Immunological Properties of the Cell Envelope Components of *Vibrio cholerae*

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Several immunobiological properties of cell envelope components of *Vibrio cholerae* such as mitogenicity, antigenicity, adjuvanticity and toxicity were tested in mice. Killed whole bacteria, spheroplasts, lipopolysaccharide and outer membrane proteins possessed mitogenic activity as determined by [³H]thymidine uptake in spleen cell cultures. All these components predominantly stimulated murine bone-marrow derived (B) lymphocytes. The mitogenicity induced by *V. cholerae* lipopolysaccharide was similar in magnitude to that observed with *Salmonella typhimurium* lipopolysaccharide. *Vibrio cholerae* lipopolysaccharide was mitogenic for gut-associated lymphocytes such as those obtained from Peyer's patches and small intestine. Antibody formation at the cellular level was detected by the haemolytic plaque assay. Plaque-forming cells to *V. cholerae* lipopolysaccharide were only detected when mice were immunized intraperitoneally with intact cells or with spheroplasts. Among the various cell envelope components, lipopolysaccharide alone possessed adjuvant properties as it increased the number of plaque-forming cells to sheep erythrocytes fourfold in mouse spleens. Also, lipopolysaccharide was the only component found to be toxic for the mouse (LD₅₀ 0.5 mg). Neither spheroplasts nor outer membrane of *V. cholerae* showed adjuvanticity or toxicity in mice.

INTRODUCTION

The cell envelope of *Vibrio cholerae* is morphologically similar to that of another Gram-negative bacterium, *Escherichia coli* (Kennedy & Richardson, 1969). It consists of three distinct layers: the outer membrane, the peptidoglycan and the inner or the cytoplasmic membrane. The outer membrane contains lipopolysaccharide (LPS), protein and lipid, while the inner or cytoplasmic membrane consists of protein and lipid. In Gram-negative bacteria, both LPS and protein moieties remain exposed on the cell surface (Shands, 1966; Kabir, 1975; Rittenhouse *et al.*, 1973) and it is most likely that these components play a role in the interaction of the bacterium and the host during infection. Among the various cell envelope components of Gram-negative bacteria, LPS has received most attention and has been found to have a variety of immunological effects in the susceptible host (Kass & Wolff, 1973). It is uncertain whether other components of the cell surface such as membrane proteins are also endowed with endotoxin-like properties.

There is evidence based on well-controlled field trials that killed whole cell vaccine, administered parenterally, is protective against cholera for limited periods of time and the immunity induced is antibacterial in nature (Mosley *et al.*, 1969). However, little is

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known about the immunological properties of the cell surface components of *V. cholerae*. Therefore, the present investigation was initiated to study in detail, in the mouse, immunobiological properties such as mitogenicity, antigenicity, adjuvanticity and toxicity induced by various cell envelope components of *V. cholerae* (intact cells, spheroplasts containing both inner and outer membranes, LPS and outer membrane).

METHODS

Animals. Female C57BL/6 mice, 6 to 8 weeks old, were used.

Bacterial strain and growth conditions. *Vibrio cholerae* strain 395 (Ogawa) was grown in Syncase medium (Finkelstein *et al.*, 1966) at 37 °C by shaking in a water bath. Cultures were harvested during the stationary phase. Bacteria were mixed with 1 % formalin for 1 h and dried by subsequent treatment with acetone. This procedure was followed to prepare killed organisms.

Media and reagents. All tissue cultures were grown in RPMI-1640 (Grand Island Biological Co., Grand Island, New York, U.S.A.) supplemented with L-glutamine (2 mM), penicillin G (100 units ml⁻¹) and streptomycin (100 µg ml⁻¹).

Isolation of LPS. LPS was extracted from bacteria by the phenol/water procedure of Westphal *et al.* (1952). The crude product was further purified to remove proteins and nucleic acids by procedures involving repeated ultracentrifugation at 105 000 g (Westphal & Jann, 1965). Protein content was estimated by the Lowry method, using bovine serum albumin as a standard. Carbohydrate was determined by the phenol/sulphuric acid method (Dubois *et al.*, 1956) using glucose as a standard.

Isolation of spheroplasts and outer membrane. Flagella were removed by shearing the bacteria at 4 °C in a Waring blender at high speed for 45 s. The suspension was diluted sixfold in 0.1 M-Tris/HCl (pH 7.8) and centrifuged at 1600 g for 15 min. The spheroplasts were prepared from the non-flagellated bacteria according to the procedure of Osborn & Munson (1975). Ten ml 0.01 M-Tris/HCl (pH 7.8) containing 0.75 M-sucrose and 0.5 ml lysozyme (2 mg ml⁻¹ in water) were added to 1 g of the non-flagellated bacteria and the mixture was incubated at 4 °C for 2 min in an ice bath. Then 20 ml cold EDTA (1.5 mM) was added slowly to the cell suspension. After 3 h incubation at 4 °C, 0.4 ml 1 M-MgCl₂, 0.3 mg DNAase and 0.3 mg RNAase were added to the mixture and allowed to stand for 10 min. The suspension was centrifuged at 20 000 g. The formation of spheroplasts was monitored by phase contrast microscopy. Spheroplasts were washed once with 10 ml 0.01 M-Tris/HCl (pH 7.8) containing 0.01 M-MgCl₂. The outer membrane was isolated according to the procedure of Schnaitman (1971). Spheroplasts prepared from 1 g bacteria were extracted with 10 ml 2 % (w/v) Triton X-100 in 0.1 M-Tris/HCl containing 5 mM-EDTA and the supernatant was discarded.

Enzyme assay. The NADH oxidase activity in the outer membrane was assayed as described by Osborn *et al.* (1972). The incubation mixture for the measurement of NADH oxidase specific activity contained 50 mM-Tris/HCl (pH 7.5), 0.12 mM-NADH, 0.2 mM-dithiothreitol and the membrane preparation (0.1 to 0.2 mg protein) in a total volume of 1.0 ml. The rate of decrease in absorbance at 340 nm at 25 °C was measured.

Preparation of outer membrane proteins. Lyophilized outer membrane (10 mg) was extracted with 1 ml of a buffer containing 0.05 M-glycine, 0.001 M-EDTA and 0.5 % (w/v) sodium deoxycholate, adjusted to pH 9 with NaOH, for 1 h at room temperature. The suspension was centrifuged at 10 000 g for 15 min. The residue contained the outer membrane proteins.

Both the outer membrane and the outer membrane proteins were subjected to immunoelectrophoresis after solubilization in the non-ionic detergent Tween 20 (Quality SD, Atlas Chemie, Essen, Germany). Briefly, 1 vol. 0.01 M-Tris/HCl buffer (pH 8.0) containing 5 % (w/v) Tween 20 was added to 1 vol. membrane suspension. After 2 h at room temperature, the mixture was centrifuged at 50 000 g. The supernatant was subjected to immunoelectrophoresis in a barbital buffer (pH 8.2) in 2 % (w/v) agarose for 1 h. The pattern of immunoprecipitation was allowed to develop against antiserum to *V. cholerae* spheroplasts. This antiserum was raised in New Zealand white rabbits by injecting each rabbit with 1 mg *V. cholerae* spheroplasts, suspended in an emulsion of 0.5 ml phosphate-buffered saline (PBS, pH 7.4) and 0.5 ml Freund's incomplete adjuvant (Behringwerke, Marburg/Lahn, Germany). The spheroplasts were administered on days 0, 14, 28 and 42. One week after the last injection blood was collected. The resulting antiserum was stored at -70 °C until used.

Spleen cell cultures. Mice were killed by cervical dislocation and their spleens were removed aseptically. Single cell suspensions from individual spleens were prepared by passing the tissue through an 80 mesh stainless steel screen into RPMI-1640 medium supplemented with 10 % (v/v) heat-inactivated foetal calf serum. Cells were washed thrice in the medium and separated by centrifugation at 200 g. Cell viability was determined by Trypan Blue exclusion. Unless otherwise stated, spleen cells (5×10^5) were cultured in a humidified atmosphere containing 5 % CO₂ at 37 °C in Micro Test II plates (Falcon Products, Oxnard,

Calif., U.S.A.) with different doses of *V. cholerae* fractions. For the last 16 h of the culture, cells were pulsed with [^3H]thymidine (1 μCi , 37 kBq). The cultures were harvested with an automatic harvester on to glass fibre strips, washed with at least 20 vol. saline solution and air-dried. After drying, the filter discs were placed in 3 ml of a xylene-based scintillation fluid and stored for 24 h in the dark at 4 °C before counting in a scintillation counter (Mann, 1978).

Separation of T and B lymphocytes using a nylon wool column. The procedure of Handwerger & Schwartz (1974) was used to separate murine spleen lymphocytes into T and B populations. The purity of the nylon adherent (B) and nylon non-adherent (T) populations was checked by immunofluorescent techniques with fluorescein-conjugated, rabbit antimouse Ig as previously described (Greaves, 1970). The nylon adherent cell populations (B) were found to be 85 % Ig-positive, while the non-adherent populations (T) contained very few Ig-positive cells (< 3 %).

Isolation of Peyer's patch and intestinal mucosal lymphoid cells. Peyer's patches were carefully excised from the small intestine and teased to obtain single cell suspensions. Approximately 5×10^6 lymphocytes were obtained from seven or eight Peyer's patches. Murine intestinal lymphocytes were isolated according to the procedure described by Cebra *et al.* (1977). The small intestine, devoid of Peyer's patches, was cut into pieces 3 cm long. The segments were everted on a Pasteur pipette and washed briefly in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free balanced salt solution (CMF-HBSS), prepared as described by Hudson & Hay (1976). They were then incubated with stirring for 15 min at room temperature in CMF-HBSS and placed in CMF-HBSS containing 0.75 mM-EDTA. The pH was kept constant between 7.2 and 7.4 by the addition of 7.5 % (w/v) NaHCO_3 . Gut pieces were then treated for 1 h with collagenase (10 units ml^{-1} , 20 ml per gut; Sigma) in RPMI-1640 at room temperature. The cell suspension was filtered through a gauze to remove debris, placed over a layer of Ficoll-Hypaque (Pharmacia) and centrifuged at 400 g for 40 min at 20 °C. About 5×10^6 lymphocytes were isolated per mouse gut.

Immunizing agents. Mice were immunized intraperitoneally with 0.5 ml of a washed sheep red blood cell (SRBC) suspension (10 %, v/v). Immunization with 0.5 ml of 10 % (v/v) SRBC suspension with different doses of the cell envelope components was used to assess their adjuvant properties. Direct splenic antibody plaque-forming cells (PFC) were counted 5 d after primary immunization.

Plaque assay. The number of PFC was estimated to two antigens, SRBC and *V. cholerae* LPS, in murine spleens. PFC to SRBC were detected by an agar plaque method similar to that developed by Jerne & Nordin (1963). In this, 10^6 nucleated spleen cells (50 μl), a suspension of SRBC (30 %, v/v; 50 μl) and 1 % (w/v) agar in minimum essential medium (MEM, Grand Island Biological Co.; 400 μl) were mixed, plated on a slide and incubated at 37 °C for 1 h. The slide was then washed and incubated with a 1:10 dilution of guinea pig complement. After further incubation for 1 h, the number of direct PFC was determined.

The technique employed to count PFC to *V. cholerae* LPS was similar to that described by Jerne & Nordin (1963) with some modifications. The LPS-coated SRBC were used as indicator cells. The coating method described here was not significantly different from that described by Möller (1965). Routinely, 5 mg LPS was dissolved in 5 ml PBS (pH 7.4) and the solution was heated at 100 °C in a water bath for 2 h. One ml of packed SRBC was added to 5 ml of the heated LPS solution and the mixture was shaken at 37 °C for 1 h. The suspension was centrifuged at 200 g, washed thrice with PBS and adjusted to 30 % (v/v) with PBS.

To confirm that anti-LPS immunoglobulins would be detected in a test involving lysis of LPS-coated erythrocytes, haemolytic tests were performed. Antiserum to *V. cholerae* LPS was raised in young New Zealand white rabbits (about 3 kg) by repeated intravenous immunization with *V. cholerae* LPS (0.1 mg ml^{-1} in PBS) according to the following schedule: day 1 0.5 ml, day 4 1.0 ml, day 8 1.5 ml, day 12 2.0 ml, day 19 2.5 ml, day 26 3.0 ml. Boosters of 3.0 ml were given fortnightly for the next 8 weeks. Rabbits were bled 1 week after the last injection. The immunoglobulins to LPS were purified by affinity chromatography using insoluble matrices of *V. cholerae* LPS covalently coupled to AH-Sepharose 4B (Pharmacia) according to the procedure of Axén *et al.* (1967). The immunoglobulins to LPS were dissociated from the column by applying 0.1 M-glycine/HCl, pH 2.3. For haemolysis, 0.1 ml anti-LPS immunoglobulins was serially diluted in PBS (pH 7.4) in microtitre plates and 0.1 ml of 2 % (v/v) *V. cholerae* LPS-coated SRBC was added to each well, followed by 0.1 ml of guinea pig complement diluted 1:10. The plates were incubated for 1 h at 37 °C and the last well showing 100 % lysis was taken as the end-point. Control experiments with uncoated erythrocytes were performed in parallel.

Toxicity test. The test substance was injected intraperitoneally in 0.5 ml PBS (pH 7.4) using groups of five mice. Deaths were recorded up to 72 h later.

Fig. 1. (a) Stimulation of $[^3\text{H}]$ thymidine incorporation by C57BL/6 spleen cells (5×10^5 per culture) in response to *V. cholerae* LPS ($0.3 \mu\text{g}$ per culture). Results are expressed as the arithmetic mean $\pm$ S.E.M. of triplicate cultures, with the unstimulated control incorporation subtracted. The uptake of $[^3\text{H}]$ thymidine (supplied during the last 16 h) in control cultures at 24, 48, 72 and 96 h was 8331 ± 557 , 5490 ± 110 , 2825 ± 72 and 1024 ± 28 , respectively.

(b) Proliferative response as a function of spleen cell density in culture. Different numbers of spleen cells were cultured for 48 h with *V. cholerae* LPS. Results are expressed as the arithmetic mean $\pm$ S.E.M. of triplicate cultures, with the unstimulated control incorporation subtracted. The uptake of $[^3\text{H}]$ thymidine in control cultures with 2.5×10^5 , 5×10^5 , 10×10^5 and 20×10^5 cells was 2540 ± 82 , 5372 ± 125 , 8212 ± 118 and 14492 ± 428 , respectively.

Fig. 2. Proliferative response *in vitro* of splenic lymphocytes from C57BL/6 mice induced by different doses of (a) killed *V. cholerae*, (b) spheroplasts, (c) LPS, (d) untreated outer membrane and (e) deoxycholate treated outer membrane. Spleen cells were cultured for 48 h and the results are expressed as the arithmetic mean $\pm$ S.E.M. of triplicate cultures, with the unstimulated control incorporation subtracted.

RESULTS

Chemical composition of the cell envelope components of V. cholerae

The LPS preparation contained 30 % carbohydrate, while the spheroplasts and the outer membrane contained 14 and 12 % carbohydrate, respectively. The LPS preparation was free of any detectable contamination with protein or nucleic acids. The spheroplasts and the outer membrane contained 44 and 80 % protein, respectively. The purity of the spheroplasts was checked by monitoring their formation by phase contrast microscopy.

Table 1. *Proliferation in vitro of murine T and B lymphocytes induced by cell envelope components of Vibrio cholerae*

Stimulants were added at the following doses per culture: killed *V. cholerae*, spheroplasts and outer membrane, each 1 μ g; lipopolysaccharide, 0.3 μ g; outer membrane proteins, 5 μ g. Results are given as the mean $\pm$ S.E.M. of two separate cell populations, each tested in triplicate, with the unstimulated control incorporation subtracted. Cells were cultured for 48 h.

Stimulant	Maximum lymphocyte proliferative response: [³ H]thymidine incorporated (c.p.m.) per 5×10^5 cells	
	T cells	B cells
Killed <i>V. cholerae</i>	1518 $\pm$ 42	69713 $\pm$ 118
Spheroplasts	5476 $\pm$ 26	86743 $\pm$ 131
Lipopolysaccharide	889 $\pm$ 29	48061 $\pm$ 150
Outer membrane	2725 $\pm$ 19	57468 $\pm$ 102
Outer membrane proteins	1153 $\pm$ 30	46745 $\pm$ 99

The outer membrane did not contain any cytoplasmic contaminant such as NADH oxidase (Osborn *et al.*, 1972). Also, it produced an immunoprecipitation reaction with the antiserum raised against the spheroplasts of *V. cholerae*. Thus, both the enzymic and immunochemical evidence support the authenticity of the outer membrane preparation.

Mitogenic effects

Proliferation in vitro of murine spleen lymphocytes induced by the cell envelope components of V. cholerae. First, the capacity of *V. cholerae* LPS to induce *in vitro* proliferation of murine spleen lymphocytes was tested. The maximum response was obtained when 5×10^5 nucleated spleen cells were cultured for 48 h (Fig. 1*a, b*). Next, the mitogenic properties of the killed whole cells, spheroplasts, outer membrane and LPS were compared. The dose-response relationships of these components are shown in Fig. 2. Each induced proliferation of murine spleen lymphocytes. The dose-response curve for whole killed cells was similar to that for spheroplasts. Increasing amounts of either component induced greater proliferation up to 5 μ g per culture; with higher doses the extent of incorporation of [³H]thymidine reached a plateau. LPS showed maximum stimulation at 0.3 μ g per culture and the outer membrane at 1.0 μ g per culture; with higher doses the incorporation of [³H]thymidine decreased.

Deoxycholate has been used to disaggregate non-covalently bound LPS from the outer membrane of Gram-negative bacteria (Kasper, 1976). To determine whether mitogenicity induced by the outer membrane was due to protein or carbohydrate moieties, the outer membrane of *V. cholerae* was treated with deoxycholate. By this treatment carbohydrate moieties were removed from the outer membrane. The outer membrane induced mitogenesis in murine spleen cell cultures. However, the dose-response curve for deoxycholate-treated outer membrane was shifted compared with that for the untreated outer membrane, the maximum response occurring at a higher concentration (5 to 10 μ g per culture).

Proliferation in vitro of murine B and T lymphocytes induced by the cell envelope components of V. cholerae. B and T lymphocytes were isolated from murine spleen cells by fractionation on a nylon wool column. The proliferative responses of these fractionated lymphocytes to various cell envelope components and their derivatives are shown in Table 1. All cell envelope components including the killed whole vibrios induced proliferation, the effect being predominantly on B lymphocytes.

Stimulation in vitro of intestinal lymphocytes by LPS. Although absorption of LPS in the intestine has been reported (Nolan *et al.*, 1977), no study involving *in vitro* stimulation of intestinal lymphocytes by LPS has been carried out. Therefore, we investigated the

Table 2. *Proliferation in vitro of murine lymphocytes from different organs induced by bacterial lipopolysaccharides*

Stimulant LPS was added at a final concentration of 0.3 µg per culture. Results are given as the mean $\pm$ S.E.M. of two separate cell populations, each tested in triplicate. Cells were cultured for 48 h.

Stimulant LPS	Maximum lymphocyte proliferative response: [³ H]thymidine incorporated (c.p.m.) per 5×10^5 cells		
	Spleen	Intestine	Peyer's patches
<i>Vibrio cholerae</i> Ogawa 395	56248 $\pm$ 139	3020 $\pm$ 139	6473 $\pm$ 40
<i>Salmonella typhimurium</i> Re 595	54213 $\pm$ 138	2096 $\pm$ 72	3967 $\pm$ 23
None	6424 $\pm$ 121	178 $\pm$ 14	182 $\pm$ 17

Table 3. *Adjuvant effects of the cell envelope components of Vibrio cholerae on anti-SRBC PFC responses in murine spleens*

SRBC suspension (10 %, v/v; 0.5 ml) was injected intraperitoneally into each mouse along with LPS (10 µg), spheroplasts (100 µg) or outer membrane (100 µg). After 5 d, direct splenic antibody PFC were counted as described in Methods. Results are given as the mean $\pm$ standard deviation.

Immunizing agent	Direct (IgM) PFC per 10^6 lymphocytes
SRBC	452 $\pm$ 112
LPS+SRBC	1656 $\pm$ 497
Spheroplasts+SRBC	649 $\pm$ 69
Outer membrane+SRBC	653 $\pm$ 292

proliferative response *in vitro* of murine intestinal lymphocytes to LPS preparations from *V. cholerae* and *Salmonella typhimurium* Re 595. In addition, we compared LPS-induced transformation of Peyer's patch cells. LPS preparations from both *V. cholerae* and *S. typhimurium* induced mitosis in intestinal lymphocytes (Table 2). The total uptake of [³H]thymidine after LPS stimulation was lower in intestinal Peyer's patch lymphocytes than in spleen cells but uptake was also lower in unstimulated enteric lymphocytes. *Vibrio cholerae* LPS induced similar mitogenicity in murine spleen lymphocytes to that demonstrated by *S. typhimurium* LPS.

Induction of antibody plaque-forming cells

To detect antibody formation to *V. cholerae* LPS at the cellular level, mice were immunized intraperitoneally with different doses of LPS, spheroplasts and killed bacteria. Spleens were assayed by the haemolytic plaque technique using LPS-coated SRBC as indicator cells. No anti-LPS secreting cell was observed when mice were immunized only with *V. cholerae* LPS. However, anti-LPS secreting cells were detected when a mouse received 125 µg of either spheroplasts or killed whole bacteria, the number of PFC (per 10^6 lymphocytes) being 150 ± 14 and 200 ± 53 (mean $\pm$ standard deviation), respectively. To confirm that coating SRBC with LPS did not prevent anti-LPS immunoglobulins from acting on SRBC, haemolysis experiments were performed. Anti-LPS immunoglobulins lysed LPS-coated SRBC in the presence of guinea pig complement suggesting that the coating of SRBC with LPS did not prevent the action of anti-LPS on SRBC.

Adjuvant action of the cell envelope components of V. cholerae

To test the adjuvant properties of the cell envelope components, mice were immunized intraperitoneally with SRBC along with spheroplasts, outer membrane or LPS. Individual spleens were assayed for anti-SRBC secreting cells. A significant action (about fourfold) was observed when mice received SRBC along with LPS (Table 3). The spheroplasts and outer membrane preparations had no adjuvant effects.

Toxicity of the cell envelope components of V. cholerae

Vibrio cholerae LPS, spheroplasts and outer membrane were tested for toxicity in mice. The LD₅₀ for *V. cholerae* LPS was 500 µg. Neither the cell wall nor the outer membrane had detectable toxicity for mice.

DISCUSSION

We observed that both intact cells and various cell envelope components (spheroplasts, LPS and outer membrane) were mitogenic for murine spleen lymphocytes. The dose-response curves for whole cells and spheroplasts were similar, with the proliferation of lymphocytes increasing with increasing concentration of the inducer. Presumably, this is due to the similarity between the presentation of functional surface groups for the activation of lymphocytes by the two materials. The results with intact cells agreed with recent studies with *Neisseria gonorrhoeae* (Brodeur *et al.*, 1977) and with whole mycobacterial cells (Bekierkunst, 1976).

The outer membrane of Gram-negative bacteria contains a complex mixture of proteins of various molecular weights. Recently, Melchers *et al.* (1975) reported that lipoprotein from the outer membrane of *E. coli* having a molecular weight of 7000 is a murine B-cell mitogen. Very little is known about whether proteins other than lipoprotein are endowed with mitogenic or other biological activities. In this investigation we observed that the outer membrane preparation was mitogenic. When residual carbohydrate moieties were removed by treatment with deoxycholate, the membrane proteins induced mitosis in spleen cell cultures suggesting that the outer membrane proteins did possess mitogenic properties. Recently, Brodeur *et al.* (1977) have also reported that membrane proteins of *N. gonorrhoeae* are mitogenic for murine spleen lymphocytes.

We observed that all the cell envelope components of *V. cholerae* stimulated murine B rather than T lymphocytes. This observation is in agreement with the general phenomenon that most of the so-called B-cell mitogens such as LPS (Andersson *et al.*, 1973), lipoprotein (Melchers *et al.*, 1975) and peptidoglycans (Damais *et al.*, 1975) have been obtained from various bacterial species.

The mechanism of action of endotoxin *in vivo* is not well understood. It has been reported that the mitogenic effects of LPS on lymphoid cells *in vivo* (Peavy *et al.*, 1978) parallel those effects observed *in vitro*. Although endotoxin may be biologically active on intact bacteria (Skarnes & Rosen, 1971), there is evidence which suggests that under certain conditions it may be released from the surface of Gram-negative bacteria (Crutchley *et al.*, 1968; Devoe & Gilchrist, 1973; Pike & Chandler, 1974) and this released endotoxin might initiate biological functions. Our results support this viewpoint. LPS is known to be absorbed in the gut (Nolan *et al.*, 1977) and we observed that *V. cholerae* LPS stimulated both the intestinal and Peyer's patch lymphocytes.

By comparing the effect of various cell envelope components of *V. cholerae* in the mouse, we found that LPS was toxic. Neither the spheroplasts nor the protein-enriched outer membrane possessed any toxic properties. In comparison with LPS of other Gram-negative bacteria such as *Salmonella* (Landy & Braun, 1964), *V. cholerae* LPS was not among the most toxic for the mouse. Its toxicity (LD₅₀ 0.5 mg) was moderate and similar to that of *Salmonella enteritidis* LPS (Landy & Braun, 1964).

Among the cell envelope components tested, only LPS possessed significant adjuvant activity, the magnitude being similar to that possessed by the LPS preparation from *Salmonella typhimurium* LT2 (Nakano *et al.*, 1975). The spheroplasts and the outer membrane were weak adjuvants. In this respect we find a correlation between adjuvant activity and toxicity. Our results indicate that although the cell envelope components of *V. cholerae* are predominantly murine B-cell mitogens, properties like toxicity and adjuvant activity are

only possessed by LPS. It is believed that the lipid A moiety of LPS, which remains buried in the cell membrane (Shands, 1973; Kabir *et al.*, 1978), mediates most of these biological properties (Lüderitz *et al.*, 1973). Hence, in the spheroplasts the lipid moiety of LPS is not available for interaction with other cell types. Therefore, the spheroplasts or the outer membrane might not possess lipid A-mediated biological properties such as adjuvant activity or toxicity in the mouse.

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