Antigens involved in resistance to mucosal association by *Vibrio cholerae*

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Summary. Resistance to growth of *Vibrio cholerae* at the mucosa of blind intestinal loops developed in rats after intestinal or parenteral exposure to live organisms or other antigenic materials. Simultaneous serological studies suggested that neither serum vibriocidal activity nor intestinal mucus antibodies are likely to provide a direct test of antibacterial immune status. Challenge of rats 4 weeks after one dose of antigen may reveal a form of immunity that is not related to antibodies in the intestine and possibly is analogous to long-term immunity expressed in man following infection with the organism. This immunity has not been attributed to lipopolysaccharide (LPS) antigen and does not appear to involve flagella-associated antigens; involvement of antigens other than LPS, such as protein antigens of the outer membranes of *V. cholerae*, has not yet been substantiated. Separation of monomeric sub-units of outer-membrane proteins by hydrophobic interaction high pressure liquid chromatography has revealed significant quantitative differences among preparations derived from the common serotypes of the organism. These differences may be sufficient to explain the better protection observed when homologous serotypes were used for immunisation and challenge in the long-term resistance model.

Introduction

Because it seems likely that antibacterial immune mechanisms prevent intestinal colonisation by *Vibrio cholerae* (Levine *et al.*, 1979), a method for quantifying its association with the intestinal mucus blanket of rats has been adapted recently to investigate the development and nature of this immunity (Cooper and Narendranathan, 1986). The efficacies of several different vaccines and immunising regimens were compared to assess the discriminatory potential of the method. Evidence suggested that, among other things, resistance to mucosal colonisation was induced by local (intestinal) immunisation, that it was not serotype specific, and that it correlated best with the presence of serum vibriocidal antibodies rather than with antibodies directed against the lipopolysaccharide or serologically dominant outer-membrane protein antigens of *V. cholerae*. The latter results, however, did not exclude a protective role for these antigens. The experiments reported here examined additional aspects of immunity to mucosal colonisation and questioned further the protective capacities of the major surface antigens of *V. cholerae*.

Materials and methods

Bacterial strains

Methods for preservation, culture and preparation of suspensions of *V. cholerae* strains have been described previously (Cooper and Narendranathan, 1986). Biotype, serotype and other characteristics as well as sources of these strains are described (see table III). *Pseudomonas aeruginosa* strain NSW467 and a recent isolate of *Campylobacter jejuni* were obtained from our culture collection.

Animals

Adult Wistar strain rats of either sex were used; they were 60–90 days old.

Immunisation

Methods for intra-intestinal (i.i.) and intra-Peyer's patch (i.p.p.) injection of bacterial suspensions into rats have been described elsewhere (Cooper and Narendranathan, 1986). When used, antigen preparations (*vide infra*) were suspended (3 mg/ml) in 0.1 M phosphate-buffered (pH 7.0) saline (PBS) homogenised with equal volumes of Freund's complete adjuvant (Commonwealth Serum Laboratories, Victoria, Australia) for Peyer's patch immunisation. In some instances, bacterial suspensions were given intravenously (i.v.) in a tail vein.

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Antigens of V. cholerae

Lipopolysaccharide (LPS) was prepared by the method of Westphal and Jann (1965) from 24-h cultures grown on Tryptose Soya Agar (TSA, Oxoid) at 37° C.

Outer-membrane proteins (OMPs) were prepared from cultures grown on TSA for 40 h at 37° C. The organisms were harvested, washed once and suspended (1g wet wt/40 ml) in 10 mM Tris-HCl (Sigma) buffer (pH 8) containing sucrose 15% w/v and 10 mM Na₂EDTA (Ajax Chemicals, Sydney, Australia). Lysozyme (0.5 mg/ml) was added and after incubation for 30 min at 20° C the suspension, chilled on ice, was sonicated for 60 sec. Unbroken cells were removed by centrifugation at 5000g for 15 min. Cell envelopes were recovered from the supernate by centrifugation at 48 000g for 30 min. The pellet was suspended in 10 volumes of 10 mM Tris-HCl buffer (pH 7.8) containing 10 mM MgCl₂. Contaminating DNA was removed by treatment with DNAase (Sigma) 10 µg/ml. Membranes were then collected by centrifugation at 48 000g for 30 min. The inner membranes were removed by extraction with Triton X-100 (Schnaitman, 1971). The pellet was resuspended in 4 volumes of Triton X-100 in Tris-MgCl₂, held at room temperature for 30 min and the outer membranes were collected by ultracentrifugation (100 000g for 60 min at 4° C) and stored in PBS at -20° C.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Stacking gel 5% w/v and resolving gel 10% w/v, containing methylene bisacrylamide 0.54% w/v, were used in the system of Laemmli (1970). Gel dimensions were 150 × 120 × 0.75 mm. Samples were solubilised by boiling for 3 min in an equal volume of sodium dodecyl sulphate (Sigma) 2% w/v in buffer (62.5 mM Tris-HCl) containing glycerol (BDH) 10% v/v and 2-mercaptoethanol (Sigma) 5% v/v. Electrophoresis was performed at 30 mA per gel until a control dye band was 1 cm from the bottom (c. 4 h). Gels were fixed overnight in methanol-acetic acid-water (10:10:80); protein bands were stained with Coomassie Blue R250 (Sigma) 0.01% w/v in fixing solution for 4 h and destained in several changes of isopropanol-acetic acid-water (25:10:65).

High performance liquid chromatography (HPLC)

OMPs were solubilised (*vide supra*) and 250-µg samples separated by hydrophobic interaction on a 280 × 5 mm µ-Bondapak C₁₈ column (Millipore-Waters, Sydney, Australia) that had been equilibrated in acetonitrile 10% v/v in water containing trifluoroacetic acid (TFA) 0.1% v/v. Proteins were eluted with a linear gradient over 15 min from 10 to 100% acetonitrile in TFA 0.1% v/v followed by elution with acetonitrile containing TFA 0.1% v/v for 10 min. Elution was monitored by absorbance at 280 nm. Retention times for each peak, area and relative abundances were calculated by a Data module connected to the absorbance monitor (Millipore-Waters).

Immunoblotting

OMPs separated by electrophoresis were transferred electrophoretically to nitrocellulose paper (Towbin *et al.*, 1979) which was then incubated for 60 min at 37° C in 25 mM Tris-HCl buffer containing 0.5 M NaCl and bovine serum albumin 3% w/v (TBS). The separated proteins were then exposed for 2 h at 37° C to appropriate rabbit antisera diluted 1 in 50 in TBS. After washing in TBS, the paper was placed in a 1 in 2000 dilution of goat anti-rabbit IgG antiserum labelled with horse-radish peroxidase (Kirkegaard and Perry Inc., Gaithersburg, MD, USA) for 4 h before washing in TBS. Labelled material was located by the method of Hawkes *et al.* (1982).

Antisera

A hyperimmune antiserum was prepared in rabbits by i.v. injection of live bacteria of *V. cholerae* strain BRL7738 of Inaba serotype. The first dose contained 5 × 10⁶ cfu; a further eight doses each increasing by 2-fold were given at weekly intervals. Sera were collected one week after the last injection, decomplexed at 56° C for 30 min, and stored at -20° C.

Antiserum raised against LPS of the same Inaba strain was also used. Rabbits were given intramuscular (i.m.) injections of 100-µg doses of this antigen in Freund's complete adjuvant at 14-day intervals for 6 weeks; sera were prepared and stored as above.

Absorption of sera with LPS

Inaba LPS (4 mg/ml) was added to antiserum diluted 1 in 2 in PBS, incubated at 37° C for 2 h, held overnight at 4° C and centrifuged at 100 000g for 2 h at 4° C. This procedure was repeated three times. Absence of anti-LPS antibodies was confirmed by failure of the absorbed material to cause agglutination of sheep red-blood cells passively sensitised with homologous LPS by the method of Auzins (1968).

Antibody measurements

Serum vibriocidal antibody titres were determined as described by Cooper and Jackson (1981) with *V. cholerae* strain BRL7738 as antigen. ELISA methods for detecting antibodies to LPS of *V. cholerae* (anti-LPS) and the serologically dominant, heat-sensitive OMPs (anti-HSSP) in serum and intestinal mucus have been reported elsewhere (Cooper *et al.*, 1984). For the latter, concentrations were expressed, on a scale from 0 to + + +, as increases in titres above those found in non-immunised rats; increases of < 4-fold (0 in tables II, IV and V) were not considered significant. Though antibodies of the three major immunoglobulin classes may be detected, serum IgM and IgG and intestinal mucus IgG and IgA antibody levels only are reported herein as previous studies showed that these classes of immunoglobulins, respectively, best reflected systemic and local output of antibodies (Cooper *et al.*, 1984).

Assessment of immunity to intestinal colonisation

Methods for infecting blind intestinal loops with *V. cholerae* and enumerating mucosa-associated bacteria have been described before (Cooper and Narendranathan, 1986). Streptomycin-resistant mutants of the strains BRL7738 (El Tor, Inaba) and O162 (Classical, Ogawa) were used for challenge infection; $(2-5) \times 10^3$ cfu were injected in each of the three or four loops prepared in each rat. The animals were killed by cervical dislocation under ether anaesthesia 14 h after inoculation; the loops were removed, opened longitudinally, washed vigorously in three changes of PBS, homogenised in 10 ml of PBS with a Teflon grinder or, in some instances, an Ultra-Turrax homogeniser. The number of cfu's per loop was estimated by the standard loop dilution method of Schaedler *et al.* (1965).

Mucosa-association resistance (MAR) index

This, the difference (in $\log_{10}$ units) between the mean cfu of mucosa-associated organisms in loops taken from non-immunised and immunised rats tested at the same time, was used to compare immunising regimens. Groups of four rats were used on each occasion and each experiment was repeated at least once. As the mean cfu of mucosa-associated organisms found in loops of immunised and normal rats on the two days of testing in each experiment were not significantly different ($p > 0.2$ by Student's *t* test) the MAR indices reported here were calculated from the means of at least 24 loops examined over 2 days. Student's *t* test was used to determine the significance of the MAR indices; under normal circumstances MAR indices of < 2.0 were not significant at the 1% level and were deemed to reflect little or no protection. Indices > 4.0 were at the upper limits of measurement and suggested strong resistance to colonisation.

Results

Duration of resistance to mucosal association

Groups of rats were given one or two doses of live bacteria of *V. cholerae* strain 569B by the i.i. or i.v. routes. Immunity to mucosal association by *V. cholerae* strain BRL7738, serum vibriocidal antibody titres, intestinal mucus anti-LPS and anti-HSSP antibody levels were determined at various times thereafter. The results shown in table I indicate that resistance developed within 2 weeks of one i.i. dose of organisms; immunity lasted from week 4 to week 12 but was lost by week 20. When two doses of antigen were given 14 days apart, strong immunity was evident within one week and lasted for at least 20 weeks. Parenteral i.v. immunisation was also effective. Antibody levels in similarly immunised rats are shown in table II; though humoral responses were evident after one

Table I. Immunity to mucosal association by *V. cholerae* strain BRL7738 after immunisation of rats with live *V. cholerae* strain 569B

Number of immunising doses	Time of challenge (weeks after last dose)	MAR indices* of rats immunised	
		i.i. ($c. 1 \times 10^9$ cfu/dose)	i.v. ($c. 1 \times 10^8$ cfu/dose)
1	1	1.72 (NS)	1.20 (NS)
	2	3.22 (S)	3.95 (S)
	4	4.58 (S)	4.73 (S)
	12	3.05 (S)	3.37 (S)
	20	1.60 (NS)	1.71 (NS)
2†	1	4.83 (S)	4.85 (S)
	12	4.90 (S)	4.53 (S)
	20	2.94 (S)	2.75 (S)

* Mucosa-association resistance index determined from mean numbers of organisms found in 24 loops prepared in groups of 8 rats.

† Doses given 14 days apart.

S = significant; and NS = not significant, at 5% level of Student's *t* test.

and two injections, in some instances as long as 12 weeks after one dose, there was resistance to mucosal colonisation when antibody titres in serum and intestinal mucus were not significantly different from those found in non-immunised rats.

Specificity of resistance to mucosa association

Rats were given single i.i. doses of $c. 1 \times 10^9$ cfu of strains representing the common biotypes and serotypes of *V. cholerae*; these rats and non-immunised rats were challenged 4 weeks later with strains BRL7738 or O162. Differences in protective capacity provided by the different bacterial strains were evident (table III). In all but three instances, however, significant immunity to challenge by both strains was found. With 12 of the 15 strains tested, the MAR indices recorded for both challenge strains differed significantly and in all cases the higher resistance index was recorded for groups immunised and challenged with a strain of the homologous serotype. The strain of Hikojima serotype, which expresses both Inaba and Ogawa serological characteristics, induced significantly better protection against challenge with the Inaba strain than with the Ogawa strain. The MAR indices recorded for the non-toxigenic strain JBK70 were significantly lower than those of the parent strain N16961 ($p < 0.01$ in both instances). In contrast, significant differences between the protec-

Table II. Antibodies in serum and intestinal mucus of rats immunised with live bacteria of *V. cholerae* strain 569B

Number of immunising doses	Weeks after last injection	Mean serum vibriocidal antibody titre*	Antibody concentrations† in intestinal mucus			
			anti-LPS		anti-HSSP	
			IgA	IgG	IgA	IgG
1	1	3.7 (4.3)‡	0 (0)‡	0 (0)	0 (0)	0 (0)
	2	3.0 (3.7)	0 (0)	0 (0)	0 (0)	0 (0)
	4	4.0 (3.9)	0 (0)	0 (0)	0 (0)	0 (0)
	12	2.3 (2.8)	0 (0)	0 (0)	0 (0)	0 (0)
	20	2.1 (2.2)	0 (0)	0 (0)	0 (0)	0 (0)
2	1	6.5 (7.3)	++(+)	++(++)	+(+)	+(+)
	12	2.5 (2.5)	+(0)	++(++)	0 (0)	0 (0)
	20	2.5 (2.5)	++(0)	+++ (0)	0 (0)	0 (0)

* In log₁₀ units; the range of titres in non-immunised rats was 1.7–2.0.

† Expressed as increases in titre, by the ELISA method, above those of non-immunised rats tested at the same time: 0 = <4-fold; + = 4–8-fold; ++ = 8–32-fold; and +++ = >32-fold.

‡ Each set of results was obtained after i.i. (or i.v.) immunisation of groups of 8 rats.

Table III. Mucosa-association resistance and serum vibriocidal antibody titres in rats four weeks after immunisation with one i.i. dose of *c.* 1×10^9 cfu of strains of *V. cholerae*

Immunising strain of <i>V. cholerae</i>		Mean serum vibriocidal antibody titre*	MAR indices* in rats challenged with		p values
Strain no.†	Biotype, serotype		Inaba strain BRL7738	Ogawa strain O162	
BRL7738	El Tor, Inaba	3.9	4.25	2.12	<0.001
N16961	El Tor, Inaba	4.2	3.80	3.37	>0.05
JBK70‡	El Tor, Inaba	3.5	2.82	1.64	<0.05
CVD107§	El Tor, Inaba	3.2	3.67	2.67	<0.05
569B	Classical, Inaba	3.9	3.91	2.94	<0.01
CVD103‡	Classical, Inaba	2.9	2.86	2.16	>0.30
35A3	Classical, Inaba	3.4	4.13	3.25	<0.05
VB1961	El Tor, Ogawa	3.2	3.08	3.84	>0.1
O162	Classical, Ogawa	3.0	2.73	4.09	<0.001
3083	Classical, Ogawa	2.8	2.48	4.46	<0.001
“Texas Star”‡	Classical, Ogawa	3.5	2.07	3.24	<0.01
111-V58	Classical, Ogawa	3.6	1.29	2.82	<0.01
111-V58NM	Classical, Ogawa	2.6	2.18	3.19	<0.05
KB112NM	Classical, Ogawa	2.3	1.19	3.93	<0.001
NW35	Classical, Hikojima	3.3	4.64	2.63	<0.001

* Vibriocidal antibody titres expressed and determined as in table II with groups of 8 rats. MAR indices shown here are the means of 24 replicates obtained from the formula $C - I$ where C = mean log₁₀ cfu of mucosa-associated organisms in 24 loops in non-immunised rats and I = log₁₀ cfu of mucosa-associated organisms in each of 24 loops in immunised rats; control and immunised rats were tested at the same time. C values for rats challenged with strains BRL7738 and O162 were not significantly different in each of the above tests.

† Strains 3083, Texas Star, N16961, CVD107 and CVD103 were obtained from Dr J. Kaper, Center for Vaccine Development, University of Maryland, USA. Other strains were from the culture collection of the School of Microbiology, NSW, Australia.

‡ The non-toxicogenic mutant strains JBK70, CVD103 and Texas Star were derived from strains N16961, 569B and 3083, respectively.

§ The non-motile strain CVD107 (with paralysed flagella) was derived from strain N16961.

|| The non-motile, non-flagellate strains 111-V58NM and KB112NM were derived from strain 111-V58.

tive capacity of the parent strains 569B and 3083 and their respective non-toxicogenic mutant strains CVD103 and "Texas Star" occurred following challenge with the homologous serotype only ($p < 0.05$ and < 0.001 , respectively). Non-motile strains of *V. cholerae* were also protective; the MAR indices were not significantly different from those recorded with the two parent strains available for testing ($p > 0.3$ for each). Though not shown in the table, antibodies were not found in the intestinal mucus of similarly immunised rats examined at the time of challenge. However, relatively low levels of serum vibriocidal activity were present in all immunised groups irrespective of immunity to the mucosal infection.

Protective activity of LPS antigen

The protection provided by LPS was investigated by injecting Inaba LPS in Freund's adjuvant by the i.p.p. route. Serum and intestinal mucus antibody levels at the time of challenge were determined in separate groups of rats. For comparative purposes, other rats were immunised i.p.p. with live organisms of the Inaba strain 569B. Though LPS was immunogenic and protective, these properties were evident only after two injections of antigen. In contrast, one injection of live organisms by the same route protected animals challenged 4 weeks later, even though anti-LPS antibodies were not detected in serum or intestinal mucus at that time (table IV).

Characteristics of *V. cholerae* OMPs

SDS-PAGE profiles of OMPs derived from several *V. cholerae* strains are shown in fig. 1. The

major protein bands had mol. wts (10^3) in the region 42–45; at least 15 other bands were resolved. Profiles of all strains of *V. cholerae* tested were generally similar and quite distinct from those of the strain of *P. aeruginosa* tested; some similarity with the profile of a representative strain of *C. jejuni* was noted.

When monomeric sub-units were separated by hydrophobic-interaction HPLC, six peaks with common retention times were found with all OMPs of *V. cholerae* tested. With different strains, however, the relative abundance of proteins isolated at each retention time varied; when results for strains of one serotype were pooled, statistical consistency for each of the protein peaks was found. Moreover, the profiles for the two common serotypes were readily distinguished whereas that of the only strain (NW35) of Hikojima serotype tested was similar to that of strains of Inaba serotype (fig. 2).

Protection by OMP preparations

Results of experiments in which rats were immunised by the i.p.p. route with OMP preparations are summarised in table V. Antibody levels in serum and intestinal mucus were determined in separate groups at the time of challenge. OMP from the challenge strain BRL7738 protected against mucosal association; however, it induced both anti-HSSP and anti-LPS antibodies in serum and intestinal mucus. Results of immunoblotting studies (fig. 3) suggested that the latter responses were most likely due to LPS associated with a number of proteins detected by SDS-PAGE, including those of mol. wt $(42-45) \times 10^3$. OMP from the rough strain NW31, on the other hand, showed little reaction

Table IV. Immunity to mucosal association induced in rats by i.p.p. injection of LPS antigen from *V. cholerae* strain 569B of Inaba serotype

Antigen	Number of doses	Day of challenge after last immunising dose	Mean anti-LPS antibody concentrations* in				MAR index*
			serum		intestinal mucus		
			IgM	IgG	IgA	IgG	
LPS	1	28	0	0	0	0	1.86 (NS)
L-O	1	28	0	0	0	0	3.32 (S)
LPS	2	5	+++	++	+	+	3.75 (S)
L-O	2	5	++	+	0	+	4.58 (S)

* See footnotes, tables I and II.

LPS = Lipopolysaccharide ($150 \mu\text{g}$ in Freund's adjuvant given in each dose).

LO = Live organisms (4×10^8 cfu of *V. cholerae* strain 569B given in each dose).

S = significant; and NS = not significant.

Fig. 1 SDS-PAGE of OMPs derived from *V. cholerae*. The strains tested in lanes 1–11 were: (1) 35A3; (2) BRL7738; (3) O162; (4) NW35; (5) 569B; (6) KB112NM; (7) 569B; (8) 35A3; (9) 111-V58NM; (10) 111-V58; and (11) N16961. Lane 12 contains mol.-wt (10^3) standards as shown on the left. OMPs of *C. jejuni* and *P. aeruginosa* strain NSW467 are shown in lanes 13 and 14, respectively.

with these antibodies, although it shared two of the three major immunogenic proteins of the strain BRL7738. Responses to HSSP, but not LPS, following injections with the OMP of strain NW31 were consistent with these observations, but this material did not induce resistance to mucosal colonisation.

Discussion

The rat intestinal loop model provides a measure of active immunity to cholera infection; it reflects the ability of immunised animals to restrict the association of the causative agent with, or proliferation at, the intestinal surfaces. As with other recently described models (e.g., Spira *et al.*, 1981; Tokunaga *et al.*, 1984), it permits assessment of the

protective capacity of live cholera vaccines or other antigenic preparations and does not rely on indirect evidence obtained from passive-immunisation studies made *in vivo* (e.g., Ujiye and Kobari, 1970) or *in vitro* (e.g., Attridge and Rowley, 1983).

Effective immunity was induced by live organisms given locally or parenterally and was retained for periods of >10 weeks after one or two immunising doses but, as with most immune responses, its onset was more rapid and its duration longer when two immunising doses were used. Results from volunteer studies have suggested that long-term resistance to cholera following clinical or sub-clinical infection may exist in the absence of antibodies other than those responsible for serum vibriocidal activity (Cash *et al.*, 1974). It is possible that the rat infection model used 4 weeks after a

Fig. 2 HPLC fractionation of OMP preparations on μ -Bondapak C18 columns. Comparison of "relative abundance of protein" profiles were obtained from the means of results recorded for: (A) *V. cholerae* Inaba strains 35A3, BRL7738, 569B, N16961; (B) *V. cholerae* Ogawa strains VB1961, O162, 3083, 111-V58, KB112NM; (C) *V. cholerae* Hikojima strain NW35. Error bars, where appropriate, are shown. Retention times (min) for the peaks were: 1.2 (1); 1.7 (2); 2.8 (3); 12 (4); 15 (5); and 19 (6).

single dose of organisms or antigen, i.e., when the same serological results are found, offers an opportunity to study the nature of this immunity and methods for its induction.

Despite evidence from earlier findings (Cooper and Narendranathan, 1986), correlations between resistance to mucosal association and antibody levels must now be questioned. As in man (Clements *et al.*, 1982), live organisms in the rat intestine leave a long-term legacy of low levels of serum vibriocidal activity. Association of this activity with resistance

may be no more than fortuitous. For instance, 7 days after one dose of organisms, rats were no more resistant than non-immunised animals despite high titres of serum vibriocidal antibody. Again, though resistance and the presence of anti-LPS antibodies in intestinal mucus after two immunising doses were seemingly related, these antibodies were not found in resistant animals that received only one dose of organisms. These and related findings (tables I and II) suggested that serological tests of serum or intestinal materials were unlikely to

Table V. Immunising activity of outer-membrane proteins prepared from strains of *V. cholerae*

OMP from strain no.	Anti-LPS antibodies* in				Anti-HSSP* antibodies in				MAR index*
	serum		intestinal mucus		serum		intestinal mucus		
	IgM	IgG	IgA	IgG	IgM	IgG	IgA	IgG	
BRL7738	+	+	+	+	+	+	+	+	3.95 (S)
NW31	0	0	0	0	+	+	+	+	0.23 (NS)
NSW467†	0	0	0	0	0	0	0	0	0.11 (NS)

* See footnotes, tables I and II. The Inaba strain BRL7738 was used as the challenge strain, 5 days after two i.p.p. injections of OMP (150 μ g in Freund's adjuvant) given 14 days apart; antibody titres were determined at the same time.

† *P. aeruginosa* strain NSW467 was included as a negative control.

Fig. 3 SDS-PAGE (lanes 1, 2) and immunoblotting (lanes 3–6) of OMPs derived from strains of *V. cholerae*. The rough mutant strain NW31 derived from Ogawa strain O162 is shown in lanes 1, 3 and 5; the strain BRL7738 of serotype Inaba is shown in lanes 2, 4 and 6. For the immunoblots, rabbit anti-LPS antiserum (lanes 3 and 4) or rabbit antiserum prepared against strain BRL7738, and absorbed with Inaba LPS (lanes 5 and 6) were used.

provide direct indication of immune status to the disease.

Indications from previous studies that the cell-wall LPS of *V. cholerae* is a protective antigen (e.g., Holmgren and Svennerholm, 1983) have been confirmed. These results and those of earlier studies (Cooper and Narendranathan, 1986), suggest that resistance to mucosal association induced by LPS depends on continued output of anti-LPS antibodies into the intestine *via* the mucosal surfaces and, perhaps, bile also (Cooper *et al.*, 1984). Although

the present results do not seem to implicate LPS in the induction of immunity to mucosal colonisation, failure to detect anti-LPS antibodies does not exclude their involvement; furthermore, absence of protective activity after a single injection of LPS could merely reflect poorer immunogenicity of the extracted material compared with the form in which it is presented in the intact organism. Nonetheless, attention is drawn to a second tier of immunity in which antigens other than LPS may be involved (Attridge and Rowley, 1983); the most

likely candidates are the heat-sensitive proteins of the organism's outer membranes.

As reported by others (e.g., Kabir, 1983; Sears *et al.*, 1984), SDS-PAGE separation and immunoblotting indicated that there were physico-chemical and serological similarities among the major monomeric sub-units of OMPs prepared from strains of the common serotypes of *V. cholerae*. Furthermore, it is obvious that LPS association with a number of these proteins precludes direct assessment of their protective capacity. The device of using an OMP prepared from a rough strain of *V. cholerae* has produced results that call into question the involvement of these antigens in resistance in the rat model. Even though the OMP derived from the rough strain contained at least two of the major immunogenic proteins associated with the surfaces of a protective strain (BRL78738) and, when injected into the Peyer's patches, caused output into the intestine of antibodies that reacted specifically with those antigens, it was no more protective than a preparation obtained from the serologically unrelated strain of *P. aeruginosa*. This result also suggested that the adjuvant itself did not enhance immunity by non-specific means. Loss of putatively protective proteins from the rough strain cannot be discounted but, if OMPs are involved in resistance to mucosa association, present serological methods seem to be either too insensitive or insufficiently discriminatory to detect them.

Whilst cross resistance was expressed in the absence of antibodies other than those causing serum vibriocidal activity, the level of resistance was significantly higher when serotypic homology existed between immunising and challenge strains. This is consistent with results from field trials with monovalent killed vaccines (Mosley *et al.*, 1973) and animal-immunisation experiments (Svennerholm *et al.*, 1975). Though the latter workers implicated type-specific antigenic determinants of LPS, absence of anti-LPS antibodies in immune rats is against this hypothesis. Until now, incrimination of the protein antigens of *V. cholerae* has not been justified because of many similarities among the OMPs obtained from different *V. cholerae* strains. Hydrophobic interaction HPLC techniques, however, have revealed for the first time

that serotype-associated differences do exist in these materials. In the absence of effective preparative methods with this separation technique, chemical and serological differences cannot be excluded but similarities of retention times of the six major proteins suggest that the differences are more likely to be quantitative. Nevertheless, they may be sufficient to account for the better performance of serotypically homologous strains in protection tests. It was interesting to find that strains of Hikojima serotype induced significantly better resistance to Inaba challenge than did Ogawa strains; the Hikojima strain also had an HPLC profile similar to that of Inaba strains.

Local immunisation by intestinal injection of live organisms has confirmed findings of Tokunaga *et al.* (1984) that the mutant strain "Texas Star" is less protective than the parent strain 3083; this is also true for the non-toxigenic mutant strain JBK70 derived from strain N16961. Whereas poorer protection has been attributed to loss of colonising and, hence, immunising ability (Tokunaga *et al.*, 1984), similarity of serum vibriocidal antibody titres in rats given parent or mutant strains suggests that this may not be so. Again, because a poorly colonising non-motile mutant strain was equally as protective as the parent strain N16961, loss of toxigenicity may be more important in reducing protective activity.

Finally, it was interesting to find that non-flagellate strains were protective. Although both non-flagellate strains colonised the rat intestine surface poorly (Cooper and Narendranathan, 1986), it was clear that their immunogenicity was not thereby affected. Passive-protection tests (Eubanks *et al.*, 1977) have implicated flagella-associated antigens in protection against cholera infection. The present results, currently being extended with non-motile strains derived from other isolates of *V. cholerae*, must question the validity of that hypothesis. Use of the rat model may resolve this and other questions relating to antibacterial immunity in cholera.

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