Lipopolysaccharide-Specific but Not Anti-Flagellar Immunoglobulin A Monoclonal Antibodies Prevent *Salmonella enterica* Serotype Enteritidis Invasion and Replication within HEp-2 Cell Monolayers

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The protective potential of immunoglobulin A (IgA) monoclonal antibodies (MAbs) directed against O and H antigens of *Salmonella enterica* serotype Enteritidis to prevent bacterial adhesion to and invasion of HEp-2 cells was evaluated. Although anti-flagellar IgA MAbs showed strong agglutinating capacities, they did not protect cell monolayers. In contrast, IgA MAbs specific for the O:9 epitope of *Salmonella* lipopolysaccharide antigen alone prevented *S. enterica* serotype Enteritidis entry and replication within HEp-2 cells, and the protection was not mediated by direct binding of antibodies to bacterial adhesins or by agglutination of microorganisms.

Immunoglobulin A (IgA) is a principal mucosal antibody isotype that can bind to surface bacterial antigens and can prevent bacterial attachment to and penetration of epithelial cells (10). Host defense against *Salmonella* pathogens at the mucous membranes is mediated by nonspecific and immunologically specific mechanisms. The complexity of the mucosal defense has made it difficult to identify the specific surface epitopes that induce protective immunity and to elucidate the role of IgA in the protection. Mucosal immunization with attenuated *Salmonella* strains induces not only local but also cell-mediated and systemic humoral immune responses (6, 7, 15, 24). Most studies have focused on *Salmonella enterica* serotype Typhimurium pathogenesis, while the pathogenesis of *Salmonella enterica* serotype Enteritidis infection is poorly understood. In recent years, *S. enterica* serotype Enteritidis has proved to be the major food-borne pathogen, and its incidence has increased dramatically worldwide (12). This necessitates the profound analysis of the virulence characteristics of *S. enterica* serotype Enteritidis clinical isolates, their pathogenesis, and the immune mechanisms of host defense against infection.

The antibodies specific for surface epitopes of *S. enterica* serotype Typhi are protective against typhoid fever, and oral immunization of humans with a live *S. enterica* serotype Typhi vaccinal strain induces protective mucosal and systemic immunity (1, 3, 20, 21). *S. enterica* serotype Enteritidis, like *S. enterica* serotype Typhimurium, can cause generalized infection in mice similar to that caused by *S. enterica* serotype Typhi in humans (2). The abilities of *Salmonella* strains to invade cell culture monolayers strongly correlate with their virulence and potential to produce disease (4, 5, 9, 16). The HEp-2 invasion

assay is a suitable in vitro model to assess the abilities of different bacteria, including *Salmonella* strains, to enter and replicate within cultured epithelial cells (19). These models make it possible to assess the protective efficacy of IgA directed against the O:9 epitope common to group D *Salmonella* strains. On the other hand, most *S. enterica* serotype Enteritidis strains, like most *S. enterica* serotype Typhi strains, are monophasic and express flagellar antigens in phase 1. This allows evaluation of the protective ability of IgA specific for a single epitope of *Salmonella* flagellar antigen. In this study, we have used monoclonal antibodies (MAbs) to evaluate the protective potentials of IgA antibodies directed against flagellar and lipopolysaccharide (LPS) antigens of *S. enterica* serotype Enteritidis.

IgA MAbs (clones 177E6 and 178) directed against LPS epitopes were produced, and their antigen specificities were characterized as described previously (8). IgA MAbs (clones 187g3, 188ND9, and 189C1) against H:g,m *Salmonella* flagellar antigen were generated after intragastral immunization with live *Salmonella enterica* serotype Suberu (3,10:g,m:−). All three MAbs were characterized as H:g epitope specific, and the production of monomeric and polymeric IgA forms was confirmed.

MAbs 177E6 and 187g3 were purified by anion-exchange chromatography on a Mono Q column (Pharmacia) as described previously (8). The other three MAbs were partially purified by ammonium sulfate precipitation of ascitic fluids. The antibody concentrations in the preparations were measured by capture enzyme-linked immunosorbent assay using purified mouse IgA MOPC 315 (Cappel) as a standard antibody, and all MAbs were sterilized by filtration through 0.22-μm-pore-size filters (Millipore).

The agglutinating properties of IgA MAbs were tested by slide agglutination tests with the *S. enterica* serotype Enteritidis type strain (ATCC 13076) and with eight *S. enterica* serotype Enteritidis clinical isolates from the collection of our diagnos-

TABLE 1. Agglutinating properties of IgA MAbs in slide agglutination test

MAb[a]	Titer[b]		Agglutinating concn (μg/ml)[c]
	0.5 μg/ml	5 μg/ml	
177E6		4–8	1.25
178H11		2–4	1.5
187g3	2–4	32–64	0.1
188ND9	4–8	32–128	0.1
189C1	4–8	128	0.05

[a] Purified MAbs were serially diluted in PBS.
[b] Endpoint titer ($1/T$) in slide agglutination test with *S. enterica* serotype Enteritidis type strain (ATCC 13076) and eight clinical isolates at indicated MAb concentration.
[c] Antibody concentration sufficient to agglutinate *S. enterica* serotype Enteritidis clinical isolate 5293 used in protection assays.

tic laboratory (the results are shown in Table 1). IgA MAbs did not reveal in vitro bactericidal activity alone or in the presence of complement (data not shown).

HEp-2 cells were cultured in 24-well plates in RPMI 1640 medium (Gibco). Confluent monolayers were infected with 10^7 exponentially growing bacteria as described previously (19). To evaluate the protective potential of monoclonal IgA, purified MAbs were diluted in fresh RPMI 1640 medium and mixed with the bacterial inoculum. The plates were incubated at 37°C for 3 h, which was sufficient time for bacterial entry into HEp-2 cells. For the last 30 min of incubation, 100 μg of gentamicin (Sigma)/ml was added to kill extracellular bacteria. Then, the monolayers were washed six times with phosphate-buffered saline (PBS) and lysed with 0.5% sodium desoxycholate (Merck) in distilled water. Serial 10-fold dilutions of cell lysates were plated on Trypticase soy agar (Difco), and the number of CFU per well was calculated after overnight cultivation. A highly invasive *S. enterica* serotype Enteritidis strain (clinical isolate no. 5293) was selected for the antibody protection assays. More than 20% of the initial bacterial inoculum
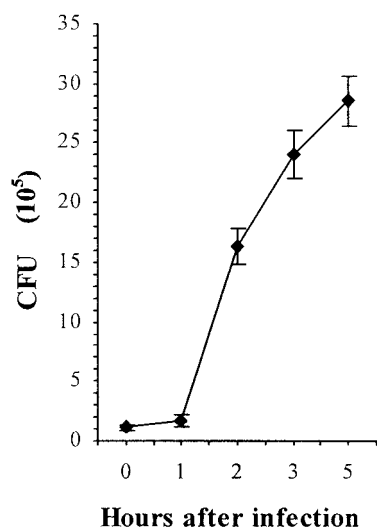

FIG. 1. Kinetics of *S. enterica* serotype Enteritidis strain 5293 growth in HEp-2 cells (in CFU per well). The confluent monolayers were inoculated with 10.6×10^6 bacteria per well, and each of the samples was run in six wells.

was protected from gentamicin killing after 3 h of infection (Fig. 1). Purified IgA MAb MOPC315 was used as an antibody isotype control. MAb 8aC10 of IgG3 isotype specific for the O:5 LPS antigen of *Salmonella* was also used as a control antibody, and *S. enterica* serotype Typhimurium strain C5 was used as a control bacterial strain. All MAbs were applied in two final concentrations—0.5 and 5 μg/ml. To determine whether bacterial LPS takes part in bacterial attachment, cell monolayers were pretreated with purified *S. enterica* serotype Enteritidis LPS for 1 h before inoculation with bacteria.

The number of bacteria recovered after 3 h of incubation with control MAbs (MOPC315 and 8aC10) and with low (0.5-μg/ml) concentrations of specific IgA MAbs was similar to that with the control without antibodies (Table 2). In concentrations of 5 μg/ml, anti-LPS IgA 177E6 and 178H11 considerably inhibited *S. enterica* serotype Enteritidis entry into HEp-2 cells. For example, the number of viable intracellular bacteria was reduced more than six times in the presence of MAb 177E6. Although the anti-flagellar antibodies possessed greater agglutinating capacity, none of the three H:g,m-specific IgA MAbs (187g3, 188ND9, and 189C1) prevented *S. enterica* serotype Enteritidis entry into and multiplication in HEp-2 cells. Invasion of monolayers by *S. enterica* serotype Typhimurium C5 was not affected in the presence of IgA MAbs, indicating that the mechanism of protection was immunologically specific for *S. enterica* serotype Enteritidis and directed against a single O:9 LPS epitope (data not shown).

Recent data suggest that centrifugation of the initial inoculum onto HEp-2 monolayers might bypass the important role of bacterial motility (23). To avoid that and to assess the abilities of IgA MAbs to prevent the initial attachment of bacteria to the cells, the assay was performed as described above but without the steps of centrifugation and gentamicin killing. The plates were incubated for 1 h at 37°C and, after six washes with PBS, were lysed with 0.5% sodium desoxycholate. A bacterial adhesion assay with centrifugation onto glutaraldehyde-fixed monolayers was performed as described previously (14).

Both anti-LPS MAbs significantly inhibited the initial adhesion of *S. enterica* serotype Enteritidis strain 5293 to HEp-2 cells (Table 2). The results were similar to those obtained with glutaraldehyde-fixed monolayers. The anti-flagellar MAbs did not prevent initial bacterial attachment to the cell monolayers. In the protective concentration of 5 μg/ml, anti-LPS MAbs agglutinated bacteria in a 1:4 dilution in a slide agglutination test (Table 1). The anti-flagellar IgA in the same concentration possessed more than 10-fold-greater agglutinating capacity (up to 1:128 for clone 189C1). Thus, the results demonstrated the absence of correlation between IgA agglutination capacity and protection of cell monolayers against bacterial entry.

In mucosal secretions, IgA directed against surface epitopes can prevent the attachment and penetration of microorganisms. The antibacterial effect may be potentiated by nonspecific humoral factors of mucosal defense, such as lysozyme, lactoferrin, and lactoperoxidase (10). IgA antibodies alone can protect epithelial cells against bacterial adherence and invasion in the absence of other immune and nonimmune protective mechanisms. IgA binding can be directed to the adhesion molecules, thus inhibiting the initial step of attachment to a surface receptor on epithelial cells. IgA binding to surface

TABLE 2. Effect of IgA MAbs on bacterial attachment and entry of *S. enterica* serotype Enteritidis into HEp-2 cells

Assay	No. of bacteria[a]								
	Control	MOPC315	8aC10	177E6	178H11	187g3	188ND9	189C1	LPS
Invasion[b]	26.0 ± 3.5	25.3 ± 2.9	26.6 ± 3.1	3.9 ± 1.1	8.1 ± 3.3	22.8 ± 2.6	23.8 ± 2.7	27.3 ± 5.7	28.2 ± 3.5
Adhesion[c]	4.2 ± 0.6	4.1 ± 0.9	4.3 ± 1.2	1.5 ± 0.3	1.5 ± 0.6	4.2 ± 1.1	3.9 ± 1.3	4.1 ± 1.2	ND
Adhesion[d]	19.7 ± 1.9	20.4 ± 2.0	ND	8.5 ± 2.1	8.6 ± 2.8	20.4 ± 3.7	22.1 ± 4.4	ND	20.6 ± 1.5

[a] Mean (10^6 CFU) per well (with standard deviation) with indicated MAb; initial inoculum, 9.2×10^6 CFU of *S. enterica* serotype Enteritidis clinical isolate 5293 per well; control, bacteria without antibodies; ND, not defined.
[b] Invasion of bacteria into cell monolayers after centrifugation of the inoculum and 3-h incubation.
[c] Adhesion assay performed like invasion assay after 1 h of incubation but without centrifugation and gentamicin treatment.
[d] Adhesion to glutaraldehyde-fixed monolayers after centrifugation of the inoculum and 3-h incubation.

epitopes may also interfere with the adhesins for steric reasons, preventing adherence by an indirect mechanism (10). In our study, anti-LPS IgA in an agglutinating concentration of 5 μg/ml prevented invasion of HEp-2 cell monolayers by a large initial *S. enterica* serotype Enteritidis inoculum (10^7 CFU per well). Pretreatment of HEp-2 monolayers with 10 μg of purified *S. enterica* serotype Enteritidis LPS/ml for 1 h prior to inoculation had no effect on bacterial entry, indicating that LPS did not take part in the initial attachment as an adhesion molecule (Table 2). Thus, one possible explanation of protection is that binding of IgA antibodies to LPS may interfere with bacterial adhesins for steric reasons.

Secretory dimeric and polymeric IgA forms are effective agglutinators. It has been reported that monoclonal IgA antibody specific for LPS antigen protected mice against oral challenge with a lethal dose of a virulent *S. enterica* serotype Typhimurium strain (13) and, in agglutinating concentrations, prevented bacterial entry into polarized MDCK cell monolayers (14). Although the reported results demonstrate a correlation between agglutinating ability and protection, there is no direct evidence for the suggested important role of IgA-mediated agglutination. The molecular structure of mouse IgA shows some differences from the conventional immunoglobulin structure (22), and generation of stabile Fab fragments is not possible (14). In our investigation, we demonstrated that strong agglutinating anti-flagellar IgA alone is not protective against *S. enterica* serotype Enteritidis invasion. Bacterial motility enables pathogens to penetrate the mucous layer and attach to host epithelial cells even when fimbriae are not present (11, 17). Very recently, it was reported that flagella are necessary for full *S. enterica* serotype Typhimurium pathogenicity (18). However, the mucosal defense is complex, and IgA specific for *Salmonella* flagellin in vivo may contribute to protection in concert with other mechanisms. Agglutination of the pathogens and inhibition of their motility within mucous secretions in cooperation with ciliary action and peristaltic clearance prevent their attachment and promote their removal.

In conclusion, IgA antibodies directed against the O:9 epitope of *Salmonella* LPS alone can prevent bacterial adhesion to and entry into epithelial cells. In contrast, IgA specific for H:g,m *Salmonella* flagellin in high agglutinating concentration is not protective in HEp-2 cell monolayers in an in vitro model, suggesting the absence of correlation between the agglutinating abilities of antibodies and protection. *S. enterica* serotype Enteritidis LPS does not mediate attachment to HEp-2 cells. Thus, the mechanism of anti-LPS IgA protection is not mediated by direct binding to adhesion molecules or by bacterial agglutination.

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