Immunochemoical Studies on Lipopolysaccharide from Agglutinable and Non-Agglutinable Vibrios

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Lipopolysaccharide (LPS) prepared from Vibrio cholerae and a non-agglutinable (NAG; not agglutinable with O-group I serum according to Gardner and Venkatraman [13]) vibrio strain, isolated from a patient with cholera-like clinical symptoms, have been compared with respect to their chemical composition and immunological behavior. In addition to a significant difference in the chemical composition between the two lipopolysaccharides, the LPS from V. cholerae, unlike that from the NAG vibrio, requires prior treatment with alkali for it to be an effective antigen in the indirect hemagglutination test with sheep cells. It has been suggested that the alkali acts by removing excess O-acetyl group from LPS of agglutinable vibrios. LPS from the NAG vibrio consistently showed a lower antibody response in rabbits in terms of agglutinin and vibriocidal titer. Also, the class of agglutinin antibody elicited by LPS of the NAG vibrio was predominantly immunoglobulin M, and that from V. cholerae was immunoglobulin G under comparable conditions.

Vibrio cholerae lipopolysaccharide (LPS) was isolated in serologically and immunologically active form by earlier workers (17, 22, 26, 27). The effectiveness of LPS as a vaccine in a field trial establishes its importance as an effective immunogen (2). Recently, the role of non-agglutinable (NAG; not agglutinable with O-group I serum according to Gardner and Venkatraman [13]) vibrios in producing disease that is very much similar, clinically, to cholera has been indicated by its isolation from patients, sometimes even as a pure culture (1, 10, 14, 20). In this context the LPS of an NAG vibrio has acquired a new importance. The present study compares the chemical composition and immunological properties of LPS from V. cholerae and an NAG vibrio, both isolated from patients with cholera-like clinical symptoms.

MATERIALS AND METHODS

Bacterial strains. Strains used were: (i) V. cholerae Ogawa 6900 isolated at the Department of Bacteriology, Cholera Research Centre, Calcutta; (ii) V. cholerae Inaba 569B isolated at Haffkine Institute, Bombay; and (iii) NAG vibrio 108 isolated from a patient at the Department of Bacteriology, Cholera Research Centre, Calcutta.

Preparation of LPS. Growth (18 h) on nutrient agar in roll bottles at 37 C were harvested in 0.85% cold saline and filtered through muslin. The filtrate was treated through Sharples super centrifuge, and the sediment was washed repeatedly in cold saline. The washed cells were digested with 50% aqueous phenol at 65 C in a water bath for 45 min. The mixture was kept overnight in a refrigerator (5 C), and the aqueous layer obtained after centrifugation was fractionated with cetyl-trimethyl-ammonium bromide and further purified as described by Pant and Shrivastava (25). Neutral LPS fraction was used in all the experiments. Prior to any experiment, the LPS preparations were confirmed to be free of protein, nucleic acid, and degraded LPS by the biuret test, ultraviolet absorption at 260 nm, and the Ouchterlony gel diffusion test, respectively.

Absorption spectrum. LPS (1 mg/ml) was dissolved in distilled water, and the absorption spectra were recorded in the ultraviolet region (250 to 280 nm) in a Hilger spectrometer. Gel diffusion test. The method described by Misra and Shrivastava (21) was followed. LPS was dissolved in water in different concentrations, and known volumes of the solutions were added to the gel diffusion plates for determining the minimum concentration required for the appearance of the precipitin line.

Precipitin activity. LPS solution of different concentrations was added to antisera in equal proportions incubated at 37 C for 4 h in a water bath, and kept overnight in the cold, and a reading was taken next morning.

Chemical analysis. Total carbohydrate as reducing sugar was determined by the orcinol method (28) after boiling the LPS in HCl for 2 h by using glucose as standard. For quantitation of lipid A (24) 300 mg of LPS was heated with 30 ml of 1% acetic acid for 3 h at 100 C. The precipitate was collected by centrifugation, washed with water, dried, and weighed to give lipid A. Total nitrogen was measured by the modified
micro-Kjeldahl method (19). The cysteine-sulfuric acid reaction (9) was used to determine total hexose by using glucose as standard. The carbazole method of Dishe (5) was followed to measure total uronic acid by using glucuronic acid as standard. Total pentose, methyl-pentose, O-acetyl, hexosamine, and keto-sugar were measured by the methods of Dishe and Schwarz (7), Dishe and Shettle (8), Hestrin (16), modified Elson-Morgan (11), and Dische-Borenfreund (6) by using arabinose, fucose, acetylcholin chloride, glucosamine, and fructose as standards, respectively.

Preparation of antisera. (i) Against whole organisms. Antisera were produced by immunizing rabbits (two for each group) intravenously with a suspension of bacteria, approximately 1,400 × 10^8 organisms per ml. Increasing doses were given twice a week for a 3-week period. The animals were bled 1 week after the last injection, and the serum was stored at −20 °C. Precipitin titer, indirect hemagglutination test, and gel diffusion studies were performed with the sera.

(ii) Against LPS. Rabbits (two for each group) were immunized with a single subcutaneous dose (5 mg), and blood from the ear veins was collected at an interval of 8 days over a period of about 2 months. The pooled sera were used for agglutination and vibriocidal tests against the whole organisms.

Agglutination test. The conventional tube agglutination technique was followed by using a serial twofold dilution of sera in normal saline by the usual method, temperature of incubation being 41 °C for 1 h.

Treatment with 2-ME. Samples of sera (0.2 cm^3) were mixed with an equal volume of 0.1 M 2-mercaptoethanol (2-ME) in phosphate-buffered saline and incubated at 37 °C for 18 h. Serum mixed with an equal volume of phosphate-buffered saline and incubated in the same manner was used as a control.

Vibriocidal test. The vibriocidal activities of the immune sera (inactivated at 56 °C for 30 min) were estimated by the plate count method (12) with 10-fold dilutions of sera. Guinea pig's complement (Difco) at a concentration of 1:40 and 1:160 were used for agglutinable and non-agglutinable strains, respectively.

Passive hemagglutination test. Sensitization of sheep erythrocytes was performed by mixing an equal volume of a 2% suspension of washed cells and a saline solution of LPS being tested. Sensitization was carried out both with untreated LPS or with LPS treated with 0.04 N sodium hydroxide for 18 h at 37 °C followed by neutralization. After incubation at 37 °C for 1 h with occasional shaking, the sensitized cells were washed three times with cold saline and resuspended to a 2% suspension. Portions (0.5 ml) of the LPS-sensitized erythrocyte suspension were added to a 0.5-ml portion of a serial twofold dilution of homologous antisera, previously inactivated at 56 °C for 30 min. The mixture was incubated for 2 h at 37 °C and kept overnight in a refrigerator before the reading was taken. The highest dilution of the serum showing hemagglutination was recorded as the titer.

RESULTS

Purity of the LPS. The preparations were biuret negative and free from nucleic acid as shown by the ultraviolet spectra (Fig. 1), and the nitrogen content was low (Table 1). A single precipitin line in the Ouchterlony gel diffusion experiment confirmed the absence of impurities or degraded LPS. The isolated LPS also revealed Ogawa LPS to be the highest in precipitin titer and to require the least amount for the formation of the alpha-band (21) in the gel diffusion test as compared to that from Inaba and NAG vibrios (Table 2).

Chemical analysis. The chemical composition of the LPS is shown in Table 1, which indicates that the main constituents of the LPS are carbohydrates and lipids, along with small amounts of nonprotein nitrogenous substances. Both LPS from V. cholerae and NAG vibrios are composed of similar components such as hexose, methyl-pentose, pentose, hexosamine, uronic acid, keto-sugar, O-acetyl, and lipid A. But there are statistically highly significant quantitative differences in the percent content of total hexose, methyl-pentose, and hexosamine between the two groups of LPS. The hexosamine content of NAG LPS is fourfold that from V. cholerae (Inaba and Ogawa). The hexose content of LPS from V. cholerae is about one-third of that from NAG LPS. The methyl-pentose content of LPS from NAG vibrios is about 2.5 times higher than that from V. cholerae. Also, the total carbohydrate, pentose, keto-sugar, and O-acetyl varies significantly between the two types of LPS. The uronic acid and lipid A components, however, are not very different.

Antibody responses in rabbits after subcutaneous injection of LPS. Serum samples were collected from rabbits immunized with a single subcutaneous inoculation with LPS from either V. cholerae or NAG vibrios at an interval of 8 days for a period of 2 months. The agglutination

![Graph showing the absorption spectra of LPS (1 mg/ml) in the ultraviolet region.](image-url)
titer was determined in the samples both with and without prior treatment with 2-ME. The vibriocidal titer was also checked. Up to the 16th day the agglutinin levels against both groups of LPS showed a steady parallel increase (Fig. 2a). After that the antibodies against LPS from NAG vibrios showed a decline, whereas that against LPS from agglutinable vibrios rose further to reach a peak on the 24th day followed by a plateau for a week. Figure 2b depicts the pattern of immunoglobulin class as detected by the agglutination test before and after 2-ME treatment. The agglutinin antibody elicited with LPS from NAG vibrios was completely 2-ME-sensitive (immunoglobulin M), whereas LPS from *V. cholerae* elicited 2-ME-resistant immunoglobulin G agglutinin antibody. Table 3 shows that the vibriocidal antibody titer rose sharply after immunization with LPS and persisted for about 2 months. LPS from NAG vibrios elicited a lower level of vibriocidal antibody than that of *V. cholerae*.

**Table 1. Chemical components of LPS from agglutinable and NAG strains of vibrios**

| Chemical constituents | Inaba 569B | Ogawa 6900 | NAG 108 | Inaba vs. Ogawa | Inaba vs. NAG | Ogawa vs. NAG |
|-----------------------|------------|------------|---------|----------------|--------------|--------------|
| Nitrogen              | 2.34       | 2.25       | 2.36    | 0.36           | 0.5           | 0.5          |
| Carbohydrate          | (2.15-2.4) | (2.15-2.4) | (2.15-2.34) | 0.36           | 0.5          | 0.5          |
| Hexose                | 31.1       | 32.7       | 39.0    | 0.36           | 0.5          | 0.5          |
| Methyl-pentose        | (30-35)    | (30-35)    | (36-42) | 0.36           | 0.5          | 0.5          |
| Hexosamine            | 12.5       | 10.2       | 36.7    | 0.36           | 0.5          | 0.5          |
| Pentose               | (10-16)    | (8-13)     | (32-42) | 0.36           | 0.5          | 0.5          |
| Keto-sugar            | 5.3        | 5.2        | 13.6    | 0.36           | 0.5          | 0.5          |
| O-Acetyl              | (4-6.5)    | (4-6.8)    | (12-17) | 0.36           | 0.5          | 0.5          |
| Uronic acid           | 3.1        | 3.9        | 5.2     | 0.36           | 0.5          | 0.5          |
| O-Acetyl              | (2.6-3.9)  | (3.6-4.4)  | (5.0-6.0) | 0.36           | 0.5          | 0.5          |
| O-Acetyl              | 2.2        | 2.6        | 0.7     | 0.36           | 0.5          | 0.5          |
| Lipid A               | (1.9-2.5)  | (2.2-2.7)  | (0.5-1.0) | 0.36           | 0.5          | 0.5          |
| Lipid A               | 2.6        | 2.3        | 1.0     | 0.36           | 0.5          | 0.5          |
| Lipid A               | (2.58-2.8) | (2.1-2.5)  | (0.96-1.33) | 0.36           | 0.5          | 0.5          |
| Lipid A               | 4.6        | 4.5        | 3.7     | 0.36           | 0.5          | 0.5          |
| Lipid A               | (3.7-5.5)  | (3.7-5.1)  | (2.5-3)  | 0.36           | 0.5          | 0.5          |
| Lipid A               | 27.9       | 29.2       | 22.8    | 0.36           | 0.5          | 0.5          |
| Lipid A               | (26-30)    | (24-31.6)  | (22-23.4) | 0.36           | 0.5          | 0.5          |

* Each result is the mean of five individual experiments. (Figures within the parenthesis indicate the ranges.)

* Significant.

* Highly significant.

**Table 2. Characteristics of vibrio LPS**

| Strain | No. of gel precipitation lines | Minimum amount required for precipitation on gel diffusion plate (μg) | Tube precipitation against homologous antisera | O-acetyl before NaOH treatment (%) | O-acetyl after treatment with NaOH (%) |
|--------|--------------------------------|---------------------------------------------------------------|-----------------------------------------------|-----------------------------------|--------------------------------------|
| Inaba 569B | 1                           | 10−*                                                          | 2.6                                           | 0.44                             | 0.44                                |
| Ogawa 6900 | 1                           | 0.5                                                          | 10−*                                          | 2.3                               | 0.44                                |
| NAG     | 1                              | 10−*                                                          | 10−*                                          | 2.6                               | 0.59                                |

* Treatment with NaOH: 0.04 N at 37 C for 18 h.

Erythrocyte-sensitizing character of LPS in indirect hemagglutination. Erythrocytes sensitized with a graded concentration of LPS preparation were used in the hemagglutination test with homologous antisera of similar agglutinin concentration. Table 4 shows that 5 μg of NAG LPS per ml was able to exhibit some hemagglutination, the titer rising with the increased concentration of the sensitizing LPS. But both of the *V. cholerae* LPS were unable to show any sensitizing effect even at a concentration of 500 μg/ml. This inability of *V. cholerae* LPS to sensitize sheep erythrocytes is overcome by alkali hydrolysis. The NAG LPS, on similar treatment, becomes a better sensitizer of erythrocytes.

It is shown in Table 1 that *V. cholerae* LPS contains twice the concentration of the O-acetyl
VOL (b) Comparison of agglutinin content.) Both isolated from components. served strains in the LPS samples in the study. However, observed that the LPS of NAG vibrios isolated from water did not show any significant difference in analytical data. The possible cause of this discrepancy may be the use of water NAG vibrios by the former workers rather than the NAG vibrios from patients in the present report. The different immunological behavior of the LPS from the NAG strain also corroborates a difference in the structure of the two types of LPS under consideration.

It is apparent from the data presented that the LPS from NAG vibrios produces a lower level of antibody in the serum than does that from V. cholerae, suggesting that NAG LPS is a less-effective antigen. Since the mode of antibody response is a complex phenomenon, the weaker immunogenical capacity of NAG LPS is consistent with the idea that, in addition to other factors affecting the antibody response during immunization, such as time, dosage, route, genetic condition of the animal (3), and serological method for antibody detection, the immunological constituents in the antigen complex also play an important role in the antibody responses.

It can be suggested from the present study that the O-acetyl groups may be responsible for interference with the uptake of LPS by erythrocytes. There is evidence (4) that, with some polysaccharides, the ability to adsorb onto red cells after alkali treatment may be related to the removal of O-acetyl groups. It has also been suggested (18, 22) that the lipid moiety of LPS may play an important part in this adsorption phenomenon. The polysaccharide moiety of LPS, when freed of lipid, does not possess this property (15). The role of lipid is discarded in the present study because there is no significant difference in the lipid content. However, this does not imply that this is the only change that

discussion

The chemical analysis data indicate a quantitative difference in the components of LPS isolated from V. cholerae and NAG vibrios. Both the LPS are, however, composed of similar components. Misra and Shrivastava (21) observed a basic similarity in the antigens present in the cytoplasms of V. cholerae and NAG strains and suggested a probable difference in

table 3. Vibriocidal antibody titer of rabbit sera immunized with LPS from agglutinable and NAG vibrios

| No. of days | Vibriocidal antibody titer |
|-------------|---------------------------|
|             | Inaba 569B                | Ogawa 6900 | NAG 108  |
| 8           | $2 \times 10^{-1}$        | $2 \times 10^{-1}$ | $4 \times 10^{-3}$ |
| 16          | $2 \times 10^{-4}$        | $2 \times 10^{-4}$ | $2 \times 10^{-3}$ |
| 24          | $2 \times 10^{-4}$        | $2 \times 10^{-4}$ | $2 \times 10^{-3}$ |
| 32          | $2 \times 10^{-4}$        | $2 \times 10^{-4}$ | $2 \times 10^{-3}$ |
| 40          | $2 \times 10^{-4}$        | $2 \times 10^{-4}$ | $2 \times 10^{-3}$ |
| 48          | $2 \times 10^{-4}$        | $2 \times 10^{-4}$ | $2 \times 10^{-3}$ |
| 56          | $2 \times 10^{-4}$        | $2 \times 10^{-4}$ | $2 \times 10^{-3}$ |
occurs, since it is entirely possible that other changes in the LPS molecules may simultaneously occur due to alkali hydrolysis, including changes in molecular rearrangement of the native structure, and that such changes may also play an important role.

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**Table 4. Effect of alkali* on the erythrocyte-sensitizing activity of the LPS from agglutinable and NAG vibrios**

| Strain          | µg of LPS per ml of 1.0% sheep erythrocytes | Hemagglutination titers* (reciprocal) |
|-----------------|---------------------------------------------|---------------------------------------|
|                 |                                             | 40         | 80         | 160        | 320        | 640        | 1,280      | 2,560      | 5,120      | 10,240     |
| LPS Inaba 569B  | 500                                         | +          | +          | +          | +          | +          | +          | -          | -          | -          |
|                 | 50                                          | +          | +          | +          | +          | +          | +          | -          | -          | -          |
|                 | 5                                           | +          | +          | +          | +          | +          | +          | -          | -          | -          |
| LPS Ogawa 6900  | 500                                         | +          | +          | +          | +          | +          | +          | -          | -          | -          |
|                 | 50                                          | +          | +          | +          | +          | +          | +          | -          | -          | -          |
|                 | 5                                           | +          | +          | +          | +          | +          | +          | -          | -          | -          |
| LPS NAG 108     | 500                                         | +          | +          | +          | +          | +          | +          | -          | -          | -          |
|                 | 50                                          | +          | +          | +          | +          | +          | +          | -          | -          | -          |
|                 | 5                                           | +          | +          | +          | +          | +          | +          | -          | -          | -          |

*0.04 N sodium hydroxide at 37 C for 18 h.

The serum employed was produced by immunization of rabbits with whole cell suspension of Inaba, Ogawa, and NAG strains. Agglutination titer of all the sera was 1:5,120.
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