Protection of Suckling Mice from Experimental Cholera by Maternal Immunization: Comparison of the Efficacy of Whole-Cell, Ribosomal-Derived, and Enterotoxin Immunogens

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The susceptibility of suckling mice to oral infection with several different *Vibrio cholerae* was determined. Mice up to 10 days of age were uniformly susceptible to oral infection with 10^8 colony-forming units of virulent organisms. Age-dependent resistance occurred thereafter to a maximum at about 15 days of age. The efficacy of selected vaccines was compared by oral challenge of 8-day-old, passively immunized CFW mice. An Ogawa-derived ribosomal antigen was found to be superior to a commercial whole-cell vaccine or to purified cholera enterotoxin. The ribosomal antigen was 50- to 100-fold more protective than the other vaccines on a weight basis against otherwise lethal challenge with Ogawa, Inaba, or El Tor Ogawa serotypes.

Oral infection of suckling mice has been reported recently by Ujiiye and associates (18, 19) and others (1) as a useful model of experimental cholera. The response to successful infection was the accumulation of fluid in the intestines, diarrhea, and death. Passive protection of suckling mice, born to appropriately immunized mothers, has been demonstrated against oral (18) and intraperitoneal (16) challenge with virulent vibrios. In contrast to the reported placental transmission of protection to the suckling rabbit (14), only protection via colostrum or milk could be positively demonstrated in the suckling mouse.

Present knowledge of the immunology of cholera is not yet sufficient to resolve the relative contributions of antitoxic and antibacterial immunity to effective prophylaxis against human cholera. However, a number of reports, particularly the extensive studies of Freter (6–8, 10), have established the importance of "local immunity" dependent upon the presence of coproantibody. Oral challenge of passively immunized suckling mice would seem to be a better measure of protection provided by cholera vaccines than the intraperitoneal challenge of the standard mouse protective test.

Considerable evidence has accumulated to suggest that immunization with subcellular preparations of a number of bacterial pathogens provides a substantial degree of protection against challenge with the homologous orga-

nism. Studies by Youmans and Youmans (28–31) have demonstrated that ribonucleic acid from *Mycobacterium tuberculosis* affords a high level of protection against experimental tuberculosis in the mouse. Immunization of mice with ribosomal and purified nucleic acid preparations from *Salmonella typhimurium* protects against subsequent challenge with the virulent organism (21–24). Effective ribosomal vaccines against *Pseudomonas aeruginosa* (26), *Staphylococcus aureus*, and *Diplococcus pneumoniae* (17) also have been reported.

Progress has been made in the development of a subcellular vaccine for cholera. Lipopolysaccharide and protein-lipopolysaccharide antigens of Verwey, in submicrogram quantities, are capable of protecting mice against intraperitoneal infection and of inducing vibriocidal antibody in animals and humans (25). An Inaba-derived protein-lipopolysaccharide was evaluated in field trials in East Pakistan (13) during 1968 and 1969. For a 3-month period postvaccination, protection was equivalent to that provided by an Inaba whole-cell vaccine. For longer periods, the subcellular preparation was significantly less effective in lowering the case rates. Actor and associates at Smith, Kline and French Laboratories have shown that mice born to mothers immunized with 20 µg of an Ogawa-derived ribosomal vaccine were highly resistant to both homologous and heterologous intraperitoneal challenge at 4 weeks of age (16). The duration of protection was related to the quantity of vaccine given to the mother.

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The production and isolation of cholera toxin in highly purified form by Finkelstein and Lo Spalluto (5) has allowed a more thorough study of its role in cholera pathogenesis and immunity. Indeed, the toxin and its naturally occurring or artificially prepared toxoid have shown promise as protective immunogens. Immunized rabbits were shown to resist intra-intestinal challenge with live vibrios (3) as well as challenge of ileal loops with toxin (11), whereas dogs immunized with toxin plus adjuvant were protected against oral challenge for 10 months (15).

This communication describes the use of the oral route of infection in establishing the susceptibility of different strains of mice to several strains of *Vibrio cholerae*. Once this was established, passively immunized suckling mice were used to test the efficacy of selected cholera vaccines.

MATERIALS AND METHODS

Mice. CFW mice, purchased from Carworth Farms (New City, N.Y.), were used in studies of the efficacy of cholera vaccines. Texas Inbred (Houston, Tex.) outbread ICR mice and CD-1 (Charles River Farms) specific pathogen-free mice, raised in departmental animal and pathogen-free facilities, respectively, were used in some experiments. The mice were housed in Iso-Cages fitted with Iso-Tops (Lab Cages, Inc., New City, N.Y.) with white pine shavings as bedding. Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) and water were available ad libitum.

Immunization. Female CFW mice, 10 to 12 weeks of age, were administered a single subcutaneous dose of each test vaccine one day prior to mating. The vaccine was administered in a total volume of 0.2 ml by injecting 0.1 ml each just anterior to the two most posterior teats.

Vaccines. Commercial bivalent vaccines (one-half of the mice received Lederle lot no. 329-383, and the other half received Lilly lot no. 6WE36A) were obtained from the Health Center, The University of Texas at Austin, and administered in doses of 2×10^8, 2×10^7, or 2×10^6 cells per mouse. These numbers of cells represented dry weights of 37.5, 3.75, and 0.375 μg, respectively, based on Verwey's (25) and our own determinations that 8×10^9 vibrios were equivalent to 1.5 mg (dry weight) of cells. Cholera toxin (NIH lot no. 1071) was rehydrated shortly before use and administered in doses of 10, 1, and 0.1 μg. An Ogawa-derived ribosomal vaccine (HS222) was administered in doses of 10, 1, and 0.1 μg. The vaccine was kindly provided by P. Actor of Smith, Kline and French Laboratories (Philadelphia, Pa.). The purification and properties of HS222 have been reported (12). All vaccines were diluted in sterile nonpyrogenic saline (Travenol, Inc.). Control animals were injected with saline alone.

Bacterial strains. Lyophilized cultures of *V. cholerae* strains CA411 (Ogawa), CA401 (Inaba), HK1 (El Tor Ogawa), and others were kindly provided by C. E. Lankford of our department. Inaba 569B was obtained as an unopened vial from R. Finklestein of

Southwestern Medical School (Dallas, Tex.). All cultures were relyophilized in multiple copies and restored as needed to minimize changes which might be encountered with cultures maintained by regular transfer.

Oral challenge. Litter size was maintained at 8 to 10 babies per mother by reducing litters in excess of 10 and adding to those below eight. This was done the day of birth. Babies to be challenged were fasted overnight when they were 7 days of age and were grouped by random assignment from litters to be used in a given test. After challenge, the mice were returned to appropriately vaccinated mothers without concern for parentage. Cultures were grown at 37 C on heart infusion agar (HIA) for 18 h. Cells were used directly after suspension and dilution in sterile nonpyrogenic saline. The viable cell number was determined by an assistant, during the time the injections were being administered, by plating on HIA. The challenge dose, which varied from 5×10^7 to 10^8 colony-forming units (CFU) contained in 0.1 ml, was injected orally into the lower esophogus through a thin vinyl tube (Intramedic, Clay Adams) connected to a blunted 23-gauge needle. This represented more than 100 and less than 1,000 mean lethal doses.

RESULTS

Effect of age. The susceptibility of ICR mice to oral infection with *V. cholerae* NIH41 (Ogawa) as a function of age is illustrated in Fig. 1. A challenge dose of 10^8 CFU was uniformly lethal within 36 h postinfection for suckling mice up to 10 days of age. Mice, 11 days of age and older, were increasingly resistant to oral infection. Similar results were obtained with *V. cholerae* Inaba (CA401) challenge (Fig. 2), although this strain was a little more virulent than the Ogawa serotype. In both cases, the most significant increase in resistance occurred in mice 15 days old. At that age, there was almost complete survival of suckling mice for 36 h; thereaf-
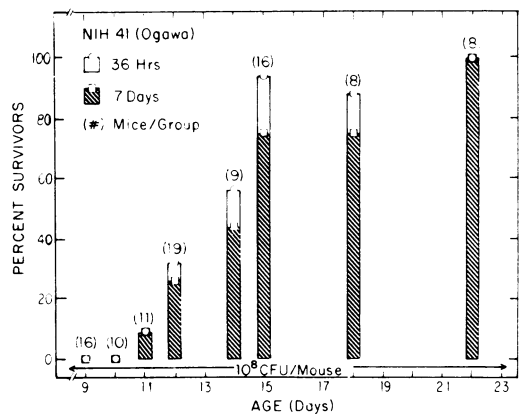

FIG. 1. *Effect of age on the susceptibility of ICR mice to oral infection with V. cholerae NIH41 (Ogawa).*

ter, there were only scattered deaths. At 18 days of age, despite a 10-fold increase in the challenge dose, mortality with the Inaba strain was only 10% at 7 days, whereas no additional deaths occurred during a total observation period of 30 days. The response of CFW mice to oral infection as a function of age was found to be similar to that observed with the ICR strain.

Comparison of the virulence of V. cholerae strains. The results of preliminary experiments to select challenge organisms and a suitable mouse strain for immunization studies are shown in Table 1. In each case, 10-day-old

FIG. 2. *Effect of age on the susceptibility of ICR mice to oral infection with V. cholera CA401 (Inaba).*

CFW, ICR, or CD-1 specific pathogen-free mice were challenged orally with 10^8 CFU of the respective organism, and deaths were recorded up to a total postinfection period of 7 days. In most cases, the majority of animals succumbed between 16 and 24 h, and only a few survived beyond 36 h. A notable exception was seen in mice infected with Inaba 569B, which proved to have low virulence for all three strains of mice employed. Although the numbers were small, the Texas Inbred outbred ICR mice raised in our animal facilities were found to be somewhat more susceptible than CFW mice to most of the strains. Deaths in these animals occurred earlier. The CD-1 mice were more resistant than CFW mice to some of the virulent strains.

The survival data of 9-day-old CD-1 mice after challenge with different doses of the two classical Inaba strains (569B and CA401) are summarized in Table 2. Similar observations (not presented) were made with CFW mice. Doses of CA401 as small as 1.5×10^7 CFU resulted in 100% mortality at 36 h. A range of challenge doses of 569B (from 1.5×10^6 to 1.5×10^8 CFU, and with up to 3×10^8 CFU in CFW mice) resulted in scattered deaths, in both the CD-1 and CFW mice, without any apparent pattern. The mean lethal dose of Inaba CA401 in CD-1 mice was found to be about three times that observed in the more susceptible CFW mice.

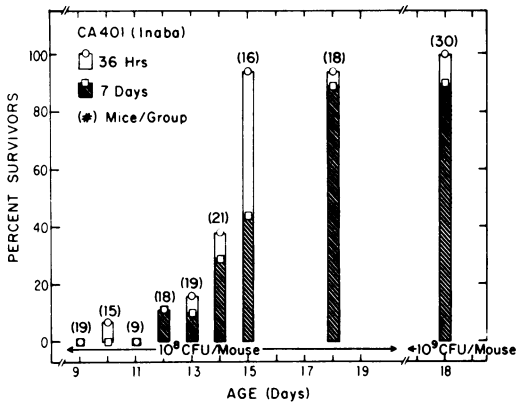**Efficacy of selected vaccines.** The oral route of infection of passively immunized suckling

TABLE 1. *Comparison of the virulence of V. cholerae strains: oral infection of 10-day-old suckling mice*

Challenge organism[a]	Biotype and serotype	Mouse strain	Survivors/total after:			
			16 h	24 h	36 h	7 days
NIH41	Ogawa	CFW	10/10	6/10	0/10	
		ICR	7/8	2/8	0/8	
CA411	Ogawa	CFW	20/20	3/20	0/20	
		ICR	5/11	2/11	0/11	
		CD-1	3/10	2/10	0/10	
569B	Inaba	CFW	10/10	10/10	7/10	7/10
		ICR	15/15	15/15	9/15	9/15
		CD-1	16/16	16/16	10/16	7/16
CA401	Inaba	CFW	21/21	13/21	1/21	1/21
		ICR	15/20	1/20	0/20	
		CD-1	8/9	3/9	1/9	0/9
HK1	El Tor Ogawa	CFW	21/22	7/22	1/22	1/22
		ICR	6/11	3/11	2/11	1/11
		CD-1	3/5	0/5		
8233	El Tor Inaba	CFW	21/22	6/22	0/22	
		ICR	8/14	3/14	1/14	0/14
		CD-1	16/22	14/22	11/22	9/22
Saline		CFW	10/10	10/10	10/10	10/10
		ICR	10/10	10/10	10/10	10/10
		CD-1	10/10	10/10	10/10	10/10

[a] 10^8 CFU per mouse.

TABLE 2. *Comparison of the virulence of classical Inaba strains 569B and CA401 in 9-day-old CD-1 mice*

Strain	Time post-infection	Survivors/total of challenge dose (CFU)					
		1.5×10^8	5×10^7	1.5×10^7	5×10^6	1.5×10^6	Saline
569B	36 h	3/9	6/10	6/9	8/10	6/9	10/10
	7 days	0/9	6/10	5/9	4/10	4/9	10/10
CA401	36 h	0/9	0/8	0/8	9/9	8/8	15/15
	7 days	0/9	0/8	0/8	7/9	8/8	15/15

mice was employed in these studies to test the efficacy of selected cholera vaccines. Although both the CFW and ICR strains of mice appeared equally suitable for immunological studies, the CFW strain was selected. The decision was not arbitrary since this strain was found to be best suited for experimental intraperitoneal cholera infections by investigators at Smith, Kline and French Laboratories (P. Actor, personal communication). The 8-day-old mouse was selected since it was more easily handled than younger mice and was significantly more susceptible to oral infection than the 9- or 10-day-old mouse (unpublished observations). Based on the data presented above, the percentage survival of suckling mice at 36 h was chosen as an end point for comparison of the efficacy of commercial bivalent vaccine, an Ogawa-derived ribosomal vaccine (HS222), and purified cholera toxin ("choleragen").

Commercial vaccine was the least effective immunogen (on a dry-weight basis) against the Ogawa challenge (Fig. 3). It protected 70% of the mice at the highest test dose of 2×10^8 cells (37.5 μg) but offered no protection at the smaller doses of 2×10^7 (3.75 μg) and 2×10^6 cells (0.375 μg). Choleragen was somewhat more protective than commercial vaccine, but far less protective than the ribosomal vaccine. HS222 at 0.1 μg was about as effective as choleragen at 10.0 μg. Each protected about 50% of the mice. Significantly, the degree of protection afforded the suckling mice by each vaccine was related to the dose of immunogen given the mother.

The degree of protection conferred by each vaccine against an Inaba (Fig. 4) and an El Tor Ogawa (Fig. 5) challenge was determined. The ribosomal vaccine again proved to be the most effective immunogen. It yielded a protection profile at the three test doses similar to that obtained against Ogawa challenge. The commercial vaccine at the median dose level protected about 70% of the mice challenged with either the Inaba or El Tor strains (see Fig. 4 and 5). The same amount of vaccine gave no protection against Ogawa challenge, as can be seen in Fig. 3. There was, however, no significant increase in protection when the largest dose of
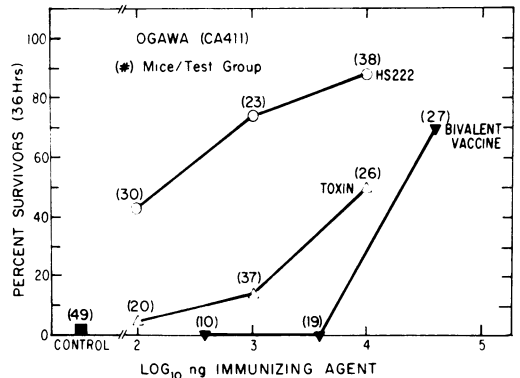

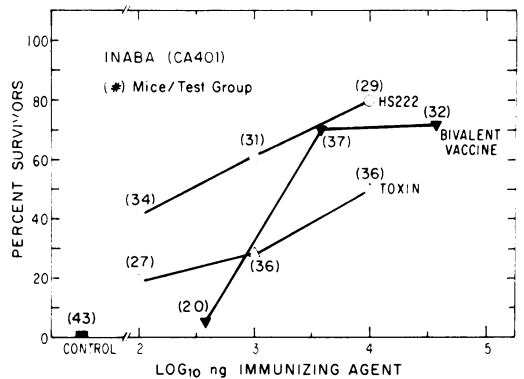FIG. 3. *Protection of passively immunized suckling mice against oral challenge with V. cholerae Ogawa CA411.*

FIG. 4. *Protection of passively immunized suckling mice against oral challenge with V. cholerae Inaba CA401.*

commercial vaccine was used in the Inaba and El Tor tests. Choleragen was perhaps superior to the commercial vaccine in protecting against Inaba and El Tor challenges, but only at the lowest test dose. It was significantly less protective at the larger dose levels.

DISCUSSION

These observations confirm and extend the original work of Ujiiye and collaborators (18–20), who demonstrated that suckling mice serve as a useful animal model in studies of

experimental cholera. After oral challenge, symptoms resembling those seen in human cholera ensue. These include distention of the intestine with fluid, copious diarrhea, and death within 24 to 48 h. Subsequently, Chaicumpa and Rowley (1) established that the vibrios undergo rapid multiplication within the confines of the digestive tract of the baby mice, whereas this fails to occur in animals that have progressed to only 3 weeks of age (2). No firm data account for such a rapid decrease in susceptibility to choleraic infection, but this does not detract from the insights to be gained with the model in testing the efficacy of vaccines.

Because antivibrio immunity, as distinguished from antitoxic immunity, seems to contribute to the resistance of animals (6–8, 10) and of humans, the latter as revealed by field trials in East Pakistan (13), the passive immunity conferred on the baby mice by a variety of vaccines promises to permit a more detailed analysis of the immunological factors involved. Freter and his associates have implicated coproantibody (6–10) as an important factor in preventing attachment of vibrios to the intestinal mucosa and have evidence that some of this may be serum derived. Protection with secretory immunoglobulin (Ig) A in the absence of demonstrable levels of IgG and IgM has also been reported (10). Finkelstein and Hollingsworth (4), on the other hand, have shown that antitoxic immunity prevents the fluid accumulation in ligated ileal loops of the mice. These findings are sufficient to establish the presence of at least two types of immunity.

Passive immunity is present in suckling mice raised by vaccinated foster mothers. Either the colostrum and/or the milk must be the source of immunoglobulin rather than that resulting from

transplacental transfer (1, 16, 18, 20). Protection against intraperitoneal challenge has been found by Pitkin and Actor (16) to persist for as long as 15 weeks, a duration longer than might be anticipated.

The relative merit of the different vaccines used in these studies depended, in part, on the antigenic dose administered the mothers. Thus the survivorship of the challenged sucklings varied over a dose range of several orders of magnitude. Although all vaccines conferred some degree of protection, the Ogawa-derived HS222 conferred immunity at the smallest dose level and was the most effective against homologous and heterologous challenge. These findings agree with the findings of Pitkin and Actor (16), who used the intraperitoneal route of infection. The material responsible for antigenicity has not been identified, but it may be dependent upon a cell wall or surface component similar to the one described by Verwey (25). Tests with his preparation are planned and the efficacy of different doses are assessed. It is also important to be in a position to look in detail at the type of immunoglobulins present in the milk and, hopefully, its localization within the intestine. In this way, it should be possible to arrive at some explanation as to why one vaccine is more effective than others.

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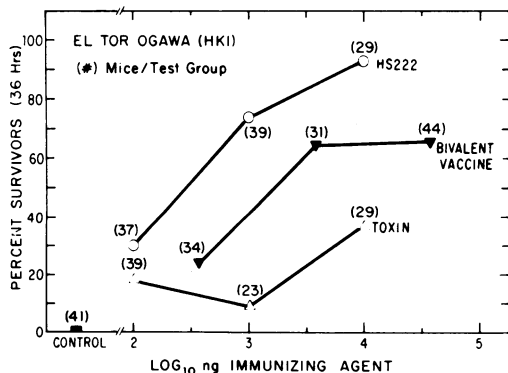8. Freter, R. 1972. Parameters affecting the association of

FIG. 5. *Protection of passively immunized suckling mice against oral challenge with V. cholerae El Tor Ogawa HK1.*

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