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Immunity to *Vibrio cholerae* in the Mouse

I. Passive Protection of Newborn Mice

DONALD PITKIN AND PAUL ACTOR

Smith Kline and French Laboratories, Philadelphia, Pennsylvania 19101

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Mice were immunized subcutaneously with either killed cells or a ribosome-containing fraction (RF) obtained from *Vibrio cholerae* Ogawa 41. At appropriate time intervals, these mice or their progeny were challenged with uniformly lethal doses of Ogawa or Inaba serotype. Half of the offspring born to mice immunized with 20 μg of RF were protected against homologous challenge at 7.5 weeks of age, and significant protection was observed up to 15 weeks of age. Similar protection was observed with heterologous challenge, but the duration of protection was reduced. The duration of protection obtained in newborns was related to the quantity of RF given to the mother. Protection was transferred from mother to young via colostrum or milk. Protection was not due to transfer of antigen, as active immunity could not be induced in newborn mice immunized with RF.

Although infection of the mouse with *Vibrio cholerae* has long been used as a method of evaluating potential cholera vaccines, the mechanism of immunity to infection in the mouse has received little study (13, 25). Vibrio-agglutinating and vibriocidal antibody are readily demonstrated, and Watanabe et al. have suggested that the mouse protective and vibriocidal antibody might be the same (32). As with other gram-negative bacteria, the vibriocidal antibodies are thought to be specific for the determinants of lipopolysaccharide (15, 19, 20, 32). Vibriocidal antibodies, directed against a protein antigenically distinct from lipopolysaccharide and common to Inaba and Ogawa strains, have been reported (22).

Passive transfer of immunity to experimental cholera infection from mother to infant rabbit has been demonstrated (24). It was concluded from this work that this protection was the result of placental transmission of cholera antibodies. It was also shown that antisera made in rabbits to live vibrio were capable of passively protecting mice and rabbits against challenge with *V. cholerae* or a toxic lysate (23). Recently, Ujiiye et al. have shown that suckling mice are susceptible to oral cholera infection (31). Mice born to immunized mothers were resistant to infection when challenged up to 10 days of age (30). Passive transfer of protection was associated with immune milk. We have confirmed and extended these findings in a preliminary report (Bacteriol. Proc., p. 102, 1971). In this study, passive transfer of protection via immune milk or serum was long lasting and related to the antigen dose given to the mother. The present communication details our work with the passive transfer of protection in newborn mice.

MATERIALS AND METHODS

Cultures of *V. cholerae* Ogawa 41 and Inaba 35A3 used in these experiments were generously provided by R. A. Finkelstein, Department of Microbiology, University of Texas Medical School, Dallas. Mice employed were CFW male and female adults purchased from Carworth Farms, New City, N.Y. All young mice were bred in our laboratory. The mice were housed in disposable plastic cages and commercial lab chow and water were supplied ad lib. *V. cholerae* challenge with either Ogawa 41 or Inaba 35A3 serotype was achieved via intraperitoneal (ip) inoculation of a mucin suspension (minimum lethal dose, 500 to 1,000). Vaccines were either heat-killed Ogawa cells or sterile Ogawa subcellular ribosomal fraction (RF) administered subcutaneously.

The details of the isolation and characterization of RF will be described elsewhere (Jensen et al. and Actor et al., Infect. Immunity, *manuscripts to be submitted for publication*) and are summarized as follows.

Cells were grown for 18 hr at 37 C on Brain Heart Infusion agar. After being washed with TM buffer [0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, 0.01 M magnesium acetate, *p*H 7.4], the cells were ruptured with high-speed shear, and debris was separated by differential centrifugation. A crude ribosomal pellet was obtained from this cell lysate by ultracentrifugation at 100,000 × *g* for 3 hr. The ribosomal pellet was suspended, clarified, layered on 5% sucrose, and ultracentrifuged. RF was isolated from this high-speed pellet as an A_{260} peak obtained from a 15 to 30% (w/v) linear sucrose gradient centrifuga-

tion. It was characterized as having 1.19 mg of orcinol-reactive material (ribonucleic acid) per ml, 0.67 mg of Lowry-reactive material (protein) per ml, A_{260}/A_{280} of 1.9, solids (16 E_{260} at 0.1%) 1.92, and a sedimentation coefficient of 70*S* in 10^{-2} M magnesium acetate which dissociated into 30*S* and 50*S* subunits in 10^{-4} M magnesium acetate.

Three basic experimental procedures were employed: (i) immunization of adults followed by mating, and challenge of the F1 generation foster-nursed on immune or nonimmune mothers; (ii) attempts to immunize young mice at selected ages; (iii) passive administration of immune serum from adult donors to newborns. The details of the immunization and challenge times are given for each experiment.

RESULTS

Maternal transfer of immunity. In the initial experiments, adults were immunized with 10^8 colony-forming units of *V. cholerae* Ogawa 41 heat-killed cells or 20 μg of RF. Control mice were given TM buffer. Fourteen days after immunization, individual males and females from each group were mated for 8 days. The males were then removed and grouped by immunization regime, and a portion was challenged with *V. cholerae* to ascertain immunity at the time of mating. The newborns were weaned at 15 days and pooled by immunization of the parent. Representative mice were challenged at intervals ranging from 3 to 15 weeks of age to determine their resistance to the Ogawa (homologous) or Inaba (heterologous) serotype. In addition, an F2 generation was obtained and challenged with the homologous serotype at 2 weeks of age.

The challenge data indicated no difference in response of male or female parents nor between male or female newborns. The data obtained from both sexes were pooled and are shown in Fig. 1 and 2. Mice born to parents immunized with either *V. cholerae* Ogawa whole cells or RF were found to be resistant to challenge with either Ogawa or Inaba serotypes. Half of the mice challenged with the homologous organism survived at 7.5 weeks of age, and significant protection was observed at 15 weeks of age (Fig. 1). Similar results were obtained with the heterologous challenge except that the duration of protection was shorter (Fig. 2). At 6 weeks of age, approximately half of the mice survived heterologous challenge, and significant protection was seen at 7.5 weeks of age. The protection observed in the mice born to immunized parents was not transferred to the F2 generation.

Maternal transfer of immunity: foster mother studies. These experiments were designed to confirm the previously observed transfer of maternal immunity and determine the origin of this immunity. RF given at 1 μg per mouse and the technique of foster nursing were employed. Adults were immunized with RF or TM buffer 2 weeks prior to mating, and individual male and

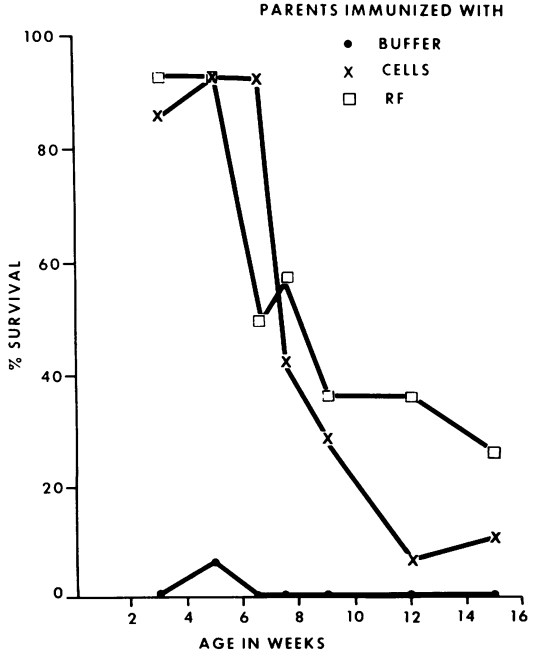

FIG. 1. *Maternal transfer of protection to newborn mice against Vibrio cholerae Ogawa 41.*

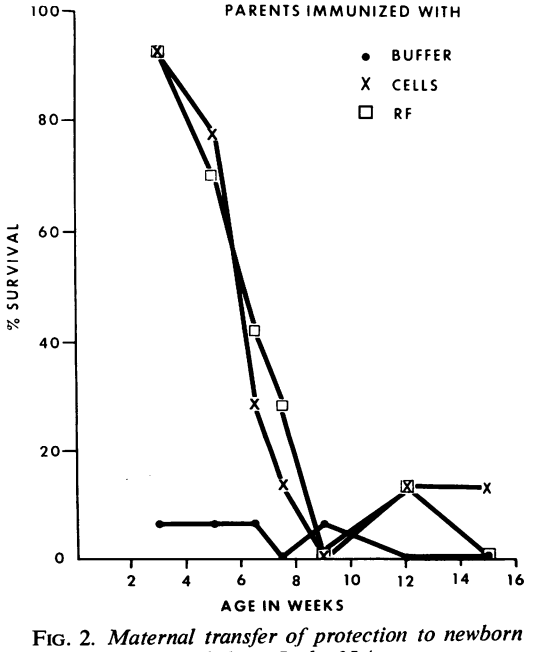

FIG. 2. *Maternal transfer of protection to newborn mice against Vibrio cholerae Inaba 35A.*

female mice were mated for 4 days. Foster nursing groups were established at birth of the F1 mice before suckling began. Four basic groups were employed: mice born to immune mothers and nursed on either immune or nonimmune mice; and mice born to nonimmune mothers nursed on either immune or nonimmune mice. The newborns were weaned and pooled by treatment at 15 days of age. Representative groups of mice from each regime were challenged at intervals ranging from 3 to 6.5 weeks with either Ogawa or Inaba serotype. The immune and nonimmune parents served as additional challenge controls at each interval. Mice born to either immune or nonimmune parents and nursed on normal mothers did not resist challenge with the homologous serotype (Table 1). Mice nursed on immune mothers regardless of the immune status of their parents were found to be protected against challenge with homologous serotype for at least 4 weeks of age. Table 2 details the results obtained with the heterologous challenge. Essentially the same results were obtained in that an immune nurse was needed to transfer protection. When protection was observed, the mice born to nonimmune mothers were protected to a greater extent than those born to immune mothers.

Immunization of young mice. The possibility of induction of active immunity by transfer of antigen from mother or nurse to newborns was examined. Young mice of various ages ranging from 3 to 18 days were immunized with a dose of RF known to protect mice (1 μg of RF). Representatives from each age group were challenged with Ogawa serotype 2 weeks after the last group was vaccinated. Production of an immunological response was measured by survival after challenge. After these results were obtained, the remaining members of each age group were boosted with the same level of RF and challenged 14 days later to determine if tolerance was induced by the initial vaccination. Table 3 describes the data obtained. Mice 8 days of age or less did not respond to primary immunization, as measured by their ability to resist challenge with Ogawa serotype. Immunization with RF at 10, 15, and 18 days of age resulted in good protection. All of the groups responded well to booster dose.

Passive transfer of immune serum. Normal newborns of various ages were injected ip with 0.1 ml of hyperimmune serum obtained from adult mice immunized with whole cells from *V. cholerae* Ogawa 41. This serum had a vibriolytic titer of 1:16,400 in a microtiter assay (2). At 22 days of age, all of the groups were challenged with

TABLE 1. *Passive protection of newborn mice against Vibrio cholerae Ogawa 41 infection by using foster nurse techniques*

Group	Per cent survival			
	Age challenged (weeks)			
	3	4	5–5.5	6–6.5
Mother				
Immune (Immune nurse)	50	50	20	0
Immune (Nonimmune nurse)	30	0	0	10
Nonimmune (nonimmune nurse)	10	0	0	0
Nonimmune (immune nurse)	100	40	30	10
Parents				
Immune	100	100	90	84
Nonimmune	20	14	0	0

TABLE 2. *Passive protection of newborn mice against Vibrio cholerae Inaba 35A infection by using foster nurse techniques*

Group	Per cent survival			
	Age challenged (weeks)			
	3	4	5–5.5	6–6.5
Mother				
Immune (immune nurse)	20	20	30	0
Immune (nonimmune nurse)	20	11	10	0
Nonimmune (nonimmune nurse)	10	0	0	0
Nonimmune (immune nurse)	80	60	40	0
Parents				
Immune	100	84	50	84
Nonimmune	20	14	0	0

TABLE 3. *Ability of young mice to respond to active immunization as measured by challenge with Vibrio cholerae Ogawa 41 infection*

Age at primary immunization (days)	Survived/total	$\bar{x}$ Age at boost (days)	Survived/total
3	2/14	40	10/11
4	0/22	42	18/20
6	2/12	46	12/12
8	1/8[a]	42	9/9
10	5/8	37	9/9
15	9/10	36	8/8
18	13/14	35	13/13
Control	0/32	Control	1/38

[a] Deaths delayed in two mice.

TABLE 4. *Passive protection of young mice given immune mouse serum and challenged with Vibrio cholerae Ogawa 41*

Age serum transferred (days)	Transfer-challenge interval (days)	Survived/tested
6	16	14/14
12	10	12/12
18	4	4/5
21	1	4/4
6 + 12 + 18	4	11/12
Control		0/6

Ogawa serotype. The results in Table 4 show that transfer of immune serum resulted in solid protection even at the longest time interval studied (16 days). Serum obtained from the survivors of this homologous challenge had a mean vibriolytic titer of 1:8.

DISCUSSION

The experiments reported here clearly show that mice born to mothers immunized with either *V. cholerae* Ogawa whole cell vaccine or RF were resistant to infection when challenged parenterally with either homologous or heterologous serotype. A similar protection has been reported for several parasitic protozoan infections in the rat (7, 9, 29, 33). Panse and Dutta have shown that 10-day-old rabbits born to mothers immunized with live cholera vaccine were immune to infection (23). Suckling mice nursed for 6 to 7 days on mothers immunized with whole cells of *V. cholerae* were resistant to oral or parenteral challenge with the homologous (Inaba) serotype (30). None of the studies with vibrios was designed to examine the duration of protection, which was found to be extended in our studies.

Significant protection with homologous (Ogawa) challenge in mice born to RF-immuned mothers was observed at 15 weeks of age. Piglets nursed on dams immune to A/Swine influenza virus were protected from experimental infection for at least 30 days (3). Zuckerman has shown that weanling mice born to hyperimmune mothers and inoculated with *Plasmodium berghei* until age 35 days totally suppressed initial infection. This strong passive immunity waned abruptly at about day 36, with 20 of 40 weanlings inoculated from day 36 to 60 succumbing (33).

It can be seen from the studies reported here that immunity to *V. cholerae* in the mouse was transferred from mother to young via colostrum or milk. Mice born to normal mothers and nursed on immune mothers were protected against infection, whereas those born to immune mothers and nursed on normal mothers succumbed to challenge. This finding is in agreement with Ujiiye and Kobari who have reported that suckling mice are protected with immune milk but not by the transplacental route (30). Immunity in piglets to swine influenza has also been shown to be transferred in the milk (3).

The protection observed in suckling mice may be due to transfer of a protective antibody in the milk. The absorption of intact globulins from the gut of young rabbits, rats, and mice has been studied extensively (4, 5, 6, 8, 16, 17). In rats, the amount of globulin absorbed was proportional to concentration and inversely proportional to the age of the recipient. No absorption was observed after 20 days of age. Mice were found to absorb antibody globulins up to 15 to 17 days of age (18, 21). Furthermore, mice nursed on immune mothers were found to have serum vibriocidal titers. Transfer of solid protection to newborns could be achieved with immune serum. This protection was evident for at least 16 days after administration of immune serum, the longest time interval studied. Passive administration of immune *V. cholerae* serum has been previously reported to be protective for rabbits and mice when administered 4 to 6 hr prior to infection (12, 24). It should be pointed out that the protection of newborns cannot be attributed to transfer of protective antigen from mother to young. Administration of RF to mice up to 8 days of age failed to protect when challenge was 2 weeks later. This dose of antigen failed to produce immune tolerance, as parallel groups given a booster dose later in life were protected against subsequent infection. Sajid et al., using a direct vibriolytic plaque assay, have demonstrated tolerance in neonatal mice given multiple low and high doses of lipopolysaccharide antigen derived from *V. cholerae* (28).

Studies of the type reported here should give insight to the dynamics of infection in the mouse and may be applicable to the development of improved cholera vaccines. The mouse model has been employed for the testing and standardization of cholera vaccines (13). The mechanism of protection in this system is not fully understood but is apparently linked to the production of a vibriocidal antibody in response to cell wall antigens (32). Furthermore, it is only recently that the dynamics of the ip infection in the normal and immune mouse have been reported (Lukasewycz and Berry, Bacteriol. Proc., p. 102, 1971). The growth of the organism in the mouse is essentially limited to the peritoneum with peak numbers seen at 12 hr. A sterile filtrate of peritoneal fluid from infected mice was toxic and immunogenic for control mice. The validity of this model has been critized because the route and method of infection, i.e., ip infection with organisms in

gastric mucin, do not simulate natural cholera. In nature, the disease is limited to the gut and produces symptoms which can be attributed to the production of an enterotoxin (choleragen) (14). The use of an animal model where the oral or intraintestinal route of infection is employed may be a more realistic approach to the study of cholera vaccines. Such systems have been reported for the infant rabbit (11, 14) and mouse (31) as well as the adult dog (26, 27) and chinchilla (1). The obvious advantages of using suckling mice as experimental animals is, in part, negated by the fact that these mice do not respond to immunization. Alternate systems have been described where suckling rabbits or mice are nursed on mothers immunized with experimental vaccines (11, 24, 31). These systems may only be of use in demonstrating immunity due to some humoral factor.

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