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ORIGINAL ARTICLE

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Evidence of M cells as portals of entry for antigens in the nasopharyngeal lymphoid tissue of humans

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Abstract The nasopharyngeal tonsils (adenoids) are prominent components of human nasal-associated lymphoid tissues (NALT). However, the role of the nasopharyngeal tonsils in antigen uptake for initiation of the mucosal immune response is unknown. The aims of this study were to describe the ultrastructure and function of the M cells of the human nasopharyngeal tonsils and to clarify their capacity for antigen uptake. Tissues obtained from eight patients undergoing adenectomy were examined by light and electron microscopy. Lymphoepithelium covers the nasopharyngeal lymphoid tissue and consists of ciliary epithelium, non-ciliary epithelial cells, M cells, goblet cells, and many intraepithelial lymphoid cells. M cells have irregular and broad cytoplasm-containing microvilli on their surface and small vesicles in their cytoplasm. Many lymphoid cells were enfolded by M cells. The uptake of horseradish peroxidase (HRP) in the tissue in organ culture was studied using histochemical techniques. Excised adenoid tissue was incubated in RPMI 1640 culture media with HRP for 10, 30, and 60 min. HRP which had adhered to the surface was taken up in vesicles and then transported in vesicles and tubules by M cells. The M cells of nasopharyngeal lymphoid tissue were ultrastructurally and functionally similar to those in human Peyer's patches and colonic lymphoid follicles. These findings indicate that NALT bears similarities to the gut-associated lymphoid tissue, and its antigen uptake capacity may be important for initiation of immunity in the upper aerodigestive tract.

Key words M cell · HRP · Nasopharyngeal lymphoid tissue · Electron microscopy

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Introduction

The mucosal membranes of the gastrointestinal and respiratory tract are protected by secretory immunoglobulin (Ig)A and secretory IgM. The nasopharynx serves as a passageway of air into the lung. The nasopharyngeal tonsils (adenoids) are largest in children of kindergarten age, whereas the palatine tonsils are often large in elementary school children. The palatine tonsils, nasopharyngeal tonsils (adenoids), and lingual tonsils constitute the major part of Waldeyer's lymphoid ring in humans.

Mucosa-associated lymphoid tissue (MALT) is composed of several components: the gut-associated lymphoid tissue (GALT) which includes the Peyer's patches, solitary colorectal lymphoid nodules, the appendix, and solitary lymphoid follicles; the bronchus-associated lymphoid tissue (BALT) which includes lymphoid follicles of the bronchus; and the nasopharyngeal lymphoid tissue (NALT) which includes the tonsils and nasopharyngeal lymphoid follicles. Other components include the mammary glands, salivary glands, lacrimal glands, genitourinary organs, and inner ear.

In Peyer's patches, M cells are specialized epithelial cells with loosely spaced surface microfolds or microvilli and a thin glycocalyx [12]. Because they contain few lysosomes [13], they can transport and deliver apparently unaltered microorganisms to the extracellular pocket which is invaginated in their basolateral membranes and occupied by migrating macrophages and lymphocytes [5]. M cells have been identified at many MALT sites, but there has been little in the literature concerning the function of the M cells of the human nasopharyngeal lymphoid tissue. The early events in antigen sampling at the level of the epithelium in the human nasopharyngeal cavity are also poorly understood. Although M cells in the epithelium of the nasopharyngeal tonsils were first described by Karchev and Kabakchiev [10] in 1984, information regarding the structures and functions of M cells in human NALT is scarce in the literature. The aims of this study were to describe the ultrastructure and function of the M cells of the nasopharyngeal tonsils in hu-

mans and to clarify the possible antigen uptake role of the M cells of NALT in humans.

Materials and methods

Tissue samples were obtained from eight patients undergoing adenotomy at Kawasaki Medical School Hospital for their chronic inflammation. Tissues were divided into three parts for light and electron microscopic studies and immunohistochemical examination under a dissecting microscope.

Processing for light microscopy and electron microscopy

For light microscopy, tissues were fixed in 10% formalin in phosphate-buffered saline (PBS) at pH 7.4 and embedded in paraffin. Sections of 4-µm thickness were stained with hematoxylin and eosin. Electron microscopic samples were fixed in 2.5% glutaraldehyde at 4°C for 2 h. Transmission electron microscopic samples were fixed in 1% osmium tetroxide for 2 h, dehydrated through an ethanol series, transferred to propylene oxide, and embedded in epoxy resin. Ultrathin sections were cut with diamond knives and a Leica Reichert Ultracut S ultramicrotome. These sections were stained with uranyl acetate and lead citrate and examined in a Hitachi H-7100 electron microscope. Samples for scanning electron microscopic observation were fixed in 1% osmium tetroxide (pH 7.4) for 2 h, in 1% tannic acid overnight to improve the quality of the image, and again in 1% osmium tetroxide for 1 h before dehydration through a graded ethanol series. After drying in a critical point dryer, specimens were coated with platinum–palladium and observed with a Hitachi S-570 scanning electron microscope.

Processing for histochemistry

Specimens for histochemistry were placed in culture media (RPMI 1640; Gibco, N.Y.) and incubated in a humid atmosphere of 95% air and CO_2 at 37°C. After a 20-min incubation, horseradish peroxidase (HRP; Sigma type II, St Louis, Mo.) at a 5-mg/ml concentration was added. After incubation for 10, 30, and 60 min, all specimens were fixed in a periodate–lysine–2% paraformaldehyde solution for 6 h at 4°C, rinsed in a 0.01-M PBS series (pH 7.6) containing graded concentrations of sucrose, and embedded in Tissue-Tek O.C.T. compound (Miles Scientific, Elkhart, Ind.). They were then sectioned at 7 µm using a cryostat, mounted on poly-L-lysine-coated glass slides, and air dried at room temperature for 3 h. After the specimens were rinsed with 0.01 M PBS, they were fixed in 1% glutaraldehyde in 0.01 M PBS. Then, they were rinsed six times in 0.01 M PBS for 5 min. The samples were reacted with 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) diluted in 0.05 M Tris buffer (pH 7.6) for 30 min at room temperature and subsequently in 0.02% DAB solution containing 10 mM hydrogen peroxide and 10 mM sodium azide for 30 min at room temperature. Next, the samples were rinsed in 0.01 M PBS and then post-fixed in 2% osmium tetroxide in PBS for 1 h. They were then dehydrated in graded ethanol solutions, embedded in epoxy resin, and allowed to stand 3 days for polymerization. Ultrathin sections were cut with a Leica Reichert Ultracut S ultramicrotome, stained with lead citrate, then observed in a Hitachi H-7100 transmission electron microscope.

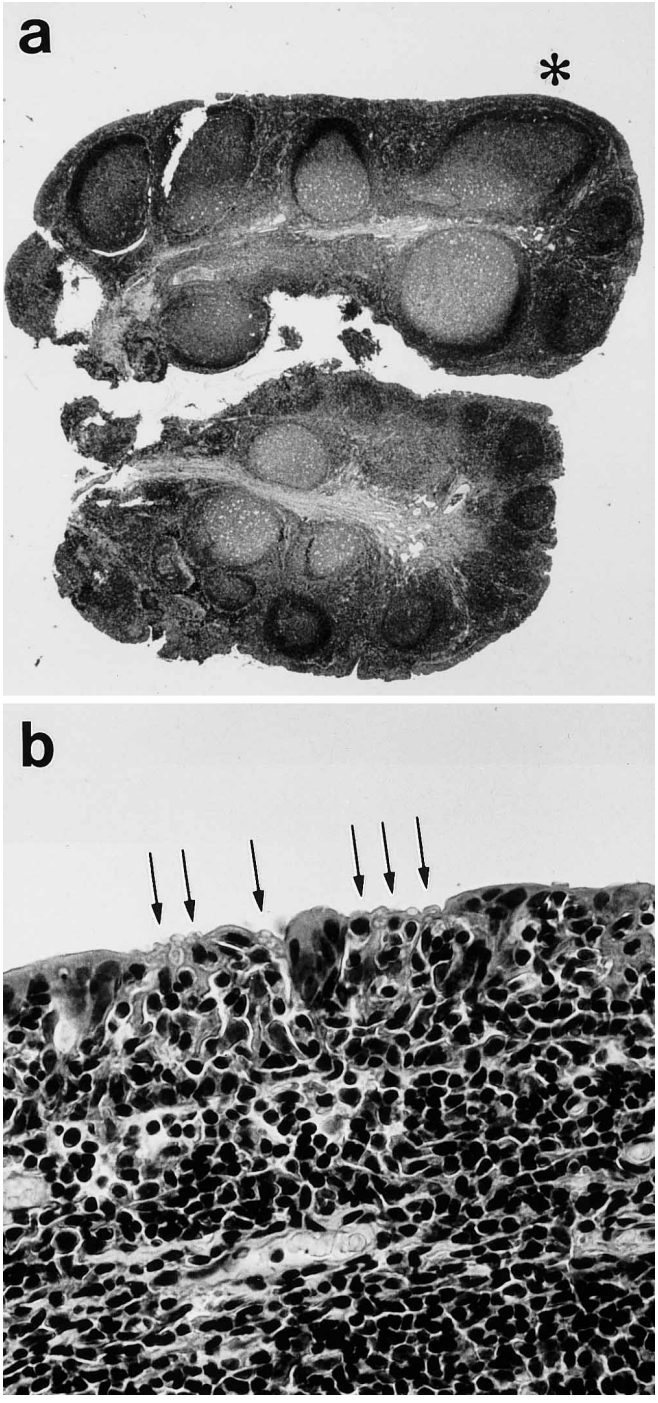

Fig. 1. a A light photomicrograph of the removed adenoid tissue (hematoxylin and eosin, ×15). The excised adenoid tissue consisted of many lymphoid follicles. **b** Enlargement of **a** (*) showing epithelium covering the lymphoid follicle. Epithelial cells associated with many lymphoid cells can be recognized (*arrows*) (hematoxylin and eosin, ×235)

Results

Dissecting microscopy, light microscopy, and electron microscopy

Light microscopy showed that the excised adenoid tissues (nasopharyngeal lymphoid tissue) consisted of many lymphoid follicles (Fig. 1a). These lymphoid follicles have distinct germinal centers in their submucosal layer and elevated surfaces facing the lumen. The covering epithelium of the lymphoid follicles was infiltrated with many migrating lymphoid cells (Fig. 1b). Dissecting microsco-

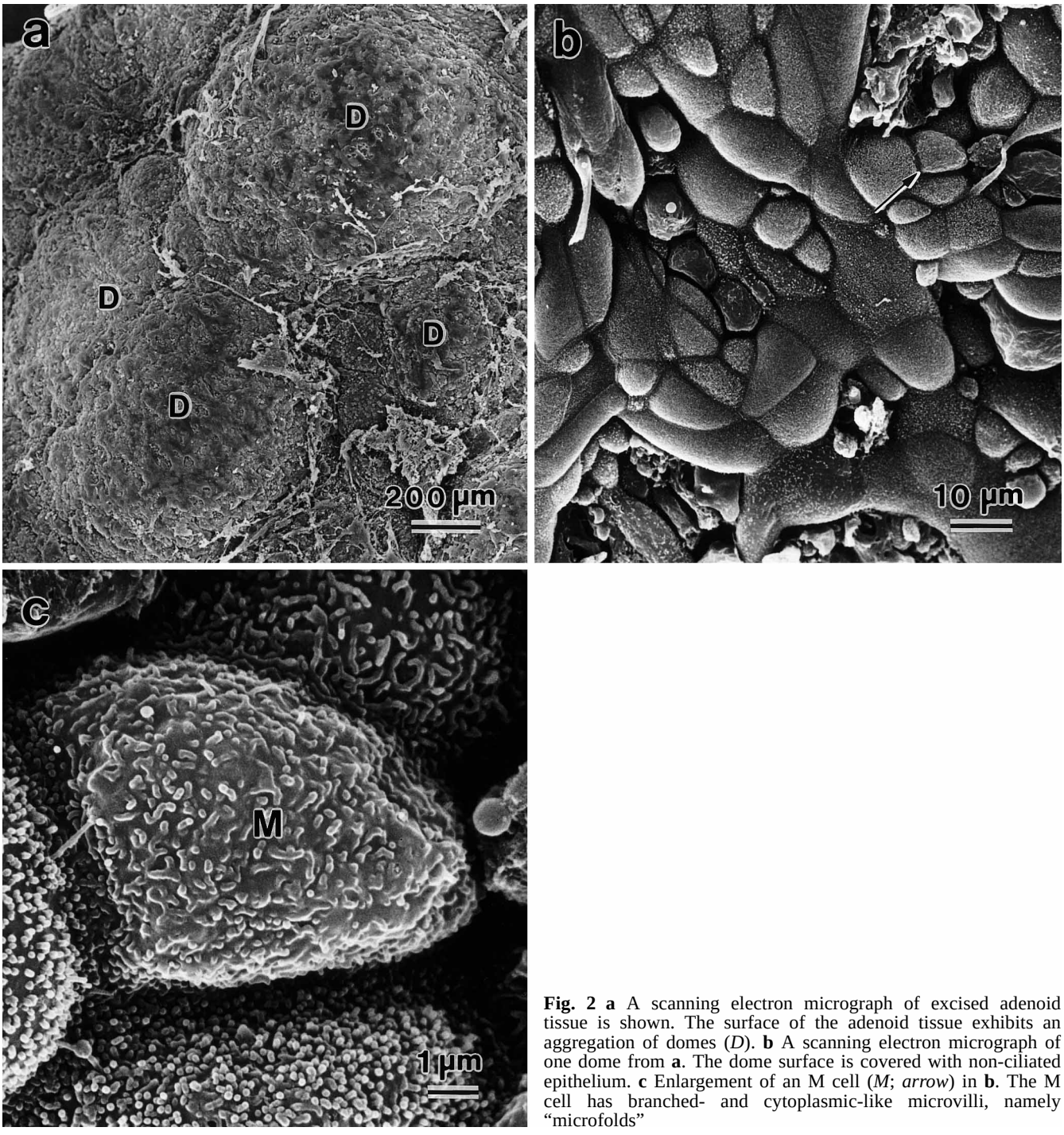

Fig. 2 a A scanning electron micrograph of excised adenoid tissue is shown. The surface of the adenoid tissue exhibits an aggregation of domes (*D*). **b** A scanning electron micrograph of one dome from **a**. The dome surface is covered with non-ciliated epithelium. **c** Enlargement of an M cell (*M*; *arrow*) in **b**. The M cell has branched- and cytoplasmic-like microvilli, namely "microfolds"

py and scanning electron microscopy (SEM) revealed an aggregation of dome-like structures on the surface of the nasopharyngeal lymphoid tissues (Fig. 2a). Most of the dome surface was covered with non-ciliated epithelium, but part was covered with ciliated epithelium (Fig. 2b). In magnified observations of the dome surface, M cells were localized near the orifice of microcrypts on the dome (Fig. 2b). M cells having branched and cytoplasm containing microvilli, namely 'microfolds', were recognized (Fig. 2c). A thin section of samples for transmission electron microscopy (TEM) showed many lymphoid cells migrating into the covering epithelium of the lymphoid follicles. TEM showed M cells in which the cytoplasm was attenuated by many enfolded lymphoid cells (Fig. 3). The microvilli were irregular and contained cytoplasm without the microfilaments seen in microvilli of adjacent cells. The surface of the M cells had cytoplasmic microvilli (so-called microfolds) and well-developed tubulovesicular systems. In addition, small vesicles were found in their cytoplasm (Fig. 4). M cells and adjacent epithelial

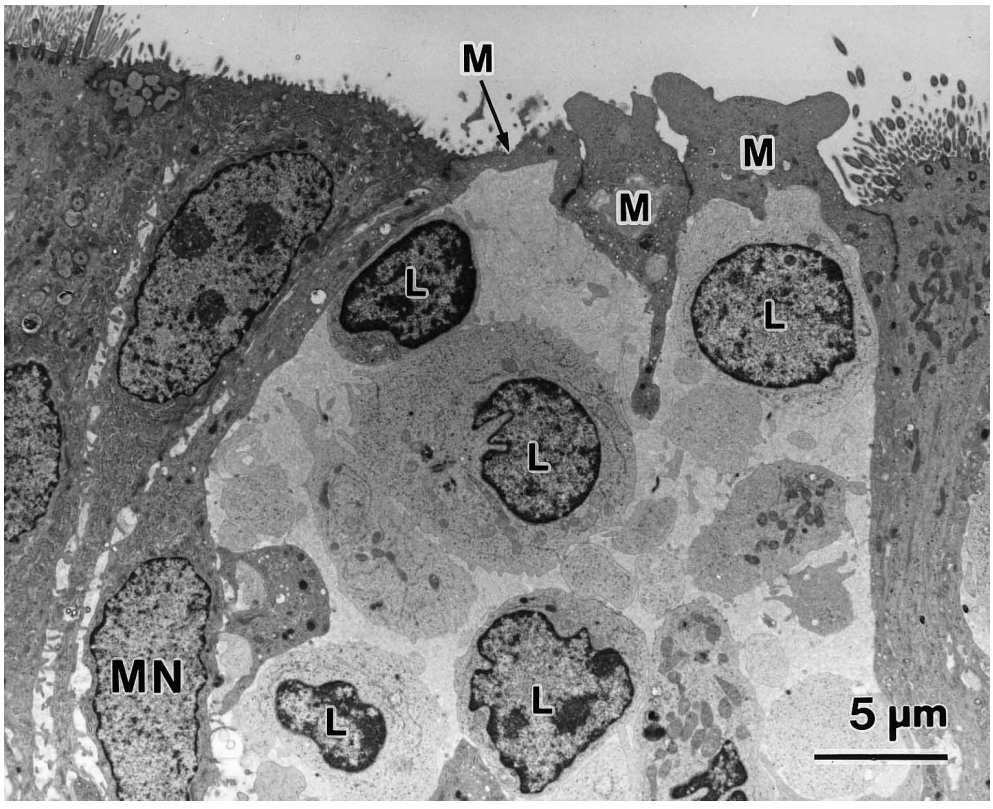

Fig. 3 A transmission electron micrograph shows M cells (*M*) that have enfolded many lymphoid cells (*L*) in the covering epithelium of the excised adenoid tissue. *MN* nucleus of M cell

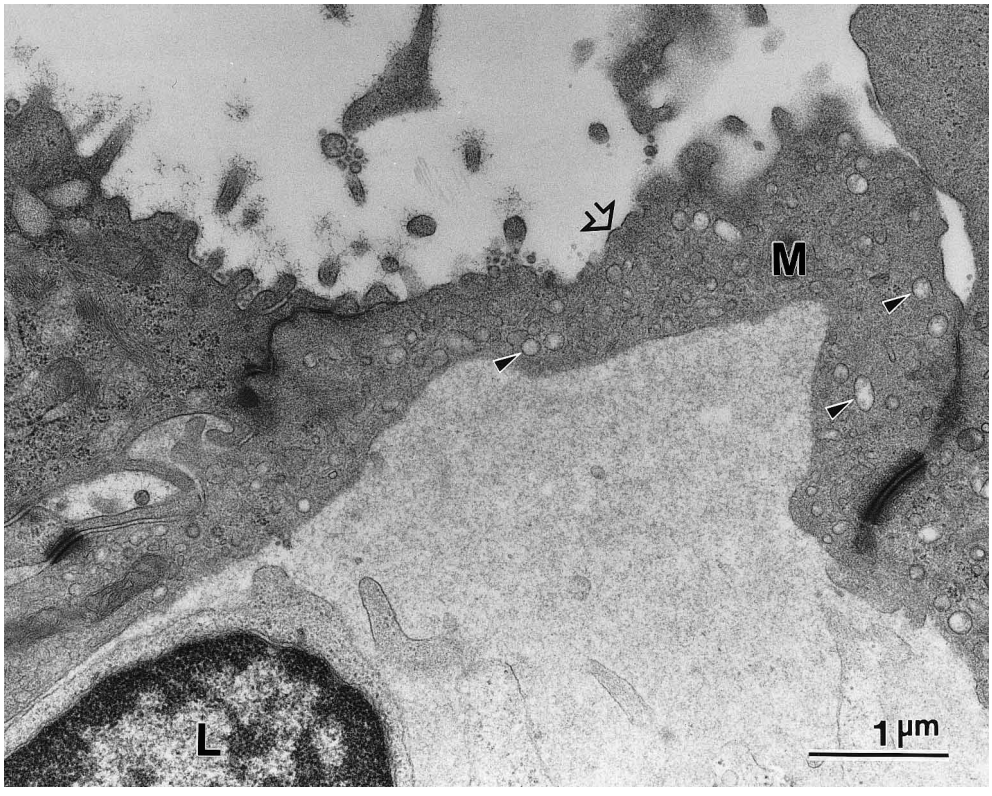

Fig. 4 An enlarged transmission electron micrograph of the apex of an M cell (*M*; *arrowed* M cell in Fig. 3). Note the cytoplasmic microvilli (*open arrow*), small vesicles (*arrows*) in the cytoplasm of the M cell and desmosomes formed with adjacent epithelial cells. *L* lymphoid cell

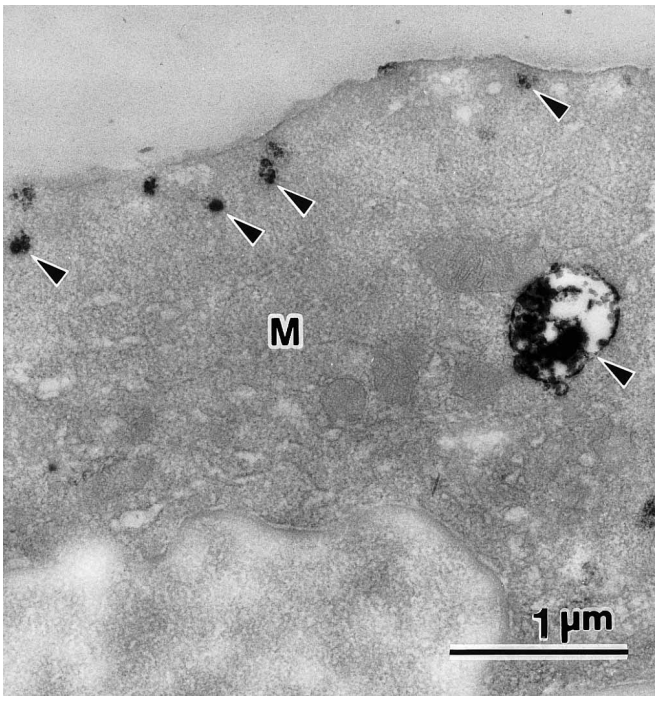

Fig. 5 After 10 min of exposure to horse radish peroxidase (HRP), a transmission electron micrograph shows HRP reaction products in the cytoplasm of an M cell (*M*; *arrowheads*)

cells made contact with each other through desmosomes and junctional complexes (Fig. 4).

Uptake of HRP by M cells

After 10 min of exposure to HRP, reaction products were observed on the surface and in small vesicles and vacuoles in the cytoplasm of M cells (Fig. 5). After 30 min and 60 min of exposure to HRP, large amounts of HRP reaction products were observed in the tubules and vesicles of the cytoplasm of the M cells, but only a small amount of HRP was observed in the cytoplasm of columnar cells (Fig. 6a, b and Fig. 7). A small amount of HRP was also observed in the cytoplasm of lymphoid cells and in the extracellular space between M cells and lymphoid cells after 60 min (Fig. 6c and Fig. 7).

Discussion

This study showed ultrastracturally that the basic structure of the M cells in the human nasopharyngeal lymphoid follicles is the same as that of M cells in the Peyer's patches [12] and colonic lymphoid nodules of humans [6] and mice [14], and that exposed HRP crosses the epithelium of the nasopharyngeal lymphoid tissue. Immunohistochemistry has revealed that M cells of the rabbit tonsils contain vimentin, which can be used as a rabbit M cell marker. Tracer experiments have shown that antigen uptake in the tonsil crypt is carried out by M cells [7]. However, the molecular mechanism of selective adherence of microorganisms to M cells is still poorly understood. In general, it is known that lectin-like adhesions on the outer membrane of various microorganisms are involved in the adherence. Recently, using confocal scanning microscopy, it was shown that M cells of the rabbit palatine tonsil bind the lectin derived from *Ulex europeus* (UEA-1) [8]. It has also been shown that the NALT M-cell lineage in hamsters is distinguished from other respiratory epithelial cells by the expression of glycoconjugates bearing terminal a(1–3)-linked galactose [9]. These features may enhance adherence and sampling of microorganisms in the initiation of mucosal immune responses. Consequently, much interest has been focused on M cells as avenues for immunization, especially using genetically engineered microorganisms as delivery agents for antigenic sequences. In human palatine tonsils, it has been reported that the incidence of M cells is lower in acute tonsillitis than in recurrent tonsillitis [15]. In patients with spondylarthropathy, the number of M cells has been seen to increase to a point where they compose up to 24% of follicle-associated epithelium in chronically inflamed mucosa [3].

Evaluation of the effect of adenotonsillectomy in humans is important for understanding of the function of NALT. In 1938, Eley et al. suggested that tonsillectomy and adenoidectomy are important predisposing factors in the pathogenesis of poliomyelitis [4]. In 1971, Ogra showed that combined tonsillectomy and adenoidectomy in children reduced the level of IgA to poliovirus three- to four-fold in nasopharyngeal secretions and delayed or abrogated the local immune response to subsequent live poliovaccine [11]. Cantani et al. observed that both salivary IgA and serum IgA were significantly reduced 4 months after combined tonsillectomy and adenoidectomy in children [2]. Furthermore, some experimental studies in rodents have demonstrated that the nasal route of antigen administration may be as effective as enteric immunization. It has also been shown that the inhalation of whole heat-killed pneumococci plus cholera toxin in saline elicits a stronger intestinal antibody response than when the antigen is administered orally or gastrically with bicarbonate to neutralize gastric acid [1]. This study showed that the surface of inflamed nasopharyngeal lymphoid tissues have M cells with the same ultrastructural and functional characteristics as M cells in Peyer's patches and colonic lymphoid follicles. Therefore, it is likely that the M cells in nasopharyngeal lymphoid follicles of the Waldeyer's ring are actually the sites of antigen uptake for induction of mucosal immunity. These findings suggest that intranasal immunization with microorganisms, such as influenza or human immunodeficiency virus (HIV) vaccine, and uptake through M cells of the nasopharyngeal lymphoid tissue might be effective for protection against these agents that are infecting the host. Since M cells are present in human nasopharyngeal lymphoid tissue and are clearly capable of transport,

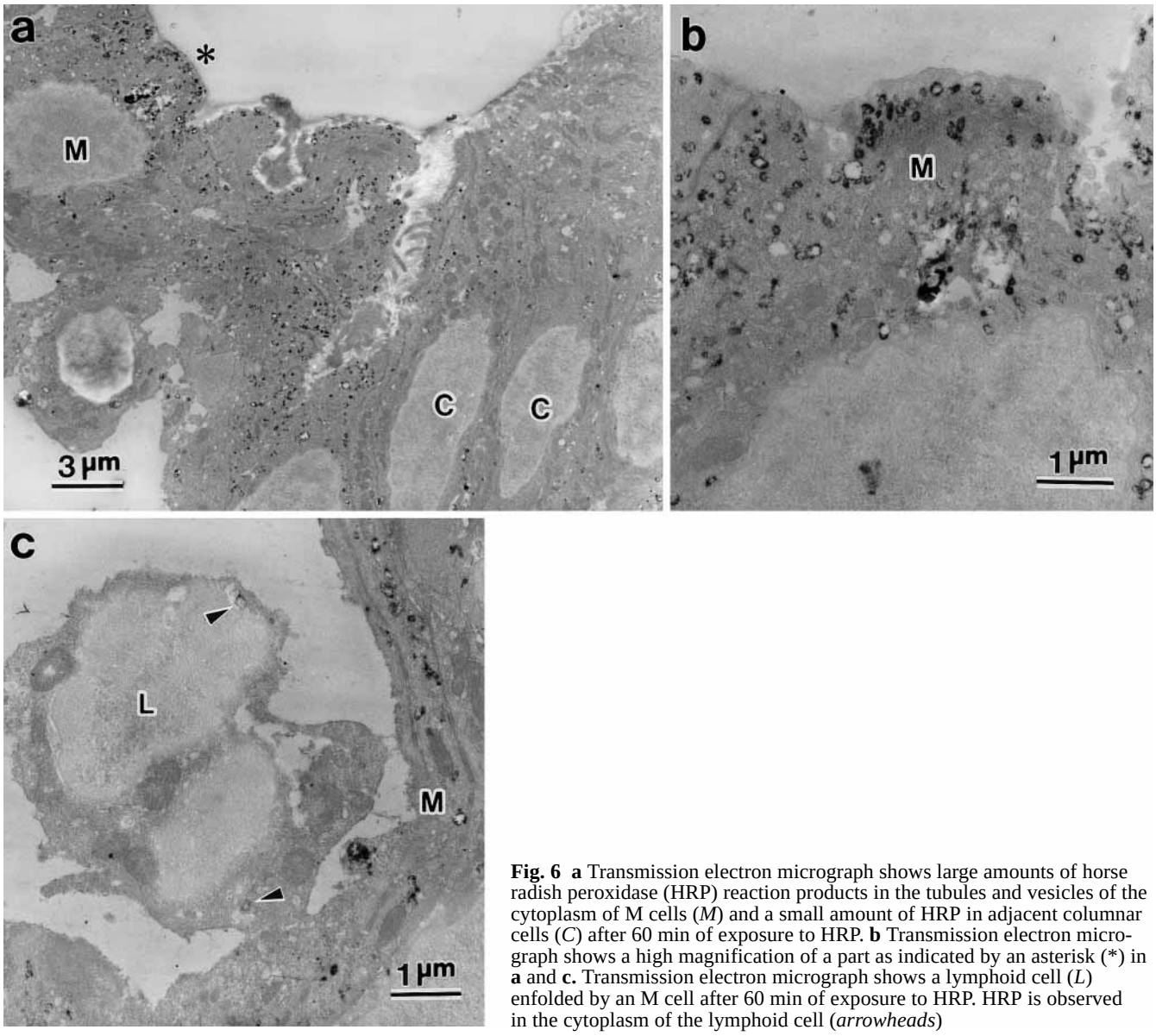

Fig. 6 **a** Transmission electron micrograph shows large amounts of horse radish peroxidase (HRP) reaction products in the tubules and vesicles of the cytoplasm of M cells (*M*) and a small amount of HRP in adjacent columnar cells (*C*) after 60 min of exposure to HRP. **b** Transmission electron micrograph shows a high magnification of a part as indicated by an asterisk (*) in **a** and **c.** Transmission electron micrograph shows a lymphoid cell (*L*) enfolded by an M cell after 60 min of exposure to HRP. HRP is observed in the cytoplasm of the lymphoid cell (*arrowheads*)

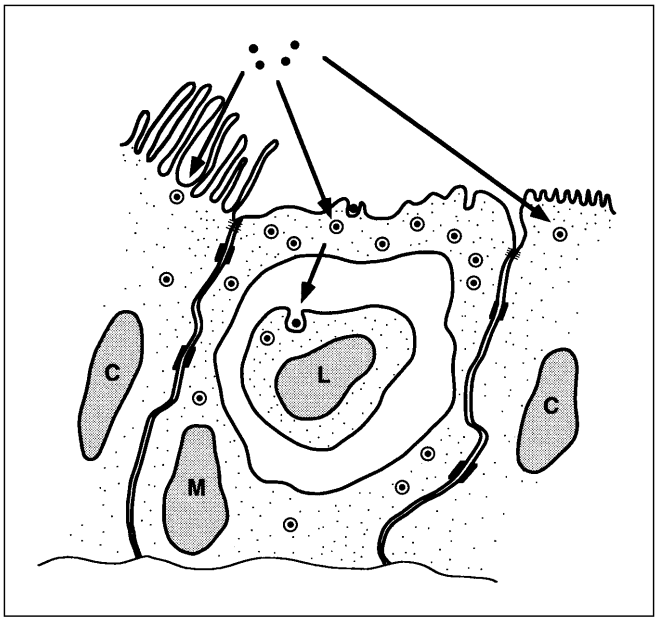

Fig. 7 Diagrammatic scheme of uptake and transport of horse radish peroxidase (HRP) by M cells (*M*) of the human nasopharyngeal lymphoid tissue. HRP are taken up by M cells after 10 min of exposure to HRP and observed in the cytoplasm of enfolded lymphoid cells (*L*) in the central hollow after 60 min. *C* columnar cell

it remains unclear why the oral route is such a low risk for inducing systemic HIV infection. The brisk uptake of HRP observed in the present study may reflect upregulation of M cell numbers and function by the chronic infection for which this nasopharyngeal lymphoid tissue was removed. Further studies are needed to elucidate whether human M cells possess surface specializations either for binding HIV or that can be exploited for drug and vaccine delivery in the future.

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