Monoclonal Immunoglobulin A Antibody Directed against Serotype-specific Epitope of *Shigella flexneri* Lipopolysaccharide Protects against Murine Experimental Shigellosis

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Summary

To determine the role of humoral mucosal immune response in protection against shigellosis, we have obtained a monoclonal dimeric immunoglobulin A (IgA) antibody specific for *Shigella flexneri* serotype 5a lipopolysaccharide (mIgA) and used a murine pulmonary infection model that mimics the lesions occurring in natural intestinal infection. Adult BALB/c mice challenged with 10^7 *S. flexneri* organisms developed a rapid inflammatory response characterized by polymorphonuclear cell infiltration around and within the bronchi and strong systemic interleukin 6 response. Implantation of hybridoma cells in the back of mice, resulting in the development of a myeloma tumor producing mIgA in the serum and subsequently secretory mIgA in local secretions, or direct intranasal administration of these antibodies, protected the animals against subsequent intranasal challenge with *S. flexneri* serotype 5a. Absence of histopathological lesion and significant decrease in bacterial load of the lungs and of systemic interleukin 6 response were the three major criteria of protection. This protection was shown to be serotype-specific and dependent on local concentration of mIgA. These data demonstrate that mucosal antibodies directed against a single polysaccharidic surface epitope of *Shigella* can protect against the disease.

Shigella flexneri, a gram-negative bacillus, is the major etiological agent of the endemic form of shigellosis, a dysenteric syndrome causing a high rate of mortality among infants, particularly in developing countries. It causes disease by invading the epithelial layer and the lamina propria of the colon (1). A major characteristic of the infectious process is the occurrence of an acute inflammatory reaction of mucosal tissues. Recently, confirming in vitro demonstration that *S. flexneri* is unable to invade the apical pole of colonic cells (2) and that polymorphonuclear cells (PMN) assist it in reaching the basal side of epithelial cells, where it can invade (3), in vivo evidence has been provided that, at the early stage of infection, *S. flexneri* enters the epithelial barrier essentially through M cells (4) that cover the dome of lymphoid follicles, and that subsequent invasion and destruction of the epithelium is primarily due to the immigration of PMN, which destroy cohesion of the epithelial barrier (5). The proinflammatory cytokine IL-1, which is released by tissue resident macrophages infected by the bacteria and killed by apoptosis (6, 7), has recently been shown to play a central role in the initiation of the inflammatory process leading to PMN cell infiltration (8).

Systemic and mucosal immune responses elicited by the host, after natural or experimental infection, are mainly directed against the LPS and some virulence plasmid-encoded proteins (9–12). Protection has been shown to occur after natural or experimental infection, but the immunological correlates of protection have not yet been clearly established (13–19). It is assumed that mucosal, rather than systemic, immunity plays a major role in protecting hosts, since *Shigella* infection remains associated with the colonic mucosa and only rarely disseminates via the systemic route. Secretory IgA (sIgA)[1] antibodies, which constitute a first line of defense against pathogens, have been found in local secretions after natural infection in humans (12, 20) and in experimentally infected monkeys and rabbits (11, 21), but their protective

[1] *Abbreviations used in this paper:* Ipa, invasion plasmid antigen; mIgA, anti-*S. flexneri* 5a LPS monoclonal IgA; PMN, polymorphonuclear cell; sIgA, secretory IgA.

role remains unclear. However, an in vitro study has reported an antibody-dependent, cell-mediated antibacterial activity of intestinal lymphocytes with *Shigella*-specific sIgA (22). The protection provided by natural infection or vaccination is considered to be serotype specific, pointing to LPS as a primary target antigen for protective immunity, but experimental demonstration has never been achieved.

Therefore, the aim of this study was to identify antibodies protecting mucosal surfaces against *Shigella* infection and to determine whether protection required the presence of these antibodies in the luminal or mucosal compartments. Three conditions had to be fulfilled to achieve this goal: (*a*) to establish a mouse infectivity model; (*b*) to generate *Shigella*-specific monoclonal dimeric IgA antibodies; and (*c*) to deliver the antibodies into mucosal secretions. Since mice do not develop intestinal infection, we adapted a previously described model of infection (23), in which mice are challenged intranasally with virulent *Shigella*. It was shown that bacteria invaded the bronchial epithelium and triggered an intense inflammatory response with acute suppurative polymorphonuclear infiltrates and epithelial necrosis that resembled the elementary lesions observed in shigellosis. An anti–*S. flexneri* 5a LPS monoclonal IgA (mIgA) was obtained by fusing Peyer's patch lymphoblasts from orally immunized mice with myeloma cells, as previously described (24), and mucosal delivery was achieved either by a "back pack" tumor procedure (25) or by intranasal administration. We show here that mIgA present in bronchoalveolar secretions protects the respiratory mucosa of mice against infection, after intranasal challenge.

Material and Methods

Bacterial Strains. The two wild-type *S. flexneri* strains, M90T (serotype 5a) and 454 (serotype 2a), were routinely grown on trypticase soy broth (TCS, Diagnostics Pasteur, Marnes la Coquette, France) at 37°C with aeration. For intranasal infection of mice, an overnight culture was diluted in sterile physiological serum to obtain a suspension of 5×10^8 bacteria/ml.

Production and Screening of IgA Hybridomas. Five female BALB/c mice were immunized orally on day 0 by gastric intubation of 10^{11} live cells of the *S. flexneri* serotype 5a strain (M90T) plus 5 μg of purified cholera toxin (Sigma Chemical Co., St. Louis, MO) in 0.2 M sodium bicarbonate. Immunizations were repeated at day 10 and day 20. On day 24, mice were killed by cervical dislocation, and the Peyer's patches (7–10 per mouse) were excised. Peyer's patch lymphocytes were isolated by collagenase digestion, pooled, washed in RPMI 1640 tissue culture medium, and fused with P3X63/Ag8U.1 mouse myeloma cells as previously described (24). After a 14-d culture, hybridoma screening for anti-*Shigella* activity was performed by ELISA, using an *S. flexneri* 5a whole-cell lysate as antigen. Positive hybridomas were subcloned by limiting dilution, and Ig isotype was determined by an isotype-specific ELISA kit (Boehringer Mannheim Corp., Indianapolis, IN) according to the manufacturer's instructions.

Characterization and Purification of Anti-LPS mIgA. Anti–*S. flexneri* 5a hybridomas were further characterized as follows: ELISA were performed using *S. flexneri* 5a LPS extracted according to Westphal (26) as antigen. mIgA were purified from ascitic fluid obtained from pristane-primed BALB/c mice in which 10^6 cloned hybrid cells had been injected intraperitoneally. After collection, ascitic were precipitated with ammonium sulfate at a final concentration of 50%. After 30 min at room temperature, the ammonium sulfate–precipitated supernatant was centrifuged at 7,000 *g* for 30 min, and the resulting pellet was resuspended in 4 ml of PBS. After overnight dialysis against PBS, the solution was applied onto an ACA3/4 column (Pharmacia, Saint Quentin-Yvelines, France). Elution was performed with 0.5 M sodium chloride in PBS. Each 5-ml fraction of elution was tested by measuring both the amount of proteins at OD_{280} nm and the reactivity in ELISA against specific LPS. Fractions corresponding to the higher reactivity in ELISA were pooled and dialyzed against PBS, and aliquots were frozen at −20°C. To determine whether mIgAs recognized a common or a serotype-specific LPS epitope, purified mIgAs were further tested in ELISA, using purified LPS from *S. flexneri* 5a or 2a as antigens.

ELISAs. ELISAs were performed as previously described (27), with the following modifications. To test the specificity of the monoclonal IgA antibodies, wells were coated with either 5 μg/well of *S. flexneri* 5a whole-cell lysates or 1 μg/well of *S. flexneri* 5a– or 2a–purified LPS. Purified *S. flexneri* 5a LPS (1 μg/well) was used to determine the concentration of mIgA in serum and bronchoalveolar wash specimens. The concentration of unknown specimens was determined from the standard regression curve constructed for each assay by using a solution of purified mIgA at 1.7 mg/ml. Concentrations were calculated for dilutions giving values in the mid-range (linear portion) of the standard ELISA curve. Alkaline phosphatase goat anti–mouse IgA conjugate (Sigma Chemical Co.) was used at a 1:2,500 dilution as secondary antibody.

In Vivo Protection Experiments. The back pack tumor model was performed as previously described (25). mIgA serum levels were measured by ELISA in mice developing a tumor. These mice were then intranasally challenged with 20 μl of a *S. flexneri* 5a or *S. flexneri* 2a culture at 5×10^8/ml. This inoculum was 10-fold less than the inoculum required for the LD_{50} in this model. For each experiment, naive BALB/c mice were concomitantly challenged with the same inoculum. 1 d after the challenge, mice were tail bled, and serum IL-6 levels were measured. Representative mice were killed, and their lungs were removed from the thoracic cavity after being filled with paraformaldehyde for histopathological analysis. For intranasal administration of mIgA, mice were inoculated with different amounts of the purified antibody in a volume of 20 μl 1 h before being challenged as described above. At 6 h after infection, serum IL-6 levels were measured, specimens were taken for histopathological analysis, and bacterial counts in lung tissues were performed. For the latter experiments, mice were killed by cervical dislocation, and lungs were dissected and placed in 10 ml of ice-cold 0.9% NaCl, and then ground with an Ultra-turrax apparatus (Janke and Kunkel, GmbH and Co., Staufen, Germany). Serial dilutions of the resulting solution were plated on Congo red agar and incubated overnight at 37°C. For each experiment corresponding to a given amount of antibody administered intranasally, a control group of naive mice was concomitantly challenged. For the back pack tumor model or the intranasally administered purified mIgA experiments, each experiment was comprised of 10 mice per group and was repeated three times.

Bronchoalveolar Wash Specimens. Mice were killed by cervical dislocation. After tracheotomy, bronchoalveolar wash specimens were obtained by injecting 1 ml of 0.9% NaCl twice. Possible blood contamination of bronchoalveolar secretions was estimated by counting the number of red blood cells and comparing it with the counts of a serially diluted blood sample (28). These specimens were stored at −20°C until tested.

IL-6 Measurement. IL-6 activity was determined by using the specific 7TD1 IL-6–dependent cell line as described previously (29).

Histopathological Studies. Lung specimens were fixed in a mixture of 0.25% glutaraldehyde and 4% paraformaldehyde in PBS, pH 7.2, for 48 h before embedding in paraffin or Lowicryl (K4M; Miles Inc., Naperville, IL) resin. Paraffin-embedded 4-mm-thin sections were stained with hematoxylin and eosin, whereas Lowicryl-embedded 2.5-mm-thin sections were stained with toluidine blue and processed for light microscopy and immunocytochemistry. To detect *S. flexneri* in infected lungs, sections were first incubated with 0.1% BSA-containing PBS to quench free aldehyde groups and then with biotinylated mIgA at a concentration of 3 μg/ml and streptavidin-FITC (Amersham Life Science, Les Ulis, France) at a dilution of 1:200. Controls included a nonrelevant biotinylated monoclonal IgA antibody used at the same concentration as the anti-LPS mIgA.

Statistics. Significant differences in bacterial counts and serum IL-6 levels after *S. flexneri* challenge were compared for the IgA-treated mice group and the naive mice group using the Student's *t* test. Probability values <0.05 were considered significant.

Results

Time Course of Lung Lesions in Mice Infected Intranasally with S. flexneri 5a. Histopathological lesions of naive mice infected intranasally with 10^7 *S. flexneri* microorganisms were characterized by a strong inflammatory reaction. During the first 6 h after infection, the morphology of the lung was indistinguishable from that of control mice (Fig. 1 *A*). At 6 h, the first signs of acute bronchopneumonia were seen, characterized by edema and diffuse polymorphonuclear and mononuclear cell infiltration of bronchial and peribronchial tissues with concomitant accumulation of debris and pus cells in the lumen of the bronchioles (Fig. 1 *B*). The inflammatory process primarily infiltrated the proximal region of the lobes, while the periphery was rarely affected. At 24 h, the bronchial and bronchiolar epithelium were partially destroyed and the subjacent mucosa appeared strongly infiltrated (Fig. 1 *C*). Inflammatory cells also were evident in the neighboring alveolar interstitium (Fig. 1 *D*). Although the maximal inflammatory response occurred 24 h after the challenge, the response was not qualitatively different from that seen at 6 h. No lesion was observed after intranasal infection with a noninvasive strain.

The fate of *S. flexneri* 5a bacteria after intranasal infection was examined by immunofluorescence labeling of bacteria within lung tissue and by electron microscopy. 2 h after challenge, the microorganisms were detected at the surface of bronchial epithelial cells (Fig. 2 *A*). 4 h later, intracellular proliferation of the bacteria was restricted to the bronchial epithelium (Fig. 2 *B*). At 24 h, intracellular microorganisms were detected in the bronchial epithelium, the lumenal pus

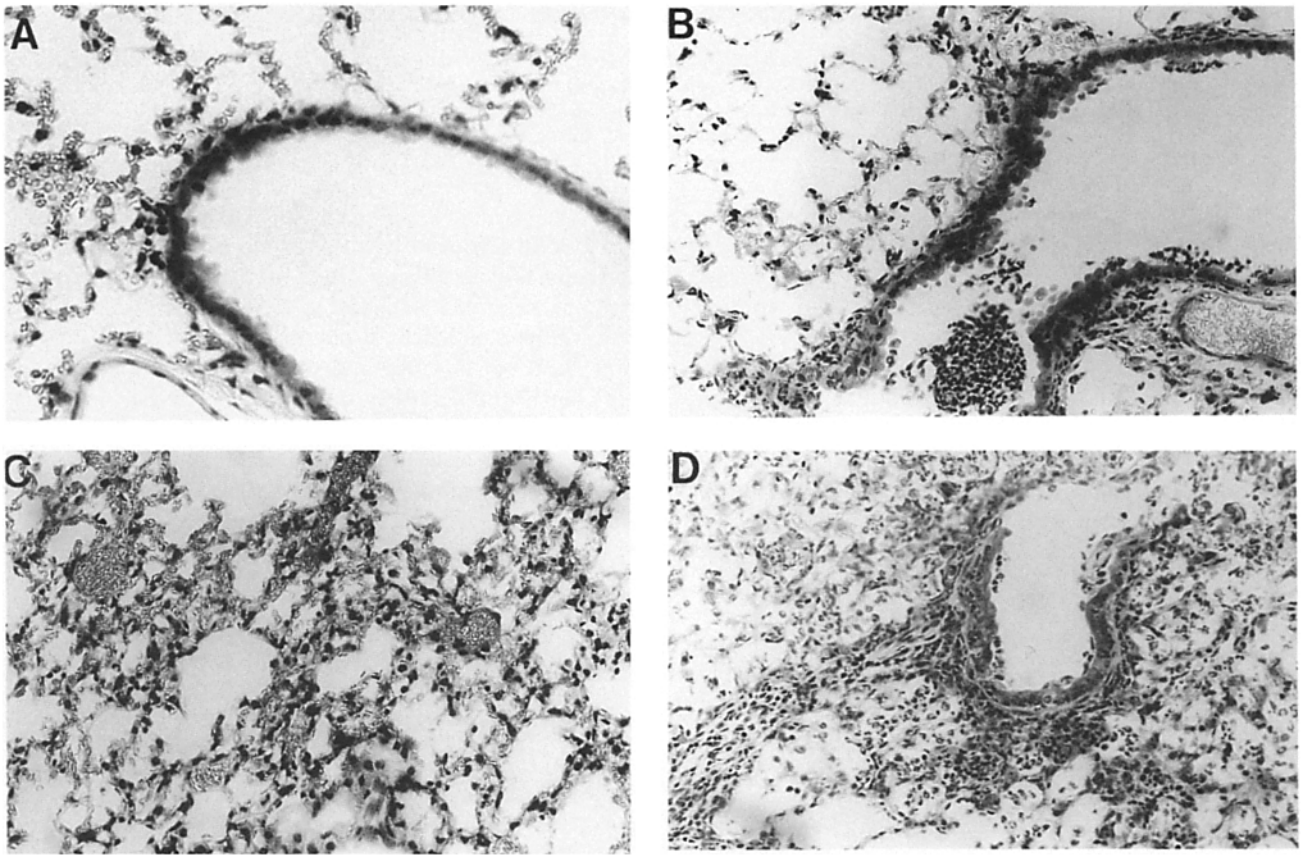


Figure 1. Histopathological lesions of mouse lungs after intranasal infection with *S. flexneri* 5a. (*A*) Uninfected mouse. Note the absence of inflammatory infiltrate around the bronchus and within alveoli. ×300. (*B*) 6 h after infection. PMN begin to accumulate around the bronchi, and pus is present in the bronchial lumen. ×200. (*C* and *D*) 24 h after infection. Heavy infiltration of inflammatory cells within alveoli (*C*) and around the bronchi (*D*). ×200.

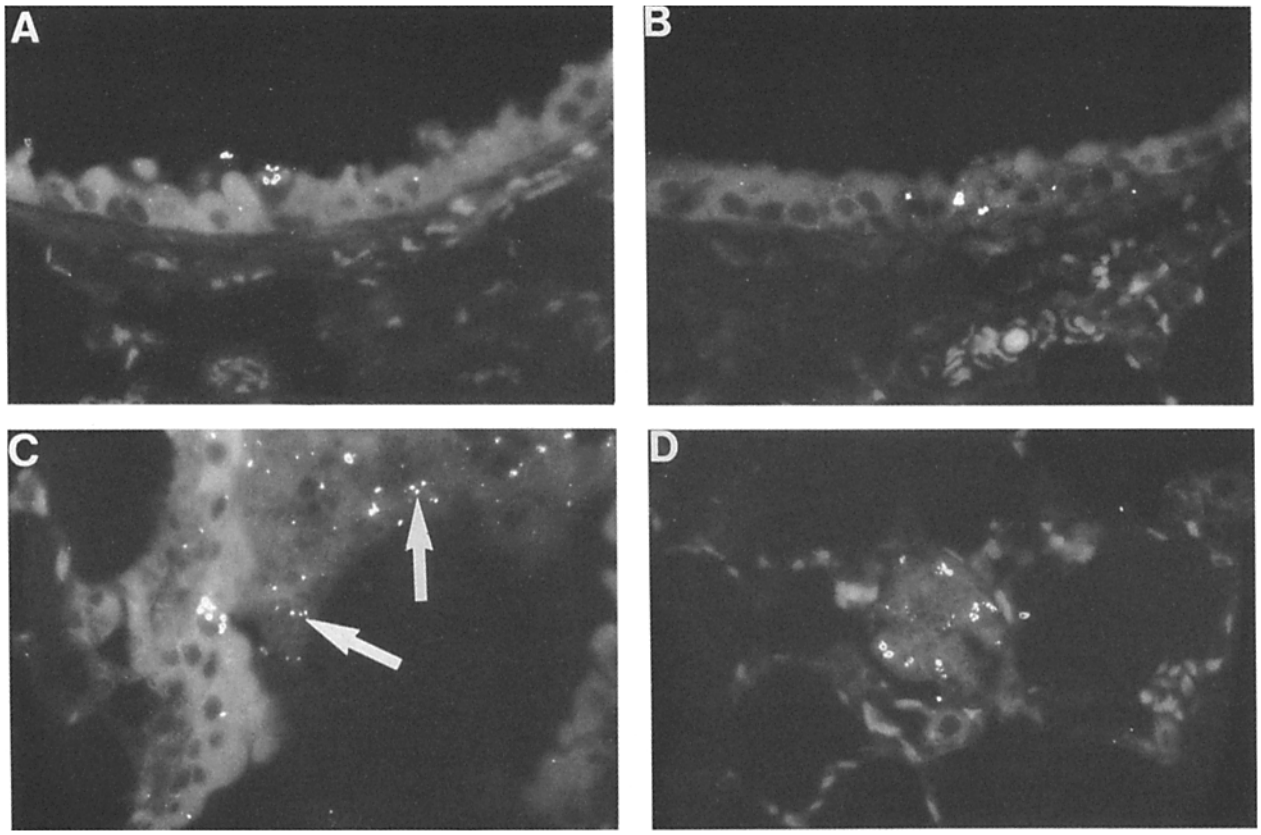


Figure 2. Localization of bacteria after intranasal infection by immunofluorescence labeling. Bacteria were revealed after incubation of lung sections with biotinylated mIgA followed by FITC-streptavidin. (*A*) 2 h after infection. Bacteria are associated with the cell surface. ×550. (*B*) 6 h after infection. Microorganisms are found in bronchial epithelial cells. ×500. (*C* and *D*) 24 h after infection. Bacteria are found as clusters within the bronchial epithelium and the lumenal pus (*C*), where bacterial debris can be visualized (*arrows*) and in the alveoli lumen (*D*).

(Fig. 2 *C*), and alveoli (Fig. 2 *D*). In addition, bacterial debris was associated with the luminal pus (Fig. 2 *C*, *arrows*).

The bacterial load in the lungs during the time course of infection was measured, and results are presented in Fig. 3 *A*. Only 5% of the inoculum (i.e., 5×10^5 bacteria) reached the lungs after intranasal administration (Phalipon, A., unpublished data). Consequently, the bacterial load measured 6 h after infection ($265 \pm 224 \times 10^5$ bacteria) reflected bacterial multiplication previously observed within the bronchial epithelium (Fig. 2 *B*). At both 24 and 48 h after infection, the bacterial load drastically decreased ($9 \pm 22 \times 10^5$ and $1 \pm 3 \times 10^5$ bacteria, respectively).

The inflammatory process was also evaluated by measuring the production of IL-6, a mediator of both acute and chronic inflammatory reaction (30). During the time course of infection, IL-6 appeared in the serum (Fig. 3 *B*). Interestingly, the serum IL-6 levels measured at 24 and 48 h after infection (2,500 ± 1,900 U/ml and 1,039 ± 910 U/ml, respectively) remained as high as those measured 6 h after infection (1,414 ± 374 U/ml) (Fig. 3 *B*), whereas the number of bacteria present within lung tissues significantly decreased during this period (Fig. 3 *A*). A large amount of bacterial debris, revealed by immunolabeling with mIgA (Fig. 2 *C*) and not observed in a control using a nonrelevant monoclonal IgA antibody, indicated that a large pool of LPS was present that was not associated with viable bacteria. This pool of LPS remained present within the bronchial epithelium during the time course of infection and was likely to account for the high IL-6 level observed in the presence of few viable bacteria.

Production of Monoclonal Dimeric IgA Antibodies Directed against S. flexneri 5a. Hybridomas producing mAbs were obtained as described in Materials and Methods. Two hybridomas were selected after screening supernatants for *S. flexneri* 5a–specific IgA antibodies by an ELISA using purified serotype 5a LPS as an antigen, and the mAb with the highest affinity was used in this study. The hybridoma was expanded and subcloned twice by limiting dilution, and antibodies were purified from ascitic fluids.

The *S. flexneri* O antigen basic structure is composed of three rhamnose and one *N*-acetyl glucosamine residue linked to each other by specific α1-3, α1-2, β1-2 links. This basic structure specifies the *S. flexneri* Y serotype. Specific linking of an additional glucosyl residue to one of the three rhamnose residues specifies the *S. flexneri* serotype 5a or 2a. To define whether the antibody recognized a common or a serotype-specific determinant on the LPS molecule (31), the

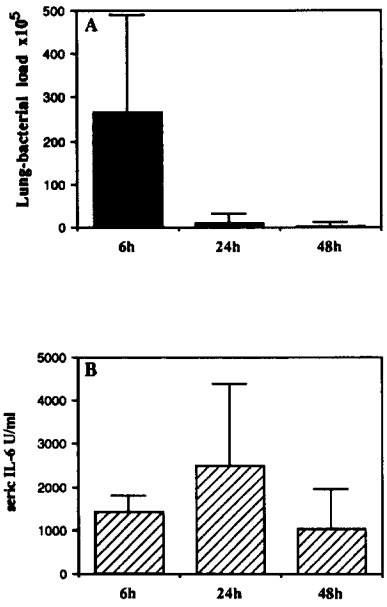


Figure 3. Bacterial load in lung tissues (*A*) and corresponding serum IL-6 level (*B*) during the time course of infection. Three groups of mice (n = 10) infected intranasally with *S. flexneri* 5a were bled for serum IL-6 measurement (*B*) and then killed to determine the corresponding lung bacterial load (*A*) at 6, 24, and 48 h after infection, respectively. Means and standard deviations are indicated.

purified mIgA was tested for its reactivity with purified LPS from *S. flexneri* serotypes 5a, 2a, and Y. The antibody recognized 5a LPS but failed to react with 2a LPS and Y LPS (Fig. 4). This suggested that mIgA was directed against a serotype-specific epitope comprising at least the glucosyl residue specifically linked to the central rhamnose of the basic structure of the *S. flexneri* LPS.

Protection against Intranasal S. flexneri Challenge by Subcutaneous Implantation of Anti-LPS Hybridoma Cells. Since receptor-mediated transport of IgA into local secretions requires antibody dimerization, we confirmed the dimeric nature of the mIgA by gel filtration. Secretion of the antibody into the systemic circulation from the hybridoma cells implanted

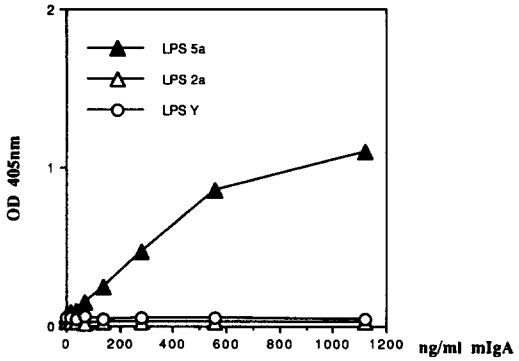


Figure 4. mIgA specificity. Dilutions of purified mIgA were incubated with purified LPS of the *S. flexneri* 5a, 2a, or Y serotype. mIgA binding was revealed using phosphatase alkaline goat anti-mouse IgA conjugate.

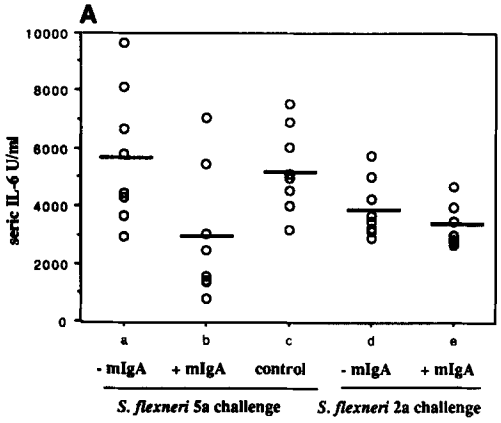
in the back of BALB/c mice was then confirmed, and efficient antibody transcytosis into bronchoalveolar secretions was demonstrated. The amount of locally transcytosed mIgA was dependent on the mIgA concentration in the serum, which was itself dependent on the tumor size (data not shown). Each tumor-bearing mouse secreted a different amount of mIgA into the serum, and consequently into local secretions.

The capacity of mIgA to prevent *S. flexneri* invasion of the bronchial epithelium was first assessed by measuring serum IL-6 levels. We previously established that IL-6 was undetectable in the serum of uninfected mice and that implantation of hybridoma cells in the back of mice did not increase serum IL-6 titers. Tumor-bearing animals were infected 21 d after hybridoma cell implantation by intranasal challenge with 10^7 *S. flexneri* bacteria, and IL-6 measurement was performed 24 h after infection. As shown in Fig. 5 *A*, after *S. flexneri* 5a challenge, a significant decrease of serum IL-6 values was observed in mIgA-secreting mice (P = 0.02) (compare groups *a* and *b*), whereas no significant difference occurred in mice secreting a nonrelevant monoclonal IgA antibody (P = 0.67) (compare *a* and *c*). Moreover, no significant difference occurred in mIgA-secreting mice (P = 0.18) (compare *d* and *e*) after *S. flexneri* 2a challenge.

Lung histopathological observations appeared to be well correlated with serum IL-6 titers. After *S. flexneri* 5a challenge, the intensity of lung lesions depended on the amount of mIgA present in mucosal secretions. No lesion was observed in mice of which mucosal secretions contained more than 100 ng of mIgA (as measured in the total of 2 ml of lung wash) (Fig. 5 *B*, *I*). In contrast, intense PMN infiltration occurred after *S. flexneri* 2a challenge, which was independent of the local amount of mIgA (Fig. 5 *B*, *II*). A similar result was obtained in mice secreting a nonrelevant monoclonal IgA antibody when challenged with *S. flexneri* 5a.

In summary, the tumor back pack procedure enabled us to demonstrate pathogen-specific IgA antibody-mediated protection of the bronchoalveolar epithelium against *S. flexneri* invasion. This protection was shown to be serotype specific and dependent on local mIgA concentration.

Intranasal Administration of mIgA before Bacterial Challenge and Analysis of Subsequent Protection. To quantify the local concentration required to confer protection and to determine whether only secreted mIgA antibodies were involved in protecting mice from *S. flexneri* invasion or whether mIgA present in the interstitium interfered with the later stages of infection, various amounts of mIgA were administered intranasally 1 h before *S. flexneri* 5a challenge. The subsequent amount of mIgA in bronchoalveolar secretions after intranasal administration corresponded to 20% of the initial inoculum. Thus, three groups of mice, whose local amounts of mIgA (expressed as the total amount of mIgA recovered from bronchoalveolar washes performed as described in Materials and Methods) were 7, 0.5, and 0.07 μg, respectively, were challenged as previously described. To observe the role of mIgA in preventing the first stages of bacterial infection, samples were collected 6 h after infection. As shown in Fig. 6, in the presence of 7 μg (*a*) as well as 0.5 μg (*b*), but not 0.07 μg (*c*) of mIgA



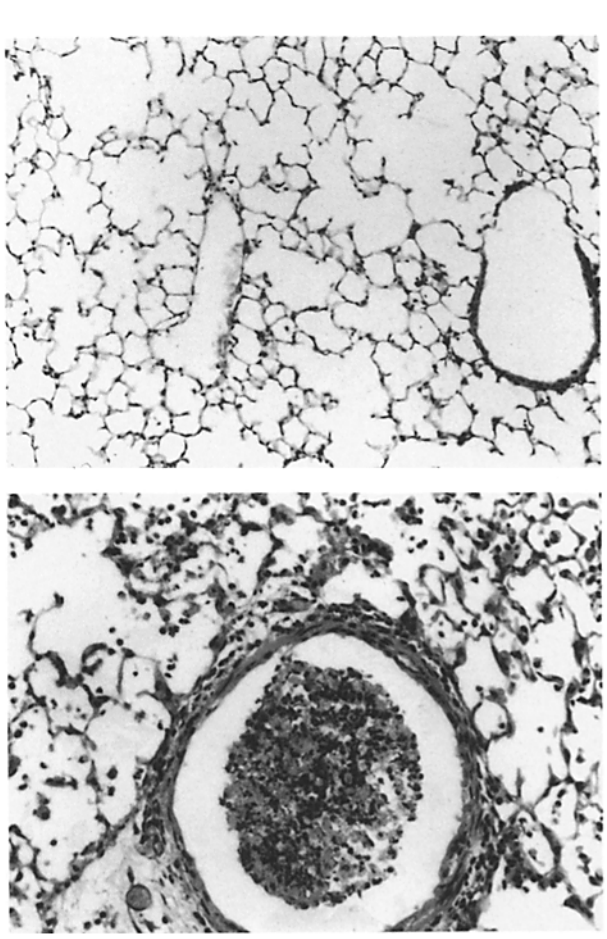


Figure 5. Protection against intranasal *S. flexneri* challenge by subcutaneous implantation of anti-LPS hybridoma cells. Protection was assessed, in hybridoma-bearing mice, 24 h after infection by measuring serum IL-6 concentration (*A*) and by lung histopathological studies (*B*). *A*, *a*–*c*, represents three groups of mice concomitantly challenged with the strain serotype 5a, in the absence of mIgA (*a*, *−mIgA*), in the presence of mIgA (*b*, *+mIgA*), and in the presence of a nonrelevant monoclonal IgA (*c*, + mIgA control). *A*, *d* and *e*, represents two groups of mice concomitantly challenged with a heterologous strain (serotype 2a) in the absence and in the presence of mIgA, respectively. The mean is indicated as a black line. (*B, I*) No lesion was observed, after homologous challenge, in mice secreting locally >100 ng of mIgA. ×100. (*B, II*) Intense inflammatory reaction after heterologous challenge. ×200.

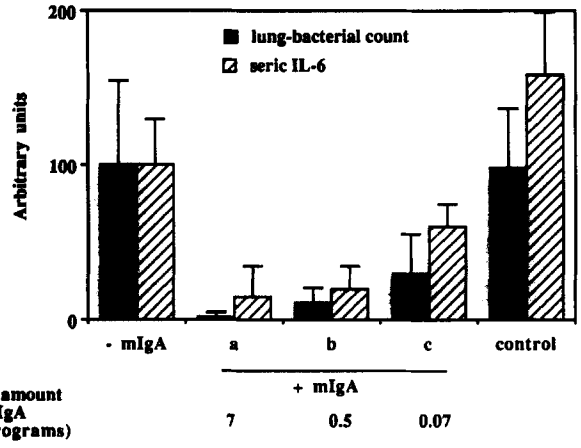


Figure 6. Intranasal administration of various amounts of purified mIgA and subsequent protection. Three different amounts of mIgA were administered intranasally, thus leading to a local amount of 7 (*a*), 0.5 (*b*), and 0.07 (*c*) μg, respectively. An anti-*Salmonella* monoclonal IgA was used as control (7 μg present locally). Serum IL-6 and lung bacterial load values obtained in the absence of antibodies (*−mIgA*) were arbitrarily defined as 100. Measurements were performed 6 h after infection. The mean and standard deviation are presented.

in local secretions, both lung bacterial load and serum IL-6 level significantly decreased compared with the control (*−mIgA*) (P = 0.001, 0.002, and 0.1, respectively. Similar P values were obtained for lung bacterial load and serum IL-6 measurements). In contrast, no significant difference was observed in the presence of 7 μg of a purified monoclonal IgA antibody directed against a carbohydrate epitope of *Salmonella typhimurium* (32) (P = 0.7 and 0.6 for lung bacterial load and serum IL-6 titer, respectively). These data demonstrated that a few hundred nanograms of mIgA were required to confer protection and that their presence in mucosal secretions only was sufficient to protect against *Shigella* invasion.

Discussion

An understanding of the basis of protective immunity against *Shigella* infection will provide fundamental information about immunity against intracellular microorganisms and will contribute towards vaccine development. In this paper, we focused our study on the role of anti-LPS IgA mucosal immunity for two reasons: (*a*) *Shigella* infection remains generally restricted to the colonic mucosa, and (*b*) LPS has been

proposed as the primary target antigen for protective immunity.

To achieve this goal, monoclonal IgA antibodies were raised, and a previously described mouse pulmonary infection model (23) was developed. This model constitutes the only available murine system, since mice do not develop shigellosis; neither clinical symptoms nor histopathological lesions are observed after oral infection at any level of the intestine. The reason for this may be that bacteria are transiently recovered from the feces, and only a few of them are recovered from Peyer's patches (Fontaine, A., and A. Phalipon, unpublished data). Antigen sampling by M cells that cover Peyer's patches is required for the induction of the mucosal immune response (33). The fact that few bacteria are found associated with the lymphoid follicles may explain the weak stimulation of B cells to commit into B cells expressing IgA at their surface, and may therefore account for the low number of IgA hybridomas obtained after fusion between myeloma cells and Peyer's patch lymphoblasts from orally infected mice.

Like the rabbit ligated intestinal loop and macaque monkey dysentery infection models (5, 34, 35), the mouse pulmonary infection model is characterized by the development of an intense inflammatory reaction with a leukocytic exudate that accumulates, in this case, in bronchi and alveoli (23). We observed that lesions occurred as early as 6 h after infection, with an increase in tissue damage reaching a maximum at 24 h after infection. In addition to the histopathological studies, the inflammatory process was evaluated by measuring the production of IL-6, a mediator of both acute and chronic inflammatory processes, which represents an accurate marker of disease progression as reported for other experimental models of infection (36). Production of IL-6 was shown to remain high, even in the presence of only few bacteria within the bronchial epithelium. Concomitantly, free LPS was detected, remaining associated with the epithelium as previously noticed in the rabbit infection model (Sansonetti, P., manuscript in preparation). Therefore, it is likely that free LPS accounts for the amplification and/or the duration of this striking inflammatory process. Recently, the persistence of inflammation was noted in *Shigella*-infected patients whose disease was clinically resolved (37). An up-regulation of cytokine-producing cells, among which are cells producing IL-6, was actually observed 30 d after the onset of disease. Whether the presence of free LPS within the enterocytes and/or the lamina propria is responsible for this phenomenon remains to be studied.

In agreement with results obtained previously using the rabbit ligated ileal loop model (5), we found a low number of intracellular microorganisms within tissues over the time course of infection. This may be due to the intense inflammatory reaction, mediated in part via activated PMN cells, which release antibacterial products (38). This observation emphasizes the double role of inflammation in shigellosis, i.e., to promote invasion as well as to act as a defense strategy.

Our results demonstrate that mucosal immunity directed against LPS plays a major role in protecting the host against shigellosis. Actually, monoclonal IgA antibodies present in the bronchoalveolar secretions of mice and directed against a serotype-specific polysaccharidic epitope of *S. flexneri* 5a LPS were necessary and sufficient to confer protection against intranasal challenge with the homologous strain. The quantity of local antibodies required for protection was estimated as a few hundred nanograms of antibody, which corresponds to the protective dose reported in previous studies of passive transfer of IgA either in the murine respiratory or intestinal tract (39, 40).

The demonstration that LPS was the main target of the protective immune response is consistent with previous epidemiological studies and vaccine field trials, which reported serotype-specific protection after *Shigella* infection (13–19). In these studies, however, antiinvasin sIgA as well as anti-LPS and antiinvasin IgG antibodies were elicited concomitantly with anti-LPS sIgA antibodies. Therefore, the actual role of each type of targets and effectors in protection has never yet really been assessed. Systemic responses are generally considered to play a minor role in protection, since *Shigella* infection remains localized to the colonic and rectal levels and only rarely disseminates systemically. Moreover, unlike IgA, few IgG antibodies are present in both intestinal secretions and lamina propria (41). However, if for any reason the local response fails to be efficient, bacterial entry through M cells would consequently occur, initiating via the release of IL-1 from apoptotic macrophages (7, 8) the characteristic inflammatory process. The latter may lead to anti-LPS and/or antiinvasin IgG antibody transudation from the serum into the lamina propria, thus contributing to a limited tissue invasion.

It has been hypothesized that the sIgA antibodies directed against the invasins invasion plasmid antigen (Ipa) B and IpaC may also contribute to protection by neutralizing the invasive process. Recent in vitro data clearly show that Ipa proteins, including IpaB and IpaC, are retained in the bacteria cytoplasm, with only small amounts being secreted. Their secretion has been shown to occur after epithelial cell contact or in response to a soluble factor sensed by *Shigella* and not yet identified (42). The exact secretion response of *Shigella* during entry into epithelial cells in vivo remains unknown. If they are not secreted from bacteria present in the lumen, but are secreted only from bacteria reaching the basolateral pole of the enterocytes where invasion occurs subsequently to the initiation of the inflammatory process (2, 3, 5), it becomes difficult to imagine a role of antiinvasin IgA antibodies as first line of protection.

In summary, we propose that anti-LPS, IgA-mediated immune response only is efficient as a primary line of protection, whereas anti-LPS IgG and antiinvasin IgG or IgA antibodies may only be effective to a lesser extent, as a second line of defense. This is reminiscent of a study on the mechanisms of neutralization of influenza virus, which reports that sIgA and monomeric IgA or IgG are able to confer protection at different stages of the viral pathogenic process (43).

Intranasal administration of purified mIgA has been useful to demonstrate that the presence of the antibody in the luminal compartment was sufficient to confer protection. This suggests that protection can occur at the initial stage of in-

fection by preventing invasion and that the presence of mIgA in the interstitial compartment for blocking the later stages of the pathogenic process is not required. It is worth noting that this phenomenon, generally known as the "immune exclusion" mechanism, is generally seen as the major mechanism of IgA-mediated protection (44). This mechanism has been reported in several studies, both in vitro and in vivo, of IgA-mediated protection against pathogens such as *S. typhimurium* and *Vibrio cholerae* (45, 46). Interestingly, in both cases, effective protection is achieved by IgA antibodies directed against a polysaccharidic epitope present on the bacterial surface. In contrast, IgA antibodies specific for a secreted protein such as cholera toxin are not efficient to confer local protection (46).

However, the reduction of the lung bacterial load in the presence of mIgA observed in this study does not necessarily mean that protection occurs exclusively via an immune exclusion mechanism. The function of IgA antibodies is considered to be augmented through interaction with phagocytic cells bearing IgA receptors. Actually, IgA-coated microorganisms at mucosal sites are able to trigger release of oxygen metabolites from monocytes or macrophages via Fcα receptors present on these cells, thus producing bacteriostatic and bactericidal effects (47). Therefore, one might imagine that sIgA-opsonized *Shigella* bacteria present in the lumen may enter the intestinal epithelium through M cells, and subsequent Fcα receptor–mediated phagocytosis by tissue-resident macrophages may trigger expression of antibacterial activities by these phagocytic cells.

In addition to immune exclusion, two additional functions for mucosal IgA have been proposed (44). One is to neutralize intracellular pathogens (i.e., virus) directly within the epithelial cells. The second is to bind antigens in the lamina propria and excrete them through the epithelium into the lumen, thereby clearing the body of locally formed immune complexes and decreasing their access to systemic circulation. Experiments will be conducted to test whether such mechanisms may achieve neutralization of the effects of free LPS within the epithelial barrier.

In conclusion, this study both contributes to our knowledge of the mucosal immune response and suggests an alternative approach to *Shigella* vaccine development. Specifically, attempts at stimulating local production of anti-LPS IgA antibodies may be sufficient to elicit protection.

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