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Identification of particular epithelial areas and cells that transport polypeptide-coated nanoparticles in the nasal respiratory mucosa of the rabbit

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Abstract

The active transcytosis of many different polypeptides (either presented free or adsorbed on latex nanoparticles), found in the respiratory mucosa of the upper nasal concha, has previously been shown to be proportional to the total volume of the lymphoid aggregates present in the tissue. By combining the use of fluorescent nanoparticles, flux measurements, confocal and scanning electron microscopy and conventional histology, it is shown in this paper that: (i) the areas of epithelium overlying lymphoid aggregates are the only transporting polypeptides; (ii) the respiratory epithelium in these areas consists mainly of non-ciliated microvillar cells, with numerous ciliated cells and rare mucous goblet cells at the periphery of the area only; (iii) non-ciliated microvillar cells are distinguishable in cells with well developed finger-like microvilli and cells with an irregularly pleated apical membrane, similar to that of intestinal and bronchial antigen-sampling M-cells; (iv) groups of polypeptide-coated nanospheres are found bound to this latter type of cells, demonstrating that these are the transporting cells, detected at the first stage of the transcytotic cycle.

Keywords: Active transport of polypeptides; Antigen sampling; Transcytosis; Lymphoid aggregate; Non-ciliated microvillar cell; M-cell

1. Introduction

Transepithelial selective transcytosis of carbocalcitonin and adrenocorticotropic hormone, which has been demonstrated to be present across the respiratory mucosa covering the upper nasal concha of the rabbit [1–4], has been proved to accept many polypeptides, with different molecular weights, also presented to the epithelium adsorbed on nanoparticles [5], and to be correlated with the total volume of lymphoid cell aggregates present in the mucosa [6]. All this seems to be in agreement with a function of antigen-sampling from the nasal lumen [6].

The aim of this paper is to localize the transport areas in the epithelium and to identify the transporting cells by combining the use of polypeptide-coated fluorescent nanoparticles, confocal microscopy, scanning electron microscopy and conventional histology.

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2. Materials and methods

Male New Zealand rabbits, weighing approx. 3 kg, were killed by cervical dislocation and the nasal mucosae from the roofs of both nostrils were mechanically excised, washed with Krebs-Henseleit saline at room temperature and mounted on frames between two teflon chambers. The features of the chambers used, salines, oxygenation, incubation temperature and the experiment protocol used to determine the unidirectional mucosa-submucosa and opposite fluxes (J_{ms}, J_{sm}) of the polypeptide-coated latex nanospheres were as described in the previous twin paper [5]. However, the latex nanoparticles used were fluorescent (0.5 μm polystyrene YG Fluoresbrite plain microspheres, produced by Polysciences, Warrington, PA, USA). The nanospheres were coated with 6.5×10^{-6} M bovine insulin (Calbiochem, Luzern, Switzerland); the coating procedure was as reported in the twin paper.

At the end of an experiment during which unidirectional fluxes of insulin-coated fluorescent nanoparticles were measured, the mucosa exposed to permeation (0.3 cm^2 area, well delimited by the scratch left by frame pressure) was cut away and fixed in 4% paraformaldehyde in PBS (standard phosphate buffer saline). *N*-Butylalcohol (60°) was employed instead of xylene in all the operations of embedding in paraffin and elimination of paraffin after section cutting to prevent the latex beads from being dissolved by xylene [7]; 8 μm thick sections were cut and mounted with glycerin again to avoid the use of mounting media containing xylene. The sections so treated were then examined with a confocal laser scanning microscope (Leica TCS NT, Leica Microscopy and Scientific Instr. Group, Heerbrugg, Switzerland) at excitation and emission wavelengths of 458 and 540 nm.

After confocal microscopy analysis had been performed, the coverslips were removed and the sections washed with distilled water and stained with hematoxylin-eosin in order to observe the presence of any lymphoid material and its organization in the mucosa (bright-light microscopy). Finally, the piece of embedded tissue immediately adjacent to a section, in which fluorescent beads were observed in the epithelial cells, was dried by graded dehydration with ethanol series, followed by replacement with hexamethyldisilazane, and processed for observation by scanning electron microscopy (Leica, ex-Cambridge, Stereoscan 250 MKZ, Leica Microscopy and Scientific Instr. Group) at 10 kV. Some nanospheres, coated in saline with insulin, then diluted in distilled water and dried, were also examined by scanning electron microscopy.

3. Results

3.1. Confocal microscopy

When the respiratory mucosa of the upper concha was exposed to fluorescent nanospheres on the submucosal side for 2 h and the submucosa-mucosa unidirectional flux (J_{sm}) determined, cross-sections of the tissue, fixed at the end of the experiment, only showed fluorescent spheroidal spots, with diameters compatible with the nanospheres used, in the subepithelial layers from the submucosal edge (Fig. 1b) to just beneath the epithelium (Fig. 1a); in the epithelium they were very rare and apparently intercellular (Fig. 1a). When the mucosa was exposed to fluorescent beads on the apical side for 2 h and the mucosa-submucosa unidirectional flux (J_{ms}) determined, the sections of tissue, fixed at the end of the experiment, generally presented no or very rare scattered fluorescent beads in the epithelium (Fig. 1c); however, in some epithelial areas fluorescent spots of different sizes were crowded together in the epithelial cells (an example is shown in Fig. 1d). Magnification of the spots (Fig. 1e) demonstrates that the smallest ones have a diameter compatible with that of the beads used, while the others are clearly aggregates of smaller fluorescent spots which are again compatible with the latex nanospheres. The conclusion of these observations seems to be that in some areas of the epithelium the insulin-coated beads can enter the epithelial cells, but only on the apical side.

It is noteworthy that the epithelium proves to be 60–85 μm high in Fig. 1a and c, a value similar to that previously observed as a mean [6]. Conversely, the epithelium containing fluorescent spots (Fig. 1d) seems to be less thick (45 μm).

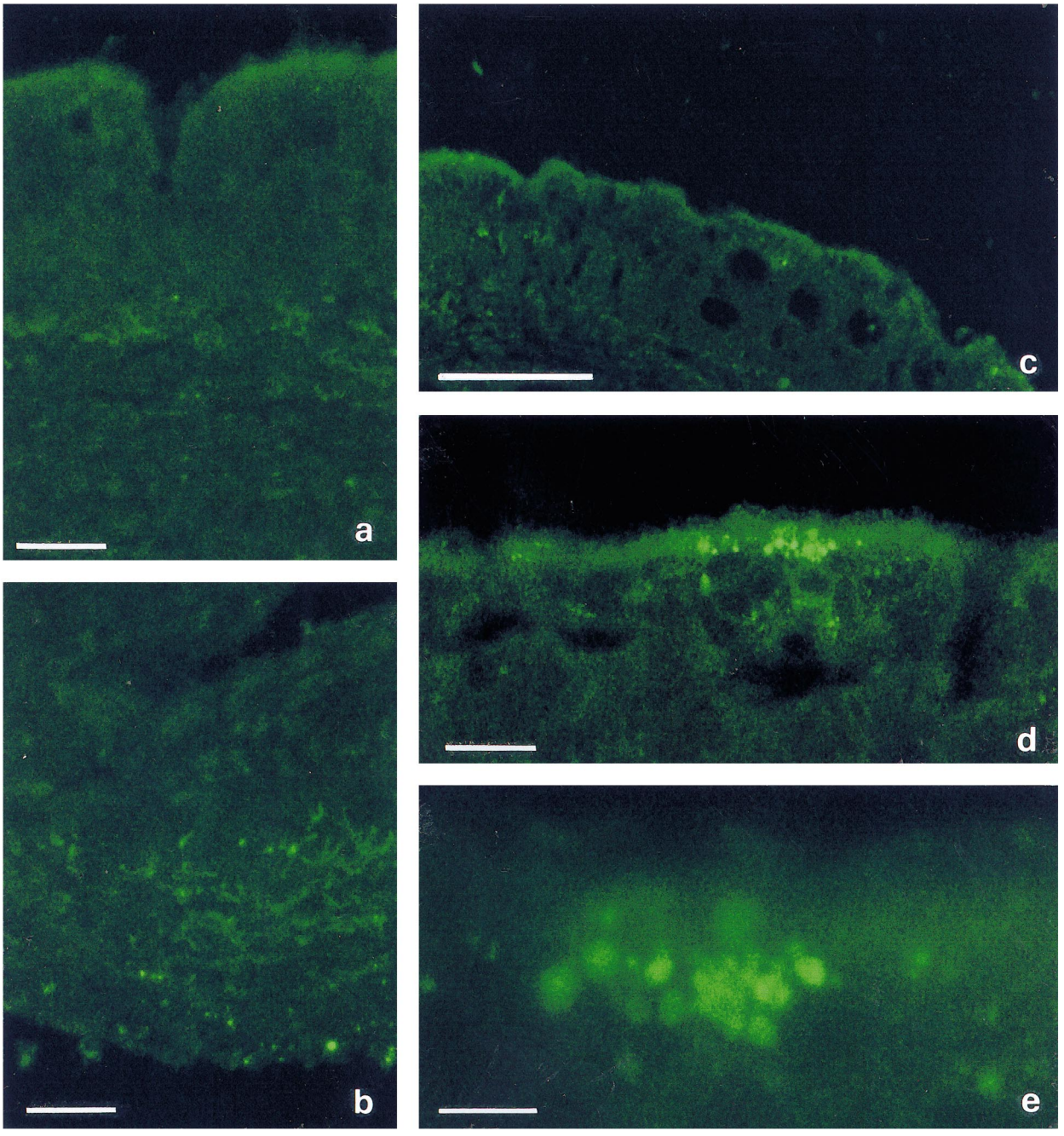

Fig. 1. Observations of the nasal mucosa (upper concha) by confocal microscopy. The tissue had previously been exposed to insulin-coated fluorescent nanospheres (diameter: 0.5 μm) on the submucosal (a,b) or mucosal (c,d) sides for 2 h. Parts of the same tissue section are shown in a and b: epithelium and subepithelial tissue are visible in a, whereas the corresponding tissue at the submucosal edge is visible in b (bar = 20 μm); the epithelium is about 60 μm high. The image of a large epithelial tract is reported in c (bar = 100 μm), showing only rare fluorescent spots with the probable exception of the left epithelial side; the epithelium is about 85 μm high. The image of a restricted epithelial region crowded with fluorescent spots has been magnified (bar = 20 μm) and reported in d (epithelium: about 45 μm thick). Some fluorescent spots of this region have further been magnified (bar = 5 μm) and reported in e.

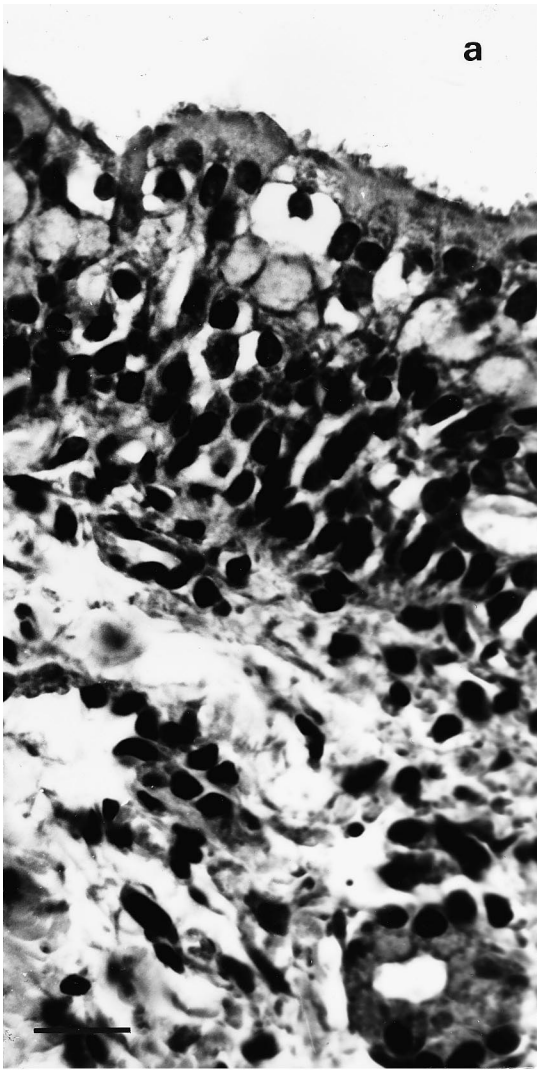

Fig. 2. Further observation of the tissue shown in Fig. 1c and d: bright-light microscopy and conventional histology. The tissue section examined by confocal microscopy has been stained with hematoxylin-eosin. The image of the central part of the epithelium, without fluorescent spots in Fig. 1c, is reported in a, with the corresponding subepithelial layer showing only rare and scattered lymphoid cells (bar = 20 μm). The image of the epithelium, crowded with fluorescent spots in Fig. 1d, is reported in b, with the subepithelial layer showing a small aggregate of lymphoid cells just beneath the epithelium and infiltrating into it at the bottom (bar = 20 μm). The epithelium is about 85 and 45 μm high, in a and b respectively.
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3.2. Conventional histology

The cross-section mainly without fluorescent spots, the confocal image of which is shown in Fig. 1c, freed from the coverslip and stained with hematoxylin-eosin, was then examined by bright-light microscopy. The central part of it, certainly without fluorescent spots, was magnified. The image obtained (Fig. 2a) was not perfectly defined in detail, due to the particular operation undergone; however, it was clear that only glands, vessels, connective fibers and scattered lymphoid cells were present in the tissue beneath the epithelium.

Conversely, when the cross-section with crowded fluorescent spots (confocal image in Fig. 1d) was freed from the coverslip and stained, the observation by bright-light microscopy showed that a small lymphoid aggregate was present just beneath the epithelium and infiltrated into it at the bottom, although the image was imperfect for the same reason as mentioned above (Fig. 2b).

The observation with conventional histology confirms that the epithelium thickness is smaller (45 μm) where fluorescent spots are contained.

3.3. Scanning electron microscopy (SEM)

After tissue exposure to beads on the apical side, a section was again found which, when observed with confocal microscopy, presented fluorescent spots in an epithelial area; the piece of tissue immediately adjacent to it was processed for observation with SEM. Fig. 3a shows the image obtained: the cross-sectioned piece of tissue is evident, with the sides of the sectioned epithelial cells visible in the foreground

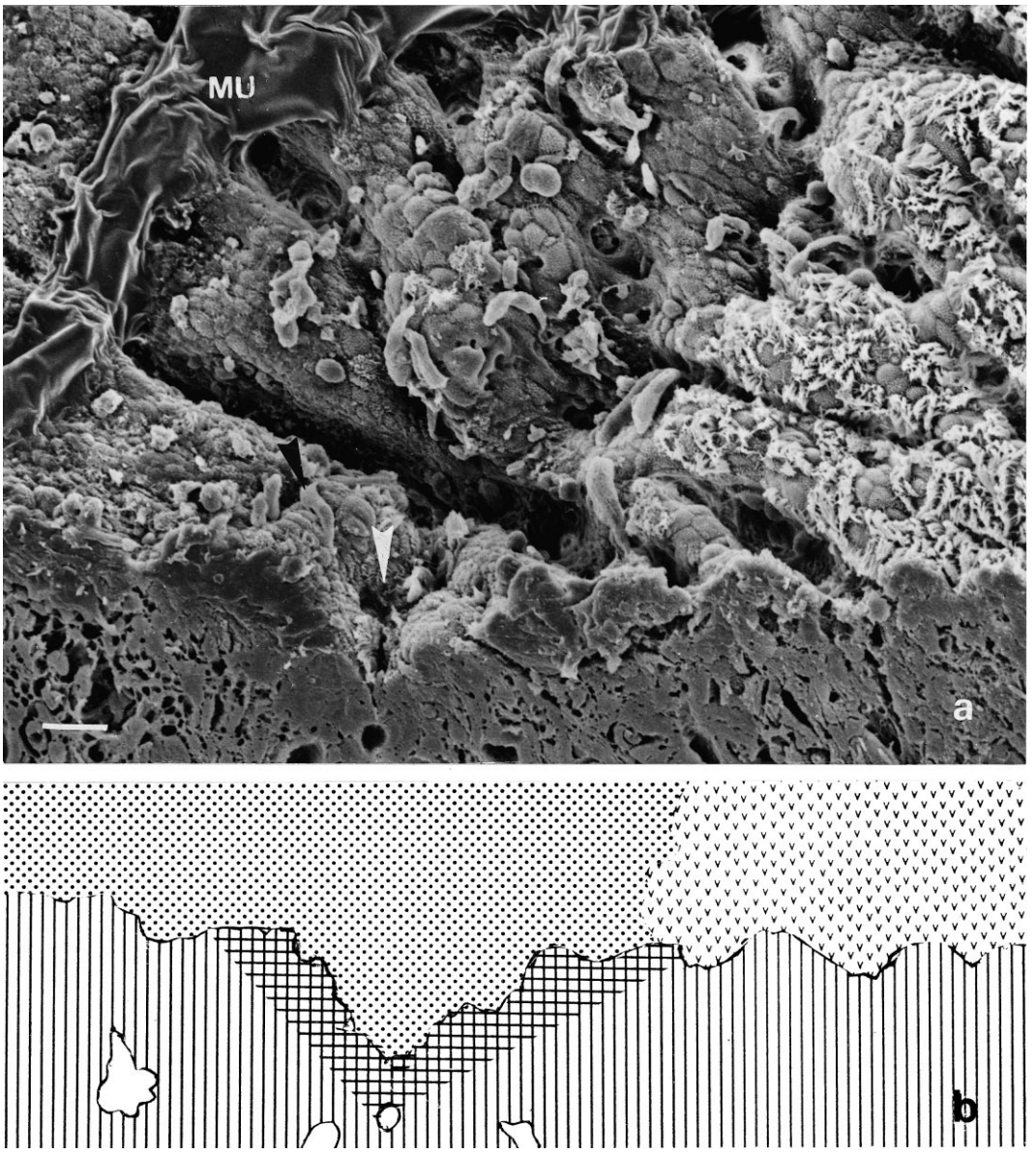
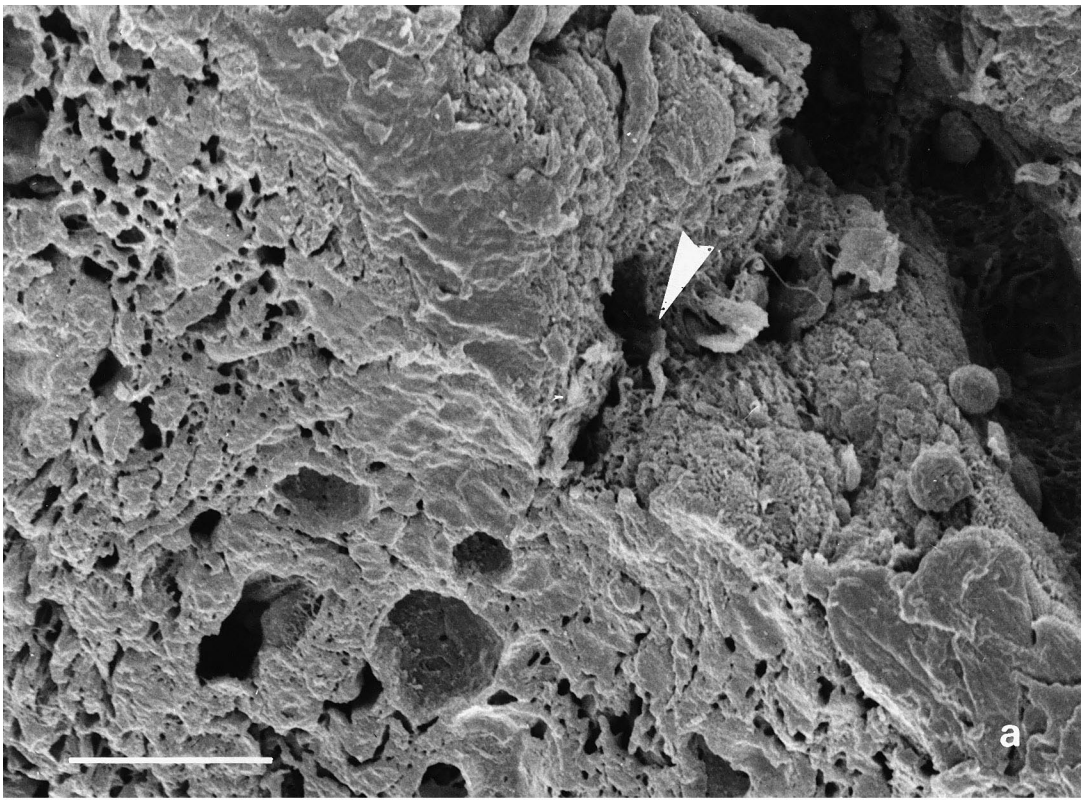


Fig. 3. Piece of tissue immediately adjacent to a section in which crowded fluorescent spots have been observed by confocal microscopy: observation with SEM. The white arrow indicates the region immediately adjacent to the area with fluorescent spots; the black arrow shows some cylindroid-like secretions of mucous goblet cells; MU indicates a coating with mucus. Bar = 20 μm. A sketch of different regions with different cells is reported in the lower part of the figure: dotted, region mainly containing microvillar cells; ticked, region mainly containing ciliated cells; vertically hatched, region showing the sides of the sectioned epithelial cells; cross-hatched, region of the sectioned cells in which crowded fluorescent spots were detected by confocal microscopy.

and the epithelial surface in the background. Many different areas can be distinguished on the epithelial surface. Some mucus formations and many mucous goblet cells, with thick cylindroid-like secretions, can be seen at the back left, whereas ciliated cells are much more numerous at the back right. The surface area in the foreground, adjacent to the section line, contains only non-ciliated microvillar cells, immediately in front of the part with fluorescent spots in the section examined by confocal microscopy. Thus microvillar cells seem to form the epithelium with fluorescent spots. Fig. 4a shows a magnified image of this area with microvillar cells.

When the area with ciliated cells, at the periphery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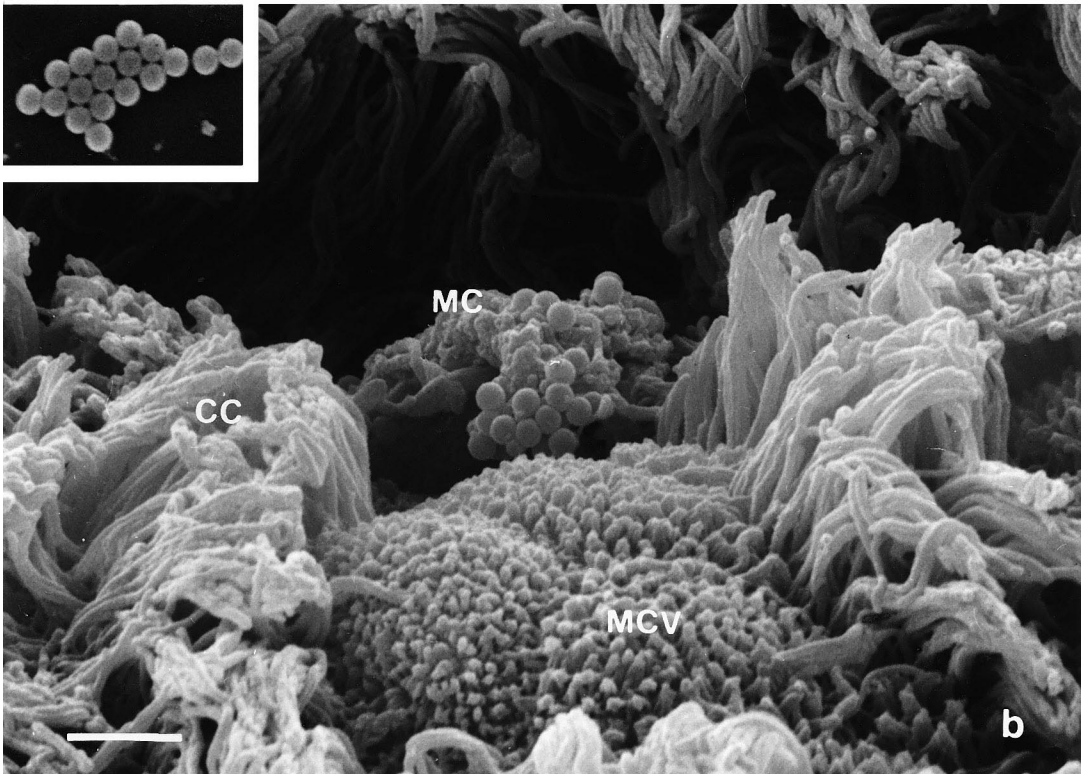
MC
CC
MCV
b

Fig. 4. (a) The region labeled by the white arrow in Fig. 3 is magnified after rotation of the piece of tissue to improve the image quality. Non-ciliated microvillar cells are clearly visible. Bar = 20 μm. (b) Microvillar cells (MCV) intermingled with ciliated cells (CC). An M-cell (MC), with microfoldings and numerous nanospheres attached, is also shown. Nanospheres are also reported in the insert for comparison (same magnification). Bar = 2 μm.
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of the area with microvillar cells, was scanned at greater magnifications, some microvillar cells scattered in small groups (4–5 cells) were observed among the ciliated cells. Fig. 4b shows one of these groups, which contains a cell with very short or microfolding-like microvilli to which many spherical bodies with a diameter of 0.5 μm are bound. In order to compare these bodies with the latex nanospheres with which the tissue had been in contact, some insulin-coated nanospheres were diluted in water, then dried and observed with SEM at the same magnification as the cell mentioned; they can be seen in the insert, which demonstrates a clear correspondence with the spherical bodies bound to the cell. Scanning of many other parts of the tissue did not detect latex nanospheres bound to other types of cells anywhere, with the exception of rare, scattered nanospheres bound to mucus cylindroids. It can reasonably be concluded that the non-ciliated microvillar cell with microfolding-like instead of finger-like microvilli and with nanospheres attached is a cell at the first stage of the transcytotic cycle, i.e. ligand binding.

4. Discussion

A positive relationship has already been observed between the extent of total polypeptide transcytosis and the total volume of lymphoid aggregates in the respiratory mucosa of the upper nasal concha and septum of the rabbit. The present paper demonstrates that transport does not extend to the entire epithelium, but is confined to limited epithelial areas lying on lymphoid aggregates. Thus, these particular epithelial areas only seem to be functionally associated with lymphoid aggregates.

Detailed studies [8,9] of the lymphoid tissue present in the nasal mucosa of the rat on the left and right sides of the septum, at the nasal entrance to the pharyngeal duct, had suggested that a nasal-associated lymphoid tissue (NALT) exists with the function of providing an immune response to the antigens, sampled by a specialized epithelium overlying the tissue, as occurs in the case of the gut- or bronchus-associated lymphoid tissue (GALT and BALT) [10–14]. The hypothesis on NALT function was based merely on histological and histochemical observations, only relating to similarities of structure, organization and cytology. In the light of the results reported in the previous and present papers [4–6] we can experimentally demonstrate a close relationship between NALT and transport using a combination of physiological and histological measurements and observations.

Whereas the majority of the respiratory epithelium is made up of ciliated cells, on the basis of the results reported here, the aggregate-associated epithelium (AAE) seems to be formed mainly by non-ciliated microvillar cells with ciliated cells at the periphery intermingled with small islets of microvillar cells binding many polypeptide-coated nanospheres; some goblet cells are also found at the periphery of the AAE. Non-ciliated microvillar cells had already been noted to cover circular lymphoid masses in the mucosal tissue of the rabbit upper concha, but no clear function had been attributed to them [15]. It is now evident that these microvillar cells, or some of them, are the cells specialized in polypeptide transport. No nanospheres were found bound to ciliated cells and only scattered nanospheres were trapped on the surface of the secreted mucus cylindroids.

In the two histological studies on the rat cited above [8,9] about 55% of the NALT-associated epithelium (lymphoepithelium) consisted of ciliated cells, while the remainder consisted of non-ciliated microvillar cells. Mucous goblet cells were almost absent. The high percentage of ciliated cells in the lymphoepithelium was ascribed by the authors to the low exposure of the animals to aeroantigens in the clean animal house, as a result of which the maturation of the antigen transporting cells was probably not optimal. This morphological analysis is in agreement with our conclusions. Moreover, the epithelial cells of the lymphoepithelium turned out to be less thick than the cells of the normal respiratory epithelium [8,9], again in accordance with our find-

ings about the smaller epithelial thickness where nanospheres are transported.

Lymphoid tissue has been observed in the bronchi, mainly at their bifurcation, below the covering epithelium, and the associated epithelium (lymphoepithelium) has been proved able to transport antigenic polypeptides; in this case too, the transporting cells were non-ciliated cells with short irregular microvilli [10]. Once again also microvillar cells scattered among ciliated cells were observed to transport polypeptides [16].

Thus along all the airways, from the nasal mucosa to the bronchi, the microvillar cells (or some microvillar cells) in the respiratory epithelium are responsible for the antigen-sampling function.

The non-ciliated microvillar cells in the rat [8,9] were present with three different forms. The first type had microvilli and bundles of filaments in the microvillus core (extending deep into the cytoplasm) as well as in the cytoplasm near the nucleus; these cells were similar to respiratory brush cells [17,18] or intestinal tuft cells [19] and could be considered immature M-cells. The second type was electron-dense, conserved finger-like microvilli but did not retain bundles of filaments in the microvillus core and in the cytoplasm; they were considered M-cells in an intermediate state of maturation. The third type of non-ciliated microvillar cell was electron-lucent like the first type, but the microvilli were reduced to an irregular outline of the apical plasma membrane and no filament bundles were conserved; these were considered mature antigen-sampling M-cells, very similar to those present in the epithelia associated with follicles of Peyer's patches [14] or bronchial lymphoid tissue [10–13].

Since we did not examine the mucosa by transmission electron microscopy, we were unable to perform detailed identification of all three cell types; however, two types of non-ciliated cells are clearly distinguishable by scanning electron microscopy, one with normally developed finger-like microvilli and the second with irregular very short pleatings of the apical membrane. It is worth noting that nanospheres are observed only bound to this latter type of cell and this provides the first experimental evidence that these are the transporting cells, i.e. actual, antigen-sampling M-cells.

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