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Cholera Toxin Modulates the Systemic Immune Responses against *Vibrio cholerae* Surface Antigens after Repeated Inoculations

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***Abstract*: The immunomodulating properties of a low cholera toxin (CT) dose over the systemic antibody response against *Vibrio cholerae* antigens after a comparatively extensive period of time were evaluated. Groups of 10 mice were injected intraperitoneally three times at 0, 30 and 86 days with 500 μl of buffer or 10^8 viable recombinant *V. cholerae* bacteria (lacking cholera toxin A subunit) with or without 100 ng of CT. Sera were obtained from inoculated mice at 0, 14, 28, 37, 58, 80, 93, 114, 236 and 356 days after the first injection. Vibriocidal activity and IgM and IgG anti-lipopolysaccharide (LPS) or outer membrane protein (OMP) antibodies levels were estimated by ELISA in sera of inoculated mice. Anti-LPS IgG subclasses were measured 2 weeks after each immunization by ELISA. Treatment of mice with CT markedly influenced the immune response to LPS but not against OMP of *V. cholerae*. Simultaneous intraperitoneal administration of CT with *V. cholerae* resulted in marked enhancement of both IgM anti-LPS and vibriocidal titers which subsisted for a relatively extensive period of time after repeated antigen administration. No differences were observed in IgM and IgG anti-OMP titers after extended periods of time between CT and control treatments. A similar pattern of IgG anti-LPS subclasses was observed in the serum samples analyzed. These results suggest that long term CT administration modulates the IgM anti-*V. cholerae* LPS response and the serum vibriocidal activity.**

***Key words*: Cholera toxin, Immunomodulation, LPS**

Cholera is considered a non-invasive disease because the causative agent *Vibrio cholerae* is confined to the lumen of the intestine (5, 10, 16, 17, 26, 31). Though some components of intestinal mucosa immunity could be expected to predict resistance to cholera, the most important immune moiety that has been correlated with protection to the disease is the serum vibriocidal activity which reliably predicts resistance to cholera (1, 3, 9, 10, 16, 23–25).

Investigation of cholera outbreaks in non-endemic settings and challenge studies in individuals with no previous exposure to cholera vaccine or infection have demonstrated increases in serum titers of both vibriocidal and anti-cholera toxin antibodies in most infected persons (1, 3, 9, 10, 23, 24, 29). It was proposed that serum vibriocidal antibodies are not only a marker of resistance to disease but confer immunity to cholera by lysing the *V. cholerae* on the epithelial surface of the small intestine (8, 12, 26).

These vibrocidal antibodies are directed against surface bacteria antigens. The outer membrane of *V. cholerae*, like other gram-negative bacteria, is composed of both lipopolysaccharide (LPS) and proteins. LPS is a unique antigen in that most of the specific antibody response is directed against the O-antigen polysaccharide determinants of the molecule, whereas the lipid A moiety can elicit a wide variety of non-specific immunological effects (22). Also, LPS is considered a helper-T-independent antigen because of its ability to induce a good antibody response in *nu/nu* mice (22).

The cholera toxin (CT) produced by *V. cholerae* is the virulence factor responsible for the massive secretory diarrhea seen in cholera disease. Additionally, CT is

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Abbreviations: BHI, Brain Heart Infusion; CT, cholera toxin; IG, intragastric; IP, intraperitoneal; LPS, lipopolysaccharide; 2-Me, 2-mercaptoethanol; OMP, outer membrane proteins; PBS, phosphate buffer saline; SC, subcutaneous.

a potent immunomodulator since mucosal stimulation by CT generates a systemic response to unrelated protein antigen when both are administered into the intestine at the same time (7) and administered intranasally, transcutaneously or parenterally, CT induces strong antibody responses against both it and co-administered proteins (11, 21, 28). An immunological attribute of CT is the adjuvant potential for both T cell-independent (TI) and T cell-dependent (TD) responses (30). CT is most potent as immune modulator when the A-B subunits are present and functional. However, the strong toxic effects of CT preclude its wider clinical use in most vaccination protocols (18) and most live vaccine strains of *V. cholerae* lack at least the catalytic A subunit responsible for the toxic effects of CT. Therefore an important immunological difference subsists between attenuated strains and wild type strains secreting functional toxin.

Since LPS and CT have strong modulator activities over the humoral immune response and both are involved in the development of the disease, it is feasible that secreted CT during repeated vibrio infection alters the typical humoral response against bacterial surface antigens. In order to examine the immunomodulating properties of CT over the systemic antibody response against *V. cholerae* antigens after relatively extensive period of time, the effects of the administration of a low dose of CT on sera vibriocidal activity and antibodies responses to LPS and outer membrane proteins (OMP) were studied. These antigens were selected because are relevant outer non-soluble antigens of *V. cholerae* which have been associated with sera vibriocidal activity.

Materials and Methods

Antigen. LPS was prepared from a commercial vaccine strain which is a recombinant *V. cholerae* classical biotype Inaba strain lacking the A subunit (Orochol, Berna) that also was used as immunogen and for vibriocidal measures. The phenol–water extraction method of Westphal and Jann (32) was followed throughout. The single extracted LPS was reextracted two more times until no protein could be detected by the method of Lowry et al. (20). OMP were extracted following the protocol of Johnston et al. (14). CT was obtained from Sigma Co. (St. Louis, Mo., U.S.A.).

Immunization of mice. Six-week-old NIH mice were used. The animals were housed in a temperature and light cycle controlled room and allowed unrestricted access to food and water. After the end of the experiments, mice were euthanized with CO_2. Experiments with animals were done according to the ethical principles formulated in the Declaration of Helsinki. In a first experimental approach, groups of 5 mice were inoculated intragastrically with vehicle solution (IG w/o), with 10^8 viable bacteria (IG), or with 10^8 viable bacteria plus 100 ng of CT (IG+CT). Also, groups of 5 mice were inoculated intraperitoneally (IP) or subcutaneously (SC) with 10^8 viable bacteria and IP with 10^8 viable bacteria plus 100 ng of CT (IP+CT). In all cases the volume inoculated was 500 µl and the vehicle solution was sterile saline solution. Blood samples were obtained from the tail vein at 0, 7, 14, 21 and 28 days after the immunization and 2 days after a second IP inoculation with 10^8 viable bacteria. Sera was stored at −20 C until use. In a second trial, 10 mice per group were injected SC or IP three times at 0, 30 and 86 days with 500 µl of immunogen containing 10^8 viable bacteria alone or plus cholera toxin. A control group also received three IP injections of 500 µl of saline on the same dates. Mice were exsanguinated at 0, 14, 28, 37, 58, 80, 93, 114, 236 and 356 days after the first injection. Sera was separated by centrifugation at $10{,}000\times g$ and stored at −20 C.

Enzyme-linked immunosorbent assay (ELISA). Flat-bottom 96-well microtiter plates (Nunc-Immunolon) were coated with OMP (10 µg/ml) or LPS (10 µg/ml) diluted in phosphate buffer saline (PBS) and kept overnight at 4 C. After six washings with 0.15 M PBS–Tween 0.05%, plates were blocked with 1% skim milk in PBS for 3 hr at room temperature. The plates were washed and serial twofold dilutions of sera in 1% skim milk in PBS–Tween 0.05% were added. Murine hyperimmune anti *V. cholerae* serum was used as the reference serum for both anti-LPS and anti-OMP IgG and IgM antibodies. Pooled non-immunized mice sera were used as negative control. Reference and negative serum and samples were assayed in duplicates. Plates were incubated for 2 hr at 37 C and washed, and the horseradish peroxidase-labeled antibody specific to mouse class or subclass IgG or IgM was added. After 1 hr at 37 C, the plates were washed, and the 4-nitrophenylphosphate substrate (1 mg/ml in 1 M Tris-HCl–3 mM $MgCl_2$ [pH 9.8]) was added. A_{492} was measured by using a Multiskan Labsystem plate reader. End-point titers were expressed as the reciprocal log2 of the last dilution that had an optical density at 492 nm of >0.1 OD unit above the value of negative control sera. IgG sublclass titers were expressed as absorbance values.

Vibriocidal assay. Bacteria for vibriocidal assay of sera were prepared transferring a single colony from Brain Heart Infusion (BHI) plate into 10 ml of BHI broth and incubating them for 2 to 3 hr at 37 C with shaking at 180 rpm. Then, 100 µl of this inoculum were transferred to 10 ml of BHI and incubated with

shaking (180 rpm) at 37 C until the culture reached approximately 10^7 cells/ml. The bacterial suspension was diluted 10^5-fold in PBS buffer. Vibriocidal assay was performed in sterile nonpyrogenic 96-well microplates (Costar, Corning, N.Y., U.S.A.). Threefold serum dilutions were mixed with equal volumes of 10^3 cells/ml in diluted guinea pig serum and incubated at 37 C for 1 hr with shaking. Plates were incubated for 4 hr at 37 C, and the bacterial growth was estimated by absorbance at 620 nm. The titer was expressed as the reciprocal of the highest serum dilution that yielded 50% reduction in the number of colonies compared to the control (complement only).

Absorption of vibriocidal activity. It was assayed by mixing equal volumes of 200 mg of LPS per ml with equal volumes of dilutions of antisera at 37 C for 1 hr prior to addition of the bacteria as described above. The inactivation of IgM was done by mixing equal volumes of sera and 2-mercaptoethanol (2-Me) 0.2 M and incubating them at 4 C overnight.

Statistics. Comparisons of the geometric means were performed with the two-sided Student *t* test or Wilcoxon analysis.

Results

Oral vs. Systemic Antigen Administration

The oral route (IG) and two systemic routes for *V. cholerae* immunization of mice were evaluated. Four weeks after inoculation, a booster dose of antigen was administered IP and antibodies responses in sera were measured. Figures 1 A and B show the IgM and IgG anti-LPS responses. IP and SC routes established secondary antibodies response but not IG.

Cholera Toxin Is a Potent Inductor of Systemic Bactericidal Antibodies

The intestine is the natural site of antigen encounter during vibrio infection but oral inoculation of mice with bacteria did not stimulate a significant response of circulating antibodies. In order to evaluate the effects of CT in vibriocidal activity after a long time, mice were administered IP with whole recombinant bacteria and CT. Vibriocidal titers were detected in sera of mice immunized against bacteria alone (Fig. 2) 14 days after the first inoculation and then fell quickly at 28 days. A second bacteria inoculation produced a fast and evident rise in titers at 7 days and then fell slowly until 50 days. A third inoculation induced a marked augment in vibriocidal activity, which endured almost 270 days. CT titers were statistically higher 7 days after the second inoculation and in all sampling times 7 days after the third inoculation (Fig. 2) showed an apparent tendency to improve serum activity after repeated antigen exposition. Sera titers of the control group injected only with saline solution did not show any increase on the dates sampled (not shown).

Cholera Toxin Induces a Higher IgM Anti-LPS Response

LPS and OMP specific antibody titers in the sera were determined by ELISA. Specific IgM and IgG antibodies titers in sera of mice immunized with recombinant vibrios alone or with CT were enhanced after each inoculation (Fig. 3). IgM anti-OMP mean titers were slightly higher in the CT group but were not statistically different between both treatments in any of the sampled dates except for 7 days after the second immunization (Fig. 3A). CT induced a statistically higher level of IgG anti-OMP only 28 days after the first immunization (Fig. 3B). LPS-specific IgM titers were statistically higher in mice immunized with bacteria plus CT when compared with mice treated with bacteria alone 50 days after the second immunization and this higher level remained until the end of the experiment (Fig. 3C). IgG anti-LPS titers in CT treatment were higher after the third antigen delivery (Fig. 3D).

Long Lasting Vibriocidal Activity Resides in IgM Anti-LPS

Vibriocidal antibodies appear to bind mostly to LPS antigen (Fig. 4A) since inhibition of activity by LPS adsorption was observed in treatment with or without CT. After the second bacteria inoculation, LPS failed to inhibit vibriocidal activity in sera from animals inoculated with bacteria+CT. Mercaptoethanol (2-Me) treatment, which destroys IgM antibodies, abolished completely the sera bactericidal activity 14 days after the first inoculation (Fig. 4B). Vibriocidal activity of sera from mice inoculated with bacteria alone 28 days after each inoculation was not sensitive to 2-Me treatment but sensitivity rose when time increased after inoculation. Sera from mice inoculated with bacteria plus CT showed no sensitivity to 2-Me after the second inoculation and the first week after the third. However, after the third inoculation sensitivity had a marked increase with time.

IgG Subtypes

IgG subtypes profiles are shown in Fig. 5. No differences were observed between CT and non-CT treatments 28 days after the first immunization (Fig. 5A) and 7 days after the third immunization (Fig. 5B). IgG3 was the predominant IgG subclass and IgG2a was the minor.

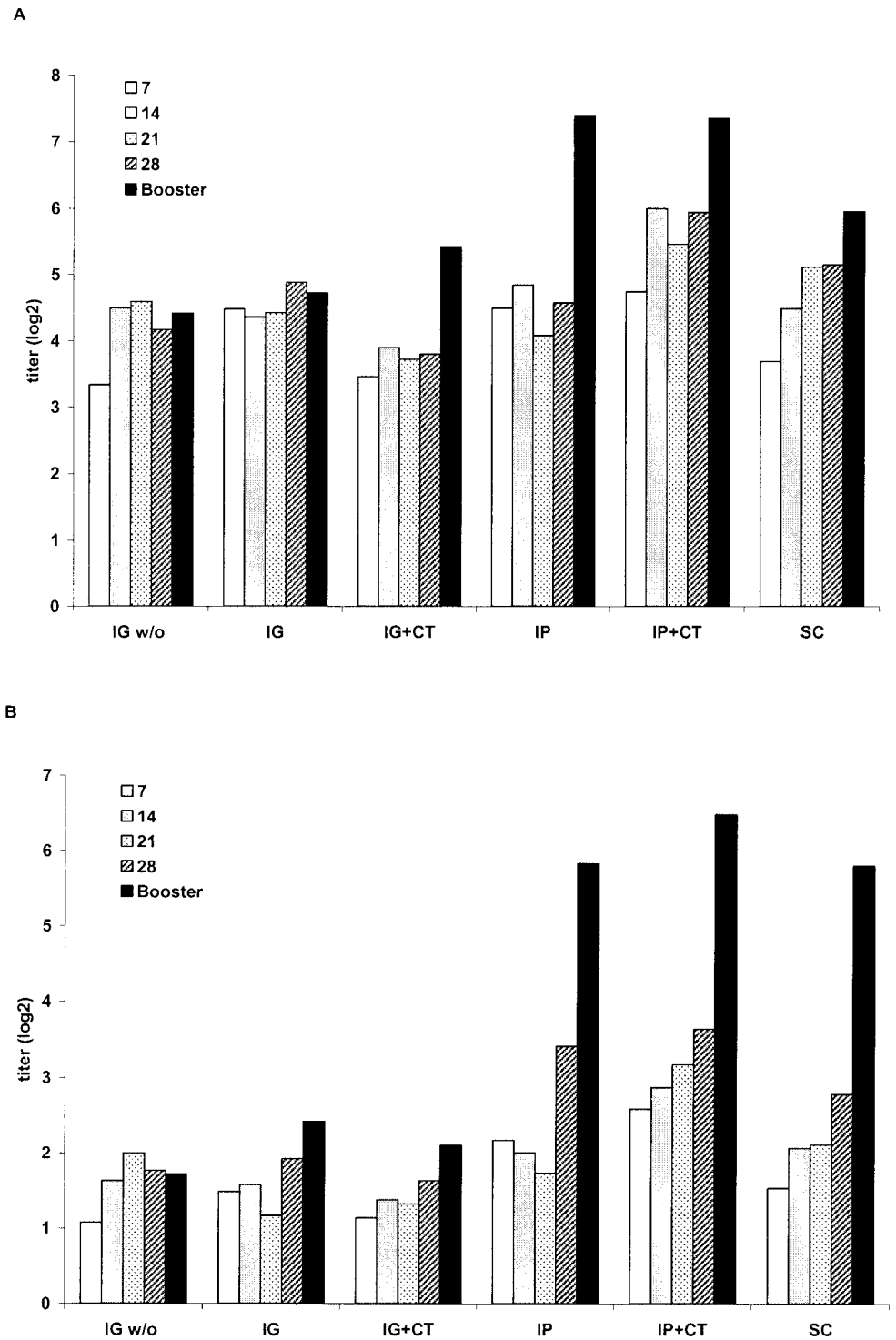

Fig. 1. Immunogenicity of recombinant *V. cholerae* in mice. Five mice were inoculated intragastrically with vehicle solution (IG w/o), with 10^8 viable bacteria (IG), or with 10^8 viable bacteria plus 100 ng of CT (IG+CT). Also, groups of 5 mice were inoculated intraperitoneally (IP) or subcutaneously (SC) with 10^8 viable bacteria and IP with 10^8 viable bacteria plus 100 ng of CT (IP+CT). Antibodies A) IgM and B) IgG against LPS were measured 0, 7, 14, 21 and 28 days after the immunization and 2 days after a second IP inoculation with 10^8 viable bacteria. These results are expressed as the mean.

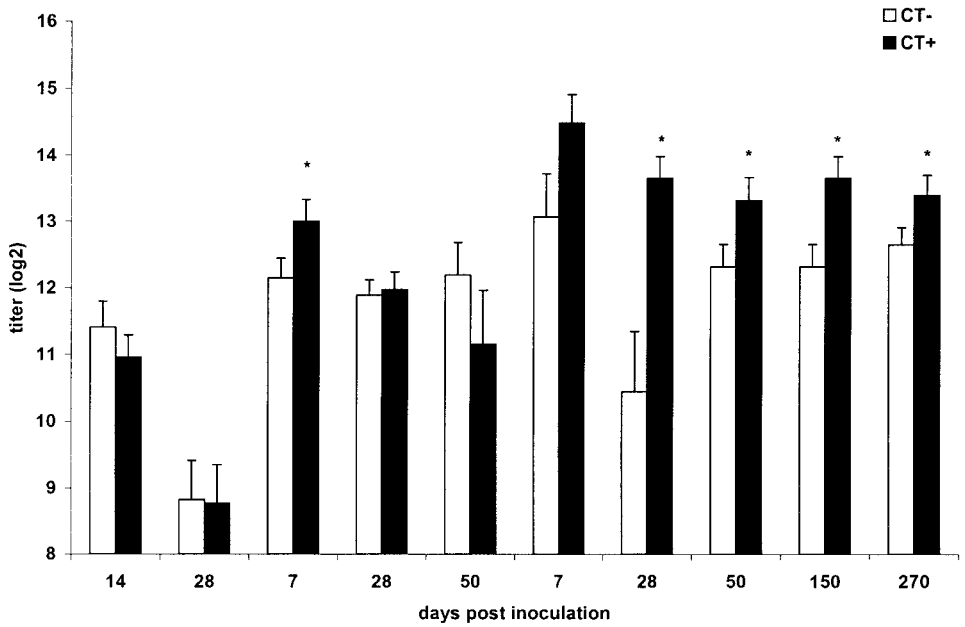

Fig. 2. The levels of vibriocidal antibodies in sera of mice during 50 weeks and three immunizations periods with 10^8 viable bacteria (IP) with or without CT. These results are expressed as the mean and standard error (10 mice per group; $*P<0.05$).

IP vs. SC Antigen Administration

Systemic antigen discharged by the IP and the SC route were compared. No statistically significant differences in IgM titers between both treatments were evident until the end of the experiment although SC inoculation produced lower titers of IgM (Fig. 6A). The IgG anti-LPS response remained close to initial levels after the first doses but IP treatment produced a significant increase of IgM titers (Fig. 6B).

Discussion

In the present report, the IP administration of CT simultaneously with *V. cholerae* whole bacteria increased the specific sera antibodies responses against surface antigens, particularly IgM anti-LPS. Sera vibriocidal activity also increased significantly in CT treatment after the third injection of antigen and it remained higher for at least 38 weeks. However, CT did not enhance IgM anti-OMP titers, neither IgG anti-OMP titers, except at a peak after the third immunization. These results show that CT increases the production and the persistence of the anti-LPS IgM antibodies and the vibriocidal activity in sera as the result of multiple antigen encounters. Concurrently, a good correlation between sera vibriocidal activity and anti-LPS IgM titer was observed in the present study, which is similar to other reports associating vibriocidal activity with anti-LPS antibodies (1, 5, 13, 17). This observation could be related to the fact that IgM has an increased efficiency in complement fixation when compared to IgG. It suggests that in most cases vibriocidal activity reside on IgM type antibodies directed against the LPS motive.

A poor immune response in mice was obtained when animals were inoculated orally with *V. cholerae*, indicating that antigen sampling on the murine intestinal mucosa was insufficient to mount a significant systemic immune response that satisfies the objectives of this report. Consequently, in order to compare the effects of CT in the sera antibodies response induced by the whole bacteria administration, a systemic route for immunization was considered. Although IP route for antigen administration is not the natural via for antigen encounter, it was considered a good enough approach to study the effects of CT over the systemic antibodies production. Also, prior studies showed that this toxin can reach other organs associated to mucosal compartments when it is absorbed from the intestine (2, 28). Experimentally, the oral administration of one dose of CT in rats resulted in an antibody immune response in the spleen 48 hr later, whereas no antitoxin antibody forming cells were found in the Peyer patches, mesenteric lymph node and lamina propria of the small intestine (2). Hence, the specific systemic immune response observed in humans exposed to vibrio infection could be due in part to an *in situ* presentation of antigens in the systemic compartments.

The specific increase and posterior decrease of IgG

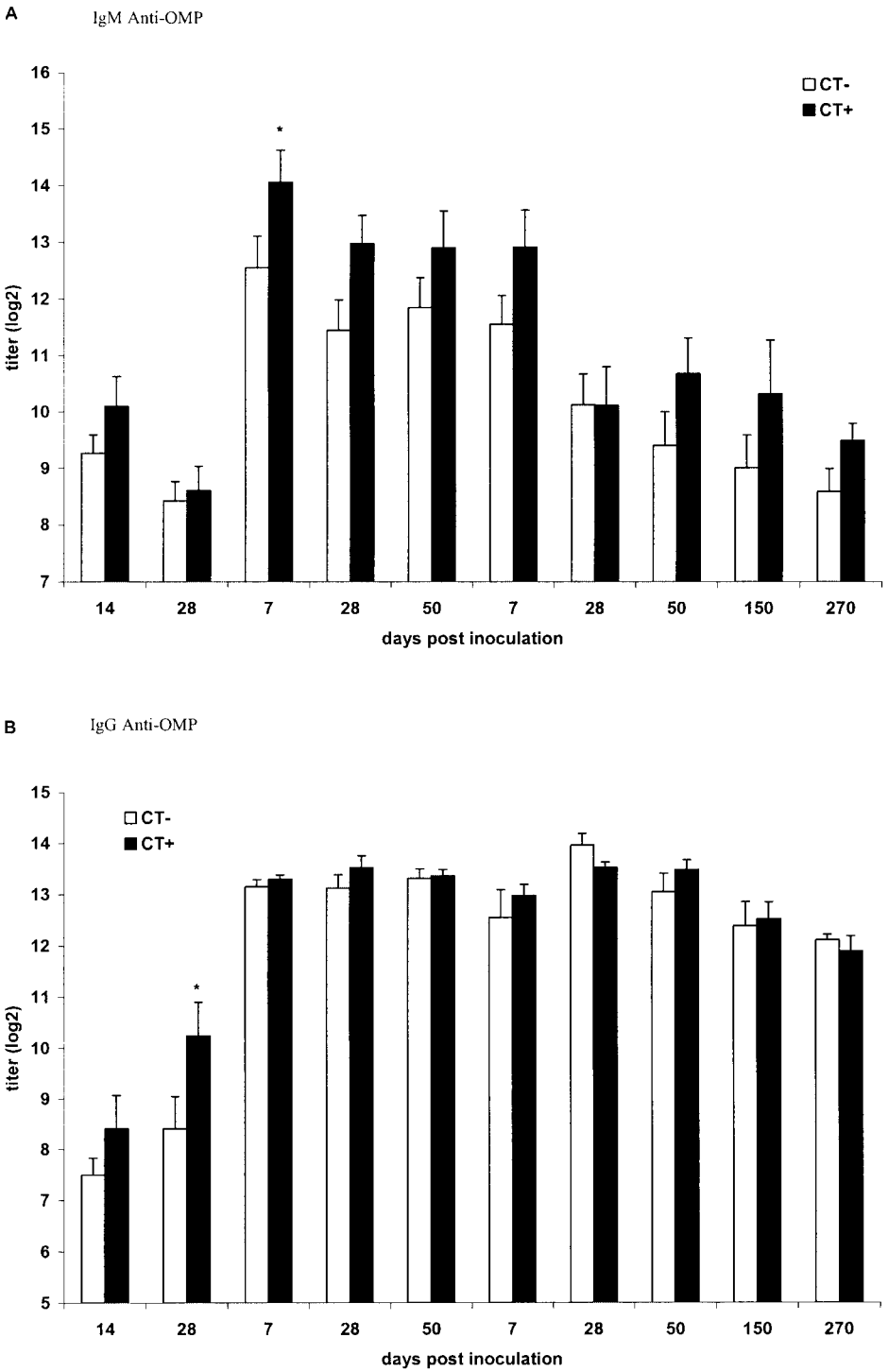

Fig. 3. Titers of antibodies in sera of mice during 50 weeks and three immunizations periods with 10^8 viable bacteria (IP) with or without CT. IgM (A) and IgG (B) anti-OMP and IgM (C) and IgG (D) anti-LPS responses. These results are expressed as the mean and standard error (10 mice per group; $*P<0.05$).

and IgM titers after secondary antigen encounter showed an apparent memory response in both CT and non-CT treatments. However, the effects of CT over the circulating immune humoral response were evident only after repeated antigen exposition at relatively prolonged times. The initial antigen administration with or without CT produced an increase of both, the vibriocidal and the antibodies titers. Posterior antigen admin-

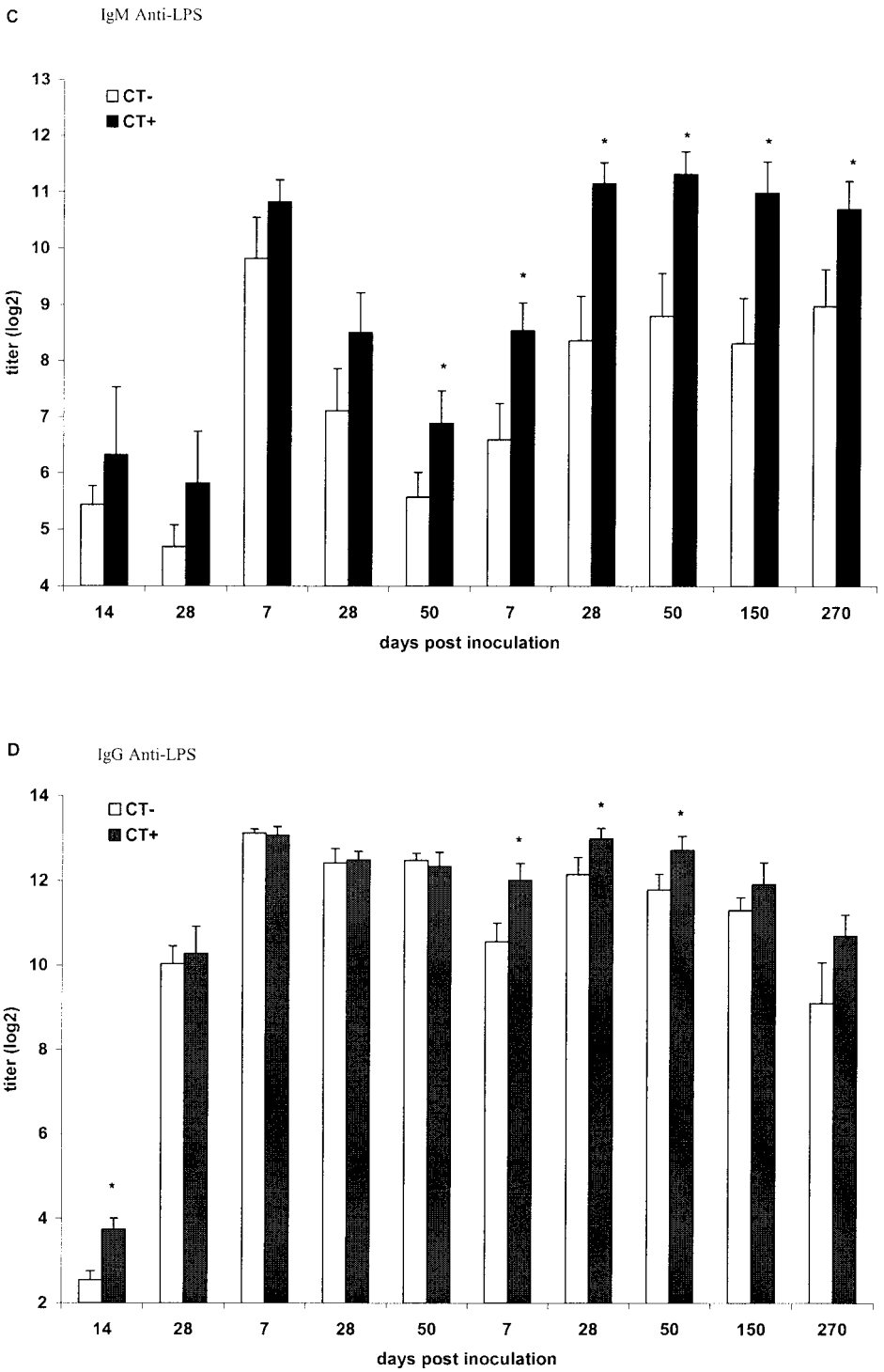

Fig. 3.

istration concurrently with CT produced a higher long lasting response when the vibriocidal and the anti-LPS IgM titers were compared to those analogous measured in sera from animals treated with antigen alone. Although the antigen used in this study is a complex one, CT seems to act like adjuvant in the specific LPS response. In a previous report, CT had showed to be adjuvant in T cell-independent responses (30). This finding reveals the potential effect of repeated administration of a relatively low concentration of CT on the immune response against vibrio infection.

The increase of IgG antibodies against LPS and OMP and the maturation of IgG response by an increase in absolute titer following secondary antigen

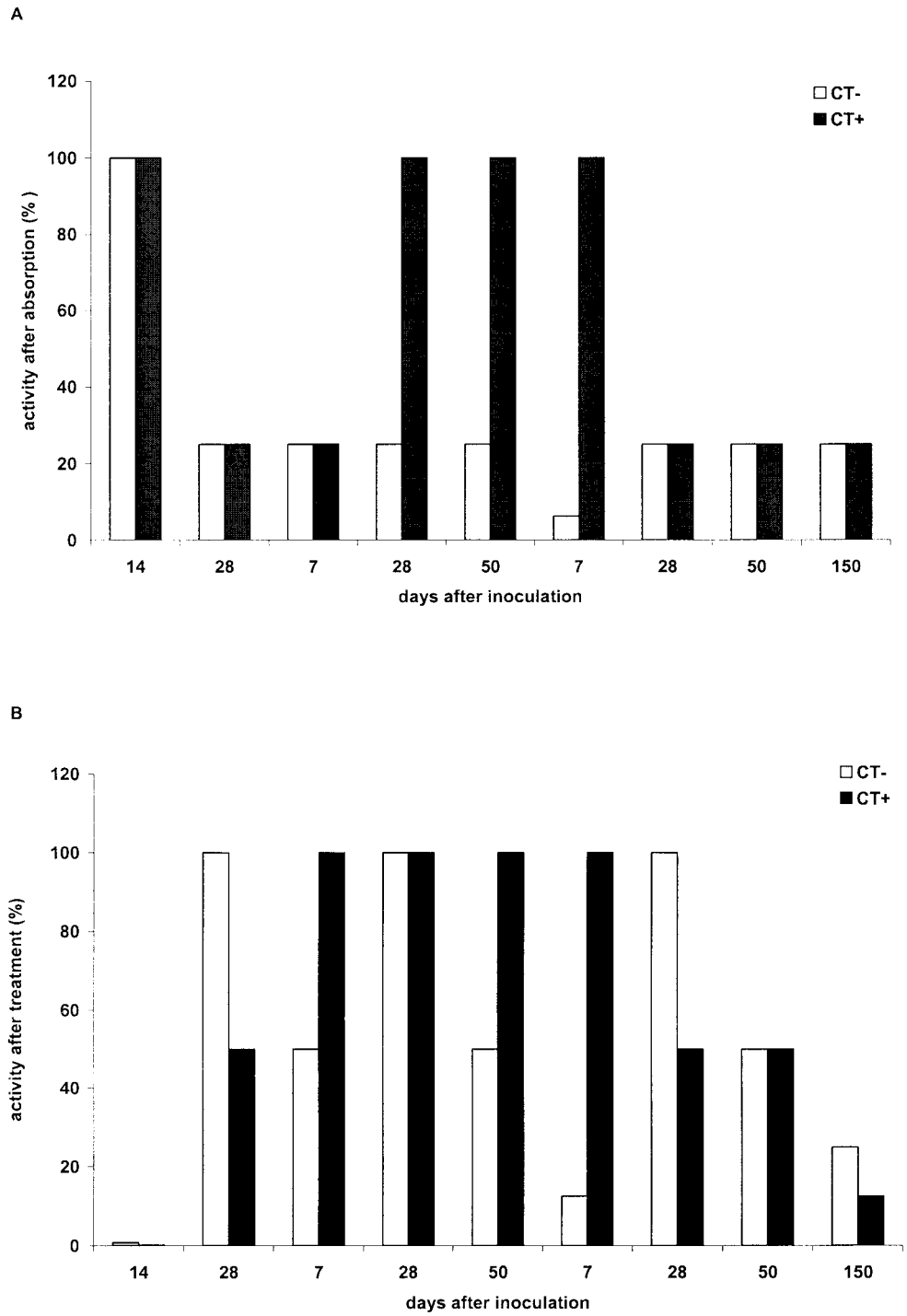

Fig. 4. Inhibition of vibriocidal activity in sera of mice immunized with 10^8 viable bacteria (IP) with or without CT. LPS adsorption (A) and 2-mercaptoethanol treatment (B). These results are expressed as % of remaining activity in treated sera.

exposition were observed at the present report. It seems that IgG contribute to vibriocidal activity at some extent and variably. However, the contribution of IgG to sera vibriocidal activity was small at the longest time, suggesting that those immunoglobulin are not enough competent to activate complement cascade. In a previous report, IgG antibodies against LPS were of little value in predicting colonization or disease (10). It suggests that most of the vibriocidal activity rely on IgM isotype and that isotype remains in circulation more actively.

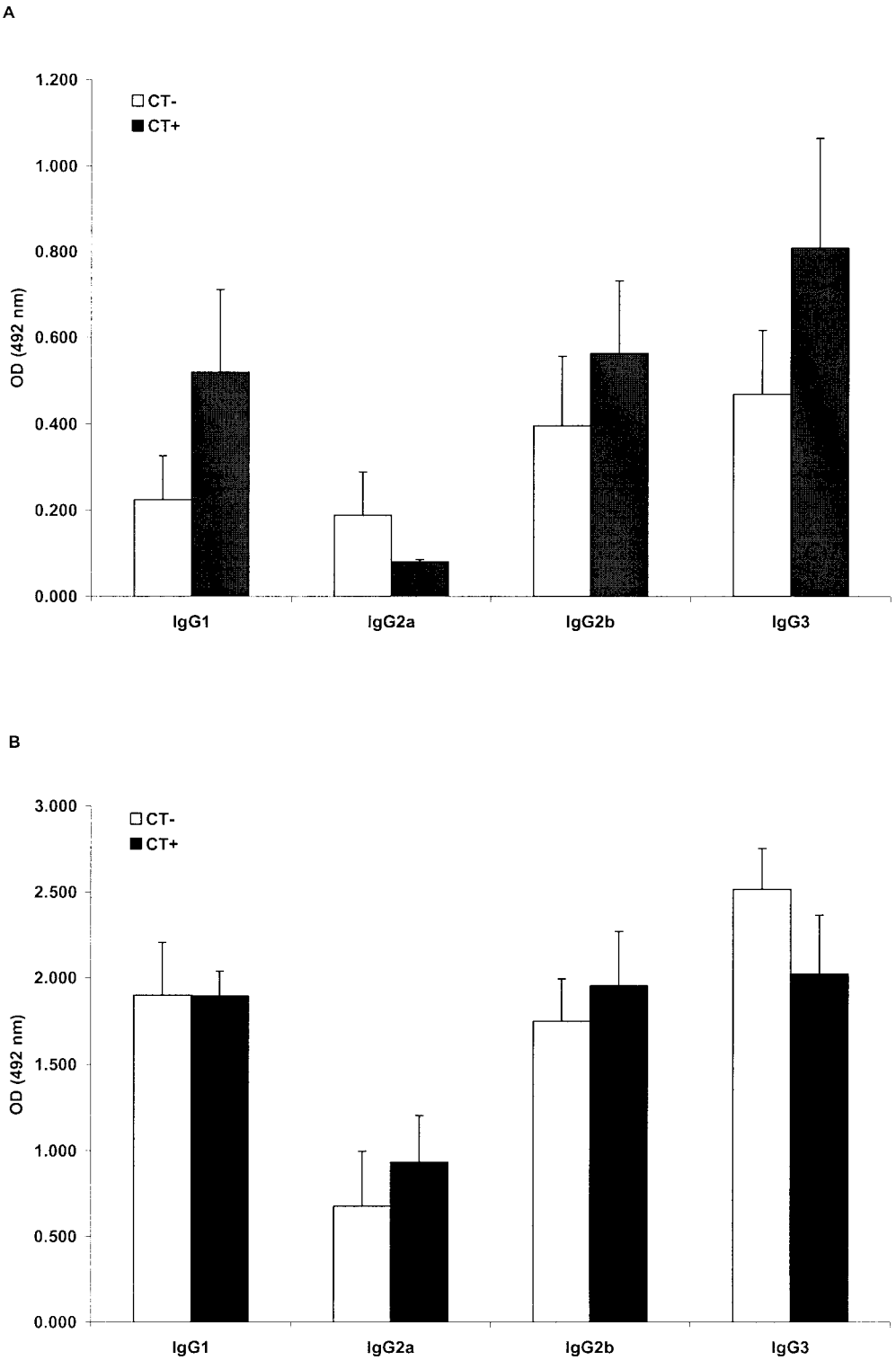

Fig. 5. The IgG subclasses levels in sera of mice 28 days after the 1 (A) and 3 (B) immunizations. These results are expressed as the mean and standard error of the OD at 492 nm.

Inhibition of vibriocidal activity with LPS and 2-Me in sera of animals inoculated with or without CT were dissimilar, particularly after the second immunization when anti-LPS IgM titer falls faster. It suggests that some competence between different antibodies class occurs. Although CT influenced neither anti-LPS IgG nor anti-OMP IgG titers, it is possible that antibodies avidity was affected. Another possibility is that IgG

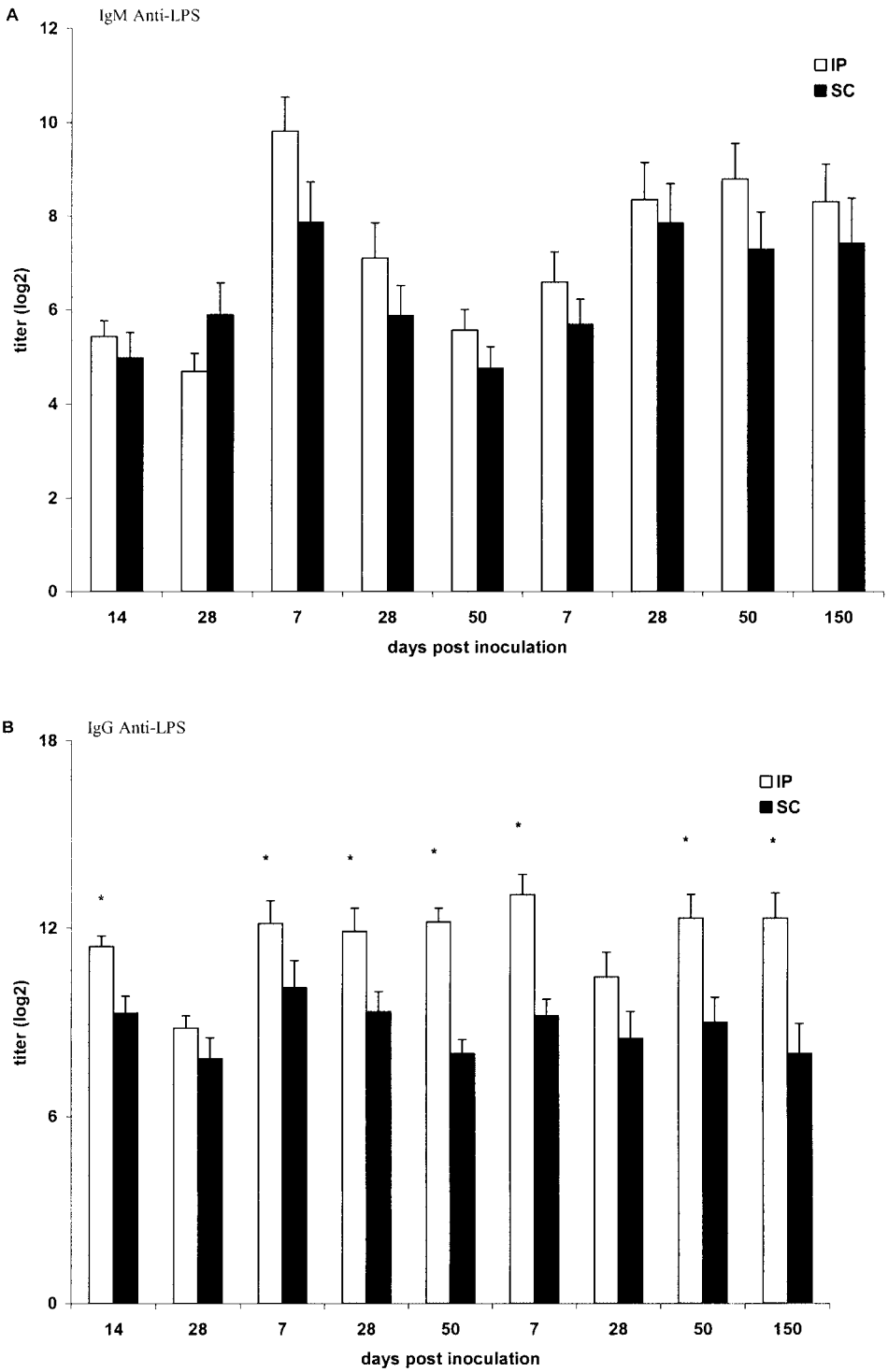

Fig. 6. Comparison of antibodies titers in sera of mice during 50 weeks and three immunizations periods with 10^8 viable bacteria without CT. IgM (A) and IgG (B) anti-LPS responses. These results are expressed as the mean and standard error (10 mice per group; $*P<0.05$).

subclasses were altered by CT treatment thus affecting complement activation. Sera LPS IgG subclasses were measured by ELISA at the IgG pike following each immunization and it was observed that IgG2a was the prevalent subclass. The pattern of subclasses remains constant at the times analyzed. IgG4 was the major component of IgG subclasses in human volunteers immunized with CVD 103-HgR (19). Among the IgG

subclasses in humans, IgG3 and IgG1 bind and activate complement cascade readily, whereas IgG2 does so poorly, and IgG4 exhibits no activity. In the mouse, IgG2a, IgG2b, and IgG3 all efficiently activate complement, whereas IgG1, the murine homologue of human IgG4, is a very poor activator.

A stronger and longer life anti-LPS response was measured in mice injected IP compared to those inoculated SC. Although unlikely, it was possible that bacteria injected in the peritoneum could survive and multiply. The efforts for recovering bacteria from the peritoneal cavity of immunized animals were performed 1 week after inoculation with negative results. Then, a potential explanation for this difference in response induced by IP and SC treatments could be founded in the characteristics of the immune system. The work from Kolb et al. (15) suggests that an antibody-forming cells (AFC) population exists and is more abundant in the peritoneum, which is specific against T-independent antigens and has characteristics of memory cells secreting IgM antibodies. These cells would be important in peritoneal cavity protection, so they could evoke a response only if the antigen was inoculated IP but not by other systemic form. Therefore, highest anti-LPS vibrio immune response would be obtained administered by this route. It is possible that this population of cells has a role in the intestinal protection against *V. cholerae* infection.

Although high titer of vibriocidal antibodies correlates with resistance to cholera infection, the role of circulating antibodies in natural infection is unclear. The reason that a serum, complement-binding antibody assay predicts protection following a noninvasive mucosal infection, such as that caused by *V. cholerae*, is currently unknown. While serum antibodies are able to penetrate into the lumen of the intestine and confer some protection against *V. cholerae*, it is not clear how much the complement system contributes to defense within the intestine. The vibriocidal antibody response, therefore, may be a surrogate marker for an as yet undefined intestinal response that is the primary mediator of protective immunity, i.e. a fraction of secreted IgA.

B1 and B2 cells are apparently implicated in innate and adaptive immunity, respectively. B1 cells are a major source for IgM antibodies specific to bacterial cell wall components. Peritoneal B1 cells could give origin to the intestinal IgA plasma cells (4). B1 cells differ from conventional B cells in their phenotype, specificity repertoire, and mode of replenishment. They have been shown to be responsible for the production of many multireactive IgM antibodies encoded by germ line genes that are directed to epitopes on microorganisms and determinants on *Escherichia coli* or comensal flora. B1 cell-derived IgA might have a role in establishing the homeostasis between the host and the normal gut flora. IgA is thought to play an important role in the humoral immune response against pathogenic microorganisms that invade the host at mucosal surfaces but many non-pathogenic commensal bacteria have been observed with coatings of IgA antibodies. Whether B1 cell-derived IgA differs functionally from conventional B cell-derived IgA is currently unknown. CT induced anti-LPS antibodies could be obliging associated to B1 cells. If that is true, then vibriocidal activity mainly coupled with IgM anti-LPS titers could be just a moiety correlated with protection to cholera without a direct role. The protection to cholera will reside on IgA isotype associated to this subset of IgM. It could be an explanation for the short time but protective effects of old anti-cholera systemic vaccines. However, this is just speculation and additional work should be done in order to prove it.

As was observed in mice after a first *V. cholerae* inoculation, in non-endemic settings and challenge studies in persons with no previous exposure to cholerae vaccine or infection, intense increases in titers of vibriocidal antibodies and the disappearance of these antibodies within a few months has been reported (6, 18, 30). In a cholera endemic area, the vibriocidal titers gradually increased with age (10, 27) and persisted throughout life. A possible explanation for these distinctive situations is that exposure to *V. cholerae* occur repeatedly with considerable frequency in endemic areas in order to boost the vibriocidal response (10). In these areas, the development of immune response prior to exposure makes these infections often asymptomatics or cause mild diarrhea. It is possible that a long lasting vibriocidal activity could be visible after a few repeated antigen administration i.e. periodic infections, and not only after continuous contacts. The results of the present report could be equivalent to the clinical cholera situations in non-endemic vs. endemic areas.

V. cholerae induces its own spreading in the environment by means of the intestinal CT secretion. This toxin also could improve the bacterial fitness through the modulation of some intrinsic host responses like IgM and IgA antibodies production providing a balanced host protection which could be essential in the life cycle. The final outcome would be to persist in human settings after reiterate cyclical individual infection. If the previous speculation is true, then the use of attenuated live vaccines in endemic locations should be critically evaluated.

The present report confirmed the good correlation between serum vibriocidal activity and anti-LPS IgM and that both are enhanced by low doses of CT after

prolonged periods of time. Further work is necessary to clarify whether repeated vibrio infection in the intestine with or without cholera toxin is also able to induce a differential immune response and to know the implication of this observation during natural infection and vaccination.

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