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### ELECTRON AND LIGHT MICROSCOPE STUDY OF INTESTINAL VASCULATURE IN CHOLERA

Final Report

by

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### 1. STATEMENT OF THE PROBLEM:

The cardinal symptom of cholera is an explosive, voluminous diarrhea which if untreated may result in dehydration, electrolyte imbalance, shock and death. The target organ of the <u>Vibrio</u> cholerae organism is the small intestine and its vascular supply. Exposure of the small intestinal mucosa to the viable organism or to cell free lysates of <u>V. cholerae</u> will result in the transfer of fluid and electrolytes from the intestinal microcirculation into the gut lumen across an anatomically intact epithelium. Since the organism is non-invasive and the cardinal signs and symptoms of the disease can be produced with cell-free lysates, absorbed exotoxins are primarily responsible for producing the disease.

To date the mechanism of action of the choleragenic toxins responsible for initiating fluid exsorption from the intestinal microcirculation is not well understood. Furthermore, the route of passage of fluid, and subsequently electrolyte concentration changes from the intestinal vasculature to lumen have not been defined.

The present experiments were undertaken in an attempt to further differentiate the effect of absorbed vibrio exotoxins on the ultrastructural (E.M.) organization of the intestinal vasculature and to determine the route and rate of passage of injected electron dense tracers in experimental cholera conditions. By comparing the results with corollary studies on a non-toxic, osmotic diarrheal model induced with MgSO<sub>4</sub>, a further understanding of the pathogenesis of cholera might be obtained.

### 2. BACKGROUND:

Most of the early workers in the field of cholera research were of the opinion that the massive diarrhea of cholera was due to the invasion and proliferation of the causative organism (V. cholerae) in the small intestinal mucosa, which resulted in necrosis, ulceration and eventual denuding of the epithelial lining causing exudation of fluid into the gut lumen in a manner similar to that of fluid loss from a burned surface. However, since 1959, it has been firmly established that the epithelial lining of the small intestine remains anatomically intact in cholera and that the diarrhea is due to the action of absorbed toxins." Several alternative explanations have been put forth to explain the fluid loss, including, alteration of the sodium transport mechanisms of the epithelium,<sup>4</sup> derangement of the molecular structure of the epithelial and vascular protein matrix<sup>5</sup> and physiological changes of the epithelial cells, in particular the crypt cells, in some manner as to initiate fluid secretion into the gut lumen. At the present time none of these hypotheses fully accommodates the observed clinical, physiological and morphological findings.

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### 3. APPROACH TO THE PROBLEM:

Previously, the Walter Reed Army Institute of Research group, Department of Experimental Pathology, under the direction of Col. Helmuth Sprinz, Chief, has studied the patho-physiology of cholera at the ultrastructural level. Using electron microscopic and electron histochemical methods, the fine structural alterations of the intestinal mucosa in response to Vibrio cholerae exotoxin intoxica-tion were elucidated.<sup>7,8,9</sup> These studies were carried out using tion were elucidated.<sup>7,8,9</sup> These studies were carried out using the preconditioned guinea pig as an experimental model.<sup>10</sup> Based on the results of these studies, we originally proposed that absorbed Vibrio cholerae exotoxins exerted an initial, profound effect on the small intestinal vasculature which was characterized by rapid capillary endothelial degeneration and rarefication, and loss of anatomic integrity, permitting an accelerated leakage of plasma, fluid and electrolytes out of the microcirculation via patent interendothelial junctions and pinocytosis. These events result in flooding of the lamina propria with a proteinaceous exudate which, in some manner, stimulated increased secretory activity of the overlying, morphologically intact epithelium and a net fluid and electrolyte exsorption into the intestinal lumen, giving rise to the characteristic diarrhea.

However, on the basis of additional experiments using an intravenously injected, relatively large molecular weight electron dense tracer (Horseradish peroxidase), it was found that the <u>route</u> of passage of the tracer out of the microcirculation was identical in three dissimilar models ([1] Normal, unchallenged controls. [2] Non-toxic, osmotic diarrhea induced by MgSO<sub>4</sub>. [3] Toxic, <u>Vibrio cholerae</u> induced diarrhea.) The <u>rate</u> of tracer loss, however, was greatly accelerated in the two diarrheal conditions. In addition, it was also noted that although vascular leakage was accelerated in the MgSO<sub>4</sub> and <u>Vibrio cholerae</u> treated animals, widespread endothelial degenerative changes were lacking.

The current study was undertaken to determine (1) if a larger molecular weight, electron dense tracer, Ferritin, injected intravenously into normal and abnormally induced diarrheal animals, would react in the same manner and follow the same routes at the same rates as the smaller molecular weight material used previously (Horseradish peroxidase); (2) whether a demonstrable extensive cyto-pathologic response by the intestinal vasculature is essential for the initiation and maintenance of abnormal fluid loss. The same experimental conditions as used in the prior studies were adhered to.

### 4. EXPERIMENTAL METHODS:

All experiments involving <u>V. cholerae</u> infection were conducted at the Biological Products Laboratory, Bldg. 501, Forest Glen Annex, WRAIR, in collaboration with Dr. G.P. Lowenthal. The experiments utilizing MgSO<sub>4</sub> only, were conducted at the Dept. of Experimental Pathology, Rm. 161A, Bldg. 40, WRAIR, in collaboration with Col. Helmuth Sprinz. Specimen preparation for light and electron

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microscopy was carried out by the Biodynamics Research Corporation personnel and laboratory facilities.

Walter Reed strain Hartley guinea pigs of both sexes were used throughout the study. The animals were preconditioned by fasting for 96 hours prior to being fed by stomach tube 10 ml nutrient broth suspension of an 18 hour culture of 10<sup>8</sup> Vibrio cholerae organisms. 0.1 ml tincture opium was given I.P. to reduce intestinal motility and insure sufficient time for the organisms to remain in the intestine. Two separate experiments were carried out, each experiment utilizing 30 animals. They were sacrificed in the following sequence: 2, 4, 6, 8 and 24 hours after intubation of the organisms. Five experimental and one normal unchallenged control were used for each stage. All animals were anesthetized by administering a 1 ml mixture of 100 mg Na Pentobarbitol (RELAXAN) and saline I.P. For the tracer study, 2.5 ml of physiologic saline containing 60 mg ferritin was injected via heart puncture, 1/2, 1-1/2, 2 and 5 minutes prior to sacrifice by exsanguination. The abdomen was opened by midline incision, and the small intestine rapidly excised. Small segments of ileum and jejunum were taken and fixed in cold 2.5% phosphate buffered glutaraldehyde for electron microscopy and in cold buffered formaldehyde-glutaraldehyde fixative for light microscopy. These fixing agents also sterilized the tissues. A corollary series of animals were treated in an identical manner except that instead of receiving V. cholerae organisms, a 10 ml solution of MgSO, (20%) was instilled by stomach tube. Two hours after feeding the hypertonic MgSO, solution, the animals were anesthetized, received 2.5 ml of Saline containing 60 mg ferritin via heart puncture, and tissue excised in the same schedule outlined above, and served as the non-toxic, osmotic diarrhea controls.

All tissue for electron microscopy was postfixed in 1% buffered Osmium tetroxide, dehydrated through a graded series of alcohols and embedded in either Araldite or BYRACOTE<sup>TM</sup> polyester plastics. The tissues for light microscopy remained in the formaldehyde-glutaraldehyde fixative for 24 hours, washed in buffer, dehydrated through alcohols and embedded in paraffin blocks. 4-6µ sections were stained with H & E, PAS-Alcian Blue and Alcian Blue alone. In addition, 1/2µ thick sections of the plastic embedded material were used for orientation purposes and light microscopic examination. Thin sections of well oriented plastic embedded material was studied in Biodynamics Research Corporation AEI-6B electron microscope. Grids from at least four blocks from each tissue biopsy taken were examined and photographed at various magnifications from 1500 to 40,000X.

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### 5. RESULTS:

### A. Normal, unchallenged controls.

The normal, unchallenged control animals were treated in an identical manner as the experimental animals except that instead of receiving viable vibrio organisms, they received a 10 ml sterile nutrient broth by stomach tube. No undue morphologic changes were seen by either light or electron microscopy. The villi were tall and slender and crypt to villus ratio was maintained at 1 to 3 or 4. The lamina propria was thin and no excessive cellular or edematous infiltrate was noted. The epithelial lining cells of the mucosa, as revealed by electron microscopy confirmed the regular, normal appearance of the epithelium. The cells were long and slender, with basally located nuclei and an even, random distribution of cellular organelles, including strands of both smooth and granular endoplasmic reticulum, ovoid mitochondria, and dispersed free ribosomes and polysomes (Fig.1). On occasion electron dense inclusions and myelin figures were noted but were not excessive. Elements of the lamina propria were also normal in appearance, including the vascular lining cells. The capillary endothelium exhibited the usual array of cytoplasmic organelles including mitochondria, sparse endoplasmic reticulum, free ribosones and numerous pinocytotic vesicles. No patent endothelial junctions were encountered. It is apparent then that injection of ferritin suspended in saline does not in itself elicit an undue pathologic response.

The ferritin tracer appeared as extremely electron dense discreet units having a tetrad configuration and measuring approximately 60Å in diameter. In all control animals studied the ferritin marker was for the most part retained within the capillary lumen, even after 5 minutes. Several animals were maintained for extended periods of time, and it was not until 9 to 12 minutes that significant ferritin was cleared from the vessels. The passage of ferritin out of the capillaries was by means of pinocytosis. Several ferritin particles were usually confined to a single pinocytotic vesicle (Figs. 2 and 3). On occasion ferritin micelles were seen to aggregate in the cisternae of the endothelial endoplasmic reticulum and rarely were they found free in the cytoplasm. At no time was ferritin observed in the interendothelial junction. The tracer also appeared in the extracellular spaces of the lamina propria (Fig. 3). It was not limited to the basement membrane area, and was randomly dispersed. It would appear then that the passage of ferritin out of the normal intestinal vasculature is slow and accomplished by means of pinocytosis.

B. Non-toxic, osmotic diarrheal controls  $(MgSO_A)$ .

The presence of hyperosmotic, non-toxic solutions of MgSO4 in the intestinal lumen causes no apparent morphologic alterations,

even at the ultrastructural level. Although an osmotic diarrhea ensues, there is no inflammatory response and the mucosa appears unaltered and confirmed our previous observations. However, there is a dramatic acceleration of ferritin particle movement as evidenced by the appearance of the tracer, both within the capillary endothelium and in the interstitial spaces of the lamina propria as early as one minute after injection. The ferritin micelles are incorporated into membrane bound pinocytotic vesicles and elements of the smooth endoplasmic reticulum (Fig. 4). Only rarely was ferritin found free in the endothelial cytoplasm (Fig.4, arrows). In contrast, the tracer outside the endothelium was always freely dispersed. Again, no ferritin micelles were seen in the interendothelial spaces. This indicates that the route followed by the ferritin macromolecules in the non-toxic, osmotic diarrhea is identical to the normal, but that the rate of transfer of the tracer is increased.

### C. Experimental Cholera.

On gross examination of experimental animals receiving <u>V. cholerae</u> organisms, there was a rapid accumulation of mucous Tlecked watery stool in the small intestinal lumen which gradually distended the intestine. Beginning at the 4 hour time period, noticeable fluid retention was apparent, and by 8 hours the small intestinal diameter was almost double that of the controls. The external (serosal) mucosal surface was dry, sticky and had a pink color due to the engorgement and distention of the splancnic vessels.

Light microscopic examination of the tissue samples revealed a scarcity of overt pathological changes. The epithelial lining was unaltered and appeared normal. There was some increase in both cellular and vascular elements of the tunica propria but not above that seen on occasion in normal controls. Hemoconcentration, plasma condensation and edematous flooding of the lamina propria was infrequently encountered.

Electron microscopic examination confirmed the light microscopic findings. No apparent cytotoxic alterations were evident in the majority of the vessels studied. The organellar structure (mitochondria, endoplasmic reticulum, ribosomes, Golgi membranes, pinocytotic vesicles, nuclei and interendothelial junctions), and organization of the vascular endothelium appeared little changed when compared to the normals. Only infrequently were degenerated, rarefied endothelial cells encountered. In these examples, the organelles were either completely absent or were in various stages of degeneration (Figs. 9, 10). The fate of the injected ferritin particles was similar to that seen in the MgSO<sub>4</sub> treated animals. The tracer rapidly (1 min.) traversed the endothelium from capillary lumen to interstitial space via pinocytotic vesicles and dilated cisternae of the endoplasmic reticulum (Figs. 5, 6), and did not escape by means of leakage out of patent interendothelial junctions. Ferritin accumulation in the endothelium was visible as early as two hours after <u>V. cholerae</u> infection, and was best seen at the 8 hour stage, which correlates with the establishment of clinical signs and symptoms of the disease. The complexity of the membraneous endoplasmic reticulum network often made the interpretation difficult as to whether the accumulation of ferritin was within the endoplasmic reticulum 'umen or free in the cytoplasmic matrix (Figs. 5 and 6). It appears that both pphenomena are present, and that sequestration within the endoplasmic reticulum is the dominant feature.

Throughout the study, at all stages examined, ppathologic changes were infrequently found. When present, they consisted of a variety of degenerative phenomena, the most striking being endothelial rarefication (Figs. 9, 10), where the ferritin tracer also rapidly traversed the endothelial barrier. However, since the endoplasmic reticulum network was absent, the ferritin particles were seen dispersed in the rarefied cytoplasmic matrix. On other occasions, seeming patent capillary fenestrae were observed with micelles of ferritin and large globules of an unknown homogeneous material apparently escaping to the extracellular spaces (Figs. 7 and 8). Similar electron dense globules were seen enclosed within dilated cisternae of the endothelial smooth endoplasmic reticulum. The composition of this material and the relationships, if any, to the disease are unknown. These findings strongly suggest that the route of ferritin loss in cholera, a toxic, non-osmotic diarrhea, is similar to that found in the MgSO<sub>4</sub> and normal control animals, and that the rate of tracer loss is greatly accelerated. These events occur, at least in these experiments, in the absence of any discernible, overt cytotoxic effects on the endothelium, tunica propria elements or epithelial lining. The route of ferritin transfer was primarily by means of pinocytosis and the smooth endoplasmic reticulum network, with some free diffusion through the cytoplasmic matrix possible. No evidence was found to suggest the loss of ferritin from patent interendothelial junctions. When cytological damage does occur, the ferritin apparently transcends the endothelial barrier by random diffusion.

### 6. DISCUSSION AND CONCLUSIONS:

The results presented here are somewhat in contradiction to the findings reported by our group previously (7,8,9,10,11). The most striking difference was the absence of a distinct inflammatory response with no widespread endothelial lesions, which corresponds to the morphological picture described for other experimental models, including the infant rabbit (12), adult rabbit (13), and dog (8). The only deviation in the experimental procedure used in the current experiments was the use of a different strain of guinea pigs than had been utilized in the prior studies. In view of our present findings, it is apparent that the increased rate of transfer of large molecular weight particles (ferritin) and, as previously shown, horseradish peroxidase, can occur in the absence of a discernible morphologic lesion. The total number of lesions

we encountered is too low to account for the fluid and electrolyte loss occurring in cholera. It is of interest to note the distinct pathway that the ferritin molecules traversed from capillary lumen to interstitial space. For the most part the micelles were sequestered into pinocytotic vesicles which either transferred the particles to, or were incorporated in toto into the smooth endoplasmic reticulum network. These events occurred rapidly and were most pronounced in the 8 hour cholera animals, as early as 1 minute after ferritin injection. This would indicate an active, fully coordinated transport system in the endothelium for capillary clearance of large molecules. Whether the passage of smaller molecular weight mater-ials, such as  $H_2O$ ,  $Na^+$ , and  $K^-$  is as orderly and by the same route, can only be inferred. Studies by Merrill and Sprinz (10,11). on the passage of injected horseradish peroxidase out of the guinea pig small intestional microcirculation showed that this smaller molecular weight (40,000) and size (30-40Å) tracer escaped via pinocytosis and interendothelial junctions with no sequestration of the tracer in the smooth endoplasmic reticulum. The findings presented here, however, do confirm a specific route for capillary transfer and an increased rate of transfer in two dissimilar diarrheal models, and that this route is essentially the same as in the unchallenged animal. It further implies that the basic, underlying patho-physiology of cholera intoxication may primarily involve the macromolecular and enzymatic rearrangement of the epithelial cells without eliciting overt morphological changes. The effects on the interstitial microcirculation could be a response to the functional epithelial changes.

### *i*. RECOMMENDATIONS:

Based on the current studies reported herein, and the results obtained previously (8,9,10,11), it would seem that the fasted guinea pig is a suitable model for studying viable <u>Vibrio cholerae</u> infections <u>in vivo</u>. The tracer experiments of this three-month study have established the route and rate of passage of larger molecular weight materials out of the intestinal microcirculation and have given further insight into the pathophysiology of choleraic and osmotic diarrheas.

On the basis of these findings it is recommended that the emphasis should now be shifted to the visualization by electron histochemical methods of the target sites of the vibrio exotoxin, especially the permeability factor. The determination of the site of action of these exotoxins has as yet not been resolved. Furthermore these findings suggest that the area of interest could be in the membranous organelles of the epithelium. However, the involvement of the capillary endothelium has not been completely discounted, and corollary studies of enzyme labeled antigen directed at both epithelium and endothelium are strongly recommended.

Another avenue for investigation is the detection and quantitation of ionic concentrations and shifts, especially the Na<sup>+</sup>,  $X^+$ , and Cl , in the epithelium and endothelium of normal, osmotic diarrhea and choleric diarrhea models. Currently the quantitative approach in local subcellular analysis utilizing the electron probe X-ray analyzer appears promising; thus such experiments could be carried out (14). Such a fundamental study, coupled with the detection of the sites of action of <u>Vibrio cholerae</u> exotoxin, would profoundly advance the knowledge of the etiology of diarrheal diseases. This information would be invaluable, not only in understanding and eventually eradicating cholera, but would apply equally as well to the broader understanding of intestinal physiology as well.



Fig. 1. Survey transmission electron micrograph of the basal portion of the epithelial lining and subjacent lamina propria from a normal guinea pig five minutes after I.V. administration of ferritin. The nuclei, endoplasmic reticulum, mitochondria and ribosomes of the epithelial cells are unaffected. There appears to be an increased number of electron dense myelin figures but this can not be correlated with ferritin accumulation. The single capillary present at the lower right margin contains an electron dense plasma precipitate containing dispersed ferritin particles. No overt lesions are apparent in the endothelial cells. X12,500 BRC-TEM 15288



Fig. 2. Higher magnification transmission electron micrograph of an intestinal capillary from a normal guinea pig five minutes after ferritin administration. Most of the ferritin particles remain in the capillary lumen. In this particular field no ferritin is seen either enclosed in pinocytotic vesicles or free in the endothelial cytoplasm. Note that several ferritin micelles (arrows) are present in the cytoplasm of the overlying pericyte. X100,000 BRC-TEM 14714



Fig. 3. Another intestinal capillary from a normal guinea pig five minutes after ferritin administration. Ferritin particles are present in the capillary lumen (L), in dilated cisternae of endothelial endoplasmic reticulum (E.P.) and in the extracellular space of the lamina propria (arrows). Note that the interendothelial junction does not contain ferritin. X75,000 BRC-TEM 14716



Fig. 4. Details of an intestinal capillary from an osmotic diarrhea control animal one minute after ferritin administration. The endothelial cells show no apparent alterations from the normal. Ferritin micelles are evident in the lumen (L), in dilated endoplasmic reticulum cisternae (ER), in pinocytotic vesicles (PV) and as free particles in the endothelial cytoplasm (arrows). In addition, ferritin is also evident outside the capillary in the basement membrane region (B.M.) X75,000 BRC-TEM 15363



Fig. 5. A portion of an intestinal capillary from an 8 hour cholera infected guinea pig 1 minute after receiving ferritin. The capillary organelles appear unaltered. Large numbers of ferritin particles are found sequestered throughout the cisternae of the endoplasmic reticulum network. Particles are also evident on occasion in pinocytotic vesicles (PV) as well as freely dispersed in the cytoplasmic matrix (arrows). Few if any ferritin particles are visible outside the capillary in this particular field. X60,000 BRC-TEM 14957



Fig. 6. Another intestinal capillary from an 8 hour cholera infected guinea pig 1 minute after receiving ferritin. The organellar arrangement of the endothelial cell shows no apparent alteration. Ferritin particles are visible primarily in the dilated cisternae of the endoplasmic reticulum network. However, ferritin is also present in the pinocytotic vesicles (P.V.) and some particles appear to be freely dispersed in the cytoplasm (arrow). Note that there is no ferritin present in the extra-endothelial spaces. X60,000 BRC-TEN 14956 14



Fig. 7. A higher magnification electron micrograph of a portion of a mucosal capillary from an 8 hour cholera animal 1 minute after receiving ferritin. The electron dense ferritin micelles are apparent in the capillary lumen (L), adhering to the red blood cell (RBC) and randomly dispersed in the extra-endothelial space (ES). Note, however, the presence of what appears to be a patent opening or fenestra in the endothelial lining (arrow). Two ferritin particles are present in this area. Such openings were rarely encountered and may represent capillary fenestrae which are out of the plane of section. Note also the aggregates of globular less electron dense material (G) on both sides of the endothelial lining. X100,000 BRC-TEM 14663

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Fig. 8. Another electron micrograph of a field similar to that seen in Figure 7. Multiple electron dense globules (arrows) are confined within the enlarged smooth endoplasmic reticulum cisternae of the endothelium, aligned along the basement membrane area between the endothelial cell and overlying pericyte and outside the pericyte cytoplasm, in the interstitial space. Note also the smaller, more electron dense ferritin particles present in the capillary lumen, accompanying the globular material and in the extracellular space. X100,000 BRC-TEM 14666



Fig. 9. A mucosal capillary 8 hours after cholera infection and 5 minutes after ferritin injection, illustrating endothelial cytoplasmic degeneration and rarefication. The mitochondrion present (M) is swollen, pale and has lost most of the cristae membranes. The usual complement of endoplasmic reticulum is sparse or absent. Several of the slightly electron dense globules are evident in the capillary lumen, endothelial cell cytoplasm and extracellular space (arrows). Note also the random distribution of ferritin micelles in the same area. X60,000 BRC-TEM 14638

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Fig. 10. A similar field of view as in Figure 9. Notice once again the lack of endothelial organelles and cytoplasmic rarefication, the random disposition of ferritin particles within the capillary lumen (L), endothelial cell (EC) and interstitial space (IS), and the presence of the slightly electron dense amorphous globules in the same areas. The crystalline electron dense aggregate in the capillary lumen (arrow) is of unknown origin. X60,000 BRC-TEM 14640

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of ferritin loss was much extended as compared to the experimen-							
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