Cellular immune responses of pigs after primary inoculation with porcine respiratory coronavirus or transmissible gastroenteritis virus and challenge with transmissible gastroenteritis virus

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Abstract

The contribution of cell-mediated immunity to protective immunity against virulent transmissible gastroenteritis virus (TGEV) infection conferred by primary porcine respiratory coronavirus (PRCV) or TGEV exposure was assessed in pigs that were challenged with TGEV 24 days after a primary oronasal inoculation with PRCV or TGEV when 11 days old. PRCV exposure induced partial protection against TGEV challenge in suckling pigs based upon a decreased number of diarrhea cases (42% vs. 90% in age-matched control pigs), limited virus shedding in feces, and increases in virus-neutralizing serum antibody titers; in contrast, all 11-day-old pigs inoculated with TGEV were completely protected after challenge. Weaned pigs were also studied to eliminate any possibility that lactogenic immunity from contact PRCV-exposed sows contributed to protection against TGEV. Once weaned, none of the PRCV-exposed or age-matched control pigs had diarrhea after TGEV challenge; moreover, both groups exhibited less rectal virus shedding than suckling pigs. Vigorous lymphocyte proliferative responses (> 96 000 counts per minute (cpm)) were detected in mononuclear cells prepared from mesenteric (MLN) and bronchial (BLN) lymph nodes of TGEV-primed pigs. Analyses of these responses indicate that virus-specific cell-mediated immune responses correlated with protection against rectal and nasal virus shedding after TGEV challenge. Primary inoculation of 11-day-old pigs with PRCV induced moderate, transient virus-specific lymphocyte

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proliferation (> 47 000 cpm) in MLN from both suckling and weaned pigs after TGEV challenge. Substantial BLN proliferative responses (> 80 000 cpm) correlated with failure to detect TGEV in nasal secretions from these pigs. Virus-specific lymphocyte proliferation in spleens was delayed in onset and of lower magnitude than that observed in MLN and BLN. Virulent TGEV exposure resulted in increased percentages of T cell subsets, especially in the lamina propria and MLN, mucosa-associated lymphoid tissues in proximity to the primary replication site of TGEV in the small intestine. Our results confirm that PRCV infection primes anti-viral immune responses and, thus, contributes to partial immunity against virulent TGEV challenge.

1. Abbreviations

ASC, antibody-secreting cells; BALT, bronchus-associated lymphoid tissue; BLN, bronchial lymph nodes; CCIF, cell culture immunofluorescence; Con A, concanavalin A; cpm, counts per minute; FCM, flow cytometric analysis; GALT, gut-associated lymphoid tissue; IEM, immune electron microscopy; IL-2R, interleukin-2 receptor; mAbs, monoclonal antibodies; MHC, major histocompatibility complex; MLN, mesenteric lymph nodes; PCD, postchallenge day; PFU, plaque-forming units; PHA, phytohemagglutinin; PID, postinoculation day; PRCV, porcine respiratory coronavirus; sIg, surface immunoglobulin; SLA, swine lymphocyte antigen; TcR, T cell receptor; TGEV, transmissible gastroenteritis virus.

2. Introduction

Transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV), two antigenically related porcine coronaviruses with distinct intestinal and respiratory tissue tropisms, present a unique model for the study of immunologic interactions among mucosa-associated lymphoid tissues. TGEV infects and destroys villous enterocytes of the small intestine leading to vomiting, severe malabsorptive diarrhea, dehydration, and mortality approaching 100% in newborn susceptible pigs (Saif and Wesley, 1992). Because effective vaccines and therapy are unavailable, the disease continues to negatively impact the swine industry. Infection of pigs with virulent TGEV elicits protective active immunity subsequent to enteric replication of the virus, stimulation of inductive sites in gut-associated lymphoid tissue (GALT), and production of virus-specific cell-mediated immunity, antibody-secreting cells (ASC), and intestinal secretory IgA (Saif and Wesley, 1992; VanCott et al., 1993; Brim et al., 1994). Protective lactogenic immunity conferred to pigs suckling sows that were immunized via natural exposure or oral inoculation with virulent TGEV is theorized to result from the migration of TGEV-specific IgA-committed B cells from GALT to the mammary glands where they mature into IgA-ASC and produce secretory IgA to TGEV in milk (Saif and Wesley, 1992). TGEV infects the respiratory tract (Kemeny et al., 1975; Furuuchi et al., 1979; O'Toole et al., 1989) and stimulates virus-specific cellular and ASC immune responses in bronchial lymph nodes (BLN) (VanCott et al., 1993; Brim

et al., 1994); however, the contribution of respiratory lymphoid tissue to protective immunity against TGEV is unclear.

PRCV was first identified in Europe in 1984 (Brown and Cartwright, 1986; Pensaert et al., 1986) and later in the USA (Hill et al., 1990; Wesley et al., 1990) as the cause of mild respiratory disease or subclinical infections without enteritis in swine. Conventional serologic tests are unable to distinguish between PRCV and TGEV, although antigenic differentiation is possible with specific monoclonal antibodies (mAbs) in a serum blocking ELISA (Callebaut et al., 1988; Garwes et al., 1988; Van Nieuwstadt and Boonstra, 1992; Simkins et al., 1992). PRCV replicates extensively in the respiratory tract, but intestinal PRCV replication is limited to a few villous subepithelial cells (Pensaert et al., 1986; Cox et al., 1990). The mechanisms by which PRCV exposure induces a variable degree of active (Van Nieuwstadt et al., 1989; Wesley and Woods, 1992; Cox et al., 1993) and passive immunity (Bernard et al., 1989; Paton and Brown, 1990; De Diego et al., 1992) to TGEV infection are unclear. A bronchus-associated lymphoid tissue (BALT)-mammary immunologic link has been proposed to account for the appearance of TGEV-neutralizing secretory IgA in milk from PRCV-exposed sows (Callebaut et al., 1990). Our recent studies with PRCV-inoculated pigs demonstrated vigorous virus-specific cell-mediated and ASC, primarily IgG, immune responses in BLN, but little stimulation of mesenteric lymph nodes (MLN) (VanCott et al., 1993; Brim et al., 1994).

Clarification of cellular immune responses to TGEV and PRCV is essential for the control of these infections in pigs. Cell-mediated immunity may play a direct role in recovery from and protection against reinfection, and the terminal differentiation of IgA-committed B cells into IgA-ASC requires virus-specific T cell help (Mestecky and McGhee, 1987). This article presents data that assesses the contribution of cell-mediated immunity to the protection conferred by primary TGEV or PRCV exposure of pigs against virulent TGEV infection. Changes in lymphocyte proliferative responses and in the distribution of mononuclear cell subsets after virus exposure were evaluated in MLN (draining lymph nodes for GALT), in BLN (draining lymph nodes for BALT and the lower respiratory tract), in the intestinal lamina propria (an effector site in GALT), and in spleens (systemic lymphoid organs).

3. Materials and methods

3.1. Viruses

The virulent Miller strain (M5C) of TGEV (Bohl and Kumagai, 1965), passaged in gnotobiotic pigs, and the PRCV strain ISU-1 (Hill et al., 1990), passaged in swine testicle cells, were titered by plaque assays in swine testicle cells before they were inoculated into pigs. The low cell culture-passaged Miller strain (M6) of TGEV (Welch et al., 1988) was processed for use as viral antigen in lymphocyte proliferation assays, as previously described (Brim et al., 1994).

3.2. Experimental design

Eleven-day-old suckling pigs from crossbred sows that were seronegative for TGEV-neutralizing antibodies were inoculated oronasally with virulent TGEV M5C (17 pigs from

three litters, 5×10^5 plaque-forming units (PFU) per pig) or PRCV (16 pigs from two litters, 2×10^8 PFU per pig). One-fifth of the total inoculum dose was administered via the intranasal route. Twenty-three age-matched uninoculated pigs from three litters served as controls. Each litter was housed in a separate isolation room during the experiments; each pig in these eight litters remained with its sow until euthanasia. On Postinoculation Day (PID) 24, 35-day-old pigs from all three groups (TGEV, PRCV, and control) were given an oronasal challenge dose of virulent TGEV (1.3×10^8 PFU per pig). The rationale for the virus doses given to these pigs was detailed elsewhere (VanCott et al., 1994). Animals were observed for clinical signs of gastroenteritis and respiratory infection, i.e. vomiting, diarrhea, nasal discharge, coughing, and dyspnea. Nasal and rectal swab specimens were collected from each pig before virus inoculation and challenge, then daily for 8 PID and 8 postchallenge days (PCD). Blood samples were drawn from selected pigs at the time of inoculation and challenge, on various PID and PCD, and from all pigs following euthanasia. Pigs were euthanized on PCD 0 (PID 24), 2, 4, 8, and 12. MLN, BLN, spleens, and segments of the small intestine were harvested for cell preparations that were tested in lymphocyte proliferation assays and flow cytometric analysis (FCM). Tissues from eight TGEV-seronegative, unexposed suckling pigs from the same herd were also available for FCM. An additional ten pigs from one litter were inoculated with PRCV as above, then weaned at 3 weeks of age (PID 10). One litter of six age-matched weaned pigs served as controls. Both litters of weaned pigs were challenged with TGEV when 35 days old (PID 24); pigs were euthanized on PCD 0, 4, and 12.

3.3. Virus neutralization test, cell culture immunofluorescence (CCIF), and immune electron microscopy (IEM)

Neutralizing antibody titers to TGEV and PRCV in serum samples were determined by a plaque reduction test (Bohl et al., 1972). Virus was detected in rectal and nasal swab samples by a CCIF test with hyperimmune porcine anti-TGEV (cross-reactive with PRCV) immunoglobulin conjugated to fluorescein isothiocyanate (FITC; Welch et al., 1988; VanCott et al., 1993). Titers were expressed as the number of fluorescing foci per milliliter of swab supernatant fluid. In addition, rectal swab specimens from PCD 2 and 3 were examined for TGEV particles by IEM (Saif et al., 1977).

3.4. Flow cytometric analysis

Mononuclear cells were isolated from MLN, BLN, spleens, and the lamina propria of the small intestines and resuspended in complete culture medium, as described previously (VanCott et al., 1993, 1994). Cell viability assessed by trypan blue exclusion was $>90\%$ for cells from each tissue. Aliquots of mononuclear cells were stored frozen up to 1 year in liquid nitrogen. The recovery of frozen cells upon thawing was 50–60% for cells from MLN, BLN, and spleens, and 20–25% for ileal and duodenal cells. Cells were incubated for 30 min at 4°C with the following mAbs specific for swine leukocyte surface antigens: PT85a (anti-Class I, monomorphic), MSA3 (anti-Class II, swine lymphocyte antigen (SLA)-DR), MSA4 (anti-CD2), 74-12-4 (anti-CD4), 76-2-11 (anti-CD8), 74-22-15 (anti-SWC3, macrophage/granulocyte), 231.3B2 (anti-interleukin-2 receptor (IL-2R)),

5C9 + 1A11 (anti-IgM), 3D11 + 3H5 (anti-IgA), and 3H2 + 3H7 (anti-IgG) (Paul et al., 1989; Bailey et al., 1992; Lunney, 1993). The mAbs were undiluted hybridoma culture supernatants except for the anti-Ig mAbs which were ascitic fluid diluted 1:100 in Earl's balanced salt solution supplemented with 1% bovine serum albumin and 5% fetal bovine serum. Following two washes, cells were stained for 30 min at 4°C with a FITC-conjugated anti-mouse Ig reagent (goat anti-mouse IgG F(ab')$_2$; Kirkegaard&Perry Laboratories, Gaithersburg, MD). An EPICS Profile-II (Coulter, Hialeah, FL) was used to perform FCM of 10 000 immunofluorescing cells (Lunney et al., 1986).

3.5. Lymphocyte proliferation

The lymphocyte proliferation assays were performed as described previously (Brim et al., 1994). Briefly, fresh mononuclear cells were plated at 4×10^5 cells per well in 96 well culture plates. Cell cultures were stimulated in triplicate with inactivated TGEV M6 viral antigen (5–20 μl), uninfected cell culture fluid (negative control), the T cell mitogen phytohemagglutinin (PHA) (positive control) (M form; GIBCO Laboratories, Grand Island, NY), or medium alone (background control). Mononuclear cells from the lamina propria were also stimulated with 1 μg per well of a second T cell mitogen, concanavalin A (Con A; Sigma Chemicals, St. Louis, MO). Doses of viral antigen, PHA, and Con A were optimized for the cell culture conditions specified. Cell cultures were incubated for 96 h (MLN, BLN, spleens) or 72 h (lamina propria) at 37°C in 5% CO_2, then pulsed with 1 μCi [^{3}H]methylthymidine per well for an additional 18 h. [^{3}H]Thymidine incorporation was determined for each tissue sample and expressed as the mean cpm of [^{3}H]thymidine incorporation for cells stimulated by TGEV antigen minus the mean cpm for cells stimulated by cell culture fluid (negative control), or as the mean cpm of PHA- and Con A-stimulated cultures minus the mean cpm of the background control which ranged from 500 to 9000 cpm. These corrected values for each pig's samples were then used to calculate group means ± standard error of the mean (SEM).

3.6. Statistical analysis

Statistical differences in proliferative responses among inoculum groups were determined by one-way analysis of variance on rank-transformed data. Fisher's least significant difference test was used to identify specific differences. A probability level of $P < 0.05$ was considered statistically significant. Proliferative responses below 40 000 cpm were defined as low, 40 000 to 80 000 cpm as moderate, and above 80 000 cpm as high.

4. Results

4.1. Clinical signs, virus shedding, and virus-neutralizing antibodies of suckling pigs

After primary inoculation with virulent TGEV M5C, 11-day-old pigs exhibited signs of severe gastroenteritis, i.e. vomiting and diarrhea, associated with the disease and shed virus in feces for 7 PID with a peak mean virus titer of 1.3×10^4 focus-forming units per ml at

PID 1 (Fig. 1(A)). Intestinal virus replication was further confirmed by positive rectal swab samples from 76% of the pigs. Respiratory virus shedding after TGEV exposure was not as prevalent because only 24% of the pigs were positive for virus in nasal swab samples and the peak mean virus titer was 1000-fold less than that in the feces (Fig. 1(B)). All three sows that were contact-exposed to virulent TGEV following inoculation of their piglets showed signs of anorexia but not agalactia from PID 3 to 5. Although clinical signs of disease were not observed in any PRCV-inoculated pigs, all of these pigs sustained respiratory infections as evidenced by virus shedding in nasal secretions; the peak mean virus titer was 2.3×10^5 focus-forming units per ml at PID 1 (Fig. 1(B)). Virus was not detected in feces from pigs given PRCV.

Pigs were challenged with virulent TGEV (1.3×10^8 PFU per pig) at 35 days of age to determine the degree of protective immunity afforded by primary PRCV exposure. Forty-two percent of PRCV-inoculated pigs developed diarrhea after TGEV challenge; one of 16 pigs and ten of 12 pigs shed virus in feces when the samples were tested by CCIF and IEM,
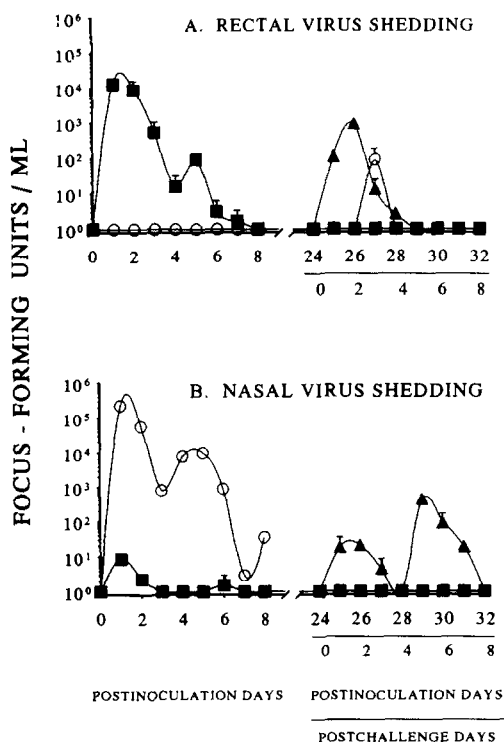

Fig. 1. Amount of virus in rectal (A) and nasal (B) swab samples measured by cell culture immunofluorescence. Pigs were inoculated with TGEV (■), PRCV (○), or no virus (▲), and samples were collected daily through PID 8. At PID 24 (PCD 0), pigs from all three primary inoculum groups (TGEV, PRCV, and no virus) were challenged with TGEV; samples were collected daily through PCD 8 (PID 32). Data are presented as the mean fluorescing focus-forming unit per ml of swab supernatant fluid of 16–23 samples (±SEM) after primary inoculation and of 6–21 samples (±SEM) after challenge. Data from samples without virus overlap and are indistinguishable from the baseline. SEM smaller than the symbols are not shown.

respectively. However, all pigs were completely protected against nasal virus shedding (Fig. 1). One PRCV-exposed pig euthanized at PCD 2 had villous atrophy in the jejunum and ileum, but intestinal lesions were not found in any pigs that were reinoculated with TGEV (R. Moxley, personal communication, 1992). After receiving a 260-fold higher dose (challenge dose) of virulent TGEV than the primary dose given to 11-day-old pigs, 35-day-old control pigs had diarrhea without vomiting; they exhibited virus shedding in feces for a shorter duration (4 days) and with a ten-fold lower peak mean virus titer than that observed in the younger pigs (Fig. 1(A)). However, about half of the 35-day-old TGEV-challenged control pigs shed virus in nasal secretions with a peak mean virus titer that was 50-fold higher than that in the younger pigs after primary TGEV exposure (Fig. 1(B)). All primary TGEV-inoculated pigs were protected against both diarrhea and virus shedding from the intestinal and respiratory tracts when challenged with virulent TGEV (Fig. 1).

The results for IEM of rectal swab samples and virus-neutralizing serum antibodies from these pigs were reported elsewhere (VanCott et al., 1994). Briefly, when rectal swab supernatants from a portion of the challenged pigs were examined at PCD 2 and 3 for virus particles by IEM, ten of 12 PRCV-inoculated pigs, one of five TGEV-inoculated pigs, and seven of nine control pigs were positive for TGEV. All virus-inoculated pigs seroconverted and produced neutralizing antibodies to TGEV, and both the TGEV and PRCV groups had similar antibody titers at PID 24 before challenge. After TGEV challenge, serum antibody titers increased nearly 100-fold in the PRCV group compared with less than a two-fold increase in the TGEV group. The control pigs challenged with TGEV seroconverted by PCD 8.

4.2. Flow cytometric analysis of immunostained cells from suckling pigs

At the time of tissue harvest, single cell suspensions preparations from each tissue were frozen; after thawing, cell subsets from TGEV-challenged pigs were compared with normal age-matched, unexposed pigs using mAbs immunostaining in FCM analysis. Details of the B cell subsets from these pigs were presented separately (VanCott et al., 1994). The phenotypic characterization of mononuclear cells from unexposed 11- to 51-day-old pigs by FCM (Fig. 2) revealed that $CD2^+$ T cells were least numerous in the ileal lamina propria (11–29%) and that the small intestine lamina propria yielded the lowest percentages of both $CD4^+$ ($<21\%$) and $CD8^+$ ($<8\%$) T cells (Figs. 2(B) and 2(C)). Percentages of $CD2^+$ (35–58%) and $CD4^+$ (22–41%) cells in MLN, BLN, and spleens were similar (Figs. 2(A), 2(D) and 2(E)). Overall, no association between the age of the pigs (11–51 days) and variation in percentages of cell subsets was noted.

After TGEV challenge, the percentages of $CD2^+$ and $CD4^+$ T cells in all five tissues generally increased over those found in age-matched unexposed pigs (Figs. 3(A) and 3(B)), but the changes in control pigs after primary TGEV inoculation were most striking in MLN and the lamina propria (duodenum, ileum), lymphoid tissues adjacent to enterocytes of the small intestine, the major replication site of TGEV. $CD8^+$ T cells followed the trends seen with $CD2^+$ and $CD4^+$ cells after virus exposure (Fig. 3(C)). The effect of time after virus exposure on T cell populations was assessed. These analyses revealed that MLN $CD4^+$ T cells apparently increased after primary TGEV inoculation of 35-day-old
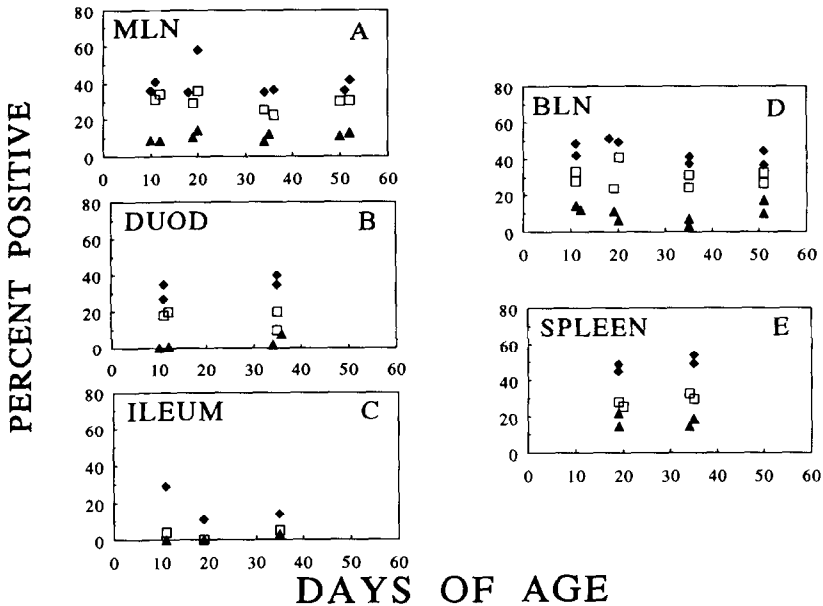
Fig. 2. Flow cytometric analysis of T cells from MLN (A), the duodenal lamina propria (DUOD, B), the ileal lamina propria (C), BLN (D), and spleens (E) of normal, unexposed 11- to 51-day-old pigs. Data are presented as the percentage positive cells after subtraction of background staining with anti-Ig-FITC. Each point represents the percentage of cells from an individual pig expressing the swine cell surface marker CD2 (♦), CD4 (□), or CD8 (▲) that specifies the T cell, helper T cell, or cytotoxic T cell subset, respectively.

control pigs; no such trends were observed in $CD4^+$ cells from BLN (data not shown).

In spleen cells from TGEV- and PRCV-primed pigs, the sum of the $CD4^+$ and $CD8^+$ cells (73% and 64%, respectively) exceeded the percentage of the $CD2^+$ cells (56% and 58%, respectively), implying that a portion of the T cell population was $CD4^+CD8^+$ (double positive) T cells (Figs. 3(A)–3(C)). The presence of $CD2^+CD4^-CD8^-$ cells in the duodenal and ileal lamina propria T cell populations was suggested by the fact that the sum of the $CD4^+$ and $CD8^+$ cells was less than the percentage of $CD2^+$ cells from unexposed pigs and from pigs in all three TGEV-challenged groups.

Percentages of macrophages ranged from < 10% in MLN and BLN to 24% in the spleen and duodenum (Fig. 4(A)). After TGEV challenge, percentages of sIg^+ B cells generally decreased in all tissues except the duodenum (Fig. 4(B)). SLA Class I antigens were expressed on > 86% of cells in all tissues and no apparent shift in intensity of SLA Class I or Class II antigen expression in response to viral exposure was evident (Fig 4(C); data not shown). Changes in percentages of cells expressing SLA Class II paralleled changes in B cell percentages. Low-level expression of IL-2R was not affected by virus exposure (Fig. 4(D)).

4.3. Virus-specific lymphocyte proliferative responses of suckling pigs

Overall, mononuclear cells purified from the mucosa-associated lymph nodes, MLN and BLN, gave vigorous virus-specific proliferative responses that were strongly associated with
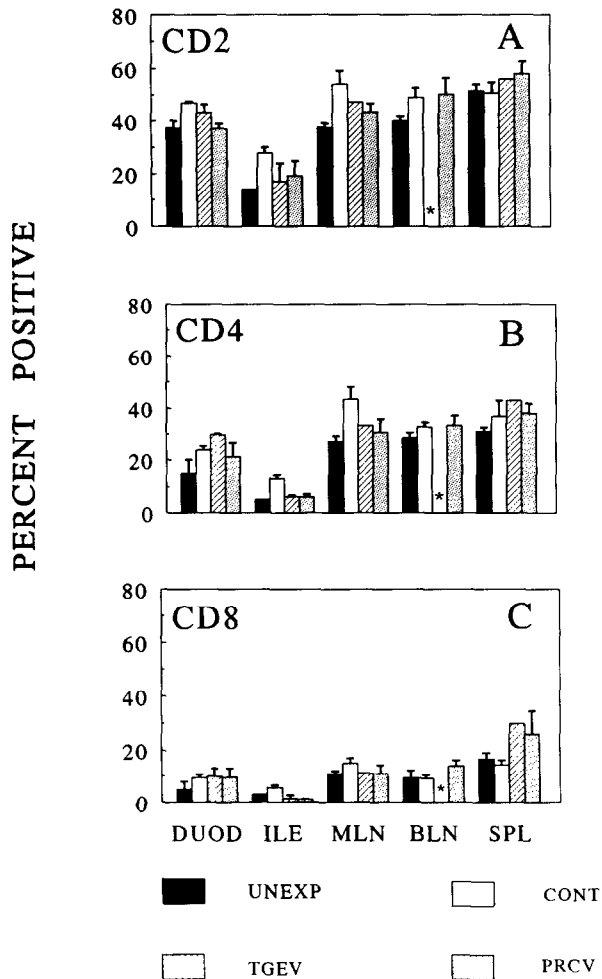
Fig. 3. Flow cytometric analysis of mononuclear cells from the duodenal lamina propria (DUOD), the ileal lamina propria (ILE), MLN, BLN, and spleens (SPL) from pigs challenged with virulent TGEV or from age-matched unexposed pigs (UNEXP, sample size (n) = 1–4). Primary virus inoculation of TGEV-challenged pigs and sample size were as follows: no virus (CONTrol), n = 3–6; TGEV, n = 1–3; PRCV, n = 2–4. Cells were immunostained for the swine cell surface markers CD2 (A), CD4 (B), and CD8 (C). Data are expressed as the mean percentage positive cells after subtraction of background staining with anti-Ig-FITC ($\pm$ SEM). *Data not available.

virus replication in the intestinal and respiratory tracts. Before challenge at PID 24 (PCD 0), MLN cells from TGEV-inoculated pigs gave high ($>$ 96 000 cpm) virus-specific proliferative responses that were significantly higher (P = 0.02) than MLN responses from the PRCV and control groups, generally persisted after TGEV challenge, and correlated with protection against diarrhea and rectal virus shedding (Fig. 5(A)). MLN responses were very low ($<$ 10 000 cpm) in the PRCV group before challenge. TGEV challenge of PRCV-exposed pigs induced a transient, moderate MLN virus-specific proliferation that peaked on PCD 4. Cells from MLN collected from control pigs challenged with TGEV showed an
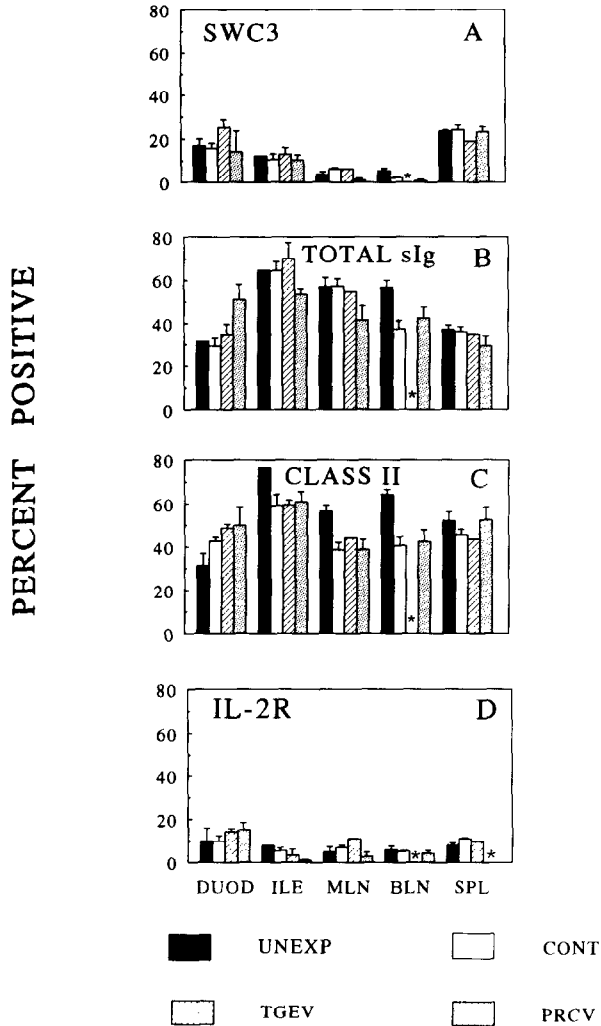
Fig. 4. Flow cytometric analysis of mononuclear cells immunostained for the surface antigen expression of SWC3 (macrophage/granulocyte) (A), total sIg (sIgM + sIgA + sIgG; B cell subset) (B), SLA Class II (C), and IL-2R (D). Sources of cells, virus exposure, sample size, and data presentation are described in the legend to Fig. 3. *Data not available.

expected primary immune response in lymphocyte proliferation that by PCD 12 exceeded 100 000 cpm and attained the magnitude of proliferation exhibited by MLN cells from TGEV-reinoculated pigs. At PCD 8 and 12, proliferative responses in MLN cells from PRCV-inoculated pigs were lower than those in MLN cells from both primary and secondary TGEV-exposed pigs.

The magnitude of virus-specific proliferation assayed in this study was highest (< 120 000 cpm) in BLN cells from TGEV and PRCV groups before challenge at PID 24 (Fig. 5(B)). BLN proliferative responses of PRCV-inoculated pigs generally remained
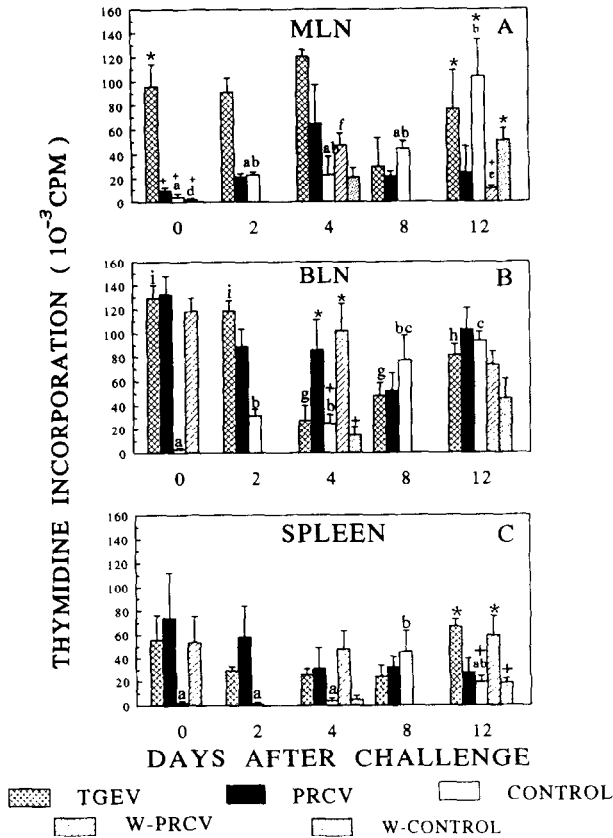
Fig. 5. Virus-specific lymphocyte proliferation in MLN (A), BLN (B), and spleens (C) from pigs challenged with virulent TGEV. Primary virus inoculation and sample size were as follows: TGEV, $n = 2-4$; PRCV, $n = 3-4$; no virus (CONTROL), $n = 2-7$; PRCV, weaned pigs (W-PRCV), $n = 2-4$; no virus, weaned pigs (W-CONTROL), $n = 3$. Data are presented as the mean virus-specific cpm for MLN, BLN, or spleen cells from the virus-inoculum groups at each time point $\times 10^{-3}$ ($\pm$ SEM $\times 10^{-3}$). Means for one tissue were compared across time within each virus-inoculum group and among groups on each day. Values that were significantly different ($P < 0.05$) among days are noted by (a, b, c) in the CONTROL group, by (d, e, f) in the weaned PRCV-inoculated group, and by (g, h, i) in the TGEV-inoculated group. In MLN cells (A) at PCD 0 and 12, in BLN cells (B) at PCD 4, and in spleen cells (C) at PCD 12, values that were significantly different ($P < 0.05$) among groups are noted by (*, +).

high after TGEV challenge and correlated with protection against nasal virus shedding. In contrast, BLN cells from TGEV-reinoculated pigs showed a significant decrease in proliferation at PCD 4 and 8 that returned to a high level on PCD 12 ($P < 0.04$); these pigs were also protected against virus shedding in nasal secretions after challenge. BLN cells from control pigs challenged with TGEV gave a primary virus-specific immune response that was significant by PCD 2 and continued to increase to a high level approaching 100 000 cpm by PCD 12 ($P = 0.03$).

Spleen cells from TGEV and PRCV groups before challenge at PID 24 gave similar moderate proliferative responses that remained fairly constant after TGEV challenge (Fig.

5(C)). The induction at PCD 8 of a primary TGEV-specific proliferative response in spleen cells from control pigs challenged with TGEV lagged behind the onset of primary responses at PCD 2 that were demonstrated in MLN and BLN cells from these same control pigs. At PCD 16 and 20, cells from MLN, BLN, and spleens of five control pigs continued to give proliferative responses consistent with the primary immune response that was observed through PCD 12 (data not shown). Trends in MLN, BLN, and spleen responses were similar when the data were also calculated as a percentage of the mitogen proliferation for each sample (data not shown). Virus-specific lymphocyte proliferation was never detected in any of the lamina propria cells purified from the duodenum, jejunum, and ileum of PRCV- and TGEV-inoculated pigs nor from TGEV-challenged pigs.

4.4. Weaned pigs: clinical signs, virus shedding, virus-neutralizing antibodies, and virus-specific lymphocyte proliferative responses

Weaned pigs were studied to eliminate any possibility that passive lactogenic immunity from contact PRCV-exposed sows contributed to protection against TGEV. Prior to weaning, 11-day-old pigs that were inoculated with PRCV responded as did other primary PRCV-exposed litters, i.e. all pigs shed virus in nasal secretions and seroconverted; none showed clinical signs of disease or shed virus in feces. After weaning (PID 10, 3 weeks of age) and virulent TGEV challenge (PID 24) these primary PRCV-inoculated pigs and their age-matched weaned controls were more resistant to disease than suckling pigs. None of the weaned pigs had diarrhea or shed virus in feces when the samples were tested by CCIF. Virus was detected by IEM of rectal swab supernatants from four pigs in each of the two weaned groups. When weaned and suckling pigs were compared, percentages of pigs with positive rectal virus shedding on PCD 2 and 3 as confirmed by IEM were: PRCV-inoculated pigs, 50% weaned vs. 83% suckling; and control TGEV-challenged pigs, 67% weaned vs. 78% suckling. All weaned pigs yielded negative postchallenge nasal swab samples except one control pig on PCD 1. Serum antibody titers in the weaned PRCV group increased about ten-fold following TGEV challenge compared with a 100-fold increase in the suckling PRCV group. Weaned control pigs seroconverted with a typical primary virus-neutralizing serum antibody response.

Mononuclear cells from MLN, BLN, and spleens of weaned pigs exhibited trends in virus-specific lymphocyte proliferative responses that were similar to those observed in suckling pigs (Fig. 5). TGEV challenge of PRCV-inoculated pigs induced significant moderate (> 47 000 cpm) MLN proliferation ($P = 0.02$) on PCD 4 that decreased to < 12 000 cpm and was significantly lower ($P < 0.05$) than MLN responses in TGEV-reinoculated pigs and both weaned and suckling TGEV-challenged control pigs on PCD 12 (Fig. 5(A)). Virus-specific lymphocyte proliferation was never detected in any of the small intestine lamina propria cells from PRCV-exposed and control weaned pigs.

4.5. Lymphocyte proliferative responses to mitogen of suckling and weaned pigs

When cellular immune function was assessed by PHA-induced proliferation of lymphocytes, responses in cells from MLN, BLN, and spleens averaged > 156, 000 cpm (Table 1). PHA responses in cells from the small intestine lamina propria were markedly lower,

Table 1
Lymphocyte proliferation responses of suckling and weaned pigs in PHA-stimulated mononuclear cell cultures

Source of mononuclear cells	Primary virus inoculum					
	TGEV		PRCV		No virus	
	Mean cpm ± SEM	n	Mean cpm ± SEM	n	Mean cpm ± SEM	n
Mesenteric lymph node[a]	212815 ± 22633	17	162426 ± 13816	26	185767 ± 14090	26
Bronchial lymph node	172592 ± 14164	11	179928 ± 12889	26	192826 ± 11317	26
Spleen	196008 ± 17038	16	156292 ± 11645	26	183797 ± 15647	25
Duodenum[b]	23578 ± 14075	7	19886 ± 4250	17	24384 ± 7392	12
Jejunum	14670 ± 5699	7	12540 ± 3765	17	10952 ± 3554	11
Ileum	61621 ± 13992	11	48431 ± 7612	18	56838 ± 13294	12

Values for each sample were calculated as the mean counts per minute (cpm) of triplicate cultures with PHA minus the mean cpm of triplicate background control cultures.
[a] Mononuclear cells from MLN, BLN, and spleens were incubated for 4 days with PHA before they were pulsed with tritiated thymidine.
[b] Mononuclear cells were purified from the lamina propria of the duodenum, jejunum, and ileum and incubated for 3 days with PHA before they were pulsed with tritiated thymidine.

ranging from < 11 000 cpm in the jejunum to about 62 000 cpm in the ileum, the latter value one-third that observed in MLN. These depressed lamina propria cell responses to PHA were directly attributable to a lower percentage of T cells present at this site and to the low numbers of viable cells present after 4 days in culture: less than 10% of the lamina propria cells vs. 50–60% of the MLN, BLN, and spleen cells survived. The 4 day incubation period of lamina propria cells with T cell mitogens was optimized for maximum PHA and Con A responses; Con A induced proliferation comparable to that of PHA-stimulated cultures (data not shown). The addition of feeder medium prepared from supernatants of Con A-stimulated porcine spleens and peripheral blood mononuclear cells to lamina propria cell cultures did not enhance cell viability. Within each of the six tissues, cells exhibited similar mitogenic reactivity regardless of virus exposure.

5. Discussion

Vigorous lymphocyte proliferative responses in MLN and BLN of TGEV-primed pigs indicate that virus-specific T cell responses correlated with complete protection after TGEV

challenge of these pigs and that these T cells augment virus-specific B cell responses (VanCott et al., 1994). Both suckling and weaned PRCV-primed pigs exhibited transient increases in virus-specific MLN proliferation at PCD 4 that were two- to three-fold greater than MLN responses in TGEV-challenged control pigs. PRCV exposure conferred partial protection against virulent TGEV in both suckling and weaned pigs based upon clinical signs, virus shedding in feces and nasal secretions, and virus-neutralizing serum antibody titers. The impact of virulent TGEV exposure on T cell subsets was most pronounced in GALT and MLN, lymphocytes in proximity to the primary TGEV replication site in villous enterocytes of the small intestine (lamina propria, i.e. GALT) or in the draining lymph nodes for GALT (MLN).

These results showing that PRCV inoculation induced partial protection against virulent TGEV challenge concur with earlier reports on pigs exposed to European strains of PRCV at 5–6 weeks of age, then challenged 4 weeks later (Van Nieuwstadt et al., 1989; Cox et al., 1993), and with one study on very young gnotobiotic pigs inoculated with the same PRCV strain (Wesley and Woods, 1992). Less than half of our challenged suckling pigs developed diarrhea compared with 90% of the challenged control pigs. Positive virus-specific MLN proliferative responses at PCD 2 were coincident with the onset of primary TGEV-specific MLN proliferation in cells from control pigs; responses from PRCV-exposed suckling and weaned pigs peaked at PCD 4 and by PCD 12 were significantly lower than those in MLN from control and TGEV-reinoculated pigs (Fig. 5). Hence, PRCV exposure did not enhance the duration of TGEV-specific lymphocyte proliferation in MLN from challenged pigs, but the magnitude of the MLN response at PCD 4 was two- to three-fold greater than that observed in MLN from TGEV-challenged control pigs. Our investigation of TGEV-specific B cell responses from these same pigs disclosed that numbers of virus-specific IgA- and IgG-ASC in MLN were greatest at PCD 12 and did not differ significantly among the three groups of suckling pigs at this time point, although significant differences were observed at PCD 0, 2, and 4 (VanCott et al., 1994). These data suggest that, despite the lack of persistent TGEV-specific lymphocyte proliferative responses in MLN of PRCV-exposed pigs, T cell help afforded to B cells was sufficient to allow virus-specific IgA- and IgG-ASC responses in these pigs to eventually approach the ASC numbers in MLN of TGEV-reinoculated and TGEV-challenged control pigs. Virus-specific BLN cell proliferative responses were high before challenge of PRCV-exposed pigs, and their continuation after challenge correlated with complete protection against virus shedding in nasal secretions from all pigs in both suckling and weaned PRCV groups.

Cells from MLN, BLN, and spleens from 35-day-old TGEV-challenged control pigs, both suckling and weaned, gave proliferative responses consistent with the induction of primary immune responses (Fig. 5). Positive TGEV-specific proliferation was noted in MLN and BLN of suckling control pigs at PCD 2, whereas significant responses in spleens were not measured until PCD 8. The decreased magnitude and delayed onset of virus-specific spleen cell proliferation in control pigs may reflect the fact that TGEV is not a systemic infection and is rarely isolated from the spleens of infected pigs (Furuuchi et al., 1978/1979), and the relocation of antigen-specific T helper cells is mucosally restricted after gut immunization (Dunkley and Husband, 1987). Age-dependent resistance to TGEV infections (Moon et al., 1973) was confirmed by milder clinical signs and less rectal virus shedding with lower titers after TGEV challenge of 35-day-old control pigs than was

observed in 11-day-old pigs. However, nasal virus titers were greater than those measured in the younger pigs which may indicate resistance to TGEV infections in older pigs depends more on maturation of the gut than the respiratory mucosa.

A comparison of rectal virus shedding from suckling pigs detected by CCIF and IEM provides indirect evidence that intestinal virus-specific antibody might contribute to the partial protection observed in PRCV-inoculated pigs. Virus detection by CCIF may have been hampered by the decreased infectivity of antibody-coated virions, whereas positive IEM only requires the visualization of virus particles. Rectal swab samples from TGEV-challenged control pigs tested by both methods gave about the same percentage of positive pigs (71–78%), implying minimal interference by intestinal antibody. In contrast, the TGEV-reinoculated pigs that were probably secreting high levels of intestinal secretory IgA (Saif and Wesley, 1992) had one positive sample of five tested by IEM and none positive by CCIF. Only one PRCV-inoculated pig had virus detected by CCIF versus 83% of the pigs were positive by IEM. In our B cell study, we described low numbers of virus-specific IgA-ASC in the small intestine lamina propria after PRCV inoculation; however, in PRCV-exposed pigs after TGEV challenge, we observed rapid, dramatic increases in virus-specific IgG-ASC in the gut lamina propria (VanCott et al., 1994). Moreover, Cox et al. (1993) detected TGEV-specific IgA in feces of PRCV-inoculated pigs following TGEV challenge. The rapid induction of these secondary antibody responses might curtail TGEV replication and may also interfer with virus detection in assays such as CCIF. The second source of intestinal antibody is passive lactogenic immunity conferred to pigs suckling sows that were contact-exposed to TGEV or PRCV. Other reports have shown that PRCV exposure of sows stimulates the production of TGEV-neutralizing IgA in milk (Callebaut et al., 1990) and provides partial protection against TGEV challenge in suckling pigs (Bernard et al., 1989; Paton and Brown, 1990; De Diego et al., 1992).

None of the 35-day-old control or PRCV-exposed weaned pigs developed diarrhea after TGEV challenge, and both groups had less rectal virus shedding detected by IEM and CCIF when compared with suckling pigs. A more rapid replacement of villous enterocytes in the intestine may be one factor resulting in increased resistance to TGEV infection in older pigs (Moon et al., 1973; Saif and Wesley, 1992). Maturation of the gut is accelerated in weaned pigs by a similar mechanism after they have recovered from the immediate postweaning damage to villous structure and function (Hall and Byrne, 1989). Although weaned pigs sustained less enteric and respiratory TGEV infection after challenge based on reduced rectal and nasal virus shedding, virus-specific lymphocyte proliferative responses were comparable between weaned and suckling pigs within the control and PRCV-exposed groups.

FCM of mononuclear cells after virus exposure revealed increased percentages of CD2^{+}, CD4^{+}, and CD8^{+} T cells in all lymphoid tissues that were most pronounced in MLN and GALT from control pigs inoculated with virulent TGEV (Fig. 3). The expansion of T cell subsets in MLN was coincident with the demonstration of virus-specific lymphocyte proliferation by cells of the same origin, although it is likely that TGEV-specific T cells were only a small part of the total T cell population. Our results are in accord with FCM data for other enteric and respiratory viruses describing increases in T cells (London et al., 1990; Kimpen et al., 1992).

The observations of a predominance of $CD4^+$ over $CD8^+$ T cells in MLN and the lamina propria, and $CD2^+$ T cell numbers about equal to those of sIg^+ B cells in the duodenal lamina propria (Figs. 2, 3 and 4) corroborate previous reports on lymphocyte subset composition in approximately 40-day-old pigs (Rothkotter and Pabst, 1989; Rothkotter et al., 1991). The excess of sIg^+ B cells over $CD2^+$ T cells in the ileal lamina propria is consistent with a description of ileal Peyer's patches in 6-week-old pigs (Rothkotter and Pabst, 1989). We did not exclude Peyer's patches from the pieces of ileum subjected to collagenase treatment and would expect them to be part of the lamina propria cell preparation. Our data showing $CD4^+$ T cells are more abundant than $CD8^+$ cells in spleens of young pigs contrast with data from spleens of adult pigs where $CD8^+$ T cells outnumbered $CD4^+$ cells (Saalmuller et al., 1989, 1990). The spleen was unique in that the presence of $CD4^+CD8^+$ spleen cells was suggested in 35- to 47-day-old pigs after TGEV challenge (Fig. 3). Double positive T cells, uncommon in mice and humans, constitute a significant subset of peripheral blood lymphocytes from adult pigs (Pescovitz et al., 1985; Saalmuller et al., 1987), but are less numerous in younger animals (Pescovitz et al., 1994). The $CD4^+CD8^+$ population has also been verified in spleens and MLN of adult pigs (Saalmuller et al., 1989, 1990).

Our FCM results demonstrating the sum of $CD4^+$ and $CD8^+$ cells was less than the percentage of $CD2^+$ cells imply that a large proportion of the duodenal (7–46%) and ileal (32–63%) lamina propria T cell population were $CD2^+CD4^-CD8^-$ cells (Fig. 3). In contrast to humans and mice, blood from young pigs and ruminants contains a relatively high number of thymus-derived null T cells ($CD2^-CD4^-CD8^-sIg^-$), many of which express the $\gamma\delta$ T cell receptor (TcR) (Hirt et al., 1990; Hein and Mackay, 1991; Binns et al., 1992). The $\gamma\delta$ TcR^+ cells are thought to provide non-MHC-restricted T cell immunity against infections of epithelial surfaces, including the gut mucosa. In swine, a subset of $\gamma\delta$ TcR^+ cells has been identified as $CD2^+CD4^-CD8^-$ T cells that selectively accumulate in spleens and MLN, but are rarely found among circulating lymphocytes (Saalmuller et al., 1989, 1990). A high proportion of T cells from the jejunal and ileal lamina propria of 1- and 5-day-old, but not 12- and 40-day-old, pigs were the $CD2^+CD4^-CD8^-$ subset (Rothkotter et al., 1991). However, our results suggest the presence of $CD2^+CD4^-CD8^-$ T cells in the lamina propria of both unexposed and TGEV-challenged pigs from 11 to 51 days of age. The mitogenic reactivity of porcine $CD2^+CD4^-CD8^-$ cells has not been described, but porcine null T cells are reported to be unresponsive to both PHA and Con A as well as to other antigenic stimuli (Binns, 1982).

The functional activity of T cells from MLN, BLN, and spleens assessed by DNA synthesis in mitogen-stimulated cultures (Table 1) concurred with our previous work revealing no variation in mitogenic reactivity based upon the source of lymphocytes or virus exposure (Brim et al., 1994). The magnitude of lamina propria cell proliferation to mitogen, less than one-third of the response for cells from MLN, BLN, and spleens, agrees with an earlier report describing PHA and Con A responses of > 10 000 cpm in lamina propria cells of 3-week-old and adult pigs (Wilson et al., 1986). Significant functional differences have been shown in the characteristics of circulating memory T cells compared with lamina propria cells. Circulating memory T cells give strong proliferative responses when resti-mulated by antigen in vitro (Sanders et al., 1988; Akbar et al., 1991), circulating naive T cells proliferate preferentially to mitogens, and lamina propria memory T cells respond to

antigenic restimulation by exhibiting effector functions (e.g. cytokine secretion and help for B cells) without marked proliferation (Zeitz et al., 1988).

Our results are consistent with the hypothesis that lymphocytes from MLN, BLN, and spleens comprised both naive and memory T cell populations that demonstrated vigorous proliferative responses to both mitogen and TGEV antigen. Lymphocytes from the lamina propria probably included significant numbers of $\gamma\delta$ TcR$^+$ T cells and memory T cells that had encountered TGEV or PRCV after primary inoculation and migrated back to the lamina propria to exert their effector function; these cell populations may exhibit weak or no proliferative responses to antigenic restimulation (memory T cells) and depressed responses to T cell mitogens ($\gamma\delta$ TcR$^+$ T cells). Many questions regarding the composition and function of the porcine lamina propria remain to be answered. The magnitude of virus-specific lymphocyte proliferation in mucosa-associated lymphoid tissues (MLN and BLN) was directly attributable to TGEV or PRCV replication that occurred in the adjacent mucosa, whereas lymphocyte proliferation in a systemic lymphoid tissue (spleen) depended on the relocalization of virus or virus-sensitized lymphocytes from the mucosa. Limited intestinal replication of TGEV and enteric disease in PRCV-exposed pigs challenged with TGEV may result from any or all of the following mechanisms: (1) active B and T cell immunity confirmed by virus-specific IgG- and IgA-ASC in GALT and lymphocyte proliferative responses in MLN; (2) passive lactogenic immunity conferred on suckling pigs from contact-exposure of sows; (3) accelerated maturation of the gut with subsequent increased resistance to TGEV infection in weaned pigs. Substantial virus-specific lymphocyte proliferation in BLN or MLN before challenge correlated with complete protection from shedding TGEV after challenge in nasal secretions or feces, respectively. These results confirm that PRCV infection primes TGEV-specific cell-mediated immune responses and, thus, contributes to partial immunity against TGEV challenge.

Acknowledgments

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