ORAL VACCINE AGAINST CHOLERA PREPARED FROM *VIBRIO CHOLERAE* ANTIGEN(S)

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INTRODUCTION

Vibrio cholerae, the causative agent of cholera in man is a non-invasive enteropathogen. Pathogenic mechanisms of the bacteria are confined to the intestinal mucosa. The ingested vibrios which survived the antiseptic barrier of saliva and gastric acidity and arrived the small intestine swim across the unstirred water layer within the intestinal lumen, penetrate the mucus layer and glycocalyx and adhere to the underlying epithelium. Penetration of the mucus and glycocalyx layers requires active motility of the bacteria, chemotactic factors and the vibrio's enzymes e.g. mucinase, fibronectinase (Freter *et al.*, 1981a, 1981b). Adherence to the intestinal epithelium is one of the most important steps in cholera pathogenensis. The phenomenon is to enable the vibrios to resist removal by intestinal peristalsis and to multiply near the site of action of cholera toxin i.e. Gm_1 receptor on epithelial cell membrane. After successful attachment at the epithelium, the organisms multiply and produce cholera toxin (CT). CT consists of 2 polypeptide subunits i.e. the A- and the B-subunits. Binding of the B-subunit to the Gm_1 receptor leads to a translocation of the A one into the epithelial cell cytosol. Subsequent activation of an enzyme adenylate cyclase ultimately results in net secretion of fluid and electrolytes (Na^+, K^+, HCO_3^-,Cl^-) into the intestinal lumen (Holmgren, 1981).

From the pathogenesis, it is speculated that three functional types of antibodies would be required for complete protection against cholera in the host intestine. The first antibody, namely the agglutinating antibody, would agglutinate the vibrios in the intestinal lumen, make them non-motile thus unable to penetrate the mucus and glycocalyx. Subsequently, these agglutinated vibrios would be removed by the intestinal peristalsis to the large intestine where they may be killed by bacterial antagonism exerted by the normal flora. Vibrio antigens which may elicit the production of the agglutinating antibodies include antigens on the surface of the vibrios among which the lipopolysaccharide (LPS) is predominant and easy to prepare. Any motile vibrios which could escape the agglutinating effect of the first antibody will penetrate the mucus and glycocalyx and make an attempt to adhere to the epithelium. At this stage, antibodies to vibrio adhesive factor(s) would be required to prevent the adhesion of the organisms to the epithelial cells. The antigens which may give rise to the functional antibodies include cell-bound haemagglutinin (CHA) (Foo and Chaicumpa, 1981). Once the vibrios have attached to the intestinal epithelium, they multiply and produce cholera toxin. The antitoxin affords protection against cholera by blocking the binding of the B subunit to the Gm_1 receptor. Immunogens responsible for the stimulation of antitoxin production include holotoxin, B subunit or procholeragenoid (P) (Pierce *et al.*, 1983).

Experiments have indicated that immunization against cholera with a combination of somatic antigen and the toxin induced a much

higher degree of protection against cholera than did vaccination with either of the antigens alone (Svennerholm and Holmgren., 1976; Pierce *et al.*, 1982), Thus, the objective of this study is to observe degrees of immunogenicity of three *V. cholerae* antigens namely LPS, CHA and P given orally either alone or in a combination to experimental animals.

MATERIALS AND METHODS

V. cholerae, El Tor biotype, Ogawa serotype, streptomycin resistant strain (O_{17}SR) was used throughout the study. Lipopolysaccharide (LPS) was prepared by phenol-water method of Westphal and Jann (1965). The single extracted LPS was re-extracted until no protein was detected by the conventional Lowry's method and by sodium dodecyl sulphate polyacrylamide gel electrophoresis with Coomassie blue stain. Cell-bound haemagglutinin was prepared as follows: the bacteria were grown in large quantities in trypticase soy agar for 48 hours at 37°C. The bacteria were harvested and washed 3 times by centrifugation at 10,000 × g at 4°C for 15 minutes in normal saline solution (NSS). The cells were suspended in 1 M NaCl and subjected to ultraturrax mixture (Janke and Kunkel, West Germany) for 2 hours at 4°C. The bacterial cells and debris were removed by centrifugation as above. The clear supernatant was filtered through a 0.45 μm membrane, dialysed against distilled water at 4°C overnight and lyophilized. The dried material was dissolved in small volume of PBS pH 7.2 (crude cell-bound haemagglutinin; cCHA was prepared). The preparation was passed through a sheep red cell stroma-Sepharose 4B column. After thorough washing to remove the non-adsorbed material, the adsorbed haemagglutinin was eluted out with triple distilled water. The eluate was concentrated and the preparation was "pure cell-bound haemagglutinin (pCHA)". Cholera toxin was purchased from Sigma Chemical Company, U.S.A., while the procholeragenoid was a gift from Dr. Furer, Swiss Serum Laboratory, Berne, Switzerland. Whole cell extract (WC) of *V. cholerae* was prepared from 48 hour culture of the organisms at 37°C on trypticase soy agar in Roux bottles. The bacteria harvested from the bottles were washed in normal saline solution (NSS) 3 times by centrifugation at 10,000 × g at 4°C for 15 minutes. Whole cells were suspended in distilled water at an optical density of 2.0 at 540 nm. and subjected to repeated ultrasonication. The preparation was kept at −20°C until use as the whole cell extract (WC).

Two formula of a combined vaccine consisted of LPS, CHA and P were prepared as follows: Crude CHA (cCHA) was diluted in cold NSS to 80 haemagglutinating units per ml. Equal volume of the preparation was mixed with 2.5% formalinized sheep red blood cells (F-SRBC) (Herbert, 1978). The mixture was kept at ambient temperature for 1 hour. The red blood cells with the haemagglutinin adsorbed on the surface (CH-SRBC) were washed three times with cold NSS by centrifugation at 850 × g at 4°C for 10 minutes and finally resuspended in Cassamino acid to 2.5% concentration. Each dose of a combined vaccine formula 1 consisted of CH-SRBC 2 ml in which 500 μg of LPS and 50 μg of P were added. Each dose of a combined vaccine formula 2 consisted of CH-SRBC 2 ml with 1,000 μg of LPS and 100 μg of P.

Young adult albino rats purchased from the National Laboratory Animal Centre, Salaya, Nakhon Pathom Province, Thailand were used as the experimental animals. They were first tested for the suitability as an animal model in the study of immunogenicity to cholera antigens. The test was performed by dividing 10 rats aged 7-8 weeks old into 5 groups of 2 rats. All of them were starved for 15 hours. Each rat was fed with 1 ml of 5% $NaHCO_3$ just prior to live *V. cholerae* chal-

lenge to reduce gastric acidity. Rats of the first 4 groups received orally 1 ml of 10^6, 10^7, 10^8 and 10^9 live *V. cholerae*, while those of the last group served as control and each of them received only $NaHCO_3$. The immunization was repeated one more time 14 days later with the same dose and route. Five days later, all rats were killed and their small intestines were removed for enumeration of anti-*V. cholerae* producing cells by double antibody sandwich method of immunofluorescence using whole cell extract of *V. cholerae* as the antigen in the assay.

The albino rats was found to be good experimental animals. Thus a new batch of 72 rats was purchased for the subsequent experiments. They were fasted for 15 hours. Each rat wa fed with 1 ml of 5% $NaHCO_3$ just prior to antigen feeding. They were divided into 9 groups of 8 rats. Rats of groups 1 and 2 served as controls of the experiments. Each rat of group 1 received 2 ml of Cassamino acid while those of group 2, each, received 2 ml of F-SRBC. Rats of groups 3, 4, 5, 6, 7, 8 and 9 were immunized with 1,000 µg of LPS, 100 µg of P, CH-SRBC, 500 µg of LPS and 50 µg of P, CH-SRBC and 50 µg of P, combined vaccine formula 1 and combined vaccine formula 2, respectively. The immunization was repeated once 14 days later with the same antigenic dose and route. Five days, thereafter, 5 rats from each group were sacrificed and their small intestines were removed for antibody producing cell enumeration. Each of the remaining rats was fed with 1 ml of 5% $NaHCO_3$ to reduce gastric acidity before challenging orally with $\simeq 2 \times 10^9$ live *V. cholerae* in 1 ml of trypticase soy broth. One rat from each group was killed at 3, 6 and 24 hours later. The small intestine was removed, clamped at both ends and washed free from blood on the serosal surface by dipping into three changes of cold NSS (excess volume). The intestine was ground up in 100 ml of cold NSS using ultraturrax mixer (Janke and Kunkel, West Germany). Appropriate dilutions in 0.1 ml volume were plated onto trypticase soy agar or TCBS agar for vibrio recovery.

Rabbit anti-whole *V. cholerae* or rabbit anti-CT conjugated to fluorescein dye: The rabbit anti-*V.cholerae* antiserum was prepared by injecting a rabbit intravenously with 10^5, 10^6, 10^7, 10^8 and 10^9 live *V. cholerae* in 1 ml of NSS at 1 week intervals. The rabbit was bled via cardiac puncture 7 days after the last immunization. The antiserum was collected and the IgG fraction was obtained by passing the serum through a DE 52 column eluted with 0.01 M phosphate buffer pH 8.0. The IgG-fluorescein conjugate (anti-VC conjugate) was prepared as described by Cherry (1970). The anti-CT was prepared by injecting 4, 10, 20 and 40 µg of CT intravenously into a rabbit at 1 week intervals. The last dose was repeated once before the antiserum was collected 7 days later. The anti-CT titre was checked by indirect enzyme-linked immunosorbent assay (ELISA) using CT as antigen to coat the ELISA plate (2 µg/ml) and goat anti-rabbit immunoglobulin peroxidase conjugate (Dakopatt,Denmark) (dilution1 :1,000), respectively. The IgG anti-CT conjugated with fluorescein dye (anti-CT conjugate) was also prepared as described by Cherry (1970).

Cells producing antibodies to LPS, CHA or CT in the intestinal lamina propria of the experimental rats were enumerated by double antibody sandwich method of immunofluorescence. The whole length of the small intestine of each rat was measured and a portion of 4 cm long at the middle was removed for cryostat sectioning. The cryostat section was fixed on a microscopic slide and refixed with 90% methanol and absolute methanol. The section was then covered with appropriate antigen (pure LPS for enumerating anti-LPS producing cells; pure CHA for anti-CHA producing cell enumeration and CT for anti-CT producing cell counting). The

reaction was allowed to take place for 30 minutes at room temperature then the non-reacted materials were washed off withP BS pH 7.2. The section was overlayed with either anti-VC conjugate (1:40) for anti-LPS or anti-CHA producing cell enumerations or anti-CT conjugate (1:50) for cell producing anti-CT detection. The preparation was kept in a humidified chamber for another 30 minutes then washed with excess PBS pH 7.2. The slide was air dried and mounted with mounting fluid (Microtrak, Syva, U.S.A.). The cells producing antibodies were counted under a Zeiss Universal fluorescence microscope at a magnification of 10 × 25. Two transverse sections of each rat's intestine were used for each specific antibody producing cell enumeration and for each section, the cells in 25 microscopic fields were counted.

RESULTS

The results on testing the suitability of the albino rats as the experimental animal model are shown in Table 1. Although the rats had been exposed to *V. cholerae* antigens, as indicated by the finding of the average of 6.6 antibody producing cells per one microscopic field in the intestinal lamina propria of the control rats which received only $NaHCO_3$ orally. The 4 groups of rats which recieved 10^6, 10^7, 10^8 and 10^9 live *V. cholerae* orally responded by having increases in antibody producing cells in the gut. This response is a dose-dependent one. Thus the average of 28, 45, 52 and 55 antibody producing cells per one microscopic field were found in the intestines, respectively.

Table 1

Number of antibody producing cells against *V. cholerae* antigens in the intestinal lamina propria of rats given various doses of live *V. cholerae* orally.

Antigen (live vibrios/dose)	Antibody producing cells per 1 microscopic field* ± SE
Control	6.6 ± 7.3**
10^6	27.5 ± 11.6
10^7	44.5 ± 12.6
10^8	52.1 ± 14.8
10^9	55.1 ± 15.0

* the antigen used in incubating with the intestinal sections was WC.

** average number from 100 microscopic fields (25 × 10) of 2 rats.

Mean and standard error of the numbers of anti-LPS, anti-CHA and anti-CT producing cells in the intestinal lamina propria of rats are shown in Table 2. The results revealed that control rats (group 1 and 2) which received Cassamino acid and F-SRBC, respectively, had the average of 11 anti-LPS producing cells, 6 and 9 anti-CHA producing cells and 8 and 10 anti-CT producing cells per one microscopic field, respectively. The rats of group 3 which received orally 1,000 μg of LPS had significant increase in anti-LPS producing cells (35 cells per one microscopic field). Rats which were fed with 100 μg of procholeragenoid (group 4) had 51 anti-CT producing cells per one microscopic field. When the two antigens (LPS and P) were fed together into a rat by reducing the amount of both antigens to 500 μg of LPS and 50 μg of P (group 6), the average number of anti-LPS producing cells was increased significantly upto 51 cells although the number of the anti-CT producing cells were statistically equal to those of group 4.

Rats which received orally CH-SRBC (group 5) had the average of 32 anti-CHA producing cells/1 microscopic field which was many more than the numbers found in the intestines of both control groups. However, when the CH-SRBC was fed together with 50 μg of P (group 7), the number of anti-CHA (30 cells/field) was equal to that of group 5

Table 2

Means and standard errors of the numbers of antibody producing cells in the intestines of rats fed various antigens and controls

Group No. antigen	Anti-LPS cells	Anti-CHA cells	Anti-CT cells
1 (Ca)	11 ± 3*	6 ± 3	8 ± 3
2 (F-SRBC)	11 ± 2	9 ± 2	10 ± 2
3 (LPS)	35 ± 4	n.d.	n.d.
4 (P)	n.d.	n.d.	51 ± 4
5 (CH-SRBC)	n.d.	32 ± 4	n.d.
6 (LPS-P)	51 ± 5	n.d.	44 ± 4
7 (CH-SRBCP)	n.d.	30 ± 4	30 ± 3
8 (formula 1 vaccine)	45 ± 4	47 ± 4	55 ± 4
9 (formula 2 vaccine)	44 ± 4	46 ± 4	43 ± 4

n.d. = not determined
Ca = Cassamino acid
F-SRBC = formalinized sheep red blood cells
LPS = lipopolysaccharide (1,000 μg)
P = procholeragenoid (100 μg)
CH-SRBC = 2.5% formalinized sheep red cells with cell-bound haemagglutinin on the surface
LPS + P = 500 μg of lipopolysaccharide + 50 μg of procholeragenoid
CH-SRBC + P = CH-SRBC + 50 μg of procholeragenoid
formula 1 vaccine = 500 μg of lipopolysaccharide + CH-SRBC + 50 μg of procholeragenoid
formula 2 vaccine = 1,000 μg of lipopolysaccharide + CH-SRBC + 100 μg of procholeragenoid
* average number from 250 microscopic fields (25 × 10) of 5 rats

and the anti-CT producing cells was only 30 cells/field which was lower than that of group 4.

The combined vaccine formula 1 which consisted of 500 μg of LPS, CH-SRBC and 50 μg of P elicited the production of cells producing anti-LPS, anti-CHA and anti-CT at the average of 45, 47 and 55 cells/field, respectively (group 8). The vaccine formula 2 which consisted of 1,000 μg of LPS, CH-SRBC and 100 μg of P induced the production of 44 and 46 anti-LPS and anti-CHA positive cells (group 9) which were not different from the numbers in group 8. The number of anti-CT producing cells of group 9 was only 43 cells which was significantly lower than that of group 8.

The results of vibrio recovery from the small intestines of rats of all groups at 3, 6 and 24 hours are not shown for it was found that there was no significant difference in the numbers recoverable from the controls and immunized groups. Three hours after the challenge, all vibrios had disappeared from the small intestines of all experimental rats.

DISCUSSION

Although the albino rats used in our experiments had background exposure to *V. cholerae* antigens, as revealed by the

presence of few antibody producing cells in the intestinal lamina propria, they still responded to the live *V. cholerae* oral challenge by having increased numbers of the antibody producing cells in the guts. The response was a dose-dependent i.e. the rats which received larger antigenic doses had more cells than those which received lower doses.

Rats which were fed orally with 1,000 μg of LPS (group 3), 100 μg of P (group 4) and CH-SRBC (group 5) had 35 anti-LPS, 51 anti-CT and 32 anti-CHA positive cells per one microscopic field, respectively. These figures are significantly higher than the numbers in the intestines of rats from both control groups (groups 1 and 2). Moreover, when LPS and P were combined in reduced amounts (500 μg of LPS and 50 μg of P) and fed into rats (group 6), the average number of anti-LPS positive cells in the intestine became 51 which was significantly higher than the average number from group 3. Besides the number of anti-CT positive cells in group 6 was equal to the number from group 4 despite the amount of P was reduced to only 50 μg in group 6. The results indicate that LPS and P are synergistic antigens which elicit better antibody responses using smaller amounts of antigens, when the two antigens are presented together than either antigen alone.

However, CHA and P when combined together and fed to rats (group 7) did not show any sign of synergism. The average number of anti-CHA producing cells per one microscopic field was equal to the number in group 5 which received CH-SRBC alone. Besides, the average number of anti-CT positive cells of group 7 was lower than that of group 4 which might imply that response to P in these rats was dose-dependent when P was given alone or together with non-synergistic antigen such as CHA.

The number of anti-LPS positive cells in groups 8 and 9 (received combined vaccine formula 1 and 2) were 45 and 44 cells, respectively. These figures were higher than that of group 3 but were not different from that of group 6. Thus, the maximum anti-LPS response was achieved when the LPS (500 μg) was combined with CH-SRBC and/or P. The maximum anti-CHA positive cells was found also in groups 8 and 9 (in the presence of 500 or 1,000 μg of LPS and 50 or 100 μg of P) but not found in group 7 (in the presence of 50 μg of P alone) indicating that LPS may act as adjuvant for CHA (in the form of CH-SRBC). The maximum anti-CT response was found in groups 8 and 4 but not in group 9 although the dose of P in groups 9 and 4 was twice as much the amount in group 8. The deduction that can be drawn from these experiments is that the oral vaccine consisted of LPS, CHA and P would elicit the best response provided that the three antigens are combined at a right proportion.

SUMMARY

Albino rats aged 7-8 weeks old purchased from the National Laboratory Animal Centre, Salaya, Nakhon Pathom, were found to be a good animal model for the study on immunogenicity of *V. cholerae* antigens. Seventy-two rats were fasted for 15 hours before feeding each one with 1 ml of 5% $NaHCO_3$ to reduce gastric acidity prior to immunization. They were divided into 9 groups of 8 rats and immunized orally with 2 ml, each, of the *V. cholerae* antigens dissolved or suspended in Cassamino acid as follows :– group 1 (control) : Cassamino acid (Ca) alone; group 2 (control) : 2.5% formalinized sheep red blood cells (F-SRBC); group 3 : 1,000 μg of lipopolysaccharide (LPS); group 4: 100 μg of procholeragenoid (P); group 5 : 80 haemaggltuinating units of cell-bound haemag glutinin (CHA) adsorbed onto the surface of F-SRBC (CH-SRBC); group 6 : 500 μg of LPS + 50 μg of P; group 7 : CH-SRBC + 50 μg of P; group 8 : combined vaccine formula 1

consisted of 500 μg of LPS, CH-SRBC and 50 μg of P and group 9 : combined vaccine formula 2 consisted of 1,000 μg of LPS, CH-SRBC and 100 μg of P.

The immunization was repeated once more 14 days later. Five days, thereafter, the rats were killed and their jejuni were removed for cryostat sectioning. Antibody producing cells against LPS (anti-LPS cells), P (anti-CT cells) and CHA (anti-CHA cells) in the intestinal lamina propria were enumerated by double antibody sandwich method of immunofluorescence using pure LPS, cholera toxin (CT) and pure CHA as the antigens in the assay, respectively. The results of the experiments indicate that LPS and P are synergistic antigens while CHA and P are not. LPS acts as CHA adjuvant. A combined oral vaccine consists of LPS, CHA and P evokes the highest production of antibody producing cells of all specificities when they are combined in a suitable proportion.

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