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Vibrio cholerae Expresses Cell Surface Antigens during Intestinal Infection Which Are Not Expressed during In Vitro Culture

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***Vibrio cholerae* O1 bacteria harvested directly from ligated or nonligated intestines of rabbits with experimental cholera expressed at least 7 to 8 novel, in vivo-specific cell envelope (env) proteins that were not found on vibrios after in vitro culture in various ordinary liquid media. At the same time, several of the env proteins ordinarily expressed in vitro had disappeared or become much reduced. The infection-induced novel env proteins were immunogenic. In immunoblot analyses, antisera raised against in vivo-grown vibrios and then absorbed with in vitro-grown bacteria of the same strain specifically stained at least eight infection-induced antigens ranging from 62 to ~200 kilodaltons; absorption with washed in vivo-grown bacteria, on the other hand, removed the antibodies reacting with these antigens, indicating that the antigens were present on the bacterial cell surface. Conversely, antiserum against in vitro-grown bacteria reacted with several env antigens in in vitro-grown bacteria that were missing in the infection-derived vibrios. These adaptational changes were strikingly similar for different strains of cholera vibrios of both classical and El Tor biotypes. Most of the in vivo-specific proteins (with apparent molecular masses of ~200, ~150, ~140, 92, 68, 62, 43, and 29 kilodaltons) were not induced during cultivation of bacteria in iron-depleted medium and are probably not related to the iron-regulated env proteins known to be involved in iron transport systems.**

Knowledge about virulence factors and protective antigens expressed by microorganisms during the infectious process is of central importance for an understanding in molecular terms of pathogenicity and immunity in infectious diseases. It is well known that bacteria can alter their metabolism rapidly in response to environmental changes and that they may exist in a variety of physiological states that can be quite different from one another. Environmental factors such as various nutrients, ions, trace metals, and vitamins, as well as temperature, oxygen tension, growth stage, etc., have been found to influence the expression of bacterial virulence factors, including fimbrial and other surface antigens of importance for adherence and colonization (8, 15), factors of importance for penetration into or across host epithelial layers (15, 20), and various toxins (15, 22, 31), extracellular proteases (5, 24), etc. However, most investigations into bacterial virulence have been carried out with organisms grown in vitro under conditions that may differ substantially from the in vivo milieu. In general, little is known about the alterations that occur in pathogenic bacteria as they adapt to and multiply in the environment found in host tissues during infection.

In *Vibrio cholerae* O1 bacteria, the causative organism of Asiatic cholera, changes in growth conditions in vitro have been found to have marked influences on the production of several factors of known or suspected significance for pathogenicity: cholera toxin (7, 12, 26), various extracellular proteases (36), outer membrane proteins (14, 30), cell-bound hemagglutinins (4, 10), and a recently described pilus which seems to be of importance for colonization and to be coregulated with cholera toxin (34). We have been particularly interested in the possibility that *V. cholerae* O1 organisms may express novel antigens during an intestinal infection that differ from those found on in vitro-grown bacteria. Changes in cell envelope (env) antigens would be of greater potential significance for protective immunity (and vaccine development) than, e.g., changes in cytosolic or periplasmic proteins resulting from the adaptation to the intestinal environment. We have therefore specifically compared the env protein and antigen composition in *V. cholerae* O1 bacteria of classical and El Tor biotypes harvested directly from the intestines of rabbits with experimental cholera with the composition after growth in vitro during different conditions. Sciortino et al. (27) observed that *V. cholerae* O1, when grown in vivo in the intestines of infant rabbits, expressed novel outer membrane-associated proteins which, in part, were similar to those observed on cholera vibrios grown in vitro under conditions of iron deprivation; however, they did not examine whether any of the novel proteins were antigenic.

MATERIALS AND METHODS

Bacterial strains and in vitro growth conditions. Six *V. cholerae* strains were used. Two of these strains were examined in greater detail: the El Tor strain T19479 (Inaba) and the classical strain Cairo 48 (Inaba) isolated from cholera patients in Bangladesh (1979) and Egypt (1949), respectively. The other four strains were X28214 (classical, Inaba), 34 (classical, Ogawa), Phil 6973 (El Tor, Inaba), and X25049 (El Tor, Ogawa). The bacteria were stored at −70°C in broth containing 20% glycerol and grown in flasks to mid-logarithmic phase at 37°C with shaking in liquid medium by using an early-logarithmic-phase preculture prepared under the same conditions as the inoculum. Three media were compared: Trypticase soy broth (TSB) without glucose (BBL Microbiology Systems, Cockeysville, Md.), LB medium (17), and Syncase (2). Some cultures were also incubated for longer times with or without shaking. Syncase without any added $FeCl_3$ and supplemented with 0.5 mg of iron-binding protein human transferrin (Sigma Chemical Co., St. Louis, Mo.) per ml was used as iron-depleted medium.

In vivo growth of *V. cholerae*. Bacteria were grown by shake-culture to mid-logarithmic phase in TSB without glucose. Washed bacteria, diluted in phosphate-buffered saline

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(PBS; 10 mM sodium phosphate, 0.15 M NaCl [pH 7.2]) to give 10^6 organisms, on the basis of measurements of optical density, were then injected into ligated intestinal segments of New Zealand White rabbits weighing less than 2 kg (33). Similarly prepared bacteria (10^5 organisms of strain T19479 and 10^8 organisms of strain Cairo 48) were also used to infect rabbits by using the RITARD technique (16). The animals were sacrificed after 16 to 20 h, and fluid from diarrhea-positive loops or, in the case of RITARD animals, from the nonligated whole small intestine was collected and immediately chilled on ice. Presence of pure cultures of *V. cholerae* was confirmed by streaking a small volume of the diarrheal fluid specimens on blood agar plates followed by incubation overnight at 37°C. In vivo bacteria were prepared without delay from the ice-cold gut fluid. The fluid was first centrifuged at 150 × *g* for 5 min to remove epithelial cell aggregates, etc. Bacteria were thereafter pelleted from the supernatant by centrifugation at 6,000 × *g* for 10 min, washed twice in PBS (all procedures were performed at 4°C), and then stored at −70°C until use.

Cell envelope preparations. Three different methods for preparing env from *V. cholerae* bacteria were used.

(i) env prepared by high-speed centrifugation (H-env). Bacteria which had been stored at −70°C in PBS at a cell density of ~5 × 10^{10} bacteria per ml were thawed and repeatedly ultransonicated in volumes of 4 ml at 22 kilocycles/s until all cells were broken (six cycles of 30 s each). After centrifugation at 3,300 × *g* for 30 min, membranes remaining in the supernatant were pelleted by ultracentrifugation at 125,000 × *g* for 2 h. After being washed in 10 mM Tris hydrochloride (pH 8.0) and after an identical round of ultracentrifugation, the membranes were dispersed by a syringe to a concentration corresponding to 2 × 10^{11} bacteria per ml. The lipopolysaccharide (LPS) content in this kind of env preparation from in vitro-grown organisms was ~1.7 mg/ml as measured by LPS inhibition-enzyme-linked immunosorbent assay (ELISA) described below. In vivo env were dispersed to give the same LPS content as the in vitro env of the corresponding strain.

(ii) env prepared by low-speed centrifugation (L-env). L-env were prepared essentially as H-env were; however, the bacteria were suspended in TM buffer (10 mM Tris, 5 mM $MgCl_2$ [pH 7.5]) before ultransonication. After centrifugation at 10,000 × *g* for 1 min in a microcentrifuge (Eppendorf Gerätebau, Hamburg, Federal Republic of Germany), env from the supernatant were pelleted by centrifugation at 10,000 × *g* for 10 min and then, after washing, dispersed in TM buffer at a concentration corresponding to ~10^{11} bacteria per ml.

(iii) Lithium chloride-lithium acetate-extracted env (Li-env). The modification of the lithium chloride-lithium acetate extraction method of Johnston et al. (9) was used. Bacteria were suspended in a buffer containing 0.2 M lithium chloride and 0.1 M lithium acetate (pH 6.0). Membrane vesicles were generated by shaking the cell suspension at 45°C for 2 h in a flask containing a layer of 3-mm glass beads. After differential centrifugation at 12,000 and 25,000 × *g* to remove whole cells and debris, the vesicles were pelleted and washed in Tris buffer (10 mM Tris, 0.1 M NaCl [pH 8.0]) by repeated centrifugation at 105,000 × *g* for 2 h. Li-env obtained from ca. 5 × 10^{12} organisms dispersed in 5 ml of Tris buffer contained ~0.13 mg of protein per ml and ~1 mg of LPS per ml.

LPS. Purified *V. cholerae* LPS was prepared from strain 569B (Inaba) by hot phenol-water extraction followed by repeated ultracentrifugation (23). The preparation was further treated in sequence with DNase, RNase, and protease (Sigma), each enzyme being added at 0.01 mg/mg of LPS. The LPS preparation was finally boiled for 10 min and ultracentrifuged twice (105,000 × *g* for 2 h per cycle). Absence of RNA A_{260} was confirmed spectrophotometrically, and the protein content was less than 0.5% as determined by the method of Bradford (1).

Radioactive labeling of bacterial surface proteins. Proteins exposed on the cell surface of *V. cholerae* bacteria of strain T19479 were radiolabeled with ^{125}I by using Iodo-Gen (Pierce Chemical Co., Rockford, Ill.) as a catalyst essentially as previously described (32). Bacteria were grown to mid-exponential phase, and after washing, cells (10^{10} bacteria in 0.5 ml of PBS) were reacted for 60 s with 250 μCi of ^{125}I in a Iodo-Gen-coated test tube and thereafter extensively washed.

Antisera against in vivo and in vitro organisms. New Zealand White rabbits were injected three times subcutaneously with ~5 × 10^9 live bacteria per dose at 2-week intervals, the first two injections being given together with Freund complete adjuvant. The in vivo bacteria used for immunization were derived from RITARD-infected rabbits, and the in vitro bacteria were grown to mid-logarithmic phase in TSB without glucose. The antisera used were prepared from bleedings taken 2 weeks after the final injection.

Absorption of anti-in vivo *V. cholerae* antiserum. The antiserum against in vivo-grown bacteria of strain T19479 was also used after extensive absorption, twice with boiled and twice with live in vitro-grown log-phase bacteria of the homologous strain. For comparisons, the antiserum was also absorbed with in vivo bacteria.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 13.5% acrylamide slab gels essentially as described by Laemmli (13) with the modification that bisacrylamide as a cross-linker was replaced by *N,N'*-dialyltartardiamide at the corresponding molarity. Pyronin Y (Sigma) was used as a marker of the electrophoretic front. All other reagents, including molecular weight reference protein kits, were purchased from Bio-Rad Laboratories (Richmond, Calif.). Proteins were stained with Coomassie brilliant blue R 250.

Immunoblotting. Immunoblot analysis of env antigens separated in SDS–13.5% PAGE gels was performed by the method of Towbin et al. (35). Nitrocellulose strips with transblotted env antigens and front marker were blocked with 1% bovine serum albumin in PBS at room temperature for 30 min and thereafter incubated for 4 h with rabbit anti-*V. cholerae* antiserum (see above) and subsequently overnight with peroxidase-labeled goat anti-rabbit immunoglobulin G (Jackson Immuno Research Laboratories, West Grove, Pa.). An orbital shaker was used during incubations and washings. Strips were washed extensively with three changes of PBS–0.05% Tween and a final rinsing with PBS without Tween before the development step, which included H_2O_2 and 4-chloro-1-naphthol (Bio-Rad). Separate strips of transblotted proteins not exposed to the subsequent immunoblotting incubations were also stained with amido black (E. Merck AG, Darmstadt, Federal Republic of Germany).

Autoradiography. Autoradiography was done on SDS-PAGE-separated cell env and whole cell sonicate of ^{125}I-labeled bacteria transferred to nitrocellulose sheets by exposure to X-Omat XS film (Eastman Kodak Co., Rochester, N.Y.), with an intensifying screen, at −70°C for 24 h.

Serological methods. Vibriocidal antibodies to *V. cholerae* T19479 were determined by a microdilution assay as previ-

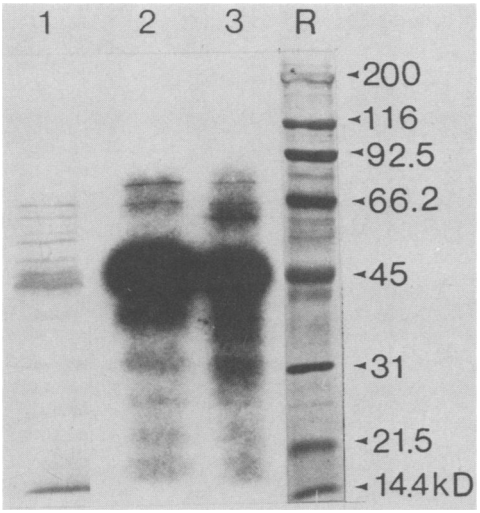

FIG. 1. Comparison by SDS-PAGE analysis of cell env preparations from unlabeled and ^{125}I-labeled intact cells of *V. cholerae* T19479. Lanes: 1, unlabeled env; 2, env from ^{125}I-labeled whole cells (80,000 cpm); 3, sonicate of ^{125}I-labeled whole cells (50,000 cpm); R, molecular mass markers (in kilodaltons [kD]): myosin, 200; β-galactosidase, 116; phosphorylase B, 92.5; bovine serum albumin, 66.2; ovalbumin, 45.0; carbonic anhydrase, 31.0; soybean trypsin inhibitor, 21.5; lysozyme, 14.4. Proteins were transblotted onto nitrocellulose before protein staining with amido black (lanes 1 and R) or autoradiography (lanes 2 and 3).

ously described (21). Antibody titers against LPS were tested by ELISA (6). The LPS content in env preparations was determined by an LPS inhibition ELISA (6) using a monoclonal anti-LPS antibody and purified LPS from 569B (classical Inaba) as a reference.

RESULTS

Comparison of env protein profile after in vitro growth and intestinal infection. We initially compared different methods for preparing *V. cholerae* env for subsequent protein analyses by SDS-PAGE: ultrasonication followed by high-speed centrifugation (for H-env) (method 1), ultrasonication with low-speed centrifugation (for L-env) (method 2), and lithium chloride-lithium acetate extraction (for Li-env) (method 3). Our aim was to establish a procedure which combined simplicity with good yields and retention of the maximum number of proteins detectable with any of the tested preparation methods. The results (data not shown) revealed that methods 1 and 2 gave higher yields of env than method 3 and also provided a richer spectrum of protein bands, including the 56- and 58-kilodalton (kDa) outer membrane proteins, which are known to be lost by several methods used for outer membrane preparation (25, 27). The protein spectra obtained with methods 1 and 2 were closely comparable and did not miss any of the proteins obtained with the lithium chloride-lithium acetate extraction method. Furthermore, env prepared by method 1 from ^{125}I-labeled intact whole cells contained all but one of the labeled surface proteins present in whole bacterial cells (Fig. 1, cf. lane 2 and lane 3); the missing protein was released in the buffer during env preparation (not shown). When not specified otherwise, method 1 was therefore used to prepare outer membrane containing cell env.

In the preliminary stage of the investigations, we also examined different culture media and growth conditions for their ability to sustain expression of env proteins by *V. cholerae* T19479 and Cairo 48. We found (data not shown) that growth (for ~3 to 5 h) to mid-logarithmic phase in TSB without glucose at 37°C with shaking provided the full set of protein bands that could be obtained with other media (LB or Syncase) or growth conditions (no shaking, extended incubation times).

We then compared the env protein profiles of optimally in vitro-grown T19479 (El Tor) and Cairo 48 (classical) organisms with those of bacteria of the same strains taken from ligated intestinal segments of rabbits infected 16 to 20 h earlier. The intestinal loops were infected by the injection of ~1×10^6 organisms, which at the time of harvest had multiplied to between 4×10^{10} and 10×10^{10} organisms and had induced the accumulation of 0.7 to 1.3 ml of diarrheal fluid per cm of intestinal segment. SDS-PAGE analysis of T19479 and Cairo 48 organisms, using env preparations from the same number of organisms (as based on LPS content) after in vitro culture or intestinal infection, respectively, showed marked differences in the protein profile (Fig. 2). In both the El Tor (T19479) and classical (Cairo 48) strains examined, the in vivo-grown bacteria contained fewer protein bands than the organisms of the same strain grown in vitro. Several of the more prominent in vitro env proteins, with estimated molecular masses of ~160, 56 (2 bands), 46, 42, 36, 30, and 22 kDa, had disappeared or become much weaker. At the same time, a number of novel, infection-induced proteins were evident in the env preparations from the in vivo-grown organisms. These novel bands represented proteins with estimated molecular masses of ~200, ~150, ~140, 92 (only Cairo 48), 68 (only T19479), 62, 43, and 29 kDa (Fig. 2).

These changes in env protein profiles between in vitro-grown and infection-derived in vivo organisms were highly reproducible also in the four other El Tor and classical strains examined (not shown). Furthermore, other experiments (not shown) demonstrated that the in vivo-induced changes in the env protein profile observed in bacteria isolated from infected ligated intestinal segments were also present in bacteria that were harvested from the nonligated small intestines of rabbits infected with the same organisms by the RITARD technique. The only appreciable differences between the ligated loop and RITARD bacteria were that the in vivo-specific 43-kDa proteins in both T19479 and Cairo 48 were more prominent in the RITARD-derived than in the ligated loop-derived organisms.

Comparison between infection-induced and iron-regulated env proteins. Iron has been described to be an important regulator of several outer membrane proteins in gram-negative organisms (3), including *V. cholerae* (27, 30), and the relative lack of iron in the intestinal milieu might be responsible for infection-induced changes in the env protein profile of cholera organisms (27). We therefore examined which of the novel, infection-induced env proteins observed in both El Tor and classical *V. cholerae* organisms could be induced in vitro by growing the organisms in iron-depleted medium. We compared the env protein profiles of infection-derived bacteria with those from bacteria grown in media with markedly different iron contents. As evident from SDS-PAGE analyses, bacteria grown in low-iron medium showed appreciable changes in their env proteins (Fig. 3, lanes 4 and 5) compared with bacteria grown in iron-rich medium (Fig. 3, lanes 2 and 3). There was a loss or decrease of a few protein bands as well as the appearance of at least three novel or strongly enhanced bands with migration rates corresponding to molecular masses of 72 to 76 kDa. However, most of the

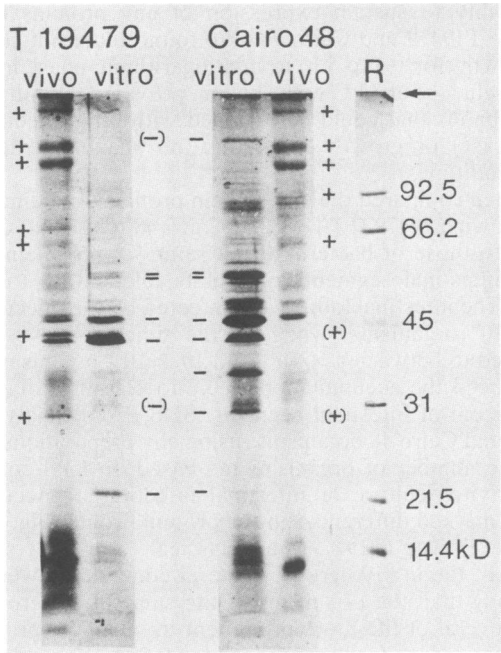

FIG. 2. Comparison by SDS-PAGE of the protein profiles of env from *V. cholerae* T19479 (El Tor, Inaba) and Cairo 48 (classical, Inaba) derived from infected rabbit intestinal "cholera loops" (vivo) or from cultures to mid-logarithmic phase in TSB without glucose (vitro). −, Protein bands that were absent or weaker in vivo than in vitro; +, novel, infection-induced protein bands (only consistently changed bands are indicated). Weak but consistently changed proteins are indicated in parentheses. Molecular mass markers used in lane R were as described for Fig. 1 (except for the 200- and 116-kDa markers, not used in this figure).

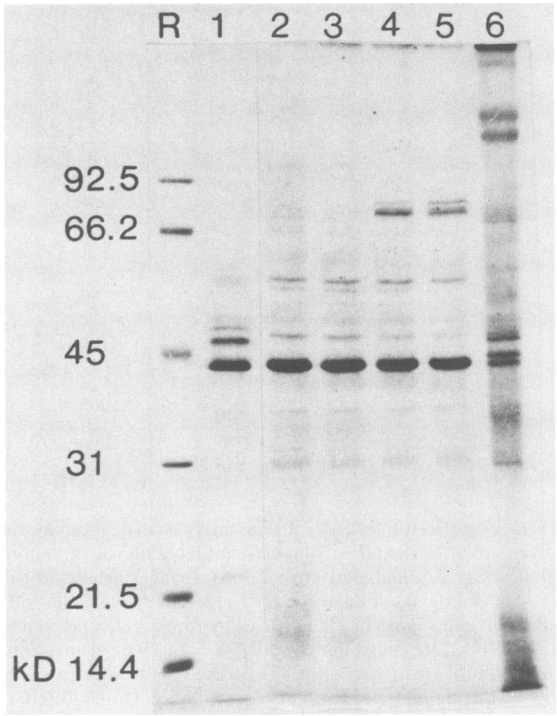

FIG. 3. SDS-PAGE analysis of env proteins from *V. cholerae* (T19479, El Tor, Inaba), comparing in vivo-induced proteins with env proteins from bacteria grown in media with different iron content. Lanes: R, molecular mass markers (as described for Fig. 2); 1, env proteins from bacteria grown in TSB without glucose; 2, Syncase (~18 μM $FeCl_3$); 3, Syncase (~3 μM $FeCl_3$); 4, Syncase (no $FeCl_3$ added); 5, same as lane 4 but with addition of human transferrin to culture medium at 0.5 mg/ml; 6, in vivo bacteria derived from rabbit intestinal loops.

infection-induced env proteins of *V. cholerae* were clearly distinct from these iron-regulated proteins (Fig. 3, cf. lanes 5 and 6). Thus, except for the possible identity of one of the main proteins induced by iron depletion with one of the infection-induced proteins in T19479 (72 kDa), we conclude that many infection-induced proteins in *V. cholerae* were not reproduced by growing the organisms in iron-depleted media in vitro.

Immunogenicity and cell surface location of infection-induced env proteins. We tested the hypothesis that the infection-induced novel env proteins included antigens against which an antibody response could be raised. We therefore immunized rabbits with washed *V. cholerae* T19479 or Cairo 48 organisms taken from nonligated rabbit intestine 16 to 20 h after infection; for comparisons, other rabbits were immunized with bacteria of the same strains after in vitro culture. The immune sera against in vivo- and in vitro-grown *V. cholerae* organisms were then reacted with env preparations from in vivo- and in vitro-grown bacteria, respectively, of the homologous strain by immunoblot technique, thus allowing identification of the antigen bands stained with either immune serum (Fig. 4). Although the anti-in vitro and anti-in vivo antisera had comparable vibriocidal and ELISA anti-LPS antibody titers (data not shown), the antiserum raised against in vitro-grown organisms detected many more env antigens in the bacteria grown in vitro than in those harvested from the infected rabbit intestine (Fig. 4, panels A, cf. lanes 1 and 2). Conversely, the antisera raised against the infection-derived organisms stained several env bands in the in vivo-grown bacteria which were missing in the organisms grown in vitro, and this difference was particularly prominent for higher-molecular-mass proteins (>60 kDa) (Fig. 4, panels B, cf. lanes 2 and 1).

Attempts were also made to separate antibodies directed against in vivo-specific env antigens from those cross-reacting with antigens present in the in vitro-grown bacteria by absorbing the anti-in vivo *V. cholerae* antisera extensively with bacteria grown in vitro. The resulting in vivo-specific antisera were then tested in immunoblotting analysis against env preparations from in vitro- and in vivo-grown organisms. As can be seen in Fig. 4, panels C, the absorption resulted in loss of the reactivity with many but not all bands in the in vitro-grown bacteria; the few remaining stained bands probably represent antigens or antigen epitopes not well exposed on the surface of the organisms that were used for absorption. However, of greater importance, the absorbed in vivo-specific antisera reacted with many additional proteins in the env preparations from in vivo-grown organisms. Thus, all the env proteins that were visible in in vivo-grown but not in in vitro-grown bacteria by amido black staining were stained with the in vivo-specific absorbed antiserum (Fig. 4, Protein panels). At least 8 apparently in vivo-specific env proteins with molecular masses of 62 to ca. 200 kDa were stained with the absorbed antiserum both in the two model strains depicted in Fig. 4 and in the four additional *V. cholerae* O1 strains examined (data not shown). Further absorption with washed in vivo-grown bacteria, on the other hand, specifically removed the antibodies reacting with these antigens (Fig. 5). We therefore conclude that most of the

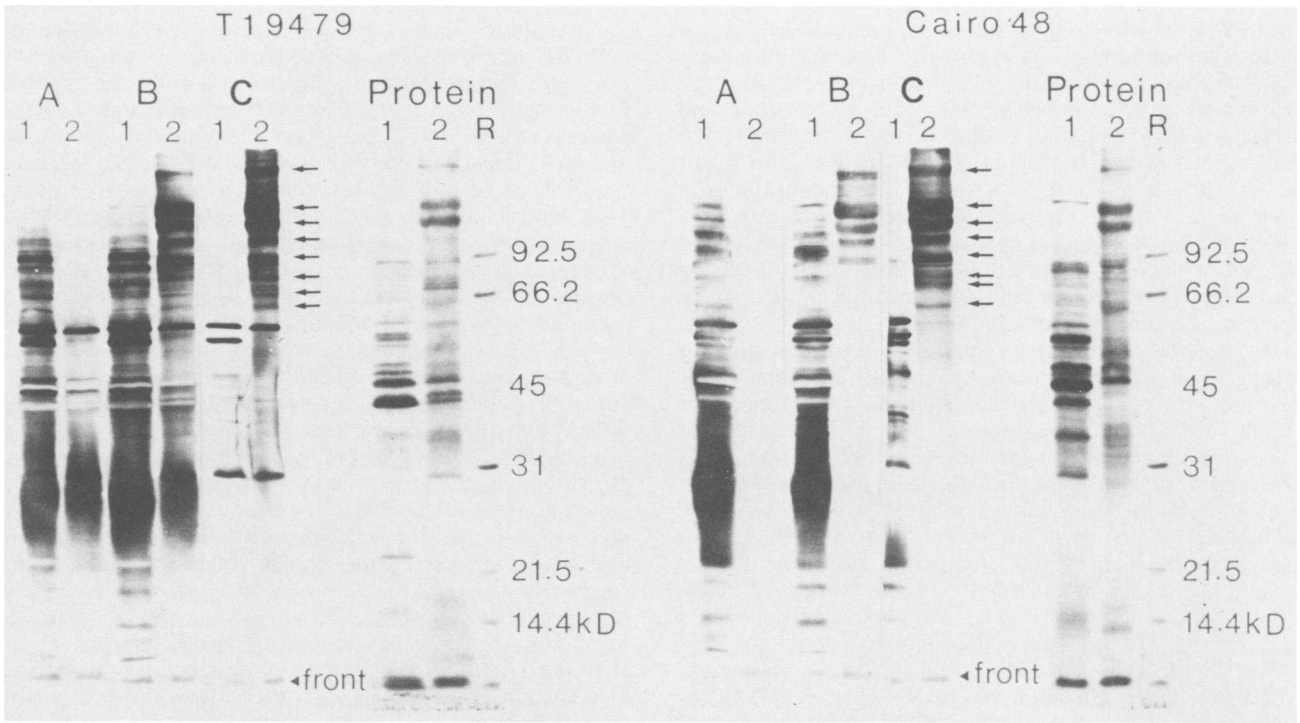

FIG. 4. Immunoblot analysis of env antigens from *V. cholerae* T19479 (El Tor, Inaba) and Cairo 48 (classical, Inaba) comparing the env antigen composition in infection-derived in vivo bacteria (lanes 2) with that in in vitro-grown organisms (lanes 1). The separated, transblotted proteins were developed with different antisera: Panels: A, rabbit anti-in vitro T19479; B, rabbit anti-in vivo T19479; C, rabbit anti-in vivo T19479 absorbed with in vitro-grown T19479 bacteria; R, transblotted env proteins and molecular mass markers (as described for Fig. 2) stained with amido black. In vivo env were prepared from rabbit intestinal loops, and in vitro env were prepared from bacteria grown in TSB without glucose. All env except from Cairo 48 (lanes 2; L-env) were prepared as H-env. ←, In vivo-specific immunogenic antigens.

infection-induced novel env proteins are indeed both immunogenic and exposed on the bacterial cell surface.

DISCUSSION

Cell env proteins of *V. cholerae* O1 bacteria probably play important, but as yet incompletely defined, roles in cholera infection and immunity. It was the aim of this study to determine possible changes in the composition of the env proteins of cholera vibrios during an intestinal infection resulting in experimental cholera in rabbits compared with the composition in bacteria after in vitro growth in various artificial media. We especially wished to examine the hypothesis that the intestinal in vivo conditions resulted in the expression of novel cell surface antigens.

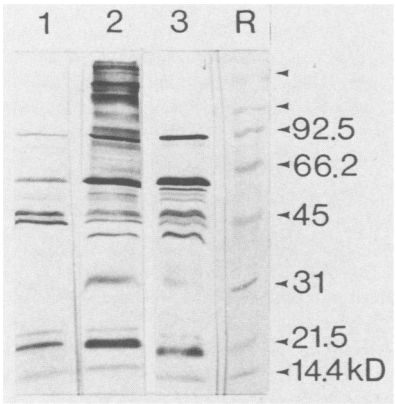

FIG. 5. Immunoblot analyses indicating the cell surface location of in vivo env antigens as studied with T19479. Lanes: 1, in vivo env developed with rabbit anti-in vivo T19479 absorbed with in vivo T19479 (after preabsorption with in vitro-grown bacteria); 2, in vivo env developed with rabbit anti-in vivo T19479 absorbed with in vitro T19479; lane 3, in vitro env developed with rabbit anti-in vivo T19479 absorbed with in vitro T19479; lane R, molecular mass markers as described for Fig. 1, stained with amido black.

Our findings that growth of different strains of both classical and El Tor *V. cholerae* O1 bacteria in the intestines of rabbits resulted in the disappearance of some env proteins and, at the same time, in the appearance of or significant increase in others indicate that the intestinal in vivo milieu is of importance for the env composition. The function of the novel infection-induced proteins and the specific factors in vivo that induce their expression are not known. The demonstration that these proteins were immunogenic in rabbits excludes the possibility that these proteins might be of host rather than bacterial origin. With possibly one exception (a 72-kDa protein) the in vivo-induced proteins observed are not the same as the env proteins induced by iron depletion as indicated by our experiments, although two additional in vivo proteins had molecular masses (62 and ~200 kDa) similar to those of iron-regulated outer membrane proteins described by Sigel and Payne (30). In this regard, our findings differ from those of Sciortino and Finkelstein (27). These investigators also observed novel outer membrane proteins from in vivo-grown compared with in vitro-grown *V. cholerae* bacteria, but they concluded that the outer membrane proteins from in vivo preparations of bacteria relatively closely resembled the outer membrane proteins

prepared from in vitro cultures of bacteria in iron-deficient media. A possible biological, rather than technical, explanation for these differences in regard to iron regulation might be that we harvested the in vivo-grown bacteria from the intestines of infected adult rabbits, whereas Sciortino and Finkelstein (27) prepared their organisms from infected infant rabbits. Possibly, the availability of iron on the gut mucosal surface and in the diarrheal fluid of adult rabbits is higher than in infant rabbits and sufficient to repress the high-efficiency iron-scavenging system(s) that might be needed for efficient colonization and multiplication in the infant rabbit intestine; baby rabbits drink milk containing the strong iron-binding protein lactoferrin.

The bacterial env were prepared by ultrasonication of bacteria followed by differential centrifugation steps, since this method gave a much better yield than the lithium chloride-lithium acetate extraction method also tested; this was an important feature when preparing env from in vivo-grown bacteria harvested directly from the intestines of infected rabbits. The apparent molecular weights of env proteins from *V. cholerae* O1 bacteria grown in vitro were mainly in agreement with outer membrane protein sizes reported previously by others (11, 19, 28). However, probably depending on slightly different gel running conditions and reference proteins used in different studies, the proteins estimated by us to have molecular masses of 30, 36, 42, and 46 kDa are referred to in some studies (14, 19, 34) as 25- (OMP V), 35-, 38- (OMP U), and 40- (OMP T) or 42-kDa proteins, respectively. Likewise, our 22-kDa protein probably corresponds to a 22-kDa minor outer membrane protein described by Manning et al. (18) but differs from the *toxR* gene-regulated 20.5-kDa pilus protein described by Taylor et al. (34). We were not able to detect any increase in expression of *toxR*-regulated proteins such as OmpU or TcpA (34) in the infection-derived bacteria. The in vivo-specific env proteins were not induced (unpublished data) by growing *V. cholerae* T19479 under in vitro conditions (pH 6.5 and at 30°C) described to maximize expression of *toxR*-regulated proteins (34), nor could we demonstrate TcpA on infection-derived T19479 bacteria (El Tor) by using specific anti-TcpA antiserum in immunoblot analyses (unpublished data). This could suggest that *toxR* was not an important regulatory factor for the many largely high-molecular-weight env proteins induced by the in vivo infection conditions.

Sears et al. (29), who examined the immune response of a large number of human volunteers after experimental infection with different strains of *V. cholerae* O1, observed antibody titer rises against env preparations from these strains grown in vitro in approximately 50% of the volunteers; in immunoblot tests, these postchallenge sera stained some eight env protein bands with apparent molecular masses of 22 to 38 kDa. In contrast, when our antisera raised against in vivo-grown organisms were tested against env preparations from in vivo-grown bacteria, a large number of different env proteins were stained, most of which had molecular masses ranging from 62 to 200 kDa. These proteins continued to be stained by using anti-in vivo antiserum which had been extensively absorbed with in vitro-grown bacteria of the same strain. We therefore conclude that during intestinal infection, *V. cholerae* O1 bacteria express a number of novel, mainly high-molecular-weight env protein antigens.

The significance, if any, of these novel antigens for protective antibacterial immunity in cholera (and for vaccine development) remains to be defined. In previous studies, when hyperimmune sera against live in vitro-grown *V. cholerae* O1 bacteria were tested, our results indicated that the protective capacity of the sera resided exclusively in the anti-LPS antibody fraction (33). However, we have recently observed that an initial intestinal infection-immunization of rabbits with rough *V. cholerae* O1 mutants confers significant protective anti-colonization immunity against subsequent challenge with fully virulent smooth cholera vibrios in the absence of any anti-O1 (anti-LPS) antibody response. This suggests a protection role for non-LPS antigens which may or may not be in vivo specific (A.-M. Svennerholm, C. Wennerås, and J. Holmgren, manuscript in preparation); our present data indicate that the in vivo-specific env antigens are indeed present on the bacterial cell surface. It also remains to be determined whether the in vivo-specific, infection-induced env antigens are expressed in sufficient amounts to elicit an immune response in the infected host; in the present study, the immunogenicity of these proteins was examined by repeated parenteral immunization with in vivo-grown organisms. However, our studies in progress examining the immunoblot reactivity of env preparations from in vivo-grown organisms with postinfection immune sera from rabbits with experimental cholera (the RITARD model) suggest that several of the novel, in vivo-specific env proteins can indeed give rise to an antibody response in the infected host also. This notion is confirmed by immunoblot analyses demonstrating antibodies staining several of the in vivo-specific env proteins in convalescent-phase sera from cholera patients (G. Jonson, A.-M. Svennerholm, and J. Holmgren, manuscript in preparation).

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