JOURNAL OF VIROLOGY, Apr. 1997, p. 2844–2850 Vol. 71, No. 4
0022-538X/97/$04.00+0

Orally Administered Microencapsulated Reovirus Can Bypass Suckled, Neutralizing Maternal Antibody That Inhibits Active Immunization of Neonates

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Received 5 November 1996/Accepted 13 December 1996

Purified reovirus serotype 1, encapsulated in biodegradable aqueous microcapsules, was found to bypass maternal antibody passively transferred by suckling to neonates. Genetically identical, immunocompetent F_1 *scid*/+ mice were generated by the reciprocal crosses of C.B17 *scid*/*scid* and normal congenic +/+ adult mice. The immunocompetent +/+ dams were either orally infected with reovirus prior to mating or not. Thus, these immunocompetent F_1 pups developed either in the absence or in presence of passively transferred maternal immunity. The F_1 mice were orally immunized on day 10 with either live virus, microencapsulated reovirus, or empty microcapsules plus live virus. The immune responses were assessed in the neonatal gut-associated lymphoid tissues (GALT). Examination of reovirus specific immunoglobulin A in the serum and GALT, taken on days 7, 14, and 21 postimmunization, clearly demonstrated that microencapsulated reovirus could bypass the normal effect of maternal antibodies, passively acquired by suckling, to inhibit active priming of neonates by oral route. These observations seem relevant to the development of efficacious oral vaccines that also allow passive, protective immunity via suckled maternal antibodies while permitting active oral immunization of neonates.

In both primates and rodents, maternal antibodies are transferred through the placenta and, after birth, via mother's milk (23). There have been convincing reports in the literature indicating that bottle-fed infants suffer from a higher incidence of diseases of enteric viral and bacterial etiology than breast-fed infants (7, 8). This protective effect of breast feeding has been attributed to the action of maternal antibodies that occur in high concentrations in mother's milk (15, 25). However, specific maternal antibodies in milk also interfere with the immunization of breast-fed infants with oral vaccines (35). This interference by maternal antibodies with the efficacy of oral vaccines remains controversial (4, 5, 14, 30).

We studied microencapsulation as a means of bypassing interference by maternal antibodies. Microcapsules are inexpensively made and resistant to breakdown by gastric acid, and they have been shown to enhance the immunogenicity of orally administered antigens. The most extensively reported method of microencapsulation is the covalent linkage of lactide and glycolide (i.e., poly-DL-lactide coglycolide [PLCG]) (13). Inoculation of animals with PLCG-encapsulated influenza virus (24), parainfluenza virus (36), or ovalbumin (31) enhanced the immune response compared to animals inoculated with unencapsulated antigen. However, the major disadvantage of PLCG microcapsules is the use of organic solvents for their preparation, which can denature conformational determinants recognized by specific B cells. We chose to encapsulate the antigen by using an aqueous-based system which involves the ionic linkage of anionic polymers and polyfunctional amines to form charged-film microcapsules (29). This aqueous-based method of microencapsulation obviates concerns about disruption of epitopes critical to induction of humoral immune responses associated with the use of organic solvents.

Mucosal immunity may play a pivotal role in mediating protection against pathogens that normally gain entry to the host through the gastrointestinal tract. We chose to study the mucosal immunoglobulin A (IgA) response to the enteric/respiratory reovirus serotype 1/Lang (T1). Reovirus T1, via interaction of the reovirus σ1 protein with an unknown ligand, exhibits a marked tropism for Peyer's patch (PP) M cells and is efficiently delivered to the mucosal system (37). In normal adult mice, enteric reovirus infection leads to rapid induction of both humoral and cellular mucosal immunity that completely resolves the infection by days 10 to 14 after challenge (22). Both placental transfer- and milk-derived maternal antibodies against reovirus T1 are protective against lethal reovirus serotype 3-mediated meningoencephalitis of newborn mice (6). Apart from a reported reduction in M cells (2), reovirus T1 infection of 10-day-old mice is generally nonpathogenic. Although *scid*/*scid* mice may become persistently infected, and can eventually succumb to liver disease, they remain in good health for 6 to 12 weeks (16). Therefore, reovirus T1 is an appropriate model antigen with which to study interference of desired neonatal mucosal immune responses by passively transferred maternal antibodies.

In this work, we used a unique model system, established in our laboratory, to understand the interference of maternal antibodies in suckling mice (19). This model is based on reciprocal crosses of immunocompetent +/+ and immunodeficient *scid*/*scid* mice to yield genetically identical, immunocompetent F_1 *scid*/+ pups (3) that then develop in either the absence or presence of passively transferred maternal immunity. The developmental time of delivery of passive immunity can be manipulated by using immunodeficient female *scid*/*scid* mice as birth and/or nurse mothers and immunized or nonimmunized, immunocompetent +/+ female mice as birth and/or nurse mothers.

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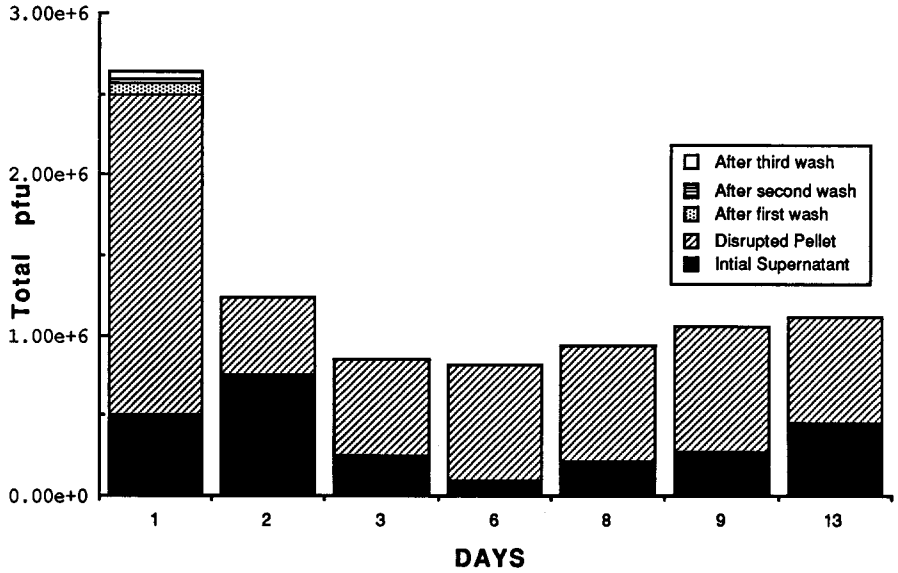

FIG. 1. Reovirus serotype 1 was captured within spermine alginate microcapsules. These microcapsules were washed several times for a period of 13 days and disrupted with 8.0 M sodium phosphate (pH 7.0). The supernatant fluid after the first wash (initial supernatant) and the fluid obtained after disruption (disrupted pellet) were tested for the presence of infectious virus by plaque titration.

MATERIALS AND METHODS

Mice. Germ-free (GF) C.B17 mice originally obtained from the University of Wisconsin Gnotobiotic Laboratory, Madison, Wis., and specific-pathogen-free C.B17 *scid/scid* mice originally obtained from the Institute for Cancer Research, Fox Chase, Pa., were used in these studies. All mice were bred and maintained within the gnotobiotic facility in the Department of Biology at the University of Pennsylvania.

To generate F_1 *scid*/+ litters, 4- to 6-week-old GF female and 10- to 12-week-old GF male C.B17 mice were transferred to a specific-pathogen-free isolator housing age-matched female and male *scid/scid* mice. This transfer was performed in order to minimize potential differences in the relative populations of gut microbes in the female mice. After 2 to 3 weeks of exposure to *scid* commensal microbes, reciprocal crosses were conducted.

Cells. Fetal green monkey kidney cells (MA-104) were grown as previously described (28).

Immunization of F_1 mice. Reovirus inoculations were conducted by using third-passage, $CsCl_2$-purified reovirus as described previously (22). Ten-day-old mice were orally intubated with polyethylene tubing (PE-10; Becton Dickinson, Mountain View, Calif.) through which 10^6 PFU of reovirus T1, microencapsulated reovirus, or placebo (preformed alginate-spermine microcapsules [empty capsules which did not contain virus] mixed with reovirus suspended in water) was administered in a volume of 100 μl. For studies involving preimmune +/+ mothers, 6- to 8-week-old C.B17 mice were orally inoculated with 3×10^7 PFU of reovirus T1 2 weeks prior to mating.

Microencapsulated virus. A 5-ml aliquot of clarified cell culture stock (1.6×10^7 PFU/ml) of reovirus T1 was added to 5 ml of 0.68 mM sodium alginate and dispersed as droplets nominally 5 μm in size into 20 ml of 0.55 M spermine hydrochloride. Putative reovirus-containing microcapsules were washed three times by centrifugation at $1,000 \times g$ and resuspended in 3 ml of distilled water. An aliquot of this preparation was centrifuged at $1,000 \times g$ and disrupted with 8.0 M sodium phosphate (pH 7.0), which does not alter detection of infectious reovirus. Supernatant fluids from washes and fluid obtained after disruption of putative reovirus-containing microcapsules were tested for the presence of infectious virus by plaque assay (Fig. 1). To determine whether virus released in this experiment was present on the surface or within the matrix of the microcapsules, 10^6 PFU of reovirus T1 was added to preformed alginate-spermine microcapsules (empty capsules) which did not contain virus (placebo beads).

GALT organ cultures. F_1 mice of the different groups were sacrificed on days 7, 14, and 21 postimmunization. Gut-associated lymphoid tissue (GALT) organ cultures were established by using PP and small intestine (SI) from the F_1 pups in a modified version of the GALT organ culture system previously used in our laboratory (19). Briefly, 4-cm segments of SI were opened longitudinally and washed three to five times to remove debris with Ca^{2+}-, Mg^{2+}-free Hanks balanced salt solution (CMF-HBSS) containing 0.1% gentamicin and 10 mM HEPES. Segments were rinsed two or three times in CMF-HBSS containing 0.05% EDTA, 0.1% gentamicin, and 10 mM HEPES to remove the mucin layer and to denude the villi of the epithelial cells. This procedure effectively removes all milk IgA antibodies but does not remove maternal IgGs bound to B cells in the lamina propria. The denuded segments were then washed five times in Iscove's modified Dulbecco's medium to remove the EDTA. Mesentery was removed, and PP were dissected from the surrounding tissues with a stereomicroscope. In addition, approximately 3-mm^2 pieces of SI adjacent to the each dissected PP were prepared; these SI fragments comprise roughly 300 villi. Intact PP or SI were washed extensively with CMF-HBSS. Intact PP or SI were cultured in a sterile flat-bottom 96-well plates (Costar, Cambridge, Mass.) in 250 μl of Kennet's H-Y medium (JRH Biosciences, Lenexa, Kans.) containing 10% fetal bovine serum, 1% L-glutamine, 0.01% gentamicin, and 1% antibiotic-antimycotic solution (100 U of penicillin per ml, 1% streptomycin, 0.25 μg of amphotericin B [fungizone] [Gibco, Grand Island, N.Y.]) for 7 days under 90% O_2–10% CO_2 at 37°C. Culture supernatants were frozen prior to assay.

RIA. The radioimmunoassay (RIA) used in our laboratory has been described elsewhere (21). For reovirus-specific antibody determinations, plates were coated overnight with 100 μl of a $CsCl_2$-purified suspension of 5×10^9 PFU of reovirus T1/ml in phosphate-buffered saline. The results are expressed in counts per minute. F_1 mice in each group which received only water were used as controls.

RESULTS

Capacity of sodium alginate-spermine hydrochloride microcapsules to capture and retain infective reovirus. To determine whether infective reovirus could be encapsulated, reovirus T1 was included in the reaction mixture leading to alginate spermine microcapsules. These microcapsules were tested for the presence and retention of infectious virus through several washes (Fig. 1). Significant infectious virus was not detected in supernatant fluids after three washes but was clearly released upon the disruption of the microcapsules. The efficiency of the encapsulation was over 70%.

Stability of the encapsulated reovirus. The stability of the microcapsules and their retention of infectious reovirus T1 were determined. The microcapsules were tested for the presence of the infectious virus by disruption, after washing, over a period of 13 days (Fig. 1). A relatively small fraction of infectious virus was detected in the supernatant fluids, but virus was clearly released upon the disruption of the microcapsules (dispersed pellet). The amount of infectious virus released over a period of 13 days remained rather constant.

F_1 *scid*/+ pups born to either *scid/scid* or +/+ dams express a humoral mucosal response to oral reovirus infection unless the +/+ mothers have been previously immunized. We com-

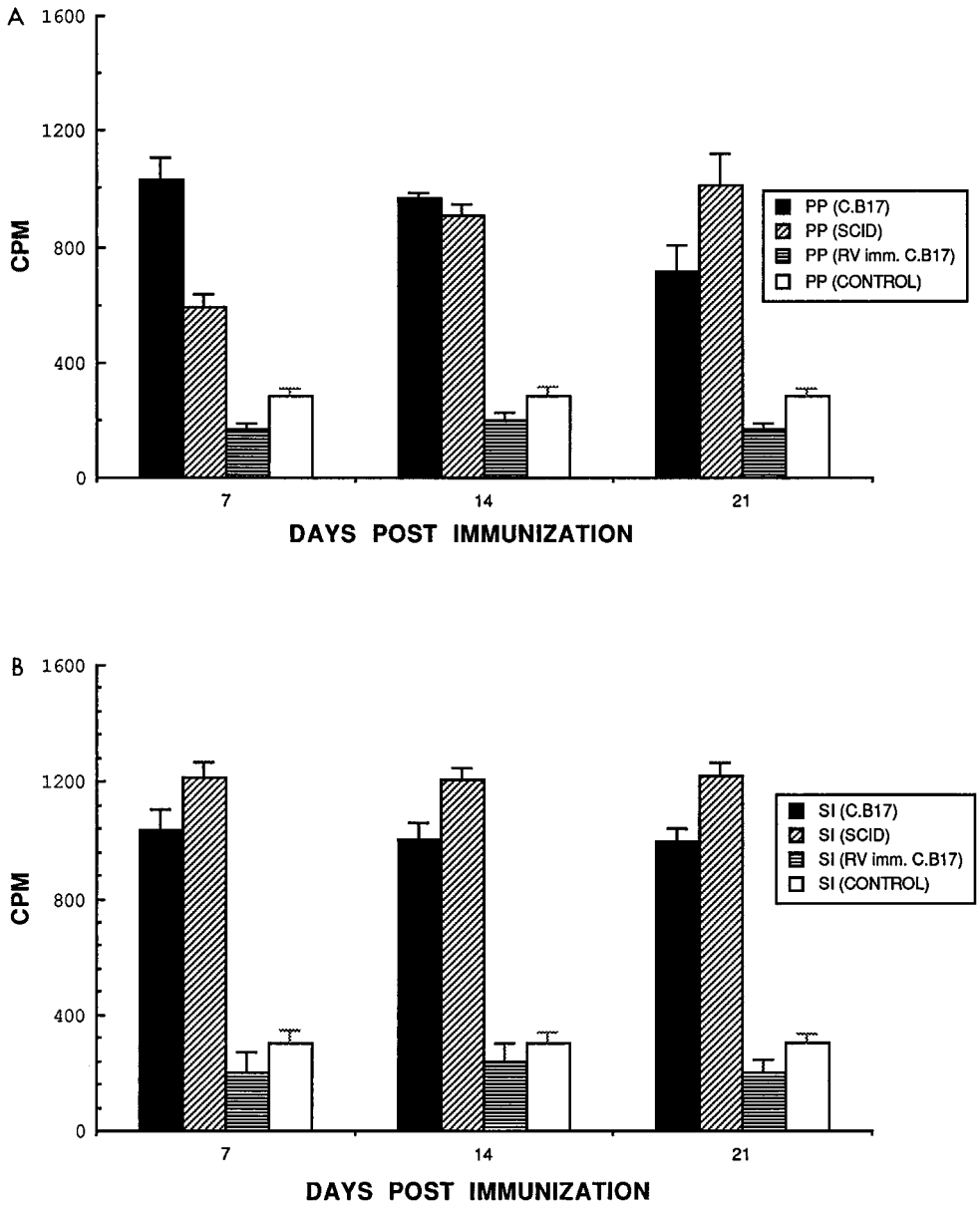

FIG. 2. Ten-day-old F_1 *scid*/+ pups born to and nursed by *scid*/*scid* (SCID), nonimmune +/+ (C.B17), or reovirus-immune (RV imm. C.B17) mothers were immunized with reovirus. Reovirus-specific IgA levels in PP (A) and SI (B) cultures are compared. Data are presented as mean counts per minute ± standard error of mean from three mice per group as determined by RIA.

pared the reovirus-specific IgA responses in the GALT cultures of PP and SI, on days 7, 14, and 21 postimmunization, in F_1 *scid*/+ suckling mice orally infected with reovirus T1 at 10 days of age. These F_1 pups were born to nonimmune, immunocompetent +/+ mothers, to immunocompetent +/+ mothers previously orally immunized with reovirus, or to *scid*/*scid* mothers. No statistically significant differences were seen in the overall kinetics of the reovirus specific antibody response in F_1 *scid*/+ pups of *scid*/*scid* and +/+ mothers for PP and SI cultures (Fig. 2). However, F_1 *scid*/+ pups of reovirus-immune mothers showed no reovirus-specific antibody in the PP and SI cultures. The reovirus-specific IgA antibodies from PP and SI cultures were compared to the sorbed IgA from the fragment cultures of nonimmunized control pups, which served as controls. These findings are in agreement with our previously published results (19).

Encapsulated reovirus bypasses the suppressive effects of maternal antibodies when used to immunize F_1 *scid*/+ pups of reovirus-immunized mothers. We compared the reovirus-specific IgA responses on days 7, 14, and 21 after oral inoculation of F1 *scid*/+ suckling pups with the spermine alginate microcapsules at 10 days of age. No statistically significant differences in the mucosal antibody response were seen between F_1 pups of *scid*/*scid* and +/+ immune or nonimmune mothers as (Fig. 3). Further, the responses expressed in both PP and SI fragment cultures to the encapsulated reovirus were of the same magnitude as observed in fragment cultures of pups of nonimmune mothers after challenge with unencapsulated reovirus (Fig. 2).

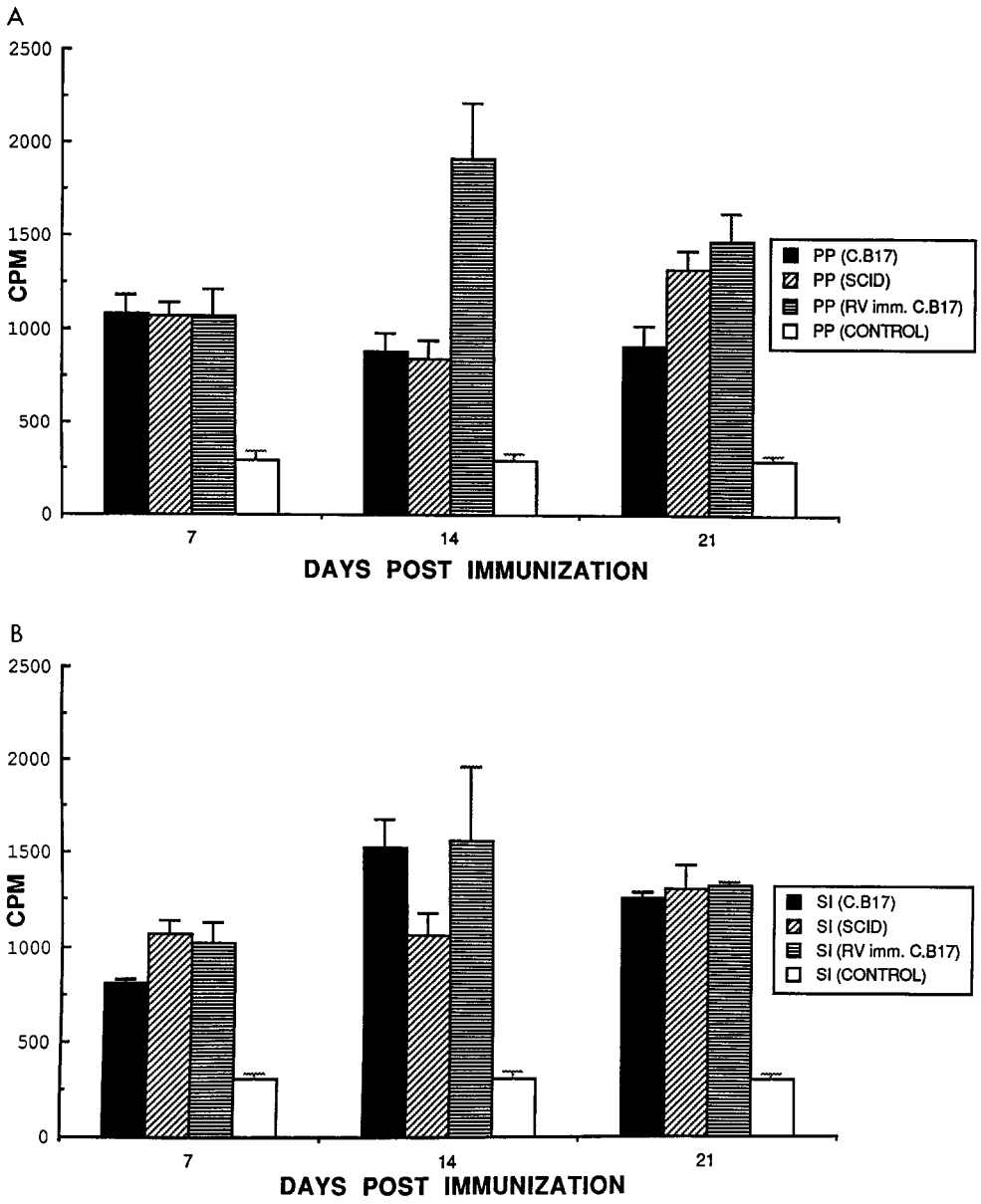

FIG. 3. Ten-day-old F_1 *scid*/+ pups born to and nursed by *scid*/*scid* (SCID), nonimmune +/+ (C.B17), or reovirus-immune (RV imm. C.B17) mothers were immunized with microencapsulated reovirus. Reovirus-specific IgA levels in PP (A) and SI (B) cultures are compared. Data are presented as in Fig. 2.

F_1 pups immunized with placebo (empty capsules) plus reovirus showed a response similar to that of F_1 pups immunized with reovirus alone as well as the suppressive effect of suckled maternal antibodies. To determine whether the virus released in the microcapsules was present on the surface or within the matrix of the microcapsules, 10^6 PFU of virus was added to the preformed spermine alginate microcapsules which did not contain the virus (placebo capsules). The F_1 *scid*/+ pups of *scid*/*scid* and +/+ nonimmune or reovirus-immune dams were immunized with placebo plus virus on day 10 after birth. Figure 4 shows the reovirus-specific IgA levels in the PP and the SI cultures from various groups of F_1 mice immunized with placebo plus reovirus or not immunized at all. The reovirus-specific IgA levels of F1 *scid*/+ pups of *scid*/*scid* dams showed an increase in the reovirus-specific IgA levels on only day 14 postimmunization in the PP and SI cultures. The reovirus-specific IgA levels of F1 *scid*/+ pups of nonimmune +/+ dams rose and remained stable over a period of 21 days following oral immunization. However, the reovirus-specific IgA levels in PP and SI cultures from F_1 *scid*/+ pups born to reovirus immune +/+ mothers were below even the basal level found in cultures from noninfected pups (Fig. 4). Thus, the placebo capsules do not protect the accompanying virus from the suppressive effects of suckled maternal antibodies. We cannot explain why the pups of *scid*/*scid* dams did not show significant mucosal responses on days 7 and day 21.

Suckling mice immunized with spermine alginate microcapsules containing reovirus show a significant increase in reovirus-specific IgA in the serum. Reovirus-inoculated F_1 *scid*/+ pups of *scid*/*scid* mothers or nonimmune +/+ mothers developed a vigorous specific IgA response in the sera upon oral inoculation with reovirus (Fig. 5). In contrast, reovirus-specific

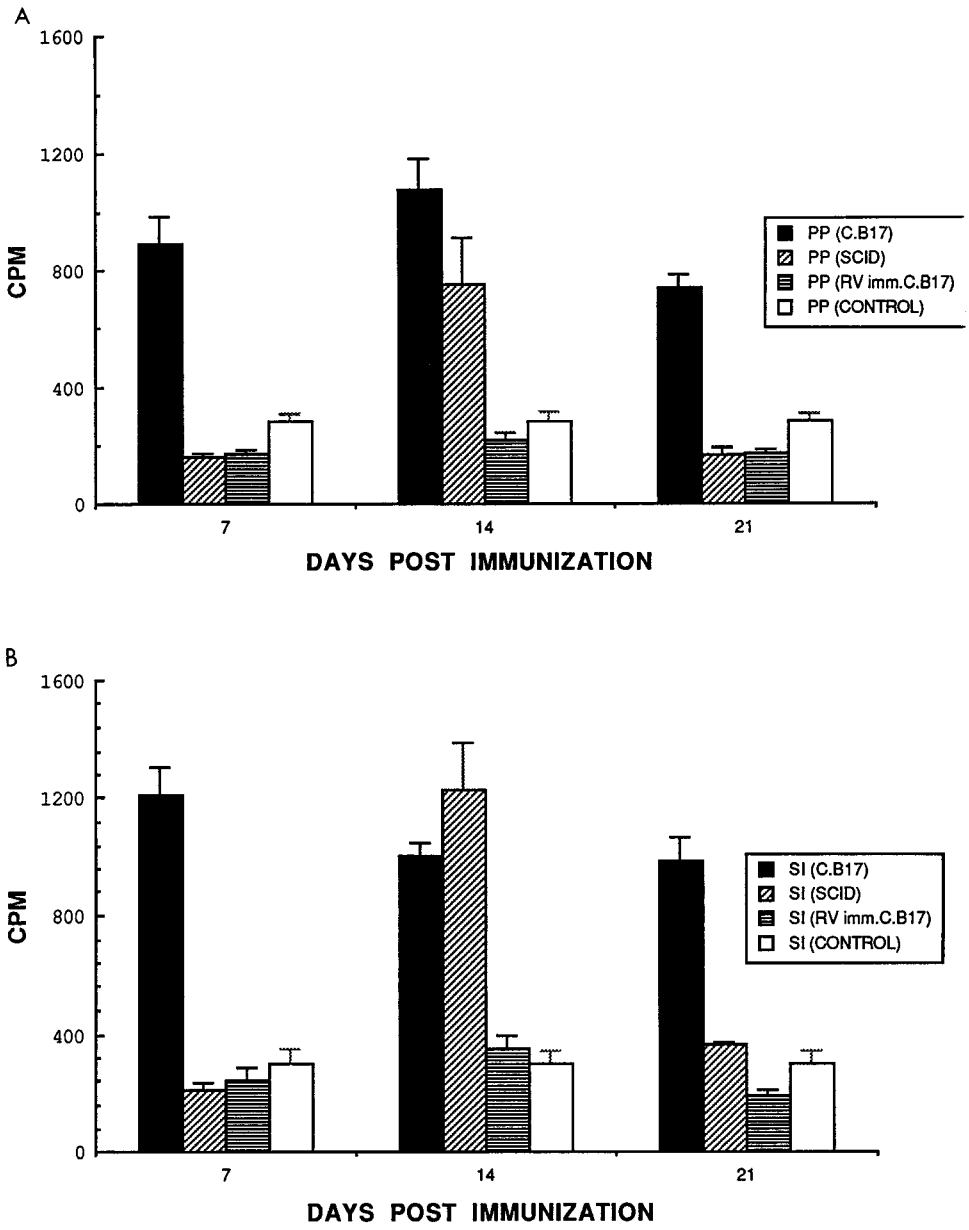

FIG. 4. Ten-day-old F_1 *scid*/+ pups born to and nursed by *scid/scid* (SCID), nonimmune +/+ (C.B17), or reovirus-immune (RV imm. C.B17) mothers were immunized with placebo plus reovirus. Reovirus-specific IgA levels in PP (A) and SI (B) cultures are compared. Data are presented as in Fig. 2.

IgA was conspicuously absent in the serum from reovirus-challenged F_1 *scid*/+ pups born and nursed by reovirus-immune dams (Fig. 5). However, F_1 *scid*/+ pups born and reared by reovirus-immune mothers and immunized with the microencapsulated reovirus could bypass the suppressive effects of maternal antibody (Fig. 5). F1 *scid*/+ pups immunized with placebo plus reovirus showed a response similar to that of F_1 mice immunized with reovirus alone.

DISCUSSION

The protective effects of breast-feeding against infections such as diarrhea, septicemia, and respiratory tract infections have been demonstrated (33, 34). The possibility that maternal antibodies, including those in mother's milk, enhance vaccine responses (33, 34) is of clinical relevance and suggests that the immunoglobulins in milk may not merely provide the neonates with passive immunity but also actively stimulate the lymphoid system of breast-fed infants.

Previous studies from our laboratory using the same model system, reciprocal crosses of +/+ and *scid/scid* mice yielding genetically identical, immunocompetent F_1 *scid*/+ pups that develop in either the absence or presence of passively transferred maternal immunity, have shown that F_1 *scid*/+ pups orally infected with reovirus and suckling on reovirus-immune mothers do not develop a mucosal immune response (20). This finding suggests that maternal antibodies not only forestall the development of IgA responses but also attenuate the magnitude of these responses once they are induced in neonates (20). Also, in our murine model, we have found that suckled maternal antireovirus antibodies protect neonates against an ordi-

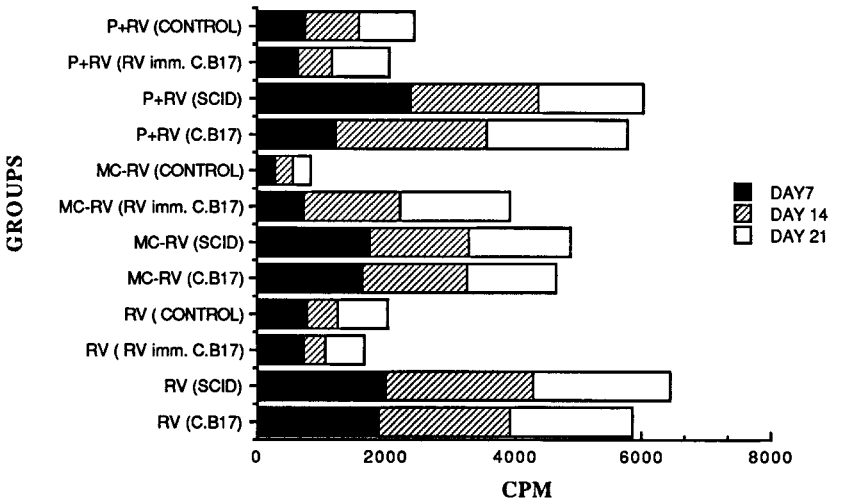

FIG. 5. Reovirus-specific antibody content in the serum of F_1 *scid*/+ suckling mice infected with reovirus (RV), microencapsulated reovirus (MC-RV), or placebo plus reovirus (P+RV) at 10 days of age. The black bars represent reovirus-specific IgA in F_1 *scid*/+ pups on day 7; diagonal bars represent IgA in F_1 *scid*/+ on day 14; blank bars represent reovirus-specific IgA in F_1 *scid*/+ pups on day 21 postimmunization born to and/or nursed by nonimmune +/+ (C.B17), *scid*/*scid* (SCID), and reovirus-immune (RV imm. C.B17) mothers. Data are for three mice per group.

narily fatal oral challenge with type 3 reovirus. However, the passively transferred antibodies interfere with active immunization by a cross-reactive, protective live vaccine (type 1 reovirus) given orally (6). These results are consistent with the findings of Offit and Clark (27), who reported that newborn mice could be protected against rotavirus by IgA or IgG antibodies contained in mouse milk from rotavirus-immune dams. Hence, a major factor associated with the failure of some pediatric vaccines is the interference of high levels of maternal antibodies (1, 35). We wanted to investigate if this interference by maternal antibodies could be bypassed by immunizing the immunocompetent F_1 *scid*/+ pups with microencapsulated virus.

There are a number of studies which demonstrate the microencapsulation of bacterial proteins or viruses (10, 24, 31, 36); however, our procedure for making the charged-film microcapsules is more likely to allow for retention of viral epitopes necessary for the induction of the humoral immune responses than procedures requiring organic solvents. We decided to test if interference by the maternal antibodies in the neonates could be bypassed by using the microencapsulated reovirus. Several other additional characteristics of charged-film microcapsules make them attractive for use in antigen delivery in our model system. (i) Microcapsules are prepared in aqueous media from materials that are safe and biodegradable. Sodium alginate, a gelling polysaccharide extracted from kelp, is commonly used as a stabilizer and thickening agent. Spermine (a derivative of spermidine) is a polyamine found in mammalian cells (17). (ii) The spermine alginate microcapsules were found to resist degradation by gastric acid (29). (iii) Microcapsules have an internal volume fraction which allows for efficient capture of the antigen. (iv) Microencapsulation is accomplished at or below room temperature; unencapsulated infectious virus can be readily recovered. We found, using this aqueous-based system, that infectious reovirus can be microencapsulated with very good efficiency (Fig. 1). These charged particles were also found to retain infectivity for a period of 13 days (Fig. 1). Thus, the microencapsulated reovirus was found to be an effective means for delivering reovirus to neonates. The results presented here convincingly show the effectiveness of microencapsulation as a means of delivering antigens by the oral route as well as the ability of orally administered microencapsulated reovirus to elicit systemic and mucosal immune responses in neonates which can bypass the passively transferred maternal antibodies. F_1 *scid*/+ pups born to reovirus-immune mothers and immunized with the microencapsulated reovirus showed a significant IgA antibody response in the serum as well as in the GALT (Fig. 3 and 5). From these data we conclude that the preexisting specific maternal antibodies in the milk, which normally would interfere with the oral immunization attempts, could be effectively bypassed in the neonates with our system of delivery of the antigen. For our attempts to actively immunize neonates, we chose a dose of infectious reovirus (10^6 PFU) based on previous findings (5a) that this was the lowest dose capable of uniformly stimulating an active immune response. We also have shown (19) that oral dose of 10^7 PFU are prevented from eliciting active immunity in neonates by suckled maternal antibodies. We are presently addressing whether much lower doses of microencapsulated reovirus (i.e., 10^5 or 10^4 PFU) are capable of inducing active immunity in neonates suckled by either nonimmune or immune, immunocompetent dams.

Possible mechanisms whereby the microcapsules are able to bypass the maternal antibody response in the neonates include the following. (i) The uptake of these microcapsules from the lumen to the gut is mediated by specialized epithelial cells (M cells) (26, 32). Analysis of the mechanism of particle uptake by M cells in the mouse gut has clearly shown that uptake is restricted to particles with diameters less than or equal to 10 μm (11, 12, 26). The average size of spermine-alginate microcapsules is 2 μm. Damage to the intestinal M cells by reovirus (2) may result in increased intestinal permeability to soluble protein antigens present in the gut lumen, as occurs in transmissible gastroenteritis virus of suckling piglets (18). Probably, the release of the reovirus from the microcapsules in M cells could lead to an enhanced immune response. (ii) The quantity of the antigen available to the antigen-presenting cells within GALT or within the peritoneum may be greater after the uptake of reovirus-containing microcapsules than that available when free virus is equally distributed throughout the mucous layer of small intestine or peritoneal fluid, respectively. (iii) The reovirus contained within the microcapsules may be

retained within antigen-presenting cells for a longer period of time than nonencapsulated antigens. (iv) Microencapsulated reovirus may be processed by antigen-presenting cells in GALT in a manner different, and perhaps more efficient, than processing antigens after natural infections.

F_1 *scid*/+ pups immunized with placebo plus reovirus showed an IgA response in the GALT and serum similar to that of F_1 pups immunized with reovirus alone (Fig. 4 and 5). Therefore, it is only the microencapsulated reovirus that can bypass the maternal antibody response in neonates.

Previously, a report by de Vries et al. (9) showed that measles virus administered in immune-stimulating complexes were immunogenic and were able to bypass the inhibitory effects of circulating passive antibodies. The present report demonstrates a mechanism to bypass maternal antibody in the gut lumen. It may be important to consider these findings in developing strategies of immunization against enteric pathogens such as cholera, and rotavirus or pathogens such as human immunodeficiency virus, that enter the host through mucosal surfaces. Our studies extend the literature on protection studies with animals immunized with microencapsulated vaccine antigens in general. Particularly, our observations in assays using microencapsulated reovirus raise the possibility that a single oral dose to neonates can confer protection at remote mucosal surfaces in the presence of suckled maternal antibodies. Indeed, preliminary experiments indicate that microencapsulated reovirus, given orally to neonates being suckled on immune dams, can protect the respiratory tract against a postweaning, intranasal challenge.

ACKNOWLEDGMENTS

This work was supported by grants AI-23970 and AI-37108 from the National Institutes of Health. S.B.P. was supported by grant AI-37108 from the National Institute of Health.

We thank Charlotte A. Moser for technical support as well as Khushroo E. Shroff and Paul Offit for helpful discussions.

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