Effects of the Route of Infection on Immunoglobulin G Subclasses and Specificity of the Reovirus-Specific Humoral Immune Response

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Reovirus serotype 1, strain Lang (T1/L), a well characterized enteric virus, elicits humoral and cellular immune responses in mice. Although orally and intradermally induced infections generate comparable reovirus-specific serum antibody titers, little is known about the effects of the route of infection on the systemic immunoglobulin G (IgG) response. To assess whether the route of exposure affects virus-specific humoral immunity, we infected various strains of mice with reovirus T1/L by the oral or intradermal routes. At day 10 following infection, virus-specific serum antibody titers and IgG subclasses were determined by enzyme-linked immunosorbent assay. Serum IgG2a and IgG2b antibodies were detected in all mouse strains independent of the route of infection. Mice of the $H\text{-}2^d$ haplotype that received an intradermal infection also had high levels of reovirus-specific serum IgG1. This dichotomy of responses was not associated with differences in the types of cytokine produced by draining peripheral lymph nodes. However, peripheral lymph node lymphocytes from C3H mice produced significantly higher levels of gamma interferon than did BALB/c, C57BL/6, and B10.D2 mice. Additionally, peripheral lymph node lymphocytes from all strains of mice produced only low levels of interleukin-5, with no detectable level of interleukin-4 or interleukin-6. Analysis of specific antibody at inductive sites of the immune response showed that orally infected Peyer's patches produced predominately IgA and intradermally infected peripheral lymph nodes produced predominately IgG2a. Western blot (immunoblot) analysis showed that virus-specific IgA, IgG1, and IgG2a reacted with reovirus structural proteins. These data suggest that the route of infection affects the isotype and IgG subclasses, but not the antigen specificity, of the local antibody response. In addition, virus-specific IgG1 generated following an intradermally induced infection is linked to the $H\text{-}2^d$ major histocompatibility complex haplotype.

Mucosal administration of antigen can elicit both local and systemic antibody responses. These humoral responses are typically characterized by the appearance of immunoglobulin A (IgA) in secretions and IgG in serum and require the presence of T helper (Th) cells. Th cells have been classified into two groups, Th1 and Th2 (44). Th2 cells secrete interleukin-4 (IL-4), IL-5, IL-6, and IL-10 which are associated with the production of IgG1, IgE, and IgA (60). Th1 cells produce gamma interferon (IFN-γ), IL-2, and tumor necrosis factor β. These factors are important for macrophage and cytotoxic T-lymphocyte activation as well as for isotype class switching to the IgG2a subclass (19, 57). It has been shown that in some disease models, such as leishmaniasis, protection may depend on the generation of the appropriate Th cell response (54). Therefore, understanding the mechanisms by which mucosal priming influences Th cell responses would aid in the development of oral vaccines that could generate the more advantageous immune response.

Humoral immune responses to viruses inhibit viral spread at the site of infection and provide immunological memory for the prevention of subsequent reinfections. Antibody responses to viruses, which are often generated against structural proteins, serve to neutralize virus and possibly mediate virus inactivation by complement or antibody-dependent cellular cytotoxicity (69). Since antibodies differ in their ability to mediate such responses, production of virus-specific IgG2a

during virus infections could prove advantageous because IgG2a is very efficient at fixing complement and mediating antibody-dependent cellular cytotoxicity (23, 28). In fact, Coutelier et al. (8, 9) demonstrated that many systemic viral infections in mice result in preferential increases in virus-specific IgG2a in serum. This preferential class switch was shown by Nguyen et al. (45) to be dependent on viral replication and is attributed in part to virus-induced production of IFN-γ. Additionally, monoclonal IgG2a that was specific for herpes simplex virus glycoprotein D was shown to be more effective at protecting infected mice than IgG1 of the same specificity (24). The mechanism for this enhanced activity has not been established.

Oral infection with enteric viruses, such as poliovirus and rotavirus, results in the production of virus-specific IgA in addition to serum IgG (41). IgA is anti-viral by a variety of mechanisms including aggregating virus, blocking adherence, and preventing attached virus from infecting cells (1, 30, 46–48). In addition, IgA may neutralize virus intracellularly (26, 32, 61). Therefore, both IgG and IgA antibody responses to enteric virus may contribute to resolution of infection and prevention of reinfection.

Respiratory enteric orphan virus (reovirus) is a nonenveloped, double-stranded RNA virus that naturally infects the host through the mucosal surface of the gastrointestinal tract. Intestinal reovirus infection induces a local IgA response in the gut as well as virus-specific precursor cytotoxic T lymphocytes from Peyer's patches (PP) and intraepithelial lymphocytes (4, 33, 35–37). Additionally, systemic reovirus infection elicits virus-specific precursor cytotoxic T lymphocytes and virus-specific serum IgG (52). In animal models of infection, neonatal mice and severe combined immunodeficient mice are suscep-

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TABLE 1. Specificity of IgG subclass-specific antibodies used in ELISAs

Specificity of secondary antibody	OD_{405}[a]		
	IgG1	IgG2a	IgG2b
IgG1	1.83	<0.01	0.02
IgG2a	0.03	1.75	0.06
IgG2b	0.06	<0.01	0.96

[a] ELISA plates were coated with 0.5 µg of goat anti-IgG (heavy and light chain specific) per ml. One hundred microliters of purified IgG1, IgG2a, or IgG2b at a concentration of 100 ng/ml was incubated as described in Materials and Methods. Reactions were developed with biotinylated goat anti-IgG1, anti-IgG2a, or anti-IgG2b. Values represent average OD values for duplicate samples.

tible to the lethal effects of reovirus infections. Protection of susceptible mice may be achieved by passive transfer of reovirus-specific immune serum (11), monoclonal antibodies (2, 62, 63), or immune lymphocytes (11, 21, 65). Furthermore, reovirus-immune dams confer passive immunity to neonates, possibly through antibodies transferred across the placenta and in milk secretions (11, 12). Some protective antibodies are serotype cross-reactive (12, 62) and inhibition of infection is believed to occur at three major steps in virus replication: (i) attachment of the virus to cells, (ii) internalization of the virus, and (iii) viral uncoating (64).

In this report, we examine the effects of the route of immunization on the systemic immune response to reovirus. Our data suggest that although the route of infection does not appear to affect the specificity of the humoral response, it does influence the production of virus-specific serum antibody IgG subclasses. The difference in serum antibody responses is characterized by the appearance of virus-specific IgG1 following intradermally induced infection. The serum IgG1 response is genetically linked to the H-2^d haplotype of the major histocompatibility complex (MHC). The results from this study suggest a possible regulatory role of the mucosal immune system on systemic immunity.

MATERIALS AND METHODS

Animals. Male BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were purchased from Charles River (Wilmington, Mass.). Male C3HeB/FeJ (H-2^k), B10.D2 (H-2^d), and DBA/2 (H-2^d) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). Unless infected with reovirus, mice were housed in specific pathogen-free conditions. All mice were used at 6 to 8 weeks of age.

Virus purification. Third-passage CsCl-gradient-purified reovirus T1/L was used in all experiments. Virus 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 (HyClone, Logan, Utah), 2 mM L-glutamine (Sigma, St. Louis, Mo.), 100 U of penicillin per ml, 0.1 µg of streptomycin per ml, and 10 µg of gentamicin (all from Whittaker Bioproducts) per ml. Virus was purified from L-cell lysates by extraction with 1,1,2-trichloro-1,2,2-trifluoroethane followed by discontinuous CsCl gradient centrifugation as previously described (51). Infectious virus titers were determined by plaque assay as previously described (12).

Animal infections. Reovirus T1/L was suspended in borate-buffered saline (pH 7.4) containing 2% gelatin (hereafter referred to as gel saline) at a concentration of 6×10^7 PFU/ml. Mice were orally infected with 3×10^6 PFU of reovirus T1/L suspended in 50 µl of gel saline with an oral feeding tube. Mice infected intradermally were given a total dose of 3×10^6 PFU of reovirus T1/L divided among four sites: each rear footpad and two sites at the base of the tail. Unless otherwise stated, mice were sacrificed at day 10 following infection.

Lymphoid tissue fragment cultures. Ten days after infection, fragment cultures of PP, mesenteric lymph nodes (MLN), and/or peripheral lymph nodes (PLN) were established by a modification of the methods of Logan et al. (34). Tissues were washed five times in RPMI 1640 (Whittaker Bioproducts) supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and antibiotics. Lymphoid tissues from each mouse were cut into halves with a sterile scalpel blade and cultured in 2 ml of tissue culture medium, which consisted of RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. Cultures were incubated at 37°C in 5% CO_2 for 5 days without further restimulation. Following incubation, the supernatants were collected, and reovirus-

specific antibody titers were determined by enzyme-linked immunosorbent assay (ELISA).

PLN culture supernatants were obtained for cytokine analysis by removing PLN from mice intradermally infected 7 days previously with reovirus T1/L. Cells were disassociated from PLN into single-cell suspensions by expressing each lymph node through a nylon mesh screen. Lymphocytes were washed three times in tissue culture medium and cultured at a concentration of 10^7 cells per ml in 1 ml of tissue culture medium. Cultures were incubated for 48 h without further restimulation, and cytokine concentrations were measured by ELISA.

ELISA for reovirus-specific antibody. Each well of 96-well immunosorbent plates (Corning Costar, Cambridge, Mass.) was coated overnight at 4°C with 2.5×10^9 particles of reovirus T1/L in 50 µl of 0.1 M $NaHCO_3$ (pH 8.0). Plates were washed twice with 200 µl of phosphate-buffered saline (PBS) supplemented with 0.5% Tween 20 (PBS-T) per well and blocked with 200 µl of 3% (wt/vol) fraction V bovine serum albumin (BSA; Sigma) per well for 2 h at room temperature. One hundred microliters of twofold serial dilutions of serum was plated in duplicate at a starting dilution of 1:50 and incubated overnight at 4°C. Plates were reacted with 100 µl of purified biotinylated goat antibody specific for murine Ig (heavy and light chain specific), IgG1, IgG2a or IgG2b (all from Southern Biotechnology, Birmingham, Ala.) at a final concentration of 0.5 µg/ml in PBS supplemented with 10% bovine calf serum. Plates were incubated for 45 min at room temperature. Following six washes in PBS-T, 0.25 µg of avidin-conjugated peroxidase (Sigma) per well was added, and the mixture was incubated for 30 min at room temperature. After eight washes with PBS-T, 100 µl of 2,2'-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid (ABTS; Sigma) was added to each well, and color development was allowed to occur for 10 to 15 min. The plates were then read with an ELISA reader at 405 nm. The results are expressed as the mean $\log_{10}$ antibody titers and were determined to be the last serum dilution that yielded an optical density (OD) of ≥0.200. Titration curves of known positive samples were found to be linear from approximately 1.0 to 0.1 OD unit. The specificities of the antibodies used in these experiments are shown in Table 1.

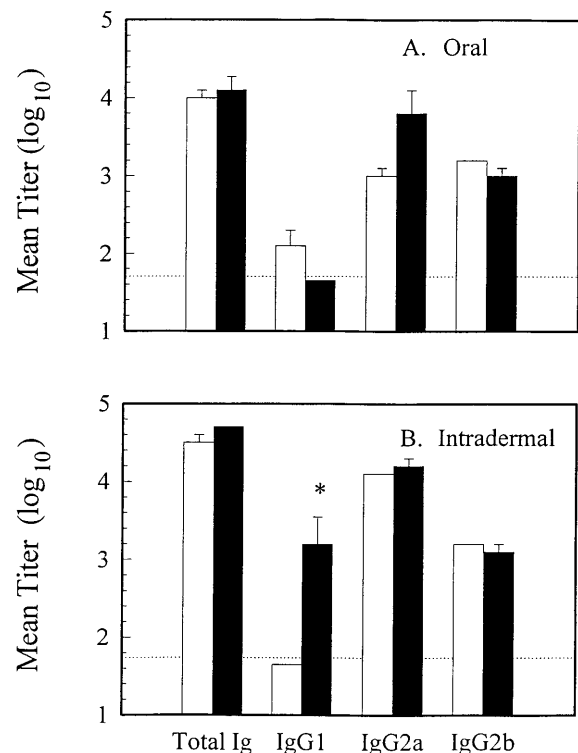Quantitation of reovirus-specific IgA, IgG1, or IgG2a concentrations in cul-

FIG. 1. Geometric mean titers of reovirus-specific IgG subclasses in serum from groups of four or five male C3H (H-2^k) (open bars) and BALB/c (H-2^d) (closed bars) mice 10 days after either oral (A) or intradermal (B) infection with 3×10^6 PFU of reovirus T1/L. Serum IgG subclasses were determined by ELISA, and specific antibody titers were determined as the last dilution of serum that yielded an OD reading of ≥0.200. The dotted lines represent the limit of detection. Error bars represent the standard errors among mice. The results represent data from one of two separate experiments that yielded similar results. An asterisk denotes a significant difference ($P < 0.05$) between C3H and BALB/c virus-specific IgG1 titers as determined by Student's t test.
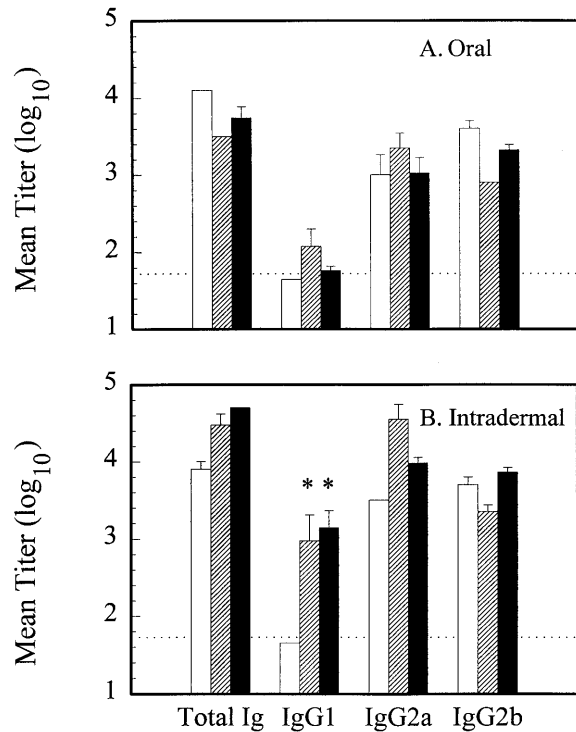
FIG. 2. Geometric mean titers of reovirus-specific IgG subclasses in serum from groups of four or five male C57BL/6 (H-2^b) (open bars), B10.D2 (H-2^d) (hatched bars), and DBA/2 (H-2^d) (closed bars) mice 10 days after either oral (A) or intradermal (B) infection with 3×10^6 PFU of reovirus. Serum IgG subclasses were determined by ELISA, and specific antibody titers were determined as the last dilution of serum that yielded an OD reading of ≥ 0.200. The dotted lines represent the limit of detection. Error bars represent the standard errors among mice. Data are representative of six experiments with various combinations of mouse strains and routes of infection. The responses in footpad-infected C57BL/6 mice and B10.D2 mice were compared in three separate experiments, with three or four mice per group in each experiment. An asterisk denotes a significantly higher ($P < 0.05$) virus-specific IgG1 titer, as determined by analysis of variance, for DBA/2 and B10.D2 mice compared with that for C57BL/6 mice.

specific for mouse IgA, IgG1, or IgG2a (Southern Biotechnology). Reovirus-specific antibody concentrations were determined by direct comparison to the appropriate standard titration curve.

Cytokine analysis in culture supernatants from PLN lymphocytes. Cytokine concentrations in the lymph node fragment cultures were measured by coating ELISA plates with either rat anti-mouse IL-4, IL-5, IL-6, or IFN-γ (all from PharMingen, San Diego, Calif.) in 0.1 M NaHCO$_3$ overnight at 4°C. Culture supernatants were added to sample wells, and the appropriate purified murine cytokine was added to the standard wells and incubated overnight at 4°C. Plates were then incubated for 45 min at room temperature with the biotinylated antibody specific for murine IL-4, IL-5, IL-6, or IFN-γ (all from PharMingen). Avidin-conjugated peroxidase was added to each well, and the mixtures were incubated for 30 min at room temperature. ABTS (100 µl) was added to each well, and color development was allowed to occur for 10 to 30 min. Quantitation of each cytokine was done by comparison to the appropriate standard curve. For the quantitation of IL-4 and IL-5 in culture supernatants, enhanced ELISAs were performed by using the ELAST system (New England Nuclear/Dupont, Boston, Mass.). Following the addition of avidin-conjugated peroxidase, biotinylated tyramide was diluted 5 µl/ml in the amplification diluent (provided by the kit), and 100 µl was added to each well. The plates were incubated for 10 min at room temperature and were subsequently washed six times with PBS-T. After the plates were washed, 100 µl of a 1:500 dilution (in PBS-T–1% BSA) of streptavidin-conjugated horseradish peroxidase was added to each well, and the wells were incubated for 20 min at room temperature. The plates were washed eight times with PBS-T, and color was visualized as described above. This assay increased the sensitivity of the cytokine ELISAs approximately fivefold.

Western blots of reovirus-specific antibodies. Gradient-purified reovirus was disrupted by being boiled for 5 min in sample buffer (0.5 M Tris-Cl, 2% β2-mercaptoethanol, 0.1% bromophenol blue, 20% glycerol, 4% sodium dodecyl sulfate [SDS]). Viral proteins were separated by electrophoresis on a 4 to 20% gradient polyacrylamide gel, and reovirus proteins were transferred overnight to a 0.2-µm-pore-size nitrocellulose membrane (Costar, Cambridge, Mass.). Following transfer, the membrane was cut into strips and blocked with 3% BSA in 100 mM Tris-buffered saline supplemented with 0.1% (vol/vol) Tween-20 for 3 h at room temperature. The blots were then reacted overnight at 4°C with supernatant from gut-associated lymphoid tissue (GALT) and PLN fragment cultures. The blots were washed and incubated for 45 min at room temperature with either alkaline-phosphatase-conjugated goat anti-mouse IgA, IgG1, or IgG2a (all diluted 1:1,000 [Southern Biotechnology]). Antigen-antibody interactions were visualized by reacting the blots with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (Sigma) until bands were visible. Dot blots with purified antibodies as substrates demonstrated that the alkaline phosphatase-conjugated developing antibodies were highly specific for the appropriate Ig isotype or subclass. Furthermore, the conjugated antibodies did not react with reovirus by Western blot.

RESULTS

Assessment of reovirus-specific serum antibody response following intestinal or intradermal infections. To determine the effects of the route of infection on virus-specific serum antibody, groups of four or five male C3HeB/FeJ (C3H) and BALB/c mice were orally or intradermally infected with reovirus T1/L. Ten days after infection, titers of virus-specific

ture supernatants or serum was determined by assaying fluids as described above, except that the wells used to generate standard curves were coated with 1 µg of purified goat anti-mouse Ig (heavy and light chain specific; Southern Biotechnology) per ml. Known concentrations of purified murine IgA, IgG1, or IgG2a (Southern Biotechnology) were added to these wells. Antibody concentrations were determined by reacting plates with purified biotinylated goat antibodies

TABLE 2. Cytokine production by lymphocytes following intradermal infection with reovirus

Source of lymphocytes	Amt of cytokine produced (pg/ml)							
	IFN-γ		IL-4		IL-5		IL-6	
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
Lymph node[a]								
C3HeB/FeJ	11,608 (2,775)*	29,882 (13,651)*	<16.0	<16.0	<8.0	8.9 (0.7)	<160.0	<160.0
BALB/c	1,548 (215)	3,836 (620)	<16.0	<16.0	<8.0	11.2 (1.1)	<160.0	<160.0
C57BL/6	2,085 (219)	2,433 (483)	<16.0	<16.0	17.6 (2.8)	11.0 (2.9)	<160.0	<160.0
B10.D2	<625	1,375 (258)	<16.0	<16.0	<8.0	9.8 (1.1)	<160.0	<160.0
Spleen[b]	15,000		57.9		12.3		<160.0	
PLN[c]	30,000		<16.0		20.0		352.0	

[a] Groups of four or five male mice were infected in each hind footpad and on each side of the base of the tail with 3×10^6 total PFU of reovirus T1/L. Seven days after infection, popliteal and inguinal lymph node lymphocytes were pooled and cultured without further restimulation. After 72 h of culture, the supernatants were tested for cytokines by specific ELISA as described in Materials and Methods. An asterisk denotes a P value of <0.05 as determined by analysis of variance. Standard deviations are indicated in parentheses.

[b] Spleen lymphocytes were stimulated with 1 µg of anti-CD3 antibody per ml for 24 h.

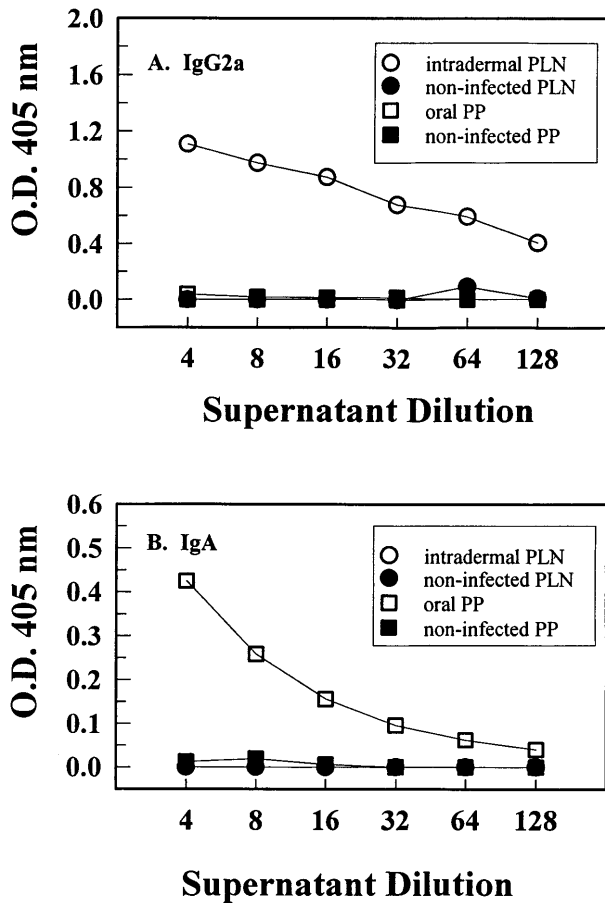[c] PLN lymphocytes were stimulated with 5 µg of concanavalin A per ml for 24 h.

FIG. 3. Reovirus-specific antibody in PLN and PP fragment culture supernatants. The levels of reovirus-specific IgG2a (A) and IgA (B) in PP culture supernatants from orally infected and noninfected mice and in PLN culture supernatants from intradermally infected and noninfected mice were determined by ELISA. Each line represents the mean OD value for a group of cultures for six individual mice.

serum antibody and IgG subclasses were determined by ELISA. All infected mice produced high titers of reovirus-specific IgG as illustrated by the mean titers of total antibody ranging from 1:10,000 to 1:40,000 (Fig. 1). The serum antibody response from orally infected mice was predominately of the IgG2a and IgG2b subclasses, with little production of IgG1 above background levels (Fig. 1A). Intradermally infected mice also produced reovirus-specific IgG2a and IgG2b, but only BALB/c mice produced high titers of serum IgG1 (Fig. 1B).

To determine whether the serum IgG1 response observed in BALB/c mice following an intradermal infection was linked to the H-2^d MHC haplotype, groups of four or five male C57BL/6, DBA/2, and congenic B10.D2 mice were infected orally or intradermally with reovirus T1/L. Ten days after infection, all mice produced virus-specific IgG2a and IgG2b independent of the route of infection (titers ranging from 1:800 to 1:40,000 [Fig. 2]). However, only H-2^d mouse strains produce high levels of serum IgG1 following intradermally induced infection (Fig. 2B). In a separate experiment, the concentrations of virus-specific IgG1 in serum from B10.D2 (H-2^d) and C57BL/6 (H-2^b) mice that were intradermally infected with reovirus 10 days previously were quantitated. The mean ($\pm$ standard errors) of samples from four individual mice per group were found to be 70.2 ± 40.2 and 4.2 ± 3.6 ng/ml, respectively.

Cytokine production by draining PLN lymphocytes following intradermal challenge with reovirus T1/L. Because differences in antibody isotype or subclass may be dependent on the differential production of Th cell-derived cytokines, we assessed the cytokines that were produced in fragment cultures of draining PLN lymphocytes from C3H, BALB/c, C57BL/6, and B10.D2 mice following intradermal reovirus infection. Table 2 shows that, compared with the results for the other three mouse strains, day 7 PLN culture supernatants from C3H mice contain significantly higher levels of IFN-γ. All draining PLN culture supernatants contained low but detectable levels of IL-5 in one of two experiments, with no measurable IL-4 and IL-6.

Reovirus-specific antibody responses in PP and PLN fragment cultures following oral or intradermal infections. To characterize the local immune responses to infection, groups of four or five male C3H mice were orally or intradermally infected with reovirus T1/L. Ten days following infection, cultures that contained the draining PLN from intradermally infected mice or PP from orally infected mice were established. Tissues were cultured for 5 days without further restimulation, and culture supernatants were then tested for reovirus-specific IgA and IgG2a by ELISA. All orally infected mice showed a significant reovirus-specific local IgA immune response in PP but produced little or no IgG2a (Fig. 3). Reovirus-specific antibody in PLN culture supernatants from intradermally infected mice contained high levels of virus-specific IgG2a, with a supernatant titer of 1:250 (Fig. 3A), and no virus-specific IgA (supernatant titer of <1:4 [Fig. 3B]).

In order to identify the site of initiation for the serum antibody response following an infection by the oral route, mice were orally infected with 3×10^6 PFU of reovirus T1/L. Ten days after infection, PP and MLN were cultured for an additional 5 days, and the concentrations of reovirus-specific IgA and IgG2a in the supernatants were determined (Fig. 4). We observed that while PP cultures produced only IgA (124.4 ng/ml), MLN produced both IgA (69.2 ng/ml) and IgG2a (8.8 ng/ml).

Reovirus-specific IgA and IgG recognize similar reovirus proteins. To determine whether reovirus-specific IgA and reovirus-specific IgG recognize similar viral proteins, groups of four male BALB/c mice were infected either orally or intradermally with reovirus T1/L. Fragments of GALT and PLN from orally or intradermally infected mice were cultured for 5 days without further restimulation. Western blotting was done with culture supernatants from GALT or PLN as a source of reovirus-specific antibodies. The blots were reacted with either goat anti-mouse IgA or goat anti-mouse IgG2a. The results from these experiments demonstrate that both reovirus-specific IgG (Fig. 5, lane 1) and IgA (Fig. 5, lane 2) recognize similar reovirus proteins. Reovirus-specific IgG and IgA recognized the λ core proteins (142 kDa), the outer capsid protein μ1c (72 kDa), the viral hemagglutinin σ1 (49 kDa), and the σ3 outer capsid protein (41 kDa). We also examined the reactivities of the virus-specific IgG1 and IgG2a in serum from a B10.D2 mouse intradermally infected with reovirus. Both IgG1 and IgG2a appeared to recognize the same proteins (Fig. 5, lanes 6 and 7).

DISCUSSION

It has long been known that oral immunization elicits IgA responses in mucosa-associated lymphoid tissue. In addition, several pathogens including rotavirus (3, 18), poliovirus (40), *Shigella* spp. (25), *Salmonella* spp. (5), and *Schistosoma mansoni* (16, 31) and antigens such as cholera toxin (22) have been
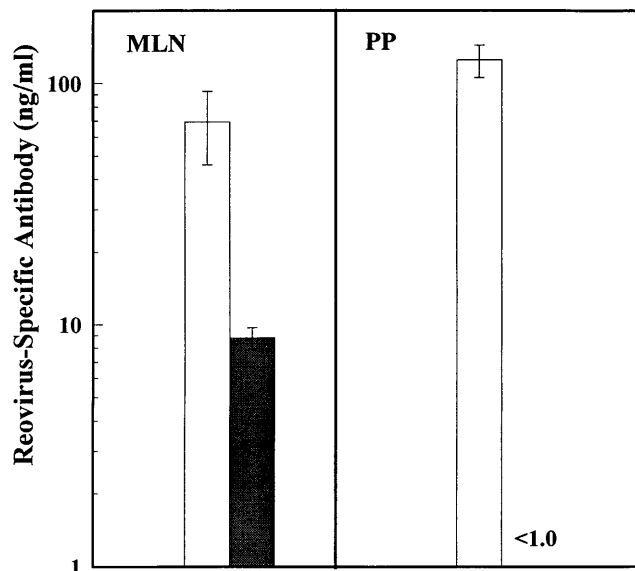
FIG. 4. Reovirus-specific IgA and IgG2a in MLN and PP culture supernatants from DBA/2 mice orally infected with 3×10^6 PFU of reovirus T1/L. Ten days after infection, PP and MLN were removed and cultured for an additional 5 days without further restimulation. Reovirus-specific IgA (open bars) and IgG2a (closed bar) concentrations were determined by specific ELISA as described in Materials and Methods. Error bars represent the standard errors of the means among four individual mice.

shown to induce serum IgG as well as local IgA following oral exposure. However, little is known about whether the route of infection or immunization affects the quality or the specificity of the humoral immune response or the ultimate function of Th cells. To answer these questions, we compared humoral immune responses following oral and intradermal reovirus T1/L infection.

Analysis of serum IgG subclasses showed that regardless of the route of infection, reovirus-specific antibody responses were predominately of the IgG2a and IgG2b subclasses. This was true for all strains of mice that we tested (Fig. 1 and 2). The presence of IgG2a in serum following intradermal reovirus infection is consistent with the results of studies conducted by Coutelier et al. (8, 9) and Nguyen et al. (45), who demonstrated that serum antibody responses to many viral infections (including infection with reovirus serotype 3, strain Dearing) is mainly of the IgG2a subclass. Our data show that an oral infection with reovirus, which initially primes the mucosal immune system, likewise results in the development of serum IgG2a. IFN-γ has been shown to induce B lymphocytes to IgG2a-producing cells. Although IFN-γ is made by other cells types, such as NK cells (49) and CD8$^+$ T cells (15), this cytokine is generally associated with Th1 cell responses (44). Irrespective of the cell source of IFN-γ, the appearance of IgG2a suggests that reovirus given systemically or orally elicits systemic Th1-associated antibody responses in serum.

We found virus-specific IgG2a in MLN fragment culture supernatants, but none was detected in the PP, a primary site for the priming of the IgA response (Fig. 4) (10). The MLN drain the GALT and are the first lymphoid tissue encountered by lymphocytes leaving the gut and entering the periphery. Therefore, the MLN is a potential site of initiation of the serum IgG2a antibody response. However, it is likely that other lymphoid sites such as the spleen and bone marrow contain specific antibody-secreting cells that contribute to the IgG levels in serum after the initiation of the response. We have found

virus-specific IgG2a-producing B cells in the spleens of enterically infected mice at 10 days postinfection (unpublished observation). It is not clear whether these cells were activated in the spleen or migrated from a distant site such as the MLN. Detailed kinetic studies of the frequencies of virus-specific IgG2a-secreting cells may provide the answer (56).

Although we found serum IgG2a in all mice tested, some strains of mice also mounted a serum IgG1 response following intradermal reovirus infection. The appearance of serum IgG1 in BALB/c, DBA/2, and congenic B10.D2 strains of mice suggests a genetic link between the production of virus-specific IgG1 and the H-2^d MHC haplotype. Because both IgG2a and IgG1 are found in serum from systemically infected H-2^d mice, the reovirus-specific antibody responses in these strains appears to be driven by both Th1 and Th2 cells. This conclusion, however, is not supported by any apparent dichotomy in cytokine production by draining PLN lymphocytes since all mouse strains produced IFN-γ and low levels of IL-5 but no IL-4 or IL-6 (Table 2). However, C3H mice, which produce essentially no serum IgG1 following systemic infection, appear to produce significantly higher levels of IFN-γ than do BALB/c, C57BL/6, and B10.D2 mice. It has been suggested by Mo et al. (43) that the antigen load can influence the production of IFN-γ by different mouse strains. The investigators demonstrated that following infection with Sendai virus, 129/SvJ mice produce higher levels of IFN-γ than does the C57BL/6J strain. However, these cytokine differences had no effect on the quality of antibody isotypes and were attributed to genotype differences as opposed to the MHC haplotype. Nonetheless, the increased IFN-γ production in the C3H mice observed in our experiments following reovirus infection may negatively regulate the IgG1 responses following intradermal infection and is a possible explanation for the absence of this antibody isotype in these mice. Additionally, the ability of H-2^d mice to produce serum IgG1 following an intradermal reovirus infection may reflect their ability to present antigen differently in association with one or more MHC molecules. Such differences in virus-specific Th cell responses have been observed by Milich et al. (42), who demonstrated that the generation of different antibody responses following hepatitis B virus infection was dependent on the strain of mouse. The investigators contended that the strain differences in T-cell help was due to recognition of
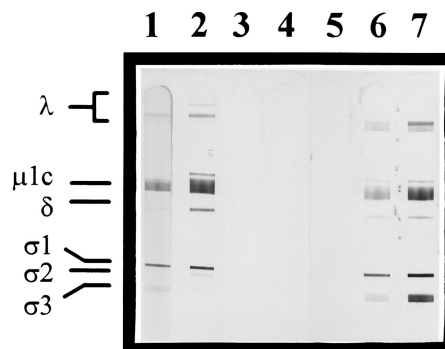

FIG. 5. Western blot analysis of reovirus-specific antibodies. Reovirus proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Lanes: 1, reovirus-specific IgA in GALT fragment culture supernatant from an orally infected mouse; 2, reovirus-specific IgG2a in PLN fragment culture supernatant from a footpad-infected mouse; 3 to 5, negative controls for nonspecific binding of the biotinylated goat-anti mouse IgA, IgG2a, and IgG1, respectively; 6 and 7, reovirus-specific IgG2a and IgG1, respectively, in serum from a footpad-infected B10.D2 mouse. The positions of the proteins are indicated on the left.

different hepatitis B virus epitopes. This response may also be influenced by the infecting virus, since systemic infections of BALB/c mice with poliovirus (38) or recombinant vaccinia virus (39) do not appear to elicit a significant serum IgG1 response.

The failure to detect production of IL-4, an IgG1 switch factor, in mouse strains that produce an IgG1 response was surprising. However, studies with IL-4-depleted (20) and IL-4 knockout (29) mice have shown that this cytokine is sufficient but not necessary for the production of IgG1. It is alternatively possible that IL-4 was produced at sites other than the PLN, although this seems unlikely since virus-specific IgG1 can be detected in culture supernatants of PLN cultures from $H\text{-}2^d$ mice by day 7 following infection (data not shown).

Interestingly, we found that the serum antibody Th2-like response, characterized by the appearance of IgG1, is absent in $H\text{-}2^d$ mice following oral reovirus infection. Several possibilities may explain this influence of the route of infection on the virus-specific IgG1 antibody response. First, the IgG1 response may be dependent on levels of viral antigenemia, which may be low following oral infection. Second, oral and intradermal reovirus infections may lead to the stimulation of different populations of antigen-presenting cells. Several studies have shown that the phenotype of antigen-presenting cells can affect isotype class switching both in vitro (13) and in vivo (14). Finally, oral infection initially primes the GALT; thus, immune responses in the gut may affect systemic immune responses. This could be due to influences of the microenvironment (66) or to differences in antigen presentation by local denritic cells (53, 58, 59) or M cells, specialized epithelial cells which cover the PP (17).

Fragment cultures of PP tissue have been demonstrated to be a source of viable IgA-secreting plasma cells (34) and have been used to assess humoral immune responses to enteric viruses (6, 27). However, fragment cultures have not been previously used to examine IgG subclasses or the specificity of the antiviral responses following different routes of exposure. As might be expected, results from PP and PLN fragment cultures show that PP from orally infected mice produce mostly reovirus-specific IgA whereas PLN from intradermally infected mice produce both virus-specific IgA and IgG2a (Fig. 3).

Similar viral proteins are recognized by specific IgA and IgG that are produced following rotavirus infections in humans (50) and rodents (7, 56). However, these studies compared IgG and IgA responses following only oral exposure to rotavirus and did not address the question of whether the route of infection affects the humoral immune response. Zhaori et al. (67, 68) demonstrated that virus-specific serum IgG as well as secretory IgA recognized VP1, VP2, and VP3 following either oral or parenteral vaccination with poliovirus. Therefore, the viral proteins recognized by poliovirus-specific IgG and IgA appear to be similar regardless of the route of administration. Our results indicate that reovirus-specific IgA from GALT, IgG2a from PLN, and serum IgG1 recognize similar proteins by immunoblot analysis. Reovirus-specific IgA, IgG2a, and IgG1 strongly reacted with λ core proteins, the outer capsid proteins, μ1c and σ3, and the viral hemagglutinin, σ1. These results are consistent with previous immunoblot analysis with human anti-reovirus IgG and IgA (55) as well as the characterization of mouse monoclonal antibodies produced by cells from systemically primed mice (63). We conclude from these data that, similar to the responses to rotavirus (7) and poliovirus (67, 68), virus-specific IgA and IgG predominately recognize structural proteins. Furthermore, antigen recognition

by the immune system seems to be unaffected by the route of infection.

In conclusion, we show that oral and systemic reovirus infections elicit systemic Th1-like (IgG2a) antibody responses in serum independent of the route of infection and local Th2-like (IgA) antibody responses in the gut following oral infection. Western blot analysis reveals that the specificities of antibodies induced by systemic or mucosal priming are similar. Collectively, these data serve to increase our current understanding of mucosal immunity and its effects on the generation of systemic immune responses.

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