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Monoclonal Antibodies to Outer Membrane Antigens of *Vibrio cholerae*

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Hybridoma-derived monoclonal antibodies were prepared against outer membrane antigens of four strains of *Vibrio cholerae* that were cultivated under iron-limited conditions, and these antibodies were partially characterized. We established a library of 66 hybridomas which produced monoclonal antibodies defining 16 different *V. cholerae* antigens. Two antigens (molecular weights, 18,000 and 112,000) were heat modifiable, whereas the reacting epitope of a third antigen (40,000-dalton–18,000-dalton doublet) was completely destroyed when it was heated at 100°C. The 112,000-dalton heat-modifiable protein was an iron-regulated outer membrane protein. This protein bound ^{59}Fe in vitro when it was combined with the *V. cholerae* siderophore-iron complex ^{59}Fe-vibriobactin; it was also found in in vivo grown *V. cholerae*, as were three other antigens. A total of 26 hybridomas produced antibody to *V. cholerae* lipopolysaccharide. Of these, 12 were cross-reactive with lipopolysaccharides of other gram-negative bacteria, including 2 which recognized lipid A. Several of these anti-lipopolysaccharide monoclonal antibodies appeared to be lipopolysaccharide region specific. Some membrane antigens were strain specific, whereas others were common to both O group 1 and non-O group 1 vibrios.

Epidemic cholera is caused by O group 1 *Vibrio cholerae* strains of both El Tor and classical biotypes and Inaba and Ogawa serotypes (14). The adherence of the cholera vibrios to intestinal microvilli and the establishment of the organisms precede the production and release of the potent cholera enterotoxin. The mechanism(s) by which the vibrios attach to the intestinal epithelial cells is not well understood (B. A. Booth, C. V. Sciortino, and R. A. Finkelstein, *in Seminars in Infectious Diseases*, in press). It has been suggested that outer membrane components may contribute to the adherence of pathogenic vibrio strains (27).

It has been clearly demonstrated in American volunteers that the disease cholera is a highly effective immunizing process (29, 30). During the disease, the host is presented with a variety of factors produced by cholera vibrios growing in vivo. It has not been established whether a single component or multiple components are primarily responsible for immunity (15, 30). The lipopolysaccharide (LPS) somatic antigen, outer membrane proteins (OMPs), flagella, hemagglutinins and other putative colonization factors, various enzymes (protease, neuraminidase, and others), and enterotoxin may be presumed to be contributory. With other gram-negative bacteria, components of the outer membrane whose functions are still not known may contribute significantly to pathogenicity and immunity (6, 9, 11, 22, 24, 31, 43, 44). Surface components are clearly very important to antibacterial immunity since it is only these components that are available for interaction with the effector arm of the host immune system. Although it has been shown that orally administered killed vibrio preparations in enormous doses do offer some protection against cholera, the degree of protection does not approach that obtained when the host has been presented with live vibrios (15, 30). Therefore, we can draw the operational hypothesis that surface components (e.g., OMPs) present in the live bacteria in vivo but not in the killed (in vitro grown) perorally administered vaccines may be important, although other factors, such as the amount and the duration of the antigenic exposure, must also be considered. Recent evidence indicates that convalescent sera from cholera patients contain antibodies to OMPs of *V. cholerae* (38).

Under conditions of iron deprivation, microorganisms initiate a sequence of events, including the production of siderophores and novel inner and OMPs (16, 34, 42), which function in the recognition, binding, transport, and reductive release of iron essential for metabolism. Similar iron-binding transport systems are present in *V. cholerae*, as pointed out by Sigel and Payne (39) in their initial report on the coordinated synthesis of iron-regulated OMPs (IROMPs) along with the production of the siderophore vibriobactin. We have previously shown that *V. cholerae* grown in vivo expresses OMPs which are produced by the same strains grown in vitro under iron-limited conditions (37). We began these studies to further characterize these IROMPs by using monoclonal antibodies (McAbs).

The serological classification of *V. cholerae* has been based upon the heat-stable somatic antigen(s) which is responsible for the predominant serological activity in agglutination and vibriocidal tests (12). The somatic antigen is extractable from cholera vibrios by conventional procedures used for isolation of endotoxic LPS from other gram-negative species (12). Organisms belonging to serogroup 0:1 are characterized by the presence of group-specific antigen A and are subdivided into serotypes Ogawa and Inaba; each of these serotypes is characterized by a type-specific antigen (antigens B and C, respectively) (7, 8, 13, 18, 28). Standard sera prepared in national laboratories have been used as references for the preparation of grouping and typing antisera in various smaller laboratories (governmental, commercial, and university). The results may be predicted to be less than completely uniform from laboratory to laboratory depending on the strains and conditions used for serum production and adsorption. This has become particularly

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significant with the increasing recognition (2) of a diverse variety of atypical O group 1 and non-O group 1 *V. cholerae* strains (formerly called non-agglutinable vibrios or non-cholera vibrios) in the environment, associated with seafood, and in human specimens (some from diseased subjects). In this regard, McAbs might be quite useful, and some have already been produced against the LPS of O group 1 *V. cholerae* (23). However, we have not found any reports on McAbs to OMPs of *V. cholerae*. In the present study, we prepared and characterized McAbs against a diversity of *V. cholerae* outer membrane components, including surface-exposed epitopes, heat-modifiable proteins, IROMPs, and LPS.

MATERIALS AND METHODS

Bacterial strains and cultivation. The four strains of *V. cholerae* which were used for mouse immunization were classical biotype strains CA401 and 569B (both Inaba serotype) and NIH41 (Ogawa serotype) and El Tor biotype strain 3083TR[2] (Ogawa serotype). These and the other strains used and pertinent information about them are shown in Table 1. All of the strains were stored frozen in 20% glycerol–peptone–saline broth at −70°C. Strains grown on meat extract agar were cultivated in low-iron "T" medium (40) before preparation of outer membrane antigens (37).

LPS preparation. LPSs were prepared from *V. cholerae* strains 569B and 7738 (El Tor biotype, Inaba serotype which was isolated by R. A. Finkelstein in Manila, The Phillipines, in 1961), strain NIH41 (= 20-A-11), and strain 416-ORa (a classical rough strain isolated in Calcutta, India, by C. E. Lankford) by the ether-water extraction procedure of Ribi et al. (36). LPS from *V. cholerae* 3083TR[2] was prepared by the phenol-water method (47) and was further purified by high-speed centrifugation at 100,000 × *g* for 4 h. *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Escherichia coli* J5 LPS preparations were donated by W. R. McCabe (Boston University School of Medicine, Boston, Mass.) and were prepared by the phenol-water method. *E. coli* 055:B5 LPS-B was purchased from Difco Laboratories, Detroit, Mich. (lot 591321). *V. cholerae* 569B LPS (lot 102692, *Salmonella minnesota* Re595 LPS (lot 405537), and *S. minnesota* Re595 lipid A (lot 309771) were purchased from Calbiochem-Behring, San Diego, Calif.

Isolation of McAbs. Our McAb isolation procedure was a modification of the techniques of Goding (19). BALB/c mice (Charles River Laboratories, Wilmington, Mass.) were immunized intraperitoneally with pooled outer membrane preparations (containing 10 μg of total protein) originating from *V. cholerae* strains 3083TR[2], NIH41, CA401, and 569B grown under iron-limited conditions. The mice were boosted intraperitoneally at 10-day intervals five times, with a final boost (5 μg intravenously) 3 days before fusion. The medium used for suspension of mouse spleen cells and growth of SP2/O-Ag14 myeloma cells was Dulbecco modified Eagle medium (GIBCO Laboratories, Buffalo, N.Y.) supplemented with 3% fetal calf serum (Dutchland Laboratories, Inc., Denver, Pa.), 1 mM pyruvate, 3 mM glutamine, 100 U of penicillin-streptomycin per ml, and 0.05 mM 2-mercaptoethanol. Fusion was performed with 2 × 10^8 spleen cells from immunized mice and 2 × 10^7 SP2/O myeloma cells by using 50% polyethylene glycol 1000 (Fisher Scientific Co., Pittsburgh, Pa.) as a fusogen. After fusion, cells were suspended at a concentration of approximately 1.1 × 10^6 cells per ml in Dulbecco modified Eagle medium containing 20% fetal calf serum, 10%

TABLE 1. *V. cholerae* strains: origins and characteristics

Biotype	Strain	Serotype	Source
El Tor	3083TR[2a]	Ogawa	Vietnam, 1964
	1196-78[b]	Ogawa	Brazil; sewage; tox$^-$
	1074-78[b]	Ogawa	Brazil; sewage; tox$^-$
	N15870[b]	Ogawa	Bangladesh; human diarrhea
	N16117[b]	Ogawa	Bangladesh; human diarrhea
	2078[c]	Ogawa	California; human diarrhea
	2633-78[d]	Ogawa	Brazil' sewage; tox$^-$
	3146-78[d]	Ogawa	Guam; human
	123-83[d]	Ogawa	Louisiana; human stool; tox$^-$
	2018[c]	Inaba	Louisiana; leg wound; tox$^-$
	2019[c]	Inaba	Louisiana; sewer; tox$^-$
	2048[c]	Inaba	Florida; oysters; tox$^-$
	2077[c]	Inaba	Louisiana; crab feces; tox$^-$
	2091[c]	Inaba	Florida; human diarrhea
	2402-78[d]	Inaba	Louisiana; human stool
	3251-78[d]	Inaba	Chesapeake Bay; cytotoxic
	872-80[d]	Inaba	Florida; tox$^-$
Classical	NIH41[e]	Ogawa	National Institutes of Health reference strain
	CA401[f]	Inaba	Calcutta, India, 1953
	569B[g]	Inaba	India, ca. 1945
Non-O group 1	9767[c]	176	Blood specimen from gastroenteritis
	9771[c]	25	Human who ate raw oysters
	9802[c]	17	New Jersey; human stool (origin, New Orleans, La., clams)
	9829[c]	11	Texas; human stool (seafood origin)
	10202[c]	15	Human stool
	10210[c]	347	Colorado; transtracheal aspiration
	10221[c]	42	Guam; human stool
	10248[c]	42	Hawaii; human throat
	10249[c]	17	Maryland; duck lung
	10305[c]	37	Louisiana; crab feces
	10322[c]	22	Pennsylvania; human sinus
	10357[c]	17	Blood from a leukemic human
	10632[c]	11	Ohio; human stool
	294-84[d]	ND[h]	West Virginia; human ear drainage
	1249-80[d]	17	Louisiana; water; tox$^-$
	1286-83[d]	31	New Mexico; human blood
	924-79[d]	14	Chesapeake Bay; water; tox$^-$
	609-84[d]	12	New York City; human stool; tox$^-$
	1368-80[d]	12	Wisconsin; human stool; tox$^-$
	335-81[d]	15	Oregon; fish tank; tox$^-$
	1351-83[d]	57	Florida; human stool; tox$^-$
	797-83[d]	17	Oregon; human stool; tox$^-$

[a] Isolated by and from the collection of R. A. Finkelstein. (TR[2], translucent colony type virulent for and passed in rabbits.)
[b] From P. A. Ristaino, Center for Vaccine Development, University of Maryland School of Medicine, Baltimore.
[c] From H. Smith, Jefferson Medical College, Philadelphia, Pa. (4).
[d] From J. J. Farmer III, Centers for Disease Control, Atlanta, Ga.
[e] From the Walter Reed Army Institute of Research; collection of R. A. Finkelstein; formerly strain 20-A-11.
[f] Isolated by C. E. Lankford; from the collection of R. A. Finkelstein.
[g] Originally from N. K. Dutta; obtained from the collection of R. A. Finkelstein.
[h] ND, Not determined.

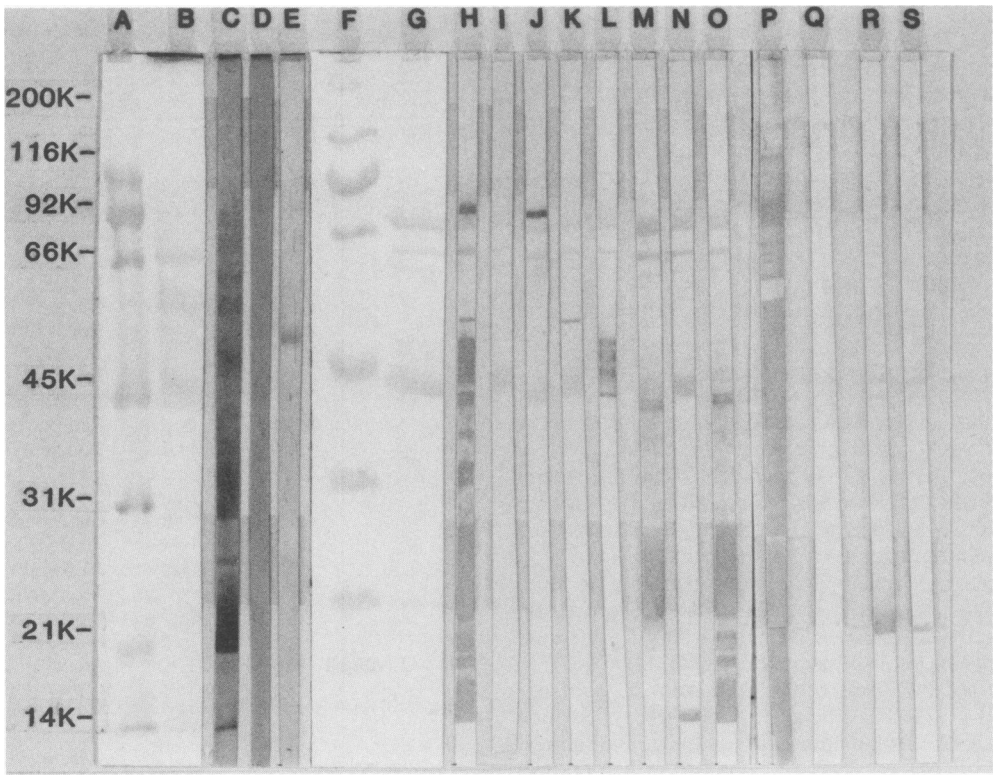

FIG. 1. Western blot analysis of outer membrane preparations (heated at 100°C and unheated) from two strains of *V. cholerae* that were immunostained by using separate McAbs. The results of three separate experiments are shown. Lanes A and F contained molecular weight markers which were stained with Ponceau S. Lanes B through E are blots of strain 3083TR[2] membranes which were heated at 100°C before electrophoresis and blot transfer. Lanes G through O are blots of strain NIH41 OMPs which were also heated. Lanes P through S are blots of strain NIH41 OMPs which were not heated. Lane B, OMPs of strain 3083TR[2] which were stained with Ponceau S; lanes C, H, and P, mouse polyclonal immune serum; lanes D, I, and Q, mouse polyclonal preimmune serum; lane E, McAb SY58/6; lane J, McAb SY72/3; lane K, McAb SY52/7; lane L, McAb SY48-46/34; lane M, McAb SY18/47; lane N, McAb SY14/52; lane O, McAb SY14/49; lane R, McAb SY40-18/5; lane S, McAb SY40-18/41. Although McAbs SY40-18/5 and SY40-18/41 appeared to recognize the same protein(s), McAb SY40-18/41 reacted only with the unheated form.

NCTC109 (MA Bioproducts, Walkersville, Md.), 0.002% Fungizone (GIBCO), 96 μg of gentamicin per ml, 13.6 μg of hypoxanthine per ml, 0.176 μg of aminopterine per ml, and 3.87 μg of thymidine per ml; 96-well tissue culture plates were precoated with BALB/c peritoneal macrophages (approximately 10^2 to 10^3 cells per well in 50 μl of Dulbecco modified Eagle medium), and 150 μl of the fused cell suspension was added to each well. Positive clones were identified initially by a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis-immunoblot analysis (45), using crude tissue culture supernatants containing McAbs derived against outer membrane antigens. Antibody-producing clones were subcloned by limiting dilution. Immunoglobulin classes and subclasses were determined by Ouchterlony immunodiffusion, using rabbit antisera (Cappel Laboratories, West Chester, Pa.). McAb concentrations were determined by using radial immunodiffusion. To obtain high yields of antibodies, the hybridomas were grown as ascites tumors in BALB/c mice which had been primed with pristane (19).

Outer membrane preparations. Outer membranes were prepared from *V. cholerae* by sarcosine solubilization, as previously described (37).

Western blot procedure. Western blot analysis was performed by the method of Towbin et al. (45), which was modified by replacing fetal calf serum with 1% Tween 20 and 3% bovine serum albumin (BSA). Outer membrane antigens were electroblotted from 8% SDS–polyacrylamide gels (0.75 mm) onto nitrocellulose sheets (pore size, 0.45 μm; (Millipore Corp., Bedford, Mass.) for 4 h at 80 V and 4°C, whereas purified LPS preparations were blotted for 1 h. Peroxidase-labeled rabbit anti-mouse antibody (Cappel) was used to detect mouse McAb, and the peroxidase enzyme reaction was mediated by O-dianisidine. Controls consisted of mouse preimmune serum, mouse immune serum, and a previously established mouse McAb to an unrelated antigen, the heat-labile enterotoxin (H-LT) of *E. coli*.

ELISA. Four different kinds of samples and four different procedures were used to coat polyvinylchloride (PVC) plates (Fisher) for the enzyme-linked immunosorption assay (ELISA) (10, 46). The samples were whole bacteria, sonicated bacteria, isolated OMP, and purified LPS. The procedures used are described below. (i) Bacteria were grown overnight, harvested, and centrifuged, and the resulting bacterial pellet was suspended in bicarbonate coating buffer (pH 9.6); 100 μl of this bacterial suspension (10^{10} cells per ml) was pipetted into 96-well microtiter PVC plates. (ii) Bacteria were harvested as described above, suspended in bicarbonate buffer, sonicated on ice for 1 min at maximum input with a cell disrupter (model W225R; Heat Systems Ultrasonics, Inc., Plainview, N.Y.), and distributed into 96-well microtiter plates (100 μl/well). (iii) Isolated outer membrane preparations were suspended in bicarbonate coating buffer (10 μg of protein per ml), and a 100-μl portion was

TABLE 2. Summary of reactivities of hybridoma-derived McAbs with O group 1 *V. cholerae* strains

No. of individual clones	Mol wt ($\times 10^3$) of the antigen(s) to which McAbs are directed[a]
2	150
1	72
1	72, 70
1	58
1	52
22	52, 31 (LPS)
2	52, 31, 18 (LPS)
5	48, 47, 46
2	46
1	44, 14 (LPS)
1	44, 18
3	40, 18
1	31
6	18
1	18 (LPS)
10	14
6	ND[b]

[a] Molecular weights were determined by SDS-polyacrylamide gel electrophoresis-Western blot analysis.
[b] ND, Not determined.

distributed to each well of a 96-well PVC plate. (iv) Each preparation of LPS was suspended in bicarbonate coating buffer at a concentration of 10 μg/ml, and the preparations were distributed in 100-μl portions to the wells of 96-well PVC plates. Purified lipid A was solubilized in a saturated solution of crystalline BSA (48) before dissolution in bicarbonate coating buffer. After antigens were coated onto PVC plates overnight, the assay wells were rinsed and then blocked with 3% BSA at room temperature for 1 h, and test McAbs were added (0.5 μg of antibody per well). The plates were incubated at 37°C for 1 h, and this was followed by addition of secondary peroxidase-labeled rabbit anti-mouse antibody (Cappel), which was allowed to react for 1 h at 37°C. The substrate used was 100 μl of 100 μM 2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid) in 10 mM Tris-hydrochloride buffer (pH 4.0). Between the steps described above, the plates were washed three times with phosphate-buffered saline (pH 7.4) containing 1% Tween 20. The optical density at 490 nm was measured with a model MR600 spectrophotometer (Dynatech Laboratories, Inc., Alexandria, Va.). An optical density of 0.1 U above the background level was considered a positive result. As a negative control, wells were coated with Dulbecco modified Eagle medium or ascites fluid from mice injected with cells of myeloma SP2/O-Ag14. In addition, LPS-reacting McAbs were added to wells which were not coated with LPS.

Isolation of vibriobactin. *V. cholerae* 569B was cultivated in low-iron T medium (37), and vibriobactin was prepared by the ethyl acetate extraction procedure described by Payne and Finkelstein (35). Vibriobactin was complexed with ^{59}Fe by the procedure used by Sokol and Woods with *Pseudomonas* siderophores (41).

Autoradiography. Radiolabeled iron (^{59}Fe) was obtained from New England Nuclear Corp., Boston, Mass., in the ferric chloride form and had a specific activity of 19.79 mCi/mg. Kodak XAR-2 film was incubated for 15 h at −70°C with the sample by using a Kodak X-Omatic cassette equipped with double intensifying screens.

Molecular weight markers. Molecular weight markers were purchased from Bio-Rad Laboratories, Richmond, Calif. These markers included myosin (molecular weight, 200,000), beta-galactosidase (116,250), phosphorylase B (92,500), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (25,500), and lysozyme (14,400).

RESULTS

Isolation of McAbs. For our records, hybridomas were designated by the molecular weight(s) of the antigen with which the McAb reacted and a library catalog number. Figure 1 shows representative immunoblots which illustrate some McAbs which recognized antigens having different molecular weights. For example (see the legend to Fig. 1), McAb SY58/6 reacted with a 58,000-dalton OMP and was the sixth clone in our library, and McAbs SY40-18/5 and SY40-18/41 were two distinct clones which reacted with membrane antigens having molecular weights of 40,000 and 18,000. Although there was some variable nonspecific background reactivity in the blots, our conclusions were based upon multiple analyses of each McAb. Some McAbs showed single sharp antigen-reacting bands (Fig. 1, lanes E, J, K, N, R, and S), and in some cases McAbs reacted with a series of bands having different molecular weights (Fig. 1, lane L). As the McAb recognition of certain epitopes was destroyed by heat, our McAb library was screened by immunoblot analysis with outer membrane preparations which were heated at 100°C before SDS-polyacrylamide gel electrophoresis (Fig. 1, lanes A through O), as well as preparations which were unheated (Fig. 1, lanes P through S).

From a single fusion experiment, 66 hybridomas which produced monospecific antibodies to various outer membrane antigens of the four immunizing strains of *V. cholerae* used were established. Most of these were of the immunoglobulin G1 subclass; only three belonged to the immunoglobulin M class. As summarized in Table 2, the

TABLE 3. Difference in McAb-defined epitopes among four strains of *V. cholerae*

McAb	*V. cholerae* strain[a]	Whole-cell ELISA[b]	Outer membrane ELISA[c]	Western blot[d]
SY52-31/12	3083TR^2	+	+	−
	NIH41	+	+	−
	CA401	+	+	+
	569B	+	+	+
SY52-31/25	3083TR^2	−	−	+
	NIH41	−	−	−
	CA401	−	−	−
	569B	−	−	−
SY48-46/35	3083TR^2	−	+	+
	NIH41	−	+	+
	CA401	−	+	+
	569B	−	−	−
SY48-46/31	3083TR^2	+	+	−
	NIH41	−	+	+
	CA401	−	+	−
	569B	−	+	−

[a] Bacterial strains were grown in liquid low-iron T medium at 37°C for 16 h before tests were done.
[b] Whole-cell ELISAs were performed by using intact micro-organisms coated onto microtiter wells (10^9 CFU/well).
[c] Outer membrane preparations of vibrios were coated onto microtiter wells (1 μg of protein per well) for ELISAs.
[d] Western immunoblot analysis was performed on purified outer membrane antigens separated by SDA-polyacrylamide gel electrophoresis and blot transferred to nitrocellulose.

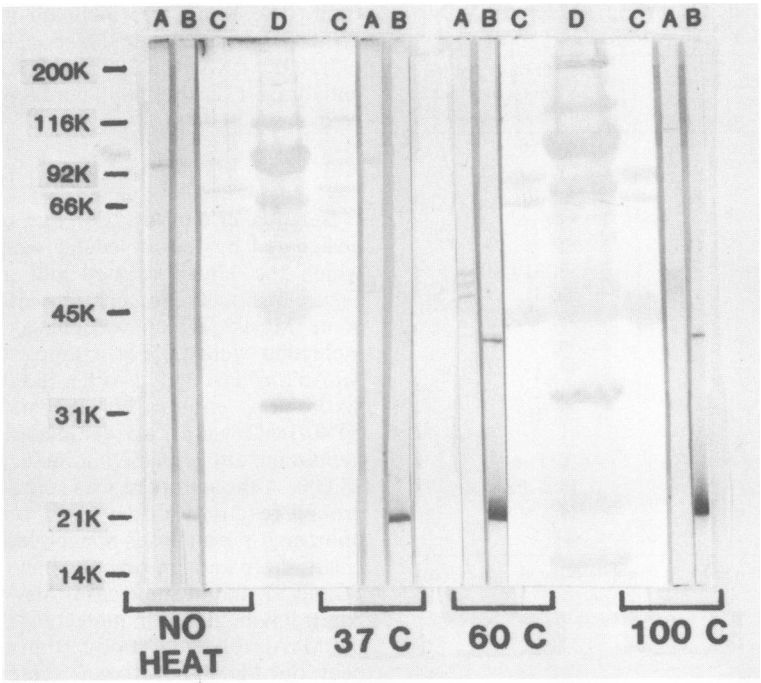

FIG. 2. Effect of heat on the reactivity of the OMPs of *V. cholerae* NIH41 with McAbs. Outer membrane antigens were heated as indicated at the bottom for 10 min, separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and detected by reaction with McAbs (lanes A and B). Proteins were also stained directly with Ponceau S immediately after transfer (lanes C and D). Lanes A, McAb SY48-46/34; lanes B, McAb SY40-18/5; lanes C, OMPs; lanes D, molecular weight markers.

hybridomas could be divided into at least 16 different groups based upon their McAb recognition of various antigens in immunoblot analyses. Interestingly, in several instances, the McAbs consistently recognized a single antigen which exhibited multiple molecular weight banding patterns when it was exposed to heat treatment (100°C). An additional six hybridomas produced antibody which was immunoblot negative but which reacted in the whole-cell ELISA. A total of 26 of the McAbs reacted with LPS. Interestingly, no hybridomas which produced antibody to the major OMP (molecular weight, 42,000 to 45,000) of *V. cholerae* (32) were detected.

Reactivity of selected McAbs in different immunoassays. The four immunizing strains of *V. cholerae* were examined by using the 66 McAbs as immunological probes in three different assays (whole-cell ELISA, purified outer membrane ELISA, and immunoblot analysis). The results obtained are too numerous to present in complete detail. However, reactions with four McAbs are shown in Table 3 as examples of the patterns of reactivity which occurred. McAb SY52-31/12 was reactive with the two Inaba serotype strains, strains CA401 and 569B, in each of the assays, but it recognized only the Ogawa serotype strains in the ELISAs. McAb SY52-31/25, which recognized the same antigen, was specific for an epitope present in strain 3083TR[2] which was apparently revealed only in its dissociated state and which was not surface exposed. McAb SY48-46/35 recognized strains 3083TR[2], NIH41, and CA401 but not strain 569B in immunoblots and in the ELISA of OMPs but not in the ELISA of whole cells. We believe that this epitope may be located on the inner surface of the outer membrane. The results obtained with McAb SY48-46/31 suggest that the location and properties of an epitope may vary in different strains; in strain 3083TR[2] this epitope was surface exposed but was altered by SDS treatment; in strains NIH41, CA401, and 569B it was not surface exposed, and it was stable to SDS only in strain NIH41.

Heat modification of proteins. The reactivity of certain OMPs with McAbs was also heat modifiable. For example, a 112,000-dalton protein (Fig. 2, lane A) was clearly rearranged into three subunits (molecular weights, 48,000, 47,000, and 46,000) by heat treatment. This protein underwent some dissociation at 37°C and was completely dissociated at 60°C and above, but reacted only weakly with antibody after treatment at 100°C.

McAb SY40-18/5 (Fig. 2, lane B) recognized a protein with a molecular weight of approximately 18,000 in unheated outer membrane preparations. This reaction intensified with heating of the outer membrane preparation, which also resulted in the appearance of a stained band at approximately 40,000 daltons. Apparently this OMP was a dimer consisting of two subunits having molecular weights of approximately 18,000 and an internal epitope that was unmasked by more complete unfolding of the protein.

IROMPs. IROMPs are expressed by *V. cholerae* in vitro (37, 39) and in vivo in the infant rabbit model (37). Although the levels of several proteins have been shown to be increased under low-iron cultivation, proteins in the molecular weight range from 48,000 to 46,000 (as determined by SDS-polyacrylamide gel electrophoresis of heated samples) were common among the four strains of *V. cholerae* examined (37). When heated, the 112,000-dalton protein described above resulted in a protein triplet which corresponded to the molecular weight region of these IROMPs. To evaluate the potential role of this protein in iron acquisition by *V. cholerae*, the homologous siderophore-radiolabeled iron complex (^{59}Fe-vibriobactin) was added to outer membrane preparations, and these mixtures were incubated for 5 min at

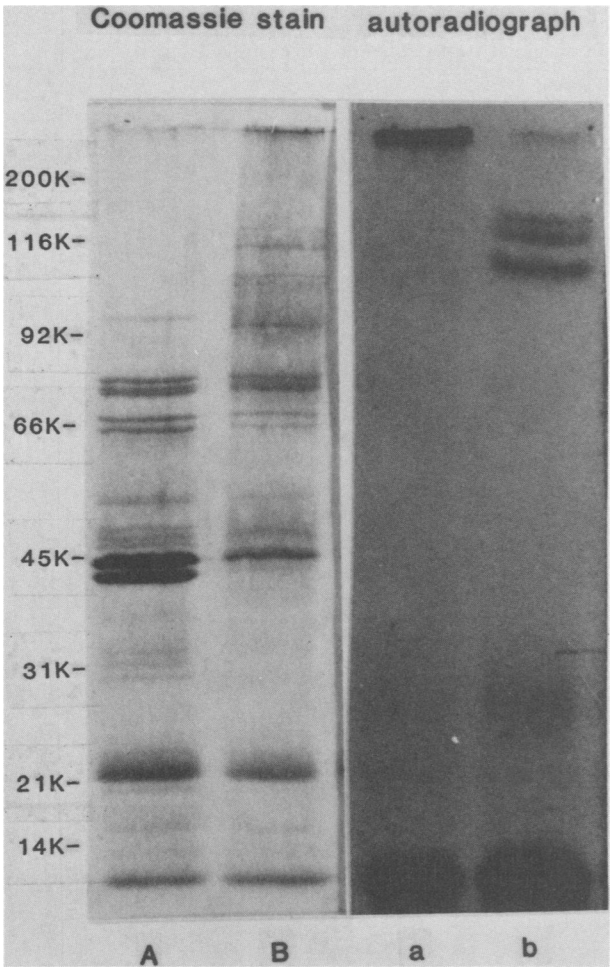

FIG. 3. Gradient (8 to 12%) SDS-polyacrylamide gel electrophoresis and autoradiography of ^{59}Fe-vibriobactin binding with OMPs of *V. cholerae* strain 3083TR^2. Lane A, Coomassie brilliant blue R stain of OMPs which were premixed with ^{59}Fe-vibriobactin and nitrilotriacetate, incubated for 5 min at 37°C, heated at 100°C for 5 min, and electrophoresed; lane B, Coomassie brilliant blue stain of OMPs as in lane A but without heating; lane a, autoradiograph of lane A; lane b, autoradiograph of lane B.

37°C and subjected to SDS-polyacrylamide gel electrophoresis before and after heating at 100°C for 5 min. In the unheated sample, the autoradiograph (Fig. 3, lane b) had a doublet (131,000 and 125,000 daltons), a darker band at 112,000 daltons, a diffuse band at 35,000 daltons, and an intense band at the low-molecular-weight dye front. Controls (data not shown) consisting of $^{59}FeCl_3$, ^{59}Fe-nitrilotriacetate, and ^{59}Fe-dihydroxybenzoate (a second siderophore produced by *V. cholerae* [21, 35]) mixed with OMPs and electrophoresed indicated that the diffuse 35,000-dalton band was ^{59}Fe-dihydroxybenzoate associated and that the lowest-molecular-weight band was ^{59}Fe-nitrilotriacetate. ^{59}Fe-vibriobactin bound to proteins having molecular weights of 112,000 to 131,000 only in unheated preparations (Fig. 3, lane b). The other iron sources did not bind to the proteins (data not shown).

After heating at 100°C (Fig. 3, lanes A and a), the 112,000- to 131,000-dalton proteins (barely visible in the protein-stained gel [Fig. 3, lane B]) disappeared coincident with the appearance of the 48,000- to 46,000-dalton triplet (Fig. 3, lane A), as shown above (Fig. 2, lane A), as well as a second major band at 42,000 daltons, which was defined previously (37) as a characteristic component of strain 3083TR^2. The ^{59}Fe-vibriobactin no longer bound to the heated preparation and upon separation from the protein became insoluble (21) and failed to enter the separating gel (Fig. 3, lane a). Attempts to immunoprecipitate the detergent (Triton X-100)-solubilized protein-siderophore-^{59}Fe complex with McAbs (directed to the heat-modifiable IROMP) were unsuccessful, presumably because of the action of the detergent on the antibodies. However, from immunoblot data it is apparent that these McAbs weakly recognized the 112,000-dalton protein in its non-dissociated form, and it is likely that they did not recognize their respective epitopes at all once the iron-siderophore complex was bound.

McAbs recognize antigens found in vivo. McAbs in our collection recognized additional vibrio antigens which were expressed in vivo. In vivo grown vibrios have been shown to be similar to vibrios grown in vitro under low-iron conditions (37). Outer membrane antigens prepared from *V. cholerae* 3083TR^2 grown in vivo as previously described (37) were separated by SDS-polyacrylamide gel electrophoresis, electroblotted, and immunostained with selected McAbs. *V. cholerae* 3083TR^2 grown in vitro under low-iron conditions was examined in parallel. The 58,000-dalton antigen, the 52,000-dalton–31,000-dalton doublet, the 48,000-dalton–47,000-dalton–46,000-dalton iron-regulated, heat-modifiable triplet, and the 18,000-dalton antigen were recognized by McAbs SY58/6, SY52-31/21, SY48-46/34, and SY18/47, respectively, in vibrios growing in vivo and in vitro under low-iron conditions (data not shown). Three additional McAbs (McAbs SY52/7, SY14/49, and SY14/54) failed to react with the in vivo preparations, but this may have resulted from the limited amount of available protein which was immunoblotted.

Interaction of McAbs with LPS. Of 26 anti-LPS McAbs, 24 reacted in the ELISA with preparations of LPS from O group 1 *V. cholerae* (Table 4). A total of 12 of these antibodies (Table 4, antibodies 13 through 24) were specific for *V. cholerae* LPS and did not react with *Salmonella*, *Pseudomonas*, or *E. coli* LPS preparations. Three of these McAbs (antibodies 20 through 22) did not recognize the rough LPS from strain 416ORa, and three (antibodies 22 through 24) failed to react with LPS from strain 3083TR^2 in LPS ELISAs. The other 12 McAbs (Table 4, antibodies 1 through 12) were cross-reactive with LPSs from other gram-negative species. Some of the cross-reactions with LPSs from different gram-negative organisms appeared to be LPS region specific. Interestingly, we found seven McAbs (Table 4, antibodies 1 through 4 and 10 through 12) which recognized *S. minnesota* Re595 LPS (representing the 2-keto-3-deoxyoctulosonic acid inner-core region and the lipid A region of LPS). Two of these McAbs recognized *S. minnesota* Re595 lipid A, and the other five did not. Of the latter five, two (antibodies 1 and 2) reacted with *Vibrio*, *Salmonella*, *Escherichia*, and *Pseudomonas* LPSs, and three (antibodies 10 through 12) reacted with *Vibrio* and *Salmonella* LPSs only. This indicates that despite the reported (25, 26) LPS inner-core difference between *V. cholerae* and members of the *Enterobacteriaceae*, some common antigenic determinants exist in this region. Three McAbs (antibodies 5, 8, and 9) which reacted with LPS from the *E. coli* mutant J5 (representing the outer-core region of LPS) shared common epitopes in the outer-core region with *Vibrio*, *Salmonella*, *Escherichia*, and *Pseudomonas*. These McAbs did not react with the strain Re595 LPS or lipid A, thus excluding the LPS inner-core region. Therefore, although *V. cholerae* LPS may differ biochemically from other gram-negative

TABLE 4. Reactions of McAbs with LPS preparations in an ELISA

Example no.	McAb Library designation[a]	Reactions with the following sources of LPS[b]: Polysaccharide chain region: *V. cholerae* O1 3083TR[2]	*V. cholerae* O1 20A11	*V. cholerae* O1 7738	*V. cholerae* O1 569B	*V. cholerae* O1 416 ORa	*P. aeruginosa*	*S. typhimurium*	*E. coli* O55:B5	*E. coli* J5 outer core	*S. minnesota* Re595 inner core	Lipid A inner core[c]
1	SY52-31/24	+	+	+	+	+	+	+	+	+	+	−
2	SY44-14/39	−[d]	+	+	−[d]	+	+	+	−	+	+	−
3	SY52-31/33	+	+	+	+	+	−	+	−	+	+	+
4	SY52-31/9	+	+	+	+	+	−	−[e]	+	+	+	+
5	SY52-31/11	+	+	+	+	+	+	−[e]	−	+	−	−
6	SY52-31/16	+	+	+	+	+	+	−[e]	+	−	−	−
7	SY52-31/14	+	+	+	+	+	+	+	−	−	−	−
8	SY52-31/29	+	+	+	+	+	+	+	−	+	−	−
9	SY52-31/10	+	+	+	+	+	+	−[e]	−	+	−	−
10	SY52-31/20	+	+	+	+	+	−	−	−	−	+	−
11	SY52-31/22	+	+	+	+	+	−	−	−	−	+	−
12	SY52-31-18/60	+	+	+	+	+	−	−[e]	−	−	+	−
13	SY52-31/8	+	+	+	+	+	−	−	−	−	−	−
14	SY52-31/12	+	+	+	+	+	−	−	−	−	−	−
15	SY52-31/15	+	+	+	+	+	−	−	−	−	−	−
16	SY52-31/17	+	+	+	+	+	−	−	−	−	−	−
17	SY52-31/21	+	+	+	+	+	−	−	−	−	−	−
18	SY52-31/23	+	+	+	+	+	−	−	−	−	−	−
19	SY52-31-18/62	+	+	+	+	+	−	−	−	−	−	−
20	SY18/59	+	+	+	+	−	−	−	−	−	−	−
21	SY0/65	+	+	+	+	−	−	−	−	−	−	−
22	SY52-31/25	−[d]	+	+	+	−	−	−	−	−	−	−
23	SY52-31/13	−[e]	+	+	+	+	−	−	−	−	−	−
24	SY52-31/19	−	+	+	−[d]	+	−	−	−	−	−	−

[a] McAbs were selected from the clone library for their reactions with LPS. Preparations of ammonium sulfate-precipitated antibody at a concentration of 25 μg/ml of phosphate-buffered saline were used at a concentration of 5 μg of antibody per microtiter well.

[b] LPS was used at a concentration of 1 μg/well. A positive reaction was defined as 0.1 optical density unit above the background level.

[c] Lipid A was solubilized in a saturated solution of crystalline BSA-bicarbonate buffer.

[d] McAb reactive in Western blots of OMP preparations from the representative organism.

[e] McAb reactive in whole-bacterial-cell ELISAs.

LPSs, it has some antigenic similarities. Some McAbs apparently recognized the repeating polysaccharide units of LPS and not the rough LPS preparations of other members of the *Enterobacteriaceae*.

The cross-reactions of the McAbs which are presumably directed against the core region of vibrio LPS are of particular interest. Various preparations of purified LPS were examined by SDS-polyacrylamide gel electrophoresis (Fig. 4, silver stain) and immunoblotting (Fig. 4, Western blot). When core-specific, cross-reactive McAb SY52-31/24 (Fig. 4, Western blot) was immunoreacted with purified electroblotted LPS preparations, it reacted only with the smooth *V. cholerae* LPS. A second core-specific, cross-reactive McAb, McAb SY44-14/39, was unreactive in a similar immunoblot. To verify that LPS was transferred to nitrocellulose during immunoblot analysis of LPS, a blot transfer of LPS preparations onto nitrocellulose was silver stained. The confirming result (data not shown) was a negative effect, indicating unstained LPS on a dark brown-stained background, which faded with time. Similar results (i.e., McAb reactions with LPS preparations in an ELISA but not in an immunoblot analysis) were recently reported by Mutharia et al. (33) with McAbs prepared against *E. coli* LPS. These authors also showed that the same antibody which reacted with immunoblots of outer membranes did not react with purified LPS preparations; we found similar reactions occurring in our experiments. It is possible that the combination of phenol-water extraction and SDS-polyacrylamide gel electrophoresis affected the reactivity of the LPS in immunoblots.

Unlike *Salmonella* LPS, which gave a ladder-like appearance in SDS-polyacrylamide gel electrophoresis preparations (Fig. 4, lane G), *V. cholerae* LPS separated into unique groups (Fig. 4, lanes A through D). These different groups were further characterized by their immunological reactions with our McAbs. As shown in Fig. 4, McAb SY52-31/24 reacted with an LPS group having molecular weights of approximately 90,000, 72,000, 52,000, and 31,000. It did not recognize the 18,000-dalton component (Fig. 4, lane A), which was recognized by McAb SY18/59 (Table 4, example 20). Components at 44,000 and 14,000 daltons which did not silver stain (Fig. 4) immunoreacted in ELISAs with McAb SY44-14/39 (Table 4, example 2).

Each of the 66 McAbs was tested for its ability to agglutinate *V. cholerae* strains 569B (classical biotype, Inaba serotype) and 3083TR[2] (El Tor biotype, Ogawa serotype) in both tube and slide agglutination tests (12). Only three McAbs (McAbs SY52-31/33, SY52-31/20, and SY52-31/15), which were directed against LPS (Table 4, examples 3, 10, 15, respectively), were agglutinative, and they agglutinated both strains.

Reactivity of McAbs with O group 1 and non-O group 1 *V. cholerae*. Each of the 66 McAbs (Table 2) was tested in the ELISA with sonic lysates of each of the 42 *V. cholerae* strains listed in Table 1 (20 O group 1 strains and 22 non-O group 1 strains).

We expected that some of the McAbs in our library would be serotype or biotype specific within the O group 1 *V. cholerae* strains. Although several McAbs in our library (McAbs SY52-31/29, SY18/59, SY52-31-18/62, SY18/65,

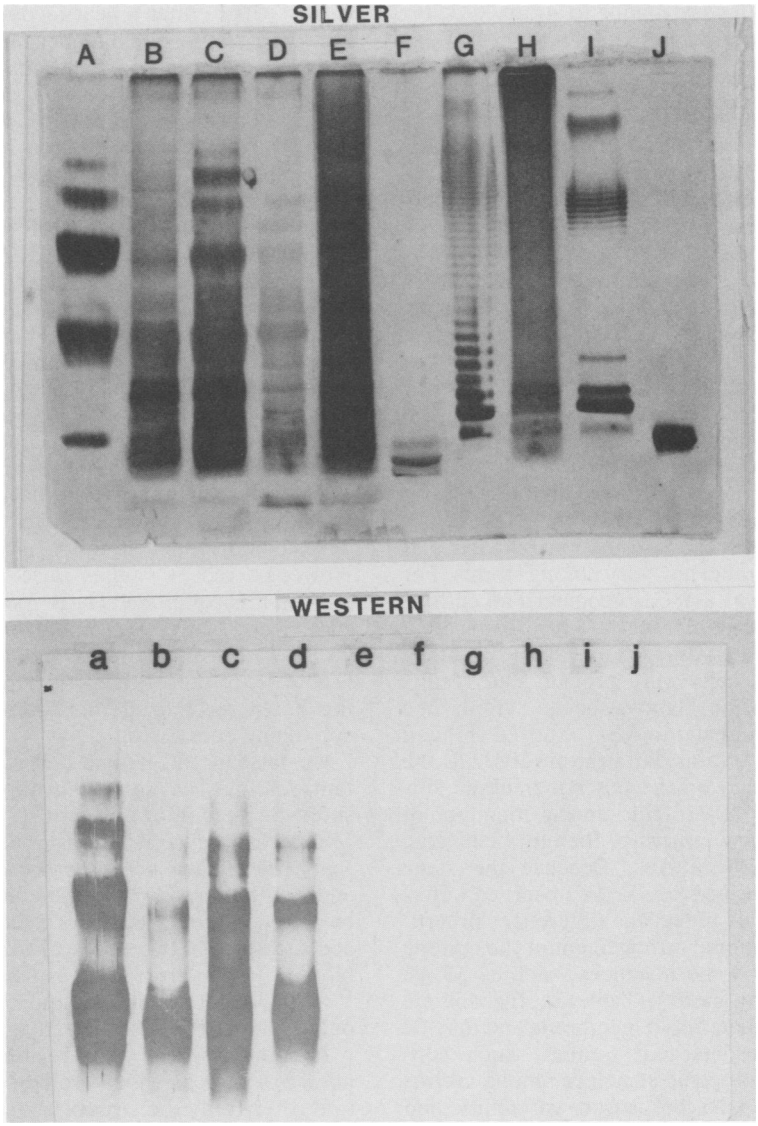

FIG. 4. Immunoblot of LPSs of *V. cholerae* and other gram-negative organisms with McAb SY52-31/24. Lanes A and a, *V. cholerae* 3083TR[2] (El Tor biotype, Ogawa serotype); lanes B and b, *V. cholerae* 7738 (El Tor biotype, Inaba serotype); lanes C and c, *V. cholerae* 20A11 (classical biotype, Ogawa serotype); lanes D and d, *V. cholerae* 569B (classical biotype, Inaba serotype); lanes E and e, *V. cholerae* 416 ORa rough LPS; lanes F and f, *S. minnesota* Re595; lanes G and g, *S. typhimurium*; lanes H and h, *P. aeruginosa*; lanes I and i, *E. coli* O55:B5; lanes J and j, *E. coli* O111:B4 (mutant J5) rough LPS. Note that the LPS of each *V. cholerae* strain except the rough LPS strain was immunoreactive with McAb SY52-31/24 (lanes a through d); however, none of the LPSs of the other gram-negative organisms were blot positive. This conflicts with data from ELISAs, in which McAb AY52-31/24 was cross-reactive with all except lipid A. Each lane contained ca. 5 μg (dry weight) of LPS.

SY52-31/8, SY52-31/22, and SY52-31/23) reacted to various degrees almost exclusively with O group 1 *V. cholerae* strains (an occasional non-O group 1 strain was reactive), none differentiated Ogawa serotypes from Inaba serotypes. One of our McAbs, McAb SY52-48-46/63, reacted only with the three classical biotype strains tested, but none reacted only with El Tor biotype strains. Some quantitative variations in ELISA optical density values among strains were observed. When non-O group 1 vibrios were examined, two McAbs (McAbs SY52-31/29 and SY18/59) reacted with a single non-O group 1 strain (strain 10210), whereas two other McAbs (McAbs SY52-31/9 and SY52-31/24, both of which were anti-LPS, recognized virtually all of the *Vibrio* strains tested (both O group 1 and non-O group 1). Of the latter, McAb SY52-31/9 was directed against lipid A and also reacted with other gram-negative strains (except *Pseudomonas*) (Table 4). McAb SY52-31/24 was directed against inner-core LPS (Table 4) and was broadly cross-reactive with other gram-negative strains, but it did not react with strain 9767, a non-O group 1 *Vibrio*. Three McAbs (McAbs SY0/64, SY46/38, and SY46/37) reacted with only two or three of the sonicates tested, whereas other McAbs apparently recognized strain-specific epitopes; two McAbs (with 31,000- and 18,000-dalton corresponding antigens) were unique to strain 569B, one McAb (with a 150,000-dalton corresponding antigen) was unique to strain NIH41, and five McAbs (each representing one of four corresponding antigens having molecular weights of 14,000, 18,000, 40,000, to

18,000, and 52,000 to 31,000) were unique to strain 3083TR[2]. Strain-specific antibodies such as these might prove useful in epidemiological studies.

DISCUSSION

This study was initiated primarily to obtain McAbs which recognize the IROMPs of *V. cholerae*. We established 66 hybridomas which produce McAbs that recognize components of the outer membrane of O group 1 *V. cholerae* strains. The reactivity of these McAbs was evaluated by using immunoblot analysis, as well as several variations of the ELISA.

From the immunoblot analysis (Fig. 1) it was evident that some McAbs reacted with a single antigen which was separated into related multiple-molecular-weight bands by SDS-polyacrylamide gel electrophoresis. A total of 16 different antigens were identified (Table 2) based upon their recognition by separate, individual McAbs. Included among these were strain-specific epitopes, heat-modifiable proteins, IROMPs (also produced in vivo), and LPS.

Our results suggest that the conformation of particular antigens in the outer membrane may vary among strains. For example (Table 3), McAb SY48-46/31 recognized an epitope which occurred in the outer membranes of four strains of *V. cholerae*; however, only one strain, strain 3083TR[2], reacted with antibody when intact cells were probed. Our results imply that detergent (sarcosine) removal of the cytoplasmic membrane from the outer membrane exposed hidden epitopes in three of the four strains, but in strain 3083TR[2] the same epitope was recognized on the outer membrane surface. The binding of SDS to proteins during immunoblot analysis affected their tertiary structure, therefore changing their immunoreactivity with McAbs. Because the same epitope was studied in the four strains, the observed differences in immunoblot analysis reflect the differences in tertiary structure and conformational arrangement of the epitope among the four strains. In some instances, such as McAb SY52-31/25 (an anti-LPS antibody) (Table 3), the epitope may be buried within the bilayer lipid membrane and may be exposed only when it is detergent treated. Such conformational differences in antigenic structure among vibrios may help to explain, in part, the strain variability and complexity of the serological reactivity of these organisms, as shown in the present study.

As shown in Fig. 2, two outer membrane antigens were clearly heat modifiable. Of these, one, a 112,000-dalton protein, was rearranged into 48,000-, 47,000-, and 46,000-dalton components by mild heat treatment. This protein was also iron regulated and bound ^{59}Fe-vibriobactin (Fig. 3), the major siderophore produced by *V. cholerae* (35). This protein was common among the four O group 1 strains tested and was present in organisms growing in vivo. Three additional McAbs recognized three other antigens (not IROMPs) which were common both to vibrios grown in vivo and to vibrios grown in vitro under iron-limited conditions. Similarly, IROMPs have been demonstrated in vivo in *E. coli* and *P. aeruginosa* (5, 17, 20). Our evidence does not establish that the 112,000-dalton protein is the surface receptor for iron-vibriobactin, but only suggests that it may play some functional role in the recognition of this iron-siderophore complex. The role of this protein in iron uptake is being studied by using the five IROMP McAbs generated in this study.

Another antigen, which shifts from a molecular weight of 18,000 to a molecular weight of approximately 40,000 upon heating, appears to be a dimer of two subunits that are similar in size. Two possibilities for this type of heat variability exist; either a heat-mediated aggregation of subunits occurs, giving rise to a higher-molecular-weight complex, or the higher-molecular-weight complex is heat denatured, unmasking an epitope such that the immunological reactivity increases. The observation of a heat-induced increase in the intensity of the 18,000-dalton band with a concurrent increase in the intensity of the 40,000-dalton band favors the latter possibility.

Although our studies were not initially directed toward the examination of *V. cholerae* LPS, we obtained 26 McAbs which reacted to LPS either in immunoblots of outer membranes or in ELISAs of purified LPS. Hybridomas which produced antibody to LPS (Table 4) reacted with components having apparent molecular masses of 90,000, 72,000, 52,000, 44,000, 31,000, 18,000, and 14,000 daltons. Among these, various region-specific McAbs were identified based upon their reactions to purified preparations of LPS and lipid A from *V. cholerae* and other gram-negative organisms.

V. cholerae is unusual in that, unlike *Enterobacteriaceae* species, it has been reported not to contain 2-keto-3-deoxyoctulosonic acid in its LPS inner-core region (25, 26). However, recent studies raise the possibility that it does contain this compound (1, 3). Two anti-LPS McAbs (McAbs 52-31/9 and 52-31/24) reacted with virtually all of the non-O group 1 vibrios tested, as well as other gram-negative LPSs (Table 4); of these, the former recognized lipid A, whereas the latter recognized the 2-keto-3-deoxyoctulosonic acid-containing core region.

Gustafsson and Holme (23) recently reported the important findings of anti-A (group-specific), anti-B (Ogawa serotype-specific), and anti-C (Inaba serotype-specific) McAbs from mice which had been immunized with purified Inaba or Ogawa serotype LPS, but their paper did not address the question of differentiating classical from El Tor biotypes. Although our efforts did not result in any serotype-specific McAbs, two of our McAbs recognized only classical biotype *V. cholerae* antigens (which were not LPS).

Of the 66 McAbs in our study, it is interesting to note that only 3 (anti-LPS McAbs) had agglutinating activity.

In summary, this study resulted in the development of a library of McAbs which are being used as molecular probes to further study the structural and functional characteristics of *V. cholerae* outer membrane components and some components of members of the *Enterobacteriaceae*.

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