J. Hyg., Camb. (1969), **67**, 539
With 1 *plate*
Printed in Great Britain

Antigenic analysis of vibrio culture filtrate and vaccine El Tor vibrio

By S. C. SANYAL, A. NARAYANASWAMI
AND S. MUKERJEE
Indian Institute of Experimental Medicine, Calcutta-32

(*Received* 3 *March* 1969)

INTRODUCTION

In the course of an investigation on live oral cholera vaccine, Bhattacharya, Narayanaswami & Mukerjee (1968) found that intra-intestinal administration of an El Tor (water) strain, ME7, produced antitoxic immunity in the intestinal tract of the adult rabbit. This suggested that although water strains of *Vibrio eltor* are not known to be toxinogenic, they possibly produce choleragenic toxin in quantities too low to be detected in presently available experimental models. Since the choleragenic toxin (toxin type 2 of Burrows (1968) classification) is known to be heat-labile and antigenically active, the present study was therefore undertaken with a view to examining whether El Tor (water) strains contain the same heat-labile antigens as those of *Vibrio cholerae* and El Tor (case) strains by means of gel diffusion and intra-gel absorption tests using an antitoxic serum raised against a culture filtrate of the 569B strain of *V. cholerae*.

Another object of the present work was to carry out an antigenic analysis of the usual toxin preparation consisting of a dialysed culture filtrate of 569B *V. cholerae*. Finkelstein, Atthasampunna, Chulasamaya & Charunmethee (1966) and Kasai & Burrows (1966) have also reported that the toxin is antigenic and stimulates the production of serum antibodies in rabbits which are capable of neutralizing choleragenic activity. Kasai & Burrows (1967) subsequently demonstrated that heat-denatured toxoid remains fully immunogenic.

It is of interest to note that both Finkelstein and Burrows used partially purified preparations of toxin in their work. Perhaps it was for this reason that Finkelstein & Atthasampunna (1967) administered the toxin with Freund's adjuvant in order to elicit protective immunity against subsequent challenge. In our laboratories we have observed that preparation of the crude toxin induces protective immunity against challenge with cholera toxin and with live vibrios in adult rabbit ileal loops only if administered along with O antigen in the form of heat-killed vibrios (S. C. Sanyal, A. Narayanaswami and S. Mukerjee, unpublished work). It would appear, therefore, that there is no advantage in using a purified toxin preparation rather than the unprocessed culture filtrate for the purpose of immunization; in fact the unpurified preparation, because of the presence of O antigen as a contaminant, may be more effectively immunogenic. The present investigation was therefore also designed to provide information about the antigenic constituents of culture filtrates in relation to their usefulness for antitoxic immunization.

MATERIALS AND METHODS

Vibrio strains

Vibrio cholerae strain no. 569B which has been widely used for production of toxin, a known toxinogenic El Tor (case) strain no. GS-9 and an El Tor (water) strain no. W 6 were used. It is proposed to use strain no. W 6 in the development of a live cholera vaccine. This strain has been found to be apathogenic in ligated intestinal loops and infant rabbit models of experimental cholera and also in human volunteers (Bhattacharya & Mukerjee, 1968; Mukerjee, 1963; Mukerjee & Sanyal, 1967). It is apparently non-toxinogenic as the filtrate prepared from it did not give rise to gut-inflammatory reactions in ligated ileal loops of adult rabbits (unpublished records) or in infant rabbits (R. A. Finkelstein, personal communication).

Preparation of cholera toxin

This was prepared from strain 569B according to the method reported by Craig (1966). The toxin in the culture filtrate was precipitated with 70% ammonium sulphate and filtered through a Buchner funnel with Whatman no. 1 filter paper. The residue was dissolved in distilled water and dialysed in the cold against distilled water with four to five changes. It was filtered through a Millipore filter and stored in the refrigerator at 4° C. The preparation will be referred to as 569B toxin. The protein content of this toxin was determined by the method of Lowry, Rosenbrough, Farr & Randall (1951) and adjusted to 1 mg./ml. with distilled water. The potency of the toxin was tested in adult rabbit ileal loops at regular intervals during the whole course of experiment. The minimum dose required for causing definite reaction in a 10 cm. loop was a volume containing 120 μg. protein and this potency remained unchanged throughout the course of the investigation.

Preparation of whole cell lysate

Overnight growth of vibrios in a Roux-flask containing papain agar medium was harvested in 10 ml. of physiological saline and centrifuged. The sediment was washed twice with saline. Finally the sediment was suspended in distilled water to obtain a concentration of 10^{11} vibrios/ml. and sonicated for 20 min. in the M.S.E. Ultrasonic Disintegrater at a frequency of 20 kc./s. The sonicated material was centrifuged in the cold. The supernatant was freeze-dried and stored at 4° C.

Preparation of live and heat-denatured antigens

Overnight growth of vibrios in a Roux flask containing papain agar medium was harvested in 10 ml. of physiological saline and centrifuged. The sediment was washed and resuspended in 10 ml. of saline. Five ml. of this was saved as live antigen and the remaining portion was heated in a boiling water bath for 1 hr. and used as heat-denatured antigen.

Preparation of antitoxic sera

The antitoxic sera were produced in adult rabbits by six injections of graded doses of toxin from 0·25 to 3 ml.; the first two doses were given subcutaneously followed by two intraperitoneal and two intravenous injections at intervals of 4 days. Blood samples were drawn 7 days after the last injection. The sera were stored in the refrigerator at 4° C.

Absorption of sera

The absorption of sera was carried out with heat-denatured and packed sediment of live vibrios. The suspensions were kept in the incubator for 2 hr. with repeated shaking and then overnight in the refrigerator. The sera were collected after centrifugation.

Serological analysis by gel-diffusion and intra-gel absorption tests

Diffusion of antigen and antibodies was carried out according to the method described by Feinberg (1958). The medium contained 0·8% Difco agar. Intra-gel absorption with soluble antigens was carried out to unmask the specific bands. The soluble antigens were mixed with equal volumes of medium and plated. Wells were cut in the medium after solidification. The precipitation bands appeared between the antigen and antibody wells on standing at room temperature in a moist chamber within 3–7 days.

Antigenic analysis by immuno-electrophoresis

The immuno-electrophoretic analysis of 569B toxin was carried out in the LKB apparatus 3290B on microscope slides using 0·8% Ionagar no. 2 and 0·87% sodium azide in the gel medium. Barbital buffer of pH 8·2 was used.

RESULTS

Plate 1, fig. 1, shows that the antitoxic serum produced four precipitin bands against the crude toxic filtrate in well 1, and nine bands against whole cell lysate of 569B strain in well 5. Bands C and D are likely to be due to the heat-labile specific antigens present in the toxin preparation, as they are absent against well 6 which contained heated toxin. Fewer precipitin bands were formed against well 4 where live cells of 569B strain was added. It is likely that although the antigens were present in the intact cells they could not all diffuse into the agar-gel in the absence of lysis. The polysaccharide preparation in well 2 had been prepared from a *V. cholerae* (Ogawa) strain by the method of Shrivastava & Seal (1937). It consisted therefore of the polysaccharide moiety of the heat-stable lipopolysaccharide antigens of wells 1 and 3. On intra-gel absorption with 569B toxin, or whole cell lysate of 569B all precipitin bands due to heat-stable as well as heat-labile antigens in the toxin disappeared.

The El Tor (water) strain no. W6 which is apparently non-toxinogenic but known to have the same antigenic make-up as *V. cholerae* and choleragenic El Tor strains (Mukerjee, 1963) was used for absorption of the antitoxic serum. When this

absorbed serum was used for gel-precipitation with the toxin, whole cell lysates of 569B, whole cell lysates of W6 and live and heat-denatured antigens of 569B and live antigens of GS-9 not a single precipitin band appeared. On the other hand when heat-denatured W6 was used for absorption of the antitoxic serum two continuous precipitin bands appeared against the toxin, whole cell lysates of 569B and of W6, and live GS-9. These bands therefore appear to be due to the heat-labile antigens in the toxin preparation Pl. 1 fig. 2 corresponding to C and D of Pl. 1 fig. 1. It appears therefore that the W6 strain contains the heat labile antigens of cholera toxin although the presence of choleragenic toxin cannot be demonstrated in culture filtrates using the adult or infant rabbit models. The presence of these heat-labile antigens in W6 is further confirmed by the results presented in Pl. 1. fig. 3 where the two bands due to the cholera toxin are seen to be continuous against wells 1, 2, 3 and 6 containing respectively 569B toxin, 569B whole cell lysate, GS-9 live antigen and W6 whole cell lysate.

Immuno-electrophoresis of cholera toxin against cholera antitoxic serum also points to the presence of two specific antigenic constituents in the toxin. The pattern of precipitation on immuno-electrophoresis of cholera toxin is shown in Pl. 1. fig. 4, and is similar to that against well 1 in Pl. 1, fig. 1. Of the four precipitin bands, those near the well may be due to the lipopolysaccharide components present in the crude toxic filtrate as a contaminant which gave bands in corresponding positions in the gel-diffusion experiment. The remaining two precipitin bands may similarly correspond to the specific antigens C and D of Pl. 1, fig. 1.

DISCUSSION

Misra & Shrivastava (1959) using ultrasonic lysates of cholera vibrios have reported that at least seven precipitin bands were formed when tested against cholera antiserum. Of these, the band near the antigen-well he named the α-band and considered it to be due to a lipopolysaccharide-protein fraction of the cell wall. The same number of precipitin bands or even more have also been obtained in the present study when whole cell lysate was titrated against serum raised in rabbits against cholera toxin. This indicates that in the toxic filtrate practically all the antigenic constituents of the vibrio cell were present, including the specific antigens due to cholera toxin.

There is now considerable evidence that the choleragenic toxin, liberated into the culture medium under suitable conditions of culture, is a heat-labile, non-dialysable compound. Coleman *et al.* (1968) have shown that purification of this component ultimately yields two lipoprotein compounds separable by fractionation on DEAE-sephadex, which are antigenically distinct. The present results confirm the presence of two distinct heat-labile antigenic constituents in the toxin, corresponding to the precipitin bands (C, D) shown in Pl. 1, fig, 1. These bands were totally absent when heat-denatured cholera toxin was used against the antitoxic serum. They were found to be completely absorbed in the intra-gel absorption test with cholera toxin.

Disappearance of the precipitin bands due to the strains 569B and GS-9 on

absorption of the serum with live El Tor W 6 but not with heat-denatured suspensions indicates that the heat-labile antigenic fractions are common to these strains of vibrios. Heat treatment denatures the labile protein antigens and the heat-treated suspensions are consequently incapable of absorbing the bands due to toxin, while their capacity to absorb precipitin bands due to heat stable fractions remains unaffected. The gel-precipitation and intra-gel absorption tests thus furnish clear proof that the toxin is present in W 6 even if in concentrations too low to produce pathogenic effects in the rabbit models. The difference between the 569 B, GS-9 and W 6 strains in their capacity to produce toxin appears therefore to be only a quantitative one.

Mukerjee (1963) showed that the apathogenic El Tor (water) strain W 6 is composed of smooth specific somatic antigens identical with those of *V. cholerae* and *V. eltor* (case) strains. From the present study it becomes evident that it also contains heat-labile antigens similar to those of *V. cholerae* and *V. eltor* (case) strains. The finding that the antigenic constituents of the proposed vaccine strain W 6 include the choleragenic toxin components is of importance in establishing that the vaccine strain is not devoid of residual virulence, an essential requirement for its use as a live vaccine. It also explains the genesis of the antitoxic immunity produced in the intestinal tract of adult rabbits following intra-intestinal administration of an apathogenic El Tor strain (Bhattacharya, Narayanaswami & Mukerjee, 1968).

SUMMARY

The antigens present in the preparation of cholera toxin consisting of the culture filtrate of 569 B strain of *V. cholerae* have been analysed by gel-diffusion, intra-gel absorption, and immuno-electrophoresis. The antiserum raised against the toxin was tested using the following antigens: whole cell lysates of *V. cholerae* 569 B and an El Tor (water) strain W 6 (proposed as a vaccine strain), suspensions of the same strains of vibrios with and without heat-denaturation, suspension of an El Tor (case) strain (GS-9) and a preparation of vibrio polysaccharide. The antitoxic serum gave four precipitin bands against the toxin preparation and nine bands against the whole cell lysate of *V. cholerae*. Two bands could be identified as being due to the heat-labile specific antigens present in the cholera toxin. These two bands were abolished on absorption of the antitoxic serum with a live suspension of W 6 but not on absorption with heat-denatured W 6. It has, therefore, been concluded that the proposed vaccine strain W 6 contains the specific antigens of cholera toxin. The significance of these results has been discussed in relation to the residual virulence of the proposed vaccine strain and antitoxic immunity produced in the intestinal tract of the adult rabbit following exposure to the vaccine strain.

We gratefully acknowledge the gift of purified vibrio polysaccharide from Dr C. V. N. Rao of the Indian Association for the Cultivation of Science, Jadavpur, Calcutta-32. The technical assistance of Mr B. Sanyamat and Mr I. Guhathakurta is also thankfully acknowledged.

REFERENCES

BHATTACHARYA, P. & MUKERJEE, S. (1968). Further studies on the development of a live oral cholera vaccine. *J. Hyg., Camb.* **66**, 307–18.

BHATTACHARYA, P., NARAYANASWAMI, A. & MUKERJEE, S. (1968). Production of antitoxic immunity by live oral cholera vaccine. *J. Bact.* **95**, 255–6.

BURROWS, W. (1968). Cholera toxins. *A. Rev. Microbiol.* **22**, 245–68.

COLEMAN, W. H., KAUR, J., IWERT, M. E., KASAI, G. J. & BURROWS, W. (1968). Cholera toxins: purification and preliminary characterization of ileal loop reactive type 2 toxin. *J. Bact.* **96**, 1137–43.

CRAIG, J. P. (1966). Preparation of the vascular permeability factor of *Vibrio cholerae*. *J. Bact.* **92**, 793–5.

FEINBERG, J. G. (1958). *Transactions of the Collegium Internationale Allergologicum*, p. 129.

FINKELSTEIN, R. A. & ATTHASAMPUNNA, P. (1967). Immunity against experimental cholera. *Proc. Soc. exp. Biol. Med.* **125**, 465–9.

FINKELSTEIN, R. A., ATTHASAMPUNNA, P., CHULASAMAYA, M. & CHARUNMETHEE, P. (1966). Pathogenesis of experimental cholera. Biologic activities of purified Procholeragen A. *J. Immun.* **96**, 440–9.

KASAI, G. J. & BURROWS, W. (1966). The titration of cholera toxin and antitoxin in the rabbit ileal loop. *J. infect. Dis.* **116**, 606–14.

KASAI, G. J. & BURROWS, W. (1967). Cholera toxoid: preparation and titration. *Fedn Proc. Fedn Am. Socs exp. Biol.* **26**(2), 801 (Abstr. no. 3042).

LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–75.

MISRA, S. B. & SHRIVASTAVA, D. L. (1959). Studies in immunochemistry of *Vibrio cholerae*. Part I. Localization of antigens in the cell. *J. scient. ind. Res.* **18**C, 209–12.

MUKERJEE, S. (1963). Preliminary studies on the development of a live oral vaccine for anti-cholera immunization. *Bull. Wld Hlth Org.* **29**, 753–66.

MUKERJEE, S. & SANYAL, S. C. (1967). Live oral cholera vaccine trial on human volunteers. *W.H.O. Cholera Information*, no. 10, p. 11.

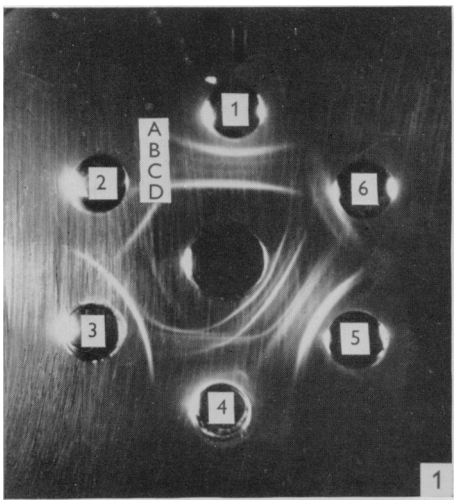
SHRIVASTAVA, D. L. & SEAL, S. C. (1937). Preparation and properties of a specific polysaccharide from a strain of *Vibrio cholerae*. *Proc. Soc. exp. Biol. Med.* **36**, 157–61.

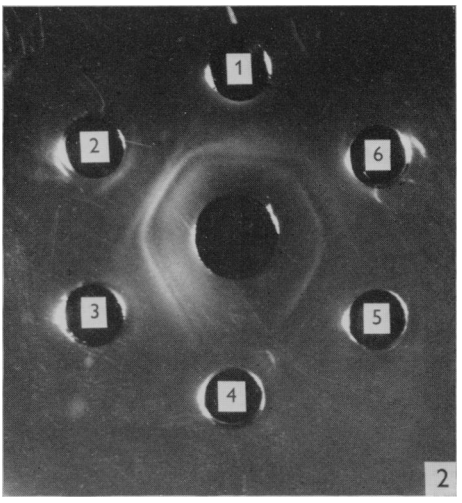
EXPLANATION OF PLATE

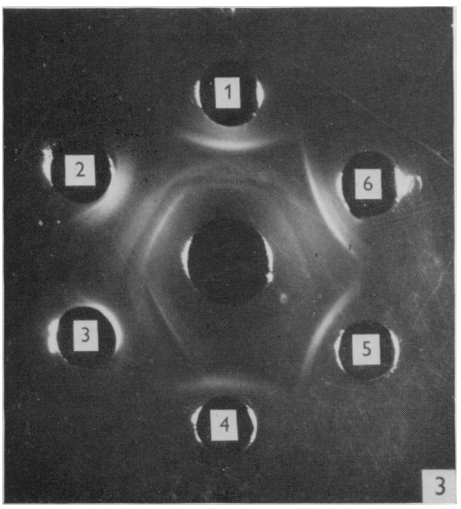
Figures 1–3.

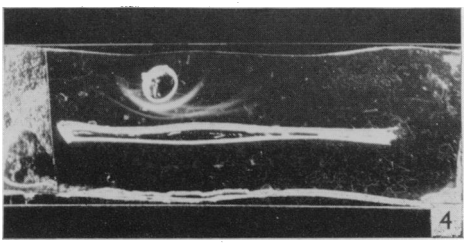
Contents of wells	Well used in Fig. 1.	Fig. 2	Fig. 3
569B antitoxin	Centre	—	Centre
569B antitoxin, absorbed with heated W6 strain	—	Centre	—
569B toxin	1	1	1
Vibrio polysaccharide	2	—	—
Heated antigen of 569B	3	—	4
Live antigen of 569B	4	—	—
Whole cell lysate of 569B	5	2	2
Whole cell lysate of W6	—	3	6
Heated antigen of W6	—	4	5
Live antigen of W6	—	5	—
Live antigen of GS-9	—	6	3
Heated 569B toxin	6	—	—

Fig. 4. Antigen well contains 569B toxin. Trough contains antiserum against 569B toxin.


A
B
C
D
1
2
3
4
5
6
1


1
2
3
4
5
6
2


1
2
3
4
5
6
3


4