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# Evaluation of the Biological Properties of Different Classes of Human Antibodies in Relation to Cholera

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Different classes of immunoglobulins (immunoglobulin M [IgM], IgG, and secretory IgA) were purified from pooled serum and milk samples of convalescent cholera patients by gel filtration and immunoadsorbent techniques. The purity of these preparations was established by immunodiffusion and the enzyme-linked immunosorbent assay, using class-specific antisera. The biological properties of antibodies present in these crude and purified immunoglobulin preparations were evaluated by tests related to cholera. Purified human IgM and IgG exhibited both agglutinating and vibriocidal properties. On the other hand, human secretory IgA was not vibriocidal (even in the presence of lysozyme), although it showed agglutinating properties. Both IgG and secretory IgA could effectively neutralize cholera toxin action in vivo, whereas such activity was virtually absent in IgM. The toxin-neutralizing capacity of IgG was, however, higher than that of secretory IgA. All three classes of human antibodies could significantly inhibit the adherence of *Vibrio cholerae* to intestinal slices in vitro. These results are discussed in relation to the protective immune mechanism during cholera infection.

Information available so far suggests that immunity during cholera infection is probably mediated by humoral factors (11). In the accompanying paper (14), we demonstrated the stimulation of various types of antibody responses in the serum and milk of cholera patients. Furthermore, these responses were marked by the production of different classes of antibodies directed against both somatic and enterotoxin antigens of *Vibrio cholerae*. It was felt, however, that for a meaningful evaluation of the available data, further information was needed on the functional properties of these classes of antibodies in relation to immunity during cholera infection. Although there are reports on the antibacterial (8, 21) and antitoxic (10, 12) properties of different classes of antibodies of a few animal species, no such information is yet available for human antibodies.

In this study, we purified immunoglobulins of immunoglobulin M (IgM), IgG, and secretory IgA (SIgA) classes from pooled serum and milk samples of convalescent cholera patients. The functional properties of antibodies present in these purified preparations were evaluated by both in vitro and in vivo tests. We believe that the results obtained from this and our other study (14) should clarify and extend our knowledge of the protective immune mechanism against the disease.

## MATERIALS AND METHODS

**Gel filtration of serum.** Sera from several convalescent cholera patients were pooled, and 5 ml was applied to a Sephadex G-200 column (2.5 by 90 cm). The column was preequilibrated with phosphate-buffered saline, pH 7.4, and the sample was eluted with the same buffer. Usually, 4 to 5-ml fractions were collected in each tube. The protein concentration in each tube was determined by measuring the optical density at 280 nm. Major protein peaks containing immunoglobulins were pooled and concentrated by negative pressure.

**Gel filtration of milk.** Milk samples were collected from a few convalescent cholera patients (lactating women) and pooled. Lipid was removed by centrifugation, and clarified milk was then decaseinated by the method of O'Daly and Cebra (17). Then 5 ml of the milk sample was fractionated through a Sephadex G-200 column (2.5 by 90 cm) by the procedure described above.

**Purification of the immunoglobulin fractions by immunoadsorbent gel.** Immunoglobulin fractions (containing, primarily IgM, IgG, and SIgA) obtained after gel filtration of serum and milk were subjected to further purification by the immunoadsorbent technique. Immunoadsorbent gels were prepared by conjugating immunoglobulin fractions of anti-human IgG ($\gamma$-chain specific), IgM ($\mu$-chain specific), and IgA ($\alpha$-chain specific) antisera to activated Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). These antisera were obtained from Meloy Laboratories. Gamma globulin fractions of the antisera were prepared after repeated precipitation with 50% saturated ammonium sulfate and directly coupled to the cyanogen bromide-activated gel in 0.2 M $NaHCO_3$ buffer, pH 9.5 (18). Coupled gel was finally placed in phosphate-buffered saline and treated with ethanolamine (1 M) before being used to block any reactive site remaining.

**Qualitative and quantitative assay of immunoglobulins.** The purity of the immunoglobulin frac-

tions was checked by Oüchterlony's immunodiffusion test, using heavy-chain- and secretory-chain-specific antisera. Class-specific antisera were also used to quantitate different classes of immunoglobulins present in these fractions by single radial immunodiffusion (15).

**Determination of antibacterial and antitoxin titers.** Antibacterial titers of the various immunoglobulin fractions were determined by bacterial agglutination, vibriocidal, and indirect hemagglutination (IHA) methods. Antitoxin titers were determined by the IHA method as well as by a toxin neutralization assay. Antibody titers of different immunoglobulin classes were determined by the enzyme-linked immunosorbent assay (ELISA) against purified lipopolysaccharide (LPS) and toxin antigens. Methodologies of these tests are described in the accompanying paper (14).

**Antiadherence test.** Antiadherence activities of purified human immunoglobulin fractions were determined by the method of inhibition of *V. cholerae* adherence in vitro to isolated slices of rabbit intestine. A highly adhering mutant strain of *V. cholerae* (Inaba), $V_{63}P^-$ (1), was used. Purified human immunoglobulin fractions containing specific antibodies to *V. cholerae* were suitably diluted in Krebs-Ringer solution (KR) to subagglutinating dilutions (two double dilutions more than their agglutination titers). Tissue slices (1 by 2 cm) were cut from the rabbit ileum and added to 4.5 ml of these antibody dilutions. To each mixture, 0.5 ml of vibrio suspension ($1 \times 10^7$ to $2 \times 10^7$/ml) in KR was added. In the control flask, tissue slices were added in 4.5 ml of KR only, followed by the addition of 0.5 ml of vibrio suspension. All flasks were incubated for 30 min at 37°C. Next, tissue slices were withdrawn, washed throughly, and suspended in 5 ml of KR. Washed tissue slices as well as the original suspending medium were homogenized in a rotating homogenizer (ADCO homogenizer, Calcutta, India), and viable counts of vibrios in both homogenates were determined by plating in nutrient agar. The percentage of adherence of vibrios in the control as well as in the inhibition experiments was determined by using the formula of Freter and Jones (7).

## RESULTS

**Serum fractionation by gel filtration.** Sephadex G-200 gel filtration of pooled serum gave rise to three major protein peaks. Tubes with immunoglobulin peaks were pooled to give four crude fractions ($S_1$ to $S_4$). Various classes of immunoglobulins present in these fractions were quantitated by radial immunodiffusion (Table 1). These results agreed well with those obtained by double-diffusion analyses.

**Milk fractionation by gel filtration.** Gel filtration of pooled milk gave a major protein peak in the void volume followed by several smaller peaks. Three crude fractions ($M_1$ to $M_3$) were pooled. Quantitative immunodiffusion analyses showed that the void volume fraction $M_1$ contained IgM and IgA but not IgG (Table 1). On the other hand, IgG was present in fraction $M_2$, which was, however, devoid of any IgM and IgA. None of these immunoglobulins was demonstrable in $M_3$. These results also agreed well with those obtained by the double-diffusion method.

TABLE 1. *Immunoglobulin content of serum and milk fractions*

| Fraction[a] | Immunoglobulin content (mg/ml) | | |
|---|---|---|---|
| | IgM | IgG | IgA |
| Pooled serum | 1.80 | 16.62 | 2.20 |
| $S_1$ | 1.40 | 0.70 | 0.28 |
| $S_2$ | ND[b] | 0.70 | 0.70 |
| $S_3$ | ND | 2.05 | 1.06 |
| $S_4$ | ND | 11.50 | 0.28 |
| Pooled milk | 0.30 | 0.70 | 2.50 |
| $M_1$ | 0.20 | ND | 2.40 |
| $M_2$ | ND | 0.50 | ND |
| $M_3$ | ND | ND | ND |

[a] Crude fractions of serum ($S_1$ to $S_4$) and milk ($M_1$ to $M_3$) after gel filtration.
[b] ND, Not detectable.

**Further purification of immunoglobulin fractions.** IgG and IgA present in serum IgM fraction ($S_1$) were removed by treating $S_1$ with anti-IgG- and anti-IgA-coupled Sepharose gels. Immunodiffusion tests showed that the purified IgM preparation ($PS_1$) thus obtained was free of any IgG or IgA. The IgM content of $PS_1$ was determined to be 0.98 mg/ml. IgA present in the crude IgG fraction ($S_4$) was removed by treating $S_4$ with an anti-IgA-coupled Sepharose gel. The purified IgG preparation ($PS_4$) was found to contain only IgG (6.6 mg/ml). Similarly, the $M_1$ fraction of milk was further purified by treating $M_1$ with an anti-IgM–Sepharose gel. The purified $M_1$ fraction ($PM_1$) was completely free of any IgM or IgG and contained only 1.5 mg of SIgA per ml (Fig. 1).

**Anti-LPS and antitoxin ELISA titers of fractions.** Table 2 shows IgM, IgG, IgA, and SIgA class-specific anti-LPS and antitoxin ELISA titers of pooled serum and milk samples and their crude and purified immunoglobulin fractions. Among the serum fractions, $S_1$ retained most of the anti-LPS activities of the IgM class present in the parent pool, whereas $S_4$ contained most of the activity of the IgG class. On the other hand, fraction $S_4$ retained most of the antitoxic activity of the IgG class of the parent pool, whereas the weak titer in $S_1$ largely belonged to the IgM class. The weak anti-LPS and antitoxin titers observed with the $S_2$ and $S_3$ fractions were mainly contributed by IgA and IgG antibodies. The purified IgM preparation ($PS_1$) gave anti-LPS (800) and antitoxin titers (50) of the IgM class only. Similarly, the purified

IgG preparation ($PS_4$) showed only IgG antibodies against both LPS (200) and toxin (800) antigens.

Most of the anti-LPS and antitoxin titers of the pooled milk were due to SIgA antibodies which were eluted in fraction $M_1$ (Table 2). This fraction also contained anti-LPS activity of the IgM class which was clearly absent in the purified SIgA preparation ($PM_1$). The anti-LPS and antitoxin titers of the SIgA class were found to be the same (200) in $PM_1$.

**Antibacterial and antitoxic activities of fractions.** Table 3 shows the antibacterial and antitoxic activities of pooled serum and milk sample as well as of their different fractions. The antibacterial activity of the pooled serum was present primarily in fraction $S_1$ and, to a lesser extent, $S_4$, whereas fractions $S_2$ and $S_3$ had weaker activities. On the other hand, antitoxin IHA results demonstrated that all four serum fractions had antitoxin titers, which were highest in $S_4$ and lowest in $S_2$. Although $S_1$ showed antitoxin titer (150 antitoxin units per ml) by the IHA method, it failed to demonstrate any toxin-neutralizing activity in either in vivo assay. Antitoxin titers of other serum fractions (particularly $S_3$ and $S_4$) determined by all three methods agreed reasonably well among each other.

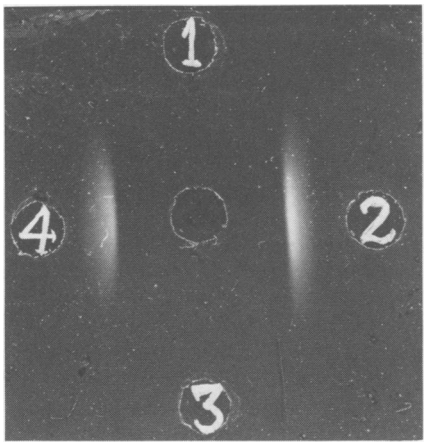


FIG. 1. *Immunodiffusion pattern of the purified immunoglobulin preparation $PM_1$. Center well, $PM_1$; well 1, anti-IgM; well 2, anti-IgA; well 3, anti-IgG; well 4, anti-SIgA. All antisera used were heavy-chain specific except anti-SIgA which was secretory-chain specific.*

Antibacterial and antitoxic activities of pooled milk were found to be present exclusively in fraction $M_1$. Interestingly, antitoxin IHA titers of the parent milk pool as well as of the $M_1$ fraction were somewhat higher than their corresponding toxin neutralization titers (Table 3).

Results obtained with purified immunoglobulin preparations (Table 3) indicated that IgM ($PS_1$), IgG ($PS_4$), and SIgA ($PM_1$) antibodies have vibrio agglutinating properties. It is interesting to note, however, the absence of vibriocidal activity in SIgA ($PM_1$), although such activity was demonstrable in $M_1$. Moreover, addition of lysozmye (10 μg/ml) to the mixture of SIgA antibody and complement failed to induce any detectable vibriocidal activity. It is also evident that IgM antibodies ($PS_1$) were unable to neutralize cholera toxin action in vivo, although these antibodies had a definite antitoxin IHA titer. The antitoxin titer of the SIgA preparation ($PM_1$) determined by the structural (IHA) method was definitely higher than those ob-

TABLE 2. *Immunoglobulin class-specific anti-LPS and antitoxin titers of crude and purified fractions of serum and milk*

| Fraction[a] | Anti-LPS ELISA titer (reciprocal) | | | | Antitoxin ELISA titer (reciprocal) | | | |
|---|---|---|---|---|---|---|---|---|
| | IgM | IgG | IgA | SIgA[b] | IgM | IgG | IgA | SIgA[b] |
| Pooled serum | 1,600 | 400 | 200 | NT[c] | 100 | 3,200 | 25 | NT |
| $S_1$ | 1,600 | 25 | 25 | NT | 100 | 25 | <25 | NT |
| $S_2$ | <25 | 25 | 50 | NT | <25 | 50 | <25 | NT |
| $S_3$ | <25 | 50 | 50 | NT | <25 | 200 | <25 | NT |
| $S_4$ | <25 | 400 | <25 | NT | <25 | 3,200 | <25 | NT |
| $PS_1$ (IgM)[d] | 800 | <25 | <25 | NT | 50 | <25 | <25 | NT |
| $PS_4$ (IgG)[d] | <25 | 200 | <25 | NT | <25 | 800 | <25 | NT |
| Pooled milk | 100 | <25 | 800 | 800 | <25 | 25 | 800 | 400 |
| $M_1$ | 100 | <25 | 400 | 400 | <25 | <25 | 400 | 400 |
| $M_2$ | <25 | <25 | <25 | <25 | <25 | 25 | <25 | <25 |
| $PM_1$ (SIgA)[d] | <25 | <25 | 200 | 200 | <25 | <25 | 200 | 200 |

[a] Crude fractions of serum ($S_1$ to $S_4$) and milk ($M_1$ and $M_2$) after gel filtration.
[b] Titers were determined by using an enzyme-antiserum conjugate specific for the human secretory chain.
[c] NT, Not tested.
[d] Purified immunoglobulin preparations.

TABLE 3. *Antibacterial and antitoxic activities of crude and purified fractions of serum and milk*

| Fraction[a] | Vibrio agglutination titer (reciprocal) | Anti-LPS IHA titer (reciprocal) | Vibriocidal titer ($-\log_{10}$) | Antitoxin titer[b] (reciprocal) | | |
|---|---|---|---|---|---|---|
| | | | | IHA | Rabbit ileal loop assay | Permeability factor assay |
| Pooled serum | 256 | 128 | 6.6 | 1,200 | 820 | 800 |
| $S_1$ | 128 | 64 | 5.8 | 150 | <30 | <30 |
| $S_2$ | 8 | 8 | 1.7 | 80 | 40 | 30 |
| $S_3$ | 4 | 4 | 2.7 | 150 | 270 | 260 |
| $S_4$ | 32 | 16 | 3.9 | 600 | 820 | 800 |
| $PS_1$ (IgM)[c] | 64 | 32 | 4.3 | 90 | <20 | <30 |
| $PS_4$ (IgG)[c] | 16 | 8 | 2.9 | 380 | 380 | 390 |
| Pooled milk | 128 | 128 | 5.8 | 600 | 100 | 110 |
| $M_1$ | 128 | 64 | 4.0 | 300 | 110 | 110 |
| $M_2$ | <4 | <4 | <1.0 | <20 | <30 | <30 |
| $PM_1$ (SIgA)[c] | 64 | 32 | <1.0, (<1.0[d]) | 190 | 60 | 50 |

[a] Crude fractions of serum ($S_1$ to $S_4$) and milk ($M_1$ and $M_2$) after gel filtration.
[b] Expressed in antitoxin units per milliliter.
[c] Purified immunoglobulin preparations.
[d] Vibriocidal data obtained by the addition of 10 μg of egg white lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml of the incubation mixture containing antibody ($PM_1$) and complement.

tained by the two functional (neutralization) methods. Titers determined by all of these methods agreed well for IgG antibodies ($PS_4$) only (Table 3).

**Antiadherence properties of fractions.** Table 4 presents the antiadherence properties of the three crude fractions ($S_1$, $S_4$, and $M_1$) of serum and milk. All of these fractions significantly inhibited vibrio adhesion to the isolated ileum slices. Similar results were obtained with the purified IgM ($PS_1$), IgG ($PS_4$), and SIgA ($PM_1$) preparations. However, antiadherence activities of IgM and SIgA antibodies were comparable to each other and slightly higher than those of IgG ($PS_4$) antibodies.

TABLE 4. *Inhibition of V. cholerae adherence to rabbit intestinal slices by crude and purified immunoglobulins preparations*

| Fraction | % of vibrio population adhering/intestinal slice[a] | Mean % of vibrio population adhering/intestinal slice |
|---|---|---|
| Crude[b] | | |
| Control | 19 | 21.0 |
| | 23 | |
| $S_1$ | 1.3 | 1.4 |
| | 1.5 | |
| $S_4$ | 3.4 | 3.5 |
| | 3.6 | |
| $M_1$ | 1.7 | 1.8 |
| | 1.9 | |
| Purified[c] | | |
| Control | 13 | 15.5 |
| | 18 | |
| $PS_1$ (IgM) | 1.4 | 1.35 |
| | 1.3 | |
| $PS_4$ (IgG) | 4.1 | 3.9 |
| | 3.7 | |
| $PM_1$ (SIgA) | 1.4 | 1.2 |
| | 1.0 | |

[a] Expressed as $[a/(a + k)] \times 100$, where $k$ is the number of bacteria per milliliter of KR present after incubation and $a$ is number of bacteria remaining on the tissue slices incubated in KR.
[b] Crude fractions of serum and milk.
[c] Purified immunoglobulin preparations.

## DISCUSSION

It was possible to isolate three major classes of human immunoglobulins free of cross-contamination. The purity of IgM, IgG, and SIgA preparations was established by the Oüchterlony test as well as by quantitative estimation of immunoglobulins during various stages of the purification procedure. These results were further substantiated by the sensitive micro-ELISA technique, which showed that $PS_1$, $PS_4$, and $PM_1$ contained only IgM, IgG, and SIgA antibodies, respectively.

Studies with purified human immunoglobulins clearly showed the superiority of IgG antibody in neutralizing cholera toxin activity. A similar property was also demonstrated by SIgA antibody, although its efficacy was somewhat less than that of IgG (Table 3). The protective role of intestinal IgA antibodies of rabbits (12) and mice (23) has been demonstrated against toxin challenge in experimental cholera. Evidently, similar protection could be mediated by human SIgA as well. The virtual absence of toxin-neutralizing ability in human IgM (Table

3) is supported by similar results obtained with rabbit IgM by Holmgren and Svennerholm (10). Since cholera toxin is known to bind the $GM_1$ ganglioside receptor on the cell surface with high avidity (2), only antibodies with high affinity for the toxin antigen will be able to prevent its ganglioside attachment, thereby effectively neutralizing its toxic activity. Thus, IgG and IgA antibodies, which usually show higher affinity toward antigen than do IgM antibodies, are likely to be more effective than the latter in the neutralization reaction.

The demonstration of toxin-neutralizing abilities in both IgG and SIgA raised the interesting question of their relative roles in protection against cholera. The functional superiority of serum IgG over local SIgA suggested serum-derived protection. Evidence in favor of this idea is available from studies (3, 19, 20) in the experimental dog model. On the other hand, the failure of high titers of circulating antitoxin to confer protection against cholera (4) suggested that a different mechanism might operate in humans. Therefore, the SIgA molecule, which is synthesized locally and is less susceptible to proteolytic digestion than IgG, seems to be better designed for effective protection against cholera toxin, whose site of synthesis and site of action within the infected host are probably close together.

Recent studies with human volunteers emphasized the role of antibacterial rather than antitoxic immunity in human cholera (13). Our results show that human antibodies of both IgM and IgG classes are agglutinating as well as vibriocidal in nature. Moreover, these data conclusively prove that human SIgA does not act as a vibriocidal antibody, even in the presence of lysozyme, although it can cause agglutination. These findings may be related to the inability of SIgA to fix complement, at least by the classical pathway (24), and are in agreement with those reported earlier with rabbit (21), mouse (8), and dog (9) SIgA antibodies to *V. cholerae*.

The vibriocidal data presented here suggest that some protective role is played by IgG and IgM antibodies provided they are present in sufficient quantities in the gut. Vibriocidal activities of pooled milk and its $M_1$ fraction (Table 3) were primarily contributed by IgM antibodies present in these samples. Nonspecific transudation of at least the IgG molecule from serum to the lumen of the gut has been shown to occur and protect infant (5) as well as adult (25) rabbits against cholera. An inverse correlation between serum vibriocidal titer and susceptibility to infection in endemic areas (16) indicates protective abilities of circulating IgG and IgM antibodies. However, it is difficult to hypothsize that the protection observed in all of these cases was mediated by a vibriocidal mechanism since very little complement may be present in the lumen of the gut (6). Moreover, the intestinal fluid of several animal species has been shown to have anticomplementary activity in an in vitro assay (22). These considerations as well as the failure of the SIgA molecule to cause vibrio lysis point to the importance of an antiadherence mechanism (6). It is evident from these data that purified human antibodies of three classes, i.e., IgM, IgG, and SIgA, could significantly inhibit vibrio adhesion to intestinal slices even at subagglutinating dilutions (Table 4). Moreover, the inhibitory capacities of SIgA and IgM appeared to be quite comparable and somewhat higher than that of IgG. Extrapolation of these data to the situation in vivo would lead one to predict the importance of both the circulating and secretory antibodies in inducing protection by inhibiting vibrio adherence to the intestinal epithelium and thereby facilitating their removal by peristalsis. The antiadherence properties of rabbit serum antibodies to *V. cholerae* have been demonstrated in vitro (7), although no information is available on the relative contributions of IgG and IgM in this respect.

Antibacterial and antitoxic properties of human IgM, IgG, and SIgA show interesting variations (Table 5). To our knowledge, this is the first report evaluating the biological properties of these classes of human immunoglobulins in relation to cholera. Unfortunately, we have been unable to correlate these properties on a more quantitative basis. Further studies with purified antibodies specific to somatic and toxin antigens of *V. cholerae* are needed to provide such information as well as to evaluate their protective functions directly. However, these data clearly identify IgG as the most potent antibody in neutralizing cholera toxin activity, whereas both IgM and IgG have effective vibriocidal and antiadherence mechanisms. Evidently, the important finding is the demonstration of toxin neutralization and antiadherence properties in human SIgA which, however, fails to act by being vibriocidal. Thus, these antibodies appear to be quite capable of inducing protection against cholera provided the kinetics of their synthesis and dynamics of their transport across the mi-

TABLE 5. *Biological properties of different classes of human immunoglobulins containing anticholera antibodies*

| Antibody class | Agglutination | Vibriocidal | Antiadherence | Toxin neutralization |
|---|---|---|---|---|
| IgM | + | + | + | − |
| IgG | + | + | + | + |
| SIgA | + | − | + | + |

croenvironment of the intestinal epithelium are sufficient to counteract colonization and toxinogenesis of *V. cholerae*.

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