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Localization of *Vibrio cholerae* O1 in the Intestinal Tissue

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SUMMARY **Colonization of *V. cholerae* O1 *in vivo* is known to be a non-invasive type which the vibrios are confined only to the intestinal tissues. The pathway by which the vibrio antigens reach the lymphoid cells and subsequently give rise to the immune responses is not entirely clear. Thus, experiments were performed in experimental rats by inoculating live *V. cholerae* O1 into the ligated ileal loops. The fate of the vibrios in the intestinal tissues was then studied by transmission electron microscopy at different times after the inoculation. It was concluded that live *V. cholerae* O1 were initially taken up by the M cells which overlay Peyer's patches and which subsequently delivered the intact vibrios to phagocytic cells in the Peyer's patches. These phagocytic cells processed (digested) the vibrios while the lymphocytes and plasma cells infiltrated around them. During the late period of infection (12-15 hours after inoculation of the vibrios), vibrios were also found passing through the loose intercellular spaces between the absorptive epithelial cells into the underlying intestinal tissues.**

Pathways of antigen handling by the gut are still to be defined. Specific antigens, particulate *vs* soluble, are handled differently and depending upon the mode of entry would lead to the generation of different immune responses. Mayer[1] concluded that there are three possible routes for antigen entry into the intestinal tissues and their possible immunologic outcomes. These are : 1) through the M cells without antigen processing. The antigen is then taken up by macrophages in the lamina propria. The macrophages process the antigen and travel to the underlying Peyer's patch where antigen priming occurs. This type of event would result in the induction of a predominant helper T cell response. 2) Antigen is taken up by the enterocytes *via* pinocytosis. Degradation occurs by activation of lysosomal enzymes. Processed antigen binds to MHC class II molecules and is re-expressed on the cell surface, where the complex can activate suppressor T cells and dampens an immune response or possibly results in a tolerant state. 3) Antigen travels between absorptive epithelial cells despite tight cell junctions. No processing occurs until the intact antigen reaches the lamina propria where macrophages take up the antigen, process and present it to the lymphocytes. This type of event would, most likely, result in helper T cell proliferation.

Studies using fluorescent antibody demonstrated that *V. cholerae* closely associated with the intestinal villi and intervillous spaces.[2] Nelson *et al*[3] showed that most of the bacteria adhered horizontally to the epithelial surface, but some were attached in an end-on manner, while their flagella extending into the lumen. The bacteria adhered *via* their surface coats directly to the tips of the microvilli, except for few vibrios that were partly embedded into the brush border. Further studies showed that live *V. cholerae* were transported to Peyer's patches

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through M cells.[4] In the ligated intestinal loop of rabbit inoculated with *V. cholerae* O1, the vibrios were found to be phagocytosed by M cells in the vicinity of Peyer's patches. They were then carried in phagocytic vesicles and discharged among the underlying macrophages.[4] However, structural degradation of the vibrios has not been observed.

In this study, colonization of *V. cholerae* O1 was investigated using ligated ileal loops of rats. The manner of vibrio adherence to enterocytes, the uptaking by M cells and the following fates of the uptaken vibrios were investigated using transmission electron microscopy.

MATERIALS AND METHODS

Bacterial strain

V. cholerae O1, biotype El Tor, Inaba strain isolated from cholera patient in 1990 was used throughout the study.

Experimental animals

Female Wistar rats were obtained from The National Laboratory Animal Centre, Mahidol University, Nakhon Pathom Province, Thailand. Twelve rats aged 7–8 weeks old were fasted for 15 hours before operation. Under ether anaesthesia, laparotomy was performed through a midline incision. Three blind ileal loops, approximately 3 cm long and 1 cm apart were made. Each loop included at least one grossly identifiable Peyer's patch. Two loops were inoculated with 0.3 ml of PBS containing 10^9 log-phase broth culture *V. cholerae* O1 while the third loop received 0.3 ml of PBS which served as a control. The abdominal cavity was closed by suturing. The animals were then kept in warm cages. At 1, 3, 6, 9, 12 and 15 hours after the inoculation, two rats were killed at each time point by cervical dislocation.

Fixation and processing of intestinal tissue for electron microscopy

At the time required, the rats were killed and intestinal loops were removed. The loops were cut opened longitudinally, washed in cold PBS and then fixed with 2.5% glutaraldehyde for 2 hours. The tissue was then cut into 1×2 mm pieces and placed in a fresh fixative at 4°C for 18 hours. They were washed three times with PBS and two times with distilled water, then dehydrated by graded acetone (30%, 50%, 70%, 90%, 100% and 100%, respectively) at room tempeature for 15 minutes each. The specimens were submerged into 2:1 and 1:2 mixtures of acetone and Spurr's resin[5] and pure Spurr's resin, respectively for 3 hours each. Subsequently, the tissue were embedded in Spurr's resin which was heat polymerized at 70°C for 8 hours.

The embedded specimens were cut with an LKB ultramicrotome with glass knives. Sections of 1 μm thick were stained with 1% toluidine blue soluton and examined by light microscopy to select proper areas for electron microscopy. Ultrathin sections with 60–90 nm thick were prepared and mounted on 100 mesh grids coated with Formvar film, stained with 2% uranyl acetate in 50% ethanol and lead citrate[6] and finally examined under a transmission electron microscope (Hitachi, HU-12A).

RESULTS

At 1 hour after the inoculation, many vibrios were found in the intestinal lumen; some of them were dividing (Fig. 1) but some were trapped in the mucus secreted by goblet cells (Fig. 2). Some vibrios adhered either horizontally or in an end-on manner to the microvilli of the absorptive epithelial cells (Fig. 3a and 3b). As seen in Fig. 4a, a group of vibrios were located over the M cell which extended its pseudopod into the lumen. Two intact vibrios were seen in the vesicle of the M cell (Fig. 4a and 4b). In a different section, intact vibrios (one with flagellum) were clearly seen in the vacuole of M cell (Fig. 5). The vibrios were not found in the absorptive epithelial cells although some were in the intervillous spaces.

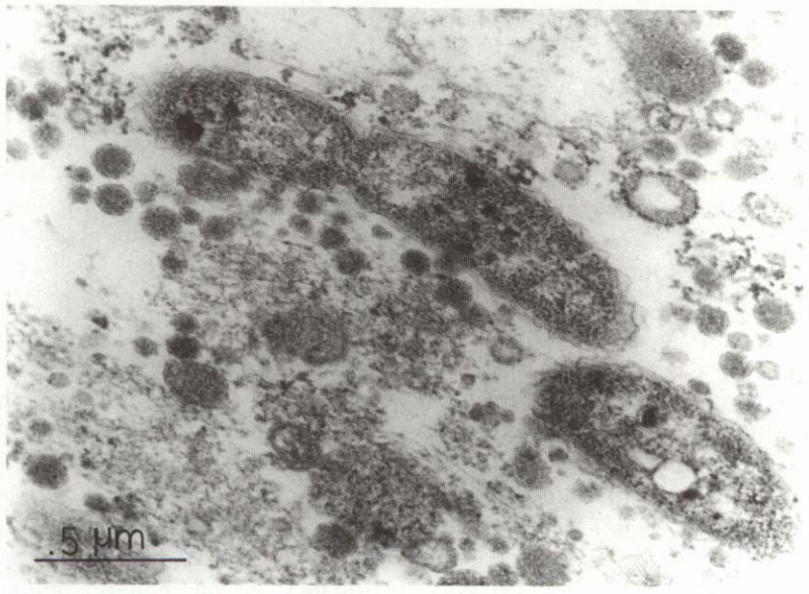

Fig. 1 Electron micrograph showing binary fission of *V. cholerae* in the intestinal lumen.

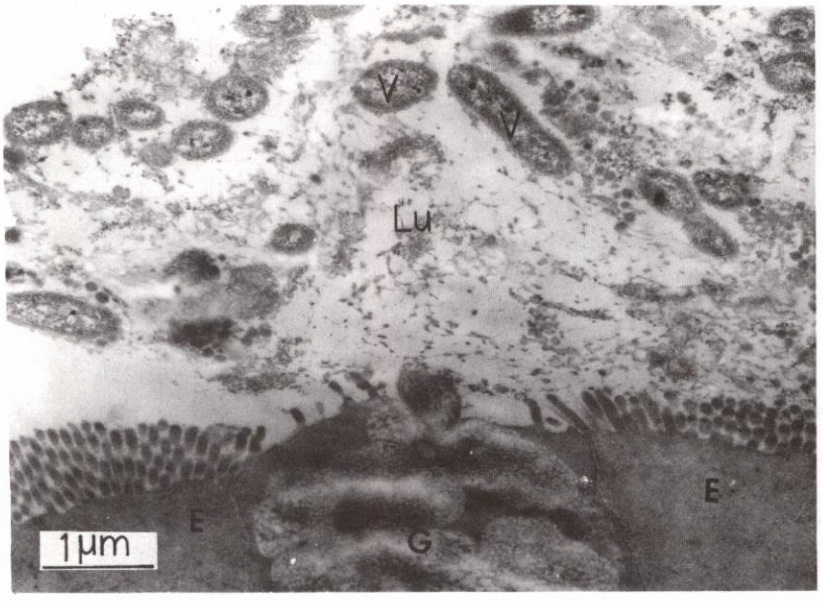

Fig. 2 Vibrios (V) are trapped in mucous secretion discharged from goblet cells (G) in the intestinal lumen (Lu); E = enterocytes.

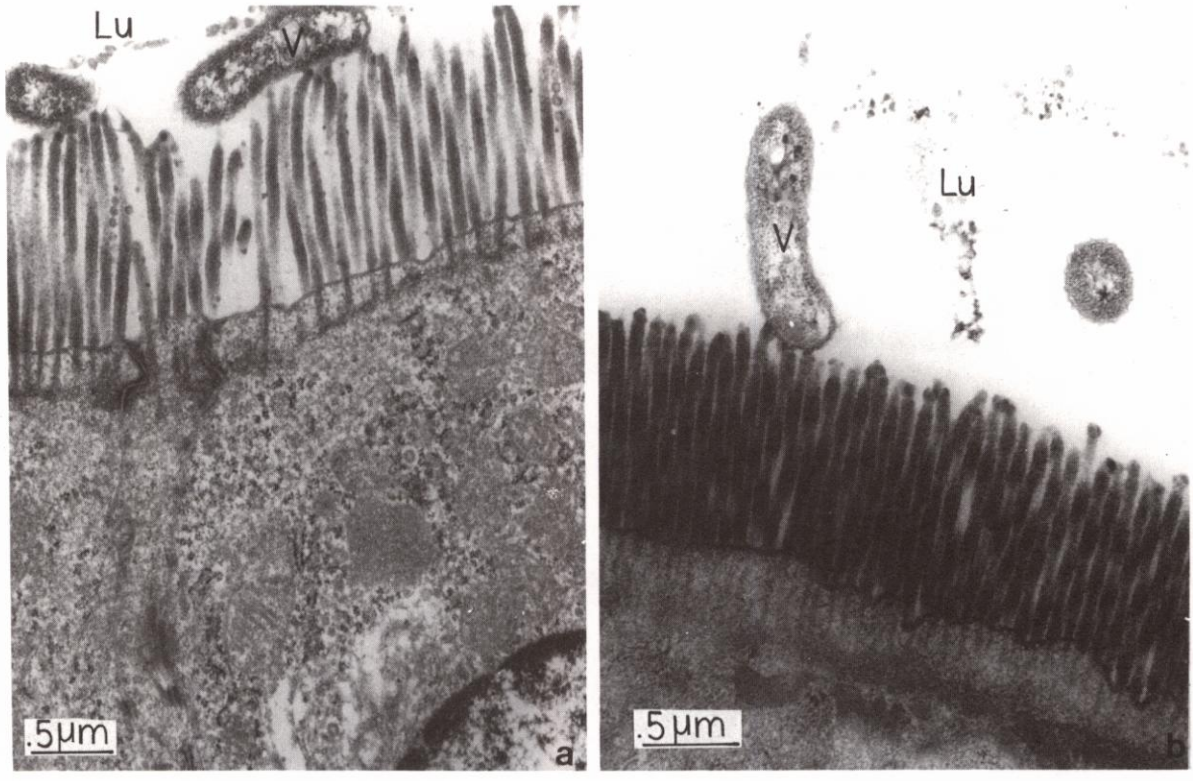

Fig. 3 Adherence of vibrios to the epithelium (a) horizontally and (b) an end-on manner. Note several microvilli bend towards and attach to the vibrios.

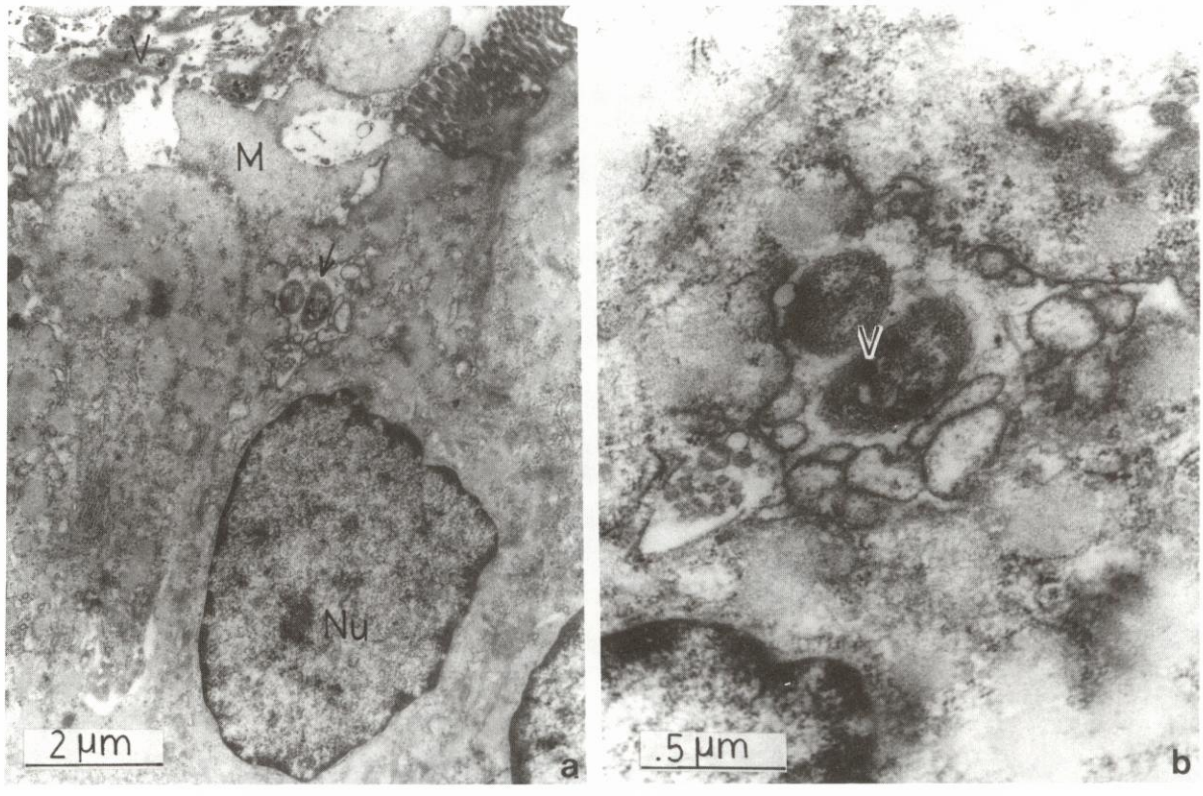

Fig. 4 At 1 hour after infection, the vibrios adhere and are engulfed by an M cell : (a) a group of vibrios (V) attached to the protruding cytoplasm of the M cell (M) while two vibrios (arrow) are in the cytoplasm of the M cell. Nu = nucleus of M cell. (b) At higher magnification, the vibrios (V) are seen surrounded by the vacuoles of an M cell.

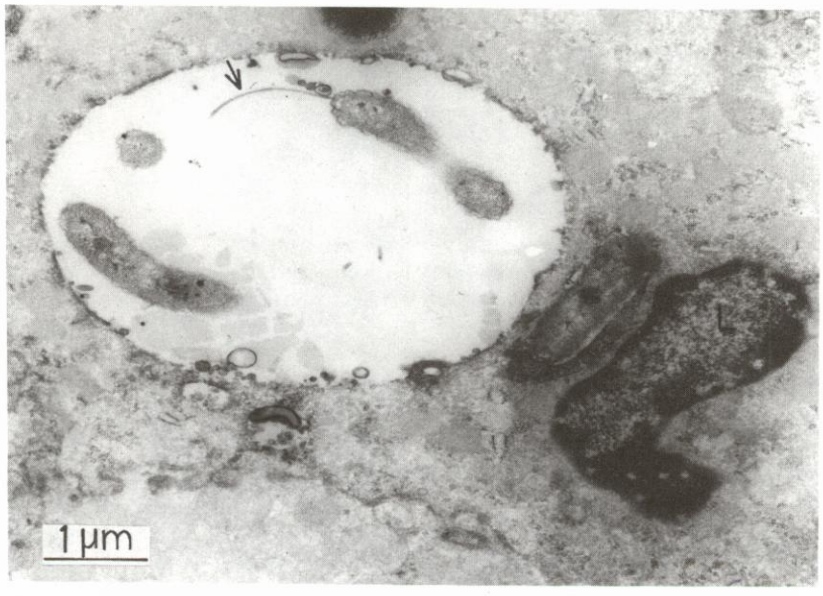

Fig. 5 M cell containing intact vibrios in its vacuole. Note one vibrio with a flagellum (arrow).

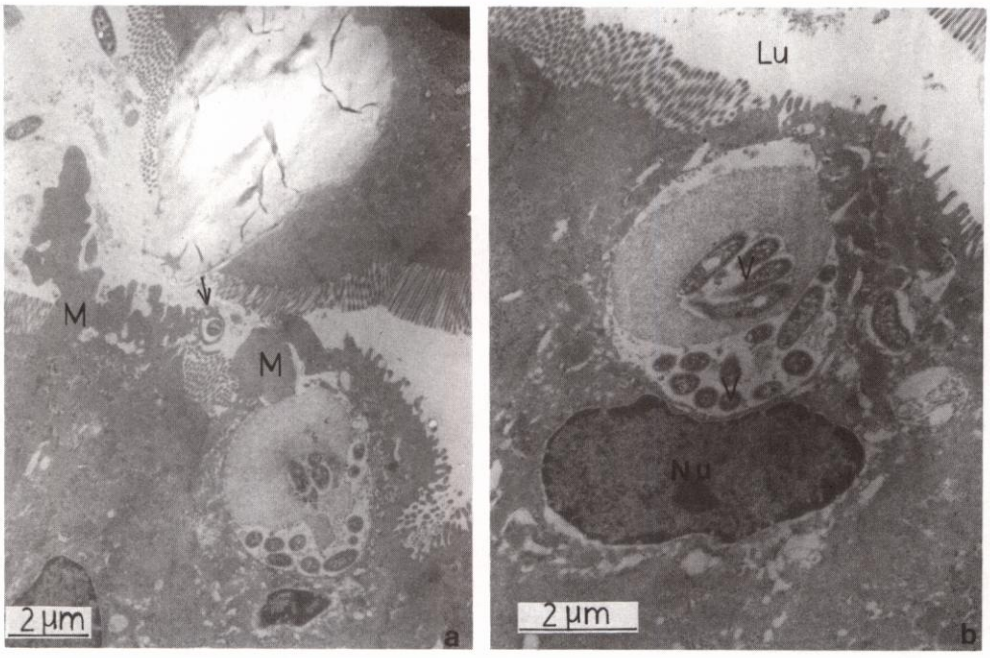

Fig. 6 At 3 hours after infection. Electron micrograph showing engulfment of vibrios by M cell
(a) one vibrio is surrounded by pseudopod (arrow) extended from cytoplasmic membrane of an M cell (M). Below, at the right corner, vibrios are seen in a large cytoplasmic vacuole of another M cell.
(b) in another section and at higher magnification, ingested vibrios are in the vacuole of an M cell
Lu = intestinal lumen.
Nu = nucleus of an M cell.

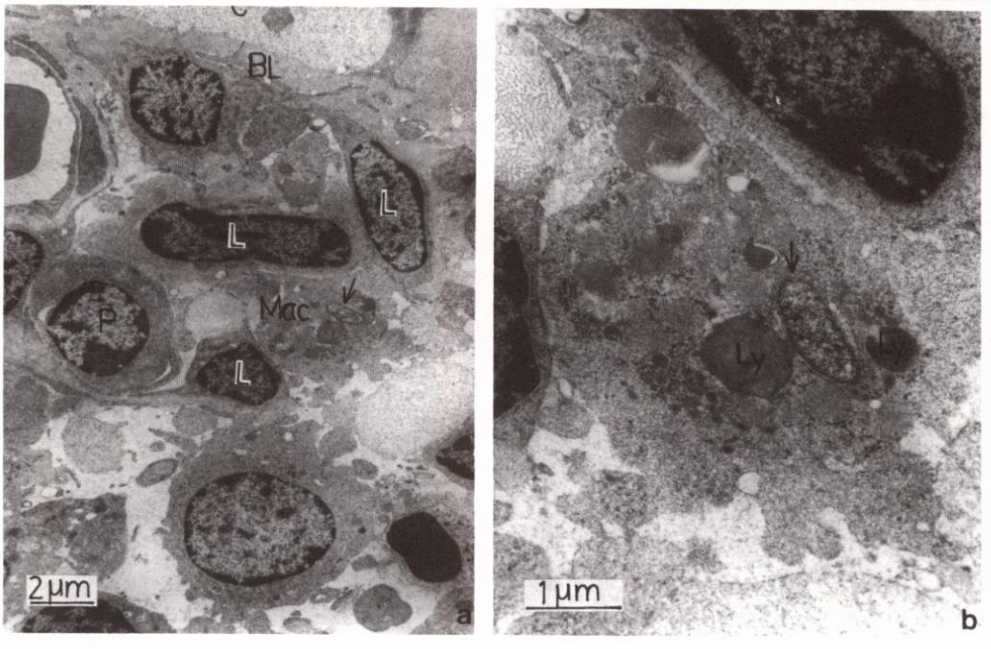

Fig. 7 At 3 hours after infection, (a) a vibrio (arrow) was found in the cytoplasm of a macrophage (Mac) located at the basal lamina (BL) beneath the columnar epithelial cell (C). Note three lymphocytes (L) and one plasma cell (P) are in vicinity (b) at higher magnification, the vibrio was seen in the phagocytic vacuole surrounded by lysosomal bodies (Ly).

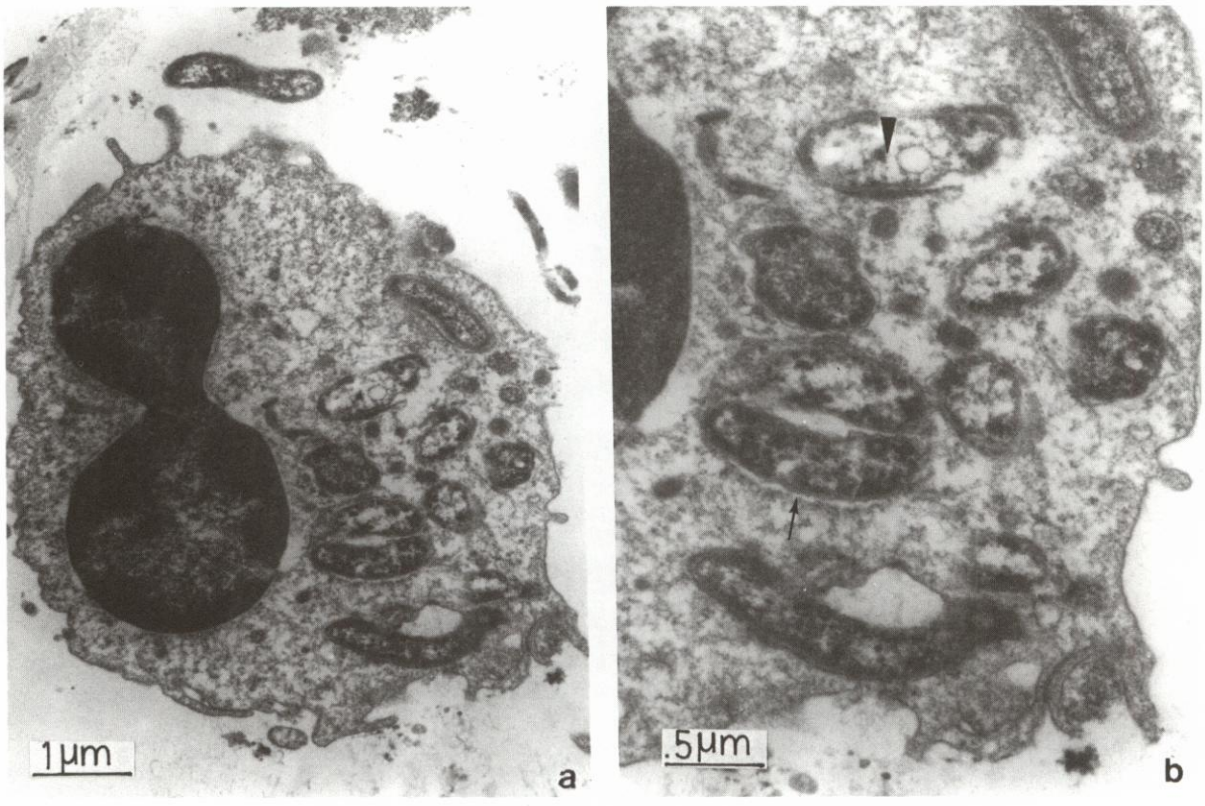

Fig. 8 Electron micrograph of a neutrophil in the intestinal lumen taken at 9 hours after infection : (a) many vibrios are in the phagolysosome of the neutrophil. (b) part of the neutrophil showing vibrios at various stages of degeneration in the phagolysosome.

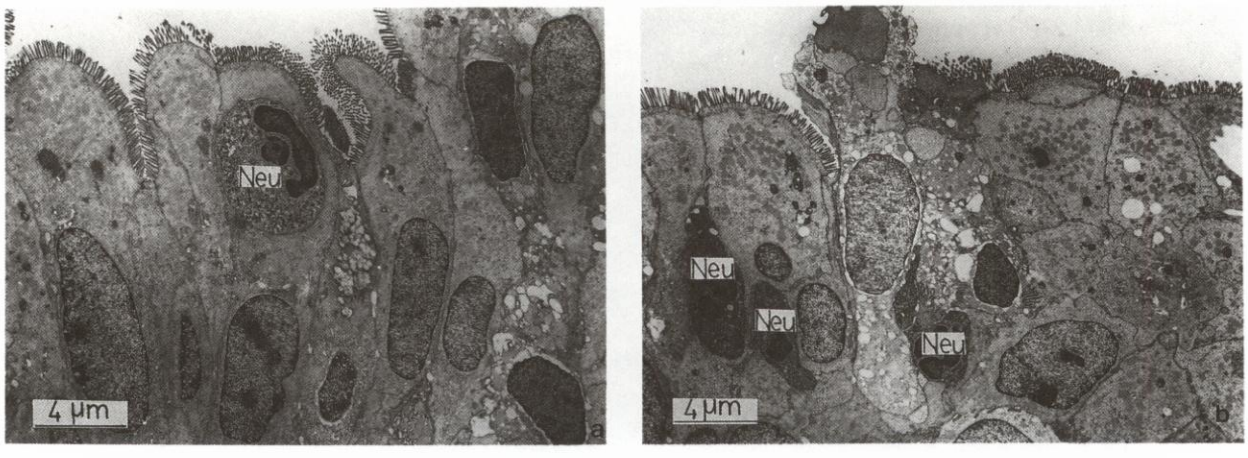

Fig. 9 Electron micrograph taken at 6 hours after infection showing direct migration of neutrophils from submucosa towards the intestinal lumen : (a) one neutrophil (Neu) is in the absorptive epithelial cell, (b) three neutrophils in the intercellular spaces.

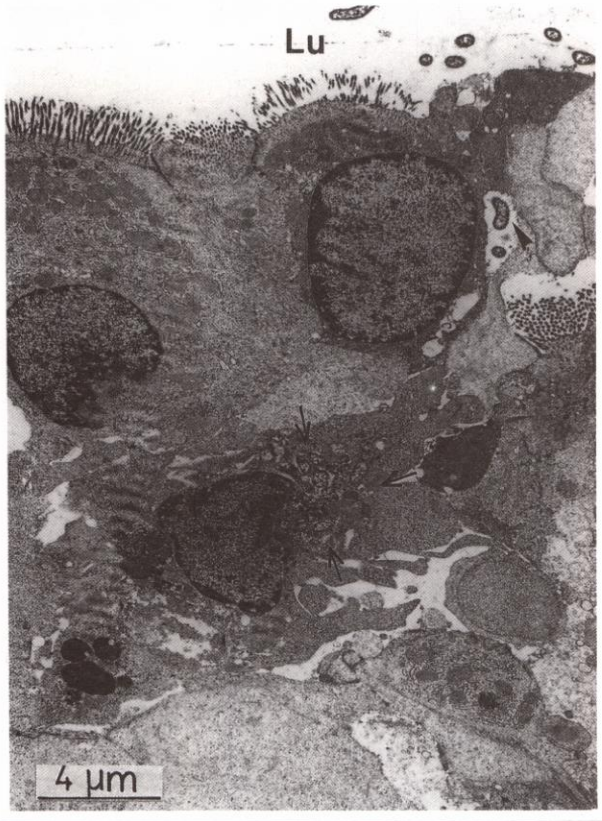

Fig. 10 Electron micrograph taken at 9 hours after infection. Several vibrios (arrows) are in the cytoplasma of a macrophage beneath the columnar absorptive cells. Some vibrios are in the intercellular space (arrow head) and lumen.

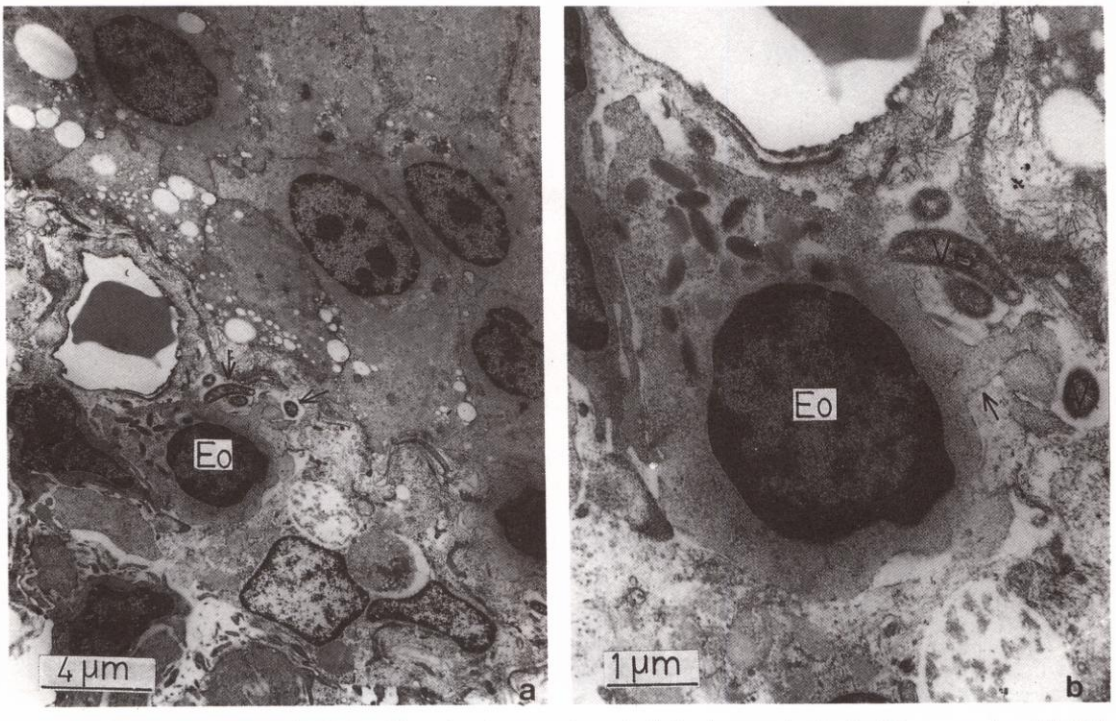

Fig. 11 Electron micrograph of the villous lamina propria : (a) vibrios (arrows) attached to an eosinophil (Eo). (b) at higher magnification, vibrios (V) are associated with a pseudopod of the eosinophil (arrow).

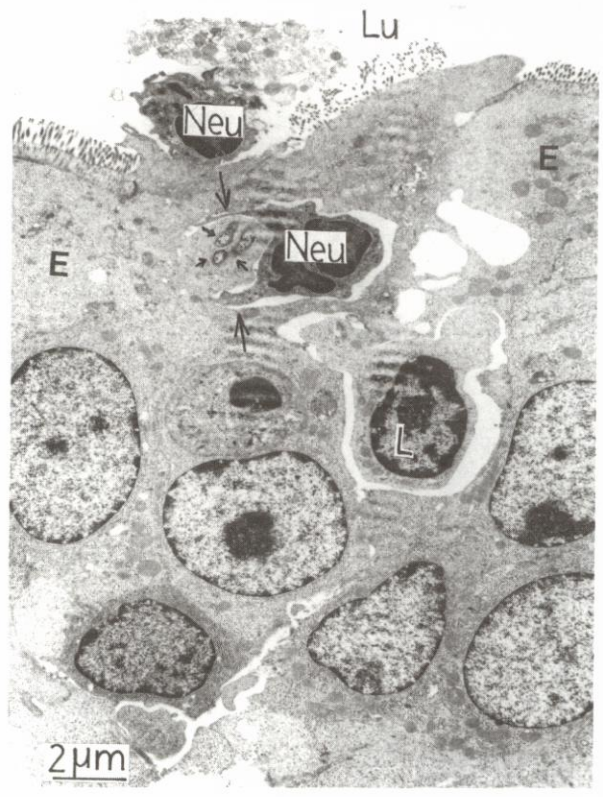

Fig. 12 Electron micrograph taken at 9 hours after infection. A neutrophil (Neu) beneath an M cell extended its pseodopodia (big arrows) to enfold the vibrios (small arrows).
E = enterocytes.
L = lymphocyte.

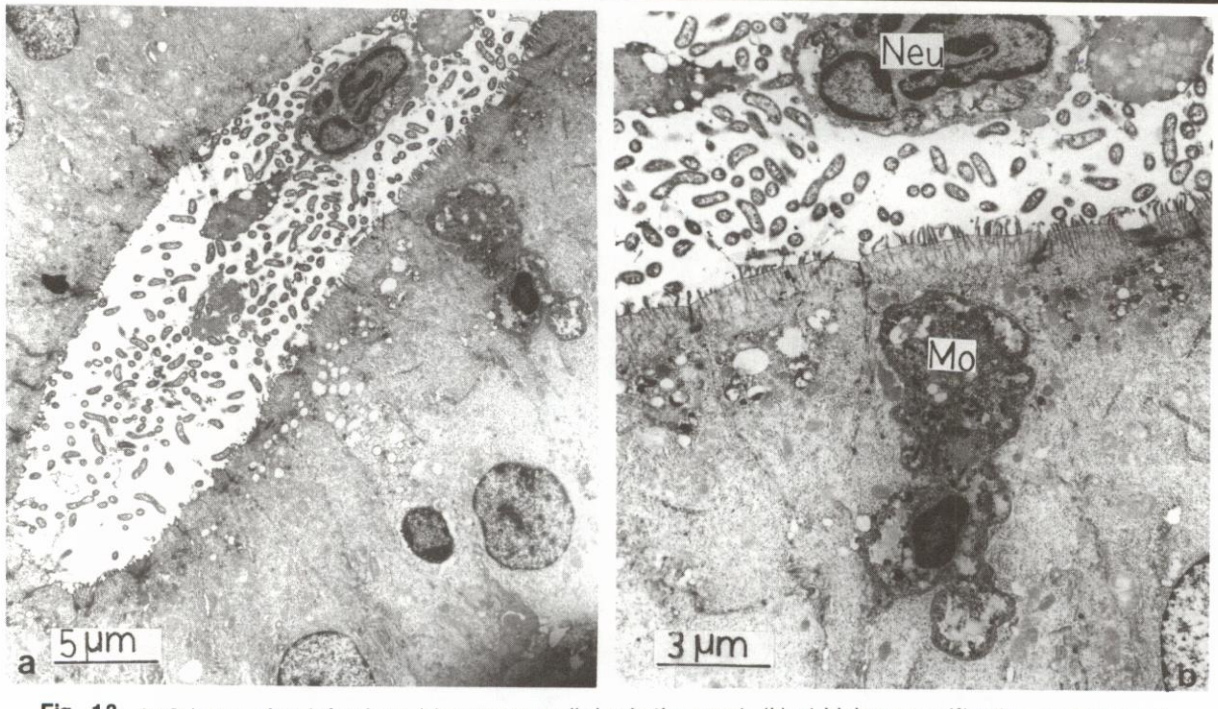

Fig. 13 At 9 hours after infection : (a) numerous vibrios in the crypt. (b) at higher magnification, a neutrophil (Neu) contains vibrios in its cytoplasm. One mononuclear cell (Mo) is making its way to the crypt via intercellular space between absorptive epithelial cells.

Fig. 14 At 15 hours after infection, the intercellular spaces are larger and many vacuoles are seen. Vibrios are found in the spaces (arrows).

Fig. 15 At 15 hours after infection, vibrios in the intestinal lumen are trapped in the secretion of goblet cells (arrow).

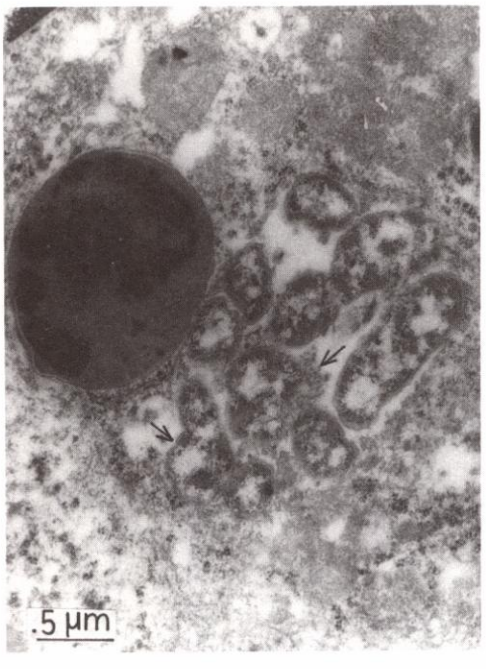

Fig. 16 At 15 hours after infection, vibrios in cytoplasm of a macrophage are degenerated (arrows).

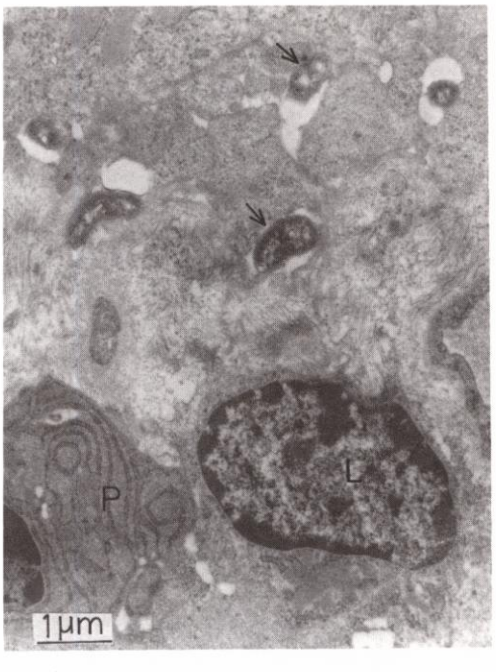

Fig. 17 Electron micrograph of lamina propria taken at 15 hours after infection showing a plasma cell (P), lymphocyte (L) and vibrios (arrows).

At 3 hours after the inoculation, large numbers of vibrios were seen in the intestinal lumen (perhaps as a result of luminal prolieration). Adherence of the vibrios to the microvilli of the absorptive epithelial cells was found to be greater than at 1 hour after inoculation. However, entering of vibrios *via* these cells was not observed. Entering was only through the M cells (Fig. 6a and 6b). Beneath the basal lamina of the columnar epithelial cells or in the dome of Peyer's paches, vibrios were found in cytoplasm of macrophages (Fig. 7a and 7b). At this time point, numerous vibrios were also found aligned in the intervillous spaces.

At 6 and 9 hours after the inoculation, the infected intestinal loops were distended by fluid accumulation while the control loops were not. However, the mucosal epithelium appeared normal. Numerous vibrios in the lumen were ingested and degraded by phagocytes predominantly neutrophils (Fig. 8a and 8b). These neutrophils migrated to the intestinal lumen *via* absorptive epithelial cells or intercellular spaces (Fig. 9a and 9b). In addition, there was an infiltration of neutrophils, eosinophils and macrophages in the areas beneath the M cells and the absorptive epithelial cells. Large numbers of vibrios were found in the cytoplasm of these cells (Fig. 10) or about to be phagocytosed by them (Figs. 11 and 12). Numerous vibrios were also seen in the intestinal crypts (Fig. 13a and 13b).

At 12 and 15 hours after the inoculation, the infected loops were more distended with fluid. The intercellular spaces were larger and the epithelial cells contained vacuoles. The vibrios were found coming along the intercellular spaces (Fig. 14). Large numbers of vibrios in the intestinal lumen were found attached to the desquamated columnar epithelial cells or in discharged secretion (Fig. 15). Macrophages infiltrated more into the subepithelial zone of Peyer's patches and they were active in phagocytosis. High magnification of the phagolysosomes revealed degenerated vibrios (Fig. 16). Some vibrios were freed in the extracellular spaces in the lamina propria where plasma cells and lymphocytes were seen nearby (Fig. 17).

DISCUSSION

From this study, although vibrios were able to attach to both the M cells and the columnar absorptive epithelial cells, only the M cells were able to take up and transport the vibrios to the underlying lymphoid tissue. M cells extended their pseudopodia to enclose the vibrios in the intestinal lumen and actively engulfed them. However, these cells, though with phagocytic activity, are not macrophages.[4] The phagocytic activity of the M cells was well established previously by the work of Owen *et al*[7] who showed that horseradish peroxidase as well as vibrios was engulfed by these cells. However, as shown in Fig. 5, the engulfed vibrios within the M cells were intact. Thus it appears that M cells transport the vibrios to the lymphoid cells in the Peyer's patches without processing antigens.

During the late period of infection (>6 hours), phagocytosis of the vibrios by neutrophils, macrophages and eosinophils was found in the area beneath the M cells and absorptive epithelial cells. During 9-15 hours of infection, the vibrios were also found in the intercellular spaces and in the crypts. This finding was different from the invasion of *Yersinia enterocolitica* which only attached to the M cells, being phagocytosed and transported from the lumen into the lamina propria.[8] Furthermore, pathogenic strain of *Y. enterocolitica* proliferated in the lamina propria within 19 hours of the infection but multiplication of vibrios was seen only in the intestinal lumen (started at 1 hour after infection).

Desquamated epithelial cells and intestinal secretion (mucus from goblet cells) seem to play natural resistance role in *V. cholerae* infection.

During late infection (9-15 hours), phagocytic cells in the lamina propria were loaded with degraded vibrios in their phagolysosomes where and when lymphocytes and plasma cells are at the immediate vicinity. Thus, it appears that M cells and the intercellular spaces are main portals of entry of the vibrios. The virios were then processed by the antigen presenting cells which presented the epitopes to the lymphoid cells and eventually lead to the immune response.

Different forms of antigens, though delivered *via* the same route result in different outcomes of the efficiency of the immune response. For cholera immunization, it is known that live attenuated strains given orally conferred high immunogenicity while an oral vaccine prepared from killed vibrios or refined antigen had low immunogenicity and multiple, spaced-doses were required to enhance the immune response. In this study as well as the study conducted by Owen *et al*[4], it is evident that live *V. cholerae* O1 were actively transported into the Peyer's patches through the M cells. In contrast, killed *V. choloerae* O1 were shown to be poorly absorbed to and transported by the cells if at all, hence the low immunogenicity. These findings emphasize the requirement of a special delivery vehicle, an oral adjuvant and/or a modification of the nature of the antigen so that the antigen uptake of a vaccine by the M cells would be facilitated.

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