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Role of Immunoglobulins in Protection Against Shigella-Induced Keratoconjunctivitis

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Various immunoglobulin preparations were tested for their ability to protect guinea pig eyes from infection by a virulent strain of *Shigella flexneri*. Secretory immunoglobulin A was effective in delaying or preventing keratoconjunctivitis in eight guinea pigs when it was used to precoat the organism, and was also placed in the eye with the inoculum. Neither immunoglobulin G nor immunoglobulin M gave any protection when used in the same way. Protection by secretory immunoglobulin A appeared to be related to the antishigella antibody content of the immunoglobulin since a low-titered preparation gave less protection than a higher-titered one.

Specific immunity to shigellosis can be induced by oral immunization (3), but not by parenteral immunization despite the fact that high levels of serum antibody may be present (14). Thus, some form of local intestinal immunity appears to exist, but the mechanism for this has remained obscure. Secretory immunoglobulin A (SIgA) appears to be well suited for mediating intestinal immunity in view of its intraluminal location (9) as well as its resistance to proteolytic enzymes (1). This immunoglobulin has been shown to be excreted in large amounts during shigellosis (12), but it fails to promote killing of shigella either by complement (10, 11), or by polymorphonuclear leukocytes (W. P. Reed, Immunology, in press). The possibility that SIgA may function with macrophages or other mucosal substances to kill shigella in or on the intestinal mucosa has not been investigated, but it appears that this immunoglobulin may either participate in killing or may inhibit growth of *Vibrio cholerae* through such a mechanism (4).

Another way in which SIgA may protect against shigellosis is through prevention of epithelial penetration by the organism. This possibility seems attractive since epithelial penetration appears to be necessary before shigella can cause clinical illness (7), and since SIgA can prevent certain other organisms from adhering to epithelial cells or mucosa (5, 6, 16). It seems likely that such adherence could be required before penetration.

The studies reported here investigate the possible role of SIgA, IgG, and IgM in preventing shigella from infecting guinea pig conjunctiva. This model has been shown to correlate well with intestinal mucosa-penetrating ability of shigella (8). Results show that SIgA is capable of preventing shigella from causing keratoconjunctivitis, whereas IgG and IgM fail to prevent the infection.

MATERIALS AND METHODS

Immunoglobulins. Human SIgA was prepared from colostrum obtained from normal postpartum Albuquerque women by the method of Tomasi et al. (15) and diluted with normal saline to a concentration of 75 mg/100 ml. Neither IgG nor IgM was detectable by radial immunodiffusion. Human IgG was separated from serum by Sephadex G-200 (Pharmacia) filtration and diluted with saline to 75 mg/100 ml. Only traces of IgA were detectable in this preparation by radial immunodiffusion. Human IgM was prepared by sucrose density ultracentrifugation, and after the sucrose was removed by dialysis, the IgM was diluted in saline to 69 mg/100 ml. The preparation contained no detectable IgA or IgG by radial immunodiffusion. Each of the immunoglobulin preparations had similar 1:8 titers against the test shigella strain using the indirect hemagglutination test (12). An additional SIgA preparation had a 1:2 antishigella titer at 75 mg/100 ml.

Shigella preparations. A strain of *Shigella flexneri* (B2) which was isolated from a patient with clinical shigellosis was passed once on nutrient agar and then lyophilized in multiple vials. This strain was shown to be reproducibly active in producing keratoconjunctivitis in guinea pigs. A fresh vial was opened for each experiment, and after overnight incubation in nutrient broth, the bacterial growth was washed in saline and suspended to an optical density of 0.39 at 620 nm (Coleman Jr. II spectrophotometer). Portions of this suspension were diluted with equal volumes of each immunoglobulin preparation and an additional

portion had an equal volume of saline added as a control. All bacterial suspensions, whether diluted with immunoglobulin or saline, were then incubated at 37 C for 30 min, and before inoculation in the guinea pig eye, measured amounts were removed for further dilution and colony counting. This assured that an equivalent number of colony-forming units was inoculated in each test, and further showed that the incubation with immunoglobulins had not reduced the colony count through clumping. The inoculating suspensions contained between 2.6×10^9 and 3.2×10^9 colony-forming units per ml. In most tests where coated organisms were used, the inoculating suspension contained the unbound immunoglobulins as well as the coated organisms.

Indirect fluorescence microscopy using shigella-absorbed fluorescein-conjugated antibody (goat) to human immunoglobulins showed that the organisms were coated only with the appropriate human immunoglobulin. Radial immunodiffusion of the supernatant after incubation with the organisms revealed that the majority of the immunoglobulin remained in the supernatant and thus was not bound to the organisms.

Guinea pig eye test. In each trial, mature albino guinea pigs had one eye inoculated with untreated shigella preparations and the other eye inoculated with immunoglobulin-treated shigella. The test used was essentially that described by Serény (13). One drop of the organism-containing suspension (approximately 1.5×10^8 colony-forming units) was placed in the conjuctival sac, and the animal was observed daily at 8 a.m. and 4 p.m. for 3 days. The reaction was considered to be positive if the cornea became opaque and purulent secretions appeared; the time at which the test became positive was recorded. Data were analyzed by comparing the day that the test and control eyes became positive. In all tests the reader was unaware of the preparation being tested. Two or more guinea pigs were tested with each of the immunoglobulin-treated shigella preparations.

RESULTS

Inoculation with a mixture of SIgA-coated shigella and free SIgA greatly prolonged the time before appearance of infection or completely prevented infection in each of eight tests (Table 1). This was especially true when the preincubation mixture contained SIgA with the higher antishigella titer of 1:8, but even SIgA with a low 1:2 titer afforded protection in that it delayed the infection for 1 day. SIgA also protected for 1 day when the inoculum was increased 10-fold, to approximately 1.5×10^9 organisms. In contrast, the IgM- and IgG-containing solutions failed to provide any protection, and in fact in all tests the keratoconjunctivitis induced by shigella pretreated with these substances appeared to be worse than that produced by untreated shigella. When all eight experiments using SIgA precoating of the organisms plus free SIgA inoculated in the eye were compared with either the four IgG or the four IgM experiments using Fisher's exact test (2), then SIgA (infection delayed or prevented in all eight) was significantly more protective than IgG (no delay in infection, $P = 0.002$) and IgM (no delay in infection, $P = 0.002$). When only the four tests using 1:8 titered antishigella SIgA and an inoculum of 1.5×10^8 organisms were compared as above, then SIgA was still significantly protective when compared with IgG ($P = 0.029$) and IgM ($P = 0.029$).

Blocking experiments were also conducted to determine whether SIgA or some other substance in the SIgA preparation afforded the protection. Antiserum to IgA was first absorbed twice with packed shigella organisms equal in volume to the serum, and this antiserum was then added to the mixture after the organisms had been incubated in SIgA. No protection occurred in two tests, so it seemed likely that the SIgA was actually responsible for the protection.

In the previously described experiments SIgA protected when it was preincubated with the organisms, but since the unbound immunoglobulins from the incubation mixture were also present in the inoculum, they may have actually been responsible for the protection. Additional experiments were performed to demonstrate whether SIgA would protect when only placed in the eye or only used to coat the organisms. There was protection in only one of four tests when the same amount of SIgA used in the previous experiments was placed in the eye 15 min before inoculation with uncoated organisms. Although preincubation with SIgA appears to afford more protection (four out of four protective using 1:8 antishigella titer in 1.5×10^8 inoculum) than simply placing SIgA in the eye before inoculation with uncoated organism (one out of four protective), these studies have not shown the increased protection to be statistically significant using Fisher's exact test ($P = 0.143$). There was no protection in two tests in which shigella were incubated with SIgA and then washed so no additional unbound antibody was placed in the eye.

DISCUSSION

Under the conditions of these studies, SIgA but not IgG or IgM protected guinea pig eyes from severe infection caused by a virulent strain of *S. flexneri*. Since the guinea pig eye test correlated well with virulence of *S. flexneri* (8, 13), it seems possible that SIgA would be capable of protecting intestinal mucosa as well. However, this should be demonstrated directly.

The blocking studies showed that it was actually SIgA and not unidentified contaminants that protected the guinea pig eye. The mecha-

TABLE 1. *Effect of preincubation with immunoglobulin on the ability of shigella to cause keratoconjunctivitis in guinea pigs*

Immunoglobulin used for preincubation	Antishigella titer	No. of organisms inoculated (×1.5)	No. of eyes tested	Day of infection in comparison with control			
				Same day	1 day later	2 days later	No infection
SIgA	1:8	10^8	4		1	1	2
SIgA	1:2	10^8	2		2		
SIgA	1:8	10^9	2		2		
IgG	1:8	10^8	4	4[a]			
IgM	1:8	10^8	4	4[a]			
SIgA[b]	1:8	10^8	2	2			
No preincubation[c]	1:8	10^8	4	3	1		
SIgA + anti-IgA	1:8	10^8	2	1			

[a] Infection more severe than control.
[b] Organisms washed after preincubation.
[c] SIgA added directly to the eye.

nism for protection was not shown by these experiments, but IgA can interfere with attachment of organisms to epithelial cells, as has been demonstrated with streptococci in the mouth (6, 16) and *V. cholerae* in the intestine (5). Other possibilities, however, have not been ruled out, and it is possible that SIgA operates along with unknown humoral or cellular factors in the conjunctiva to prevent proliferation of the organisms or to kill them. It is clear that SIgA alone did not kill or even significantly agglutinate the organisms during a 30-min incubation. Previous studies have shown that SIgA does not act with complement or polymorphonuclear leukocytes to kill shigella.

Since the higher-titered SIgA preparation was more effective and since the only consistently protective preparations contained both SIgA-precoated organisms and unbound SIgA, it appears that either fairly large amounts of the immunoglobulin were required or else prolonged contact with it was necessary. SIgA-coated organisms can still proliferate in vitro, and after several generations the immunoglobulin coating the organisms would presumably be diluted to levels too low to offer protection. It seems possible that free SIgA in the eye could allow additional coating of proliferating organisms with resulting prolonged protection. There may also be a requirement that both organisms and the conjunctival epithelium must be coated for protection to occur.

It seems unlikely that the protection was simply due to steric hindrance preventing the bacteria from coming close to the epithelial cell, since subagglutinating quantities of IgA were effective and also since IgG and IgM which reacted equally in the hemagglutinating test failed to provide protection. A similar phenomenon was observed with *V. cholerae* in the intestinal tract by Fubara and Freter (5), who concluded that an active metabolic step was involved since iodoacetate prevented the SIgA induced protection.

The lack of effectiveness of IgG and IgM in protecting conjunctiva cannot be due to lack of reactivity of these immunoglobulins with the shigella strain used since all three immunoglobulins reacted in the indirect hemagglutination test. In fact, the concentrations of each immunoglobulin were chosen to provide equal reactivity in this test. The apparent increase in virulence of IgG- and IgM-coated organisms was not anticipated. The infection with these organisms occurred on the same day as with the control organisms, but the increased severity of infection with the IgG- and IgM-coated organisms was remarkable.

Prior studies which have shown SIgA to have no role in complement or polymorphonuclear leukocyte killing of shigella have, nevertheless, shown both IgG and IgM to participate in the killing (11). Therefore, SIgA may prevent the initial epithelial penetration by shigella, but once penetration has occurred the other immunoglobulins act together with complement, the properdin pathway, and phagocytes to kill the invading organisms.

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