Correlation Between Intestinal Synthesis of Specific Immunoglobulin A and Protection Against Experimental Cholera in Mice

ANN-MARI SVENNERHOLM,* STEFAN LANGE, AND JAN HOLMGREN

Institute of Medical Microbiology, University of Göteborg, Göteborg, Sweden

Received for publication 29 December 1978

The importance of locally and systemically formed antibodies of various classes for protection against experimental cholera has been studied in mice immunized with cholera toxin. Groups of mice were given various numbers of peroral or intravenous immunizations, or a combination of both. Serum antibodies and antibodies synthesized by spleen and small intestine in vitro during tissue culture were measured by the enzyme-linked immunosorbent assay, and protective immunity against intestinal toxin challenge was determined by means of a small-bowel loop assay. Regression analyses showed a close correlation between the magnitude of intestinal synthesis of specific immunoglobulin A (IgA) antibodies and protection ($r = 0.98$), whereas neither the local formation of IgG or IgM nor the production of antitoxin antibodies of any immunoglobulin class by spleen showed any significant correlation with protection. The serum titers of IgG and IgM antibodies did not show any such relation, whereas the level of specific IgA in serum, probably mainly derived from the intestine, correlated significantly ($r = 0.90$).

In cholera and other noninvasive enteric infections only antibodies present in the gut lumen or at the epithelial surface confer protection against disease. Such antibodies may be either locally produced in the lamina propria of the intestine or derived from the circulation (6). A protective role of serum antibodies against experimental cholera has been indicated in rabbits (3) and dogs (1, 11) by transfusion experiments. The significance of locally produced antibodies for protection has, however, been difficult to establish unambiguously. Since both the enteral and parenteral routes of immunization may give rise to local as well as systemic antibody formation, the leakage of serum into the intestine has prevented accurate evaluation of the proportion of locally produced antibodies in the intestinal fluid.

It was recently found that enteral immunization was much more effective than parenteral immunization in inducing protection against experimental cholera in mice (8). This suggested that the local immune response was of prime importance for protection in this species. The aim of the present study was to characterize in greater detail the functional significance of local and systemic antibody formation in various immunoglobulin classes. This was done by determining the synthesis of immunoglobulin G (IgG), IgA, and IgM antitoxin antibodies in spleen and small intestine as well as the serum antibody titers after various immunization schedules and analyzing their relationship to the level of protective immunity measured.

**MATERIALS AND METHODS**

**Animals.** Inbred C57Bl/6 J(H-2*) mice of both sexes were used. Their age varied between 8 and 12 weeks at the onset of immunization.

**Immunization.** Groups of mice were given various numbers of intravenous (i.v.) or peroral (p.o.) immunizations with purified cholera toxin (CT) (Schwarz/Mann, Orangeburg, N.Y.). The i.v.immunizations were performed by injecting 1 µg of CT in 0.1 ml of phosphate-buffered saline (PBS) into a lateral tail vein, and the p.o. immunizations were done by instillation of 5 µg of CT dissolved in 0.5 ml of PBS supplemented with 3% (wt/vol) NaHCO₃ into the stomach or upper duodenum by means of a baby-feeding catheter introduced p.o. to a mark 4 cm from the end (8). The first two immunizations were administered at an interval of 10 days, and subsequent immunizations were given every 6th day. Mice injected i.v. with PBS or given bicarbonate-PBS solution p.o. served as controls and were analyzed simultaneously with the immunized animals.

**Protection tests.** Protection against intestinal challenge with CT was studied by using a "ligated loop assay" (3, 8). The procedure has been described in detail in a previous report (8). At least five mice from each immunization group and an equal number of control animals were tested. In each animal two 6- to
8-cm-long, small-bowel loops were ligated, into which were injected in alternating positions graded amounts of a crude CT (1 mg equivalent to 0.3 μg of purified toxin). Routinely, five animals were each challenged with 5 and 25 mg of crude toxin. When immunized animals gave different responses than the control mice to either or both doses, additional animals were challenged with appropriate doses to enable estimation of the 50% effective dose. The fluid accumulation per centimeter of loop was measured 5 to 6 h after challenge, and the dose of toxin giving rise to 50% maximal fluid accumulation (50% effective dose) was estimated by interpolation. The protective effect of immunization—the protection factor—was determined as the ratio between the 50% effective dose values of immunized and concurrently tested control groups (13). Protection was regarded as significant when the fluid response to one or more of the challenge doses was significantly lower in immunized than in concurrently tested control animals (P < 0.05, Student’s t test).

Serum sampling. Sera were collected from three to five animals in each group on the day corresponding to that for the protection test (4 days after the last antigen administration unless specified otherwise). The samples were pooled groupwise, stored at −20°C, and then heated at 56°C for 30 min immediately before use.

Antibody synthesis. Spleens and small intestines were quickly excised from three to five animals in each group. The capacity of these tissues to synthesize specific antibodies in vitro was, with some modifications, determined as earlier described (14). Approximately 500 mg of small intestinal tissue from each animal and the groupwise pooled spleens, 300 to 500 mg, were thoroughly washed in Eagle medium and minced into 1- to 2-mm2 pieces. The minced tissues were incubated at 37°C for 24 h in 10 ml of freshly prepared, sterile Eagle medium, pH 7.2, supplemented with 5% (wt/vol) heat-inactivated normal rabbit serum and 200 U of penicillin-streptomycin per ml. The pH was kept at 7.2 to 7.4 by bubbling a 96% O2-5% CO2 mixture through the medium. After incubation the medium was centrifuged at 3,000 × g for 10 min, and the supernatant was dialyzed against PBS for at least 24 h and stored at −20°C until used.

**Antibody determinations.** Specific antitoxins of various immunoglobulin classes in serum and in diazylated incubation medium were determined by means of the enzyme-linked immunosorbent assay, using CT as solid-phase antigen (5). The immunoglobulin fractions of class-specific goat antiserum to mouse IgG2b, IgA, and IgM (Meloy Laboratories, Springfield, Va.) were purified by means of an immunosorbent column containing mouse gamma-globulin, (mixture of IgG, IgA, and IgM) covalently coupled to Sepharose (14) and then used for conjugation with alkaline phosphatase (5). Since other studies of antibody-forming cells in various lymphoid tissues had shown very little variation with regard to the various subclasses of IgG (IgG1, IgG2a, and IgG2b), only IgG antibodies of the IgG2b subclass were assayed (S. Lange, unpublished data). Antibody titers were determined as the interpolated dilutions of serum or incubation medium giving rise to an absorbance at 400 nm of 0.2 above background after reaction of the tube-bound enzyme with substrate for 100 min (serum) or 300 min (incubation medium).

<table>
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<th>Route</th>
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* ELISA, Enzyme-linked immunosorbent assay.
* PF, Protection factor; ratio between 50% effective dose values of immunized and concurrently tested control animals.
* 5 μg of CT per injection.
* 1 μg of CT per injection.

**RESULTS**

Protection after p.o. and i.v. immunization. Two, but not one, p.o. immunizations gave rise to a significantly increased resistance against intestinal toxin challenge as tested 4 days after the last immunization (Table 1). Two further booster doses by the same route increased the protective immunity markedly to a level which was not significantly enhanced by up to five additional enteral immunizations. With an i.v. immunization regimen earlier shown to give maximal serum antibody levels (8), no protec-
tion was achieved even by repeating the immunizations up to five times. A low level of immunity, similar in magnitude to that obtained by two p.o. vaccinations, could, however, be induced by eight i.v. injections (Table 1).

**Antibody synthesis by intestine.** The formation of antitoxin antibodies in intestine was determined by letting tissue specimens from immunized animals synthesize protein in vitro and then measuring the titers of specific antibodies of various classes in the incubation medium. The IgG and IgM antibody titers were usually higher after i.v. than after an equal number of p.o. immunizations, whereas the intestinal synthesis of specific IgA antibodies was always considerably higher after immunization by the p.o. route (Table 1). Those immunization schedules that were most effective in inducing protection, i.e., four to nine p.o. immunizations, also gave rise to the highest intestinal IgA antitoxin titers. Schedules which resulted in protection of less magnitude, i.e., two p.o. and eight or nine i.v. immunizations, gave rise to only slightly elevated IgA antibody titers. The titers of IgA antibodies in intestinal medium of nonprotected immunized animals did not exceed the background level obtained with unimmunized controls (Table 1).

It was important to determine to what extent the antibodies in incubation medium were newly synthesized during the in vitro incubation or represented preformed immunoglobulins merely released into the medium. This was tested by comparing the amounts of antibody in the medium after simultaneous incubation of the tissue at 37 and at 0 to 5°C. Since incubation in the cold has been found to decrease protein synthesis by 90 to 95% (14), the titers observed in medium of ice-bath-incubated tissue should mainly represent preformed antibodies of either local or systemic origin and increase in titer on incubation at 37°C should represent the de novo synthesis. Table 2 shows that, as tested with intestine of animals immunized four times p.o. as well as four times i.v., incubation in the cold resulted in considerably lower antibody amounts in medium than did incubation at 37°C.

**Antibody synthesis in spleen.** Incubation medium of tissue-cultured spleen from i.v. immunized mice contained considerably higher levels of IgG and IgM antibodies than did spleen medium from p.o. immunized animals, unless many immunizations had been given (Table 1). The synthesis of specific IgA antibodies, on the other hand, did not differ significantly between enterally and parenterally immunized mice.

**Serum antibodies.** After a single p.o. immunization, no antibodies were detected in serum. A booster immunization by the same route induced significant levels of IgG and IgM as well as IgA antibodies, which increased markedly by two further immunizations (Table 1). In all instances the i.v. immunizations induced higher titers particularly of IgG, but also of IgM, antibodies than were found after a corresponding number of p.o. vaccinations. Already two i.v. immunizations induced very high IgG antibody titers, which did not increase significantly by further antigen injections. Also, the IgM antibody level was almost maximal after only two parenteral immunizations. In contrast, more than four i.v. immunizations were needed to induce detectable IgA antibody titers in serum (Table 1).

**Course of the immune response.** The magnitude of protective immunity and its relation to specific antibody production was studied in mice at various intervals after four p.o. immunizations. Whereas the animals were very resistant to intestinal toxin challenge on day 4 after vaccination, no significant protection was seen after 2 or 10 days, and only slightly increased resistance was found on day 7 (Fig. 1). The time course of the protective immune response correlated well with the intestinal synthesis of specific IgA, which showed a similar peak on day 4 after immunization. Also, the IgA titers in serum were at their maximum at this time. In contrast, optimal IgG antibody formation in intestine as well as maximal serum levels of specific IgG were not attained until 7 days after vaccination, when there was almost no protection. The maximal intestinal IgM antibody production, in contrast to IgM antibody titers in serum, coincided with the time for optimal protection but decreased at a slower rate than did the protective immunity and the intestinal IgA antibody synthesis (Fig. 1).

**Boosting i.v. of p.o. primed intestine.** Recent studies in rats (10) and in humans (15) have indicated that parenteral immunization, even when incapable itself of eliciting local antibody formation, may boost a mucosal immune response in intestine already naturally or artifi-
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Correlation between intestinal IgA formation and protection. Linear regression analyses were performed by groupwise plotting of the protection factor values observed after the various immunization regimens against the antibody titers of different immunoglobulin classes measured in serum or tissue culture medium. A highly significant correlation was found between protection and intestinal IgA antibody synthesis (Fig. 2), the $r$ value being 0.98. In contrast, no significant correlation between protection and intestinal IgG ($r = 0.12$) or IgM ($r = 0.10$) antibody formation was found. Nor did the synthesis of antitoxin of any immunoglobulin class by spleen correlate significantly with protection ($P > 0.20$ in all instances). With regard to serum antibody titers, there was no correlation between the IgG or IgM titers and protection. In contrast, the IgA titer correlated significantly with the protection factor values ($r = 0.90$), but these antibodies seemed to be of predominantly an intestinal origin since they correlated significantly with the IgA antibody synthesis in intestine ($r = 0.88$) but not with that in spleen ($r < 0.20$).

DISCUSSION

A previous study suggested that protective immunity against toxin-induced experimental...
cholera in mice depended primarily on local antibody formation (8). This hypothesis is strongly supported by the present investigation, in which the protection is shown to correlate closely with the in vitro synthesis of specific IgA antitoxin in intestine. Thus, the various protective immunization schedules, i.e., two or more p.o. antigen administrations, eight to nine i.v. injections, or a combination of p.o. and i.v. immunizations, all gave rise to significant synthesis of IgA antibodies in the small intestine, the magnitude of which corresponded to the level of in vivo protection. In contrast, local IgA antitoxin synthesis was consistently absent after immunization schedules which did not result in detectable protection. This included repeated parenteral antigen administrations, which induced high levels of IgG antitoxin in serum. Furthermore, the course of the intestinal IgA response was similar to that of the protective immunity; i.e., they both showed a peak about 4 days after the last immunization followed by a rapid decrease. A similar short duration of the mucosal immune response has been observed by Pierce and Gowans (10), who studied antitoxin-containing cells in lamina propria of immunized rats.

In vitro incubated intestinal tissue from immunized mice was shown to release IgG and IgM antitoxin antibodies also. In contrast to the IgA antibodies, the IgG levels especially, but also the IgM levels, were higher after the i.v. than after the p.o. immunizations. Some of the IgG and IgM antibodies registered were no doubt due to release from the tissue of preformed serum immunoglobulin. However, the observation of considerably higher antibody levels by incubation of intestine at 37 rather than 0 to 5°C indicates that the major part of the antibodies represented local de novo synthesis. At variance with the intestinally formed IgA, the levels of neither locally produced IgG nor IgM antibodies correlated with protection; i.e., although the intestine at least in vitro is capable of specific antibody formation, including IgA and IgG as well as IgM, only the IgA antibody synthesis is important for the in vivo protection. This probably reflects that in vivo only the locally produced IgA by virtue of its J-chain is transported from its site of synthesis in the lamina propria across the epithelium to reach the intestinal mucosa surface. Some IgM may also possess J-chain to facilitate transport across epithelium but is readily degraded by intestinal enzymes because of its inability, different from IgA, to stabilize its interaction with degradation-protective secretory component (L. A. Hanson and P. Brandtzaeg, in E. R. Stiehm and V. A. Fulginiti [ed.], Immunologic Disorders in Infants and Children, 2nd ed., in press).

Our data indicate that the effect of systematically produced antibodies in mice is minimal compared to that of locally formed IgA antitoxin. Thus, the serum antitoxin titers, which were predominantly of the IgG class, were up to 1,000-fold higher after nonprotective i.v. than after effective p.o. immunization. Moreover, the in vivo IgG and IgM antibody synthesis by spleen was greater from i.v. than from p.o. immunized mice. A significant correlation was seen between the serum IgA titers and protection. However, these antibodies probably reflected a "spillover" from the intestinal immune response, since the serum IgA titers correlated significantly with the IgA antibody synthesis in intestine but not with that in spleen. Fujita and Finkelstein (3) observed some protection in mice against challenge with live vibrios 3 weeks after a single subcutaneous toxin immunization, probably resulting primarily in systemic IgG and IgM antitoxin. The immunity attained, however, was insufficient to give detectable protection against intestinal challenge with toxin. We have confirmed the complete absence of protection in mice against toxin after one or two subcutaneous immunizations (unpublished data). The observations indicate that serum antitoxins diffusing into the intestine, although capable of mediating partial neutralization of the toxin gradually released from multiplying vibrios, in contrast to local IgA, are unable to protect against the more massive challenge represented by a bolus of toxin. The poor protective capacity of systemic antibodies in mice is at apparent variance with the situation in dogs and rabbits. In these animals an important protective effect of serum-derived antibodies, particularly of the IgG class, has been demonstrated (1, 4, 11). It is possible that this difference is due to more rapid degradation of serum immunoglobulins in mice than in the other species. Thus, Fubara and Freter (2) observed that intestinal IgA but not serum IgG or IgM antibacterial antibodies conferred protection against cholera infection in mouse intestine; in contrast, it has been shown that serum IgG and IgM antibodies are highly effective in similar studies in rabbits (12).

The extent to which various animal models reflect the protective immune mechanisms against cholera in humans needs further investigation. An observed close correlation in field trials between the degree of protection and the serum vibriocidal titers after parenteral cholera vaccination with killed vibrios (9) could be taken as support for a protective role of serum antibodies. On the other hand, the correlation between serum titers and protection might not necessarily be of a causal nature, since a recent
study showed that subcutaneous cholera vaccination of individuals in endemic areas stimulated local secretory IgA formation in addition to the serum antibody response (15).

A contributing role of serum antibodies does not detract from the obvious aim of vaccination programs against enteric infections to stimulate a maximal local immune response in the intestine. The described mouse system appears to provide a suitable model for further studies of the optimal conditions to induce local formation of protective antibodies by immunization with cholera toxoid (7).

LITERATURE CITED