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Enhanced Mucosal and Systemic Immune Responses to Intestinal Reovirus Infection in β2-Microglobulin-Deficient Mice

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Enteric infection of mice with respiratory enteric orphan virus (reovirus) type 1, strain Lang elicits both humoral and cellular immune responses. To investigate the role of $CD8^+$, α/β T-cell receptor $(TCR)^+$ T cells in mucosal immunity to an enteric pathogen, we examined immune responses and viral clearance following enteric reovirus infection in C57BL/6, B6129F2, and β2-microglobulin-deficient (β2m−/−) mice. Analysis of Peyer's patch and lamina propria culture supernatants revealed a two- to threefold increase in levels of reovirus-specific immunoglobulin A in β2m−/− mice compared to normal controls. These data corresponded to a similar increase in the frequency of virus-specific immunoglobulin A-producing cells in Peyer's patches and lamina propria and an increase in immunoglobulin G-producing cells in spleens from β2m−/− mice compared to controls. These increased humoral immune responses were not due to a difference in B-cell populations because cell counts and flow cytometric analyses showed that β2m−/− and control mice had similar numbers and percentages of B cells in mucosal and systemic tissues. Analysis of cytokine message by reverse transcriptase-PCR 5 and 10 days after infection revealed no difference in message level for transforming growth factor beta, gamma interferon, interleukin-4, interleukin-5, or interleukin-6 for all mouse strains. Virus tissue titers determined by plaque assay at 5 and 10 days after infection demonstrated that β2m−/− mice cleared reovirus from the small intestines with the same efficiency as control mice. Collectively, these data suggest that $CD8^+$, α/β TCR^+ T cells may regulate mucosal and systemic humoral immune responses to oral infection with reovirus.

A large proportion of T cells in the gut-associated lymphoid tissue (GALT) of normal mice are $CD8^+$. These $CD8^+$ T cells are distributed throughout the three major lymphoid compartments in the gastrointestinal tract, constituting approximately 2 to 5% of Peyer's patch (PP) lymphocytes and 15% of lamina propria (LP) lymphocytes (5, 20, 27). Among the lymphocytes in the intraepithelial compartment, over 90% are $CD8^+$ (24, 25). Antigen-specific $CD8^+$ T cells are activated following enteric exposure to a wide range of pathogens such as *Listeria monocytogenes* (18, 50), *Salmonella typhimurium* (60), rotavirus (49), reovirus (38), *Toxoplasma gondii* (9), and *Cryptosporidium* spp. (43). The generation of antigen-specific $CD8^+$ T cells in GALT is thought to be important in resistance to infection either by lysing infected cells (18, 43, 49, 60) or by producing cytokines such as gamma interferon (IFN-γ) (8, 18, 57). However, despite the hypothesized protective role for antigen-specific $CD8^+$ T cells, protection against intestinal infections with pathogens such as rotavirus (21), reovirus (1), and *Cryptosporidium muris* (44) occurs in the absence of functional $CD8^+$ T-cell-mediated immunity. Therefore, the exact role of $CD8^+$ T cells in clearing pathogens or preventing reinfection of the intestine is still unclear.

In addition to the hypothesized role of $CD8^+$ T cells in protection against enteric infection, there is evidence that intestinal $CD8^+$ T cells may also regulate mucosal immune responses in vivo and in vitro. $CD8^+$ T cells have been implicated in the induction of tolerance to orally administered antigens (63). In experimental autoimmune encephalomyelitis, adoptively transferred $CD8^+$ PP and spleen (SPL) cells from animals fed myelin basic protein mediate resistance to disease in naive animals (10, 37, 56). These $CD8^+$ T cells appear to exert their immunosuppressive effects, at least in part, by the production of transforming growth factor beta (TGF-β) (11, 56). In addition, it has been suggested that $CD8^+$ T cells can inhibit T helper (Th) cell responses in vitro. Harriman et al. (26) found that expansion of keyhole limpet hemocyanin (KLH)-specific $CD4^+$ LP T cells was inhibited when $CD8^+$ LP T cells were present in culture. Subsequent depletion of $CD8^+$ T cells prior to culture resulted in the generation of long-lived, antigen-specific Th cell lines. Similarly, Hornquist et al. (27) described a possible role for $CD8^+$ T cells in the inhibition of antigen-specific immunoglobulin A (IgA) and $CD4^+$ T-cell responses in GALT. Following oral administration of KLH and cholera toxin, $CD8^+$ T-cell-deficient mice had enhanced KLH-specific IgA responses as well as increased IFN-γ production in LP cell culture supernatants. It has also been hypothesized that $CD8^+$ T suppressor cells are selectively activated by major histocompatibility complex (MHC) class II molecules expressed on the surface of intestinal epithelial cells (4, 42). While these studies demonstrate that antigen-specific humoral and cellular immune responses to soluble protein may be regulated by $CD8^+$ T cells, little is known about the immunoregulatory effects of $CD8^+$ T cells following enteric infection with a replicating antigen. A candidate agent for such studies is respiratory enteric orphan virus (reovirus), a well-characterized enteric pathogen that has been extensively used to study intestinal immune responses.

Enteric infection with reovirus elicits cellular and humoral immune responses in GALT (16, 39, 41). Humoral mucosal immunity is characterized by the production of virus-specific IgA in PP and LP (39). $CD8^+$, α/β T-cell receptor $(TCR)^+$ virus-specific precursor cytotoxic T lymphocytes (CTL) also

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develop in PP and the epithelium following reovirus infection (15, 38–40). In addition, oral administration of UV-inactivated reovirus induces oral tolerance (54).

In this study, we measured the local and systemic humoral immune responses in reovirus-infected β2-microglobulin-deficient (β2m−/−) mice. β2m−/− mice are deficient in expression of MHC class I and therefore lack the majority of $CD8^+$, α/β TCR^+ T cells (32, 65). Thus, these animals provide a model with which to study immune responses in the absence of conventional $CD8^+$ T cells. Although systemic infection with lymphocytic choriomeningitis virus (LCMV) (2, 36) and pulmonary Sendai virus infection (29) resulted in increased humoral immune responses in β2m−/− mice, it is not well known whether the intestinal immune response, with its poorly understood mechanisms of regulation, is similarly enhanced in β2m−/− mice. Our data suggest that $CD8^+$, αβ TCR^+ T cells may inhibit mucosal and systemic antibody responses following enteric virus infection.

MATERIALS AND METHODS

Animals. Male C57BL/6, B6129F2 (F2), and β2m−/− mice were purchased from the Jackson Laboratory. β2m−/− mice were also bred in our animal facility from parental stock purchased from the Jackson Laboratory. Mice were housed in specific-pathogen-free conditions, and reovirus-infected mice were housed in a separate room in a laminar flow hood. All animals were used between 8 and 12 weeks of age. The β2m−/− mice used in these studies are hybrids of the C57BL/6 and 129 mouse strains (32, 65). Therefore, we have used both C57BL/6 control mice and the additional control strain F2 (30a).

Virus purification. Reovirus type 1, strain Lang stocks were grown in L929 cells at 34°C in a spinner flask in Eagle's minimum essential medium for suspension cultures (Whittaker Bioproducts, Walkersville, Md.) containing 5% fetal bovine serum (FBS; HyClone, Logan, Utah), 2 mM L-glutamine (Sigma, St. Louis, Mo.), and 100 U of penicillin, 0.1 μg of streptomycin, and 10 μg of gentamicin (all from Whittaker Bioproducts), per ml. Third-passage reovirus was purified from L929 cell lysates by extraction with 1,1,2-trichloro-1,2,2-trifluoroethane followed by CsCl gradient centrifugation. Plaque assays were used to determine titers of purified virus as described by Cuff et al. (16).

Animal infections. Purified reovirus was suspended at a concentration of 6×10^8 PFU/ml in borate-buffered saline (pH 7.4) containing 2% gelatin (gel saline). Mice were orally infected with 3×10^7 PFU of reovirus in 50 μl, using an oral feeding tube.

Virus titers. Five and ten days after infection, β2m−/− and control mice were sacrificed by cervical dislocation, and PP, small intestines (SI), mesenteric lymph nodes (MLN), and SPL were harvested. Tissues were placed in 3 ml of gel saline and frozen and thawed three times. Tissues were then homogenized (Brinkmann, Westbury, N.Y.) and sonicated (Misonix, Inc., Farmingdale, N.Y.). Serial 10-fold dilutions of tissues were made in gel saline, and 100 μl of each dilution was incubated on confluent L929 cell monolayers in 12-well plates (Costar, Cambridge, Mass.) for 45 min at 34°C. Virus-infected monolayers were overlaid with an equal volume of 2% agar and 2× medium 199 (Whittaker Bioproducts) containing 10% FBS, 4 mM L-glutamine, and antibiotics. Plaque assays were cultured for 7 days at 34°C, with additional overlays every 3 days. The final overlay contained 0.02% neutral red (Sigma) to visualize plaques.

Lymphoid tissue fragment cultures. PP were cultured as previously described previously (41). PPs were removed from the gut and intestines were cut longitudinally. Intestinal contents were removed by washing the tissue at least three times in Hanks' balanced salt solution (HBSS) containing 0.1 M HEPES, 0.2% $NaHCO_3$, and 10 μg of gentamicin per ml. Tissues were then suspended in 5 mM EDTA–HBSS and incubated for 30 min at 37°C. SI devoid of epithelium (hereafter referred to as LP) were then cultured in 5 ml of tissue culture medium (TCM) consisting of RPMI 1640 (Whittaker Bioproducts) supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics in six-well plates (Costar). Cultures were incubated at 37°C and 5% CO_2 for 5 days without further restimulation. Following incubation, the supernatants were collected and reovirus-specific antibody concentrations were measured by enzyme-linked immunosorbent assay (ELISA).

ELISA for reovirus-specific and total antibody. Each well of 96-well ELISA plates (Costar) was coated overnight at 4°C with 50 μl of either 5×10^{10} particles of purified reovirus or 1 μg of goat anti-mouse Ig (heavy and light chain specific; Southern Biotechnology, Birmingham, Ala.) per ml in 0.1 M $NaHCO_3$. Plates were washed twice with phosphate-buffered saline (PBS) supplemented with 0.5% Tween 20 (PBS-T) and blocked with 3% (wt/vol) bovine serum albumin (Sigma) in PBS for 2 h at room temperature. Following two washes with PBS-T, serial twofold dilutions of culture supernatants or serum were added to sample wells (100 μl/well) and incubated overnight at 4°C. Plates were washed four times with PBS-T and were reacted for 45 min at room temperature with 100 μl of biotinylated goat antibody specific for murine IgA, IgG2a, or IgG2b (Southern Biotechnology) at a concentration of 0.5 μg/ml in PBS supplemented with 10% FBS. Following incubation, plates were washed six times, avidin-conjugated peroxidase (0.25 μg/well; Sigma) was added, and the plates were incubated for 30 min at room temperature. Plates were washed eight times with PBS-T, 100 μl of the substrate 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (Sigma) was added to each well, and color was developed for 20 min. Plates were read at 405 nm on an ELISA microplate reader (Bio-Tek Instruments Inc., Winooski, Vt.). Quantitation of virus-specific and total antibody was accomplished by adding serial twofold dilutions of known concentrations of purified mouse IgA, IgG2a, or IgG2b (Southern Biotechnology) to wells coated with goat anti-mouse Ig (heavy and light chain specific) to generate standard curves. Specificities of antibodies were previously confirmed by ELISA and immunoblot analysis (41).

Lymphocyte isolation. Spleen lymphocytes were isolated by expressing organs through a nylon screen (Tetko Inc., Briarcliff Manor, N.Y.) and lysing erythrocytes with Tris-buffered ammonium chloride (46). Single-cell suspensions were washed three times with RPMI 1640 containing 5% FBS and antibiotics (hereafter referred to as wash medium) and resuspended in TCM.

PP lymphocytes were isolated by dissecting PP from the SI and mechanically disassociating lymphocytes from the tissue using 18-gauge needles. Single-cell suspensions were passed over nylon wool columns to remove tissue debris, and recovered cells were washed three times in wash medium.

LP lymphocytes were isolated by using modifications of previously published methods (33). The epithelium was removed from the SI by using EDTA as described above. SI were then incubated in RPMI 1640 supplemented with 10% FBS and 0.1% gentamicin for 30 min at room temperature. Tissues were digested with collagenase (50 U/ml; Sigma), dispase grade II (0.3 to 0.5 mg/ml; Boehringer Mannheim, Indianapolis, Ind.), soybean trypsin inhibitor (0.2 mg/ml; Sigma), and DNase (1.6 μg/ml; Sigma) in RPMI 1640 supplemented with 10% FBS and antibiotics for 60 min at 37°C. After digestion, tissue suspensions were passed over nylon wool columns and washed twice with HBSS. This process was repeated on the remaining undigested tissue. Lymphocytes from both digestions were pooled and enriched on a discontinuous Percoll (Sigma) gradient. Cells were resuspended in 42% Percoll, underlaid with 70% Percoll, and centrifuged at $600 \times g$ for 20 min at 4°C. Lymphocytes were removed from the 42%/70% interface and washed three times in wash medium.

ELISPOT assays. Enzyme-linked immunospot (ELISPOT) analyses were performed by using modifications of methods of Kramer and Cebra (33). Each well of 96-well nitrocellulose plates (Millipore, Bedford, Mass.) was coated overnight at 4°C with 50 μl of either 2.0×10^{11} particles of reovirus or 10 μg of goat anti-mouse Ig per ml in sterile PBS. Each well was washed three times with 200 μl of sterile PBS and blocked for 2 h at 37°C with 200 μl of RPMI 1640 containing 10% FBS. SPL and PP cells were diluted to 10^6, 10^5, and 10^4 cells/ml, and LP cells were diluted to 10^5, 10^4, and 10^3 cells/ml, in TCM. One hundred microliters of the cell suspensions was placed in the wells, and the plates were incubated for 4 h at 37°C and 5% CO_2. Plates were washed six times with PBS-T and incubated overnight at 4°C with 100 μl of either alkaline phosphatase-conjugated anti-mouse IgA and IgG2a or biotinylated goat anti-mouse IgG2b, diluted 1:250 in 1% bovine serum albumin–PBS-T, per well. Plates were washed eight times with PBS-T, and IgA and IgG2a plates were developed with substrate solution containing 0.15 mg of 5-bromo-4-chloro-3-indolylphosphate per ml, 0.30 mg of nitroblue tetrazolium per ml, and 5 mM $MgCl_2$ in 100 mM Tris buffer (SIGMA *FAST* BCIP/NBT; Sigma). Plates incubated with biotinylated goat anti-mouse IgG2b were incubated with 100 μl of avidin-peroxidase (2.5 mg/ml) per well for 90 min at room temperature before development with substrate solution containing 3,3′-diaminobenzidine (0.7 mg/ml) and urea H_2O_2 (2.0 mg/ml) in 0.06 M Tris buffer (SIGMA *FAST* DAB; Sigma). Plates were developed for 15 to 20 min, and the reaction was stopped with distilled H_2O. A dissecting microscope was used to count antibody spot-forming cells (SFC).

Flow cytometric analysis. Cell suspensions of PP, LP, and SPL were stained with either fluorescein isothiocyanate-conjugated antibody specific for murine B220 or CD8α (both from Caltag Laboratories, San Francisco, Calif.) or phycoerythrin-conjugated antibody specific for murine CD4 or Thy1.2 (both from Pharmingen, San Diego, Calif.). Cells were incubated with antibodies for 30 min at 4°C in the dark. Following two washes with 1% FBS in PBS (vol/vol), cells were fixed in 100 μl of 0.4% paraformaldehyde in PBS (wt/vol) and analyzed with a FACscan (Becton Dickinson, San Jose, Calif.). PP cells contained ≤1% γ/δ TCR^+ cells, indicating that PP lymphocyte preparations were not significantly contaminated with intraepithelial lymphocytes.

RT-PCR for cytokine mRNA in PP. Total cellular RNA was isolated by using modifications of methods described by Chirgwin et al. (12). Five and ten days after infection, total RNA from β2m−/− and C57BL/6 PP was isolated by placing whole tissue in 1.0 ml of RNAzol-B solution (Tel-Test Inc., Friendswood, Tex.) containing guanidinium thiocyanate and phenol. RNA was extracted from dissolved tissues by adding 100 μl of chloroform to each sample tube. Five micrograms of purified RNA was treated with 1 U of DNase per sample as instructed by the manufacturer (Promega, Milwaukee, Wis.) for 1 h at 37°C. First-strand cDNA was synthesized in a final volume of 10 μl by reacting each sample with 12.5 U of RNase inhibitor, 0.5 μl of deoxynucleotide triphosphate (dNTP) mixture (containing 10 μM each ATP, CTP, GTP, and TTP), 0.5 μl of random primer (0.1 A_{260} units/μl), 10 U of reverse transcriptase (RT), and 2 μl of reaction buffer (all from Boehringer Mannheim). Samples were reacted for 1 h at 42°C and were stored at −20°C until used in PCR analyses.

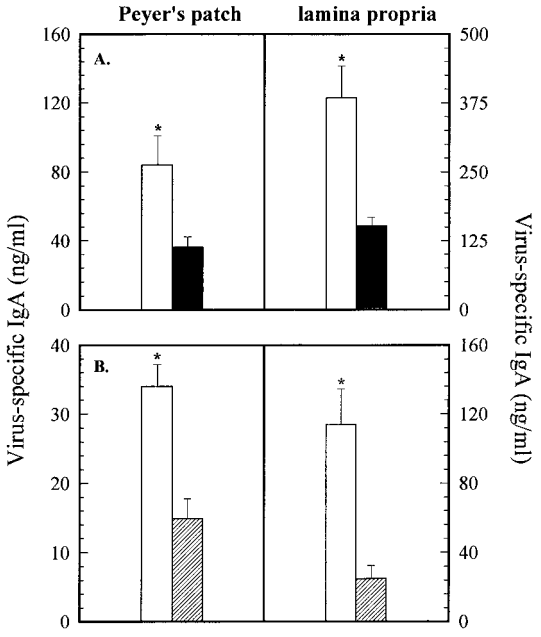

FIG. 1. Reovirus-specific IgA concentrations in PP and LP fragment culture supernatants from β2m−/− (open bars), C57BL/6 (closed bars; A), and F2 (hatched bars; B) mice. Ten days after oral infection with reovirus, fragment cultures of PP and LP were cultured for 5 days without further restimulation. Reovirus-specific IgA concentrations were determined by specific ELISA. Bars represent the means and standard errors for four to five individual mice per group. Data shown are from two representative experiments of six comparing β2m−/− to either the C57BL/6 or F2 control mouse strain. An asterisk denotes significantly higher virus-specific IgA concentrations as determined by Student's *t* test ($P < 0.05$).

For PCR, 2 μl of cDNA was reacted in a total volume of 25 μl with 0.125 U of AmpliTaq polymerase per ml, 2 μl of dNTP mixture (2.5 mM each dNTP), 0.4 μM 5′ primer, 0.4 μM 3′ primer, and 2.5 μl of 10 × PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin, pH 8.3 [Perkin Elmer, Norwalk, Conn.]). Commercially available primers for IFN-γ, TGF-β, interleukin-4 (IL-4), IL-5, and IL-6 (Clontech Laboratories, Palo Alto, Calif.) were used. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were prepared by the West Virginia University DNA Core Facility and consisted of the GAPDH 1 primer sequence 5′ TGA AGG TCG GTG TGA ACG GAT TTG G 3′ and the GAPDH 2 primer sequence 5′ ACG ACA TAC TCA GCA CCA GCA TCA C 3′ (55). Positive controls consisted of reaction mixtures containing 1 μl of specific template for each cytokine, which was provided by the manufacturer. Conditions for PCR consisted of one cycle of 94°C for 4 min, 55°C for 2 min, 72°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min and one cycle of 94°C for 45 s, 55°C for 1 min, and 72°C for 7 min. All PCRs were performed with an excess of enzyme, primers, and deoxyribonucleotides. Samples were stored at 4°C until they were analyzed by gel electrophoresis on a 1.5% agarose gel. Specific bands were visualized by examining ethidium bromide-stained gels under UV light. Specificity of the reaction was confirmed by comparing band size to positive controls provided by Clontech and Southern blot analysis of PCR products by using specific probes.

RESULTS

Reovirus-specific IgA responses in GALT from β2m−/− and control mice. Virus-specific IgA was significantly increased in PP and LP fragment culture supernatants from β2m−/− mice compared to controls (Fig. 1). While there was variation in antibody output between experiments, six separate experiments demonstrated that virus-specific IgA concentrations were two- to threefold higher in culture supernatants from β2m−/− mice than in control strain culture supernatants. There was no difference among the three strains of mice in the amount of total IgA in the culture supernatants (data not shown).

Cellularity and phenotype distribution in secondary lymphoid tissues from β2m−/− and control mice. Lymphocyte cell counts and flow cytometric analyses were used to determine whether the increase in virus-specific IgA in fragment culture supernatants was due to increases in cellularity or B-cell distribution. PP, LP, and SPL from β2m−/− mice contain cell numbers similar to those in C57BL/6 and F2 controls (Table 1). The percentages of $B220^+$ cells were similar for β2m−/− and controls in SPL and PP; however, F2 mice had a significantly higher percentage of $B220^+$ cells in LP compared to β2m−/− and C57BL/6 mice. Percentages of $Thy1.2^+$ cells in SPL, PP, and LP from β2m−/− and control mice were similar. However, β2m−/− mice had an increased percentage ($P < 0.04$) and absolute number ($P < 0.02$) of $CD4^+$ T cells in SPL (Table 1). In addition, in two of two separate experiments, the percentage of $CD4^+$ T cells in PP from β2m−/− mice was higher than in controls, although the difference was not statistically significant when the data from both experiments were combined.

Increased frequencies of reovirus-specific IgA antibody-producing cells in β2m−/− mice. The increase in virus-specific IgA without an increase in the number of B cells suggested that

TABLE 1. Cellularity and lymphocyte distribution in tissues from reovirus-infected mice

Tissue	Mouse strain	Cell count (10^6) (±SEM)	% Positive cells (±SEM)[a]			
			B220	Thy1.2	CD4	CD8
SPL	β2m−/−	90.0 (9.0)	41.9 (2.3)	29.3 (2.4)	33.5 (2.5)[b]	<1.0
	C57BL/6	76.8 (14.4)	41.8 (3.3)	33.7 (2.4)	21.0 (0.0)	10.5 (2.5)
	F2	80.0 (4.3)	39.5 (2.4)	38.5 (7.5)	22.0[d]	8.0[d]
PP	β2m−/−	6.2 (0.7)	63.0 (2.2)	20.3 (1.9)	25.0 (4.0)	<1.0
	C57BL/6	6.1 (0.6)	68.7 (1.8)	19.7 (7.7)	16.0 (8.0)	4.0 (2.0)
	F2	6.1 (0.8)	67.8 (3.1)	15.5 (2.5)	ND[e]	ND
LP	β2m−/−	1.8 (0.6)	44.6 (8.7)	36.2 (4.8)	ND	ND
	C57BL/6	1.6 (0.5)	41.4 (6.0)	43.4 (4.4)	ND	ND
	F2	0.7 (0.4)	67.8 (3.1)[c]	41.0 (7.2)	ND	ND

[a] Surface phenotypes of pooled lymphocytes from reovirus-infected mice were determined by flow cytometric analysis (Materials and Methods). Except where indicated, data represent the means and standard errors for two to eight separate experiments.
[b] Value is significantly greater than C57BL/6 control value ($P < 0.04$) as determined by Student's *t* test.
[c] Value is significantly greater than β2m−/− and C57BL/6 values ($P < 0.05$) as determined by analysis of variance.
[d] Value obtained from one experiment.
[e] ND, not determined.

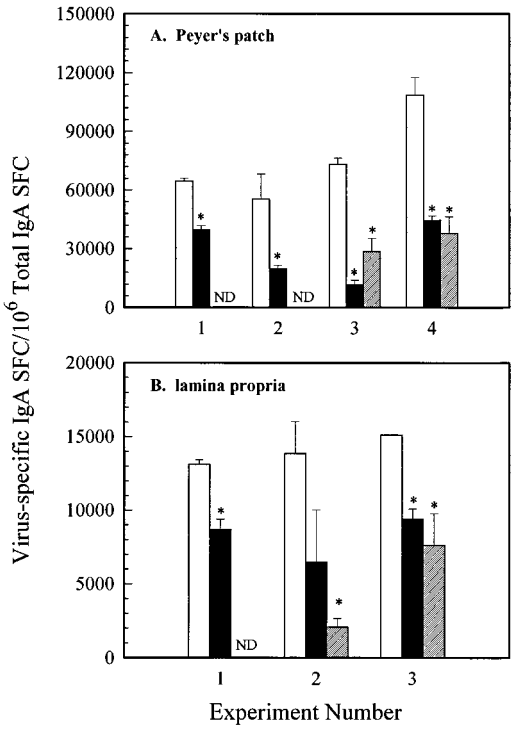

FIG. 2. Frequencies of reovirus-specific IgA SFC per 10^6 total IgA SFC in PP (A) and LP (B) from β2m−/− (open bars), C57BL/6 (closed bars), and F2 (hatched bars) mice. PP and LP lymphocyte pools from three mice per group were assayed by ELISPOT for reovirus-specific IgA-secreting cells 10 days after oral infection with reovirus. PP data represent four separate experiments comparing β2m−/− to C57BL/6 mice in which two experiments include the F2 control. LP data represent three separate experiments comparing β2m−/− to C57BL/6 mice in which two experiments include the F2 control. Bars represent the means and standard deviations for duplicate wells. An asterisk denotes a significantly lower frequency of virus-specific IgA SFC for control mice compared to β2m−/− mice as determined by Student's *t* test (A, experiments 1 and 2; B, experiment 1 [$P < 0.05$]) or analysis of variance (A, experiments 3 and 4; B, experiments 2 and 3 [$P < 0.05$]). In all experiments, there was no difference among β2m−/− and control mice in the frequency of total IgA SFC from both PP and LP (data not shown). ND, not determined.

there may be a higher frequency of reovirus-specific antibody-producing cells in PP and LP in β2m−/− mice. Therefore, we performed ELISPOT assays to determine frequencies of virus-specific antibody-producing cells. PP from β2m−/− mice had a two- to sevenfold higher frequency of virus-specific IgA SFC compared to C57BL/6 and F2 controls (Fig. 2A). In addition, LP lymphocytes from β2m−/− mice had 1.5- to 7-fold higher frequencies of virus-specific IgA SFC compared to controls (Fig. 2B). Similar results were found at day 17 postinfection (data not shown).

Systemic humoral immunity following reovirus infection of β2m−/− and control mice. Because the intestinal immune responses were greater in β2m−/− mice than in controls, we determined whether β2m−/− mice also exhibited augmented systemic virus-specific IgG responses. The frequencies of virus-specific IgG2a- and IgG2b-producing cells in pooled splenocyte populations were determined by ELISPOT 10 days after infection. β2m−/− mice had significantly higher frequencies of both virus-specific IgG2a and IgG2b SFC compared to control mice (Fig. 3). Virus-specific serum antibody concentrations did not correspond to the increased frequency of virus-specific IgG SFC observed in β2m−/− mice. β2m−/− mice had similar concentrations of virus-specific IgG2a (1,096.7 ± 328.7 ng/ml) compared to C57BL/6 (588.3 ± 50.4 ng/ml) and F2 (1,301.0 ± 988.3 ng/ml) controls. Virus-specific IgG2b responses were approximately 10-fold lower in β2m−/− mice (3,783.3 ± 354.4 ng/ml) compared to C57BL/6 mice (33,650.0 ± 6,818.5 ng/ml) but were similar to responses in F2 mice (4,780.0 ± 1,370.9 ng/ml) (Fig. 4).

RT-PCR analysis of cytokine-specific mRNA in PP from reovirus-infected mice. The increased frequency of virus-specific B cells in β2m−/− mice led us to hypothesize that altered cytokine production in β2m−/− mice resulted in enhanced antigen-specific humoral immunity. Relative levels of mRNA for TGF-β, IFN-γ, IL-4, IL-5, and IL-6 were assessed by RT-PCR in PP, the initial site of reovirus infection and initiation of the IgA immune response (14, 64). Specific mRNAs for TGF-β and IL-4 were present 5 and 10 days after infection (Fig. 5 and data not shown). In addition, no mRNA for IFN-γ, IL-5, or IL-6 was detected in PP 5 or 10 days after infection in either mouse strain. Image analysis of scanned gels revealed no significant difference in relative TGF-β and IL-4 mRNA signals between β2m−/− and C57BL/6 mice 5 and 10 days postinfection (data not shown).

Reovirus titers in lymphoid tissue. To determine whether $CD8^+$, α/β TCR^+ T cells are necessary for efficient clearance of replicating reovirus from mucosal and systemic lymphoid tissues, reovirus titers were determined in PP, SI, MLN, and SPL 5 and 10 days after infection. Virus titers in PP revealed that β2m−/− and C57BL/6 mice clear reovirus later than day 10, whereas F2 mice had no measurable virus 10 days after

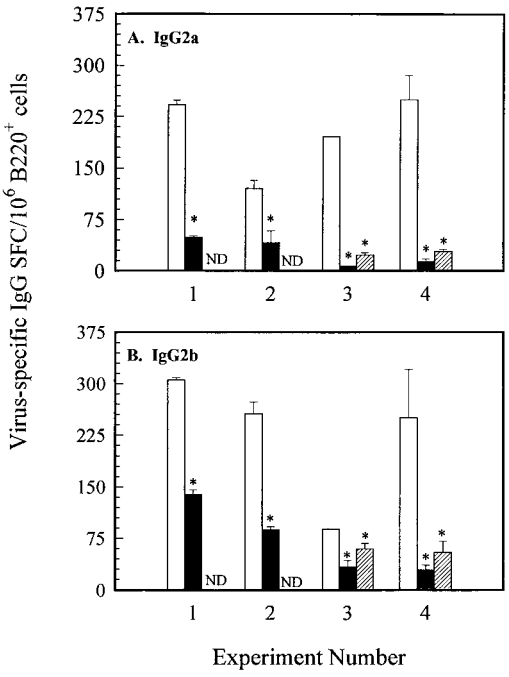

FIG. 3. Frequencies of reovirus-specific IgG2a (A) and IgG2b (B) SFC per 10^6 $B220^+$ cells from β2m−/− (open bars), C57BL/6 (closed bars), and F2 (hatched bars) mice. Spleen lymphocyte pools from three mice per group were assayed by ELISPOT for reovirus-specific IgG-secreting cells 10 days after oral reovirus infection. The data are from four separate experiments comparing β2m−/− to C57BL/6 mice and/or F2 controls. Bars represent the means and standard deviations for duplicate wells. An asterisk denotes a significantly lower frequency of virus-specific IgG SFC for control mice compared to β2m−/− mice as determined by either Student's *t* test (experiments 1 and 2 [$P < 0.05$]) or analysis of variance (experiments 3 and 4 [$P < 0.05$]). In all experiments, there was no difference among β2m−/− and control mice in the percentage or absolute number of $B220^+$ cells in SPL calculated from the data in Table 1. ND, not determined.

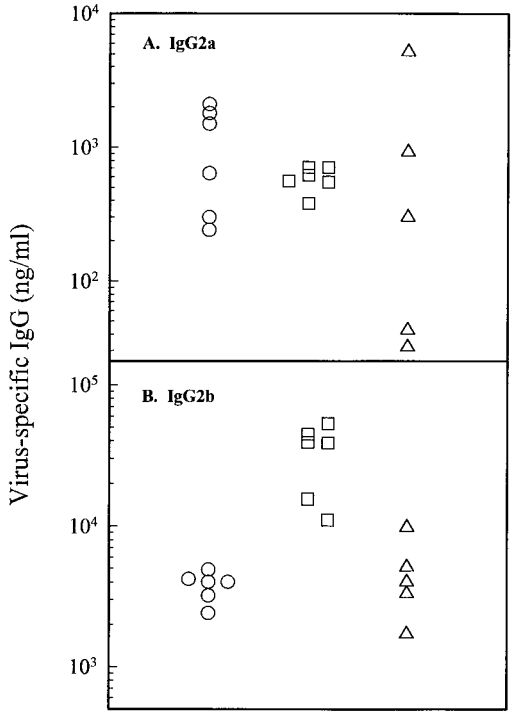

FIG. 4. Reovirus-specific IgG2a (A) and IgG2b (B) in serum from β2m−/− (circles), C57BL/6 (squares), and F2 (triangles) mice. Virus-specific IgG concentrations were determined by ELISA 10 days after oral reovirus infection. Each symbol represents an individual mouse, and data shown are combined from two experiments. Virus-specific antibody concentrations were determined by comparison to the appropriate standard curve as described in Materials and Methods.

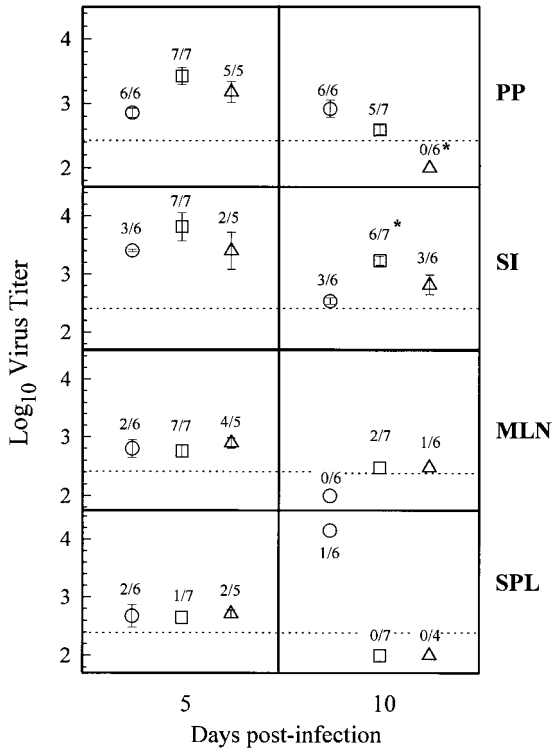

FIG. 6. Reovirus titers in tissues from β2m−/− (circles), C57BL/6 (squares), and F2 (triangles) mice 5 and 10 days after oral infection. Symbols and error bars represent the means and standard errors of positive virus titers as determined by plaque assay for six to seven mice per group. Numbers above symbols denote the numbers of positive animals per total number of animals assayed. The dotted line represents the limit of detection for the plaque assay ($10^{2.48}$ PFU/organ). An asterisk denotes significantly different virus titers as determined by analysis of variance.

infection (Fig. 6). In SI, C57BL/6 mice had significantly higher virus titers ($\log_{10}$ 3.23 ± 0.08) 10 days after infection compared to β2m−/− mice (three of six positive; $\log_{10}$ 2.54 ± 0.06). However, SI virus titers in β2m−/− mice were not different from those in F2 control mice (three of six positive; $\log_{10}$ 2.82 ± 0.17). Both β2m−/− and control mice demonstrated little replicating virus in the MLN and SPL 5 days after infection. Most of the mice cleared virus from these tissues within 10 days after infection.

DISCUSSION

We examined the immune responses to enteric reovirus infection in $CD8^+$, α/β TCR^+ T-cell-deficient β2m−/− mice in order to gain a better understanding of the role of $CD8^+$ T cells in generating or regulating virus-specific intestinal immunity. PP and LP fragment culture supernatants from β2m−/− mice had two- to threefold-higher concentrations of virus-specific IgA, but no difference in total IgA, compared to both C57BL/6 and F2 controls 10 days after infection. The increase in virus-specific IgA was not due to differences in cellularity or B-cell percentages in PP and LP but corresponded to increases in the frequency of reovirus-specific IgA-producing cells per 10^6 total IgA-producing cells in GALT. These data are consistent with other studies that demonstrate increases in antigen-specific IgA in mice lacking $CD8^+$ T cells. Hornquist et al. (27) found that $CD8^+$ T-cell-deficient mice had a higher frequency of KLH-specific IgA-producing cells in LP following oral exposure to KLH and cholera toxin. In addition, Hyland et al. (29) reported that intranasal infection with Sendai virus results in an increase in virus-specific IgA antibody-forming cells in the SPL, mediastinal lymph nodes, and bone marrow from β2m−/− mice. In both of these studies, the authors suggested that the $CD8^+$ T-cell population may regulate the production of antigen-specific IgA (27, 29). The mechanism(s) of such regulation remains to be elucidated.

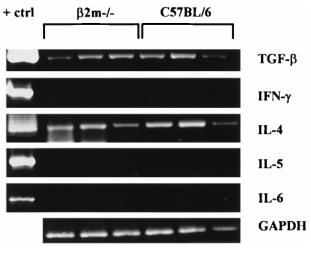

FIG. 5. RT-PCR analysis of cytokine message in PP from β2m−/− and C57BL/6 mice 5 days after infection with 3×10^7 PFU of reovirus type 1, strain Lang. Total RNA was extracted from the PP of three mice per group. Following generation of first-strand cDNA, cytokine and GAPDH messages were amplified by reaction with specific primers as described in Materials and Methods. Amplified DNA was electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide. ctrl, control.

In contrast to those reports and the present study, intranasal influenza virus infection in β2m−/− mice resulted in no enhancement of virus-specific antibody 3 weeks after infection (3). Franco and Greenberg (21) found a delayed appearance of virus-specific IgA in rotavirus-infected β2m−/− mice, but there was no significant difference in rotavirus-specific IgA by

day 10 after infection. Because we did not examine virus-specific IgA prior to day 10, it is unknown whether a kinetic difference in antibody appearance exists in β2m−/− mice following enteric reovirus infection. However, serum IgG appears by 7 days postinfection in both β2m−/− and control mice (data not shown). Therefore, it is possible that the production and/or enhancement of virus-specific IgA is dependent on the type of virus as well as the initial route of infection.

In addition to increased frequencies of virus-specific IgA-producing cells in the GALT, we observed enhanced frequencies of IgG2a and IgG2b SFC in SPL. The frequencies of IgG1-producing cells was not determined because oral reovirus infection in *H-2*^{b} mice does not elicit substantial virus-specific serum IgG1 (41). Reovirus-specific serum antibody responses were not elevated in β2m−/− mice. The significantly elevated IgG2b responses in C57BL/6 mice compared to β2m−/− and F2 mice did not correspond to an increased antibody-forming cell response in SPL. This difference might suggest that other lymphoid tissue such as bone marrow or MLN substantially contribute to the IgG2b serum levels in C57BL/6 mice. Alternatively, strain differences in IgG2b catabolism might account for the increased concentrations of serum antibody in C57BL/6 mice because IgG2b has a shorter serum half-life than other IgG subclasses (51). We did not examine total IgG2b concentrations in these experiments.

Alterations in systemic antibody responses to other virus infections have been found in the absence of CD8$^+$ T cells. Sendai virus infection in β2m−/− mice resulted in increased frequencies of IgG2a antibody-forming cells in mediastinal lymph nodes without a concurrent increase in serum antibody titer (29). Likewise, infection with LCMV resulted in increased numbers of IgG-producing cells in SPL from CD8$^+$ T-cell-deficient mice with no significant difference in serum antibody (2, 36). In contrast to these reports and our study, Spriggs et al. (59) found that intraperitoneal vaccinia virus infection in β2m−/− mice resulted in decreased virus-specific serum IgG responses. Additionally, influenza virus-specific antibody responses are decreased in β2m−/− mice 9 days and 3 weeks after intranasal immunization with recombinant vaccinia virus (3, 19). These latter studies did not report frequencies of virus-specific antibody-producing B cells in lymphoid tissue. It has been hypothesized that serum antibody homeostasis is maintained by recirculating Ig that is bound to an Fc receptor that closely resembled the neonatal Fc receptor (FcRn) and is constitutively expressed in most tissue of the adult mouse (7, 23, 30, 45, 51, 52, 58). Like the MHC class I molecule, the FcRn requires association with β2-microglobulin to be efficiently expressed on the cell surface (58). β2m−/− mice do not have normal expression of the FcRn, and as a consequence, serum IgG concentrations and half-lives are reduced (23, 30). Thus, the lack of parallel increases in virus-specific IgG-producing cells in SPL and virus-specific IgG serum levels in β2m−/− mice may not be due to the absence of MHC class I-restricted CD8$^+$ T cells, rather the lack of β2-microglobulin and appropriate FcRn function. Increases in the frequency of virus-specific IgG-producing cells in the periphery do correspond to increases in IgA responses in GALT. Therefore, if CD8$^+$ T cells regulate mucosal immune responses, they might also regulate systemic immunity.

We found an increase in the percentage of CD4$^+$ T cells in the SPL and PP in β2m−/− mice compared to controls. Additionally, the absolute number of CD4$^+$ T cells was increased in the spleens, reflecting the absence of CD8$^+$ T cells. This increase in CD4$^+$ T cells could account for the increased antibody responses by providing more T-cell help for humoral immunity. This hypothesis would be supported only if there is an increase in the frequency of antigen-specific Th cells in the spleen or other lymphoid tissue. Experiments are in progress to determine the frequency of virus-specific helper cells and mechanisms of T-cell function in β2m−/− mice.

One mechanism by which CD8$^+$ T cells might regulate immune responses is through the production of cytokines (31). Our laboratory (6) and others (35) have found that PP CD8$^+$ T cells make IFN-γ. Although IFN-γ is not thought to be directly involved in IgA responses in GALT, it can down regulate Th2-associated cytokines such as IL-4, IL-5, IL-6, and IL-10, which, together with TGF-β, are hypothesized to be integral in the maturation of IgA-producing B cells (34). Therefore, we originally hypothesized that IFN-γ produced by PP CD8$^+$, α/β TCR$^+$ T cells might serve to inhibit antibody responses in GALT. Thus, we expected to observe decreases in IFN-γ and increases in other IgA-associated cytokines in β2m−/− mice compared to controls. In two separate experiments, RT-PCR analyses revealed that β2m−/− and C57BL/6 mice had similar levels of relative mRNA for TGF-β and IL-4. No mRNA for IL-5, IL-6, or IFN-γ was detected at day 5 or 10 after infection. Amplification of cytokine mRNA has been used to assess cytokine function in GALT (35, 48, 61, 62) even though mRNA levels may not correspond precisely to protein levels. We failed to detect substantial differences in cytokine mRNA for several cytokines that have been implicated in regulating IgA responses in the PP, the initial site of infection by reovirus (64). Analysis of mRNA for other cytokines such as IL-10 or IL-12, or cytokine analysis of other lymphoid tissues, may provide information regarding the mechanism of the enhanced IgA response in CD8$^+$, α/β TCR$^+$ T-cell-deficient mice. Additional studies using cytokine knockout mice or manipulation of cytokine levels would also be useful in defining the role of particular cytokines in the immune response to enteric virus infection.

Although β2m−/− mice are deficient in the ability to generate virus-specific CTL responses (53), they efficiently clear reovirus from GALT, and virus did not disseminate substantially. The F2 control mice appeared to clear virus more rapidly from PP than C57BL/6 and β2m−/− mice. Virus in the PP at day 10 could contribute to the enhanced IgA response in β2m−/− mice by providing prolonged antigen exposure to the PP. This explanation is unlikely because there was no difference between the virus titers in β2m−/− and C57BL/6 mice, yet there was a difference in IgA responses in those mice. Our results are consistent with those of Barkon et al. (1), who examined infection of β2m−/− mice with reovirus clone 9, a strain of serotype 3 reovirus. However, they did not examine viral clearance in PP and intestines separately. In addition, we examined virus titers in MLN and SPL, which are likely the sites of priming of the systemic antibody response to oral reovirus infection (41). Barkon et al. (1) cautioned against assuming that virus-specific CD8$^+$ precursor CTL are not important in protection against reovirus and suggested that compensation of the immune response could be responsible for efficient clearance in these mutant mice. Our data support this conclusion and suggest that increased production of virus-specific IgA may allow β2m−/− mice to clear reovirus at rates similar to those of control mice. In addition, β2m−/− mice develop MHC class II-restricted CD4$^+$ CTL in pulmonary lymphoid tissue following infection with influenza virus (3, 17) and Sendai virus (28) and in SPL following LCMV infection (47). Whether CD4$^+$ CTL develop in GALT as a compensatory mechanism in β2m−/− mice following reovirus infection is not yet known. However, it is conceivable that such T cells do develop and may function to clear virus from infected tissues.

In addition to CD4$^+$ CTL, it is possible that CD8$^+$, γ/δ

TCR^+ T cells, which make up the majority of the intraepithelial lymphocytes in β2m−/− mice (13), kill reovirus-infected cells or provide increased help for antibody responses. Because γ/δ TCR^+ T-cell-deficient mice have decreased IgA responses to tetanus toxoid and cholera toxin (22), it is thought that γ/δ TCR^+ intraepithelial cells may enhance IgA immune responses. An increase in the relative frequency of γ/δ TCR^+ intraepithelial cells might provide additional T-cell help to amplify the virus-specific antibody response in β2m−/− mice. However, it should be again noted that although we observe increases in virus-specific IgA-producing cells, the numbers of total IgA-producing cells are the same in β2m−/− and control mice.

Various hypotheses have been proposed to explain the mechanism(s) by which $CD8^+$ T cells regulate antigen-specific humoral immunity. $CD8^+$ CTL may down regulate antibody responses during viral infection by lysing antigen-presenting cells or antibody-producing B cells (2). Reovirus-specific $CD8^+$ CTL may lyse virus-infected cells, thus reducing viral load and decreasing the activation of Th and B cells. Because no difference in virus titers was observed among β2m−/− mice and control animals, increases in virus-infected cells would not explain augmented IgA responses in mutant mice. Inefficient clearance of viral antigen could result in prolonged activation of virus-specific Th cells in β2m−/− mice, which may then enhance virus-specific B-cell responses. Alternatively, $CD8^+$ T cells have been described as suppressors of immune responses (31, 63). Harriman et al. (26) demonstrated that KLH-specific Th cells from LP could be expanded only if $CD8^+$ T cells were first depleted, suggesting that $CD8^+$ T cells from GALT inhibit Th cell proliferation. We are currently conducting studies to examine the immunoregulatory effects of GALT $CD8^+$ T cells on MHC class II expression, antigen presentation, and the frequency of virus-specific Th cells.

In conclusion, intestinal reovirus infection induces enhanced virus-specific mucosal IgA and systemic IgG responses in β2m−/− mice compared to control animals. These enhanced immune responses are not directly related to differences in the absolute number of B cells in lymphoid tissue and are not due to differences in TGF-β, IFN-γ, IL-4, IL-5, or IL-6 production in PP. Collectively, these data support the hypothesis that MHC class I-restricted $CD8^+$, α/β TCR^+ T cells can act as immunoregulatory cells of humoral immune responses during enteric infection.

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