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Clearance of *Pseudomonas aeruginosa* from the Murine Gastrointestinal Tract Is Effectively Mediated by O-Antigen-Specific Circulating Antibodies

GERALD B. PIER,* GLORIA MELULENI, AND JOANNA B. GOLDBERG

Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

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The colonization of mucosal surfaces by *Pseudomonas aeruginosa* can lead to local or disseminated disease. Secretory immunoglobulin A (IgA) has been assumed to be responsible for preventing mucosal colonization by interfering with the binding of bacterial ligands to epithelial surface receptors. However, the efficacy of this mechanism of immunity derives little actual support from in vivo experiments. In an investigation of the role of local and systemic immunization strategies in reducing colonization of the gastrointestinal tract of mice by *P. aeruginosa*, the bacterial antigens that were potential targets for immune effectors promoting mucosal clearance were identified. Levels of gastrointestinal colonization were reduced when immunity to homologous O antigens, but not that to pili or flagella, was elicited. Oral vaccination with attenuated *Salmonella typhimurium* expressing *P. aeruginosa* serogroup O11 antigen elicited mucosal and serum IgA antibodies and serum IgG antibodies specific for the recombinant antigen. Oral challenge of immunized mice with *P. aeruginosa* serogroup O11 demonstrated protection against gastrointestinal colonization. Intraperitoneal immunization with a serogroup O11 high-molecular-weight O-polysaccharide antigen elicited only serum IgG and IgM antibodies yet was as effective as oral vaccination in protecting mice against gastrointestinal colonization. This finding was confirmed by the demonstration that intraperitoneal immunization with purified lipopolysaccharide was also protective against mucosal surface colonization. These results call into question the need for local immune effectors, particularly secretory IgA, directed at bacterial ligands for epithelial surface components, in protecting a mucosal surface from bacterial challenge.

Although it is not readily appreciated as such, *Pseudomonas aeruginosa* is often a mucosal pathogen, either causing local disease at sites such as the eye (50, 55), the urinary tract (4), and the bronchial mucosa (37) or colonizing the gastrointestinal (GI) tract, from which it can disseminate to infect the bloodstream (24, 62). It has been suggested that infection could be prevented if colonization of mucosal surfaces could be interrupted. Immunologic strategies that interrupt mucosal colonization could provide an important approach to immunotherapy. However, the immunologic mechanisms that prevent or limit bacterial colonization of a mucosal surface have been defined mostly on the basis of logical speculation as opposed to actual experimentation. Local or secretory immunoglobulin A (sIgA) is often invoked as a prime effector of mucosal immunity (11, 21, 36), but few studies have shown that local sIgA, in the absence of systemic IgG, protects against mucosal surface colonization and subsequent infection and disease.

Recent studies have shown that very high levels of monoclonal sIgA antibodies specific to the O1 lipopolysaccharide (LPS) antigen of *Vibrio cholerae* can prevent experimental infection by this bacterium when delivered to the GI tract (64). Similarly, monoclonal sIgA specific for the LPS of *Salmonella typhimurium*, when delivered to the GI tracts of mice, protected against infection when each mouse was challenged with 10^7, but not with 10^9, CFU of *S. typhimurium* (41). In these studies, it was unclear whether these levels of sIgA reflected those that could reasonably be attained under normal physiologic circumstances. Even more importantly, these studies failed to determine whether mice with high titers of systemic IgG were equally or less well protected against mucosal surface infections in comparison with the animals given sIgA. Indeed, protective immunity against diseases such as cholera and group A streptococcal pharyngitis is associated with bactericidal and opsonic serum IgG levels (28, 39, 52). The contention that serum IgG serves as a marker for protective local sIgA (34) has not been shown experimentally, and Avery and Gordon (3) have demonstrated that it is the IgG, and not sIgA, in breast milk that mediates effective opsonization of bacteria. What is lacking in the literature are good comparative studies of the roles of local IgA and of local and systemic IgG in protecting against mucosal surface colonization by bacterial pathogens.

We have previously described an animal model of mucosal surface colonization by *P. aeruginosa* (48). This model can be used to study the immunologic mechanisms that prevent bacterial colonization. We have also developed high-molecular-weight O-polysaccharide vaccines against the major O-antigen serogroups of *P. aeruginosa* that elicit systemic circulating IgM and IgG antibodies after intraperitoneal (i.p.) immunization of animals (30). Finally, we have described the cloning of the *rfb* locus encoding O-antigen production from serogroup O11 (Fisher immunotype 2) *P. aeruginosa* in *Escherichia coli* (29). In these studies, we documented both expression of the *P. aeruginosa* antigen by this enteric organism and induction of local IgA antibodies after oral immunization, and we indicated that the *P. aeruginosa* O antigen can also be expressed by *S. typhimurium* (29). These are the basic tools and findings that we used in this study to determine (i) whether antibodies specific for *P. aeruginosa* O antigens, like sIgA specific to *V. cholerae*

* Corresponding author. Mailing address: Channing Laboratory, 180 Longwood Ave., Boston, MA 02115. Phone: (617) 432-2269. Fax: (617) 731-1541. Electronic mail (Internet) address: gpier@warren.med.harvard.edu.

TABLE 1. Bacterial strains, plasmids, and bacteriophage used in this study

Bacterial strain plasmids or phage	Relevant characteristics[a]	Reference and/or source
P. aeruginosa		
PAK	Clinical isolate, serogroup O6	S. Lory, Seattle, Wash.
15921	Clinical isolate, serogroup O6	Clinical microbiology laboratory, Brigham and Women's Hospital
9882-80	Clinical isolate, serogroup O11	
1244	Clinical isolate, serogroup O7	J. Sadoff, Washington, D.C.
PAO1	Clinical isolate, serogroup O2/O5	M. Vasil, Denver, Colo.
E. coli HB101	Plasmid host	12
S. typhimurium		
CS019	*phoN2 zxx*::6251Tn*10*d-Cam	42; S. I. Miller, Boston, Mass.
SL3261	*aroA*	33; S. I. Miller
Plasmids		
pRK2013	Helper plasmid (Km^r); tra^+	25
pLAFR1	Broad-host-range cosmid (Tet^r); mob^+ *tra*	27
pLPS2	Recombinant plasmid containing *rfb* region of serogroup O11 *P. aeruginosa* in pLAFR1 (Tet^r)	29
Bacteriophage P22	General transducing phage	S. I. Miller

[a] Km^r, kanamycin resistance; Tet^r, tetracycline resistance.

and *S. typhimurium* O antigens, can protect against mucosal surface colonization and (ii) whether circulating antibody alone can promote clearance of *P. aeruginosa* from the GI mucosal surface or whether induction of local antibodies, achieved, for example, by oral immunization with recombinant *S. typhimurium* expressing *P. aeruginosa* serogroup O11 antigen, is required for high-level immunity.

MATERIALS AND METHODS

Bacterial strains, recombinant plasmids, and vectors. The strains of *P. aeruginosa*, *E. coli*, and *S. typhimurium*, and the recombinant plasmids and vectors, used are described in Table 1.

Plasmid mobilization. Plasmids from *E. coli* were introduced into *P. aeruginosa* and *S. typhimurium* CS019 via triparental mating. In brief, *E. coli* HB101 containing either pLAFR1 or pLPS2 was grown overnight in L broth with tetracycline (10 μg/ml), and *E. coli* HB101 containing the helper plasmid pRK2013 was grown overnight in L broth with kanamycin (30 μg/ml). *P. aeruginosa* and *S. typhimurium* CS019 were grown overnight in L broth. For *S. typhimurium*, cells were washed once in saline and resuspended in L broth and a 100-μl volume mixed with 100-μl volumes of the appropriate *E. coli* strains and deposited onto an L-agar plate to transfer either pLPS2 or pLAFR1 to *S. typhimurium*. To transfer pLAFR1 to *P. aeruginosa*, a 200-μl volume of the various strains of this bacterium was mixed with 200-μl volumes of each of the needed *E. coli* strains, which was then deposited onto a filter (pore size, 0.22 μm) by using a syringe, and the filter was then placed on an L-agar plate. After incubation overnight at 37°C, the mixtures from the plates or filters were either streaked onto L agar containing 10 μg of tetracycline per ml (to select for the transfer of the plasmid) and 25 μg of chloramphenicol per ml (to select for *S. typhimurium* CS019) or spread onto cetrimide agar (Difco Laboratories, Detroit, Mich.) containing 50 μg of tetracycline per ml (to select for *P. aeruginosa* bearing pLAFR1). To confirm their identity as *S. typhimurium*, colonies were subcultured on Hektoen agar (Becton Dickinson, Cockeysville, Md.). Individual colonies of *P. aeruginosa* were picked and rescreened on cetrimide agar with tetracycline.

Plasmids were transferred from *S. typhimurium* CS019 to *S. typhimurium* SL3261 via P22 phage transduction (22) and selection on L agar containing tetracycline. To confirm the presence of plasmids in *S. typhimurium*, DNA was isolated from bacterial strains either by the method of Birnboim and Doly (8) or by the use of Magic or Wizard Minipreps (Promega Corporation, Madison, Wis.) and was subsequently digested with *Eco*RI (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as suggested by the manufacturer. Fragments were then separated by electrophoresis in 0.7% agarose in Tris-acetate-EDTA buffer. After electrophoretic separation, DNA in gels was visualized on a UV light box after ethidium bromide staining.

Antigens. For use in enzyme-linked immunosorbent assay (ELISA) and immunoblot analyses and for immunizations, *P. aeruginosa* LPS was isolated by the phenol-water extraction technique (63) and purified further by ultracentrifugation and chromatography as described elsewhere (31). High-molecular-weight O polysaccharide was isolated as described previously (30). Purified pili from strain PAK, and type a *P. aeruginosa* flagella, were obtained from William Paranchych, Edmonton, Alberta, Canada, and Stephen Lory, Seattle, Wash., respectively (48).

For use in immunoblots, LPS from *S. typhimurium* was released by lysis from the pellet of 100 ml of bacteria grown in L broth. Lysis was carried out by suspending the bacterial cells for 1 h at 37°C in 10 ml of 10 mM Tris (pH 7.5) containing 1% sarcosyl. Cellular debris was removed by centrifugation at 20,000 × *g* for 30 min, and the LPS was pelleted in an ultracentrifuge by centrifugation at 100,000 × *g* for 3 h. The pellet was resuspended in water and lyophilized. Prior to application to a sodium dodecyl sulfate (SDS)-polyacrylamide gel, a weighed aliquot was dissolved in water and digested with DNase (50 μg) and RNase (5 μg) overnight at 37°C, after which 50 μg of pronase was added and incubation for 2 h at 56°C was carried out.

Immunoassays. For the detection of the expression of O antigens of *S. typhimurium* and *P. aeruginosa* on appropriate bacterial strains, immunoblots of sarcosyl-extracted antigens and LPS-specific antibodies were used as described previously (29, 31). For detection of *Salmonella* O antigen by immunoblot assay, Bacto *Salmonella* O-antiserum factor 4 (Difco) was used.

Serum antibody titers were calculated following ELISA as described previously (30, 47). Briefly, antigens in either 0.04 M phosphate (pH 7.2) (LPS, 10 μg/ml) or 0.1 M carbonate (pH 9.6) (pili and flagella, 2 μg/ml) were used to coat Immunolon II ELISA plates (Dynatech, Chantilly, Va.). Dilutions of serum or single dilutions (1:20) of fecal homogenates were added in duplicate, incubated for 2 h at 37°C, and washed away, and heavy-chain-specific anti-mouse IgM, IgG, or IgA conjugated to alkaline phosphatase was used as the secondary antibody. The antibody titer was determined by using regression analysis (30, 47) to calculate the reciprocal of the serum dilution giving an optical density of 0.2 at 405 nm after 60 min of incubation of the enzyme-substrate combination.

Immunization of mice. Systemic antibodies were elicited in female BALB/c mice (6 to 8 weeks old) by i.p. immunization with monovalent preparations containing 50 μg of purified high-molecular-weight O polysaccharide or 10 μg of purified LPS. High-molecular-weight O-polysaccharide and LPS vaccines were administered three times with intervals of 5 to 6 days between doses. For oral immunization with recombinant *S. typhimurium* strains, mice were fed (from a micropipette tip) 100 μl containing 10^9 CFU of bacterial cells suspended in 2% sucrose–2% bicarbonate. Water was removed from the animals' cages about 4 h before each dose of bacteria to encourage drinking of the bacterial suspension. Mice received three or four oral doses of recombinant *S. typhimurium* at intervals of 3 to 4 days.

Virulence of recombinant *S. typhimurium* strains in mice. In an evaluation of the safety of the recombinant *S. typhimurium* strains, groups of five female BALB/c mice (6 to 8 weeks old) were challenged orally, by the procedure described above for oral immunization, with these strains at doses of 10^1 to 10^{10} CFU per mouse. In addition, groups of five outbred 6- to 8-week-old female Swiss Webster mice were challenged by i.p. inoculation with increasing doses of recombinant *S. typhimurium* strains. Mice were monitored multiple times each day for signs of morbidity likely to lead to death (inability to reach food or water, shaking chills, and ruffled fur). For the purposes of analysis, moribund animals were killed and counted along with animals found dead in their cages as dead as a result of infection.

Chronic GI tract colonization by *P. aeruginosa*. Chronic *P. aeruginosa* colonization of GI tracts of female BALB/c mice (6 to 8 weeks old) was established as described previously (48). One modification of the method used previously to eradicate facultative bacterial flora from the GI tract was the addition of 1 mg of

gentamicin sulfate along with 1 mg of streptomycin sulfate per ml of drinking water (48). After 4 days (24 h before the addition of *P. aeruginosa* to drinking water), the addition of gentamicin was discontinued, and only streptomycin sulfate was added to drinking water for the remainder of the protocols.

Oral immunization, bacterial clearance, and challenge with *P. aeruginosa*. To determine which *P. aeruginosa* antigens were effective targets of immune-mediated clearance of bacteria from the GI tract, 25 female BALB/c mice underwent depletion of their indigenous facultative colonic flora as described above, and chronic colonization of the GI tract with a streptomycin-resistant clone of *P. aeruginosa* PAK was established by the addition of 10^8 CFU/ml to drinking water for 5 days. Thereafter, only 1 mg of streptomycin sulfate per ml was present in the drinking water, and the level of chronic colonization was monitored via fecal culture as described previously (48). After 2 weeks of colonization by strain PAK, the mice were given drinking water containing 1 mg of gentamicin sulfate per ml for 5 days, and clearance of *P. aeruginosa* from the GI tract was monitored by thrice-weekly fecal cultures over the ensuing 2 weeks. Samples of serum and feces were obtained as described elsewhere (48) for antibody determinations. After this latter 2-week period, mice were divided into groups of five and were orally challenged with various strains of *P. aeruginosa*. Matched groups of mice depleted of their indigenous facultative GI bacterial flora by antibiotic treatment but not colonized by *P. aeruginosa* PAK served as nonimmune controls for colonization experiments. All challenge strains were resistant to streptomycin; moreover, all contained pLAFR1, which encodes resistance to tetracycline, and we were thus able to incorporate this antibiotic into the media to monitor fecal counts. Samples were also plated onto selective media lacking antibiotics to ensure that reduced counts were not due to spontaneous plasmid loss. The original colonizing strain of *P. aeruginosa*, PAK, is sensitive to tetracycline. The challenge strains, and their relationship to strain PAK, were as follows: PAK(pLAFR1), original strain, now resistant to tetracycline; 15921(pLAFR1), serogroup O6 strain (as is strain PAK), unknown pilus and flagellum type; 1244(pLAFR1), serogroup O7, LPS heterologous to strain PAK, cross-reactive pilus and flagellum type; and strain PAO1(pLAFR1), serogroup O5, LPS heterologous to strain PAK, different pilus type, cross-reactive flagellum type. For challenge, the *P. aeruginosa* strains (at a concentration of 10^7 CFU/ml) were added to drinking water for 5 days; thereafter, the only addition to the drinking water was 1 mg of streptomycin sulfate per ml. Mice were housed individually, and feces were obtained two or three times per week for culture.

Statistical analysis. Fifty percent lethal dose (LD_{50}) estimates and 95% confidence intervals were determined by probit analysis (49). Differences in fecal counts of *P. aeruginosa* were assessed by analysis of variance (ANOVA), with the Stat-View SE + Graphics software (Abacus Concepts, Inc., Berkeley, Calif.). For results from cultures in which no colonies of *P. aeruginosa* were detected, a value of zero was used, although the lower limit of detection is 100 CFU/g of feces. The Fisher exact formula was used to compare the proportion of mice colonized at a level of >100 CFU/g of feces among groups receiving different immunization reagents.

RESULTS

Specificity of mucosal immunity for *P. aeruginosa* antigens. We first sought to determine whether the LPS O-polysaccharide antigen of *P. aeruginosa*, like those of *V. cholerae* and *S. typhimurium* (41, 64), was an effective target for mucosal immune effectors. The findings with the latter two organisms conflict with the frequent claim that the inhibition of binding of surface structures such as pili and flagella to epithelial targets is the mechanism of antibody-mediated immunity at a mucosal surface. After depletion of the indigenous facultative colonic flora of 25 mice by treatment with gentamicin sulfate and streptomycin sulfate for 4 and 5 days, respectively, no bacterial colonies grew on aerobically incubated MacConkey's or cetrimide agar inoculated with 100-μl suspensions of fecal homogenates (0.1 g/ml). The animals were then given drinking water containing 10^8 CFU of streptomycin-resistant *P. aeruginosa* PAK (LPS serogroup O6) per ml for 5 days in order to colonize their intestines; thereafter the drinking water contained only 1 mg of streptomycin sulfate per ml. Cultures of fecal samples obtained three times per week over the next 2 weeks indicated that all of the mice became colonized with *P. aeruginosa* PAK (10^3 to 10^6 CFU/g of feces in individual samples). Next, the mice were given 1 mg of gentamicin sulfate per ml of drinking water for 5 days to kill the colonizing *P. aeruginosa* PAK organisms, after which the drinking water contained only 1 mg of streptomycin sulfate per ml. Fecal cultures obtained three times per week over the next 2 weeks yielded no colonies of *P. aeruginosa*.

TABLE 2. Serum and fecal antibody responses to *P. aeruginosa* antigens following 2 weeks of GI colonization with *P. aeruginosa* PAK

Antigen	Postcolonization serum antibody titer (SEM)[a]		OD_{405} nm of fecal samples[b]			
	IgG	IgA	IgG		IgA	
			Pre	Post	Pre	Post
Serogroup O6 LPS	1,277 (125)	270 (47)	0.068	0.502	0.060	0.343
Flagella	2,390 (298)	2,140 (413)	0.016	0.234	0.020	0.318
Pili	1,319 (158)	1,423 (122)	0.067	0.746	0.073	1.054

[a] Mean antibody titer of 10 sera. Titers were calculated by regression analysis as the reciprocal of the serum dilution yielding an optical density at 405 nm (OD_{405}) of 0.200. All precolonization serum antibody titers were <40 against all antigens tested.

[b] A pool of 10 fecal samples was tested at a single 1:20 dilution. Pre and Post denote pre- and postcolonization samples, respectively.

Serum and fecal antibody determinations indicated that GI colonization elicited both IgG and IgA antibodies to the LPS of strain PAK, along with antibodies to purified flagella and pili (Table 2). Titers were comparable to those previously reported for similarly colonized mice (48). These mice were designated immune and were divided into five groups; four groups were challenged with four different *P. aeruginosa* strains, while the fifth group served as a sentinel group in which the GI tract flora was monitored to ensure that strain PAK did not recur. An additional four groups of nonimmune mice that had had their facultative GI flora depleted but were not previously colonized with strain PAK were challenged with the four different *P. aeruginosa* strains initially given as a suspension of 10^7 CFU/ml of drinking water for 5 days.

During the next 3 weeks, none of the sentinel mice had a fecal culture positive for *P. aeruginosa*. Immune mice challenged for 5 days with 10^7 CFU of the LPS-heterologous strains 1244(pLAFR1) and PAO1(pLAFR1) per ml of drinking water had high levels (mean, 10^3 to 10^4 CFU/g of feces) of fecal *P. aeruginosa* that were not significantly different from those in corresponding nonimmune animals (data not shown). Mice challenged with the same dose of the homologous serogroup O6 strain 15921(pLAFR1) or the immunizing strain PAK(pLAFR1) had substantially lower fecal counts of these strains by day 16 after colonization, and these counts were significantly lower than those in nonimmune controls (Fig. 1) ($P \leq 0.02$, ANOVA). These data indicate that colonization of the GI tract with *P. aeruginosa* can effectively immunize mice against mucosal challenge by other LPS-homologous *P. aeruginosa* strains, although it is possible that immune responses to antigens other than LPS O side chains that are shared by strains PAK and 15921 may have contributed to mucosal clearance.

Expression of *P. aeruginosa* serogroup O11 antigen by *S. typhimurium* and its effect on virulence. We next investigated the properties of strains of *S. typhimurium* bearing pLPS2 to determine if they expressed *P. aeruginosa* serogroup O11 antigen and whether their virulence for mice was altered. Both *S. typhimurium* CS019(pLPS2), which is derived from a wild-type, virulent strain, and *S. typhimurium* SL3261(pLPS2), which is derived from a poorly virulent strain auxotrophic for aromatic amino acid synthesis, expressed *P. aeruginosa* serogroup O11 antigen (Fig. 2A); in contrast, when these strains carried only the cloning vector pLAFR1, they did not produce detectable *P. aeruginosa* O antigen. All of the *S. typhimurium* strains also

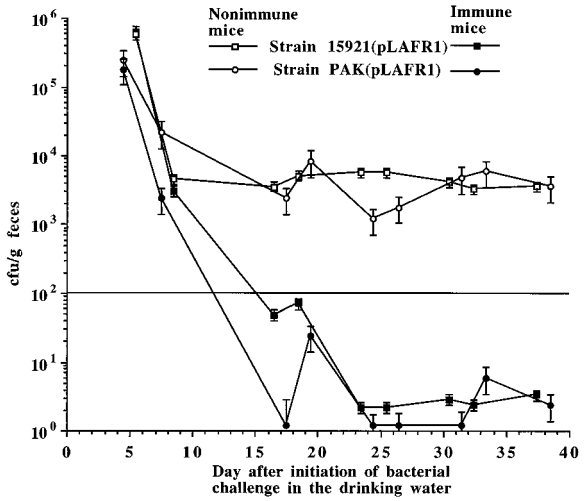

FIG. 1. Clearance of *P. aeruginosa* from the murine GI tract after challenge with strain 15921(pLAFR1) or strain PAK(pLAFR1). Immune mice were colonized with and then cleared of *P. aeruginosa* PAK before rechallenge, while nonimmune mice were not previously colonized with *P. aeruginosa*. Each point represents the geometric mean number of CFU per gram of feces detected by culture of samples from four or five animals, and error bars show the standard errors of the means. From day 17 on, the bacterial levels detected were significantly lower in samples from immune mice than in samples from the corresponding group of nonimmune mice ($P \leq 0.02$, ANOVA). Solid horizontal line indicates lower limit of detection of *P. aeruginosa* counts in fecal suspensions. Points below this line were derived by using a value of zero for a result from cultures in which no *P. aeruginosa* colonies grew on agar media.

expressed endogenous serogroup D *Salmonella* O antigen (Fig. 2B). Analysis of the virulence properties of these strains when either fed to BALB/c mice or injected i.p. into Swiss Webster mice revealed no effect on the virulence of the auxotrophic

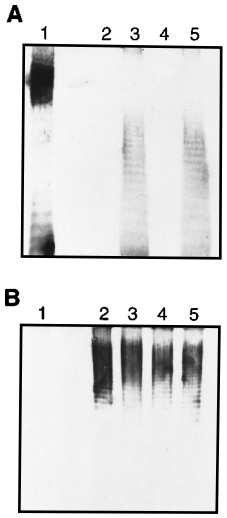

FIG. 2. Immunoblot of sarcosyl extracts from recombinant *S. typhimurium* strains expressing *P. aeruginosa* serogroup O11 antigen (A) or *Salmonella* group D antigen (B) in the presence of either recombinant plasmid pLPS2 or the cloning vector pLAFR1. The following antigens are shown: lane 1, LPS from *P. aeruginosa* 9882-80 (serogroup O11 control); lane 2, *S. typhimurium* CS019 (pLAFR1); lane 3, *S. typhimurium* CS019(pLPS2); lane 4, *S. typhimurium* SL3261 (pLAFR1); lane 5, *S. typhimurium* SL3261(pLPS2). In lane 1 of each immunoblot, 10 μg of *P. aeruginosa* LPS was applied to the original SDS-polyacrylamide gel; in lanes 2 to 5 of immunoblot A, 70 μg of sarcosyl extract from recombinant *S. typhimurium* strains was applied; in immunoblot B, 14 μg of the sarcosyl extract from each recombinant *S. typhimurium* strain was applied in lanes 2 to 5.

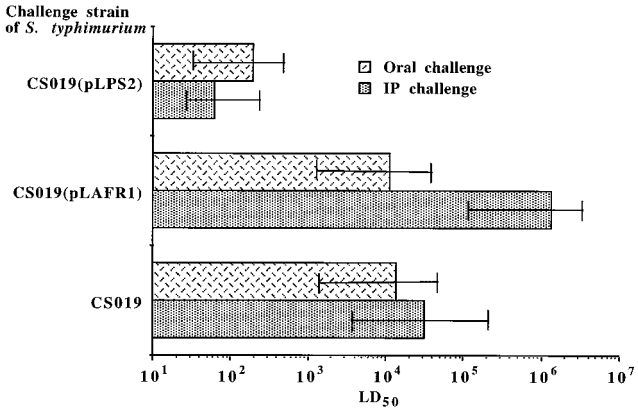

FIG. 3. LD_{50} after oral challenge of groups of five BALB/c mice or i.p. challenge of groups of five Swiss Webster mice with the derivative of wild-type, virulent *S. typhimurium* CS019 indicated on the *y* axis. Each bar represents the point estimate of the LD_{50} value, and error bars indicate the 95% confidence intervals. The LD_{50} value for *S. typhimurium* CS019(pLPS2) was significantly lower than values for the control strains ($P < 0.05$, probit analysis).

mutant *S. typhimurium* SL3261 due to expression of *P. aeruginosa* O antigen. No mice died after i.p. or oral challenge with up to 10^{10} CFU of *S. typhimurium* SL3261(pLPS2). Interestingly, expression of *P. aeruginosa* O antigens by the wild-type, virulent *S. typhimurium* CS019 strain decreased the LD_{50} by ~2 to 4 log units in both orally and i.p.-inoculated animals (Fig. 3). This finding suggests that coexpression of a *P. aeruginosa* O antigen increases the virulence of this wild-type strain of *S. typhimurium*.

Immune response to oral immunization with recombinant attenuated *S. typhimurium* strains and i.p. immunization with purified high-molecular-weight O polysaccharide. Oral immunization of mice with *S. typhimurium* SL3261(pLPS2) induced both serum and mucosal IgA antibodies to *P. aeruginosa* serogroup O11 antigen (Fig. 4A). In addition, oral immunization with recombinant *S. typhimurium* elicited circulating IgG antibodies (Fig. 4B). Controls fed *S. typhimurium* SL3261 (pLAFR1) had no immune response to *P. aeruginosa* O antigen (data not shown). Mice immunized i.p. with purified high-molecular-weight O11 polysaccharide produced circulating LPS-specific IgG and IgM antibodies (Fig. 4C), but no increase from preimmunization levels of serum or mucosal IgA antibody was evident (data not shown).

Clearance of *P. aeruginosa* from the murine GI tract after oral immunization with recombinant *S. typhimurium*. In the first experiment conducted after oral immunization of mice with recombinant *S. typhimurium* SL3261 carrying either pLPS2 or pLAFR1, we found greater clearance of the challenge strain of *P. aeruginosa* by mice with antibodies to *P. aeruginosa* serogroup O11 antigen than by mice lacking these antibodies (Fig. 5). Six of 10 immune mice had no detectable colonies of the challenge strain in their fecal cultures (<100 CFU/g of feces) by 15 days after challenge, whereas fecal cultures of all 10 nonimmune mice were positive ($P = 0.005$, Fisher exact test). Furthermore, the number of CFU of *P. aeruginosa* per gram of feces was significantly lower in colonized immune animals than in colonized nonimmune controls ($P < 0.01$, ANOVA). These results indicate that the recombinant strain of *S. typhimurium* expressing *P. aeruginosa* serogroup O11 antigen elicited immunity that protected mice against mucosal colonization by an LPS-homologous *P. aeruginosa* strain.

Since the mice in the first experiment had both local and

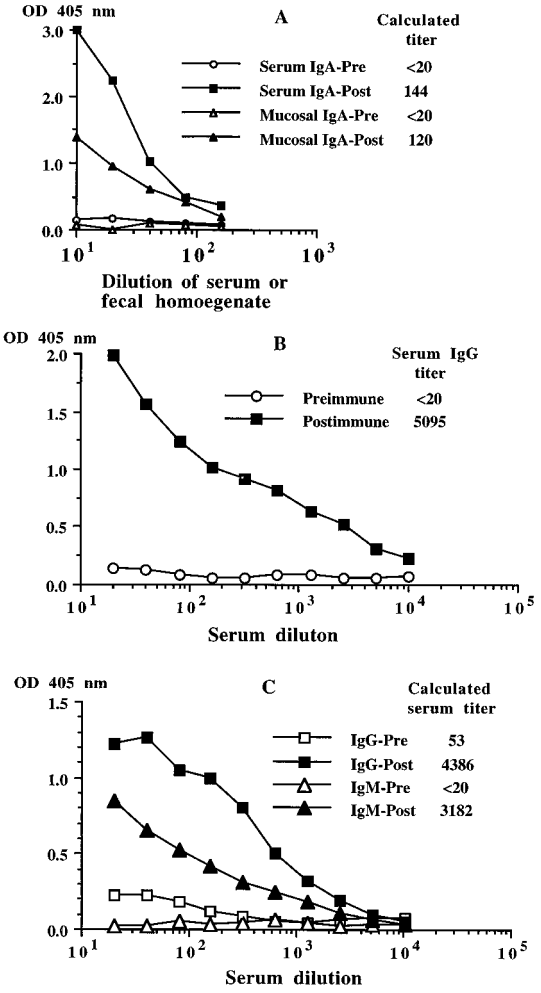

FIG. 4. Antibody responses to *P. aeruginosa* serogroup O11 antigen after oral immunization of mice with recombinant *S. typhimurium* SL3261(pLPS2) (A and B) or i.p. immunization with serogroup O11 high-molecular-weight O polysaccharide (C). Each point represents the mean of duplicate determinations. Titers were calculated by regression analysis as described previously (30). OD, optical density; Pre and Post, pre- and postimmunization.

systemic antibodies to *P. aeruginosa* O antigen before mucosal challenge, we next determined whether a local antibody response was needed to promote *P. aeruginosa* clearance from the GI tract or whether circulating antibody was as effective as antibody induced through mucosal immunization. Mice were immunized orally with recombinant *S. typhimurium* SL3261 bearing either pLPS2 or pLAFR1 or were immunized i.p. with purified high-molecular-weight O polysaccharide, which induced measurable circulating IgG and IgM antibody only (Fig. 4C) and, as noted above, no detectable serum or mucosal IgA. As a control for circulating antibody specificity, we immunized an additional group of mice with an irrelevant high-molecular-weight O polysaccharide from serogroup O3 *P. aeruginosa*. Results are shown in Fig. 6. There were no significant differences in colonization level on any day among the three control groups: mice immunized orally with *S. typhimurium* SL3261 (pLAFR1), mice immunized i.p. with serogroup O3 high-molecular-weight O polysaccharide, and mice given nothing before oral challenge. The results from these three control groups were pooled for the comparative analysis of bacterial loads in animals immunized either orally with *S. typhimurium*

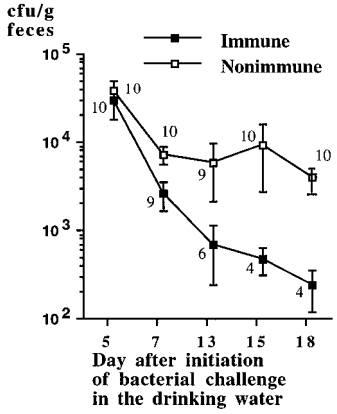

FIG. 5. Clearance of *P. aeruginosa* 9882-80 (serogroup O11) from the murine GI tract after oral vaccination with either *S. typhimurium* SL3261(pLPS2) (immune) or *S. typhimurium* SL3261(pLAFR1) (nonimmune). Each point represents the mean of fecal culture results for four or five animals, and error bars show the standard errors of the means. The number next to each point indicates how many mice had fecal cultures yielding >100 CFU of *P. aeruginosa* 9882 per g. From day 13 on, the number of CFU of *P. aeruginosa* per gram of feces measured in the immune group was significantly lower than that measured in the nonimmune group ($P \leq 0.05$, ANOVA).

SL3261(pLPS2) or i.p. with serogroup O11 high-molecular-weight O polysaccharide. ANOVA indicated a trend by day 14 or 15 ($P = 0.07$) toward lower fecal *P. aeruginosa* counts in both immunized groups than in the combined control groups; the *P* values were 0.008 on day 22 and 0.03 on day 26. When analyzed separately, both immunized groups were found to be colonized with significantly fewer *P. aeruginosa* organisms than

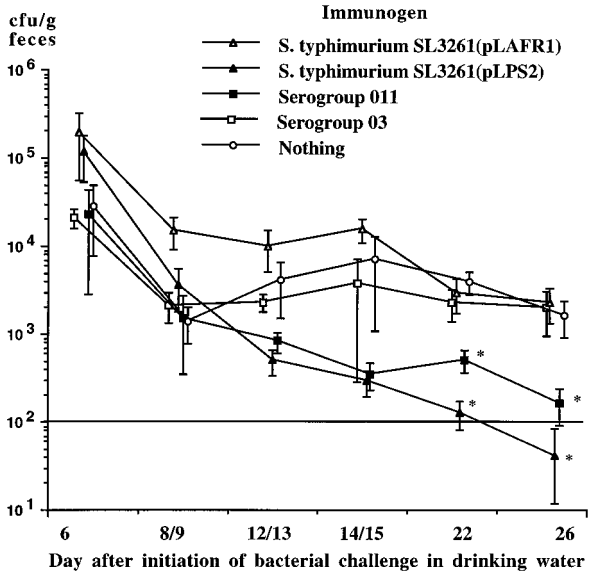

FIG. 6. Clearance of *P. aeruginosa* 9882-80 (serogroup O11) from the murine GI tract after oral vaccination with either *S. typhimurium* SL3261(pLPS2) or *S. typhimurium* SL3261(pLAFR1), i.p. immunization with high-molecular-weight O polysaccharide from *P. aeruginosa* serogroup O3 or O11, or no prior immunization. Each point represents the geometric mean of fecal culture results for eight animals, and error bars show the standard errors of the means. Asterisks indicate measurements significantly lower than the number of CFU per gram of feces measured in the three control groups combined ($P \leq 0.02$, ANOVA). The horizontal line indicates the lower limit of detection of *P. aeruginosa* counts in fecal suspensions. Points below this line represent mean values for groups of animals in which cultures from some of the animals yielded *P. aeruginosa* colonies and others did not.

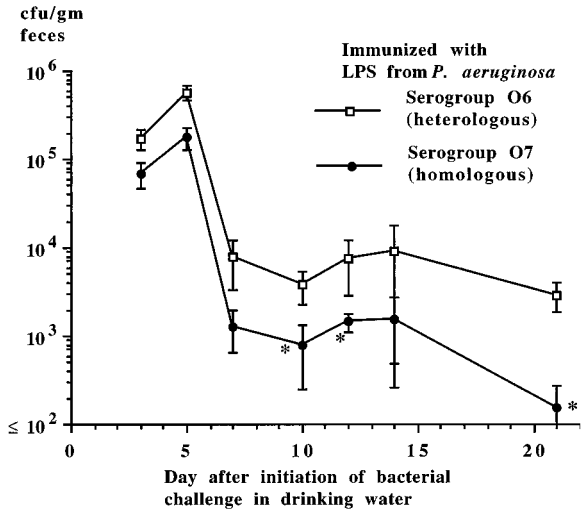

FIG. 7. Clearance of *P. aeruginosa* 1244 (serogroup O7) from the murine GI tract after i.p. immunization with LPS from the strain indicated in the key. Each point represents the geometric mean of fecal culture results for eight animals, and error bars the standard errors of the means. Asterisks indicate counts for the serogroup O7-immunized group that are significantly lower than the number of CFU per gram of feces in the serogroup O6-immunized animals ($P \leq 0.05$, ANOVA).

controls by day 22 ($P \leq 0.05$, ANOVA). Moreover, the two immunized groups did not differ significantly ($P > 0.05$) from each other in terms of bacterial fecal counts. By day 21, only 1 of 22 control mice had fecal cultures yielding <100 CFU of the challenge strain of *P. aeruginosa*, compared with five of eight such cultures from animals immunized i.p. with serogroup O11 high-molecular-weight O polysaccharide ($P = 0.002$, Fisher exact test) and six of eight such cultures from mice immunized orally with *S. typhimurium* SL3261(pLPS2) ($P < 0.001$, Fisher exact test).

To confirm that the enhanced clearance of *P. aeruginosa* from the murine GI mucosal surface after elicitation of circulating antibody to LPS by i.p. immunization was not limited to *P. aeruginosa* from the O11 serogroup nor a property of the reagents and bacterial strains used in the experiments described above, additional mice were immunized i.p. with *P. aeruginosa* LPS isolated from serogroups O6 and O7 and then challenged orally with *P. aeruginosa* 1244, a serogroup O7 isolate previously shown (48) to be capable of chronically colonizing the murine GI tract. LPS was used here because a high-molecular-weight O-polysaccharide vaccine cannot be prepared from serogroup O7 *P. aeruginosa* (30). Mice immunized i.p. with serogroup O7 LPS developed IgM and IgG antibody titers to the homologous O antigen at levels that were comparable to those shown in Fig. 4. These animals did not have detectable serum or fecal IgA or fecal IgG (data not shown), indicating a lack of preinfection mucosal antibodies. Ten days after oral challenge with *P. aeruginosa* 1244, mice with antibodies to the homologous O7 antigen had cleared this strain from the GI tract significantly better than animals immunized with the heterologous O antigen ($P < 0.05$, ANOVA; Fig. 7). By day 21 after challenge, five of eight mice immunized i.p. with serogroup O7 LPS had fecal cultures that grew <100 CFU of strain 1244, compared with none of the eight controls immunized with serogroup O6 LPS ($P = 0.01$, Fisher exact test).

DISCUSSION

Our results indicate the *P. aeruginosa* LPS O antigens, like those of *V. cholerae* and *S. typhimurium* (41, 64), can serve as targets for antibodies that mediate protection against mucosal colonization. Furthermore, there appeared to be no need to induce antibodies by mucosal immunization to promote clearance of *P. aeruginosa* from the murine GI tract. Circulating antibodies, induced by i.p. immunization with purified antigens, were as effective in promoting mucosal clearance as was the combination of mucosal and circulating antibodies, induced by oral immunization with recombinant *S. typhimurium* SL3261(pLPS2). These animal studies indicate that local antibodies, particularly sIgA, may actually play little to no role in protecting mucosal surfaces against bacterial colonization. The concept that sIgA or other local immune effectors are needed for effective immunity against mucosal pathogens may need to be reevaluated.

The animal model of chronic GI colonization with *P. aeruginosa* uses antibiotic treatment to alter the natural flora and allow for establishment of *P. aeruginosa* infection. This model mimics to a large degree a situation commonly encountered among patients at risk for *P. aeruginosa* infection (1, 10, 56). As Pollack has recently written (51), "Asymptomatic large-bowel colonization resulting from prolonged exposure to the hospital environment and the selective pressure of antibiotics may be a silent source of [*P. aeruginosa*] organisms that subsequently invade the bloodstream." Thus, evaluating therapeutic interventions in mice whose indigenous GI flora has been depleted with antibiotics to allow *P. aeruginosa* to establish mucosal colonization is both a useful and representative model of human infection. This strategy has been used in similar animal models and employed by investigators throughout the world (17, 19, 46, 59).

Although our results are derived from a limited set of experiments in animals, they nonetheless raise questions about the commonly expressed dogma that sIgA is the major effector of immunity to infection at a mucosal surface. As long as there are no deficiencies in other immunoglobulin classes, selective IgA deficiency is most often a benign condition, manifesting no clinical consequences; specifically, 60 to 90% of individuals lacking IgA are clinically healthy (38, 43, 54, 58). Even with long-term IgA deficiency, adults can remain healthy (38), and most children with an IgA deficiency leading to clinical problems also have deficiencies in other antibody isotypes (most commonly IgG [60]) as well. Selective IgA immunodeficiency is very common, occurring at a rate of 17 per 100,000 white, non-Hispanic people (18). (As a basis for comparison, the corresponding figure for patients with AIDS was 30 per 100,000 in the United States in 1993 [16].) These figures question the biologic role and importance of IgA if so many people can live without it and remain healthy. If, as has been argued, IgA-deficient individuals have compensatory IgG and IgM on mucosal surfaces, then these antibodies, and not sIgA, may be the truly important mediators of mucosal immunity, even in persons with normal levels of sIgA. Finally, even though IgA deficiency is the most frequent of the antibody deficiency syndromes, recurrent sinopulmonary infection, bronchiolitis, and bronchitis are more commonly associated with IgG deficiency than with IgA deficiency in a variety of populations of patients (15, 23, 44, 57, 61), and most of the affected individuals have normal levels of IgA.

One of the areas that we tried to address in this study was the relative efficacies of circulating and local antibodies in promoting bacterial clearance from a mucosal surface. Few studies have compared the clearance of a bacterial pathogen

from a mucosal surface in the presence of only local or only systemic antibodies. A mouse model of pharyngeal infection with group A streptococci has been used to address some of the relevant issues (6). For example, while intranasal immunization with peptides of M protein coupled to the cholera toxin B subunit was protective, this vaccine induced significant levels of serum IgG. The investigators could not exclude the possibility that this immune effector accounted for the protective immunity observed (5, 7, 13, 14). In another study, it was shown that serum IgG was inferior to sIgA in protection against mucosal infection (6) when these immune effectors were both delivered by the intranasal route. However, no animals with serum IgG passively infused into their blood were evaluated. A subsequent study by Fischetti and colleagues, using active immunization with a vaccinia virus-M protein recombinant vector, demonstrated that intranasal delivery of streptococcal antigens by this vector elicited protective immunity in association with high levels of serum IgG and undetectable levels of local IgA (26). Thus, the immune effector responsible for protection against pharyngeal colonization by group A streptococci was not clearly identified. While these data indicate that sIgA can provide better protection against pharyngeal infection than IgG when both immunoglobulin isotypes are delivered by the intranasal route, they do not clarify whether serum IgG also provides protection or whether it can be synergistic with local IgA. Thus, it has not been firmly established that either local immunization or sIgA is needed for the clearance of bacteria from a mucosal surface.

There is currently a strong interest in developing a variety of vaccines for delivery by application to a mucosal surface, with interest focused on the use of genetically attenuated mucosal pathogens as delivery vehicles for antigens that can elicit protective immunity (2, 20, 33, 35, 65). The advantages of this route of immunization include low cost, ease of vaccine preparation and delivery, low toxicity, and what is often assumed to be a need to induce local immunity. Thus, it was gratifying to observe that *P. aeruginosa* O antigens can be expressed at a level sufficient to provoke protective antibodies by an attenuated *S. typhimurium* strain without changes in the virulence of that strain. However, oral immunization invariably elicits circulating IgG as well as local immune effectors, as was shown in our studies with the *P. aeruginosa-S. typhimurium* hybrid construct. Thus, it is not valid to conclude solely on the basis of studies using a mucosal immunization route that local antibodies such as sIgA, and not circulating IgG, are the mediators of protective immunity. An oft-cited example of the superiority of local immunity to systemic immunity is derived from the comparison of live attenuated oral poliovirus vaccines with killed inactivated vaccines. Oral vaccine is claimed to impart greater intestinal, and hence greater overall, immunity, and there is evidence that recipients of live oral polio vaccine subsequently challenged with live vaccine virus strains excrete less virus in their feces than do recipients of inactivated polio vaccines (45). However, the inactivated vaccine also protects against virus excretion by the fecal route and is even more effective than the live vaccine in reducing oropharyngeal excretion of live virus (32, 40). Furthermore, the exclusive use of killed polio vaccine in the United States through 1962 reduced the incidence of polio by 90% in nonvaccinated individuals (53), and the exclusive use of killed vaccine in Finland, Holland, Iceland, and Sweden eliminated circulating pathogenic polioviruses in these countries (9). These results indicate that the systemically administered killed vaccine effectively reduces mucosal transmission of wild-type virus. The fact that the sIgA induced by oral poliovirus vaccines has never been documented to be the mediator of reduced virus excretion again emphasizes the paucity of evidence that any advantage of oral vaccination is due to elicitation of sIgA antibodies.

Future studies must validate the potential of local immune effectors to clear mucosal pathogens and must determine whether these local effectors are needed for full immunity. For such studies, passive therapies employing variable-region identical monoclonal antibodies of different isotypes would be optimal, with the amounts of local and circulating antibodies controlled and a quantitative evaluation undertaken. The development by Neutra and colleagues of an effective method for delivering IgA antibodies to the GI tracts of mice (41, 64) should facilitate comparisons of the roles of different antibody isotypes in protective immunity.

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