CURRENT CONCEPTS OF THE PATHOGENESIS OF CHOLERA INFECTION *

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Field trials with humans as well as research with various animal models have demonstrated that both antibacterial and antitoxic immunity can protect against the fluid secretion of cholera infection. These two types of immunity involve different mechanisms and must act at different points in the sequence of events that consitutes the overall pathogenesis of cholera. Empirical efforts to improve anti-cholera immunization have not been very successful. This emphasizes the need to understand the host-parasite relationships that collectively make up the pathogenesis of this infection. For some time data have been coming from a number of laboratories which, when considered together, may provide a basis for more explicit concepts.

This presentation is an effort to synthesize some of these informations into a unified picture of the possible pathogenesis of cholera and the ways in which immunity may interfere in this process. Much of the data have come from work with animal models and there are points where information is lacking. It is also likely that some may disagree with various interpretations and speculations that will be made. However, it is hoped that both the gaps in information and disagreements will stimulate research leading to further progress.

A primary consideration in cholera pathogenesis is the nature of the lumen of the small intestine itself.

This first slide is a section of the small intestine of a rabbit prepared by the usual histological techniques. It shows the intestinal villi, widely gapping intervillous spaces and the crypts of Lieberkuehn. It also shows the epithelial covering of the villi and mucus-producing goblet cells.

However, does the small intestine really look like this? The next slide is from a normal rabbit ileal loop that was injected with carbon particles and after one hour was carefully frozen and sectioned by a slight modification of the method of Savage et al. Here the villi are plump, the intervillous spaces are very narrow and it can be seen that the lumenally injected carbon is separated from the villous area by a layer of mucus. The examination of many such sections from all levels of the small intestine shows that this mucus is quite continuous and, although variable in thickness, usually covers the villi to a depth of 100 microns or more. Its top surface, where it appears to be released into the free lumenal contents, shows globules and channels of the sort described by Jones et al. in *in vitro* experiments but in the intervillous spaces and areas immediately above the villi it is quite continuous. The next two scanning electron microscope pictures were provided by Dr. Schrank. The first is a washed preparation of mouse small intestine showing the villi, intervillous spaces and the openings of the crypts of Lieber-

* Since the full text was not available at the time of compilation, only the manuscript for oral presentation is reproduced here (Editors).

kuehn. The second is a picture of tissue prepared by careful handling and special fixation techniques to keep the mucus layer intact. Even though some of the mucus layer has been removed in preparation, only the tips of two villi are seen. It would appear that a mucus existing in the intervillous spaces is a reality of the normal adult intestine and that bacteria approaching the villous mucosa must do so by penetrating this gel.

This mucus layer is continually sloughing off into the free lumen, and is being replenished by upward flow through the intervillous spaces. Calculations based upon a stylized model indicate that because of the small dimensions of these spaces, if this mucus layer is maintained, the linear velocity of the flow near the villous tips is about five times greater than the rate with which mucus is being sloughed off into the free lumen. Therefore, anything entering the intervillous spaces must move against a considerable current in the opposite direction. Rapid penetration into the mucus layer by bacteria would appear to require some form of mechanical force and the ability to colonize this gel appears to be a property of relatively few kinds of organisms. *Vibrio cholerae* appears to be one of these.

Williams et al. found that *V. cholerae* could penetrate soft agar gels to produce macro-colonies. The next slide shows colony formation in 0.8, 0.6, and 0.4% agar. Phase microscopy sutides of the leading edges of expanding macro-colonies in 0.4% agar demonstrated that penetration was accomplished by multiplication, separation of cells, and random motility of the daughter cells, with process being repeated continuously so that the vibrios spread out through the gel. As seen in the next slide, gel penetration could be inhibited by antibody to boiled cells, non-flagellated cells, or to a Boivin-type LPS-protein somatic antigen complex, which had already been proven to be highly protective for humans in the 1968/1969 field trial in Bangladesh. When antibody is present, instead of separating, the cells remain clumped together through successive divisions spinning erratically but making no progress. This climping has been referred to as growth agglutination and an agglutinate clump may represent the progency of a single vibrio. It does *not* depend upon the aggregation of cells preexisting in large numbers as studied by Freter and Jones. The few cells that separate from the clumps are only weakly motile or non-motile as described by Benenson et al. some time ago. Therefore, inhibition of gel penetration appears to consist of two components, clump formation by growth agglutination and inhibition of motility. Steel et al. have shown that the crosslinking ability of the $F(ab')_2$ portion of anti-cholera antibody is protective in cholera infections in baby mice. Inhibition of motility by an anti-somatic antibody was puzzling at first until it was recognized that the somatic antigen covers both the cell and its flagellum. This slide is an electron microscope picture of a cholera vibrio sensitized with anti-somatic antigen rabbit antibody and exposed to ferritin-labelled anti-rabbit globulin goat serum. The ferritin label is on both the cell and its flagellum.

Schrank and Verwey studied the early events in the protection of rabbit ileal loops using the freezing and sectioning methods of Savage et al. and determined the location of the vibrios with fluorescein-labelled anti-somatic antigen antibody. In other loops in the same rabbits they counted viable vibrios in gentle washings of the intestinal lumen and in the mucus-plus-tussue areas after homogenization in a manner similar to that employed by Freter. In non-immunized animals considerable multiplication took place in the free lumen and the organisms were well separated as shown in this slide. Also, penetration of the mucus layer occurred and here again the organisms appear as isolated fluorescent rods at the tips of the villi in the mucus cell. In animals passively immunized with intravascular anti-somatic antigen serum, multiplication in the free lumen occurs, but the organisms remain clumped together as fluorescent aggregates. Though some penetration of the mucus layer occurs as in this next slide, in general organisms tend to remain clumped together at the tips of the villi. As

ileal loops from normal animals are sectioned longer after initial infection it appears that the organisms make their way down through the intervillous spaces against the direction of the flow of mucus. This slide was from a loop sectioned four hours after infection, with increasing time, tissue sections show increasing numbers of organisms in the intervillous spaces. In immunized animals penetration of intervillous spaces did not occur over the full nine hours in which these experiments were carried on. In other experiments covering 18 hours, fluid accumulation did not occur in immunized animals.

The next slide shows the differences in logarithmic mean vibrio population between lumenal counts and tissue-plus-mucus counts in immunized and non-immunized animals. The lumenal counts are about the same, but the tissue-plus-mucus counts show wide differences which average to indicate about 60 times more organisms in the tissue-plus-mucus areas of normal animals than in immunized animals. Freter obtained similar data but interpreted this to indicate a killing effect of antibody plus tissue. The interpretation suggested here is the failure of orgasnims to penetrate the mucus layer and enter intervillous spaces in the presence of anti-somatic antibody.

Guentzel et al. have recently done similar experiments using the open gut model represented by eight day old mice suckled by normal or immunized mothers. They have confirmed observations concerning aggregation of vibrios in the free lumen and failure of vibrios to penetrate well into intervillous spaces correlated with protection of immunized mice. In addition, in some immunized mice they could find no vibrios in the free lumen suggesting that in the open gut model the organisms may be swept into the large intestine. While the experiments of Schrank and Verwey have used a variety of antigens all but one of which could be expected to contain the protein-lipopolysaccharide somatic antigen complex. This one is a flagellar core preparation. Antibody to this antigen protects baby mice but not as well as antibodies to crude flagella. Yang et al. also have isolated flagellar cores. Antibody to these reacts with intact flagella but not with cell bodies or LPS, forms loose aggregates of vibrios and renders motility rotational and non-directional. This antibody also protects ileal loops when given intralumenally.

There appear to be two protective antigens now described whose antibodies diminish motility and contribute to growth agglutination—one the protein-LPS complex and the other a flagellar core-associated antigen.

It should be emphasized that these experiments are relevant to penetration into intervillous spaces and its inhibition by antibody. Sequentially, this is considered to be the first or primary step in pathogenesis. If vibrios can not reach the mucosal cells, questions of adherence or short range toxin delivery become immaterial.

Immunity is quantitatively variable and in the non-immunized or poorly immunized host colonization of the mucus layer and penetration into the intervillous spaces does occur. Association of vibrios with mucosal cells has been described by many investigators. Recently Jones et al. have demonstrated adherence to isolated intestinal epithelial brush borders. This has been confirmed by Schrank using suspensions of isolated guinea pig whole mucosal cells. Surprisingly, adherence is temporary and is followed by release. Jones and his co-workers believe that the flagellum is the organelle of attachment and have coined the name "adhesin" for the substance that they speculate may be carried on the flagellum. Since L-fucose and various glycosidic moieties containing L-fucose block *in vitro* adherence, they postulate that this sugar may be a functionally dominant component in the mucosal cell binding site. Schrank has found that Inaba protective somatic antigen also blocks adherence. These ideas, combined, offer the possibility that adherence of vibrios may be to a mucosal cell site containing L-fucose and the substance on the vibrio reacting with the receptor is the protective somatic antigen. To date, I know of no *in vitro* experiment with isoalted mucosal cells showing whether anti-somatic

antigen antibody interposed between the vibrio and the brush border binding sites would inhibit vibrio adherence. The *in vitro* adherence situation is somewhat confused since Freter and Jones could demonstrate no blocking effect from L-fucose in adherence studies with intestinal tissue slices nor did they observe vibrio disassociation. However, they were able to show blocking by an intestinal tissue digest and the inhibition of adherence by antisomatic antibody. Freter reported similar antibody effects with intestinal tissue slices in previous experiment. Though somewhat clouded by technical problems, there is a strong suggestion that anti-somatic antigen antibody inhibits vibrio adherence. This could be an important second effect in preventing pathogenesis.

Nelson and Finkelstein have recently done electron microscope studies of the colonization of intestinal epithelium by *V. cholerae* using both ligated ileal loops of adult rabbits and the open gut infant rabbit model. They have demonstrated the occurrence of adherence beginning about one hour after infection and reaching a maximum between four and seven hours. There was then a rapid decline in the number of bound organisms until after twelve to sixteen hours only a few vibrios could be found on the mucosal surface. Though differing in time frame from the *in vitro* studies, these workers also appear to have domonstrated adherence followed by disassociation. Schrank has also demonstrated adhesion *in vivo* and the next slide is a scanning electron microscope picture of mucosal cells at the base of a villus in the adult mouse model, clearly showing vibrios adhering flatly to the villus. This tends to support the view of Nelson and Finkelstein and suggestion of Schrank that adherence is not necessarily a function of an exclusively flagellar substance.

It is obvious that any toxin produced by these cells would be delivered almost directly to the receptors on the mucosal cells through which cholera toxin is known to exert its effects in producing hyper-activation of the fluid and electrolyte secreting mechanisms. There is a lag period prior to the initiation of fluid secretions but once the cells have been influenced by cholera toxin this intoxication is long lasting.

It could be speculated that possibly about the time when disassociation of vibrios takes place, fluid secretion into the intervillous spaces may begin. This would be expected to accelerate the removal of both mucus and detached organisms and empty the material into the free lumen where it is swept into the large intestine. Fluid secretion would be expected to continue even in the absence of further toxin input.

In a synthesis of all of the information that has been discussed up to this point it is suggested that the pathogenesis of cholera infection may be approximately as follows:

1) Lumenal multiplication
2) Mucus layer invasion
3) Penetration of intervillous spaces
4) Adherence of vibrios to mucosa
5) Toxin production near mucosal receptors
6) Disassociation of vibrios from mucosa
7) Beginning of fluid secretion
8) Removal of organisms and mucus
9) Continuation of fluid secretion
10) Mucosal cell replacement

It is not suggested that the time for this to occur throughout the whole gut is as short as observed with animal models where large numbers of organisms are put into small areas of intestine. Rather, it is visualized to be a sequence that in the human exposed to nautral inocula is initiated and terminated in a time-variable manner in different areas throughout the gut which may take several days to reach its maximum, and organisms may continue to be shed in relatively large numbers for several days more.

Non-flagellated toxigenic vibrio variants can produce infection in various animal models but 100 times or more larger doses are required than their parent motile strains. These variants do not penetrate soft agar gels. They are much less able to penetrate into intervillous spaces and apparently are less adherent to the brush borders or mucosal cells. They are, therefore, at a disadvantage both in mucus gel penetration and in ad-

herence so may not be as useful in separating the mechanisms of vibrio pathogenesis as was hoped originally.

Cholera toxin placed in the free lumen of the small intestine causes fluid accumulation. Although Williams has shown that *in vitro* toxin production is not inhibited by antibacterial antibody, conditions that limit vibrios to the free lumen do not permit fluid accumulation even when the lumenal vibrio population is very large. Fluid secretion occurs only when vibrios come into close association with mucosal cells. At present, antibacterial antibodies are the only known inhibitors of mucus gel and intervillous penetration, and vibrio adherence. Yet, complete protection against overt cholera infection has been produced with cholera toxoid essentially free of bacterial antigens. The *in vivo* behavior of vibrios in the presence of antitoxin alone is yet practically uninvestigated. There would seem to be little opportunity for antitoxin action on delivered by vibrios on mucosal cells as seen in a previous slide and as demonstrated by Nelson and Finkelstein. However, as a speculation, toxin as it is secreted is probably a component of the cell wall and as suggested by Nelson and Finkelstein, may even assist in adherence through the mucosal toxin receptors. Therefore, in addition to tis neutralizing effect on free toxin, antitoxin may have to some degree properties of an antibacterial antibody in preventing mucus gel penetration and adherence. Antitoxin and antibacterial antibody also may collaborate in interfering with adherence. All of these antibacterial-type actions would tend to dimensionally separate toxigenic vibrios from the mucosal toxin receptors and improve the opportunities for toxin neutralization. Obviously, much more information is needed concerning the role of antitoxin in the prevention of overt vibrio infection.

However, regardless of speculations, Svennerholm and Holmgren have reported an approximate 100 times greater protection of rabbit ileal loops against live vibrio challenge when somatic lipopolysaccharide and choleragenoid are used as co-immunogens. This has now been confirmed by Peterson using toxoid. Both groups agree that the two antigens do not seem to interact as adjuvants. Therefore, it appears that enhanced protection may result from separate but co-operative actions of antibacterial and antitoxic antibodies in the intestine. This form of co-immunization has been envisioned at least since the conference in Hawaii in 1965. It would now appear that there is experimental evidence to justify intensive study including the consideration of field trials in human populations. The degree and duration of any enhanced protection can not be predicted from animal model systems.

The mechanisms of cholera pathogenesis discussed here have implications beyond considerations of cholera immunity.

Some of these provide connection with other non-immunological problems and observations concerning cholera. Ryder et al. have found that persons recovering from non-cholera diarrhea (where their intervillous spaces were probably cleared of mucus and their goblet cells exhausted) are very much more susceptible to cholera. Mucus may be a natural protective mechanism and the functional location of whatever antibody immunity the host may have. The question of other diarrheal disease prior to cholera infection has not yet been investigated systematically as a possible determinant of who becomes infected and who does not in an environment containing cholera vibrios.

Palmer et al. have found that malnourished persons have a significantly prolonged duration of cholera diarrhea whether they receive tetracycline or not. They suggest that the slower replacement of intoxoicated mucosal cells may be the malnutrition effect that is responsible for this prolongation of diarrhea.

Gastric hypo-acidity is already associated with susceptibility to cholera. In view of the hypothesis of cholera pathogenesis that has been assembled here other clinical conditions, particularly those associated with malnutrition and malabsorption should be investigated in relation to cholera susceptibility.

The techniques for investigations both in models and to some extent in the human

are available. It now becomes a question of time and investigator interest to obtain additional answers.

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DISCUSSION

Dr. Murphy: Can you tell me if L-fucose will chemotactically attract vibrio?

Dr. Verwey: No, I cannot.

Dr. Finkelstein: I think you gave very nice and dramatic presentation of potential sequence of events, and I have only trivial arguments with entire sequence. But I find it difficult to understand how antibody against vibrio flagellin will inhibit motility if, in fact, cholera vibiro flagellum is a sheathed organ. So I think one would have to look carefully at those reports to determine whether or not flagellin was entirely free of very antigenic immunogenic somatic antigen.

Dr. Verwey: The unpublished paper of Yang, Gordon and Freeman takes that point quite specifically. It is true that there is this coat material, if you might say somatic antibody, but I demonstrated that antibody against the flagellum core material will apparently react even through that, and they have used labled antibody to show that it does not react with cell body itself.

Dr. Finkelstein: My understanding is that they have extremely purified flagellin and found that anti-flagellar antibody does not react with intact flagellum.

Dr. Verwey: This is surprising observation to Dr. Shrank who started out the work feeling the same way.

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