ANTIGENIC RELATIONSHIPS AND TOXICITY OF MUCINOLYTIC PREPARATIONS FROM *VIBRIO COMMA* AND RELATED VIBRIOS

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The products of infection with *Vibrio comma* (*Vibrio cholerae*), which are responsible for the severe purging diarrhea characteristic of the disease, have not yet been clearly demonstrated. To provide a rational basis for development of more efficient immunization practices, such information on the relation of vibrio products to specific pathology is required. Various kinds of toxic materials from the vibrio cell have been described by Burrows *et al.* (1944*a*, 1944*b*, 1944*c*), which may be implicated in pathologic processes of cholera, and recent observations focus attention on enzymatic activities of vibrios. Burnet and Stone (1947), Singer *et al.* (1948), Narayanan *et al.* (1953), and Gurkipal *et al.* (1953) have reported that filtrates from vibrio cultures cause sloughing of epithelium from isolated strips of guinea pig ileum, and that this effect is neutralized by specific antiserum. Also, Lam and Mandle (1954) have demonstrated that such filtrates increase the permeability of isolated intestinal loops from the mouse. Further studies of these activities have led to immunological characterizations of vibrio mucinases (Jensen, 1953; Freter, 1955) and to consideration of the possible effect of their incorporation in cholera vaccines.

Thus, the relationship of vibrio mucinolytic activity to pathogenesis currently is a key question. Determination of whether there is a rise in titer of mucinase-inhibiting antibody in sera of cholera patients would seem to be a reasonable approach to a study of the problem. However, the variable results obtained by others (Goodner, 1955 *personal communication*) suggested that, perhaps, before meaningful results could be expected, the testing procedures themselves needed investigation. It seemed possible that antigenic differences in mucinases of different strains of vibrios could explain some of the observed variability of results, and that the choice of preparations of mucinase to use for tests with human sera might be of critical importance.

An attempt has been made, therefore, to study mucinase preparations from a number of vibrio strains as to their antigenic relationships as well as their other properties, including particularly toxicity for animals.

MATERIALS AND METHODS

Vibrio strains. Mucinolytic preparations obtained from cultures of the following strains were studied: (a) Four strains of vibrios, Ogawa type, isolated in 1953 from cases of cholera in Calcutta, were received from Dr. William Burrows, Chicago. Strain C 441 was from a fatal case; and strains C 172, C 173, and C 175 were all from one patient who recovered later. (b) Three Inaba type strains were obtained from National Institutes of Health: Strain 20-A-14 (Hong Kong, 1939), 20-A-10 (Calcutta), 4Z (Teheran). (c) Other strains studied included El Tor strain 40 from N. I. H., and two strains of water vibrios isolated in 1953 in the vicinity of Chicago (LC and JP) obtained from Dr. Burrows.

Test preparations. Organisms were grown on brain-heart infusion agar for 24 hr at 37 C. Whole agar cultures were crushed and pressed in gauze; fluids were collected and residues discarded. In early experiments, bacterial growth was carefully removed from the surface of the agar prior to pressing, but this step was shown to be unnecessary and, therefore, omitted. Collected fluids were centrifuged at 3,000 rpm for 30 min; the sediment was discarded and the supernatant retained. This preparation, referred to as fraction V (according to a reference system developed during a series of experiments seeking to concentrate and purify mucinase), is similar to preparations described by Burnet and Stone (1947). Concentration and partial purification of fraction V was obtained by precipitation with 60

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per cent acetone at 4 C. The precipitate was dissolved in physiological saline (0.85 per cent NaCl), and the resultant solution was called fraction VII.

Fraction I was prepared by extracting washed, intact vibrio cells in the cold (5 to 8 C) with M/2 trichloracetic acid according to the method of Burrows (1944); this was called by him "endotoxin."

All preparations were preserved with merthiolate (1:10,000) and stored after lyophilization.

Antisera. Rabbits were injected intravenously with increasing amounts (0.25 to 1.0 ml) of fractions V or VII at 2 to 3 day intervals for 3 weeks. Animals were bled 10 to 14 days after the last injection.

Standard ovomucin. Substrate for mucinase activity of the fractions was derived from fresh hens' eggs, prepared essentially as described by Young (1937), and standardized to test concentration as previously reported (Jensen 1953).

Test for mucinase activity. A test for mucinolytic activity against ovomucin is basic to the present experiments. A procedure was described by Burnet (1949) in which unaltered ovomucin was demonstrated by precipitation with protamine sulfate. This method was followed with a few modifications. Serial dilutions of test materials in buffered (0.01 M phosphate, pH 7.2) physiological saline were mixed with ovomucin and incubated at 37 C for 30 min. Addition of protamine sulfate did not induce a precipitate in tubes which contained sufficient mucinase to alter the ovomucin. Thus, one unit of mucinase activity was defined as that contained in the highest dilution of test material which destroyed ovomucin.

Mucinase inhibition. To measure mucinase inhibitory antibody, serial dilutions of serum were mixed with an equal volume (0.25 ml) of a mucinase preparation standardized to contain 4 units of mucinolytic activity. After incubation at 37 C for 30 min, the mixture was tested for residual mucinase by adding 0.25 ml of the ovomucin. Ovomucin precipitating with protamine sulfate indicated the presence of sufficient antibody to inhibit the mucinase, while failure to inhibit was indicated when no precipitate developed. Antigenic comparisons of fractions from several strains of vibrios were readily carried out in this manner.

RESULTS

Serological relationships of mucinases. Table 1 presents results from a typical experiment. Here mucinase from vibrio strain 4Z was inhibited by homologous antiserum at a titer of 1:128; and by antiserum C 175 at 1:32. In contrast, mucinase C 175 was not inhibited by antiserum 4Z, although it was effectively neutralized by its homologous serum. This clearly indicates that mucinases from the two strains are antigenically related, but distinct.

When dilutions of rabbit antisera prepared against fraction V from eight different strains were tested with these mucinolytic materials, the titers obtained with heterologous fractions ranged from less than 1:2 to 1:256. The absence of inhibitory effect in certain antisera produced against fractions obtained from other strains of vibrios was interpreted to mean that significant antigenic differences exist between these materials. Results of all cross tests completed indicate that mucinases from the various strains studied appear to fit into three serologically distinct groups, designated M1, M2, and M3 in table 2. Reactions obtained with the products of strains antigenically characteristic of each group are listed. This grouping appears to be independent of antigenic types based on somatic and flagellar antigens of the bacterial cells, since both Inaba and Ogawa types produce mucinases which may belong in either M1 or M2 group. The two strains of water vibrio produced mucinases which were antigenically similar to M2 mucinases, but were separated into group M3 by failure of their

TABLE 1

Mucinase inhibition tests

Mucinase* Derived from Vibrio Strain No.	Antiserum against Fraction V	Neutralization† of Mucinases							
		Serum dilutions							
		2	4	8	16	32	64	128	256
4Z	4Z	+	+	+	+	+	+	+	0
	C175	+	+	+	+	+	0	0	0
C175	4Z	0	0	0	0	0	0	0	0
	C175	+	+	+	+	+	+	0	0

* Standard test dose: 4 units of mucinase activity.

† Neutralization was indicated by ovomucin precipitate which, in turn, indicated removal of mucinase by antiserum.

TABLE 2

Grouping of mucinases based on results of cross inhibition tests

Group	Mucinase Derived from Strain	Antisera against Fraction V of Strains in the 3 Groups							
		Group M1			Group M2			Group M3	
		4Z	40	20-A-14	C441	C175	Inaba 1	LC	JP
M1	4Z Ogawa	128	64	32	64	32	32	0	0
	40 E1 Tor	32	64	16	128	16	16	0	0
	20-A-14	64	—	64	256	64	—	—	—
M2	C441 Ogawa	0	0	0	256	256	64	32	256
	C175 Ogawa	0	0	0	256	64	64	256	256
	Inaba-1	0	0	—	64	32	64	32	64
M3	LC water vibrio	0	0	0	128	128	128	128	16
	JP water vibrio	0	0	0	256	256	256	256	256

0, no inhibition at 1:2 dilution; —, not tested.

antisera to react with group M1 mucinases. These classifications were based on results of tests which included only 12 strains and further studies may demonstrate additional groups. Perhaps these data could be interpreted to provide evidence for the hypothesis advanced by Freter (1955) that two mucinases occurring in varying combinations in preparations from different strains are responsible for antigenic variation. The schematic interpretation in table 3 will serve to illustrate this point. The previously discussed results suggest that antigenic experience with strains producing M1 mucinase would not induce antibody reactive with M2 or M3 mucinases. Similarly, M3 mucinases did not stimulate antibody inhibitory for M1 mucinase. In contrast, antisera produced against M2 mucinase inhibited mucinases produced by any of the strains studied. These relationships appear explainable if it is assumed that groups M1 and M3 comprise preparations containing purely types A or B mucinase and group M2, a mixture of types A and B. Thus, antisera for group M1 preparations containing only anti-type A mucinase would not completely inhibit representatives of groups M2 or M3, but antisera prepared against M2 mucinases would inhibit any combination of types A and B mucinases. Reactions between the M2 and M3 groups present an interesting challenge to this hypothesis. That is, antisera against M3 mucinases (B) would not be expected to completely inhibit a mixture of A and B mucinases suggested as the case for group M2 preparations. If the mixtures were predominantly B mucinase, however, the test reagent diluted to the 4 unit level might contain only B mucinase and, therefore, give reactions with anti-B mucinase sera. Thus the data obtained indicate that mucinolytic preparations from vibrio cultures may be classified into 3 different groups, of which one group is characterized as containing varying mixtures of two antigenically distinct mucinases.

TABLE 3

Schematic interpretation of antigenic relationships among mucinase groups

Mucinase Group	Antisera Produced against Mucinase Groups		
	M1 (A)	M2 (A + B)	M3 (B)
M1 (A)	+	+	0
M2 (A + B)	0	+	+
M3 (B)	0	+	+

+, neutralization; 0, none.

In any case, it is evident that the selection of mucinases for the detection of antibody in human sera should be governed by these observed antigenic differences. The above data suggest that inclusion of one mucinase from group M1 and one from group M2 in the test antigen should suffice to demonstrate antibody produced in response to antigenic experience with mucinase from any strain of vibrio. In addition, it appears

that human experience with a mucinase of group M2 should be expected to provoke anti-mucinolytic activity against any strain of vibrio.

Toxicity of preparations. Other studies aimed at defining the action of vibrio culture fractions on the intestinal epithelium of laboratory animals have led to the observation of a previously undescribed toxin. Mucinolytic preparations from many strains have been observed to produce a diffuse peritonitis after intraperitoneal injections in monkeys, rabbits, guinea pigs, and mice (table 4). Deaths occurred in 12 to 48 hr with characteristic hemorrhagic lesions on the peritoneum and visceral surfaces. A toxic effect was also demonstrable by intracerebral injections in mice or by allantoic inoculations of embryonated chicken eggs. Intradermal injections in rabbits and guinea pigs provoked acute inflammatory reactions with ulceration and sloughing. Although fraction I (the endotoxin of Burrows) also killed mice, the pathological lesions it produced were not as described above. Furthermore, the endotoxin could be differentiated from the presently described toxin in fraction V or VII on the basis of physical-chemical properties. Burrows (1944*a*) described the endotoxin as a polysaccharide-lipid complex which was soluble in ether, stable in acids but destroyed at high pH and readily dialyzable. In contrast, the toxic component of mucinolytic preparations is not soluble in ether or precipitable by acetone and alcohol, and it is unstable at low pH and non-dialyzable.

In general, this toxicity was observed only with preparations which contained mucinolytic activity. An exception was noted with one vibrio strain (4Z) in which fractions were not toxic even though high levels of mucinase were demonstrated. In table 5 it is noted that fraction VII from this strain had a mucinase titer of 1:512, while no intracerebral toxicity for mice was found at 1:2 dilution. Preparations of the same type from strains C 175 and JP had lower mucinase titers but were definitely toxic for mice. Similar results were obtained with fraction V from these strains. Non-toxic preparations containing mucinase activity have been reported previously (Jensen, 1953), and were obtained from a strain similar in this respect to 4Z.

Toxicity of the preparations can be specifically neutralized by antisera as shown in table 6. Here antisera prepared against fraction V from the indicated vibrio strains were tested with fraction VII from C 175 which contained both mucinase and toxic activities. Each antiserum except that for the 4Z fraction precipitated with test materials and neutralized mucinase and toxicity. Although precipitation and neutralization of toxicity were obtained only with low dilutions of antisera (1:4), pre-immunization sera, in all instances, failed to neutralize either mucinase or toxicity. These results suggest at least two pos-

TABLE 4

Toxicity of preparations with mucinolytic activity

Animals	Injection Route	Results
Mice, monkeys, rabbits, guinea pigs	Intraperitoneal	Death (12–48 hr) with hemorrhagic peritonitis
Mice	Intracerebral	Death (12–24 hr) with cerebral hemorrhages
Chick embryos	Allantoic	Death (12 hr) with diffuse petechial hemorrhages
Guinea pigs and rabbits	Intradermal	Acute inflammatory reactions in 4–6 hr

TABLE 5

Mucinolytic activity and toxicity of fraction VII from three strains of vibrios

Fraction VII, Vibrio Cultures		Intracerebral Toxicity in Mice				
Strain number	Mucinase titer	D/T* with different dilutions of fractions				
		1:2	4	8	16	32
4Z	512	0/5	0/5	0/5	0/5	0/5
C175	128	5/5	5/5	5/5	2/5	0/5
JP	64	5/5	5/5	2/5	0/5	0/5

* Number of mice dying/total injected.

TABLE 6

Neutralization of toxic and mucinolytic activities

Antisera against Fraction V from Vibrio Strain No.	Fraction VII, C175: Neutralization of	
	Mucinase	Toxin
C175 (M2)	+	+
C441 (M2)	+	+
JP (M3)	+	+
4Z (M1)	0	0

TABLE 7

Mucinolytic and toxic titers of Fraction VII from growth of Vibrio Strain C 175 on various media

Growth Media	Activities of Fraction VII	
	Ovomucinase titer	Toxicity titer*
Nutrient broth	2	0
Broth + 0.1% agar	64	2
Broth + 1.0% agar	1024	8
Broth + 0.5% caragen	128	8
Broth + 2.0% gum ghatti	512	8
Broth + silica gel	128	4

* Reciprocal of highest dilution at which mice died after intracerebral injections.

sibilities: (1) the fraction from 4Z contained no toxin and, therefore, no antitoxin was stimulated, and (2) antigenic dissimilarity of mucinases is related to the presence or absence of an antigenic radical which is toxic. A limited series of experiments has been carried out to characterize the general chemical natures of the two activities. Thus, each activity is destroyed by heating at 80 C for 10 min and by digestion with trypsin and neither one is dialyzable or ether soluble. Both factors can be concentrated by acetone precipitations, while trichloracetic acid precipitates and destroys both activities. It is possible, however, to treat gently with 0.3 per cent formaldehyde (6 to 12 hours at 24 C) and cause a marked reduction of toxicity without affecting mucinase titers. These results suggest that toxicity and mucinolytic activity derive from molecules of somewhat similar chemical natures; however, much more work must be done before a conclusion can be drawn regarding the precise relationship of these activities.

The evidence at hand concerning the nature of the toxic factor does not permit definite conclusions as to whether this is an endotoxin or true exotoxin. Toxicity is found in fluids extracted from the agar medium after the vibrio culture has been removed, suggesting a material released into the agar by the vibrios and, therefore, an exotoxin. On the other hand, these organisms are known to be autolytic and the toxicity might then be due to products of autolysis. Neutralization of toxicity by antiserum also suggests a true toxin, but the low antitoxin titers are not entirely convincing. It is hoped that results of future studies will permit a clearer characterization of the toxin.

Factors influencing production of mucinase and toxin. Data listed in table 7 indicate the status of information about certain factors influencing the production of mucinase and toxin in vibrio cultures. Broth cultures of vibrios did not produce significant levels of either activity, but the addition of small amounts of agar or certain other polysaccharides stimulated production of both activities. These results suggested that suitable substrates might call forth the mucinase as an adaptive enzyme. However, growth on a silica gel medium, which did not contain such substrates, also resulted in fluids which were both mucinolytic and toxic, suggesting that certain physical factors may be involved. Perhaps the agar or silica gel provides an absorbent for the products of vibrio metabolism and protects these active materials from denaturation. It may also be postulated that perhaps both suitable substrate and physical conditions needed for elaboration of mucinase and toxin are present in the intestines of the cholera patient.

SUMMARY

Evidence has been presented which indicates that ovomucinases produced by strains of vibrios can be antigenically differentiated. Active preparations from the 12 strains studied comprise three different groups. A previously undescribed toxic activity of vibrios has been found in fractions containing mucinolytic activity. Further work is required to determine the precise relationship between mucinolytic and toxic activities, and whether they are involved in the production of the disease cholera and, most importantly, if these factors are of significance in the immunology of cholera.

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