rats and sheep are similar to those described in previous studies (*op. cit. supra*), and are compatible with the description of similar cells from cattle (Emery *et al.*, 1987). The larger question of the relationship of lymph-borne dendritic macrophages to inter-digitating cells in lymph nodes, Langerhans' cells in the skin, or dendritic cells isolated from murine spleens, etc., cannot be pursued here, although reviews of the subject have been attempted (Steinman & Nussenzweig, 1980; Austyn, 1987). Few would doubt though that the abundance of class II antigens on dendritic cells and the close associations that they make with lymphoid cells indicate that they are probably important accessories in the business of antigen presentation (Chain, Kay & Feldmann, 1986). Similarly, our observation of their derivation from a precursor in peripheral blood is in accordance with others (Knight *et al.*, 1986). It is known that this precursor is a non-lymphoid cell of bone marrow origin (Pugh *et al.*, 1983) and the blood monocyte seems a likely candidate. This would be consistent with the increased numbers of dendritic cells in cutaneous lymph that follow xylene-induced hyperaemia and the coincident occurrence of monocyte-like cells, but conclusive proof is still lacking. The exact mechanism of the xylene effect is unclear; insofar as serial applications give identical responses it does not involve sensitization in an immunological sense, and in any case it was much more rapid than the response to genuine skin sensitizing chemicals like oxazolone (Hall, 1980).

The relatively large amounts of surface Ig on the dendritic cells has been noted by others who work on sheep (Miller & Adams, 1977; M. Brenan and B. Morris, personal communication) but is puzzling that this has not been noted in other species, in spite of the batteries of sophisticated immunological probes that have been employed. We have striven to avoid being misled by technical artefacts but, even if our results were caused by the adventitious binding of Ig reagents to fixative-resistant Fc receptors of unusually high avidity, it would merely underline the close relationship between the surfaces of these cells and immunoglobulins in general. However, we were dealing only with lymph-borne cells and, *in vivo*, this phase of the cells' existence lasts only a few seconds before, for the most part, they become incorporated into a regional node. Because the cells were always coated with Ig, the presence of conventional Fc receptors on the cytoplasmic membrane is difficult to answer. It should not be assumed that the surface Ig has a crucial functional role; dendritic macrophages in the lymph sinuses of fetal nodes have been observed being actively phagocytic long before immunoglobulins, or even functional lymphocytes, have developed (Morris & Al Salami, 1987).

The question of phagocytosis by dendritic cells has always been a vexed one. There is abundant evidence from observations of these cells *in vivo* in sheep which shows them to be actively phagocytic, but for nearly 30 years attempts to show rapid phagocytosis of red blood cells and bacteria *in vitro* have failed. The use of 'latex' microspheres provided us with the first acceptable evidence of phagocytosis *in vitro*, and it might be explained by the known affinity of particles of this type for the immunoglobulins on the cells' surfaces. Even so, the phagocytosis appeared relatively slow and unimpressive to those who worked with peritoneal exudate cells from rodents. Given this background the very rapid and abundant phagocytosis of virus particles *in vitro* was a striking finding. The 'flu virus used was able to agglutinate suspensions of sheep red blood cells and lymphocytes and it seems plausible that it attached itself to a similar neuraminic acid receptor on the dendritic cells. No doubt the T4 phage used an analogous receptor system and it is difficult to decide whether the virus 'recognized' the dendritic cell, or *vice versa*. None of the cells came from specifically immune animals and there is no reason to believe that specific antibodies were involved. Clearly, much remains to be discovered about the kinetics of virus uptake, the time at which processed viral epitopes appear on the plasma membrane of the cells, and the range of virus types that are susceptible to phagocytosis by these cells. This work has been put in train but, already, the marked ability of these cells both to engulf viruses and to express class II antigen suggest a pivotal role for them in the induction of effective anti-viral immunity. In this context, it should be borne in mind that although most of the dendritic lymph cells appear to be retained by the first node they come to, their arrest is not quantal. Small but significant numbers do escape into intermediate lymph (De Martini, Fiscus & Pearson, 1983) and this number is increased during the course of vigorous or damaging reactions in the lymph nodes (Hall, 1984).

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