Journal of General Virology (2001), **82**, 2271–2274. Printed in Great Britain

SHORT COMMUNICATION

Distribution of rotavirus-specific memory B cells in gut-associated lymphoid tissue after primary immunization

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We found previously that mice inoculated orally with simian rotavirus strain RRV developed virus-specific memory B cell responses 16 weeks after immunization that were greater than those found 6 weeks after immunization. Memory B cell responses were defined as the quantity of virus-specific IgA detected in small intestinal lamina propria (LP) fragment cultures of immunized mice at various intervals after challenge. Enhanced memory B cell responses correlated with enhanced protection against shedding. In order to understand better the delayed onset of rotavirus-specific memory B cell responses, a method was developed to determine the frequencies of rotavirus-specific memory B cells in gut-associated lymphoid tissues (GALT). We found that protection against rotavirus challenge was determined by the frequency of rotavirus-specific memory B cells in GALT LP.

Rotaviruses replicate solely in mature villous epithelial cells of the small intestine (Sheridan *et al*., 1983; Starkey *et al*., 1986). Therefore, protection against rotavirus disease is likely to be mediated by immunological factors present at the intestinal mucosal surface. Studies in both experimental animals and humans found that rotavirus-specific IgA, present at the intestinal mucosal surface, can either prevent or modify disease (Coulson *et al*., 1992; Feng *et al*., 1994; Franco & Greenberg, 1995; Matson *et al*., 1993; McNeal & Ward, 1995; McNeal *et al*., 1995; Moser *et al*., 1998). Whereas complete protection against rotavirus disease is likely to be mediated by high titres of virus-specific IgA present at the small intestinal surface at the time of challenge (Coulson *et al*., 1992; Matson *et al*., 1993), modification of rotavirus disease is probably mediated by virus-specific IgA derived from memory B cells in gut-associated lymphoid tissue (GALT) (Moser *et al*., 1998; Offit, 1996).

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We developed a murine model for rotavirus infection that distinguishes the relative protective capacity of virus-specific IgA present at the intestinal mucosal surface at the time of challenge from virus-specific IgA derived from memory B cells after challenge (Moser *et al*., 1998). We found that mice immunized with murine rotavirus strain EDIM or a high dose ($1{\cdot}9 \times 10^7$ p.f.u.) of simian strain RRV produced high levels of virus-specific IgA up to 16 weeks after immunization and were completely protected against shedding following EDIM challenge. In contrast, mice immunized with a lower dose of RRV ($1{\cdot}9 \times 10^6$ p.f.u.) did not produce virus-specific IgA at the intestinal mucosal surface, but were still partially protected after EDIM challenge. Protection against shedding in mice immunized with a lower dose of RRV was mediated by virus-specific IgA. Virus-specific IgA was most likely produced by effector cells generated from virus-specific memory B cells and not from effector cells that continued to produce IgA after immunization. The magnitude of virus-specific IgA produced after challenge by mice immunized 16 weeks previously with a low dose of RRV was greater than that produced by unimmunized mice or by mice immunized 6 weeks previously.

These studies were performed in order to understand better the delay in development of virus-specific IgA memory B cell responses in GALT following infection of mice with RRV. We used a method, similar to that of Slifka & Ahmed (1996), that employed *in vitro* stimulation and antibody-secreting cell (ASC) frequency analyses to determine the site and presence of rotavirus-specific memory B cells in GALT after primary immunization. At various intervals after immunization, mice were sacrificed and single-cell suspensions were prepared from various GALT and non-enteric lymphoid tissues. Cells were either tested immediately for the presence of rotavirus-specific IgA-secreting cells (primary effector B cells) or incubated *in vitro* with or without rotavirus. After 5 days in culture, viable cells were tested by ELISpot assay to determine the number of rotavirus-specific IgA-ASC derived from memory B cells (secondary effector B cells).

Adult, 5- to 6-week-old, female BALB/c mice, obtained from Taconic Breeding Laboratories (Germantown, NY, USA),

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Table 1. Generation of rotavirus-specific IgA-ASC from virus-specific memory B cells in GALT at various times after immunization

Values are geometric means of the number of virus-specific IgA-ASC per 10^6 cells, with the mean of the $\log_{10}$-transformed data $\pm$ 2 SD per group given in parentheses.

Tissue	Unimmunized	Immunized with RRV	
		4–8 weeks	14–18 weeks
PP	0	275^{a*} ($2{\cdot}44 \pm 1{\cdot}19$)	35^{b} ($1{\cdot}54 \pm 1{\cdot}52$)
MLN	0	0	0
Spleen	0	138^{a} ($2{\cdot}14 \pm 0{\cdot}50$)	47^{c} ($1{\cdot}68 \pm 1{\cdot}32$)
LP	0	0	$174^{b,c}$ ($2{\cdot}24 \pm 0{\cdot}45$)
BM	0	0	0

* Statistical significance is indicated by: *a*, $P \leqslant 0{\cdot}001$ compared with unimmunized mice; *b*, $P \leqslant 0{\cdot}05$ compared with mice immunized 4–8 weeks prior to the experiment; *c*, $P \leqslant 0{\cdot}05$ compared with unimmunized mice.

were inoculated orally by proximal oesophageal intubation with $1{\cdot}2 \times 10^6$ p.f.u. of rhesus rotavirus strain RRV in a volume of 200 µl. RRV was originally obtained from N. Schmidt (Berkeley, CA, USA) and was grown and titrated in foetal green monkey kidney cells (MA-104) as described previously (Offit *et al*., 1983). At various intervals after inoculation, single-cell suspensions were prepared from tissues including Peyer's patches (PP), mesenteric lymph nodes (MLN), spleen, lamina propria (LP) and bone marrow (BM) as described below. PP, MLN and spleens were removed and disrupted using two L-shaped 21-guage needles. LP cells were isolated enzymatically using a method described previously (Offit *et al*., 1991). In order to obtain BM, femurs were removed from mice and flushed with RPMI CM [RPMI 1640 (Mediatech) containing 10% FBS (BioWhittaker), 1% HEPES (Gibco BRL), 1% glutamine (BioWhittaker), 20 U/ml penicillin, 20 µg/ml streptomycin, 50 µg/ml gentamicin (all antibiotics from Gibco BRL) and 0·003% 2-mercaptoethanol (Sigma)] using a 26-gauge needle. Clumps were drawn into a 1 ml syringe to separate cells. In the case of all tissues, cell suspensions were washed with RPMI CM and counted by trypan blue exclusion.

Initial studies were performed to determine the presence of primary rotavirus-specific effector B cells by subjecting single-cell suspensions to assay by ELISpot, performed as described previously (Khoury *et al*., 1994). Samples were considered to be positive if there were at least five spots per 10^6 cells in a well and at least 2-fold more spots in wells coated with purified RRV than in wells coated with BSA. Rotavirus-specific IgA effector B cells were not detected in the PP, MLN, spleen, LP or BM of unimmunized mice or mice immunized 5–6 weeks or 16 weeks after immunization. However, at 3 weeks after immunization, 55 rotavirus-specific IgA-ASC were detected in the MLN and 180 rotavirus-specific IgA-ASC were detected in the LP.

In order to determine whether secondary rotavirus-specific effector B cells were present, either 4–8 or 14–18 weeks after immunization, groups of three to ten immunized or unimmunized mice were sacrificed and single-cell suspensions were prepared as described above. Cells (1×10^6) were placed into individual wells of 96-well round-bottomed plates with 100 µl of either medium alone or medium containing 80 ng caesium chloride-purified RRV. Quantities of purified rotavirus were determined by spectrophotometric analysis. Plates were incubated at 37 °C in 5% CO_2 for 3 or 5 days prior to assay by ELISpot. In the ELISpot assay, 5×10^4 viable cells from each lymphoid culture were tested in duplicate wells coated with either purified rotavirus, goat anti-mouse IgA (to determine the total number of IgA-secreting cells as a measure of cell viability) or 1% BSA. By normalizing viable cells recovered following stimulation *in vitro*, equal numbers of cells potentially capable of secreting antibodies could be assayed by ELISpot and the results compared. Samples were considered to be positive if there were (i) at least five spots per 10^6 cells in a well, (ii) at least 2-fold more spots in wells coated with purified RRV than in wells coated with BSA and (iii) at least 2-fold more spots in wells containing virus-stimulated compared with unstimulated cells. Data were adjusted by subtracting the mean number of spots in BSA-coated wells from that found in virus-coated wells. These numbers were adjusted further by subtracting numbers calculated for unstimulated cells from the same tissue. Each experiment was performed three to five times and data were $\log_{10}$-transformed and subjected to statistical analysis using two-sample *t*-tests. Whereas quantities of rotavirus-specific IgA-ASC derived from secondary B cells decreased in PP and spleen between 4–8 and 14–18 weeks after immunization, cell numbers increased in the LP (Table 1). Mechanisms that might account for the development of increased frequencies of virus-specific memory B cells in the LP

between 6 and 16 weeks after immunization include the following. Firstly, a decrease in virus-specific memory B cells in the PP and spleen was associated with an increase in virus-specific memory B cells in the LP. These changes may represent a migration of memory B cells from PP and spleen to the LP. Secondly, virus-specific T cells, capable of stimulating virus-specific memory B cells, might migrate slowly to and accumulate in the LP. Thirdly, virus-specific memory B or T cells, already residing in the LP, might increase in frequency over time. Finally, virus-containing antigen-presenting cells, capable of generating or stimulating virus-specific memory B or T cells or both, might migrate to the LP over time. Because the numbers of B cells, T cells and antigen-presenting cells were preserved from each tissue, and not normalized to one specific cell type, none of these mechanisms can be ruled out. Future experiments will help to sort out the likelihood of each of these proposed mechanisms.

IgA-ASC were not detected in the MLN or the BM after stimulation *in vitro*. While this result was at variance with another study of rotavirus-infected mice (Williams *et al*., 1998), the studies are not directly comparable. Specifically, Williams *et al*. (1998) studied mice inoculated orally with a murine strain of rotavirus, whereas our studies were based on mice inoculated orally with RRV, a rhesus strain of rotavirus that is less well adapted to growth at the intestinal mucosal surface. Previous studies comparing murine and non-murine rotaviruses found differences in the relative abilities of these strains to induce rotavirus-specific IgA responses (Moser *et al*., 1998; Feng *et al*., 1994; Ward *et al*., 1992): IgA responses in LP induced by murine rotaviruses were of higher magnitude and longer duration than those induced by non-murine rotaviruses. Ridderstad & Tarlinton (1997) also found memory B cells residing in bone marrow. However, their studies were based on observations in mice inoculated parenterally with a non-mucosal immunogen [the hapten 4(hydroxy-3-nitrophenyl)-acetyl coupled to keyhole limpet haemocyanin]. The low levels of memory cells detected by Ridderstad & Tarlinton (1997) and the undetectable quantities in our studies of mice inoculated with a non-murine strain of rotavirus support the findings of Benedetti *et al*. (1998), that few memory B cells actually reside in the BM. It is more likely that memory B cells, stimulated outside the BM, migrate to the BM and differentiate to ASC. Virus-specific IgG-secreting cells found after stimulation *in vitro* of the PP, MLN, LP, spleen or BM were not significantly different from those found in unimmunized mice (data not shown).

In order to determine whether virus-specific ASC were generated *in vitro*, cells were harvested 3 and 5 days after stimulation *in vitro*. Rotavirus-specific IgA effector B cells derived from virus-specific memory B cells were not detected 3 or 5 days after stimulation of cells from unimmunized mice or 3 days after stimulation of cells from immunized mice. However, in mice immunized with RRV 8 weeks previously, stimulation of PP cells *in vitro* for 5 days generated 190 rotavirus-specific IgA-ASC and stimulation of splenic cells *in vitro* generated 80 IgA-ASC. No rotavirus-specific IgG-ASC were detected. Several observations support our detection of *in vitro*-activated, secondary, rotavirus-specific B cells. Firstly, rotavirus-specific effector B cells were detected 5 but not 3 days after culture with RRV *in vitro*. Secondly, virus-specific B cells were detected in immunized but not unimmunized mice. Thirdly, primary virus-specific effector B cells were not detected in GALT 16 weeks after immunization when cells were tested immediately at the time of harvest. These findings are also supported by observations from published studies, that memory cells are activated and differentiate to effector cells between 3 and 5 days after stimulation (Arpin *et al*., 1995, 1997).

Virus-specific memory B cells were detected about 4 months after a single oral inoculation of mice with RRV. However, RRV is not detected as infectious virus in GALT or non-enteric tissues beyond 5 days after inoculation (Offit *et al*., 1991). As has been shown for a number of antigens (Gray & Skarvall, 1988; reviewed in Sprent, 1994), maintenance of long-term memory in PP and spleen might be mediated by the presence of rotavirus antigens in follicular dendritic cells. Since follicular dendritic cells are not found in the LP, memory B cells in the LP would more likely be derived from memory cells migrating from other sites, such as the PP or spleen.

We found previously that, after a single inoculation of mice with RRV, protection against challenge correlated with development of virus-specific IgA in LP fragment cultures after challenge; virus-specific IgA responses in the LP following challenge were greater 16 weeks compared with 6 weeks after immunization (Moser *et al*., 1998). In these studies, we showed that virus-specific memory B cells were present in the PP and spleen early after immunization, but decreased in frequency several months after immunization. In contrast, virus-specific memory B cells were detected in the LP late, but not early, after immunization. These findings support the hypothesis that protection against shedding is dependent upon the presence of memory B cells in the small intestinal LP. Activation of memory B cells present in the PP or spleen alone is not adequate to ensure protection against mucosal challenge. Strategies of immunization that hasten the onset and enhance the longevity of local virus-specific memory B cells are likely to be important in protection against mucosal pathogens.

We thank Kurt Brown, Jeff Brubaker, Stephanie Clark, Susan Coffin, Douglas Dolfi, Jenny Kriss and Kristine Macartney for their helpful discussions. We also thank Michele Lutz for her technical assistance. This work was supported in part by RO1 grant AI-26251 from the National Institutes of Health to P.A.O.

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Received 13 March 2001; Accepted 8 June 2001