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Antibody and Cytokine Responses in a Mouse Pulmonary Model of *Shigella flexneri* Serotype 2a Infection

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A murine pulmonary model was used to study the mucosal immune response to *Shigella flexneri* serotype 2a infection. Inoculation of BALB/cJ mice with shigellae via the intranasal route resulted in bacterial invasion of bronchial and alveolar epithelia with concomitant development of acute suppurative bronchiolitis and subsequent development of lethal pneumonia. The pathology of pulmonary lesions resembled the colitis that characterizes shigellosis in humans and primates. Significant protection against a lethal dose of *S. flexneri* 2a was observed in mice previously infected with two sublethal doses of the homologous strain. Immunity against lethal challenge was associated with decreased bacterial invasion of the mucosal epithelium. Over the course of two sublethal challenges, which constituted primary and secondary immunizations, mice developed pulmonary and serum immunoglobulin G and A antibody recognizing both lipopolysaccharide and invasion plasmid antigens IpaB and IpaC. Immune mice and naive control mice differed in lung lavage cytokine levels following lethal challenge. Immune mice developed significantly elevated levels of pulmonary gamma interferon within 6 h of challenge, while naive control mice developed elevated levels of this cytokine later during the initial 24-h period. Both groups had elevated levels of gamma interferon during the 24- to 48-h period of infection. Both groups also had elevated levels of tumor necrosis factor alpha within 6 h of challenge, but the control mice had significantly higher levels at the 48- and 72-h time points. Elevated levels of interleukin-4 were observed only in immunized mice. This cytokine appeared within 24 h and receded between 48 and 72 h. Fluorescence-activated cell sorter analysis of lung parenchymal cells showed that both groups experienced an initial influx of monocytes, but the proportion of this cell type began to recede in immunized mice after 48 h of infection, while peak levels were maintained in the control animals. These studies suggest that elements of local B lymphocyte activity, as well as Th_1 and Th_2 lymphocyte activity, may contribute to the survival of immune mice after intranasal challenge with shigellae.

Mus musculus, the laboratory mouse, is the animal of choice for most experimental medical research. Inbreeding of mouse strains enhances experimental reproducibility, and strains with well-described genetic anomalies facilitate the characterization of the immune response to infection on a molecular level. Unfortunately, utilization of the mouse for studies of the immunobiology of shigellosis has been complicated by the inability of virulent *Shigella* spp. to colonize the intestinal tract or to elicit overt disease in rodents. Streptomycin treatment renders mice susceptible to transient asymptomatic intestinal colonization with dysentery bacilli (6), and there is a progressive decrease in the duration of fecal shedding when antibiotic-conditioned mice are given multiple intragastric inoculations of a streptomycin-resistant *Shigella flexneri* strain (13). Although the latter studies helped to establish the principle of mucosal immunity in protection against shigellosis, the requirement for streptomycin-resistant strains and the absence of overt intestinal disease have discouraged the use of intragastric challenge of mice as a model of bacillary dysentery.

While attempting to develop a lagomorph model of intestinal shigellosis, it was discovered that organisms accidentally introduced into the lungs of rabbits elicit focal pneumonia. Voino-Yasenetsky and Voino-Yasenetskaya subsequently found that pneumonia could be induced experimentally by intranasal challenge of mice (26). Virulent strains were isolated in pure culture from diseased lungs, and the lethality of *Shigella* strains administered intranasally correlated with virulence in humans. Shigellae were also detected within tracheal and bronchial epithelial cells of infected mice (26). Invasion of intestinal epithelial cells was subsequently established as an essential virulence mechanism of *Shigella* species (10, 25).

The mouse lung can be considered a simplified model for the study of the pathogenesis and immunobiology of *Shigella* infection. For example, the pulmonary epithelium in the tracheobronchial tree is simple ciliated columnar epithelium with transition to simple cuboidal epithelium in the alveolar sacs (2). In addition, the bronchial wall contains occasional lymphoid follicles or aggregates of follicles that are similar to the Peyer's patches of the intestine (22). Thus, the bronchus constitutes a mucosal surface with some characteristics of the intestinal epithelium. Like the intestine, the lung is a lymphoid organ with antigen-presenting cells, T helper and suppresser lymphocytes, and B lymphocytes (22). The local and systemic immune response to *Shigella* infection can be accurately evaluated after intranasal challenge of mice because the host is immunologically naive with respect to *Shigella* antigens, and the absence of a resident bacterial flora in the bronchial lumen means that lung tissue is not primed by previous exposure to other gram-negative bacteria.

The intranasal challenge model is amenable to many techniques that are employed in the evaluation of systemic and local immune responses (24). In the current study, for example, inflammation elicited by *Shigella* invasion of the pulmonary

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epithelium was evaluated histologically and by fluorescence-activated cell sorting (FACS). Local and systemic antibody responses against either the serotype 2a polysaccharide somatic antigen (associated with serotype-specific immunity in intragastrically challenged rhesus monkeys) (5) or proteinaceous invasion plasmid antigens (Ipa) (3) were studied after primary and secondary stimulation with *S. flexneri* serotype 2a. In addition, cytokine responses (gamma interferon [IFN-γ], tumor necrosis factor alpha [TNF-α], and interleukin-4 [IL-4]) in naive and immunized mice were compared. These studies illustrate the potential of the mouse intranasal challenge model for characterization of cellular and humoral immune responses protecting against *Shigella* infection in mucosal epithelia.

MATERIALS AND METHODS

Bacterial inoculum. *S. flexneri* 2a strain 2457T (10) was used for immunization or challenge of mice. The inoculum was subcultured from lyophilized, Serény test-positive cultures (7) that were streaked for isolation on Trypticase soy agar plates (Difco Laboratories, Detroit, Mich.) containing 0.01% Congo red. The plates were incubated overnight at 39°C, and Congo red-binding colonies (denoting the expression of plasmid-encoded *Shigella* virulence determinants) (20) were transferred to 50 ml of Penassay broth (Difco). The cultures were incubated with shaking at 37°C for 4 h and harvested by centrifugation. The bacteria were then resuspended in normal saline and standardized spectrophotometrically.

Intranasal inoculation of mice. Eight-week-old BALB/cJ female mice weighing approximately 25 g (Jackson Laboratory, Bar Harbor, Maine) were sedated by intramuscular injection of a mixture of 0.3 mg of xylazine hydrochloride (Rompun; Mobay Corp., Shawnee, Kans.) and 1.0 mg of ketamine hydrochloride (Ketaset; Aveco Company, Fort Dodge, Iowa) in 50 μl of saline. A bacterial suspension (30 μl) was applied drop-wise on the external nares of each mouse with a 100-μl Hamilton syringe. Groups of 15 to 20 mice were challenged twice with approximately 10^6 CFU. This inoculum of invasive shigellae typically caused a transient weight loss of 2 to 4 g with catheresis and cutis anserina as described for mice inoculated with attenuated *S. flexneri* vaccine strains (12); however, fatal pneumonia ensued in only about 10% of the challenged animals. Control mice were inoculated intranasally with 30 μl of normal saline. The animals were inoculated on day 0, or on days 0 and 28, for studies of local antibody and antibody-secreting cells (ASC). Time course studies of cytokine production and FACS analysis of pulmonary inflammatory infiltrate were performed on groups of animals that had been inoculated on days 0 and 28 and challenged with approximately 5×10^6 CFU 35 days later. For studies of the immune protection, mice were inoculated on days 0 and 28 and challenged with approximately 10^7 CFU (2.2 50% lethal doses) 35 days later. Protective efficacy was calculated as {[(percent deaths of controls) − (percent deaths of immunized mice)] ÷ [percent deaths of controls]} × 100. This research was conducted in compliance with the Animal Welfare Act and with other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals* (18a).

Sampling of immune response. For antibody detection, lung lavage samples were collected 1, 2, and 4 weeks after the first sublethal intranasal challenge and 1, 2, 5, and 6 weeks after the second sublethal challenge. For cytokine studies, lavage fluid was collected at selected intervals within 72 h postchallenge. Mice from experimental and control groups were euthanized by CO_2 inhalation. Blood for serology was collected by cardiac puncture, and the lungs were removed from the chest cavity. Pulmonary lavage was performed by inflating the lungs with 0.6 ml of cold RPMI 1640 (Gibco, Gaithersburg, Md.) and by withdrawing the fluid through the trachea. Lavage fluids were maintained at 4°C and centrifuged to remove cellular debris. Sera and lavage fluids were stored at −70°C until used for antibody and cytokine studies. Splenocytes for ASC studies were harvested from spleens of euthanized mice by mincing and teasing the tissue, which was then forced through 80-mesh screens to disrupt large fragments and treated with ammonium chloride to lyse erythrocytes. Splenocytes were washed in RPMI 1640 with 50 μg of gentamicin per ml and used in enzyme-linked immunospot (ELISpot) assays. Lung parenchymal cells for FACS analysis were prepared by finely mincing pooled lungs from each experimental group. The resultant tissue suspension was processed in the same manner as that for teased spleens except that phosphate-buffered saline was used for the washing steps.

Enzyme-linked immunosorbent assay (ELISA). Test wells of 96-well polyvinyl microtiter plates were coated with 0.5 μg of hot water-phenol (27)-extracted *S. flexneri* 2a lipopolysaccharide (LPS) in 50 μl of carbonate buffer (pH 9.6); blank wells were coated with buffer alone. Lung lavage fluid was added in 50-μl volumes to paired test and blank wells. Sera were serially diluted in twofold steps in paired wells beginning with a dilution of 1:20. The microtiter plates were then incubated overnight at 4°C. Bound antibodies were detected by incubation with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG), IgA, or IgM and then by development with *p*-nitrophenyl phosphate substrate (Kirkegaard & Perry, Gaithersburg, Md.). The optical density at 405 minus 570 nm was recorded, and blank well readings were subtracted from corresponding test well readings to yield net optical density results. For sera, the endpoint titer was the highest dilution yielding a net optical density of 0.100 or greater (1).

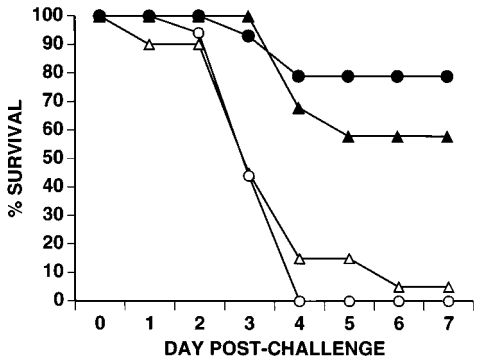

FIG. 1. Survival of mice challenged intranasally with a lethal 10^7 CFU dose of *S. flexneri* 2a (see Materials and Methods). Symbols: ● and ▲, immunized mice surviving two sublethal intranasal challenges with 10^6 CFU of *S. flexneri* 2a; ○ and △, control mice sham inoculated twice with saline. Results of two separate experiments are presented.

ASC. Spleen cells were resuspended to a density of 2.5×10^6/ml in complete medium consisting of RPMI 1640 supplemented with 2 mM L-glutamine, 50 μg of gentamicin per ml, and 10% fetal bovine serum (Gibco). ASC were detected by a modification of previously published ELISpot methods (4). One-hundred-microliter volumes of the cells were allowed to incubate for 4 h at 37°C in microtiter wells (Immunoplate I; Nunc, Roskilde, Sweden) coated previously with 1 μg of *S. flexneri* 2a LPS in carbonate buffer. Each sample was tested in quadruplicate. The plates were washed, and the wells were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG, IgA, or IgM overnight at 4°C. Spots representing ASC were visualized by the addition of molten agarose (type I, low EEO; Sigma Chemical Co., St. Louis, Mo.) containing 100 μg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate (Sigma) per ml. Spot-forming cells were counted with a stereomicroscope and recorded as the number of ASC per 10^6 splenocytes.

Western blots (immunoblots). Whole-cell lysates of *S. flexneri* serotype 5 strains M90T and M90T-55 (virulence plasmid negative) (17) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose sheets (Hoefer Scientific Instruments, San Francisco, Calif.). The blots were incubated either in serum pooled from five animals (diluted 1:200 in casein filler) or in pooled lavage fluids (diluted 1:5 in casein filler), then in goat anti-mouse IgG or IgA conjugated to alkaline phosphatase and, finally, in fast red substrate (Sigma). Ipa proteins recognized by serum or lavage antibodies were identified by comparing immunoblot patterns of Ipa-expressing strain M90T with those of strain M90T-55, which does not express these proteins.

Cytokines. Commercially available kits were used to quantify TNF-α, IFN-γ (Genzyme, Cambridge, Mass.), and IL-4 (Endogen, Boston, Mass.) in lung lavage fluids and in sera.

FACS analysis. Suspensions of lung cells were stained with anti-Mac-1 monocyte-specific antibody (Boehringer-Mannheim Corp., Indianapolis, Ind.) and analyzed on a FACSort (Becton Dickinson Immunochemistry Systems, San Jose, Calif.). Isotype control reagents (Biomedical Products, Morris Plains, N.J.) were employed for specificity control.

Histology. Animals were euthanized by CO_2 inhalation. The lungs were removed and perfused with 10% buffered formalin phosphate (Fisher, Pittsburgh, Pa.), dehydrated, and processed in paraffin. Sections were cut at 3 μm and stained with hematoxylin and eoxin or Giemsa.

Statistics. Frequencies were compared by chi square, and continuous variables were compared by the Wilcoxon signed-rank test by use of StatView (Abacus Concepts, Inc., Berkeley, Calif.). *P* values of <0.05 were considered statistically significant.

RESULTS

Protection against lethal pneumonia after intranasal challenge with *S. flexneri*. Mice that had survived two sublethal challenges with *S. flexneri* 2a were protected against subsequent intranasal challenge with a 10^7 CFU dosage that caused lethal pneumonia in control mice (Fig. 1). In replicate experiments, levels of protection of 56 and 79% were demonstrated. A single sublethal pulmonary *Shigella* infection did not elicit

significant protection against a subsequent intranasal challenge with a lethal dose of shigellae (data not shown).

Histology of mouse lung tissue after *Shigella* challenges. After two sequential intranasal challenges with *S. flexneri* 2a, mice typically had residual lymphohistocytic aggregates and infiltrates in the interstitium surrounding the airways and vessels that were indicative of prior antigenic stimulation (Fig. 2B). Twenty-four hours after a third challenge with 10-fold more shigellae, acute suppurative bronchiolitis was observed in the lungs of both naive and previously challenged mice (Fig. 3). At this time, numerous intracellular bacteria were present in the bronchiolar and alveolar epithelia of control mice (Fig. 3A), while intracellular bacteria were rarely present in the epithelia of immunized mice (Fig. 3B). No bacteria were seen in sections of lungs from mice that were challenged with saline alone and sacrificed at the same time as the mice that were challenged with *S. flexneri* 2a (data not shown). Both immunized and control mice were characterized by focal areas of pneumonia and hemorrhage with necrosuppurative bronchiolitis 48 h after challenge; however, few intraepithelial bacteria were observed in the lungs of either group at this time (data not shown).

Antibody response against somatic antigen. Both primary and secondary mucosal immune responses were evaluated because significant protection against a lethal intranasal challenge dose of *S. flexneri* 2a was observed only after two sublethal pulmonary infections. The relative levels of local antibody recognizing *S. flexneri* 2a LPS were determined by ELISA with undiluted lung lavage aspirates. These samples were collected from euthanized animals after either one or two pulmonary infections (Fig. 4). A primary IgM response was detected 2 weeks after a single intranasal challenge, and within 4 weeks, all three isotypes were detectable at low levels (Fig. 4A). A second group of animals was challenged twice (day 0 and day 28), and classic secondary IgG and IgA immune responses were seen 2 weeks after the second challenge (Fig. 4B). Elevated levels of IgG were maintained in lung lavage fluid for more than 6 weeks, while the IgA and IgM levels waned within 5 weeks (Fig. 4B). The reciprocal geometric mean titers of serum antibody recognizing *S. flexneri* 2a LPS 2 weeks after a second sublethal intranasal challenge were 1,691 (IgG), 80 (IgA), and 183 (IgM) (data not shown).

ASC. Trafficking of antibody-secreting B-cell lymphoblasts (ASC) to distal tissues after one or two pulmonary *Shigella* infections was measured in spleen cells with *S. flexneri* 2a LPS as the antigen. A single infection generated a primary response, consisting mainly of IgM ASC, which was detectable within 1 week. This primary response peaked at 2 weeks and was still detectable at 4 weeks (Fig. 5A). Two infections stimulated a secondary response characterized by a transient increase in IgM ASC and by a dramatic increase in trafficking IgA ASC (Fig. 5B). Surprisingly, trafficking IgG ASC were not detected after one immunization, and after two challenges, only very low numbers (<5 ASC per 10^6 cells) were found.

Immunoblot analysis of anti-Ipa response. After a single sublethal challenge with *S. flexneri* 2a, serum IgG recognizing IpaB was readily detectable, and this response was enhanced after a second challenge (Fig. 6). Interpretation of the IpaC response was confounded by IgG recognizing a protein of similar molecular mobility in the plasmid-negative *S. flexneri* 5 control strain, but the intensity of antibody binding at the expected molecular mobility of IpaC in the lysates of plasmid-positive strains suggested that primary and secondary IgG responses were also developed against this protein. Serum IgA recognizing IpaB was detectable only after a second sublethal challenge with *S. flexneri* 2a. Immunoblots of lung lavage fluid gave evidence of a primary IgG response against IpaB with a weak response against IpaC. A prominent IgG secondary immune response against IpaB and IpaC was seen after a second sublethal challenge (Fig. 7). Mucosal IgA recognizing IpaB and IpaC was also detectable after a second challenge.

Pulmonary cytokine levels. Lung lavage fluid was collected prior to challenge (0 h) and 6, 24, 48, and 72 h after intranasal challenge of immunized or control mice with a lethal dose of *S. flexneri* 2a. TNF-α responses were detected in both naive and immunized mice 6 h after challenge, with higher values in the latter animals (Fig. 8A). After 24 h, TNF-α levels began to decline in the immunized animals, while levels in control mice peaked. Controls maintained significantly higher values at 48 and 72 h. IFN-γ was detectable in all immunized mice and in some of the control mice 6 h after challenge (Fig. 8B). At this early time point, IFN-γ levels in immune mice were significantly higher than those in controls. This cytokine peaked at 24 h and began to decline within 48 h, with no significant differences between immunized and control mice. The detection of TNF-α and IFN-γ indicated that the *Shigella* infection activated the Th_1 type of T helper cell response in both naive and immunized mice. Production of IL-4 was monitored in lung lavage fluid to determine whether the Th_2 type of response was also stimulated during infection. Only the immunized mice produced a local IL-4 response to infection, with significantly higher values detectable at 24 and 48 h (Fig. 8C). This cytokine peaked at 48 h and returned to baseline by 72 h. Serum cytokine levels were measured at the same time points, and values above baseline were detected at 6 h for TNF-α and at 48 h for IL-4 only in immunized animals (data not shown).

FACS analysis. As suggested by histological evaluation (Fig. 2), FACS analysis indicated that the resident Mac-1-positive monocyte population in the immunized mice was eightfold greater than that in control animals (Fig. 9). The influx of monocytes was enhanced during the first 24 h in the immunized group, and this cell type peaked at 48 h in both immunized and control groups. After 72 h, monocyte levels decreased in the immunized mice while remaining at the peak level in naive controls.

DISCUSSION

Previous studies have described the histological features of pulmonary *Shigella* infection (26), and we have confirmed that shigellae invade the bronchiolar and alveolar epithelia in this model. Cultivation of homogenized lung tissue from moribund mice yielded pure cultures of *S. flexneri* 2a, while no shigellae were cultured from the spleen, liver, intestine, or blood of these animals (unpublished data). Thus, shigellae remain localized in the pulmonary epithelium, and this localized infection elicits an inflammatory response that is reminiscent of intestinal shigellosis (21). In the current studies, intranasal challenge established sublethal *Shigella* infections in mice, and the protective mucosal immunity elicited by these infections was demonstrated and analyzed. Two intranasal challenges with sublethal doses of *S. flexneri* 2a resulted in significant survival of mice challenged with a dose that was lethal for a group of naive control mice. Mice immunized by sublethal intranasal *S. flexneri* 2a challenge were not protected from challenge with *Shigella sonnei* expressing heterologous LPS. Intraperitoneal immunization with live *S. flexneri* 2a elicited high titers of circulating antibody that did not protect against intranasal challenge with homologous organisms (unpublished data). Thus, locally produced, LPS-specific antibody is apparently crucial for protection against lethal pneumonia in this model.

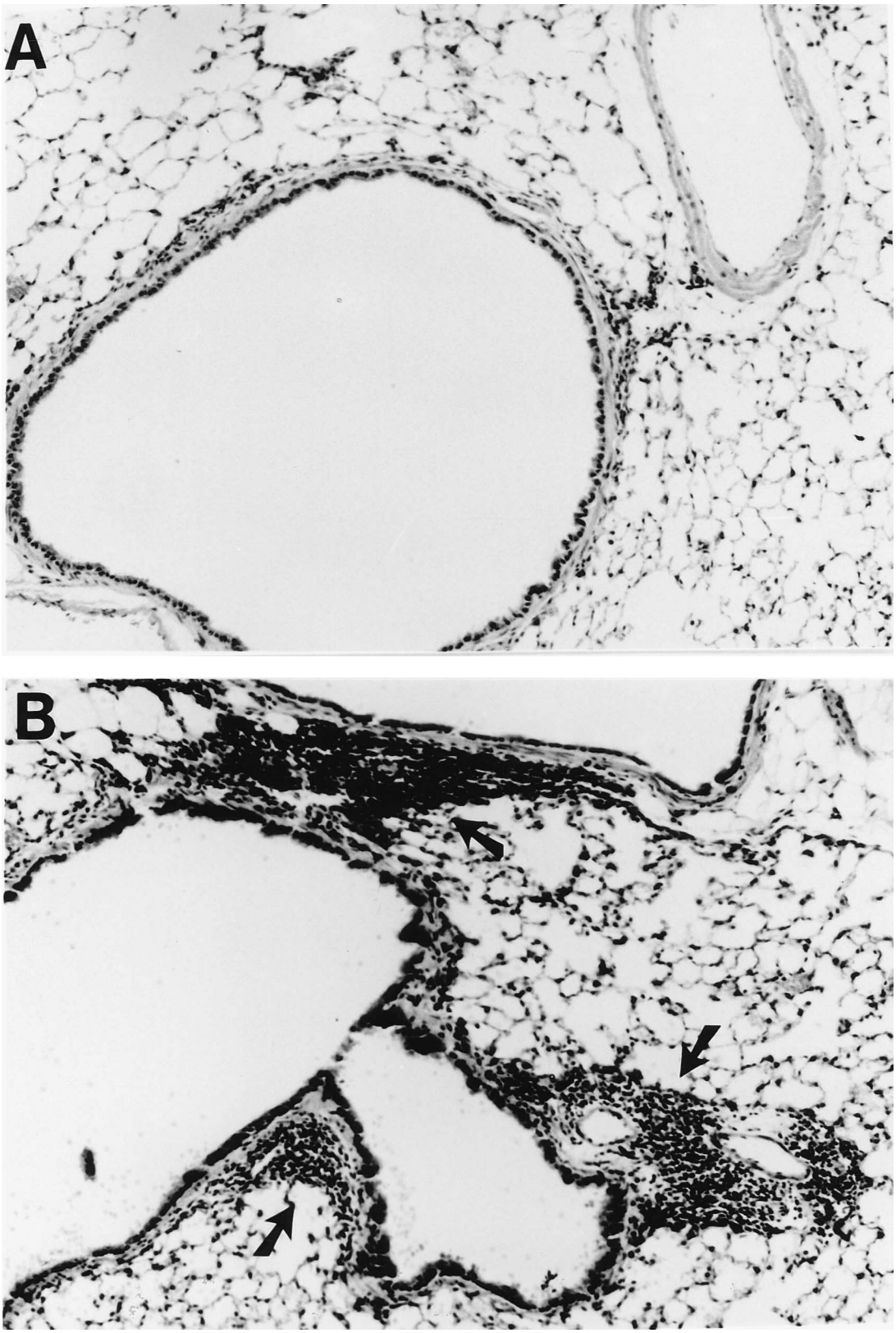

FIG. 2. Histology of mouse lung tissue 35 days after the second of two sham inoculations with saline (A) or after a second sublethal intranasal challenge with *S. flexneri* 2a (B). The control mouse is essentially normal, while the immunized mouse has extensive peribronchiolar and perivascular aggregates consisting of macrophages and lymphocytes (arrows) associated with more than 50% of the bronchioles. Magnification, ×90.

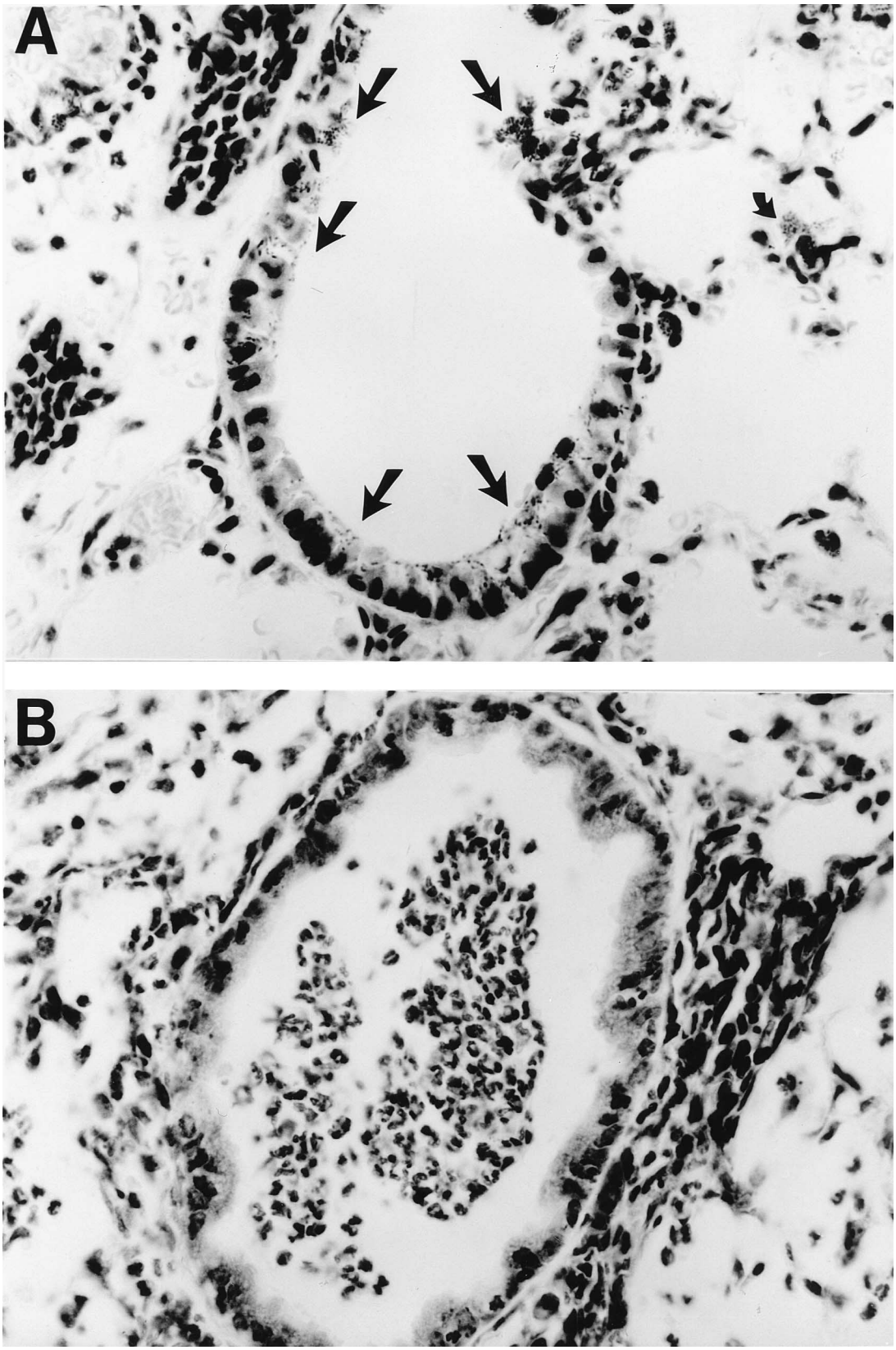

FIG. 3. Histology of mouse lung tissue 24 h after intranasal challenge with a lethal dose of *S. flexneri* 2a. (A) Numerous bacteria are visible in the bronchiolar and alveolar epithelia (arrows) of the control mouse. (B) Bacteria were rarely seen in the epithelia of immunized mice. Both control and immunized mice experienced acute suppurative bronchiolitis progressing to focal pneumonia at 24 and 48 h. Magnification, ×540.

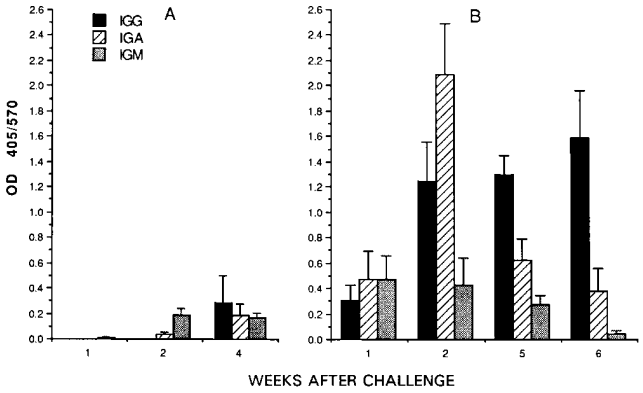

FIG. 4. Development of pulmonary antibody recognizing *S. flexneri* 2a LPS following one (A) or two (B) sublethal challenges. Antibody was assayed in undiluted lung lavage samples by ELISA. Each bar is the mean of samples from six animals; error bars represent 1 standard error.

Intranasal inoculation with sublethal doses of *S. flexneri* 2a stimulated high levels of pulmonary IgG and IgA and moderate levels of IgM antibodies specific for LPS. Local and humoral IgG and IgA antibody recognizing IpaB or IpaC proteins, the determinants of the invasive phenotype (9), were also stimulated by pulmonary *Shigella* infection. In contrast, IpaA, IpaD, and the IcsA proteins were not recognized by antiserum from mice challenged with *S. flexneri*. Since the latter virulence-associated proteins are typically recognized by convalescent-phase serum from monkeys or humans (19), the immune response of BALB/cJ mice against the Ipa proteins differs somewhat from that evoked by shigellosis in monkeys or humans.

In previous studies involving intranasal vaccination of mice with live attenuated *Shigella* vaccines (12), it was found that serum LPS antibodies are chiefly of the IgG and IgM isotypes while serum IgA levels tend to be very low. A similar serum antibody isotype distribution was observed in the current study, suggesting that the predominance of IgA found in pulmonary lavage fluid reflects IgA-secreting B cells residing in the lung mucosa. As an alternative to collecting lung lavages, trafficking ASC in the spleen were monitored as a reflection of antibody levels in the lungs. The ASC results showed that IgA- and IgM-secreting cells, which were induced as a result of pulmonary infection, traffic through the spleen and populate the

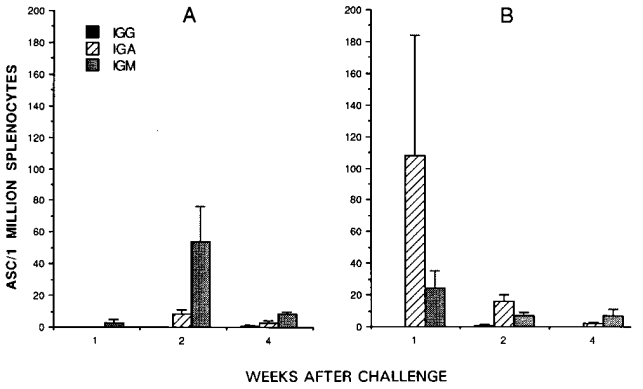

FIG. 5. Development of splenic ASC specific for *S. flexneri* 2a LPS following one (A) or two (B) sublethal challenges. ASC were detected by an ELISpot assay. Each bar is the mean of results from six animals; error bars represent 1 standard error.

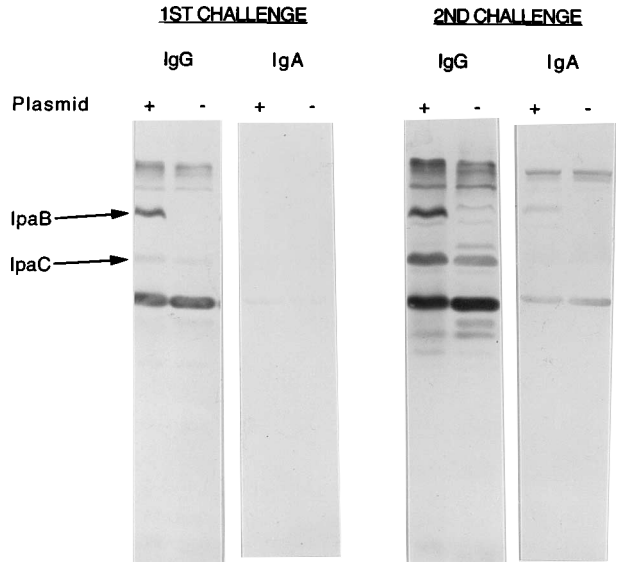

FIG. 6. Immunoblot analysis of serum pooled from mice that were bled 2 weeks after an initial sublethal challenge with *S. flexneri* 2a or 2 weeks after a second challenge. Serum IgG recognized the IpaB plasmid-encoded protein after the first challenge, and serum IgA recognizing this protein was detected after the second challenge. The whole-cell bacterial antigen lysates were prepared for plasmid-containing (+) and plasmid-cured (−) isogenic strains of *S. flexneri* serotype 5.

common mucosal system. On the other hand, IgG-secreting cells were detected at comparatively low levels even though high levels of IgG were detectable in lung lavages. It is possible that IgG ASC traffic predominately through peripheral lymphoid tissues following immunization. In guinea pigs challenged by the conjunctival route, the majority of LPS-specific ASC found in the superficial and posterior cervical nodes following immunization are of the IgG isotype (8). In any event,

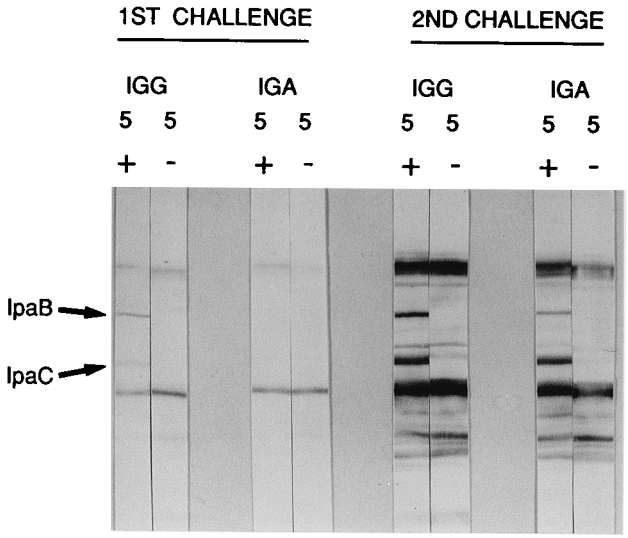

FIG. 7. Immunoblot analysis of lung lavage fluid pooled from mice euthanized 2 weeks after an initial sublethal challenge with *S. flexneri* 2a or from mice euthanized 2 weeks after a second challenge. IgG recognizing both the IpaB and IpaC plasmid-encoded proteins was detected after the first challenge, and IgA recognizing these proteins was detected after the second challenge. The whole-cell bacterial antigen lysates were prepared for plasmid-containing (+) and plasmid-cured (−) isogenic strains of *S. flexneri* serotype 5.

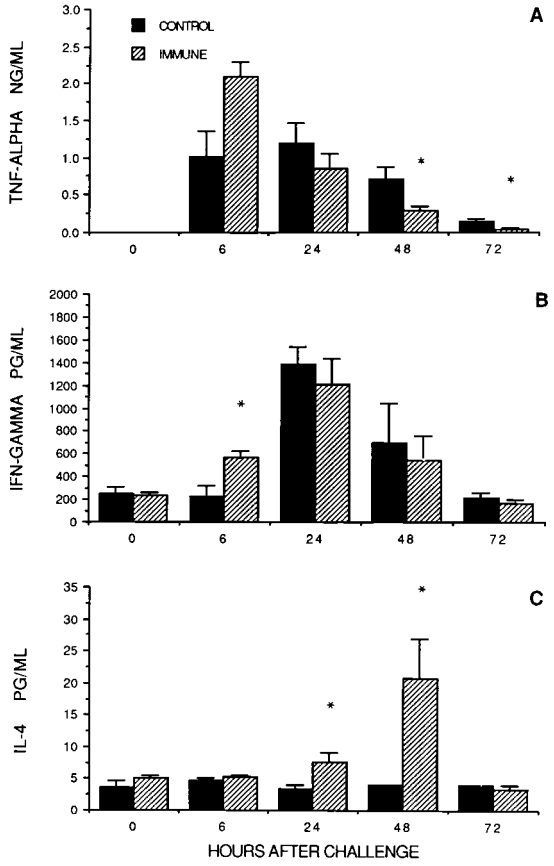

FIG. 8. Time course of cytokine expression following lethal challenge of mice that had been previously challenged twice with sublethal doses of *S. flexneri* 2a (immune) or sham inoculated with saline (control). TNF-α (A), IFN-γ (B), and IL-4 (C) were assayed by ELISA with undiluted lung lavage samples. Each bar is the mean of samples from three animals; error bars represent 1 standard error. Asterisks denote time points at which significant differences were found between the immune and control groups ($P < 0.05$).

direct measurement of antibody levels in lung lavage fluid provided the most accurate reflection of local antibody response.

The time course of local cytokine production reflected the immune status of the animals at the time of challenge. For example, the sustained elevation of TNF-α levels that was seen in the control mice compared with that in immunized mice correlated with FACS analysis showing a prolonged increase in the proportion of monocytes or macrophages in the lung tissue. TNF-α secreted by these infiltrating macrophages may elicit tissue damage. Crohn's disease is an inflammatory colitis mediated by locally synthesized cytokines such as TNF-α (11), and increases in circulating levels of TNF-α and IFN-γ are also seen in humans challenged with *S. sonnei* (17). Thus, *Shigella* infection has some of the characteristics of endotoxic shock (14, 23), with elements of the Th_1 T-cell response (15) playing a prominent role.

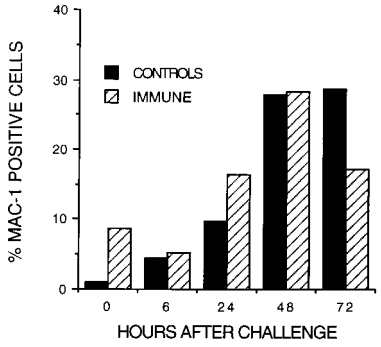

FIG. 9. FACS analysis of the percentage of Mac-1-positive cells in the lungs of mice euthanized at various times after intranasal challenge with a lethal dose of *S. flexneri* 2a. Immune mice had been challenged twice with a sublethal dose of the same strain, while control mice had no previous exposure to shigellae.

IFN-γ levels were elevated in the lungs of both immunized and control mice, but the elevation was detected at an earlier time point in the former animals. This rapid IFN-γ response could serve to increase the microbicidal activity of infiltrating macrophages (18). Such activated macrophages would have enhanced bactericidal activity (18), and this cell-mediated response, in conjunction with secretory IgA, may account for the decreased bacterial load seen in histological sections of lungs from immunized mice. In contrast to the inflammatory cytokines, the IL-4 response was detected only in immunized animals, indicating that previous *Shigella* infection activated Th_2 lymphocytes. The presence of IL-4 at 24 and 48 h after challenge may have inhibited the further production of TNF-α, thus curtailing the damaging effects of excessive production of the latter cytokine (16).

Characterizing immune responses which protect against shigellosis, and distinguishing these from responses which contribute to pathogenesis, has been difficult in intestinal models of infection. In the mouse intranasal challenge model, much of the pathology appears to be due to the effects of an extended inflammatory response. This response is similar to that observed in intestinal shigellosis, and both the cellular and humoral responses elicited by pulmonary infection are also similar to the human response to bacillary dysentery. Thus, the intranasal challenge model should be useful for evaluation of the protective immune response and for characterization of specificity of Th_1 and Th_2 T-cell subsets activated by *Shigella* infection.

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