Uptake of BCG into Immunized and Nonimmunized Rabbit Peyer's Patches
—— Morphology and Quantitative Analysis ——

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ABSTRACT. In an attempt to compare the uptake and transport of BCG by M cells between percutaneous immunized and nonimmunized rabbits, we administered BCG suspension into ligated ileal loops which containing a Peyer's patch in two groups of rabbits. Fluorescent microscopic and electron microscopic observations revealed that BCG was actively phagocytized by the membranous cells (M cells) in the Peyer's patches and transported to intraepithelial macrophages in adult rabbits despite percutaneously immunization. A quantitative analysis was made by using the quadrat method of spatial pattern analysis of fluorescent photomicrographs. As a result, we confirmed the fact that the uptake and transport of BCG were more enhanced in percutaneously immunized rabbits than nonimmunized rabbits.

Key words: BCG — M cells — Peyer's patches — immunized rabbits — phagocytosis

The covering epithelium of Peyer's patches consists of absorptive cells, goblet cells which are less abundant than the neighboring villi and a unique kind of epithelia known as membranous[1] or microfold cells (M cells).[2] Recently, it has been clarified that the M cells have special microvilli[1,3] which were cytoplasm-like processes on the surface and that they play an important role in "initiation" of the intestinal immune response such as endocytosis of macromolecule antigens[4-7] and microorganisms[8-15] in the intestinal lumen.

In 1986, the author[10] have clarified that BCG (Bacilli Calmette-Guérin) administered into ligated ileal loop of nonimmunized rabbits specifically adhered to M cells and were phagocytized by the M cells and transported to intraepithelial macrophages.

At present, the factors regulating phagocytosis and transport of the M cells are still unknown, though M cells are the major entrance for the uptake of antigen in the intestinal lumen. Moreover, quantitative analyses have not yet to be made on the uptake of antigens through the M cells under parenterally immunized conditions. In the present study, the authors made morphological observations and analyses of the quantitative kinetics of uptake and transport of BCG administered to the intestinal lumen using Peyer's patches in the terminal ileum of both percutaneously immunized and nonimmunized rabbits.

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MATERIALS AND METHODS

Preparation of BCG

Dried BCG vaccine (Japan BCG laboratory) was cultured in 1% Ogawa medium for 3-4 weeks. Then the suspension of the bacteria was centrifuged and rinsed 3 times with physiological saline and then suspended in 30 ml of physiological saline. The suspension contained $8\text{-}25\times10^8$ viable bacilli/ml.

Experimental animals

Eighteen male New Zealand White rabbits, 8 weeks of age and weighing 2.0-2.5 kg were used. The tuberculin skin tests (PPD 30 μg) were performed on the tips of both earflaps. After confirming the reactions to be negative, the animals were then divided into two groups, namely, the immunized group and nonimmunized group. Each group was again divided into 3 subgroups of 2, 4 and 8-hour administration groups, each consisting of 3 rabbits. For immunization, a dose of a mixture of 20 mg of BCG and 2 ml of Freund's incomplete adjuvant was administered 4 times by subcutaneous injection to the outer thigh. On the 40th day after the commencement of immunization, the tuberculin test's change to positive was confirmed. At that time, the serum antibody levels rose from 8 to 2084-fold in the agglutination test.

Preparation of intestinal loops[10]

Both the immunized and nonimmunized groups were anesthetized intravenously with pentobarbital (0.4 ml/kg) and laparotomy was performed. The Peyer's patches in the terminal ileum were macroscopically found on the serosa side. Ileal loops of 3-5 cm in length containing Peyer's patches were ligated. The blood supply to the loop was kept intact. BCG (2.0-2.5 ml) suspension was injected into the ligated ileal loop and their abdominal walls were sutured. After 2, 4 and 8 hours administration, the ligated loops were excised and prepared for fluorescent and electron microscopic observations.

Electron microscopic observation

The luminal surface of samples was rinsed with physiological saline and the mucosa was examined under a dissecting microscope. The specimens were prepared from the lymphoid follicles of the Peyer's patches being cut in 1 mm cubes. Each specimen was fixed in 2.5% glutaraldehyde for 2 hours and in 1% osmium for 2 hours, and then dehydrated with graded ethanols. For transmission electron microscopic observation, the specimens were transferred to propylene oxide and embedded in epoxy resin. Ultra-thin sections were prepared with a glass knife using a Porter-Blum MT 2-B type microtome. They were then double-stained with uranium acetate and lead citrate and observed under a HITACHI H-500 transmission electron microscope. For observation by scanning electron microscope, the specimens were transferred to isoamyl acetate, dried to the critical point, and coated with gold-palladium, and observed with a HITACHI S-570 scanning electron microscope.

Tissue processing for fluorescence and quantitative analysis

The specimens for fluorescent microscopic observation were rinsed with physiological saline on the luminal surface. They were fixed with 10% buffered formalin, dehydrated and then embedded in paraffin. Nonserial paraffin sections were cut 6 μm in thickness with every other slice being examined. The sections were double-stained with Rhodamine B-auramine and observed under

a Nikon (incident-light) fluorescent microscope. Three lymphoid follicles were randomly selected from the Peyer's patches of each animal. The follicles were observed (×200) centering on the dome epithelium of the follicle, then colored photomicrographs (×500) were prepared. The size of the lymphoid follicle domes were 200–450 μm. Eight to fifteen photomicrographs were selected at random for each animal. Fluorescing a bright yellow, the BCG located in the lymphoid follicles were easily discernible. BCG locations in the photomicrographs were plotted on tracing paper. Dividing the mucosal layer into 4 layers by the covering epithelial thickness on the tracing paper, restricted areas from each layer were defined by dividing the thickness with a curve parallel to the margin and to the inner part of the lymphoid follicle. The area of each restricted area was measured by a Nikon image analyzer (COSMOZONE 98-1S) to determine the number of BCG per unit area. The BCG taken up and transported were distributed within restricted areas. Subsequently, the spatial distribution pattern of BCG in the epithelium and inner layers was analyzed by the quadrat method. Thus, BCG uptake in the immunized group vs. the nonimmunized group, namely, the spatial distribution of BCG was compared.

RESULTS

Electron microscopic observation

The dome epithelium in the Peyer's patches was observed by scanning electron microscope 2 hours after BCG administration. Even in the immunized group, many bacilli specifically adhered to the M cells and were captured by the microfolds of the M cells (Fig. 1). The scanning electron microscope revealed no apparent difference in BCG adherence to M cells between the immunized and nonimmunized group 2 and 4 hours after administration. Transmission electron microscopic observation revealed vigorous capture of the bacilli by the microfolds in the 4-hour immunized group (Fig. 2). In transmission electron micrographs of the 4-hour immunized group, the bacilli were found in abundance in the intraepithelial macrophages enfolded within M cells and the phagosomes of the macrophages immediately beneath the dome epithelium. The observations of many other sections of immunized groups revealed that uptake of BCG occurred only in the M cells of the dome epithelium, but never in the neighboring absorptive epithelial cells nor goblet cells. Moreover, it was found that BCG, first phagocytized by M cells, had then been transported into intraepithelial macrophages.

Spatial distribution of fluorescent BCG and kinetics of uptake

According to fluorescent microscopic observation, BCG uptake in both the immunized and nonimmunized groups was found in the lymphoid follicles but not within the adjacent villi. A fluorescent photomicrograph of the lymphoid follicles and BCG distribution which plotted of three cases in the immunized group (Fig. 3a,b) and the nonimmunized group (Fig. 3c,d) of 8 hours after administration is shown. The mean BCG count ($N/\mu m^2 \times 10^5$) per unit area in each layer of the immunized and nonimmunized group is presented in Table 1 and shown in graph form in Fig. 4.

The BCG count in each layer in the immunized and nonimmunized group: 2 hours after administration, in the 1st layer (dome epithelium), where M cells

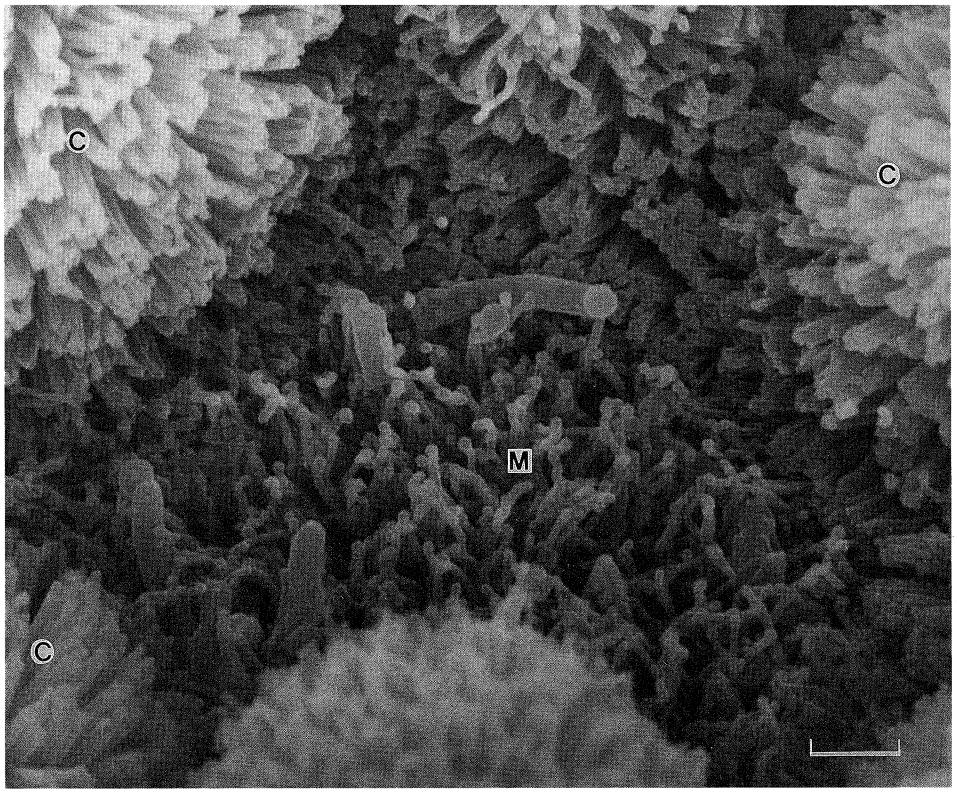

Fig. 1. Scanning electron micrograph 2 hours after BCG administration in the immunized rabbit. Many bacteria specifically adhered to an M cell (M) and were captured by the microfolds of an M cell. Columnar cell (C). Bar 1 μm.

TABLE 1. The mean BCG count ($N/\mu m^2 \times 10^5$) per unit area in each layer of the immunized and nonimmunized group.

Layer \ Time		After 2 hours	4 hours	8 hours
1st	N	45.3±30.8 (n=42)	64.7± 35.5 (n=33)	30.4± 20.5 (n=41)
	Im	63.8±52.5 (n=45)	164.6± 82.7 (n=25)	83.1± 61.9 (n=40)
2nd	N	41.2±28.7 (n=42)	68.6± 46.3 (n=33)	96.4± 52.5 (n=43)
	Im	71.6±62.5 (n=45)	292.3±114.0 (n=25)	310.6±204.7 (n=40)
3rd	N	15.6±16.8 (n=42)	33.3± 24.7 (n=30)	54.0± 41.2 (n=42)
	Im	26.6±39.7 (n=44)	197.1± 96.6 (n=25)	280.0±183.5 (n=40)
4th	N	9.8±11.8 (n=31)	34.7± 23.4 (n=21)	49.3± 48.8 (n=33)
	Im	14.0±28.0 (n=37)	173.3± 74.1 (n=15)	158.6±136.8 (n=39)

N : Non-immunized rabbits
Im : Immunized rabbits

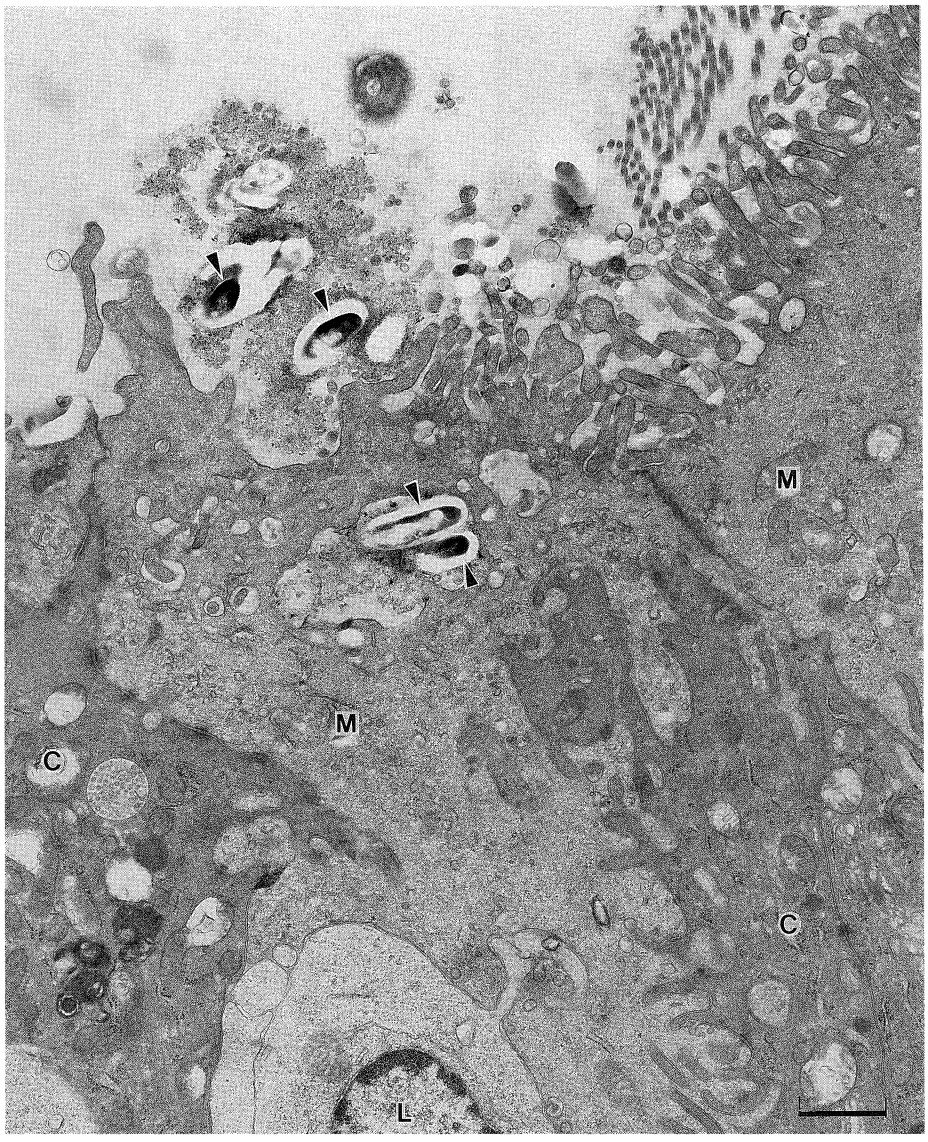

Fig. 2. Transmission electron micrograph of an M cell (M) 4 hours after administration of immunized group. Many bacteria (arrows) were captured and phagocytized by an M cell. Columnar cell (C). Lymphocyte (L). Bar 1 μm.

are located, the mean count was 45.3 for the nonimmunized group and 63.8 for the immunized group; 4 hours after administration, the count was 64.7 and 164.6; 8 hours after administration, the count was 30.4 and 83.1, respectively. Thus the count was consistently higher in the immunized group. In the same fashion, the count in the immunized group was higher in the 2nd to 4th layer. The results examined by the unmatched pair test of Wilcoxon is shown in Table 2.

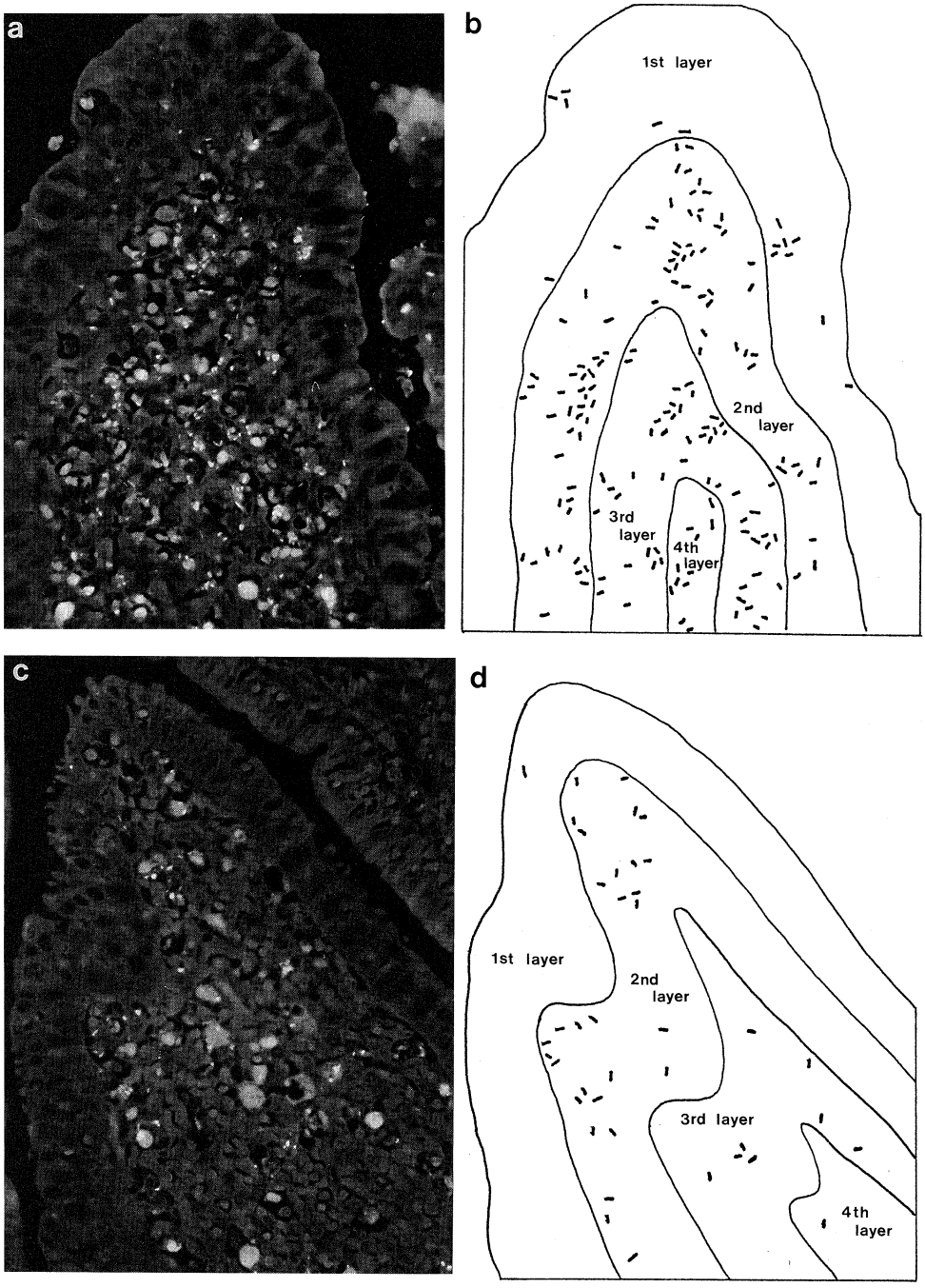

Fig. 3. a. A fluorescent photomicrograph shows a lymphoid follicle in the immunized group of 8 hours after administration. Many fluorescent bacteria are recognized in the lymphoid follicle. (Rhodamine B-auramine double staining. ×500)
b. A drawing schema shows BCG distribution which plotted in Fig. 3a.
c. A fluorescent photomicrograph shows a lymphoid follicle in the nonimmunized group of 8 hours after administration. (Rhodamine B-auramine double staining. ×500)
d. A drawing schema shows BCG distribution which plotted in Fig. 3c.

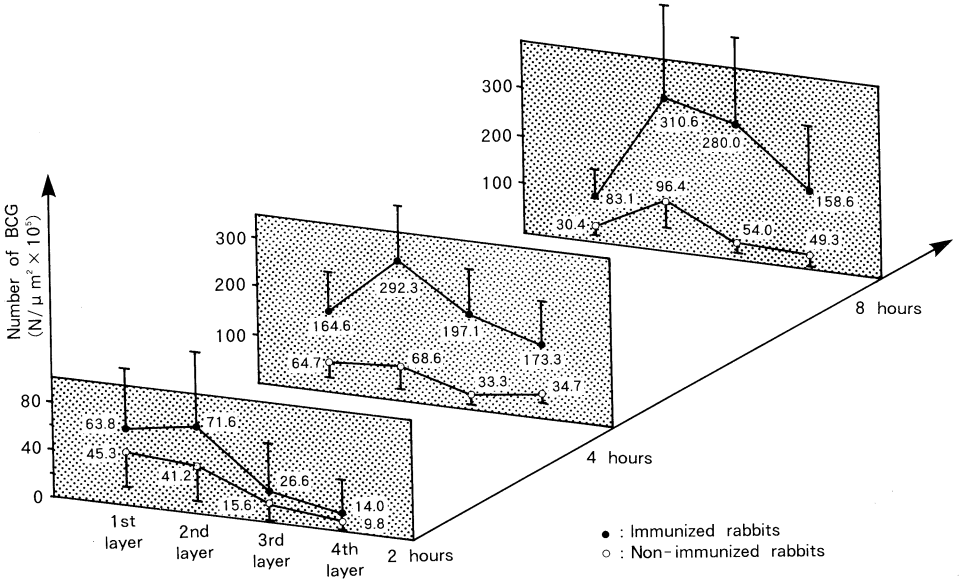

Fig. 4. The mean BCG count ($N/\mu m^2 \times 10^5$) per unit area in each layer of the immunized and nonimmunized group in graph form.

TABLE 2. The results of Table 1 examined by the unmatched pair test of Wilcoxon.

Time / Layer	After 2 hours	4 hours	8 hours
1st	NS	**	**
2nd	*	**	**
3rd	NS	**	**
4th	NS	**	**

** : $P<0.01$ * : $0.01<P<0.05$

At 2 hours after administration, there was no significant difference between the groups. However, at 4 and 8 hours after administration, a clearly significant difference of $P<0.01$ was observed, not only in the 1st layer but in other layers for the immunized group. This indicated that the BCG uptake was markedly greater in the immunized group than in the nonimmunized group. 80% of BCG microbes were found in the 2nd to 4th layers, namely the parafollicular area, at 4 hours after administration in the immunized groups. However, the ratio was lower (68%) in the nonimmunized groups.

DISCUSSION

There have been few quantitative models of uptake of substances by the covering epithelium of the Peyer's patches. In 1983, Keljo & Hamilton[16]

clarified that the epithelium of the Peyer's patches transport more soluble antigen than do the neighboring villi. In 1989, Pappo & Ermak[17] for the first time, suggested the uptake dynamics of the M cells in their study using latex microspheres, a nonsoluble antigen. In 1988, Owen *et al.*[18] demonstrated the M cells are still capable of take up vibrio cholerae in adult rabbits despite oral immunization by using autoradiography. In the present study, the authors examined and compared the uptake and transport through the covering epithelium of the Peyer's patches in rabbits which were percutaneously immunized vs. nonimmunized rabbits. Percutaneous immunization with BCG activates cellular immune reaction and elevates the level of BCG antigen in blood. In our ultrastrutural studies, we found that BCG was vigorously phagocytized by the M cells and transported to macrophages even under percutaneous immunization. Moreover, spatial pattern analysis revealed that the uptake and transport of BCG from the dome epithelium of the Peyer's patches was more enhanced under the percutaneous immunized condition than under the nonimmunized condition. This suggests that a subcutaneous BCG vaccination might not inhibit the M cells' uptake of tubercle bacillus in the ileal Peyer's patches.

There have been many reports on the effect of immunological sensitization on intestinal absorption. Walker *et al.*[19,20] and Andre *et al.*[21] reported that absorption of antigens such as horseradish peroxidase, and cow and human serum albumin was reduced in rats which were parenterally or orally immunized. Brandtzaeg *et al.*[22] demonstrated that transport of albumin through lingual mucous membranes was less in rabbits immunized with albumin than those nonimmunized, but that transport of transferrin increased in the immunized.

On the other hand, there have been a few reports which indicated the possibility that antigen uptake might be enhanced after immunization. Bockman *et al.*[23] made electron microscopic observations of intestinal absorption of ferritin in parenterally immunized hamsters and reported that the absorption of antigen increased. Kimura[24] orally administered killed Salmonella typhi treated with 10% formalin to intravenously immunized animals and found uptake of many of the microbes in the covering epithelium of Peyer's patches and in subepithelial macrophages.

In 1987, Nakura *et al.*[25] made immunohistochemical observations using the Peyer's patches and solitary lymphoid follicles in the human ileum which was obtained by right colectomy for cancer. According to their findings, lymphocytes and dendritic cells existed abundantly between the covering epithelial cells. Moreover, they reported that the cells were HLA-DR (Ia) positive, the majority of which expressed Leu 14 and 16 positive with a few expressing IL-1, and that the surface of the M cells was also stained positive with HLA-DR. These findings gave recognition to the dendritic cells and M cells as antigen-presenting cells. Lampert *et al.*[26] and Masson *et al.*[27] reported that Ia antigen reacted positively in keratinocytes, epidermis and mucous epithelium of the intestine and large intestine as a result of graft-versus-host disease (GVHD) appearing after bone marrow grafting in rats. Barclay *et al.*[28] found in their experiment using rats that stimuli by *Trichinella spiralis* and those by contact with dinitrofluoro-benzene in addition to GVHD made the stimulated intestinal epithelium and epidermis Ia antigen positive. Bensussan

et al.[29] reported that the intestinal epithelium was made Ia antigen positive in vitro by γ-interferon released by activated T cells in rats. However, the details of interaction between the M cells and HLA-DR are unknown yet. There is the possibility that BCG phagocytosis by the M cells might be enhanced as a result of changes in the membranous properties of the surface of the cells due to immunization.

Recently, Kato[30] immunohistochemically studied secretory IgA of the covering epithelium of rabbit ileal Peyer's patches using the immunoelectron microscope and found that the surface of the M cells showed strong positive response to both secretory IgA and IgA although the cells had less glycocalyx than the neighboring absorptive cells which did not demonstrate positive reaction. In 1982, Shorter *et al.*[31] reported that small quantities of IgG existed on the mucous surface of the intestine and that there is not only increased transudation of IgG from serum but also an invasion of the mucosa by IgG producing plasma cells when mucosal inflammation occurs. Phagocytosis of the M cells is presumably accelerated by the antigen-antibody reaction and opsonization on the surface of the intestinal lumen.

On the basis of these findings, it is speculated that increase in the uptake and transport of antigenic substances by the M cells of the Peyer's patches due to parenteral immunization might be an immune response of the intestinal epithelia to the emergence of antibodies in circulating blood.

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