

# STUDY OF INTESTINAL IMMUNITY AGAINST *V. CHOLERAE*: ROLE OF ANTIBODY TO *V. CHOLERAE* HAEMAGGLUTININ IN INTESTINAL IMMUNITY

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## INTRODUCTION

After oral infection with *V. cholerae*, the five to six day old mice develop signs of cholera resembling those of human infection (Ujjiye *et al.*, 1968; Chaicumpa and Rowley, 1972). Complete protection, without the occurrence of cholera symptoms can be achieved by either oral or parenteral administration of specific antibody prior to or simultaneous with the cholera challenge. The protective ability of the antibody in this baby mouse model appears to be antibacterial (Chaicumpa and Rowley, 1972). However, phagocytosis and complement mediated killing seems to be of minor importance in this antibody dependent antibacterial mechanism (Steele *et al.*, 1974). The prevention or the reduction of adsorption of *V. cholerae* to the intestinal epithelium by cross-linking property of the specific antibody has been proposed by Rowley and his colleagues (Bellamy *et al.*, 1975). The antibody might act by agglutinating the bacteria or by masking the "adherence receptor" on the bacterial surface.

Bellamy *et al.*, (1975) has demonstrated that the reduction in adsorption of *V. cholerae* to the epithelial cells correlated with the degree of agglutination for a given antibody preparation. The intact antibodies protected infant mice from cholera only at concentrations that agglutinate the bacteria and the purified antibodies to flagella antigens protected mice from cholera. The reduction of bacterial adsorption to the intestinal epithelium by antibody via its masking effect on

bacterial adhesive factor has not yet been clarified.

One of the adhesive factors of choleraogenic vibrios namely "a slime sheath or cell-bound haemagglutinin", was described by Lankford and Legsomburana (1965) and later by Chulasamaya (1970). Studies were undertaken to determine the ability of the haemagglutinin antiserum in protection against experimental cholera in infant mice.

## MATERIALS AND METHODS

**Bacterial Strain:** *Vibrio cholerae* biotype E1 Tor, strain streptomycin resistant Ogawa 17 (017SR) were maintained as described previously (Chaicumpa and Rowley, 1972). For experimental use, the organisms were streaked on streptomycin nutrient agar (100 µg of streptomycin/ml of agar). The agar plate was incubated at 37°C for 18 hours. A loopful of the bacteria was inoculated into nutrient broth and a late-log phase of shaking culture was used either for the baby mouse protection test or for inoculation of the Roux bottles (each containing 250 ml of trypticase soy agar).

**Assay for *V. cholerae* haemagglutinin:** Haemagglutinating activity of the haemagglutinin preparation was determined with a 2.5% washed sheep red blood cell suspension. Serial dilutions of the haemagglutinin were performed in a haemagglutination tray using normal saline as a diluent. Equal volume of the sheep red blood cells was subsequently added to each dilution. Mixed, and the tray

was incubated at 37°C. The haemagglutination end point was the highest dilution of the haemagglutinin preparation at which 50% of the red cells were agglutinated.

***V. cholerae* lipopolysaccharide:** *V. cholerae* lipopolysaccharide (LPS) was extracted and purified as described by Neoh and Rowley, (1970). For quantitative estimation of the LPS in the haemagglutinin preparation the technique of inhibition of LPS-anti-LPS passive haemagglutination was employed (Crumpton *et al.*, 1958; Neoh and Rowley, 1970; Chaicumpa and Rowley, 1972).

**Protein estimation:** Protein concentration of the haemagglutinin preparation was determined with micromodification of Folin-Ciocalteu method (Kabat and Mayer, 1961).

**Haemagglutinin antiserum:** Rabbits were subcutaneously immunized with 1 ml of crude haemagglutinin (P<sub>1</sub>) containing 3-5 mg dry weight mixed with equal volume of complete Freund adjuvant. The next dose was repeated at 2 week interval. Fourteen days after the second injection, the animals were bled and their sera were heated at 56°C for 30 minutes before they were kept at -70°C in 5 ml aliquots without preservative.

**Infant mouse protection test:** The test was done as described previously (Ujiye *et al.*, 1968; Bellamy *et al.*, 1975). Five-to-six-day-old white mice were orally inoculated with  $2 \times 10^7$  *V. cholerae* 017 SR in a total volume of 0.1 ml of either normal rabbit serum or appropriate dilutions of antisera. The dilution of the antiserum that protected 50% of the infant mice (PD<sub>50</sub>) from death (at 48th hour after infection or at a time when 90% of the control mice had died) was calculated by the method of Reed and Muench (1938). Recovery of the bacteria from the gastrointestinal tracts of the baby mice was as described previously (Chaicumpa and Rowley, 1973).

**Immuno-electrophoresis:** Modified slide technique of immunoelectrophoresis of Scheidegger was used as previously described (Savanat and Chaicumpa, 1969).

## RESULTS

### Preparation of cell bound haemagglutinin from *V. cholerae*

The method of Chulasamaya (1970) was followed with modification. After incubation at 37°C for 48 hours, the bacteria from 15 Roux bottles were removed from the surface of agar using glass beads and cold physiological saline and the final volume was brought up to 1,200 ml with the same solution. They were washed three times in physiological saline by centrifugation at 9,000 G at 4°C for 15 minutes. The bacteria were finally resuspended to 600 ml. Equal volume of 0.05 M cyclohexylaminopropane sulfonic acid (CAPS) was added to the suspension. The mixture was incubated at 37°C with shaking for one hour. The bacteria and cell debris were removed by centrifugation at 9,000 G at 4°C for 15 minutes. The supernatant (CAPS extract) was dialysed extensively against at least four changes of cold distilled water within 24 hours, follow by millipore filtration (0.45 μ membrane) to remove any residual bacteria. The extract was concentrated to 15-20 ml using PM10 amicon membrane ultrafiltration, and then dialysed against 0.05 M Tris buffer pH 8.6 for at least two hours. The concentrated CAPS extract was recentrifuged at 9,000 G at 4°C for 15 minutes. The supernatant was passaged through Sephadex G200 column (90 cm × 2 cm) equilibrated with 0.05 M Tris buffer pH 8.6. The flow rate was maintained at 6.4 ml per hour and 6 ml fractions were collected.

The optical density of Sephadex fractions were measured at 280 nm using Perkin-Elmer model 124 spectrophotometer and quartz cell with 1 cm light path, and the haemaggluti-

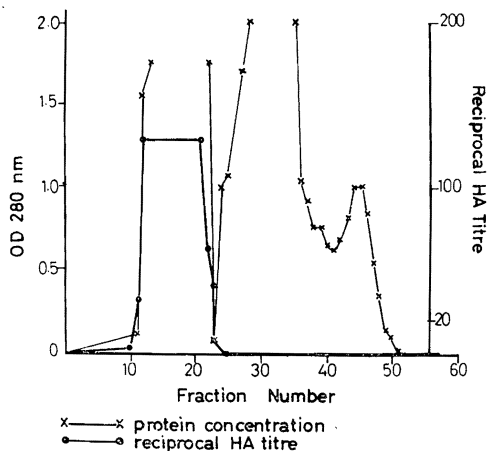


Fig. 1—Chromatography of *V. cholerae* 017SR CAPS extract.

Concentrated CAPS extract (15 ml) was layered on the top of 2 cm × 90 cm column of Sephadex G-200 equilibrated with Tris buffer pH 8.6 at 4°C. The flow rate was maintained at 6.4 ml per hour and 6.0 ml fractions were collected. Fractions were assayed for total protein by optical density at 280 nm and the haemagglutinating activity by haemagglutination of 2.5% sheep red cells.

nating activity assayed. The relationship between the haemagglutinating activity and the protein profile is shown in Fig. 1. The activity is associated with the first peak. Fractions of the first peak were pooled and

concentrated by amicon membrane ultrafiltration. It was called “crude haemagglutinin” or “P<sub>1</sub>” and was used to immunize the rabbits for the preparation of crude haemagglutinin antiserum (anti P<sub>1</sub>). Table 1 shows a summary of the preparation procedure.

More than 50% (W/W) of the P<sub>1</sub> was LPS as detected by the ability of the P<sub>1</sub> to inhibit the LPS-anti-LPS passive haemagglutination.

Immuno-electrophoresis of the P<sub>1</sub> against the rabbit anti P<sub>1</sub> serum reveals at least two precipitin bands designated band A and band B (Fig. 2). Band A was located near the starting well. In certain preparations of the P<sub>1</sub> IEP slide, there were more than one band in this region. Band B was more anodic and was likely to be the haemagglutinin band as revealed by the evidence to be mentioned in the later section. When the LPS from 017SR was used in the IEP only band A was apparent suggesting that band A was most likely due to the LPS.

**Rabbit haemagglutinin antiserum:** The immuno-electrophoresis slides of the P<sub>1</sub>-anti

Table 1

Preparation of crude haemagglutinin.

Preparation	Volume (ml)	Total HA activity (units)	Specific activity (units/mg protein)	Recovery (%)
Bacteria in saline	1,200	6,144,000	819	100
Bacteria in CAPS buffer (after incubation)	1,200	6,144,000	819	100
**CAPS extract (supernatant after removal of bacteria and cell debris)	1,200	96,000	160	1.6
Concentrated CAPS extract	18	92,160	280	1.5
Sephadex G200 Peak 1	10	51,200	1,138	0.83

\*\* Addition of more CAPS buffer to the bacterial suspension or longer incubation of the mixture at 37°C resulted in higher recovery of the haemagglutinin. However, there was a lysis of the bacteria hence released the RNA & DNA which interfered with the subsequent column chromatography.

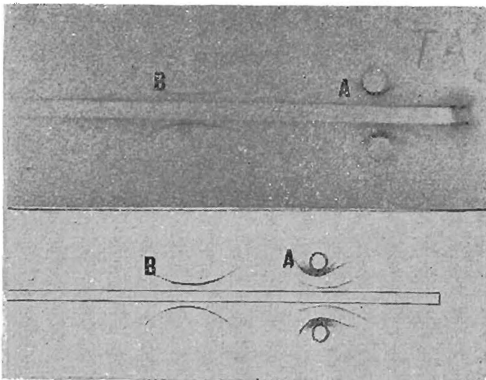


Fig. 2—Immuno-electrophoresis of the Sephadex G200 Peak 1 ( $P_1$ ) against crude haemagglutinin antiserum (anti  $P_1$ ).

$P_1$  were washed thoroughly with many changes of physiological saline over a period of more than one week. This is to remove all of the non precipitating materials. Pieces of the agar containing band A and band B were cut and homogenized separately in 5 ml of cold saline. Each homogenate containing pool of at least 16 precipitin bands in agar was injected subcutaneously into rabbits. The rabbits were reimmunized 2 weeks later. Fourteen days after the second infection, the animals were bled, and their sera collected. The sera were heated at  $56^\circ\text{C}$  for 30 minutes before use.

Incubation of the anti-band B serum with the  $P_1$  followed by high speed centrifugation (9,000 G at  $4^\circ\text{C}$  for 1 hour) resulted in the removal of the haemagglutinating activity from the supernatant. IEP of the supernatant against crude haemagglutinin antiserum revealed only band A (Fig. 3), suggesting that the nature of this band was due to the haemagglutinin. Deliberate spinning of the  $P_1$  at this speed, temperature and time can not remove the haemagglutinating activity from the supernatant. Both band A and band B were recovered from the deposit after centrifugation of the  $P_1$  at 100,000 G at  $4^\circ\text{C}$  for 3 hours.

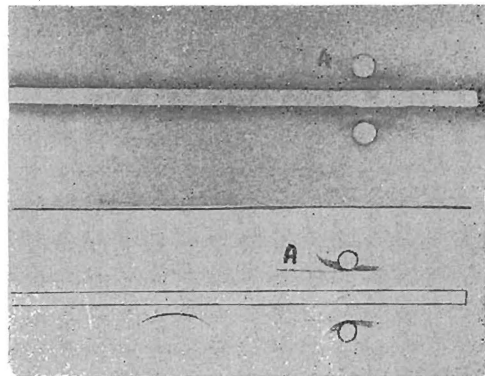


Fig. 3—Immuno-electrophoresis of the  $P_1$  (lower well) and the  $P_1$  absorbed with haemagglutinin antiserum (upper well) against the crude haemagglutinin antiserum (anti  $P_1$ ).

#### Protective effect of haemagglutinin anti-serum

Ten fold dilutions of the antiserum were made in physiological saline. To each 0.9 ml of every dilution, 0.1 ml of log phase broth culture 017 SR was added. After incubation of the preparations at  $37^\circ\text{C}$  for 15 minutes, aliquots of 0.1 ml were given orally into 5-6 day old mice. The  $PD_{50}$  of the antiserum was calculated. The bacterial recovery from the gastrointestinal tract was performed on streptomycin agar plates. Table 2 shows the  $PD_{50}$  of the anti-crude haemagglutinin, the anti-haemagglutinin and the so called anti-LPS sera. At the protective dilutions, all of the three antisera also give bactericidal effect to the challenged organisms as revealed by the reduction in number of the bacterial recovery.

Table 2

$PD_{50}$  of the crude haemagglutinin, the haemagglutinin and the LPS antisera.

Antisera	$PD_{50}$
Crude haemagglutinin	1:113
Haemagglutinin	1:10
LPS	1:87

## DISCUSSION

Recent studies using scanning and transmission electron microscopy (Nelson *et al.*, 1976) have shown that the majority of the challenged vibrios in the intestines of the experimental animals exhibited an aligned horizontal adherence with the intestinal epithelium. The minority of the vibrios were attached in an end-on manner, with their flagella extending into the lumen. The findings once again confirm the significance of the vibrio adhesive factor(s) in attachment prior to disease.

The haemagglutinin antiserum obtained from rabbits immunized with the complexes of the vibrio cell-bound haemagglutinin and its antibody showed definite protection. The protection was confirmed by the reduction in numbers of the bacteria recovered from the gastrointestinal tracts of the baby mice at 24 hours after infection. Though the PD<sub>50</sub> of the serum was low (1:10) this may merely indicate the small quantity of the specific antibody in the serum and not the low protective efficacy of the antibody against the haemagglutinin.

The experiments will be done to purify the haemagglutinin and to raise high titre antiserum against it. The quantitative comparison of PD<sub>50</sub> of this antibody and antibodies to other *V. cholerae* antigens will also be performed.

## SUMMARY

Cell-bound haemagglutinin as an adhesive factor of *Vibrio cholerae* has been partially purified from E1 Tor vibrios using 0.05 M cyclohexylaminopropane sulfonic acid buffer pH 9.5 and gel filtration column chromatography. Rabbits were immunized with the precipitin complexes of the haemagglutinin and its antibody. The antiserum is tested for the protective ability against the oral challenge

with *Vibrio cholerae* strain of which the haemagglutinin has been prepared. The results indicate definite protection of the haemagglutinin antiserum.

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