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IMMUNOLOGICAL RESPONSE TO INFECTION

Immunogenicity and protective role of an IgA reactive 31-kDa antigen of *Vibrio cholerae* O139

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Immunoglobulin A (IgA) is important in protective immunity against infection by *Vibrio cholerae*. In this study, the immune response to and protective role of a 31-kDa antigen of *V. cholerae* O139, reacting with IgA antibodies present in the sera of cholera patients and common to *V. cholerae* strains O139 and O1 was evaluated in BALB/c mice. From the various antigens of *V. cholerae* O139 and *V. cholerae* O1 which reacted with IgA antibodies in sera of a cholera patient, a 31-kDa common antigen was selected and purified by DEAE-Sepharose CL 6B column chromatography. Oral administration of live *V. cholerae* O139 in BALB/c mice elicited an IgA response to the 31-kDa antigen in serum and intestinal fluid, and a proliferation of the splenic lymphocytes on stimulation with the same antigen. The cytokine profile of these splenic lymphocytes revealed a shift from a mixed Th1 and Th2 response – interleukin-10 (IL-10) and interferon-γ – in the first week after infection to a Th2 type of response – IL-10 – in the third week. In passive protection studies, hyperimmune serum to the 31-kDa antigen was able to protect infant mice against challenge with O139 and O1 strains. These results demonstrate the ability of the 31-kDa antigen of *V. cholerae* O139 to induce humoral and cellular immune responses in mice, and its immunoprotective nature.

Introduction

After its emergence in late 1992, *Vibrio cholerae* O139 Bengal caused large epidemics of cholera in the Indian subcontinent. Subsequently, it spread to many other countries in Asia and imported cases were also reported from several developed countries. This organism is indistinguishable from *V. cholerae* O1 El Tor strains biochemically and in major virulence characteristics, especially in producing cholera enterotoxin (CT) and toxin co-regulated pilus (TCP) [1, 2]. Remarkable similarity has been shown between the outer-membrane protein (OMP) profiles of O1 and O139 *V. cholerae* [3]. However, *V. cholerae* O139 does not produce the O1 lipopolysaccharide (LPS) [2, 4].

A strong protective immunity is conferred by an infection with *V. cholerae* [5], but the identity of crucial protective antigens and the nature of the protective immunity are still not clear. Studies have shown the importance of antibacterial over antitoxic immunity in *V. cholerae* O1 and O139 [5–7]. Many antigens, including LPS, OMP, TCP and mannose-sensitive haemagglutinin (MSHA), are believed to be involved in the induction of protective immunity against cholera [8–15]. Attridge and Rowley [9] compared the protective activities of antibodies to LPS and non-LPS antigens of *V. cholerae* strain 569B and concluded that the latter protected infant mice more efficiently from challenge with strain 569B.

In non-invasive enteric infection like *V. cholerae,* secretory immunoglobulin A (S-IgA) antibody appears to be the main, although not the only, protective molecule [16] and serum vibriocidal antibodies are regarded as the marker for the presence of intestinal antibodies against critical bacterial surface antigens. Although most of these are directed against the LPS, antibodies against protein antigens also exist and have been the subject of investigation. IgA antibodies to MSHA and OMPs have been reported to appear in the sera of volunteers and cholera patients [14, 17, 18].

This study searched for the antigens reactive with IgA

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by the immunoblotting technique with sera from cholera patients and whole-cell sonicated antigens of *V. cholerae* O139 and O1 and identified a 31-kDa protein common to different strains of both serogroups. This protein was purified and its roles in humoral and cellular immune responses against *V. cholerae* O139 infection in adult BALB/c mice and passive protection against challenge with *V. cholerae* O139 and O1 in an infant mouse model were investigated.

Materials and methods

Bacterial strains

V. cholerae strains used in this study were MO45 (O139), S-776 (O139), NIH41 (O1 classical Ogawa) and V86 (O1 E1 Tor Inaba). Of these, strain S-776 was a clinical isolate obtained from the enteric laboratory of this department and the others were provided by the Director, Central Research Institute (CRI), Kasauli, India.

Collection of sera

Sera were collected from 12 bacteriologically confirmed (stool culture) cholera patients, who were admitted to the Nehru Hospital, PGIMER, Chandigarh, India. Three of the cases (one acute and two convalescent) were infected with *V. cholerae* O139 and the others (all acute) with *V. cholerae* O1 El Tor Ogawa. Sera from eight healthy controls who had no history of diarrhoea in the previous 3 months were also included in the study. Venous blood (3–5 ml) was collected from each subject; serum was separated and stored at −20°C until used.

Preparation of whole-cell sonicate (WCS) antigens

Bacteria were grown overnight at 37°C in 5 ml of Brain Heart Infusion (BHI) Broth (Hi Media, Mumbai, India) with pH adjusted to 8.4. The growth was harvested by centrifugation at 10 000 *g* for 15 min, washed twice with 0.01 M phosphate-buffered saline (PBS, pH 7.2) and lysed by sonication (SONIPREP-150). The lysate was centrifuged at 10 000 *g* for 15 min at 4°C to obtain clear cell-free lysate. Protein concentration was determined by the Lowry method and antigenic preparations (WCS) were stored at −20°C.

Analysis of protein by SDS-PAGE

The protein profiles of WCS antigens of different *V. cholerae* O139 and O1 strains were analysed by SDS-PAGE [19] in acrylamide 10% separating gel with a BioRad Mini Protean II electrophoresis apparatus, following the manufacturer's instructions. The gels were stained either with Coomassie Brilliant Blue R 250 or by the silver staining method of Merril *et al.* [20].

Immunoblotting

The WCS proteins, separated by SDS-PAGE, were transferred on to nitrocellulose paper (NCP, BioRad) as described by Towbin *et al.* [21] with a BioRad Mini Trans Blot apparatus according to the manufacturer's instructions. After blocking with 15 ml of bovine serum albumin 3%, fraction V (Sigma) at 4°C overnight, the blotted sheet was treated with sera from patients infected with *V. cholerae* strain O139 or O1 or normal healthy sera (NHS) in BioRad Mini Protean II multiscreen apparatus. Different dilutions of sera were used initially in the screening experiments and finally blots were treated with serum at a dilution of 1 in 50. After incubation for 1 h at 37°C and overnight at 4°C, the NCP was washed (15 min, four changes) with PBST (0.01 M PBS, pH 7.2, containing Tween-20 0.05%). Anti-human IgA-horseradish peroxidase conjugate (Sigma) diluted 1 in 5000 was added to NCP and incubated at 37°C for 1 h to detect the antigens reacting with IgA antibodies. After washing as above, NCP was developed with diaminobenzidine hydrochloride (SRL) and H_2O_2.

Purification of the 31-kDa protein by DEAE-sepharose CL 6B column chromatography

The 31-kDa protein was purified by the method of Nagai *et al.* [22]. Briefly, WCS protein concentrate (100 mg) prepared from strain MO45 was applied to a DEAE-Sepharose CL 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) column (bed volume 10 ml) with 30 mM Tris hydrochloride buffer (pH 8.7) containing methyl cellosolve 3%. During standardisation, DEAE-Sepharose CL 6B slurry equilibrated with 30 mM Tris buffer at pH 8.5–9.0 was found to show maximum absorption of the protein (verified by SDS-PAGE); therefore, in subsequent work a mean pH of 8.7 was chosen for the equilibrating buffer. After loading, the column was washed thoroughly with elution buffer and protein was eluted with increasing salt concentration (NaCl, 50–300 mM, with 50 mM unit interval), at a flow rate of 20 ml/h. The protein content of each fraction was determined by measuring absorbance at 280 nm. Peaks were pooled and the protein in each peak was concentrated by Amicon® ultrafiltration (10-kDa cut-off). The protein profile of each peak was analysed by SDS-PAGE. Peak II protein concentrate (6 mg) containing the 31-kDa protein was taken to the second stage of DEAE-Sepharose CL 6B column chromatography (bed volume, 3 ml) for further purification and was eluted with 50–100 mM salt gradient (with 10 mM unit interval). The pooled peaks, concentrated by Amicon® ultrafiltration, were analysed by SDS-PAGE. The purified 31-kDa protein was stored at −20°C.

Raising of hyperimmune sera

Hyperimmune sera were raised against WCS and 31-

kDa antigens of strain MO45 in adult New Zealand White rabbits by the method of Vaitukaitis [23]. Pre-immunisation sera were collected and stored at −20°C. Antigen (20 μg) was emulsified with an equal volume of Freund's complete adjuvant and injected intradermally at 8–10 sites in the back of each rabbit. The procedure was repeated three times with protein emulsified with an equal volume of Freund's incomplete adjuvant at intervals of 14 days. Test bleeding was done 1 week after the last dose and any rise in titre was assessed by ELISA and immunoblotting.

Immunological studies in mice

Immunological studies were done in BALB/c mice (4–6 weeks old) obtained from CRI. Each of a test group of 28 animals was given $(1–2) \times 10^6$ cfu of strain MO45 in 0.2 M sodium bicarbonate by mouth on days 0, 14 and 21 [24]. Colonisation of mouse intestine by vibrios was confirmed in five animals on the following day by coproculture and intestinal content culture. Another group of 24 mice under similar conditions, inoculated orally with suspending medium without any bacteria, served as control. Blood was collected from a group of five mice every week for 4 weeks by sampling from the retro-orbital plexus under ether inhalation anaesthesia. Subsequently, the mice were killed and the intestinal fluid and spleens were collected. For the collection of intestinal fluid, the abdomens of mice were opened and the intestine was removed and flushed with 1 ml of sterile PBS (0.01 M, pH 7.2) to remove all the contents. The intestinal effluent was centrifuged at 5000 *g* for 20 min at 4°C to remove the debris and the supernate was collected. Both serum and intestinal fluid were stored at −20°C until used to assess the IgA antibody response by ELISA. Spleens were removed from the mice and immediately placed in 5 ml of RPMI-1640 in a sterile plastic petri plate (Tarson) for further processing to isolate the lymphocytes.

ELISA

Microtitration plates (96-well, flat-bottomed; Nunc) were coated with 31-kDa protein (1 μg in 100 μl/well) in 0.05 M carbonate buffer (pH 9.6) and incubated at 37°C for 1 h and at 4°C overnight. The plates were washed twice with PBST with a microtitration plate washer (812-SWI, SLT Instruments, Austria) and blocked with blocking buffer (PBS containing BSA 1%) 200 μl/well then incubated at 37°C for 2 h. This was followed by five washes and addition of 100 μl/well of sera or intestinal fluids diluted 1 in 10 in blocking buffer. After incubation for 1 h at 37°C, the plates were washed five times and 100 μl of anti-mouse IgA-horseradish peroxidase conjugate (Sigma; diluted 1 in 29 000) were added to each well. After incubation for 1 h at 37°C, plates were washed five times and freshly prepared substrate solution (100 μl) consisting of ortho-phenylenediamine (0.4 mg/ml) and H_2O_2(6%, 0.4 μl/ml) in 0.01 M citrate buffer was added to each well. The plates were incubated at room temperature for 15–20 min and the reaction was stopped by the addition of 50 μl of 2.5 N H_2SO_4 to each well. Absorbance was measured at 492 nm (against a reference filter of 620 nm) in an ELISA reader (ATC 340, SLT Instruments).

Lymphocyte proliferation

Lymphocytes were isolated from spleens by the method of Breiman and Horwitz [25]. Spleens were ground to pulp which was strained through a layer of nylon mesh to remove fibrous material. The spleen cells, passing through the mesh, were collected by centrifugation at 800 rpm for 10 min at 4°C. Residual erythrocytes were lysed with ammonium chloride 10% w/v. The pellet of lymphocytes obtained was washed twice with RPMI 1640 (pH 7.4 ± 0.2) and finally with RPMI-1640 containing fetal calf serum (FCS) 10% v/v. The viability of lymphocytes was checked by staining with trypan blue dye and the lymphocyte count was adjusted to $(1–2) \times 10^6$/ml.

Lymphocyte activation was studied by the method of Kabir [26]. The cells from individual mice were cultured in triplicate in flat-bottomed 96-well microtitration plates (Nunc). Cells (100 μl) were added to each well. This was followed by the addition (100 μl/well) of 31-kDa antigen, 6 or 8 μg/ml. Parallel sets of wells containing lymphocytes exposed to medium alone and to Concanavalin A (Con A; Sigma) 2.0 μg/ml were also included in all experiments as negative and positive controls. The culture plates were incubated at 37°C in a humidified atmosphere with CO_2 5% for 72 h. The cultures were pulsed for a final 16 h with [^{3}H]thymidine (Bhabha Atomic Research Centre, Mumbai, India; specific activity 6.7 Ci/mM) 1.0 μCi/well. The cells were harvested with a PHD cell harvester (Cambridge Technology) on to glass fibre disks. The disks were air-dried and placed in 5 ml of xylene-based scintillation fluid. The radioactivity incorporated was measured as counts/min (cpm) with a liquid scintillation counter (LKB Wallace 1214, Rackbeta Turku, Finland) and the results of lymphocyte proliferation were expressed as stimulation index (SI, ratio of mean cpm of stimulated cultures to the mean cpm of unstimulated cultures).

Cytokine assays

For the cytokine assays, 200 μl of splenic lymphocytes from each mouse were cultured in duplicate in 24-well tissue-culture plate (Laxbro, India). This was followed by the addition of 100 μl of 31-kDa protein (6 μg/ml) and 200 μl of RPMI-1640 with FCS 10%. Appropriate negative controls containing medium alone were also set. The culture plates were incubated at 37°C for 72 h in humidified air with CO_2 5%. Culture supernate from each well was collected and centrifuged at 6000 *g* for 15 min at 4°C. Supernate was collected and stored at

−20°C until used for interleukin-10 (IL-10) and interferon-γ (IFN-γ) assays. Mouse Interleukin-10 ELISA Kit Intertest-10X and Mouse Interferon-γ ELISA Kit-Intertest (Genzyme Diagnostics, USA) were used according to the manufacturer's instructions.

Protection studies

The lethal dose 50 (LD50) was determined and protection studies were done by the passive protection method with *V. cholerae* strains MO45, NIH41 and V86 in 5–7-day-old infant BALB/c mice obtained from the Institute of Microbial Technology, Chandigarh, India. For both the experiments, mice were removed from their mothers about 6 h before use to ensure that stomach contents were emptied. The LD50 of *V. cholerae* strains was determined as described by Ujiiye *et al.* [27]. Bacteria from 4-h BHI broth culture grown at 37°C, washed once in physiological saline and resuspended in 0.2 M $NaHCO_3$ were used as inoculum. Serial 10-fold dilutions were made corresponding to 10^8–10^4 cfu/dose. A dose of 100 μl was administered orally to each mouse with the help of a gel loader tip fitted on the 100-μl micropipette. Each dilution was inoculated into a group of 8–10 mice. Then, 48 h after challenge, the number of animals surviving in each group was noted and the LD50 was calculated by the method of Reed and Muench [28]. Passive protection studies were performed by the method of Albert *et al.* [29]. Different dilutions of polyclonal antiserum to 31-kDa antigen (1 in 50, 1 in 100 and 1 in 200) were mixed in 100-μl amounts with 10 LD50 doses of *V. cholerae* O139 strain MO45. After incubation at 37°C for 30 min this mixture was administered orally to each member of groups of 8–10 mice for each dilution. The mice were observed for 48 h for death. The controls included a group of mice inoculated with 1 in 100 antiserum alone, 1 in 100 pre-immune serum with 10 LD50 bacteria and with 10 LD50 of strain MO45. Protection studies were also done with strains NIH41 and V86.

Statistical analysis

The unpaired Student's *t* test (two-tailed) was used for comparing the humoral and cellular immune responses between mice infected with *V. cholerae* O139 strain MO45 and control mice.

Results

SDS-PAGE

The protein profile of WCS antigens of *V. cholerae* strains O139 and O1 was grossly similar, with minor quantitative differences in the intensity of some of the bands (Fig. 1). The major proteins of these strains had mol. wts of *c.* 79, 71, 64, 59, 54, 47, 44, 36, 31 and 24 kDa.

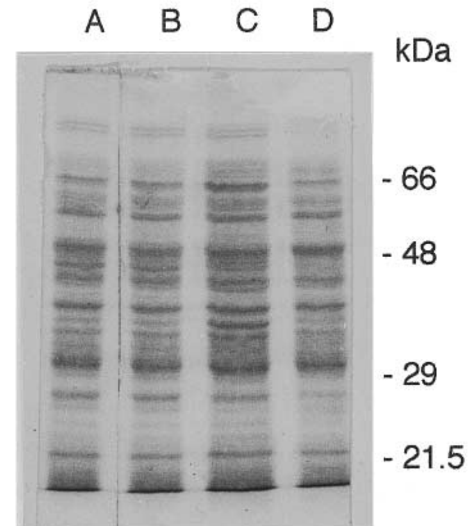

Fig. 1. SDS-PAGE protein profile of *V. cholerae* O139 clinical isolate S-776 (lane **A**), O139 strain MO45 (**B**), O1 El Tor Inaba strain V86 (**C**) and O1 classical Ogawa strain NIH41 (**D**).

Immunoblotting

Immunoblotting results of WCS antigen of *V. cholerae* O139 strain MO45 with individual cholera patient sera or NHS are shown in Fig. 2. Convalescent sera from O139 cholera patients revealed several IgA specific immunoreactive bands with mol. wts of *c.* 110, 71, 41, 36 and 31 kDa (lane A), 61 kDa and 24 kDa (lane B). Two bands of >200 kDa and three faint bands of *c.* 31, 29 and 27 kDa appeared with acute serum from an O139 cholera patient (lane C). The number of immunoreactive bands highlighted with sera from strain O1 patients were fewer and had mol. wts of

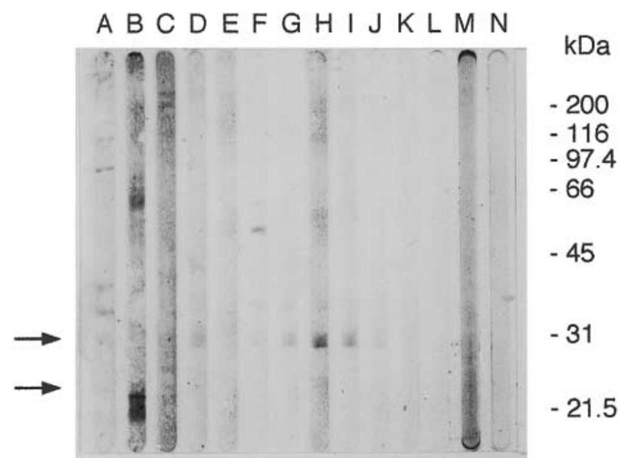

Fig. 2. Immunoblotting showing IgA reactive bands in sera from patients with *V. cholerae* O139 (lanes **A**–**C**), O1 patients (**D**–**L**) and normal healthy controls (**M** and **N**).

52 kDa (lane F), 36 kDa (lanes F and G) and 31 kDa (lanes D, F, G, H, I and J). Control sera from normal healthy donors did not show any reaction with WCS antigen of strain MO45 (lanes M and N). The results were further confirmed by using the pooled sera from cholera patients and NHS against the various *V. cholerae* strains. The immunoblot showed a few bands additional to those visible in Fig. 2 (results not shown).

Purification of proteins by DEAE-Sepharose CL 6B chromatography

A total of six peaks was obtained by broad-range (50–300 mM NaCl gradient) chromatography (Fig. 3). The protein profiles of the peaks were analysed by SDS-PAGE and the 31-kDa protein was present in peak 2. Narrow-range separation of peak 2 showed four further peaks (Fig. 4) and the 31-kDa protein was obtained in peak I. The purity of the protein was confirmed by SDS-PAGE (silver staining) against mol. wt markers (Fig. 5).

Raising of hyperimmune sera

A significant rise in titre against WCS and 31-kDa protein of *V. cholerae* O139 strain MO45 was observed by ELISA. The WCS and 31-kDa antigens (10 μg/ml) treated with a serum dilution of 1 in 50, had an absorbance of 0.022 (pre-immunisation sera) and 1.021 and 1.134, respectively (post-immunisation sera). The purity of the hyperimmune serum was confirmed by immunoblotting (Fig. 6).

ELISA

In both serum and intestinal fluid, a significant ($p < 0.05$) rise in IgA antibodies to 31-kDa antigen

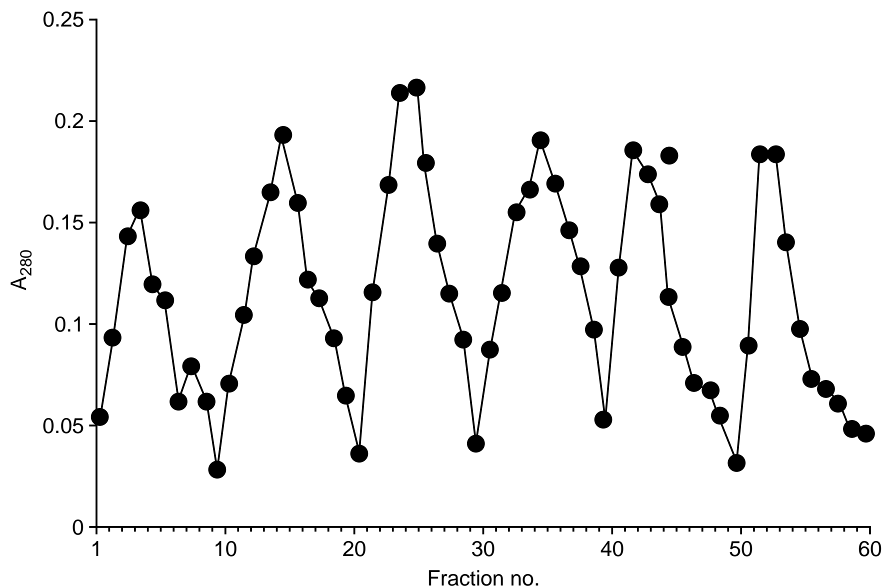

Fig. 3. Elution profile of DEAE-Sepharose CL 6B chromatography (broad-range), showing six peaks.

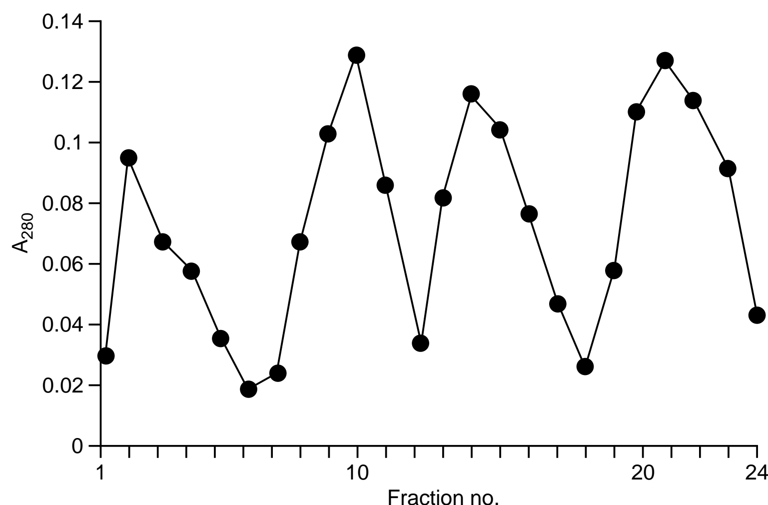

Fig. 4. Elution profile of DEAE-Sepharose CL 6B chromatography (narrow-range), showing four peaks.

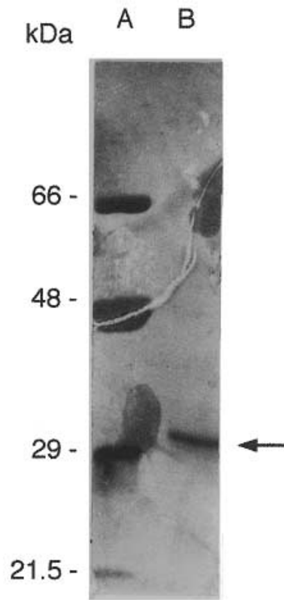

Fig. 5. SDS-PAGE profile of purified 31-kDa protein (silver stain); mol. wt markers (lane **A**) and 31-kDa protein (**B**).

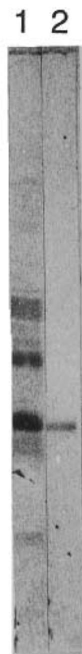

Fig. 6. Immunoblotting of SDS-PAGE-resolved WCS (lane **1**) and 31-kDa (**2**) proteins of *V. cholerae* O139 by homologous hyperimmune sera.

was observed in test mice compared with the control mice (Fig. 7). The serum titre reached a peak in the second week after infection and remained almost static thereafter. The levels of IgA antibodies in intestinal fluid of test mice were almost twice that in serum and showed a gradual increase from the first to the third week after infection.

Lymphocyte proliferation assay

In general, the SI and cpm values obtained in the proliferation experiments were low. The ranges of cpm values were 800–1500 in control mice, 1000–6000 in test mice stimulated with 31-kDa antigen and 4000–10 000 in both test and control mice stimulated with Con A. The proliferation of the lymphocytes increased as the concentration of the 31-kDa antigen was increased up to 8 μg/ml. A significant increase was observed in the proliferation of splenic lymphocytes from the test mice as compared with the control mice and maximum values were obtained in the third week after infection (Fig. 8).

Cytokine assays

On stimulation with 31-kDa antigen, significantly higher amounts of IL-10 ($p < 0.001$) were produced from the splenic lymphocyte culture supernates of test mice in the first week after infection (Fig. 9). A second peak was obtained in the third week after infection. IFN-γ, like IL-10, was produced maximally in the first week after infection (Fig. 9).

Protection studies

LD50 of strains MO45, NIH41 and V86 was determined in infant mice and strain V86 was found to be the most lethal (LD50, 5.5×10^6 cfu) followed by MO45 (6.6×10^6 cfu) and NIH41 (1.2×10^7 cfu). The protective abilities of the antiserum to the 31-kDa antigen were determined in infant mice by orally administering a mixture of *V. cholerae* organisms and antiserum. Protection was observed at 1 in 50 and 1 in 100 antiserum dilution against all three challenge strains (Table 1). However, at 1 in 200 dilution a slightly better protection was observed against *V. cholerae* O1 (60%) than O139 (50%).

Discussion

To detect an IgA specific antigen of *V. cholerae* O139 common to serogroups O1 and O139, whole-cell sonicate antigens of four *V. cholerae* strains were analysed by SDS-PAGE. The protein profiles of these strains were found to be grossly similar (Fig. 1). As the WCS antigens were used in SDS-PAGE in this study, it is likely that the predominant proteins were from the outer membrane. Remarkable similarities have been shown previously among the OMP profiles of various *V. cholerae* O1 strains belonging to both biotypes and serotypes [11, 17]. A comparison of envelope and OMP profiles between O1 and O139 isolates also failed to show any significant differences [3]. In this study, the major proteins of 79, 71, 64, 59, 54, 47, 44, 36, 31 and 24 kDa were found in different strains of *V. cholerae*. Many proteins, with similar mol. wts, have been described in the earlier studies as the major (77, 66,

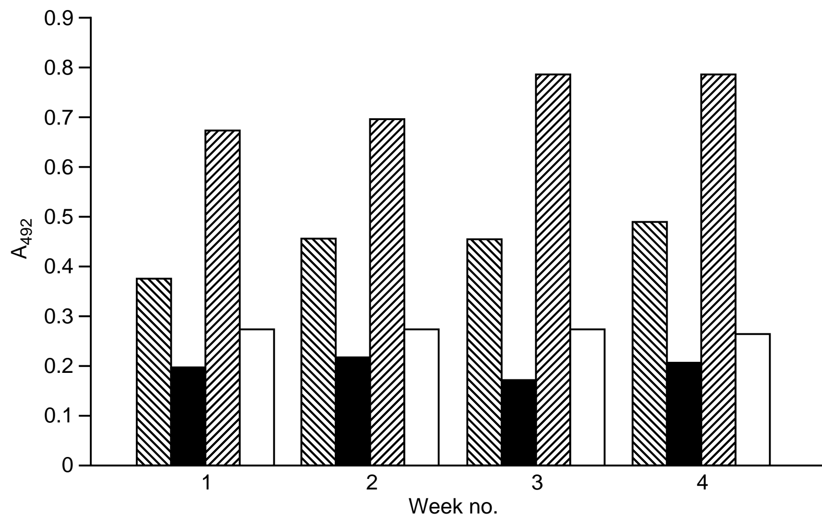

Fig. 7. IgA response in BALB/c mice to 31-kDa antigen in serum (▧) and intestinal fluid (▨) compared with serum (■) and intestinal fluid (□) of control mice.

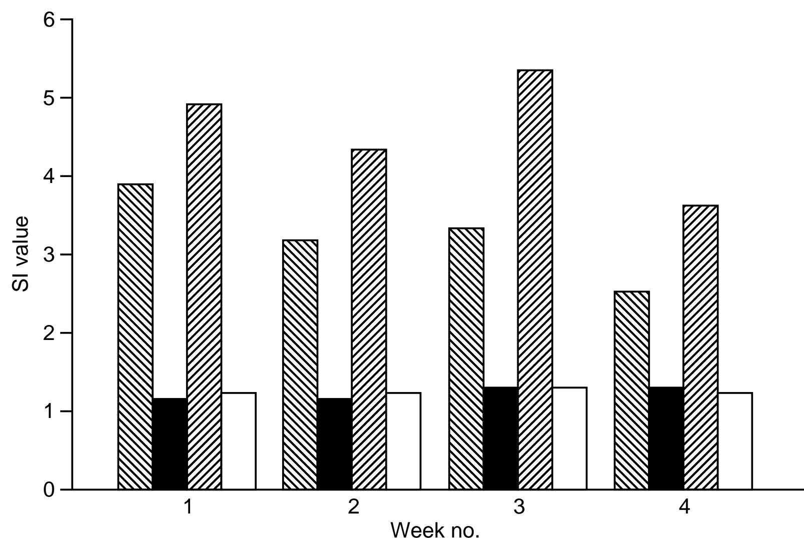

Fig. 8. Proliferation of murine splenic lymphocytes stimulated with 31-kDa antigen (6 μg/ml, ▧; 8 μg/ml, ▨) compared with controls (6 μg/ml, ■; and 8 μg/ml, □).

48–50, 40–43, 35–36, 30, 25 kDa) or minor (31 kDa) OMPs of *V. cholerae* [12, 17, 30–33].

In the immunoblotting experiments, a number of IgA specific immunoreactive bands were highlighted (Fig. 2). IgA antibodies to 31-kDa antigen could be detected in sera from at least six of nine O1 cholera and two of three O139 cholera patients at 1 in 50 dilution. The 31-kDa protein was also detected in the three strains of *V. cholerae* O139 and O1 (except strain S-776, which was not tested) in reactions with sera from both O139 and O1 cholera patients (results not shown). Hence, the 31-kDa protein was selected as a common, species-specific antigen for immunological and protective studies. Sears *et al.* [17] reported a 31-kDa protein, as a minor OMP in *V. cholerae* O1, which was common to the strains of both biotypes and serotypes. An OMP of 30 kDa and a cell surface protein of 33 kDa have also been described [12, 34]. Therefore, it is possible that the 31-kDa protein identified in this study is an OMP. Here, it should be noted that the results of the present study show that the 31-kDa antigen reacted with IgA antibodies, but the possibility of this antigen reacting with other classes and subclasses of antibodies present in the sera from cholera patients cannot be ruled out. Also, although this study did not look for LPS contamination by any direct method, the two-stage anion-exchange chromatography procedure used for purification and the absence of any band other than the 31-kDa protein in the silver-stained gel of the purified protein preparation probably exclude significant contamination with LPS.

Adult BALB/c mice were used to evaluate the role of species-specific 31-kDa antigen in the immune response to cholera in the present study. Fujisawa *et al.*

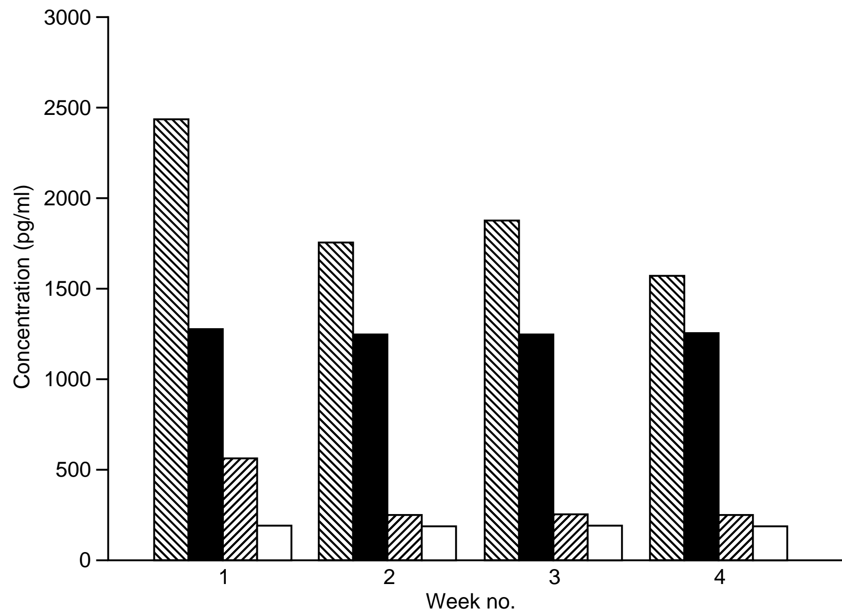

Fig. 9. IL-10 and IFN-γ levels in splenic lymphocyte culture supernates of test (IL-10, ▧; and IFN-γ, ■) and control (IL-10, ▨; and IFN-γ, □) mice.

Table 1. Protective efficacy of hyperimmune serum against the 31-kDa antigen in experimental *V. cholerae* infection

	Deaths/total challenged (percent protection) with strain			
Serum dilution	MO45	V86	NIH41	None
Test				
1 in 50	0/10 (100)	0/10 (100)	0/10 (100)	ND
1 in 100	0/10 (100)	0/9 (100)	0/10 (100)	ND
1 in 200	4/8 (50)	4/10 (60)	4/10 (60)	ND
Control				
No serum	10/10 (0)	ND	ND	ND
Hyperimmune serum 1 in 100	ND	ND	ND	0/8 (100)
Pre-immune serum 1 in 100	10/10 (0)	10/10 (0)	9/9 (0)	ND

ND, not determined.

[35] have previously advocated adult inbred mice as a useful tool to analyse the immune response of bacterial antigens participating in protection against *V. cholerae* infection. Other workers have reported the adult mice to be poorly colonised by vibrios. In the present study, multiple doses were administered to the BALB/c mice to obtain maximum colonisation and immune response. Coproculture and intestinal content culture of infected mice were done on the day after administration of the first dose and *V. cholerae* could be isolated from both samples, confirming the colonisation of gut mucosa.

In the first week after infection, a significant ($p < 0.05$) rise in the IgA antibodies to the 31-kDa antigen was observed in the sera of *V. cholerae* O139-infected mice as compared with the control mice (Fig. 7). The response showed an upward trend until the second week and thereafter reached a plateau. No reports are available on the kinetics of IgA or IgG antibody response to *V. cholerae* antigens in the serum or intestinal fluid of mice for comparison. Fujisawa *et al.* [35] detected IgA antibodies by the single radial immunodiffusion method, in the intestinal content of mice immunised orally with live vibrios, but they could not detect IgA antibodies in the serum by the same method. In this study, in terms of OD values, the IgA antibodies to 31-kDa protein in the intestinal fluid were found to be almost two-fold higher than that in serum of *V. cholerae* O139-infected mice (Fig. 7). The kinetics of the IgA antibody response in intestinal fluid were comparable to those in serum and a plateau was observed in the third week after infection.

T-cell proliferation and production of cytokines upon in-vitro culture in the presence of specific antigens are the two parameters most frequently used for identifying antigen-specific T cells [36]. In the present study, proliferation of the mouse splenic lymphocytes was found to depend upon the concentration of stimulating antigen. These results are consistent with those of Lewis *et al.* [37], who investigated the proliferative response of peripheral blood mononuclear cells

(PBMCs) obtained from CT-B immunised subjects to CT-B, and Kabir and Mann [38], who studied the proliferation of murine splenic lymphocytes on stimulation with LPS or OMP of *V. cholerae*. The low SI and cpm values of proliferative responses recorded in the present study probably reflect the fact that oral administration of *V. cholerae* O139 strain MO45 induced only relatively small numbers of antigen-specific T- or B-cell precursors in the circulation. Furthermore, a significant ($p < 0.01$) rise in the proliferation of splenic lymphocytes obtained from the test mice as compared with those of controls is suggestive of cell trafficking (Fig. 8). The demonstration of specific antibody-producing cells in peripheral blood after oral cholera vaccination in man [39] and adoptive transfer of gut mucosal antitoxin memory by B cells isolated from the spleens of mice orally immunised with CT support the notion of recirculating mucosal memory cells with trapping in various lymphoid tissues [40]. The kinetics of the proliferative response to the 31-kDa antigen show that it peaked in the third week after infection and was evident ($p < 0.01$) even in the first week (Fig. 8). A similar bimodal proliferative response in PBMCs of human volunteers immunised with CT was observed by Lewis *et al.* [37] with the first peak between days 17 and 25 and the second around 35 days after immunisation.

Production of high levels of IL-10 and IFN-γ was observed in response to the 31-kDa antigen by the splenic lymphocytes of test mice in the first week after infection (Fig. 9). This may indicate a possibility of the presence of antigen-specific B cells and Th2 cells (producing IL-10) and Th1 cells (producing IFN-γ) in the spleen in the first week after infection. A peak of IL-10 and an insignificant increase in IFN-γ in the third week may reflect a decrease in Th1 cells during this period. Overall, it appears that the 31-kDa antigen has more Th2-type cell epitopes.

The infant mouse model is an established animal model to study the protective nature of various antigenic components of *V. cholerae* and has been used in various previous studies [13, 29, 41]. The antiserum to the 31-kDa antigen, at higher concentrations (1 in 50 and 1 in 100), was able to protect the infant mice against challenge with *V. cholerae* O139 and O1. It was expected to do so because the 31-kDa antigen was detected in both O1 and O139 *V. cholerae* strains in reactions with homologous and heterologous cholera patient sera, in the immunoblotting experiments. At lower dilution, the 31-kDa antigen provided slightly better protection against O1 (60%) than O139 vibrios (50%). We are unable to explain this at present. A cell surface antigen of similar mol. wt (33-kDa) to that in the present study was reported by Jacob *et al.* [34], who found it to be immunogenic and protective in the rabbit ileal loop model.

Data from immunological and protection studies show the potential usefulness of the 31-kDa antigen in terms of subunit vaccine, yet the protein demands more detailed characterisation structurally (amino acid sequencing of amino-terminal end), functionally and immunologically. Further, it is important to note that the studies have been done only in an infant mouse model, and that the validity and extrapolation of these findings for the development of an improved cholera vaccine for human use remain to be examined.

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