Development of a Germfree Mouse Model of *Vibrio cholerae* Infection

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A mouse model of *Vibrio cholerae* infection was successfully developed with germfree mice. Three- to four-week-old germfree mice were orally inoculated with strains of *V. cholerae* to be tested and then moved to normal housing after inoculation. Stool culture, measurement of serum vibriocidal antibody titers, and determination of immune responses to the cholera toxin B subunit demonstrated that germfree mice are readily colonized by *V. cholerae* and develop systemic and mucosal immune responses to antigens expressed by these organisms. Immune responses to the B subunit of Shiga toxin 1, which was expressed from a *V. cholerae* vaccine vector, were less pronounced. This model should be valuable for studying immune responses to *V. cholerae* infection and immunization, including responses to heterologous antigens expressed by cholera vector strains.

Although a large variety of animal models have been used in cholera research, no single animal model reproduces all the aspects of the human disease (12). *Vibrio cholerae* can infect suckling animals because of their immature host defenses and reduced intestinal floras, but infection of suckling animals (such as neonatal mice) is often lethal and is a poor model in which to study the development of immune responses. A variety of nutritional, chemical, antibiotic, and surgical treatments permits the colonization of adult animals by *V. cholerae*, but such manipulations may not be physiologic (12). Neutralization of gastric acid and paralysis of intestinal motility in adult rabbits allows colonization with *V. cholerae* after oral inoculation (5). This model allows measurement of specific immune responses to the infecting organism (2, 8) but is cumbersome and expensive; only a small number of rabbits can easily be included in each experiment.

We have been particularly interested in developing animal models in which to study the immune response to heterologous antigens carried by *V. cholerae* vaccine vectors (2, 3). A model of *V. cholerae* infection in older mice would have many advantages over models of *V. cholerae* infection in neonatal mice and in adult rabbits for measuring immune responses to heterologous antigens. Neonatal mice are rapidly killed by *V. cholerae* infection and have an immature immune system. Older mice would provide a mature immune system in which to assess immune responses to oral immunization, and the mouse model would be more versatile than the rabbit model in studying protection induced by *V. cholerae* vectors expressing heterologous antigens because of the ease of studying more animals, the availability of different genetic backgrounds of mice (including knockout mice), and the usefulness of mice for studying protection in cases in which mice provide the standard animal model of disease, such as infection with *Salmonella typhimurium* or *Bordetella pertussis*. However, we were unable to colonize 5- to 6-week-old female Swiss-Webster mice (Tac-

onic Laboratories, Germantown, N.Y.) with *V. cholerae* after oral inoculation. We reasoned that use of germfree mice might allow colonization by orally inoculated strains of *V. cholerae*, with subsequent measurement of immune responses.

To test this hypothesis, 3- to 4-week-old female NIH Swiss germfree mice (Taconic Laboratories) were removed from a germfree shipper and were either orally inoculated immediately with 10^{10} streptomycin-resistant *V. cholerae* in a volume of 0.5 ml of 0.5 M NaHCO₃ per mouse or kept uninoculated as controls. *V. cholerae* strains with chromosomally encoded streptomycin resistance were used to allow the monitoring of stool cultures for the shedding of *V. cholerae* against the background of other fecal floras. The *V. cholerae* strains tested were the wild-type El Tor strain C6709, the vaccine vector JRB12, which expresses the B subunit of *Escherichia coli* Shiga toxin 1 (StxB1) (formerly called Shiga-like toxin I) (C6709 $\Delta attRS1$ *irgA::irgP-stxB1 lacZ::htpG-stxB1*) (2), and the wild-type *V. cholerae* O139 strain MO10 (18); three to six mice were inoculated per group. Mice were not kept in germfree conditions after removal from the germfree shipper. Normal (not germfree) 5- to 6-week-old female Swiss-Webster mice were identically treated to compare colonization models. No animals became ill after inoculation.

Fresh stool pellets were collected from each animal on days 1, 3, 5, 7, 14, and 21; single pellets were resuspended in 0.5 ml of Luria-Bertani medium (14), vortexed, and allowed to settle. One hundred microliters of supernatant was then plated onto Luria-Bertani agar with streptomycin (100 μ g/ml) to evaluate the persistence of viable streptomycin-resistant *V. cholerae* in the stool. The identities of the streptomycin-resistant colonies were confirmed as *V. cholerae* by subsequent plating on thio-sulfate-citrate-bile salts-sucrose agar, since direct plating of stool pellets on this highly selective agar led to lower colony counts.

Blood was drawn from tail arteries on days 0, 14, and 21, and sera were separated by centrifugation, prior to storage at -70°C . Stools were collected on days 14 and 21, and samples were prepared for use in immunoassays as follows. Single stool pellets were resuspended in 1 ml of phosphate-buffered saline

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(PBS)-EDTA-soybean trypsin inhibitor (type II-S; Sigma Chemical Co., St. Louis, Mo.) at a ratio of 3 parts PBS, 1 part 0.1 M EDTA, and a final concentration of 0.1 mg of soybean trypsin inhibitor per ml. The stools were vortexed and then centrifuged at 1,800 rpm (Eppendorf Microcentrifuge 5415 C) for 10 min. The supernatant was removed and recentrifuged at 12,000 rpm for 10 min. Twenty microliters of 100 mM phenylmethylsulfonyl fluoride (Sigma) was added to the supernatant, and samples were stored at -20°C . Bile (approximately 4 to 6 μl) was collected following sacrifice and gallbladder aspiration on day 21. Rodents have a polymeric immunoglobulin A (IgA) receptor on their hepatocytes, allowing bile to be a relevant marker of secretory IgA in mice (1).

Serum vibriocidal antibody titers were measured in a microassay as follows. The endogenous complement activities of test sera were inactivated by heating the sera at 56°C for 1 h. Fifty microliters of serial twofold dilutions of the test sera in 0.15 M saline (1:25 to 1:25,600) were placed in the wells of sterile 96-well tissue culture plates. Fifty microliters of a 10^8 CFU/ml culture of *V. cholerae* C6709 (for testing sera from animals inoculated with C6709 and JRB12) or *V. cholerae* MO10-T4 (an unencapsulated *V. cholerae* Bengal strain used as the target strain for vibriocidal assays on sera from animals inoculated with *V. cholerae* MO10 [4, 17]) in 0.15 M saline with 22% guinea pig complement (Gibco BRL Life Technologies, Gaithersburg, Md.) was added to the serum dilutions, which were then incubated for 1 h at 37°C . Brain heart infusion broth (150 μl ; Difco Laboratories, Detroit, Mich.) was added to each well, and the plates were incubated for approximately 2 h at 37°C . The optical densities at 650 nm were then measured; the vibriocidal antibody titer was calculated as the dilution of serum causing a 50% reduction in optical density compared with the optical densities of wells containing sera from control (uninoculated germfree) animals drawn on the same day.

Mouse anti-cholera toxin B subunit (anti-CtxB) IgG and IgA were measured as follows. Microtiter plates were coated with 10 μg of type III gangliosides (Sigma Chemical Co.) per ml in 50 mM carbonate buffer, pH 9.6, and then incubated with 1 μg of purified CtxB (List Biological Laboratories, Inc., Campbell, Calif.) per ml in carbonate buffer, pH 9.6. Plates were blocked with PBS-1% bovine albumin (Sigma) and then washed with PBS-0.05% Tween 20 and stored dry at 4°C . Duplicate serial twofold dilutions of serum (1:40 to 1:20,480), stool (1:1 to 1:512), and bile (1:200 to 1:102,400) samples in PBS-0.05% Tween 20 were added to the microtiter wells. Serum anti-CtxB IgG was assayed with goat anti-mouse IgG conjugated to biotin (Zymed Laboratories, Inc., South San Francisco, Calif.) at a 1:2,000 dilution in PBS-0.05% Tween 20 and then with streptavidin horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) at a 1:4,000 dilution in PBS-0.05% Tween 20. Reactions were developed with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Sigma) with 0.1% H_2O_2 (Sigma), and the optical densities at 405 nm were measured. Stool and bile anti-CtxB IgA titers were determined with goat antiserum to mouse IgA conjugated to biotin (Zymed) and then streptavidin-horseradish peroxidase and ABTS- H_2O_2 as described above. The antibody titer was calculated as the highest dilution of test sample producing an optical density greater than three times the arithmetic mean of the optical densities of control lanes containing PBS-0.05% Tween 20. Geometric mean titers and standard errors of the mean were plotted, and statistical significance was analyzed by the two-tailed Student *t* test for the comparison of means.

To assess the ability of the germfree mice to respond to a heterologous antigen expressed by a *V. cholerae* vaccine vector, serum and mucosal antibody responses to StxB1 expressed by

JRB12 were measured by enzyme-linked immunosorbent assays (ELISA) in similar fashions with globotriosyl ceramide, the natural receptor for Stx1, as the capture molecule. Microtiter plates were coated with 10 μg of globotriosyl ceramide (Matreya, Inc., Pleasant Gap, Pa.) per ml in methanol, which was allowed to evaporate, incubated with 1 μg of purified StxB1 (gift of David Acheson) per ml in carbonate buffer, pH 9.6, and blocked. Duplicate serial twofold dilutions of serum (1:20 to 1:10,240), stool (1:1.25 to 1:640), and bile (1:150 to 1:76,800) samples were added, and serum anti-StxB1 IgG titers and stool and bile anti-StxB1 IgA titers were determined as described above.

The nongermfree adult mice shed *V. cholerae* in stools on day 1 after inoculation but not thereafter; in comparison, the germfree mice continued to shed *V. cholerae* at 21 days postinoculation (Fig. 1). One hundred percent of mice inoculated with the wild-type strains C6709 and MO10 continued to shed vibrios in stools up to 7 days after inoculation, while the *attRS1* deletion strain JRB12 colonized the germfree mice less well. This observation is in accord with the reduced colonization of JRB12 relative to that of C6709 previously demonstrated with a suckling mouse model (2). Nongermfree mice did not develop significant serum vibriocidal antibody titers to the inoculated strains, consistent with the poor colonization seen (Fig. 2). In contrast, the germfree mice developed substantial vibriocidal titers at days 14 and 21 after inoculation with the two El Tor strains C6709 and JRB12 and the Bengal strain MO10 (Fig. 2), demonstrating immune response to the inoculated *V. cholerae*. The nonencapsulated mutant of MO10, MO10-T4, was used as the target strain in the vibriocidal assay of sera of mice inoculated with MO10, as vibriocidal antibody titers following oral inoculation with MO10 have been found to be greatly increased when MO10-T4, rather than MO10, is used in the assay (4).

After inoculation of germfree mice with the wild-type El Tor strain, C6709, no serum anti-CtxB subunit IgG titers or stool anti-CtxB IgA titers were detectable (Fig. 3 and 4). However, a substantial mucosal anti-CtxB IgA antibody titer was observed in bile specimens at day 21 (Fig. 5). The *attRS1* deletion strain JRB12 lacks the *ctx* genes and served as a negative control in this experiment. Oral inoculation of germfree mice with the O139 strain MO10 produced a significant serum anti-CtxB subunit IgG antibody response at day 21 (Fig. 3), as well as significant stool and biliary anti-CtxB subunit IgA antibody responses at day 21 (Fig. 4 and 5).

The *V. cholerae* vaccine vector strain JRB12, which contains two copies of the gene encoding the B subunit of Shiga toxin 1 (*stxB1*), each of which is under the control of the *V. cholerae* heat shock promoter *hspGp* (2), was used to assess the immune response to a *V. cholerae*-vectored heterologous antigen in the germfree mouse model. No significant serum anti-StxB1 IgG titers or stool anti-StxB1 IgA titers were detected in animals inoculated with JRB12 (data not shown). Two of the four mice orally inoculated with JRB12 had sufficient bile collected to allow testing for biliary anti-StxB1 IgA. One of the two mice had a strongly positive response in the anti-StxB1 IgA ELISA at the starting dilution of 1:150 (data not shown). StxB1 has previously been shown to be a poor oral immunogen in comparison with CtxB (2), perhaps because of inherent antigenic differences between the two subunits or because less StxB1 than CtxB is available to the mucosal immune system, since StxB1 is confined to the periplasm until released by cell lysis (3).

Although germfree mice have been used to study the chemotaxis and adhesion of *V. cholerae* to the intestinal mucosa in vivo (7), few prior studies have utilized a germfree mouse model of *V. cholerae* colonization to assess immune responses

FIG. 1. Percentages of mice with *V. cholerae* recovered from stool cultures over several days following a single oral inoculation. Symbols shown on the right from top to bottom correspond to bars in the figure from left to right. Values for nongermfree mice appear only for day 1.

to infection; all have kept the animals germfree throughout the course of experimentation (9, 10, 13, 15, 16). In one study (13), 3- to 5-week-old germfree mice were inoculated with 10^6 CFU of classical *V. cholerae*. All became colonized with vibrios throughout the gastrointestinal tract, but they did not become ill. High vibrio concentrations in the feces were observed throughout the 3 months of observation; animals developed

serum agglutinating antibodies as well as type-specific antibodies. In contrast, under the protocol described here, mice were moved to normal housing following inoculation, greatly increasing the ease of handling, since no special facilities were required, and resulting in substantial savings. The subsequent colonization of the gut by aerobic and anaerobic floras still allowed *V. cholerae* colonization for several weeks.

FIG. 2. Vibriocidal antibody assays following oral inoculation. See the text for details. The geometric mean titer (GMT) of each test group was compared with that of the uninoculated germfree animals. Bars depict the standard errors of the mean. *, $P < 0.05$; #, $P < 0.01$; +, $P < 0.001$.

FIG. 3. Serum anti-CtxB IgG ELISA results at days 14 and 21. See the text and the legend to Fig. 2 for details. The geometric mean titer (GMT) of each test group was compared with that of the control strain, JRB12, and that of the uninoculated germfree animals.

FIG. 5. Bile anti-CtxB IgA ELISA results at day 21. See the text and the legend to Fig. 2 for details. The geometric mean titer (GMT) of each test group was compared with that of the control strain, JRB12, and that of the uninoculated germfree animals.

V. cholerae is a mucosal, noninvasive organism not only in a human host but also in a germfree mouse (13). *Vibrios* selectively adhere to the M cells of the gastrointestinal tract, thus delivering a strong stimulus to the mucosal immune system (6, 11). Our observations with germfree mice support the finding that *V. cholerae* specifically induces mucosal immune responses. Mice orally inoculated with the Bengal strain MO10 developed strong biliary and stool anti-CtxB IgA responses in addition to a systemic IgG response, while those inoculated with the El Tor strain C6709 developed a strong biliary anti-CtxB IgA response even in the absence of systemic anti-CtxB IgG. It is also suggestive that one of two mice orally inoculated with the vector strain JRB12 developed a biliary anti-StxB1 IgA response in the absence of systemic anti-StxB1 IgG.

Our results suggest that strong antigens, such as the surface lipopolysaccharide epitopes that stimulate vibriocidal antibody responses or the highly immunogenic, secreted cholera toxin,

are most likely to produce detectable serum and mucosal immune responses in the germfree mouse model. Repeated oral inoculation of germfree mice with *V. cholerae* vector strains, which have previously enhanced immune responses in an adult rabbit model of *V. cholerae* infection (8), may also lead to greater immune responses in the mouse model than those seen with a single dose.

The germfree mouse model of *V. cholerae* infection has several shortcomings. Germfree mice are more expensive to purchase than nongermfree animals, but since the mice do not remain germfree, the cost of maintaining the animals is low. The volume of bile that can be obtained from a mouse may be too small for some applications and requires sacrifice of the animal; alternatively, several stools can be collected from mice at a variety of time points and processed for secretory IgA as described in this report. Finally, gut physiology and intestinal immune responses in germfree mice may not be entirely normal. However, the model does allow measurement of systemic and mucosal immune responses to antigens expressed by *V. cholerae* in vivo and may be very useful for studies of cholera-vectored delivery systems. Oral inoculation and serum, stool, and bile collection in mice are rapid, simple techniques. Therefore, for studies in which the mouse provides the optimal experimental system, we suggest that oral inoculation of germfree mice with *V. cholerae*, with subsequent housing in nongermfree conditions, may be an effective model.

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FIG. 4. Stool anti-CtxB IgA ELISA results at days 14 and 21. See the text and the legend to Fig. 2 for details. The geometric mean titer (GMT) of each test group was compared with that of the control strain, JRB12, and that of the uninoculated germfree animals.

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