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Large mononuclear Ia-positive veiled cells in Peyer's patches

I. ISOLATION AND CHARACTERIZATION IN RAT, GUINEA-PIG AND PIG

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Summary. The antigen presenting cell system in the skin is extensively studied, and is supposed to consist of Langerhans cells in epidermis and dermis, veiled cells in skin lymph and interdigitating cells in skin lymph nodes. In order to detect whether a similar cell system is present in the gut, we studied Peyer's patch cell suspensions.

Mononuclear cells with long actively moving cytoplasmic veils were found in cell suspensions from Peyer's patches of rat, guinea-pig, and pig, and in cell suspensions from small intestinal villi of guinea-pig and pig, but not of rat. These veiled cells are strongly Ia-positive. Because of their Ia positivity, their enzyme cytochemical staining pattern, and their ultrastructure, these cells resemble the antigen presenting cells of the skin, skin lymph and lymph node.

Abbreviations: DC, interdigitating cell; LC, Langerhans cell; Ia, immune response associated; EMEM, Eagles minimal essential medium; Ca-Mg-free, calcium- and magnesium-free; HBSS, Hanks's balanced salt solution; FCS, foetal calf serum; HE, haematoxylin-eosin; AP, acid-phosphatase; ATP, adenosine-triphosphatase; NSE, non-specific esterase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TEM, transmission electron microscopy.

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INTRODUCTION

Skin lymph contains a group of cells with actively moving cytoplasmic processes, the so-called veiled cells (Drexhage, Mullink, de Groot, Clarke & Balfour, 1979), which are thought to present antigen to T cells (Søeberg, Sumerska, Binns & Balfour, 1978). The destination of these cells seems to be the T-dependent area of the lymph node and they are the likely precursors of a special type of cell in this area, the interdigitating cell (IDC; Kamperdijk & Hoefsmit, 1978a). In the epidermis another type of cell is known for its accesory function, the Langerhans cell (LC Silberberg-Sinakin, Thorbecke, Baer, Rosenthal & Berezowsky, 1976). Veiled cells and interdigitating cells resemble the epidermal LC in many respects; their enzyme cytochemical staining pattern is very similar (Drexhage *et al.*, 1979); they are all strongly positive for immune response-associated antigen (Ia-antigen; Stingl, Katz, Clement, Green & Shevach 1978). The Birbeck granule, a typical cell organelle of the LC (Birbeck, Breathnach & Everall, 1961) has also been found in veiled cells and in IDCs, mainly after immune stimulation (Kamperdijk, Raaymakers, de Leeuw & Hoefsmit, 1978b). Moreover it has been found that LC carry antigen from the skin via the skin lymph to the subcapsular sinus of the lymphnode (Silberberg-Sinakin *et al.*, 1976). Therefore epidermal LC, lymph-borne veiled cells and paracortical IDCs have been

regarded as belonging to the same family of antigen presenting cells (Drexhage *et al.*, 1979). Such a cell system has not yet been identified in the gut, although this is a site with ample antigen contact.

In the present study we describe the presence of Ia-positive veiled cells in suspensions obtained from Peyer's patches and other gut tissues. These cells possess an enzyme staining pattern resembling that of their proposed epidermal counterparts.

MATERIALS AND METHODS

Wistar rats of R-Amsterdam strain (both sexes, weighing 400 g) were obtained from C. P. B. Zeist, The Netherlands. Hartley strain guinea-pigs (both sexes weighing 500 g) were obtained from T. N. O. Leiden, The Netherlands. Pigs weighing approximately 60 kg were purchased from a stock farm.

Media

Eagles Minimal Essential Medium (EMEM) and calcium- and magnesium-free Eagles Minimal Essential Medium (Ca-Mg-free EMEM) were obtained from Flow Laboratories (Irvine) and were supplemented as described by Bland, Richens, Britton & Lloyd (1979), using 15% foetal calf serum (FCS Flow Laboratories, Irvine) instead of 10%. For tissue washings we used Hanks's balanced salt solutions (HBSS) without further supplementations.

Preparation of cell suspensions.

Rats and guinea-pigs were killed by ether intoxication. Pigs were killed by intravenous potassium chloride injection during neuroleptic anaesthesia. The entire small intestine was removed. Peyer's patches were dissected out, and extensively washed with HBSS, to remove faecal contents. Segments of approximately 3 cm in length between the Peyer's patches were also removed and cut into small pieces of approximately 0·5 cm^2. The superficial layers of both Peyer's patches and other gut tissues were removed by magnetic stirring in Ca-Mg-free EMEM for 15 min at room temperature. The obtained supernatant was centrifuged (250 ***g***, 10 min) and the cell pellets were washed twice in Ca-Mg-free EMEM (250 ***g***, 10 min). The leucocyte fraction of these cell suspensions was obtained using a percoll gradient (see below). The remaining tissue parts, devoid of the superficial layers were incubated in EMEM containing 174 u. collagenase/ml (0·1% Worthington CLS III) and magnetically stirred at 37° for 15 min. Cells in the obtained supernatant were washed three times in EMEM. In some instances cell suspensions were purified by using a discontinuous percoll gradient (B.M. Balfour, personal communication): cells in EMEM were suspended on a percoll layer of a density of 1·050 (32% percoll, density 1·130, Pharmacia Fine Chemicals Sweden; 10% Medium TC 199 ten times concentrated, Difco Laboratories, Detroit, Mich., U.S.A.; 58% aqua bidest.), which was on top of a layer with a density of 1·070 (48% percoll, density 1·130, 10% Medium TC 199 ten times concentrated, 42% aqua bidest.). The columns were spun to equilibrium at 2000 ***g*** for 15 min at room temperature. The 1·070 band was harvested, washed twice and used for further experiments. Cell suspensions were kept for at least 1 hr at 37° in EMEM before further experiments were done.

Time-lapse cinematography of living isolated cells

Cell suspensions at a maximum concentration of 5×10^5 cells/ml in EMEM were transferred directly into a Sykes-Moore chamber and time-lapse cinematographic studies were performed as described before (Drexhage *et al.*, 1979). Standing frames were projected one by one, and drawings of the outlines of cells were made. These drawings will be shown.

Membrane collection and haematoxylin-eosin staining of prefixed isolated cells

As cytoplasmic extensions of dendritic cells are destroyed by smearing or making cytospin preparations, a technique was used to collect prefixed cells on millipore membranes and thereafter stain them with haematoxylin-eosin (HE) (Drexhage *et al.*, 1979).

Cytochemistry of prefixed isolated cells on millipore membranes

Cytochemistry of the prefixed cells collected on millipore membranes was performed as described (Drexhage *et al.*, 1979) with minor modifications: membranes with adherent cells were placed directly in the incubation media, left there for 60 (acid-phosphatase), 20 (non-specific esterase) or 15 min (adenosinetriphosphatase), rinsed in distilled water and counterstained by immersion in haematoxylin or methyl-green. After being washed the membranes were dehydrated, cleared, mounted in malinol and covered with a coverslip.

Preparation of isolated cells for transmission electron microscopy

Cells were incubated after isolation for 1 hr at 37° to allow veiled cells to develop veils. Cold glutaraldehyde 2% in 0·1 M phosphate buffer, pH 7·4 was added and cells were fixed for 1 hr at 4°. Cells were then centrifuged for 5 min at 250 *g* and washed. 1·33% OsO_4 in S-collidine buffer was then added at 4° and cells were centrifuged after 7 min. After being washed the pellet was mixed with agar and centrifuged at 1800 *g* for 20 min. Cell pellets were dehydrated through graded ethanols and embedded in Epon-812. Sections were cut on an LKB-Ultratome III, mounted on copper grids, stained with uranyl acetate and lead citrate, and examined in a Zeiss-109 electron microscope.

Preparation of isolated cells for antiserum labelling by peroxidase staining for transmission electron microscopy

Cells were incubated after isolation and purification for at least 1 hr at 37° to allow veils to develop. Cold glutaraldehyde in 0·1 M phosphate buffer pH 7·35 was added to a final concentration of 0·2%, and cells were prefixed at 4° for 15 min, washed twice in 0·01 M phosphate-buffered saline (PBS) pH 7·4 and spun down. The pellet was incubated with commercially obtained mouse anti-rat Ia serum (Sera Lab. MAS 029C) in a final dilution of 1:1600 in PBS with 0·5% bovine albumin (PBS/BSA) for 30 min on ice (dilutions were tested for each serum batch). After centrifugation, cells were further incubated with rabbit anti-mouse IgG peroxidase conjugate (PO; Miles Yeda, Israel) in a dilution of 1:400 in PBS/BSA for 30 min on ice. Cells were washed twice and stained for peroxidase activity with 3·3′-diaminobenzidine-tetra-HCl (Sigma, U.S.A.) in 0·5 mg/ml Tris-HCl pH 7·6 containing 0·01% H_2O_2, for 10 min at 4°. Cells were washed five times and postfixed in 1·5% glutaraldehyde in 0·09 M phosphate buffer overnight. As a control, cells were incubated with preimmune mouse serum, PBS/BSA or incubated in all media in the absence of conjugate, and examined for non-specific staining.

RESULTS

Magnetic stirring in calcium and magnesium free EMEM removed the superficial layers (epithelial and subepithelial zones) from the Peyer's patches. Applying this simple technique we were able to obtain suspensions of cells originating from these zones. The remaining tissue parts (the dome, the follicles and the interfollicular areas) were treated by collagenase. Parts of the gut in between Peyer's patches were treated in a similar fashion (magnetic stirring followed by collagenase treatment). These parts of the gut, not including Peyer's patches will further on be referred to as 'villous tissues'. Viability of cells tested by trypan blue exclusion was 80% for the superficial layers of Peyer's patches, 65% for superficial layers of villous tissues, and 90% for the collagenase treated tissue parts.

Percoll purification removed dead cells and epithelial cells and thus improved the viability to over 95%.

Peyer's patch cell suspensions

Cinematography of living cells. Time-lapse phase contrast cinematography films of Peyer's patch cell suspensions showed cells with long actively moving cytoplasmic protrusions, which were thin and flat and strongly resembled the veils of the veiled cells in skin lymph. They were sometimes as long as the cell itself, and they appeared at different parts of the cell. The cells, however, were smaller than lymph veiled cells, and in addition the number of veils present was smaller. Figure 1 shows drawings of Peyer's patch veiled cells taken from the time-lapse film at intervals of 10 sec. As can be seen the cell is capable of withdrawing its veils completely in this 10 sec time interval and to project some others at other parts of its cell membrane (Fig. 1A).

In doing so the cell is constantly changing its shape. During the process of protrusion and retraction vacuoles are formed. The nucleus is present in the central part of the cell and stays there, despite the movements of the cell. The cells do not seem to move directionally. Sometimes Peyer's patch veiled cells make contacts with other cells present in the same cell suspension, mostly with lymphocytes (Fig. 1B). Consequently they form cellular aggregates, which are in some cases quite stable during 3 hr periods of observation. Cells may also make contacts for only a short time, thereafter swimming away. Veiled cells were found in cell suspensions of Peyer's patches of rat, guinea-pig and pig. They were present in both the superficial and collagenase-treated layers. Purifications by a percoll gradient did not influence the movements of veiled cells. An advantage of the use of this gradient was that less epithelium and cellular debris contaminated the cell suspensions, making them more suitable for cinematographic studies.

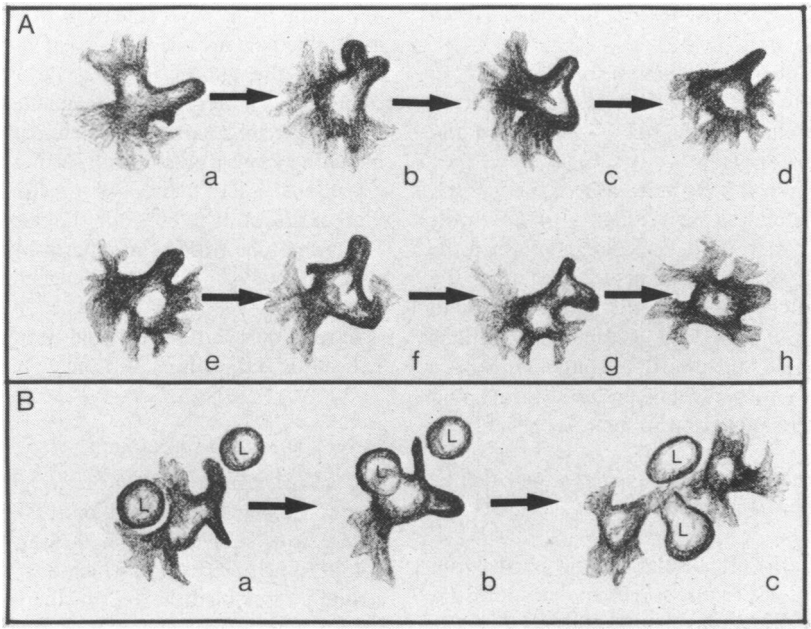

Figure 1. Drawings of living Peyer's patch veiled cells, taken from standing frames of phase-contrast cinematography. (A) The movements of one single cell. The pictures were taken with time intervals of 10 s. (B) Peyer's patch veiled cell in contact with lymphocytes (L). (Magnification × 1000.)

Differential cell counts on HE stained millipore membranes. Veiled cells could easily be recognized on HE-stained millipore membrane preparations (Fig. 2) due to their pale staining cytoplasm, their protrusions, and their lobulated nucleus. There is a slight difference in percentage of veiled cells between species studied: guinea-pig contained about 12%, pig contained about 8% and rat about 6%. Equivalent figures were obtained when studying superficial layers or deeper collagenase-treated tissue parts. In HE-stained millipore membrane preparations aggregates could again be recognized. These appeared to contain veiled cells, lymphocytes, eosinophilic leucocytes and in some instances cells with the appearance of macrophages, i.e. large, round, foamy, vacuolated cells. The contacts of veiled cells with lymphocytes were the most predominant ones.

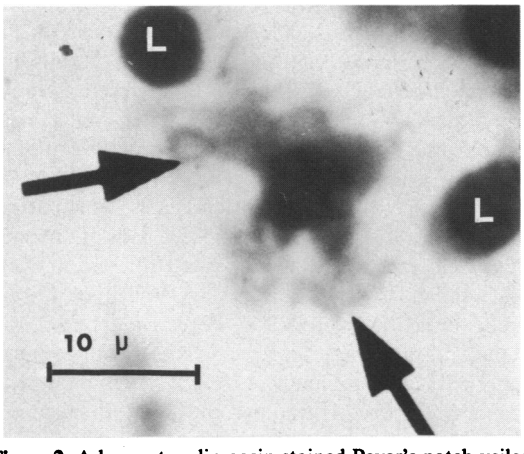

Figure 2. A haematoxylin-eosin stained Peyer's patch veiled cell, collected on a millipore membrane. The veils are clearly visible (arrows). At one pole the cell is in contact with a lymphocyte (L). (Magnification × 2000.)

Enzyme equipment of prefixed cells on millipore membranes. In suspensions of both superficial and deeper layers of Peyer's patches of rat and guinea-pig, veiled cells were detected which had a strong adenosine-triphosphatase activity (ATP-ase). This activity was bound to the cell membrane and consequently the veils were clearly visible (Fig. 3). About half of the veiled cells showed this ATP-ase activity. Approximately 40% were non-specific esterase (NSE) positive with a variable degree of enzyme activity, mainly concentrated near the nucleus in the central part of the cell; the actual protrusions themselves were not NSE

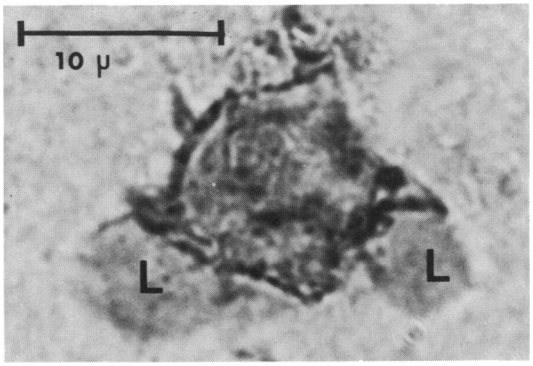

Figure 3. The adenosine triphosphatase activity (ATP-ase) of a Peyer's patch veiled cell. It is clearly visible that the cell is strongly active and that the enzyme activity is membrane bound. The two contacted lymphocytes are ATP-ase negative. (Magnification × 2800.)

positive. Nearly all veiled cells were weakly acid-phosphatase (AP) positive. As with NSE, this enzyme was again concentrated in the central part of the cell.

Transmission electron microscopy. The veiled cells had a lobulated or horseshoe-shaped nucleus, and electron translucent cytoplasm with several cytoplasmic processes. The cytoplasm contained many free ribosomes, a few scattered polyribosomes and some cysterns of rough endoplasmatic reticulum. Usually a well developed Golgi complex was present. Furthermore, the majority of the veiled cells contained many mitochondria, some microtubules, lysosomes and vesicles (Fig. 4). Some had phagolysosomes. No difference was evident in transmission electron microscopy (TEM) between veiled cells of rat and guinea-pig. Suspensions of pig cells were not studied in TEM.

Veiled cells processed for the detection of Ia antigens showed a heavily labelled cell membrane (Fig. 5). The cytoplasmic vesicles were sometimes positive, suggesting that these vesicles might be formed by invagination of the cell membrane. The presence of Ia determinants was more pronounced on veiled cells than on lymphocytes bearing Ia antigens, probably B cells, present in the same cell suspensions. All veiled cells observed were Ia-positive. No non-specific staining activity was seen in the controls (see methods).

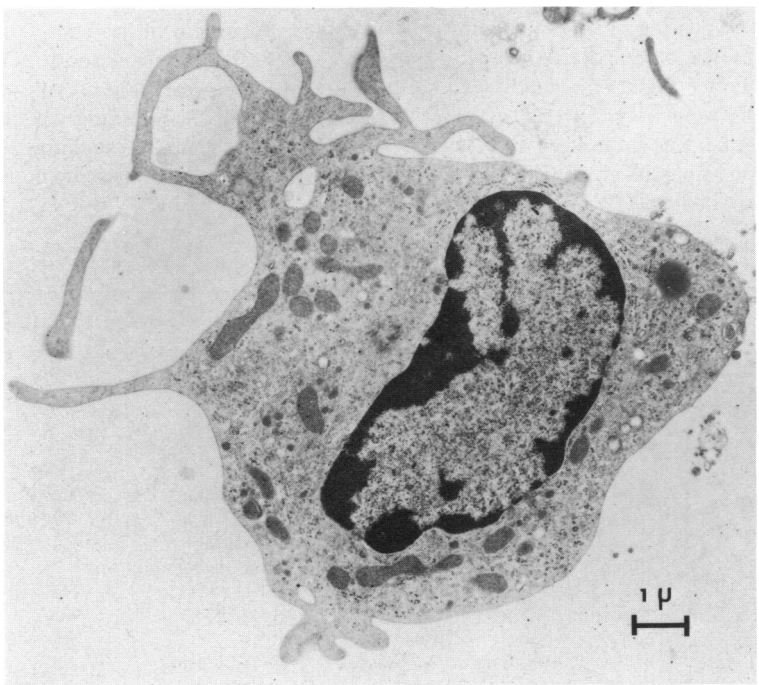

Figure 4. The ultrastructure of a Peyer's patch veiled cell. The cell shows an electrontranslucent cytoplasm, and is rich in mitochondria, small vesicles, and free and poly-ribosomes. There is an indication of a small amount of rough endoplasmic reticulum. Very few lysosomes are present. The Golgi complex is visible in the hof of the nucleus, which is kidney shaped. (Magnification × 6500.)

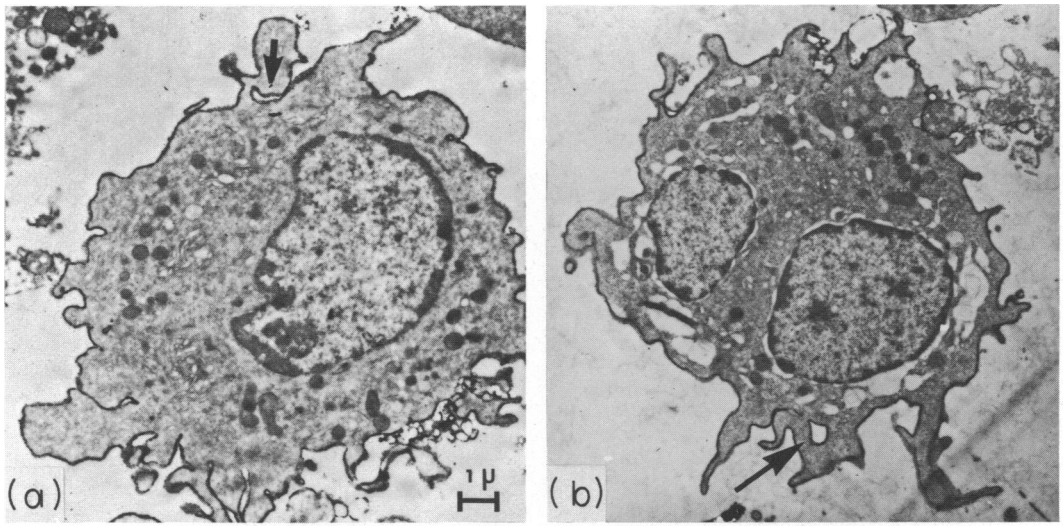

Figure 5. Ia staining of Peyer's patch veiled cells, using immune peroxidase techniques. The cells are strongly positive and the Ia structures are bound to the surface membrane (dark lining). Some vesicles are positive, on some occasions in contact with the outer membrane (arrows). (Magnification × 5000.)

Villous tissue cell suspensions

Time-lapse films of cell suspensions from superficial and deeper layers of guinea-pig and pig small intestinal villi showed the presence of veiled cells, which were similar to those described for Peyer's patches. In rat, however, veiled cells were only detected in Peyer's patches and not in other gut areas, even after several purification steps using a percoll gradient. This finding was in agreement with our figures obtained with differential counts using HE stained millipore membranes: guinea-pig villous tissue contained about 23% veiled cells, pig villous tissue about 7% and no veiled cells could be detected in rat villous tissue. The veiled cells in the small intestinal cell suspensions had an enzyme cytochemical staining pattern similar to Peyer's patch veiled cells. In none of the examined

Table 1. Comparison between LC, veiled cell IDC, and gut veiled cells

	LC	Veiled cell	IDC	Gut veiled cell
Cytoplasm	Clear	Clear	Clear	Clear
Long cytoplasmic projections	+	+	+	+, but shorter
Nucleus	Lobulated	Lobulated	Lobulated	Lobulated
Microfilaments	+	+	+	+
Lysosomes	+/−	+/−	+	+/−
Phagocytosis	Not primary function	Small proportion	Not primary function	Not primary function
Birbeck granules	+/−, Species dependent	+/−, Species dependent	Rat:after immune stimulation	Rat, guinea-pig: absent
Acid-phosphatase	+ in lysosomes	Diffuse, weak	+ in lysosomes	Half of the cells diffuse, weak +
Non-specific esterase	+	Diffuse, weak	+	40% varying degree +
Adenosine triphosphatase	+, plasma membrane	+, veils	+, plasma membrane	All +, plasma membrane
Contacts	+	+	+	++
Ia antigens	+++	+++	+++	+++
After Drexhage *et al.*, 1979				See results

species were veiled cells observed in the large intestinal tissues.

DISCUSSION

Cells with long actively moving cytoplasmic extensions were found in suspensions of Peyer's patches of rat, guinea-pig and pig. They were present in the epithelial and subepithelial zones, as well as in the deeper structures. They were also found in small intestinal tissues not containing Peyer's patches, but their presence here was species dependent: they were found in guinea-pig and pig, but not in rat. Similar species differences have also been described for skin lymph veiled cells (Drexhage *et al.*, 1979). The cinematographic behaviour of Peyer's patch veiled cells appears to be very similar to that of veiled cells found in skin lymph, which have been suggested to be involved in antigen presentation to T cells. The strong Ia positivity of the Peyer's patch cell membrane suggests a similar function. Besides their characteristic movements and Ia positivity Peyer's patch veiled cells share many ultrastructural and enzyme cytochemical features with Langerhans cells, veiled cells and interdigitating cells, which is indicated in Table I.

Besides the similarities between Peyer's patch veiled cells and skin lymph veiled cells, there are also differences: Peyer's patch veiled cells are smaller, their veils are usually shorter and less numerous, and in general gut veiled cells are more active in contacting other cells and in forming aggregates. Aggregate formation has been reported to be an important process in the induction of contact sensitivity of DNCB, both *in vivo* (Drexhage *et al.*, 1979) as *in vitro* (Balfour, Drexhage, Kamperdijk & Hoefsmit, 1981). In Peyer's patch veiled cells we could not detect Birbeck granules (Birbeck *et al.*, 1961) though they have been described in skin lymph veiled cells (Silberberg-Sinakin *et al.*, 1976). It has been suggested that the vicinity of keratinizing cells determined the presence of the cellular organelles (Thorbecke, Silberberg-Sinakin & Flotte, 1980) and our results might support this view.

Steinman & Cohn (1973) reported 0·1% of Peyer's patch cells to adhere to glass and to resemble the dendritic cell of the spleen in phase contrast microscopy. Steinman's spleen cells contain a few acid-phosphatase reactive lysosomes close to the nucleus, no membrane-bound adenosine-triphosphatase activity, and they are all glass-adherent. The Peyer's patch veiled cells as described by us were not glass-adherent and strongly ATP-ase positive. Species difference between the mouse studied by Steinman and the three animal species studied by us, might play a role, but it may also be that there are two populations of dendritic cells in Peyer's patches, one glass-adherent and one non-glass-adherent with long ATP-ase positive cytoplasmic veils.

The relationship between Peyer's patch veiled cells and macrophages is not clear. In contrast to Peyer's patch veiled cells, macrophages usually have only short ruffles; they adhere strongly to glass and show active phagocytosis. On the other hand, the enzyme cytochemistry of Peyer's patch veiled cells resembles that of macrophages (NSE and AP positivity), though the distribution patterns of these enzymes are different.

Dendritic cells similar to Peyer's patch veiled cells were found in the afferent gut lymph of rat (McPherson, 1980). Mason, Pugh & Webb (1981) have described a technique by which they isolated these lymph-borne gut-derived dendritic cells by cannulating the thoracic duct after removal of the mesenteric lymph nodes. These dendritic cells were strongly Ia positive and appeared to be very potent stimulators of allogeneic T cells in mixed lymphocyte reactions. Lechler & Batchelor (1982) found the same cells to be capable of reconstituting transplantation reactions of rat kidney grafts. Recent experiments of Pugh (1982) showed that dendritic cells isolated by the same technique, originated from the bone marrow and had a rapid turnover. Their amounts of NSE varied. These cells resemble our Peyer's patch veiled cells strongly. We have the impression that they belong to the same family of cells.

In conclusion, our results indicate the presence of Ia-positive veiled cells in gut tissue, showing strong similarities with the antigen presenting cells present in skin, skin lymph and lymph nodes.

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