IMMUNOLOGIC ASPECTS OF CHOLERA

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It may well be that cholera can be controlled only by the combined devices of education, sanitation and immunization. The immunologic approach still offers many difficulties both theoretical and practical. It is the purpose of this report to present studies based upon the immunologic analyses of the sera of cholera patients and of the sera of animals immunized by various methods. A preliminary statement has already been made (Goodner *et al*, 1960).

The possibilities for serologic diagnosis of cholera have been greatly neglected. Possible explanations are (a) discouraging early findings, due perhaps to the use of bacterial suspensions in which the antigenic patterns had been altered by some method of "preservation," (b) poor choice of strains for agglutinin tests, (c) lack of proper laboratory facilities in cholera areas, or (d) lack of opportunity for securing proper serum specimens from convalescent patients.

The historic aspects of this subject are reviewed by Pollitzer (1959) in his comprehensive treatise. The early observations by Greig (1915), Shiiba and Oyama (1920) and Tagami and Watanabe (1920) gave an excellent basis for the serologic approach but this aspect came into neglect possibly because many subsequent workers found rather low titer of antibodies. Most writers on the subject of cholera omit this approach entirely and state that the diagnosis must be made bacteriologically. A second system of laboratory diagnosis would, however, have a considerable value if it would assist in (a) determining the etiologic relationship of vibrios sometimes isolated in instances of mild diarrhea, or (b) by establishing validity of the diagnosis in instances in which there was failure of direct isolation for any reason. In any cholera epidemic the diagnosis is all too often on purely clinical grounds. A basic confusion arises from the fact that clinical diagnosis gives a case rate about five times the truth during epidemics and about one fifth of the truth in non-epidemic periods.

Obviously if the older concepts as to the appearance of antibodies in convalescence should prove faulty and it were possible to actually demonstrate the development of antibodies, it should be possible to analyse the antibody response with reference to the antigens of *Vibrio cholerae* and thereby approach directly the question of an artifical immunization appropriate to mimic the consequences of natural infection.

Experimental

The clinical material here employed was derived from the cholera epidemic in Bangkok in 1959. The patients involved were studied by R. Freter, H. L. Smith and F. J. Sweeney at the Chulalongkorn Hospital. Bacteriologic examination of stools was carried out in the field,all isolates being returned to this laboratory for verification. A sample from each fecal specimen was streaked on four types of gelatin-agar, media, brain-heart infusion agar, desoxycholate agar, and Salmonella - Shigella agar. The gelatin-agar media, modified from the medium of Smith and Goodner (1958), were employed for differentiation of gelatinase-positive organisms. After incubation of cultures at 37°C, colonies resembling *V. cholerae* were selected and checked for identity by slide agglutination using *V. cholerae* Inaba and Ogawa antisera. Those colonies which gave positive agglutination were picked, subcultured and returned to The Jefferson Medical College. In the base laboratory, the isolates were examined both as to fermentative and immunologic properties. It may be stated that *V. cholerae* was isolated from fecal samples in only 21 of 80 "cholera" admissions. There was in the field some doubt as to the success of the methods employed because of this low rate of recovery of the organism. However, as will be shown, the methods proved entirely adequate.

Serum samples obtained from patients were immediately frozen and were so maintained until examined by us.

The vibrio cultures selected for immunologic work were representatives from our collection of over 500 strains. Of these some 300 are regarded as *V. cholerae*. The individual characteristics of these have been exhaustively studied by both biochemical and serologic methods.

A long series of preliminary agglutinations was undertaken to establish optimal procedures. Suspensions of vibrios were prepared with preservation by formalin, phenol, alcohol and heat. None of these, in our hands, gave satisfactory results with sera of rabbits

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immunized with various experimental vaccines. The subject achieved clarity only when suspensions of living vibrios were employed. This conclusion was reached by D. C. Lahiri (1959) at about the same time.

Cultures for agglutination procedures were grown at 33°C for 18 hours in broth consisting of trypticase, 1 per cent; sodium chloride, 1 per cent; pH 7.1. These suspensions were diluted with sterile broth to attain a turbidity level of McFarland standard No. 1.

At this point it became apparent that even with living vibrios a second element was of considerable importance, i. e., choice of strains. Apparently the antigenic composition of strains varies quantitatively and perhaps in matters of stability. Certain it is that strain selection is an important point. The strains employed during phases of this work are present in Table 1.

Agglutinations were set up in the standard fashion using equal volumes of serum dilutions and diluted 18 hour broth cultures of appropriate vibrios. The combinations were held at 41°C for 1 hour and the results were then read. It should be noted that agglutination occurs very rapidly. These were well delineated and were in all respects representative of O-type antigen-antibody reactions. Check readings were made after incubation at 4°C for 18 hours.

Sera were titered for both group and type specific antibodies using the agglutination procedure just described. For determining the group antibodies, the patients' sera were tested with the six Ogawa and five Inaba strains listed in Table 1. Two strains of water vibrios were employed as controls. The group antibody titer was the mean of the individual titers against the 11 cholera strains. Each of the cholera strains was readily agglutinable by antisera containing group antibody as determined with thirty specific antisera (rabbit).

For agglutinin absorption 0.5 ml serum samples were mixed with 9.5 ml of living bacterial suspensions. Suspensions were prepared by washing the growth of a given strain from agar cultures using broth cultures of the same organisms. The absorption then was with organisms from both liquid and solid culture media. The trypticase - NaCl medium is considered essential to successful work. At this point the primary serum dilution was 1-20. The material was then held at 4°C for 24 hours; centrifuged and the supernatant decanted to another tube containing a packed mass of the same bacterial cells from both liquid and solid cultures. After a futher holding at 4°C for 24 hours this last procedure was repeated. Thus each serum was absorbed 3 times at a primary dilution level of 1-20.

Since the serum samples were obtained in the Bangkok epidemic of 1959 it seemed appropriate for absorption to use *Vibrio cholera* strains isolated there. The Ogawa used for absorption is designated in our series as "C", in Inaba as "H". Each serum sample was absorbed with both Inaba and Ogawa. Either vibrio should remove the group antibody from the serum but only the homologous type antibodies. It is therefore possible to estimate relative content of these antibodies within the limits imposed by the technical procedures.

Table 1

Vibrio strains employed

Designation	Type	Source
A	V. cholerae - Ogawa type	Campbell Hospital Calcutta 1953
B	V. cholerae - Ogawa type	NIH 41; India 1941
C	V. cholerae - Ogawa type	Bangkok Epidemic 1958
D	V. cholerae - Ogawa type	Bangkok Epidemic 1958
E	V. cholerae - Ogawa type	Ogawa type original, Japan
F	V. cholerae - Ogawa type	El Tor 34 D-9 (NTCC6550)
G	V. cholerae - Inaba type	NIH 35A3; Kasauli
H	V. cholerae - Inaba type	Bangkok Epidemic 1958
K	V. cholerae - Inaba type	P93A, Old stock culture, origin unknown
L	V. cholerae - Inaba type	Inaba type, original, Japan
M	V. cholerae - Inaba type	El Tor; Tor A
N	Water vibrio, Heiberg I	Old stock
P	Water vibrio, Heiberg II	Isolated by Freter in Chicago

Table 2

Agglutinin titers of patients' sera against various vibrios

No. Csae	Remarks	Serum Sample Day of Disease	*V. cholerae Ogawa* A	D	F	*V. cholerae Inaba* K	H	M	Water vibrio N	P
23	No history of immunization	1	<40	<40	<40	40	<40	<40	<40	<40
	V. cholerae	4	<40	<40	<40	160	<40	<40	<40	<40
	Ogawa isolated on days 2,	7	640	640	640	640	160	160	<40	<40
	5; not thereafter	10	640	2560	2560	2560	2560	2560	<40	<40
		13	2560	2560	640	640	640	640	<40	<40
63	Claimed immunization	1	40	<40	40	80	<40	<40	<40	<40
	injection 10 days before onset.	4	<40	<40	40	40	<40	<40	<40	<40
	V. cholerae Ogawa isolated	6	40	40	160	640	40	160	<40	<40
	on days 1, 2; not thereafter	13	640	160	640	640	160	640	<40	<40

Results

The problem of obtaining suitable serum samples during the convalescent phase proved particularly difficult due to the local necessity of freeing bed space. In only 27 of 80 patients was this effort successful but in many of 27 a good series of samples was obtained. To illustrate the general type of immunologic findings, the results of attempted retrospective diagnosis are shown in Table 2.

The results shown would indicate the following possibilities:

1. A significant rise in antibody titer occurs in cholera.
2. This begins on the sixth or seventh day.
3. The type of infection (Ogawa or Inaba) cannot necessarily be inferred by direct agglutination.
4. In persons previously immunized there appears to be a complete absence of anamnestic effect.

These impressions as described with these two patients are completely borne outby observations on the sera of all 27 patients. Many inferences can be drawn.

The total diagnostic experience with these 27 patients is shown in Table 3. From these results it is seen that a complete correlation exists between serologic diagnosis and that made by careful isolation studies. The results are mutually supporting. It would not have been surprising had the positive serologic series been larger than the positive isolation series but it should be recalled that the isolation effort was elaborately intensive in this instance. It is usually not so.

Antibody patterns of diarrhoeal patients on admission. In Thailand an intensive program of immunization had been carried out using largely an Ogawa vaccine prepared with recently isolated strains. There had been a considerable propaganda effort; the people were therefore quite conscious as to this subject and the historical record of immunization or non-immunization was probably of significance.

From the results shown in Table 4, it will be noted that among 12 persons who claimed to have avoided immunization only 1 showed antibodies to *V. cholerae*. In this instance there were no type specific antibodies. With 44 persons who claimed immunization there was a significant level of group antibody in 39 instances but

Table 3

Diagnostic serologic results as compared to results of isolation methods

History of Immunization	Vibrio cholerae isolated from fecal samples	Patients in group	Patients showing four-fold rise in agglutinin titer
None	Positive	6	6
	Negative	2	0
Claimed	Positive	9	9
	Negative	10	0

Table 4

Admission antibody patterns Cholera wards; all patients)

	Number	Group		Ogawa		Inaba	
		+	−	+	−	+	−
Claimed non-mimunized	12	1	11	−	12	−	12
Claimed immunized	44	39	5	4	40	−	44

type-specific antibody (Ogawa) was found in only 4. It is therefore apparent that immunization with the usual cholera vaccine is likely to produce group specific antibodies in many individuals but that significant type-specific antibodies appear in only a small proportion. Obviously this point requires further study.

When this matter is approached from another angle, that of diagnosed disease, a more important concept is developed as shown in Table 5. Twenty of these individuals suffered from cholera as judged by both bacteriologic and clinical evaluation. Of these, 11 showed significant titers of group antibody on admission; none possessed type specific antibody. Of 36 non-cholera patients, 29 possessed group antibody and 4 type specific antibody. From these results one might question the significance of the presence of group antibody as protective against the disease. One might suspect protection to be associated with type-specific antibody.

Antibody patterns in convalescence from cholera. With the 20 patients who had cholera it was possible to collect convalescent serum specimens in only 14 instances but from some of these a number of specimens were obtained. In all of these cases the total antibody response was 4-fold or better. The data presented in Table 6 show that a rise in group antibody occurred in all and that a significant proportion developed type specific antibody for Ogawa, the type associated with their disease.

A quantitative evaluation as to proportional antibody content was attempted. Even though this study is open to some criticism it is clear from results shown in Text-fig 1 that a reasonable proportion of the total convalescent antibody was in fact type specific.

These findings reinforce the suggestion that the critical antibody feature may be type-specific.

Antibody response in animals with variously prepared vaccines. Commercial type vaccines employed in Thailand and obtained there were used in comparative immunization of rabbits. In addition vaccines were prepared in this laboratory from 40 differing strains of *V. cholerae* and by a wide variety of procedures. The evaluation of the antisera so obtained is still in progress but the results now warrant certain conclusive statements with respect to a hypothetical vaccine which will give a high type specific antibody response.

1. Treatment of vibrio suspensions with preservatives or with heat tends to have an adverse influence on antigenicity as compared with similar suspensions of living vibrios. Some antigenic elements appear to be more labile than others and there appears to be a definite order of destructiveness as between the various methods of vaccine preparation. Living vibrios give antisera with a high proportion of type specific antibody. It is difficult to reproduce this effect quantitatively with killed vaccines.

Table 5

Diagnosed Cholera with reference to admission immunologic status

	Number	Group Antibody		Type Antibody	
		+	−	+	−
Cholera	20	11	9	0	20
Non - cholera	36	29	7	4	32

Table 6

Patients showing significant antibody increase in convalescence.

	Number	Group Antibody		Ogawa		Inaba	
		+	−	+	−	+	−
Claimed non - immunized	6	6	−	5	1	−	6
Claimed immunized	8	8	−	6	2	−	8

2. A basic necessity in vaccine preparation is the selection of strains. Whereas all living vibrios give excellent titers of type antibody the effect of preservatives differs markedly with each strain. The reasons are completely unknown.

3. The relationship of chemical preservative to type-antigen stability remains rather mysterious. With some strains a given preservative may eliminate type antigenicity altogether, with others the antigenicity of the group antigens is greatly depressed. At this time there is no theoretical approach. It is simply a matter of trial and error as to which strain and which preservative are appropriately matched to bring out group or type responses as desired.

4. If one were forced at this time to select one given preservative for the generality of strains and for the preservation of type antigenicity that selection would of necessity be formalin. Curiously enough this is the one method which also gives preservation of morphologic integrity.

5. It is likely that the strain composition of vaccines should be inclusive of :

(a) Recognized strains known to give type-antigen stability with the chosen preservative. One might well employ three Ogawas and three Inabas.

(b) Additional strains from among current isolates even though antigenic stability had not been determined.

6. It could not be claimed by any seasoned observer that a single injection of even the best theoretical vaccine would lead to immunity in the face of an epidemic. Two injections properly spaced should have an excellent effect. If it is possible to give only one injection this injection of vaccine should be accompanied with an impressive amount of wisdom. There is no substitute for individual indoctrination as to personal hygiene and sanitary measures.

Summary

1. Retrospective serologic diagnosis of cholera has been demonstrated.

2. It would appear that antibodies against the group O antigen of *V. cholerae* may not be protective in man.

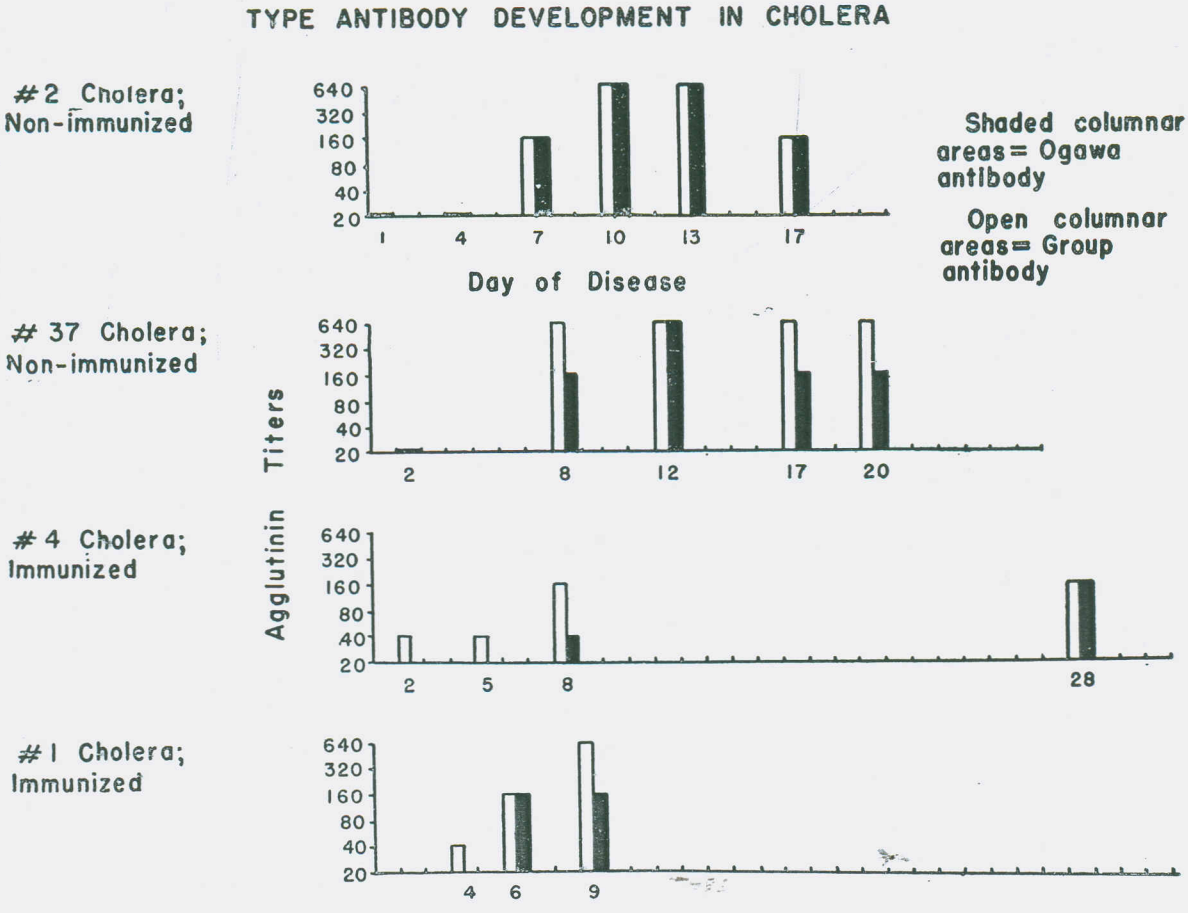

Fig. 1

3. Recovery from clinical cholera is frequently associated with the rise of type specific antibody.

4. The problems of an effective cholera vaccine have been enumerated.

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