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Effect of Water-Based Microencapsulation on Protection against EDIM Rotavirus Challenge in Mice

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We determined the capacity of microcapsules formed by the combination of sodium alginate, an aqueous anionic polymer, and spermine hydrochloride, an aqueous cationic amine, to enhance protection against rotavirus challenge in mice. Adult BALB/c mice were orally inoculated with either free or microencapsulated rotavirus (simian rotavirus strain RRV) and challenged 6 or 16 weeks later with murine rotavirus strain EDIM. Virus-specific humoral immune responses were determined at the time of challenge and 4 days after challenge by intestinal fragment culture. We found that spermine-alginate microcapsules enhanced protection against challenge 16 weeks after immunization but not 6 weeks after immunization. Quantities of virus-specific immunoglobulin A produced by small intestinal lamina propria lymphocytes were correlated with the degree of protection against challenge afforded by spermine-alginate microcapsules. Possible mechanisms by which microcapsules enhance protection against rotavirus challenge are discussed.

Development of new vaccines or improvement of existing vaccines may depend on adjuvants that target antigens to specific tissues, eliminate the need for booster dosing, contain multiple antigens, or protect antigens from acids and proteases produced at mucosal surfaces. One of the most widely studied adjuvant systems is microencapsulation (3, 12, 19, 22). This technique involves capturing antigens in particles typically 1 to 10 μm in size that can be delivered by various routes.

We developed a water-based system of microencapsulation using anionic polymers (e.g., alginate, chondroitin sulfate, or carboxymethyl cellulose) and cationic amines (e.g., spermine or decylamine) that react in a salt exchange to form precipitated microcapsules (18). We found that water-based microcapsules captured infectious virus (18), resisted breakdown by gastric acid (18), and enhanced virus-specific humoral immune responses after oral or intramuscular inoculation (1, 7, 13, 14, 18).

In these studies, we extended our previous findings to include a study of the capacity of water-based microcapsules to enhance protection against rotavirus challenge in mice. To evaluate the relationship between humoral immunity and protection against challenge, we used an intestinal fragment culture assay to measure quantities of virus-specific and total antibodies produced by small intestinal lamina propria lymphocytes (LPL) both at the time of challenge and 4 days after challenge.

MATERIALS AND METHODS

Mice. Adult, 6- to 8-week-old, female BALB/c mice or pregnant Swiss-Webster mice were obtained from Taconic Breeding Laboratories (Germantown, N.Y.) and housed in separate isolation units.

Viruses. Rhesus rotavirus strain RRV (P5[3]G3), originally obtained from N. Schmidt (Berkeley, Calif.), was grown and titrated by plaque assay in fetal green monkey kidney cells (MA-104) as previously described (17). Murine strain EDIM (P[18]G3), originally obtained from R. Ward (Children's Hospital Research Foundation, Cincinnati, Ohio), was propagated in 7-day-old Swiss-Webster mice. Small intestines were removed 3 to 4 days after oral inoculation, and 10% (wt/vol) suspensions were prepared in BHK cell medium (11; Wistar Institute, Philadelphia, Pa.). Suspensions were homogenized (PowerGen 125 tissue homogenizer; Fisher Scientific, Pittsburgh, Pa.) and stored at −70°C.

Determination of SD_{50}. Groups of five adult, female BALB/c mice were orally inoculated with one of the following dilutions of EDIM prepared as an intestinal homogenate: 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, or $1:10^6$. An average of three fecal pellets were collected from each animal daily during the 6 days following inoculation. Samples were stored in 0.5 ml of Earle's balanced salt solution (Gibco Life Technologies, Grand Island, N.Y.) at −20°C and later tested for rotavirus antigen by enzyme-linked immunosorbent assay (ELISA). The 50% shedding dose (SD_{50}) was calculated to be 1:60,250 by the method of Reed and Muench (20).

Microencapsulation. Five-milliliter aliquots of RRV were suspended in sodium alginate and precipitated in spermine hydrochloride to form spermine-alginate (SA) microcapsules as previously described (18). Microcapsules were washed and diluted in distilled water. Aliquots representing approximately 2% of the entire preparation were disrupted in 2% sodium chloride, and the quantities of rotavirus released were determined by plaque assay as previously described (18). Approximately 14% of the initial quantity of RRV to be microencapsulated was captured within SA microcapsules. Unencapsulated rotavirus was diluted in distilled water to correspond to the quantities of microencapsulated rotavirus used to immunize mice.

Inoculation of mice. Groups of 20 mice were orally inoculated with either 2.2×10^7 or 6.0×10^6 PFU of RRV per mouse in SA microcapsules, equivalent quantities of unencapsulated RRV, or distilled water. All preparations were inoculated in a volume of 100 μl by proximal esophogeal intubation.

Challenge of mice. At 6 or 16 weeks after inoculation, five mice per group were challenged orally with 1.2×10^5 SD_{50}s of EDIM virus per mouse in a volume of 200 μl. On each of the 6 days following challenge, an average of three fecal pellets were collected from each mouse and placed in 0.5 ml of Earle's balanced salts solution. Samples were stored at −20°C prior to determination of rotavirus antigen content by ELISA.

ELISA to detect rotavirus in feces. To determine the quantities of rotavirus antigen in feces, 96-well, high-binding, flat-bottom plates (Costar, Cambridge, Mass.) were coated with a 1:2,000 dilution in sodium carbonate buffer (1.5 mM sodium carbonate and 3.5 mM sodium bicarbonate) of serum obtained from cows hyperimmunized with bovine rotavirus strain WC3 (obtained from H Fred Clark, Philadelphia, Pa.). Plates were covered and incubated overnight at 4°C in a humidified chamber. On the day of the assay, plates were washed (MultiReagent Plate Washer; Dynatech, Chantilly, Va.) four times with wash buffer (1.73 M NaCl, 0.03 M KH_2PO_4, 0.13 M Na_2HPO_4, 0.25% Tween 20; Sigma, St. Louis, Mo.) and two times with distilled H_2O (dH_2O). Individual wells were blocked with 200 μl of blocking buffer (0.5% gelatin [Sigma] containing 0.05% Tween 20) and incubated at room temperature for 1 h. Known concentrations of purified rotavirus were tested in duplicate during each assay to establish a standard curve from which to determine quantities of rotavirus antigen in feces. Plates were washed four times with wash buffer and twice with dH_2O, after which 50 μl of undiluted

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TABLE 1. Average quantities of rotavirus antigen detected in feces at various intervals after challenge in mice immunized 6 or 16 weeks previously with either RRV or RRV in SA[a]

Postinoculation time and dose	Microcapsule	Quantity (ng/ml) of virus shed at postchallenge day:							*P* value[c] vs:	
		1	2	3	4	5	6	Total	Same dose of free virus	dH_2O
6 wks										
2.2×10^7	SA	<10[b]	20	16	6	13	<10	55	NS[d]	<0.0001
2.2×10^7		24	8	<10	<10	<10	<10	32		<0.0001
6.0×10^6	SA	11	59	121	115	38	<10	344	<0.05	NS
6.0×10^6		33	61	10	20	36	<10	160		<0.005
dH_2O		<10	195	130	105	83	31	544		
16 wks										
2.2×10^7	SA	<10	21	<10	<10	<10	<10	21	<0.01	<0.0001
2.2×10^7		16	60	89	63	39	<10	267		<0.001
6.0×10^6	SA	<10	128	190	160	79	43	600	NS	<0.01
6.0×10^6		11	185	147	139	177	<10	659		<0.05
dH_2O		120	381	213	252	161	74	1,201		

[a] Groups of mice were orally inoculated with either RRV or RRV in SA microcapsules. At 6 or 16 weeks after inoculation, the mice were challenged with EDIM. On each of the 6 days following challenge, fecal pellets were collected and later tested for the presence of rotavirus antigen by ELISA.

[b] Mice which did not shed detectable quantities of rotavirus were assigned a value of 0 for statistical calculations. The lower limit of detection for the ELISA was 1 ng/ml.

[c] Statistical analyses were performed by Student's *t* test; *P* values of ≤0.05 were considered significant.

[d] NS, not statistically significant.

sample, purified rotavirus, or blocking buffer was added to each well. A negative control well containing only blocking buffer was assayed to correspond to each individual sample. Plates were incubated for 1 h and then washed four times in wash buffer and twice with dH_2O. One hundred microliters of a 1:2,000 dilution of rabbit antiserum to bovine rotavirus strain WC3 (obtained from H Fred Clark) was added to each well. Plates were incubated for 1 h at room temperature. After washing, 100 μl of a 1:2,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG; Cappel, Durham, N.C.) in blocking buffer was added to each well. After 1 h at room temperature, plates were washed and 100 μl of developing solution (1 M diethanolamine and *p*-nitrophenyl phosphate solution; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added to each well. One hour later, optical densities (OD) were read at a wavelength of 405 nm (Dynatech MR4000; Dynatech). Samples were considered to be positive if the OD in experimental wells were at least 0.1 U and twofold greater than those of the corresponding control wells. Standard curves of purified rotavirus were determined by exponential fit (correlation coefficient, >0.90), and quantities of antigen in each sample were calculated by using the net OD. Averages were calculated for each group on each day. *P* values, obtained by Student *t* tests, of ≤0.05 were considered to be statistically significant.

Intestinal fragment cultures. At 6 or 16 weeks after inoculation of mice, fragment cultures of small intestines were established both at the time of challenge and 4 days after challenge as previously described (6). Briefly, intestines were removed from each animal, cut open longitudinally, and placed in Hanks balanced salt solution (HBSS; GIBCO) on ice for 2 h. Intestinal fragments were then washed twice in HBSS, once in HBSS containing 0.05% EDTA (to remove the intestinal epithelial layer), and twice with HBSS. Sixteen 1- to 2-mm fragments were isolated from each group and placed in individual wells of a 24-well plate (Becton-Dickson, Lincoln Park, N.J.) containing 1 ml of GALT medium (Kennett's HY medium [JRH, Lenexa, Kans.], 10% fetal bovine serum [GIBCO], 10 mM HEPES, 4 mM L-glutamine, 100-μg/ml streptomycin, 50-μg/ml gentamicin, 0.025-μg/ml amphotericin B [all additives other than fetal bovine serum were obtained from JRH]). Samples were placed in a 95% O_2–5% CO_2 environment at 37°C for 5 days. Supernatants were collected and stored at 4°C prior to determination of virus-specific and total antibodies.

Use of intestinal fragment cultures eliminated concerns associated with collection of intestinal contents, such as variable dilution of antibodies by osmotic catharsis, entrapment of antibodies in the intestinal mucin layer, breakdown of antibodies during collection or storage, and complexing of antibodies with antigen following challenge. In addition, intestinal fragment cultures allowed the preservation of antigen-presenting cells and antibody-secreting cells in a native microenvironment. Previous studies (15) have demonstrated the fragment culture technique to be more sensitive than intestinal lavage. Specifically, antibodies produced by LPL were not always detected at the surface by lavage; however, the reverse was never observed.

ELISA to determine virus-specific and total antibodies in intestinal fragment cultures. Quantities of virus-specific and total IgG and IgA were determined as previously described (6). Samples were considered to be positive if OD values in experimental wells were at least 0.1 U and twofold greater than those in the corresponding control wells. Quantities of antibodies were determined by comparison with standard curves constructed for either IgA, IgG, or IgM by using purified murine antibodies (IgA-kappa chain, IgG1-kappa chain, or IgM-kappa chain [Sigma]). Standard curves were established during each assay, subjected to exponential fit, and used only if the correlation coefficient was greater than 0.90. The lower limit of detection was 1 ng/ml for all antibody isotypes. Standard errors were calculated based on relative percentages of virus-specific and total antibodies among samples in each group.

Statistical analysis. Correlations between reduction of shedding and production of antibody were determined according to Spearman's rank correlation coefficient, r_s, using Pearson's equation and a two-tailed probability.

RESULTS

SA microcapsules enhanced protection against rotavirus challenge 16, but not 6, weeks after immunization. Mice orally inoculated 16 weeks previously with 2.2×10^7 PFU of RRV in SA microcapsules shed less rotavirus antigen than did animals inoculated with the same dose of unencapsulated virus (Table 1). However, protection against shedding in mice immunized 6 weeks previously with microencapsulated RRV was either similar to or greater than that observed in animals that received identical doses of free virus (Table 1).

Enhanced protection by SA microcapsules was associated with production of virus-specific IgA by small intestinal LPL. Mice inoculated 16 weeks previously with RRV in SA microcapsules developed quantities of virus-specific IgA 4 days after challenge that were approximately 10-fold greater than those found after challenge in animals inoculated with unencapsulated RRV ($P \leq 0.0001$; Table 2). Conversely, animals inoculated 6 weeks previously with RRV in SA microcapsules produced less virus-specific IgA 4 days after challenge than did mice inoculated with unencapsulated virus (Table 2), although these differences were not statistically significant ($P = 0.11$).

Lesser quantities of virus-specific IgG detected after challenge were also associated with reduced protection against challenge in animals immunized 6 weeks previously with microencapsulated compared with unencapsulated virus (Tables 1 and 2). However, the presence of virus-specific IgG was not clearly associated with enhanced protection against challenge afforded by microencapsulation 16 weeks after immunization

TABLE 2. Percentages and quantities of virus-specific and total antibodies detected by fragment culture at the time of and 4 days after challenge in mice immunized previously with RRV or RRV in SA[a]

Postinoculation time and dose	Microcapsule	Mean % of VS IgA ± SEM (VS/total quantities)		Mean % of VS IgG ± SEM (VS/total quantities)	
		At challenge	4 days postchallenge	At challenge	4 days postchallenge
6 wks					
2.2×10^7	SA	0 (0/2,835)	0.70 ± 0.16 (16/2,283)	0 (0/16)	0 (0/17)
2.2×10^7		0.11 ± 0.01 (4/3,455)	1.07 ± 0.37 (41/3,842)	0 (0/14)	14.8 ± 5.20 (4/27)
6.0×10^6	SA	0 (0/3,444)	0 (0/2,900)	0 (0/19)	0 (0/33)
6.0×10^6		0 (0/4,508)	1.94 ± 0.67 (66/3,405)	0 (0/25)	9.43 ± 1.44 (5/53)
dH_2O		0 (0/3,826)	0 (0/3,410)	0 (0/24)	0 (0/19)
16 wks					
2.2×10^7	SA	0 (0/1,863)	20.3 ± 5.26 (384/1,896)	0 (0/23)	21.7 ± 8.60 (47/217)
2.2×10^7		0.1 ± 0.03 (3/2,961)	2.0 ± 0.80 (37/1,848)	17.0 ± 5.19 (8/47)	28.3 ± 9.00 (13/46)
6.0×10^6	SA	0.1 ± 0.02 (2/1,921)	1.37 ± 0.40 (30/2,197)	0 (0/23)	1.7 ± 0.82 (1/58)
6.0×10^6		0 (0/3,582)	0 (0/1,503)	0 (0/168)	0 (0/134)
dH_2O		0 (0/1,523)	0 (0/1,697)	0 (0/35)	0 (0/57)

[a] Groups of mice were orally inoculated with either RRV or RRV in SA microcapsules at various doses. At 6 or 16 weeks after inoculation, the mice were challenged with EDIM. Small intestinal fragment cultures were performed at the time of challenge and 4 days after challenge. Supernatant fluids from fragment cultures were tested for the presence of virus-specific (VS) and total IgA and IgG by ELISA. Virus-specific IgA and IgG were not detectable at quantities of less than 1 ng/ml. Undetectable quantities are shown as 0. Quantities of virus-specific and total antibodies are expressed in micrograms per milliliter. Standard errors of the mean are based on relative quantities of virus-specific and total antibodies from 16 samples.

(Tables 1 and 2). Virus-specific IgM was not detected either at the time of challenge or within 4 days of challenge in any group (data not shown).

Virus-specific IgA produced on day 0 was not significantly correlated with reduced shedding ($P \leq 0.2$); however, virus-specific IgA produced on day 4 was correlated with reduced shedding ($P \leq 0.001$).

Immunization of mice with simian strain RRV induced protection against challenge with murine strain EDIM, and protection was not necessarily associated with production of virus-specific IgA by small intestinal LPL. Mice orally inoculated 6 or 16 weeks previously with unencapsulated RRV shed less EDIM in the days immediately following challenge than did unimmunized animals (Table 1). Protection against challenge was not necessarily associated with production of virus-specific IgA, IgG, or IgM at the time of challenge or within 4 days after challenge (Table 2).

DISCUSSION

Microencapsulation of 2.2×10^7 PFU of RRV in SA microcapsules enhanced protective immune responses 16 weeks after immunization (Table 1). While protection was not associated with virus-specific IgA or IgG at the time of challenge (Table 2), enhanced effector B-cell responses derived from memory B cells in the days immediately following challenge were observed. Specifically, 4 days after challenge, virus-specific IgA production was 10-fold greater in mice immunized with RRV in SA microcapsules than in those immunized with unencapsulated RRV (Table 2).

There are a number of possible mechanisms by which microencapsulation in SA enhanced protection against challenge. First, microcapsules may protect virus from the acids and proteases encountered in the digestive tract. However, both RRV and RRV encapsulated in SA microcapsules are completely inactivated by exposure to simulated gastric acid (pH 1.2) at room temperature for 1 h (data not shown). It is possible that although the microcapsules are resistant to breakdown by acid in vitro, influx of protons through pores in the microcapsule wall allows inactivation of the virus within microcapsules. In addition, intramuscular immunization of mice with rotavirus in SA microcapsules enhanced rotavirus-specific IgG responses (13). Second, microencapsulation of rotavirus in SA microcapsules may select for antigen-presenting cells different from and perhaps more efficient than those involved following infection with unencapsulated virus. We found that fluorescently labelled SA microcapsules are taken up by dendritic cells in Peyer's patches to a greater extent than macrophages or B cells after oral inoculation of mice (10); dendritic cells have been found to be more efficient at processing and presenting antigens than other professional antigen-presenting cells in a number of systems (4, 8, 9). Conversely, oral inoculation of mice with EDIM resulted in the detection of rotavirus-specific proteins primarily in Peyer's patch macrophages rather than either dendritic cells or B cells (2). Therefore, one possibility is that by delivering the virus within microcapsules, dendritic cells rather than macrophages have the opportunity to process and present the antigen, thereby leading to an alteration in the generation of the immune response. However, the relative capacity of macrophages and dendritic cells to present rotavirus antigens remains to be determined. Third, SA microcapsules may delay, prolong, or otherwise alter rotavirus processing and presentation by antigen-presenting cells. Several pieces of evidence support this hypothesis. First, we previously found that virus-specific IgM was produced by LPL 60, but not 21, days after oral inoculation of suckling mice with inactivated rotavirus in SA microcapsules (7). In addition, microencapsulation of RRV in SA microcapsules ablated protective immune responses 6 weeks after immunization (Table 1), suggesting that animals that received microencapsulated virus had not been exposed to enough virus to generate a protective response equivalent to that of those that received unencapsulated virus.

Determination of the immunologic mechanisms by which microencapsulation enhances protection against rotavirus challenge depends, in part, upon understanding protective immune responses which occur after immunization with free virus. Immunization of mice with simian rotavirus strain RRV induced a reduction in virus shedding (partial protection) following challenge with murine rotavirus strain EDIM. At a dose of 2.2×10^7 PFU of RRV per mouse, protection was associated with production of virus-specific IgA both at the time of challenge and 4 days after challenge. However, at a lower dose of RRV (6.0×10^6 PFU of RRV

per mouse), protection against challenge detected 16 weeks after immunization occurred in the absence of production of virus-specific IgA or virus-specific IgG at the time of challenge or within 4 days of challenge (Table 2). Therefore, mechanisms other than virus-specific humoral immune responses play a role in protection against challenge.

The immunologic basis of protection against rotavirus challenge in the absence of production of virus-specific IgA or IgG is unclear. Reduction of virus shedding, which occurred within 2 days of challenge, is probably not mediated by virus-specific cytotoxic T lymphocytes (CTLs). First, following immunization with RRV, virus-specific CTLs are not detected at the intestinal mucosal surface (among intraepithelial lymphocytes) beyond 6 days after infection (16). Second, generation of CTLs from CTL precursors is unlikely to occur within 2 days (5, 16). Therefore, another effector mechanism, possibly secretion of antiviral cytokines by memory T cells, may be associated with early reduction of shedding after challenge.

While the mechanisms responsible for enhanced protection against rotavirus challenge after immunization with microencapsulated virus are not completely understood, these findings may impact vaccine design. Although it remains to be determined whether microencapsulation provides a commercially feasible approach to enhancing vaccine-specific protective immune responses, a delay in protective immune responses by microencapsulation may allow the use of unique combination vaccines that decrease the need for booster doses. Potential applications of microcapsules that alter the kinetics of virus-specific immune responses will be a subject of future study. In addition, a broader range of doses, increased time intervals, and, perhaps, less stringent challenge doses may help us to gain a better understanding of the boundaries of the protection afforded by microencapsulation.

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