ELSEVIER

FEMS Immunology and Medical Microbiology 15 (1996) 143–148

FEMS IMMUNOLOGY AND MEDICAL MICROBIOLOGY

Electrophoretic mobility and immune response of outer membrane proteins of *Vibrio cholerae* O139

Sangita Pal [a], D. Sasmal [a], B. Guhathakurta [a], G.B. Nair [a], S.K. Bhattacharya [a], R. Mallick [b], A. Datta [a,*]

[a] *Department of Biochemistry, National Institute of Cholera and Enteric Diseases, P-33, C.I.T. Road, Scheme XM, Beliaghata, Calcutta 700010, India*

[b] *Department of Botany, University of Calcutta, Calcutta 700019, India*

Received 15 January 1996; revised 11 June 1996; accepted 12 June 1996

Abstract

The outer membrane (OM) protein components of a *Vibrio cholerae* O1 and four *V. cholerae* O139 strains, collected from cholera patients, were analysed by SDS-PAGE. A protein of 69 kDa molecular mass was observed only when the OMPs were prepared from strains grown in synthetic broth. As a result of passage in the rabbit ileal loop (RIL), virulence was enhanced, and a protein component around 18 kDa of the *V. cholerae* O139 OM became the major protein component. On immunoblot analysis with rabbit antiserum against *V. cholerae* O139 OM, it was shown that, apart from the major protein component of *V. cholerae* O1 OM of around 45 kDa and that of *V. cholerae* O139 OM of around 38 kDa, all other minor protein components were cross-reactive between the two serogroups. In immunoblot assays with convalescent sera obtained from *V. cholerae* O139-infected patients, it was observed that in addition to the lipopolysaccharide (LPS)-induced antibody, only the 38 kDa major protein component elicited considerable levels of antibody in the patient. Minor OM components of 18 kDa were detected in the immunoblot analysis by LPS-directed antibody, however, as the OM proteins are known to be associated with LPS.

Keywords: *Vibrio cholerae* O139; Outer membrane protein; *Vibrio cholerae* O1; Lipopolysaccharide

1. Introduction

Evidence suggests that the outer membrane (OM) proteins of *Vibrio cholerae* O1 play an important role as protective antigens in the disease [1]. The genesis of a new serogroup, currently classified as *V. cholerae* O139 Bengal, and capable of causing cholera, formed the impetus for this study to examine the involvement of OM in O139-mediated infections. Serologically distinct, the O139 serogroup possesses a capsular polysaccharide which distinguishes the O139 serogroup from the O1 serogroup and reportedly confers increased virulence to the O139 strains [2]. Chemical analyses also revealed that the LPS of O139 Bengal contains (among other sugars) colitose (3,6-dideoxy-L-galactose), a sugar constituent not found in the LPS of other members of the Vibrionaceae and further O139 lacks per-

* Corresponding author. Tel: +91 350-1176; Fax: +91 350-5066.

PII S0928-8244(96)00056-9

osaminea characteristic component sugar of *V. cholerae* O1 LPS [3]. The OM of *V. cholerae* O139 as compared to OM of the O1 serogroup has been less extensively examined. This paper attempts to record some observations relating to OM proteins of *V. cholerae* O139. It is also intended to observe the effect of variation in the growth media and animal passage of the strains on the OM protein profile of the *V. cholerae* O139 strains in term of their electrophoretic mobility. A further objective of the study was to examine the immune response to OM proteins of *V. cholerae* O139 using sera from *V. cholerae* O139 infected patients, OM-immunised rabbits and to study the immunological cross-reactivity of the *V. cholerae* O139 OM proteins with that of *V. cholerae* O1 by immunoblot technique.

2. Materials and methods

2.1. Bacteria and media

Four strains of *V. cholerae* O139 and one strain of *V. cholerae* O1 biotype El Tor from patients with watery diarrhoea were included in the study. Bacterial strains were grown in different media as tryptic soy broth (TSB), nutrient broth (NB), synthetic broth (SB) (ammonium sulphate 7.5 mM, glucose 5.5 mM, magnesium sulphate 0.8 mM, sodium chloride 0.085 mM, dipotassium hydrogen phosphate 5.7 mM, pH 8.0) [4], and peptone water (NaCl 0.5%, peptone 1%).

2.2. Serial passages in rabbit ileal loop (RIL)

The rabbit loop test was performed according to the method of De and Chatterjee [5]. *V. cholerae* O139 strains were introduced into the ileal loops (1×10^2 cfu/loop). The volume of fluid accumulated in the loops in ml was measured (V) and also the length of the loop in cm (L). V/L ratio was calculated as an index of virulence after each passage. After draining a loop of the accumulated fluid the intestinal tissue was incubated in peptone water for 3–4 h at 37°C before reisolating the bacteria by plating on thiosulphate citrate bile salt sucrose agar (TCBS; Eiken). The reisolated strains after serological confirmation, using *V. cholerae* O139 antisera raised in rabbits, were again passaged three times in the RIL.

2.3. Preparation of outer membranes

Outer membrane (OM) was prepared essentially following the method of Kabir [6] from 18 h culture of different bacterial strains grown at 37°C with shaking. Bacterial cells were washed and suspended in 0.1 M Tris-HCl (pH 7.4) followed by shearing of flagella and treatment with lysozyme (0.1 mg/ml of cell suspension). The spheroplast so formed, was lysed by osmotic shock. After removal of the unlysed cells by centrifugation, the total membrane fraction was recovered from the osmotic lysate by centrifugation at $48\,000 \times g$. The inner membrane was removed by treatment with 2% Triton X. OM was prepared from one of the passaged strains grown in synthetic broth for immunisation, ELISA and immunoblot.

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

SDS-PAGE of OM proteins was performed by the method of Laemmli [7] at 12.5% (w/v) acrylamide in the presence of 2-mercaptoethanol. Electrophoresis was carried out in vertical slab gel apparatus (Atto, Japan) at constant current using 15 mA on stacking gel and 20 mA on the resolving gel. The gel was stained with 0.2% Coomassie brilliant blue G.

2.5. Preparation of OM-antisera

Adult male albino rabbits were immunised with OM preparations from *V. cholerae* O139 by four intramuscular injections (500 μg of protein per dose) once a week, initially with Freund's complete adjuvant (Sigma, St. Louis, MO) followed by three booster doses with Freunds incomplete adjuvant. The animals were bled ten days after the last injection.

2.6. Preparation of LPS

V. cholerae O139 LPS was prepared by treatment of the bacteria with phenol at 65°C [8], followed by removal of nucleic acid and acidic polysaccharide by precipitation with cetyl trimethyl-ammonium bro-

mide. The LPS was finally collected after removal of protein impurities with pronase, by alcohol precipitation.

2.7. Patient sera

Sera were collected from patients admitted to the Infectious Diseases Hospital, Calcutta, with diarrhoea due to *V. cholerae* O139, on admission (acute) and after 21 days (convalescent).

2.8. LPS binding

Convalescent patient sera and rabbit anti-OM sera were absorbed with *V. cholerae* O139 LPS to remove anti-LPS antibodies by incubating with 100 μg of LPS per ml of sera. Removal of the anti-LPS antibodies was confirmed by ELISA with LPS as solid phase antigen.

2.9. ELISA

IgG serum antibodies to OM were determined by standard ELISA method [9] with 5 μg of OM per well as solid phase antigen. Diluted sera (1 in 10 for human sera and 1 in 80 for rabbit sera) were incubated with the antigen-coated well followed by colour development with peroxidase labelled goat anti-IgG conjugate (1 in 1000 dilution) and O-phenylenediamine along with hydrogen peroxide, was measured at 492 nm.

2.10. Immunoblot

The SDS-PAGE protein profile of *V. cholerae* O139 and *V. cholerae* O1 OM preparations were transferred electrophoretically to nitrocellulose membrane [10]. The proteins on the membrane were allowed to react with anti-OM rabbit sera (1 in 20 dilution), human patient sera acute and convalescent (both before and after absorption with LPS). The reaction was visualised by using peroxidase-conjugated anti-rabbit/anti-human IgG antisera. Colour was developed by treatment with a solution of 3,3′ diamine-benzidine hydrochloride (1 mg ml^{-1}, Sigma) in 1 mM Tris buffer saline containing H_2O_2 at a concentration of 0.3 μl ml^{-1}.

3. Results

The composition of the growth medium affected the *V. cholerae* O139 protein profile as observed in SDS-PAGE and recorded in Fig. 1. A cluster of protein bands around 69 kDa was observed consistently in all the strains when the bacteria were grown in synthetic media, which were absent when the peptone water, nutrient broth and tryptic soy broth were used to grow the strains. The 28 kDa protein in the OM was best expressed in synthetic broth. The major protein band of *V. cholerae* O139 OM was observed to be at 38 kDa in all the growth media tested.

Fig. 2 depicts the effect of serial passage of *V. cholerae* O139 in RIL on the protein profile of its OM in SDS-PAGE. As expected the virulence of the strains were enhanced on RIL passage as indicated by the increased fluid accumulation as measured by V/L index (data not shown). The expression of the 18 kDa protein was enhanced considerably so that it appeared to be the major protein component of the OM of the animal passaged strains.

Table 1 records the IgG antibody response against OM antigens as a result of *V. cholerae* O139 infection in humans, being measured by ELISA using

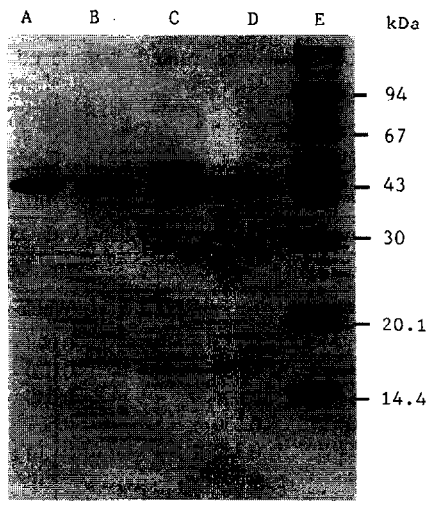

Fig. 1. SDS-PAGE of the OM proteins of *V. cholerae* O139 strain grown in different media. Lane A: peptone water; lane B: nutrient broth; lane C: synthetic broth; lane D: tryptic soy broth; lane E: standard marker (Pharmacia).

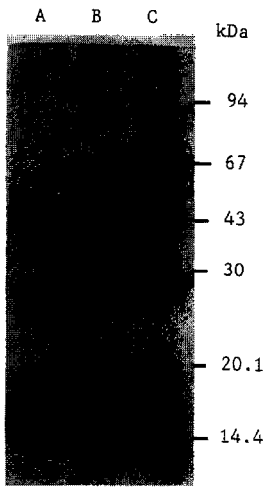

Fig. 2. SDS-PAGE of OM protein of *V. cholerae* O139 strain. Lane A: after third passage in RIL (V/L = 1.24); lane B: before RIL passage (V/L = 1.0); lane C: standard marker (Pharmacia).

paired sera (acute and convalescent). Four out of six patients manifested a significant rise in the antibody level against homologous *V. cholerae* O139 OM. The ELISA results with LPS-absorbed sera indicated a significant rise in the level of antibodies against heterologous *V. cholerae* O1 OM.

Table 2 indicates that both the LPS and the protein components of the *V. cholerae* O139 OM were highly immunogenic in rabbit. The residual protein specific antibody level in the LPS-absorbed OM-immunised sera was raised compared to the preimmunised sera. A considerable level of antibody was also directed against heterologous *V. cholerae* O1 OM.

In the immunoblot of the SDS-PAGE of the *V. cholerae* O1 and *V. cholerae* O139 OMs with rabbit antisera raised against the latter (Fig. 3) four bands in the region of 69 kDa, 38 kDa, 28 kDa and 18 kDa were visualised. The *V. cholerae* O1 OM did not show any band corresponding to the 38 kDa protein component of *V. cholerae* O139 OM. Other protein components of the *V. cholerae* O1 OM of 69 kDa, 28 kDa and 18 kDa reacted well with the heterologous sera. Fig. 4 indicates the effect of removal of

Table 1
Homologous and heterologous serum antibody response to OM measured in *V. cholerae* O139 infected patients by IgG ELISA

Patient no.	Rise * in net absorbance (492 nm) with OM antigen		
	Homologous		Heterologous
	Whole sera	LPS absorbed sera	Whole sera
1	0.307	0.152	0.098
2	0.049	0.025	0.020
3	0.056	0.025	0.050
4	0.304	0.250	0.064
5	0.514	0.352	0.116
6	0.506	0.305	0.116

Net absorbance = absorbance of test well − absorbance of background well.
* Rise in net absorbance = net absorbance with convalescent sera − net absorbance with acute sera.

Table 2
IgG response measured by ELISA in *V. cholerae* O139 OM immunised rabbit sera

Mean net absorbance at 492 nm with:								
Homologous OM						Heterologous OM		
Whole sera			LPS absorbed sera			Whole sera		
P	I	I−P	P	I	I−P	P	I	I−P
0.108	1.903	1.795	0.049	0.555	0.506	0.070	0.375	0.305

P = Preimmunised sera.
I = Immunised sera.
Net absorbance = absorbance of test well − absorbance of background well.
Rise in net absorbance = net absorbance with immunised sera − net absorbance with preimmunised sera (I−P).

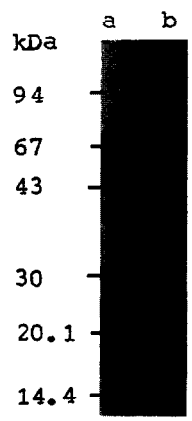

Fig. 3. Immunoblot of SDS-PAGE of *V. cholerae* O1 and *V. cholerae* O139 OMs with rabbit antisera immunised with *V. cholerae* O139 OM. Lane a: *V. cholerae* O1 OM antigen; lane b: *V. cholerae* O139 OM antigen.

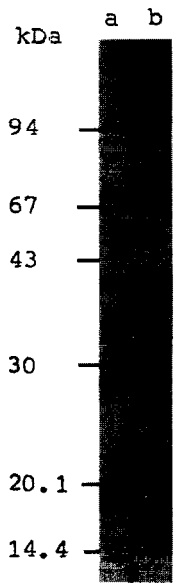

Fig. 4. Immunoblot of *V. cholerae* O139 OM with rabbit anti-*V. cholerae* O139 OM sera raised in rabbit. Lane a: antisera absorbed with LPS; lane b: unabsorbed antisera.

the LPS-specific antibody from rabbit anti-*V. cholerae* O139 OM serum on the reactivity of the homologous OM proteins. The immunological reaction of the 18 kDa protein band became considerably reduced in intensity with LPS-absorbed sera. Removal of the LPS-specific antibody affected the intensity of the 38 kDa immunoblot band marginally, if at all.

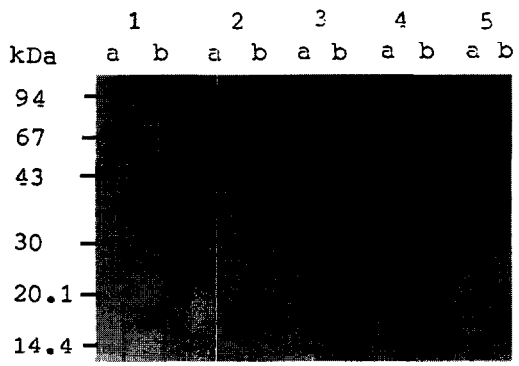

Fig. 5. Immunoblot with convalescent sera of *V. cholerae* O139 infected patients against OM of *V. cholerae* O1 and *V. cholerae* O139 OM. Lane a: with heterologous *V. cholerae* O1 OM; lane b: with homologous *V. cholerae* O139 OM.

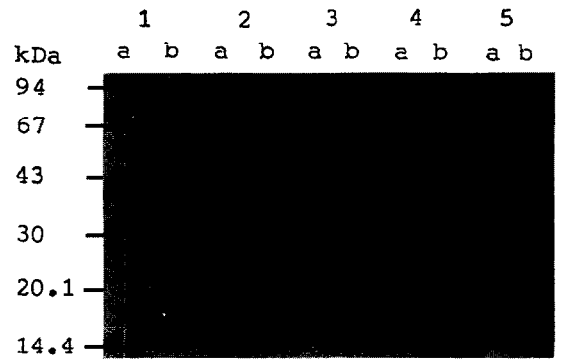

Fig. 6. Immunoblot of *V. cholerae* O139 OM with convalescent sera from *V. cholerae* O139 infected patients. Lane a: LPS absorbed; lane b: LPS unabsorbed.

Convalescent sera from *V. cholerae* O139 infected patients contained antibody towards the 38 kDa and 18 kDa component of *V. cholerae* O139 OM (Fig. 5, lane b). In one patient (No. 4) proteins below 38 kDa, except for 18 kDa, also reacted with *V. cholerae* O139 convalescent sera. *V. cholerae* O1 OM protein antigens did not react with *V. cholerae* O139 convalescent sera, except sera No. 4 which reacted faintly (Fig. 5, lane a). Immunoblot with the acute sera from the same patients did not react at all with either *V. cholerae* O139 or *V. cholerae* O1 OM. LPS-absorbed *V. cholerae* O139 infected convalescent patient sera could detect only the 38 kDa protein in *V. cholerae* O139 OM (Fig. 6).

4. Discussion

The variation of the OM protein profile of *V. cholerae* O139 with media of growth was noted. Similar observations with *V. cholerae* O1 OM proteins were reported earlier [6]. The major protein component of the *V. cholerae* O139 OM was observed to be of 38 kDa molecular mass instead of 45–48 kDa reported for *V. cholerae* O1 by several workers. This difference in the molecular mass of the major protein bands of *V. cholerae* O139 and *V. cholerae* O1 has been reported earlier [11]. The present observation of enhanced expression of 18 kDa protein on passage in RIL possibly reflects a

virulence related in vivo situation. A low iron-regulated, protective 18 kDa OM protein has been described in *V. cholerae* O1 which is expressed in vivo [12]. Our observation indicates that both in *V. cholerae* O139 OM immunised rabbits and in convalescent *V. cholerae* O139 infected patient sera antibody directed against both LPS and OM protein antigens were present. The ELISA titers of the LPS-absorbed sera against *V. cholerae* O139 OM is indicative of the total anti-OM protein antibody (Tables 1 and 2). The antibodies directed against the OM-protein antigens which are immunologically cross-reactive between the two groups of strains are indicated by the ELISA titer of the convalescent patient sera and OM-immunised rabbit sera against the heterologous *V. cholerae* O1 OM (Tables 1 and 2).

By immunoblot technology we could detect the OM protein antigens against which the antibody response was directed. Protein specific antibody in OM-immunised rabbit and convalescent patients sera detected four and one (38 kDa) protein bands respectively from the SDS-PAGE of *V. cholerae* O139 OM. It is suggested that in natural infection all the protein components of the OM may not be suitably exposed to elicit maximum antibody response which was not the case in immunisation of rabbits with isolated OM. Our observation of the immunoblot with both OM-immunised rabbit and *V. cholerae* O139 infected patient sera indicates that the major protein components of 38 kDa and 45 kDa of *V. cholerae* O139 and *V. cholerae* O1 respectively are immunologically unrelated. The OM proteins other than the major protein components of 38 kDa and 45 kDa of the two groups of strains are immunologically related as is evident from the immunoblot with OM-immunised rabbit sera. The 18 kDa component of *V. cholerae* O139 OM was detected in the immunoblot analysis by LPS-directed antibody as the OM protein is generally known to be associated with LPS. Further investigation of the 38 kDa antigen of *V. cholerae* O139 OM for its role, if any, in inducing protective immunity may yield interesting information.

Acknowledgements

We gratefully acknowledge the technical assistance of S.R. Choudhury, B.K. Pal and M. Ghosh.

References

[1] Svennerholm, A.-M., Jonson, G. and Holmgren, J. (1994) Immunity to *Vibrio cholerae* infection. In: *Vibrio cholerae* and cholera (Kayewachsmuth, I., Blake, A.P. and Olsvik, O., Eds.), p. 260, A.S.M. Press, Washington.

[2] Weintraub, A., Widmalm, G., Jansson, P.E., Jansson, M., Hultenby, K. and Albert, M.J. (1994) *V. cholerae* O139 Bengal possesses a capsular polysaccharide which may confer increased virulence. Microb. Pathog. 16, 235–241.

[3] Hisatsune, K., Kondo, S., Isshiki, Y., Iguchi, T., Kawamata, Y. and Shimada, T. (1993) O-antigenic lipopolysaccharide of *Vibrio cholerae* O139 Bengal, A new epidemic strain for recent cholera in the Indian subcontinent. Biochem. Biophys. Res. Comm. 196, 1309–1315.

[4] Bhaskaran, K. and Rowley, D. (1976) Nutritional studies on *Vibrio cholerae*. J. Gen. Microbiol. 15, 417–422.

[5] De, S.N. and Chatterjee, D.N. (1953) An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membranes. J. Pathol. Bacteriol. 66, 559–562.

[6] Kabir, S. (1980) Composition and immuno chemical properties of outer membrane proteins of *Vibrio cholerae*. J. Bacteriol. 144, 382–389.

[7] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227, 680–685.

[8] Westphal, O. and Jann, K. (1965) Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. 5, 83–91.

[9] Young, C.R., Levine, M.M., Craig, J.P. and Robins-Browne, R. (1980) Microtiter enzyme-linked immunosorbent assay for immunoglobulin G cholera antitoxin in humans: method and correlation with rabbit skin vascular permeability factor technique. Infect. Immun. 27, 492–496.

[10] Towbin, H., Stachelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.

[11] Sasmal, D., Guhathakurta, B., Ghosh, A.N., Pal, C.R. and Datta, A. (1995) Studies on adhesion, haemagglutination and other biological properties of *Vibrio cholerae* O139. FEMS Immunol. Med. Microbiol. 10, 199–206.

[12] Sciortino, C.V. (1993) Prevalence of a *Vibrio cholerae* 18 kDa antigen in Vibrios. FEMS Microbiol. Lett. 111, 49–56.