Are There M Cells in the Cecal Tonsil of Chickens?

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(Received 11 November 1991/Accepted 18 June 1992)

ABSTRACT. A unique morphological cell type, "microfold or membranous (M) cell-like cell", was detected electron-microscopically in the cecal tonsil epithelium of the chicken. M cell-like cells possessed a few short microvilli of irregular arrangement and a large number of lymphocytes and macrophages wedged into their basal surfaces. *Triticum vulgaris* was found to bind to M cell-like cells. With horseradish peroxidase (HRP) treatment, M cell-like cells showed an active HRP uptake just as did the neighbouring usual absorptive epithelial cells. No uptake of colloidal carbon particles from the intestinal lumen was recognized in any part of the intestinal epithelium. These results suggest that M cell-like cells of the chicken possess some M cell-characteristic morphological and histochemical features, but that their active uptake of foreign materials is not so developed as in mammalian M cells.—**KEY WORDS:** chicken, M cell-like cell, WGA

J. Vet. Med. Sci. 54(5): 999–1006, 1992

In the follicle-associated epithelium (FAE) of intestinal Peyer's patches, there exist specialized epithelial cells named microfold or membranous (M) cells, as first described by Owen and Jones in the human [30]. Many researchers have taken an interest in these cells [2, 8], and now M cells have been discovered in many species including mice, rats, rabbits, guinea pigs, dogs, and turtles [5, 7, 9, 10, 15, 17, 20, 21, 24, 33, 35]. M cells have been found not only in gut-associated lymphoid tissues, but also in bronchial-associated lymphoid tissues [6, 32].

M cells are characterized in morphology by small microvilli or microfolds of irregular arrangement and by a large number of lymphocytes and macrophages wedged into their basal surfaces [4, 12, 17, 30]. M cells transport many kinds of macromolecules from the intestinal and bronchial lumen to the lamina propria [7, 9, 11, 16, 19, 30, 31, 35, 39, 40]. Except for suckling animals [14, 18, 36, 38], such uptake of macromolecules has not been found in normal absorptive intestinal epithelial cells.

Studies on M cells have been carried out mainly in mammals, but very few in birds. Befus *et al.* [3] described M cells of chicken Peyer's patches but not in great detail, and Fagerland and Arp [13] reported that in the bronchial-associated lymphoid tissues of turkeys, there were no epithelial cells which showed the typical morphological characteristics of M cells. In chickens, it is considered that the FAE cells of the bursa of Fabricius (BUR) are similar to mammalian M cells [8].

In the present paper, an attempt was made to examine whether M cells would exist in the chicken

cecal tonsil (CT) and as a result, epithelial cells morphologically similar to mammalian M cells were found. These epithelial cells were termed "M cell-like cells", tentatively by their morphology for the convenience of description. The functional significance of these M cell-like cells is also discussed.

MATERIALS AND METHODS

Animals: Thirty four male and female White Leghorn chickens (GG-strain) bred in our own colonies were used. They received commercial food and tap water *ad libitum*.

Histology: For light microscopy, the duodenum and the CT were obtained from 4 chickens over 2 months of age and the BURs were obtained from 4 chickens of 1–4 weeks of age. The tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. They were cut into 3–4 μm sections, and stained with hematoxylin-eosin (H-E). For electron microscopy, the same tissues described above were obtained from 6 chickens and fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 2 hr, later postfixated in 1% osmium tetroxide at 4°C for 2 hr, dehydrated with ethanol series and then embedded in Quetol 812. Ultrathin sections were double-stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (Hitach HU-12A) at 75 KV.

Histochemistry: For light microscopy, 4 chickens were used. Three–4 μm paraffin sections of the duodenum, CT and BUR were stained with PAS reagent and with a glyco-histochemical method

Table 1. Lectins used for identification

Lectin	Abbreviation	Concentration ($\mu\text{g/ml}$)
<i>Triticum vulgaris</i> (wheat germ)	WGA	5
<i>Canavalia ensiformis</i> (jack bean)	ConA	5
<i>Pisum sativum</i>	PSA	10
Succinylated WGA	sWGA	10
<i>Bandeiraea simplicifolia</i> I	BS-I	10
<i>Ricinus communis</i> I	RCA-I	10
<i>Glycine max</i> (soybean)	SBA	5
<i>Lens culinaris</i>	LCA	5
<i>Phaseolus vulgaris</i> E	PHA-E	5
<i>Araichis hypogoea</i> (peanut)	PNA	5
<i>Dolichos biflorus</i>	DBA	10
<i>Phaseolus vulgaris</i> L	PHA-L	5
<i>Ulex europeus</i>	UEA-I	10
<i>Sophora japonica</i>	SJA	10

(ABC method) using 14 biotinylated lectins (Vector, Burlingame, U.S.A.). The lectins used in this study are shown in Table 1.

According to the results of light microscopic examination, an electron microscopic histochemical staining (pre-embedding method) using biotinylated *Triticum vulgaris* (WGA) was applied. Briefly, each CT from 6 chickens over 2 months of age was fixed in a mixture of 1% glutaraldehyde and 3% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 2 hr. The tissue block was rinsed with the same buffer and then incubated with biotinylated WGA, ABC-complex and finally diaminobenzidine (DAB)-H₂O₂. After incubation, it was post-fixed in 1% osmium tetroxide at 4°C for 1 hr, dehydrated with graded ethanols and embedded in Quetol 812. Ultrathin sections stained with lead citrate alone and non-stained sections were observed with the electron microscope as described above.

Injection of colloidal carbon and horseradish peroxidase: Ten chickens over 2 months of age were used (4 chickens for light microscopy and 6 chickens for electron microscopy). They were anesthetized by an intramuscular injection of Ketamine hydrochloride (Ketalar 50, 1 ml/kg; Sankyo Co., Tokyo, Japan). The intestinal region containing the ceca was exposed by right sub-abdominal incision, and in order to make a pouch the proximal and distal sides of the unilateral CT were ligated with silk. Ten % colloidal carbon diluted with 0.75% physiological saline for light microscopy, or 10 mg of HRP (type II, Sigma, St. Louis, U.S.A.)/ml of 0.75% physiological saline for electron microscopy were injected into the intestinal pouch with disposable needles. The non-ligated CT was not treated but left intact as

a control. The incision was closed. Ten, 30 and 60 min after the injection, the CT was removed, fixed in 10% neutral buffered formalin for light microscopy, cut into 3–4 μm paraffin sections and stained with H-E. For electron microscopy, the tissue was fixed in a mixture of 1% glutaraldehyde and 3% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 2 hr, sliced into 40 μm with a microslicer DTK-3000 (Dosaka E. M., Kyoto, Japan), incubated with DAB for 4–6 hr and DAB-H₂O₂ for 20 min, and finally embedded in Quetol 812. Ultrathin sections stained with lead citrate and non-stained sections were observed with the electron microscope as described above.

RESULTS

Histology: In light microscopy, large folded villi were recognized in the CT. The lamina propria of the villi were filled with lymphoid tissues including germinal centers; however, no polarity or division in cell arrangement was observed. The CT epithelium covering the lymphoid tissues of the lamina propria was composed of simple cuboidal or columnar type cells, and many lymphocytes were observed in it. In the chicken, the CT epithelium did not show any of the domeshaped structures as typically seen in the mammalian Peyer's patches, and seemingly, did not include M cells.

In electron microscopy, however, M cell-like cells possessing a few irregular microvilli with incomplete terminal webs were observed in the CT epithelium. These cells were combined with adjacent M cell-like or usual absorptive cells by junctional complexes. Their apical surfaces were studded with some

Fig. 1. M cell-like cells in the CT epithelium. This M cell-like cell (M) possesses a few irregular microvilli (arrowheads). Many vesicles and thin filaments (arrows) exist in the apical cytoplasm. Lymphocytes (Ly) are wedged into the basal side of the cell. E: absorptive epithelial cells, Lu: intestinal lumen, N: nucleus of M cell-like cell. $\times 6,750$.

Fig. 2. M cell-like cell (M) connecting with absorptive epithelial cells (E) by tight junctions (arrows). Luminal surface of this M cell-like cell is almost smooth. Mitochondria, thin filaments and vesicles are observed in the apical cytoplasm. Mc: macrophage. $\times 7,800$.

irregular cytoplasmic processes or were almost smooth. The apical cytoplasm contained thin filaments, free ribosomes, mitochondria, rough endoplasmic reticulum and many vesicles (Figs. 1, 2). The nuclei of M cell-like cells appeared smoothly or roughly oval in shape similar to those of normal absorptive cells. The cytoplasm of most M cell-like cells was less electron-dense than that of normal absorptive epithelium. Besides typical M cell-like cells, there were epithelial cells having intermediate features of M cell-like cells and normal absorptive epithelial cells. These intermediate cells possessed microvilli which were not so irregular but small in size. The cells were not in contact with lymphocytes and macrophages. On the other hand, almost all of M cell-like cells were in contact with lymphocytes and macrophages on their basal and lateral surfaces, but they were not invaded deeply by clusters of these immuno-competent cells.

The FAE of the BUR possessed small irregular cytoplasmic processes just as did M cell-like cells. The apical portion of the FAE cells was rich in vesicles. Numerous lymphocytes infiltrated into the FAE of the BUR.

Fig. 3. Sections of the duodenum (a), the BUR (b) and the CT (c) stained with PAS reagent. (a) Brush border of the duodenum is clearly PAS-positive. $\times 144$. (b) Luminal surface of interfollicular epithelium (I) is stained with PAS, but the reaction of FAE (F) is very weak. $\times 240$. (c) Luminal surface of the CT epithelium is almost PAS-negative. $\times 300$.

Table 2. Lectin-staining pattern of glycocalyx of the intestine and the bursa of Fabricius

	WGA	ConA	sWGA	GSL-I	RCA-I	PHA-E	PNA	PSA	SBA	LCA	DBA	PHA-L	UEA-I	SJA
DUO	2	3	1	1	3	3	2	0	0	0	0	0	0	0
CT	1	2	1	1	1	2	0	0	0	0	0	0	0	0
IFE of BUR	2	2	1	2	3	2	3	2	3	2	2	1	0	0
FAE of BUR	0	1	0	0	0	1	0	0	0	0	0	0	0	0

Numbers show grade of reaction intensity. 0: negative, 1: weak reaction, 2: medium reaction, 3: strong reaction. Abbreviation refer in the text and Table 1.

Histochemistry:

1) PAS reaction: In the duodenum, highly PAS-positive brush borders were clearly observed (Fig. 3a). In the BUR, the surface of the interfollicular epithelium was PAS-positive, but in contrast, the FAE was weakly positive or negative (Fig. 3b). In the CT, the epithelium was also weakly positive or negative (Fig. 3c), showing little glycocalyx as did the FAE of the BUR.

2) Lectin histochemistry: The results of lectin histochemistry are summarized in Table 2. In the duodenum, brush borders of the epithelium reacted with many lectins and the intensity of reaction tended to decrease from the top of villi to the crypt. In the BUR, the interfollicular epithelium reacted with many lectins except UEA-I and SJA, whereas the FAE reacted very weakly with a few lectins (Fig. 4). The CT epithelium reacted also weakly. WGA was demonstrated exclusively on the surface of a few CT epithelial cells (Fig. 5a). In electron microscopic histochemistry, these WGA-positive cells corres-

Fig. 4. Section of the BUR reacted with WGA. Luminal surface of FAE (F) is WGA-negative, whereas that of interfollicular epithelium (I) is strongly positive. $\times 240$.

Fig. 5. Sections of the CT reacted with WGA. (a) WGA-positive reactions are scatteringly seen on the surface of the CT epithelium (arrows). $\times 375$. (b) Luminal surface of M cell-like cells (M) react with WGA. The surface of normal enterocytes (E) is WGA-negative (non-stain). $\times 3,600$.

Fig. 6. The CT epithelium of HRP-treated chicken. HRP exists in intercellular spaces of CT epithelium. Release of HRP from cytoplasmic vesicles to intercellular spaces can also be seen (arrow). E: absorptive epithelial cells, M: M cell-like cells, Lu-intestinal lumen. $\times 3,400$.

ponded to M cell-like cells (Fig. 5b). Usually, no WGA reaction was detected on the surrounding epithelial cells provided with compact microvilli.

Injection of foreign materials: With the intestinal intraluminal injection of HRP, reaction products were found in the intercellular spaces and in the apical vesicles of the CT epithelial cells including M cell-like cells. Some features of HRP release from

the cytoplasmic vesicles into the intercellular spaces were also observed (Figs. 6, 7). Although M cell-like cells showed an active HRP uptake, the activity seemed to be not different from that of the neighbouring absorptive cells.

When colloidal carbon particles were introduced into the CT lumen, they were recognized neither in M cell-like cells nor in absorptive cells.

Fig. 7. The CT epithelium of HRP-treated chicken. HRP uptake by a M cell-like cell is observed in vesicles of cytoplasm (arrowheads). Apical cytoplasmic membrane is also HRP-positive (non-stain). $\times 22,500$.

DISCUSSION

The foregoing observations revealed that in the CT of chickens, there existed peculiar epithelial cells morphologically similar to but functionally different from mammalian M cells. Mammalian M cells represented by human and mice, are provided with (1) short irregular microvilli, (2) incomplete terminal webs containing many vesicles and mitochondria, (3) lightly stained and deeply indented cytoplasm by aggregates of lymphocytes and macrophages [9, 29]. No conspicuous difference can be seen between species except ruminants. M cells of ruminants show a peculiar distribution in Peyer's patches in such a way that they cover almost all area of the surface of Peyer's patches [21]. In the present study, M cell-like cells of chickens possessed a few short cytoplasmic processes of irregular arrangement on their luminal surfaces, and vesicles in their apical cytoplasm as did mammalian M cells, but they did not contain mitochondria in the area of incomplete terminal webs. Lymphocytes and macrophages were in contact with M cell-like cells along the basal surface, but unlike in mammals, few of these immuno-competent cells were clustered and deeply wedged into the cytoplasm of M cell-like cells. In mammals, there are reports of lymphocyte migration into M cells [4, 17]. Some researchers have conceived that the characteristic morphology of M

cells is due to the sub-cellular migration of these lymphocytes [34, 35]. Others, however, have argued against this concept [12].

In the present study, some M cell-like cells showed no fundamental morphological differences from usual absorptive epithelial cells, except the presence of relatively shorter microvilli, seemingly to be in a process of transformation from absorptive cells to M cell-like cells. As to the origin of mammalian M cells, there are two hypotheses, differentiation from crypt cells [9] and that from neighbouring absorptive cells [27, 35]. Recently, another hypothesis has been reported that M cell-equivalent FAE of the BUR originates from the mesenchyme [23]. The morphological features of the FAE of the BUR observed in the present study were well in agreement with those reported by Bockman and Cooper [8] who compared the fine structure and micropinocytotic capability of FAE cells in the chicken BUR, rabbit appendix and mouse Peyer's patch [8].

It has been reported that mammalian M cells take up India ink [8, 33], HRP [27], ferritin [8, 9, 26], bovine serum albumin [26], IgA [37], some types of toxin [25] and latex particles [22]. Furthermore, in chickens, the uptake of ferritin by the FAE of the BUR has also been reported [8]. In the present study, HRP uptake by M cell-like cells was observed throughout the CT epithelium though not specific. Thus, such undeveloped uptake function of chicken M cell-like cells is the reason why these cells are termed not "M cells" but "M cell-like cells" in the present paper.

Some bacteria and viruses [16, 31, 39, 40] are also transported by M cells from the lumen to the lymphoid tissues in the lamina propria. Since the lysosomal system of M cells is undeveloped [28], these microorganisms are saved from lysosomal digestion and come in contact with immunocompetent cells without losing the antigenicity. From a medical viewpoint, oral vaccination may be possible on the basis of these characteristics of M cells. M cells also may serve as the preferential portal for pathogenic micro-organisms. Furthermore, M cells often ingest intestinal bacteria in non-treated animals, and this situation is considered to represent a natural tolerance to a bacterial antigen and a maintenance of intestinal microbial flora [7, 24]. In the present study, uptake of intestinal bacteria by M cell-like cells was not clarified.

The present results of PAS staining and lectin

histochemistry revealed that the CT epithelium and the FAE of the BUR have little glycocalyx as compared with the duodenal epithelium. Reactions of glycocalyx for lectin histochemistry in the duodenum were almost in line with the results reported by Alroy *et al.* [1]. It should be noted that M cell-like cells in the CT epithelium showed a specific reaction for WGA. Neutra *et al.* [26] have reported that *in vivo* or in culture, WGA was bound to a surface receptor of M cells to be taken up. M cell-like cells of chickens may also have such a receptor for WGA, indicating the presence of β -(1, 4)-N-acetylglucosamine. Although it is not clear what kind of function the WGA positive reaction reflects, WGA may at least become a useful marker for M cell-like cells in the CT epithelium of chickens. As some CT epithelial cells with relatively compact microvilli were also stained with WGA, it is suggested that these cells are transforming from normal absorptive cells to M cell-like cells.

It has been known that mammalian M cells are lacking in alkaline phosphatase (ALP) activity [15, 29]. Though unpublished data, our immunohistochemical studies using anti-ALP serum showed that brush borders of numerous absorptive cells of the CT and FAE of the BUR were positive, but that some of epithelial cells of the CT were completely negative. Considering their incidence, ALP-negative cells of the CT seemed corresponding to M cell-like cells, suggesting that M cell-like cells have histochemical features for ALP similar to those in mammalian M cells. In order to test this suggestion, some series of electron microscopic ALP-histochemical observation must be carried out.

From these results, it is difficult to conclude that M cell-like cells in chickens are specialized enough for uptake function, and that they have an important role in the intestinal immune system as do mammalian M cells. Judging from the WGA-reaction and HRP treatment, M cell-like cells of chickens are also considered to belong to a cell type comparatively different from the FAE cells of the BUR.

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