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Apoptosis by CD95 (Fas)-Dependent and -Independent Mechanisms in Peyer's Patch Lymphocytes in Murine Retrovirus-Induced Immunodeficiency Syndrome

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CD95 (Fas)/CD95 ligand (CD95 L)-mediated apoptosis is thought to be involved in the delayed progression of murine AIDS (MAIDS) induced by LP-BM5 murine leukemia virus (MuLV). We show evidence of apoptosis in lymphocytes of Peyer's patches (PP) at the early stage of MAIDS. Both T and B cells in PP expressed CD95 at the early stage of MAIDS and decreased in number thereafter. The decrease in T cells was not evident in CD95-mutated *lpr* mice with MAIDS, suggesting that CD95/CD95 L interaction is involved in the apoptosis of T cells in PP during the course of MAIDS. On the other hand, the number of B cells was also decreased in PP of *lpr* mice with MAIDS. The proliferative ability of B cells in PP of MAIDS mice in response to immunoglobulin M cross-linking or lipopolysaccharide was severely impaired, while the B cells normally proliferated in response to anti-CD40 monoclonal antibody. These findings imply that aberrantly activated B cells in PP undergo apoptosis independently of the CD95/CD95 L system during the course of infection with MAIDS virus.

Murine AIDS (MAIDS) is induced by a defective LP-BM5 murine leukemia virus (MuLV) and has many symptoms similar to those found in patients infected with human immunodeficiency virus (HIV) (5, 18, 19, 35–37, 50). The disease is characterized by pronounced splenomegaly and lymphoadenopathy, polyclonal B-cell activation, impaired T- and B-cell functions, aberrant regulation of cytokine production, and late B-cell lymphomas (35). The lymphoproliferation of both T and B cells in the peripheral lymphoid tissues of MAIDS mice is a feature different from that of AIDS patients, whereas the suppressed mucosal lymphocyte population is observed in the gut-associated lymphoid tissue (GALT) such as the Peyer's patches (PP) and intestinal lamina propria (ILP) of MAIDS mice (32). There are several lines of evidence that mucosal $CD4^+$ T cells are decreased in GALT with advance of AIDS (21, 44). GALT plays important roles in intestinal protection against opportunistic infection (34). It was shown that MAIDS mice exhibited impaired resistance against gastrointestinal pathogens such as *Cryptosporidium parvum* and *Giardia muris* (7, 41). Analysis of mechanism underlying suppression of mucosal lymphocytes in MAIDS may be useful for understanding the enteropathy and depressed resistance to intestinal opportunistic infection in AIDS patients.

CD95 (Fas) is a cell surface protein belonging to the tumor necrosis factor/nerve growth factor receptor family (20). CD95 and CD95 ligand (CD95 L) interaction mediates apoptosis in various types of cells, including T and B cells (30, 38). Kobayashi et al. (29) have reported that HIV-infected cell lines express CD95 and are sensitive to the cytolytic activity of an anti-CD95 monoclonal antibody (MAb). CD95 expression on CD4 and CD8 T cells reportedly increases during the course of HIV infection (8, 24). Furthermore, it is suggested that CD95-mediated apoptosis contributes to $CD4^+$ T-cell depletion in AIDS. We reported that the level of CD95 is increased on splenocytes in MAIDS mice (15). Recently, Kanagawa et al. have demonstrated that *lpr* mice deficient in CD95 exhibited drastically accelerated disease development, suggesting the involvement of the CD95 system in the regulation of the lymphoproliferation in MAIDS mice (23).

CD40 is an another member of tumor necrosis factor receptor family, and a signal through CD40 is important for B-cell activation, including proliferation, differentiation, and isotype switching (10). Tsubata et al. have indicated that the presence or absence of T-cell help through CD40 is crucial in determining whether antigen-stimulated B cells are activated or whether they die by apoptosis (46). On the other hand, B cells stimulated via only CD40 reportedly undergo apoptosis induced by CD95 (42, 43). Thus, the CD40 system is also closely associated with the induction of apoptosis in B cells.

To investigate whether the CD95 and/or CD40 systems are involved in suppressing the lymphocyte population in PP during the course of MAIDS, we examined the expression and function of CD95 and CD40 on the lymphocytes in PP during the course of MAIDS. We show here that CD95/CD95 L is involved in the apoptosis of T cells in the PP in MAIDS mice whereas that of B cells may occur independently of the CD95/CD95 L system.

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MATERIALS AND METHODS

Mice and viruses. Female C57BL/6 (B6) and C57BL/6J-*lpr*/*lpr* (B6 *lpr*) mice were purchased from Japan SLC (Shizuoka, Japan). LP-BM5 MuLV stocks were prepared from cell-free supernatants of chronically infected SC-1 cells (35). Four-week-old mice were injected intraperitoneally with 0.1 ml of LP-BM5 viral

stock containing 10^4 XC PFU of ecotropic virus and 10^2 mink cell focus-inducing units. According to spleen weight, subcervical lymph node (LN) weight, and fluorescence-activated cell sorting analysis (13, 28), 4-week-infected mice developed stage 1 to 2 disease and 8-week-infected mice developed stage 3 disease, although the profiles of individual mice considerably varied (data not shown).

Antibodies and reagents. Fluorescein isothiocyanate (FITC)-conjugated anti-Thy1.2 MAb, phycoerythrin (PE)-conjugated anti-CD4 MAb (L3T4), and biotin-conjugated anti-CD8 MAb (Lyt2) were purchased from Becton Dickinson (Mountain View, Calif.). FITC-conjugated anti-CD3-ε MAb, FITC-conjugated anti-immunoglobulin M (IgM) MAb (rat IgG2a, II/41), PE-conjugated anti-T-cell receptor α/β-chain (TcRαβ) MAb, PE-conjugated anti-CD95 MAb (hamster IgG, Jo-2), and purified anti-Bcl-2 MAb (hamster IgG, 3F11) were purchased from PharMingen (San Diego, Calif.). FITC- and PE-conjugated anti-B220 MAb and FITC-conjugated anti-hamster IgG was obtained from Caltag (San Francisco, Calif.). Streptavidin-RED613 was purchased from Gibco BRL (Gaithersburg, Md.). The anti-FcγRII/III-specific MAb (2.4G2) was obtained from the American Type Culture Collection (Rockville, Md.). The biotin-conjugated anti-CD40 MAb, HM40-3, was prepared by immunizing Armenian hamsters with WEHI 231 cells and screening for reactivity with CHO cells transfected with mouse CD40 cDNA (49). Purified anti-CD40 MAb (rat IgG2a, 3/23) was obtained from Serotec. Purified $F(ab')_2$ anti-IgM MAb was purchased from Organon Teknika. Lipopolysaccharide (LPS) was purchased from Sigma Chemical Co. (Munich, Germany).

Cell preparation and cell count. RPMI 1640 (Life Technologies, Inc., Grand Island, N.Y.) medium supplemented with 10% heat-inactivated fetal calf serum (CSL Limited, Victoria, Australia), L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U of penicillin per ml, and 100 μg of streptomycin per ml was used in all experiments. PP and mesenteric LN (MLN) were collected after sacrifice under ether anesthesia, and mononuclear cells were obtained by gently teasing the tissues through stainless steel wire mesh screens in RPMI 1640 medium. The single-cell suspensions were washed twice with cold medium; viability was determined by trypan blue exclusion and adjusted to give the desired viable cell concentrations (10^6/0.1 ml per tube) for lymphocyte surface marker determination. In some experiments, $B220^+$ B cells were isolated from PP or MLN by sorting using Coulter EPICS Elite ESP (Coulter Co., Miami, Fla.).

Detection of the defective LP-BM5 MuLV genome integration by PCR. Template DNAs extracted from the sorted $B220^+$ cells (5×10^4) of PP and MLN of the mice at 4 weeks after virus inoculation were amplified by PCR as described previously (16, 40). The PCR primers were 5′-CCTCTTCCTTTATCGACACT-3′ and 5′-ATTAGGGGGGGAATAGCTCG-3′. These primers correspond to sequences in the defective LP-BM5 MuLV *gag*-encoded genes, p15 and p12, respectively. We amplified template DNAs by PCR with 100 pM each primer plus 2.5 U of Ampli-Taq (Perkin-Elmer Cetus, Norwalk, Conn.) in a total volume of 100 μl and subjected them to 30 cycles of amplification. Reaction buffer consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 200 μM deoxynucleoside triphosphate. PCR cycle was carried out for 1 min at 95°C (5 min for the first cycle) followed by 3 min at 55°C and 1 min at 72°C. Five-microliter aliquots of PCR products were electrophoresed on a 1.8% agarose gel and visualized with ethidium bromide. The PCR products were 237 bp.

Histological analysis. PP specimens were fixed in 20% formalin, and then 3-μm paraffin-embedded sections were cut and stained with hematoxylin and eosin.

In situ labelling of apoptotic cells. Paraffin was recovered from embedded PP from uninfected and LP-BM5-infected mice. Degraded DNA was then labelled by using the Oncor Apoptag system as instructed by the manufacturer (Oncor, Gaithersburg, Md.). Residues of digoxigenin-labelled nucleotides were added to the DNA by using terminal deoxynucleotidyltransferase, and new nucleotide polymers were detected with a peroxidase-labelled antidigoxigenin antibody and diaminobenzidine.

Flow cytometry. Single-cell suspensions of MLN or PP from either uninfected or LP-BM5-infected mice were incubated with culture supernatant from 2.4G2 (anti-FCγRII/III-specific MAb-producing hybridoma) to prevent nonspecific staining. After washing, the cells were stained with various MAbs. When cells were stained with biotin-conjugated anti-CD8 MAb or biotin-conjugated anti-CD40 MAb, they were followed by treatment with streptavidin-RED 613. Flow cytometry was performed with a FACScan Lysis II (Becton Dickinson). We gated cells by forward and side light scattering of live lymphocytes. The percentage of fluorescence-positive cells was determined by integrating the profiles determined on the basis of 3×10^4 live lymphocytes. To analyze Bcl-2 protein expression, cells were fixed in 4% paraformaldehyde for 30 min, washed, and permeabilized with saponin 0.1% in Hanks balanced salt solution containing 0.01 M [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) for 30 min. After washing, the cells were stained with PE-conjugated anti-B220 MAb and purified anti-mouse Bcl-2 MAb (hamster IgG) and then treated with FITC-conjugated anti-hamster IgG (Caltag). Fluorescent analysis was performed with a FACScan.

Proliferation assays. In vitro MAb stimulation was performed as described by Aoki et al. (2). Cells were cultured in 96-well tissue culture plates (Nunc, Roskilde, Denmark) in quadruplicate at 2×10^5 cells per well and stimulated with either anti-CD40 MAb (5 μg/ml), $F(ab')_2$ anti-IgM MAb (5 μg/ml), or LPS (10 μg/ml) in 0.2 ml of RPMI 1640 medium. The plates were placed in a humidified 5% CO_2 atmosphere at 37°C. Cultures were harvested 48 h later after an 8-h pulse with 1 μCi of [^{3}H]TdR (Amersham, Buckinghamshire, England) per well. Radioactivity was determined in a liquid scintillation beta counter.

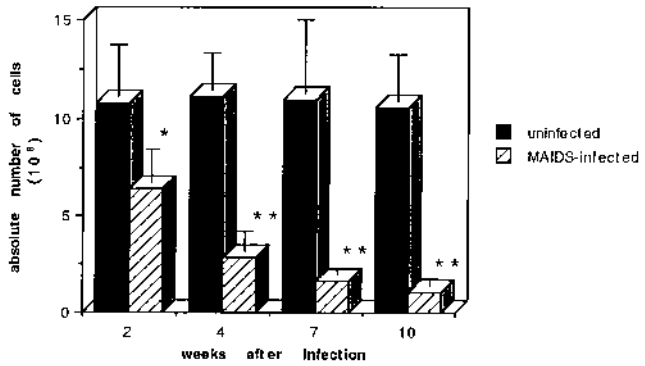

FIG. 1. Absolute numbers of cells in PP in uninfected and LP-BM5-infected mice. PP were collected after sacrifice under ether anesthesia, and mononuclear cells were obtained by gently teasing the tissues through stainless steel wire mesh screens in RPMI 1640 medium containing 10% fetal calf serum. The single-cell suspensions were washed twice with cold RPMI, and absolute numbers of cells were determined by trypan blue exclusion. Data were obtained from 10 mice per each group and are shown as means ± standard deviations. *, $P < 0.01$; **, $P < 0.001$ versus uninfected controls. Results from one representative experiment of three are shown.

Statistical analysis. The statistical significance of the data was determined by Student's *t* test. A *P* value of less than 0.05 was considered significant.

RESULTS

Decrease in numbers of both T and B cells in PP during MAIDS is partly due to apoptosis. The kinetics of the total number of leukocytes were examined in the MLN and PP during the course of MAIDS. As shown in Fig. 1, infection with LP-BM5 caused a significant decrease in the total number of cells in the PP at 2 or 10 weeks after infection compared with age-matched uninfected control mice, whereas the total number of leukocytes gradually increased in MLN. For example, at 6 weeks after infection, the development of lymphoadenopathy in infected mice was observed as an 8- to 10-fold increase in the number of MLN cells compared with that in uninfected controls (data not shown). To determine which cell populations were decreased in PP during the course of MAIDS, we examined the expression of CD3, TcRαβ, Thy1, CD4, CD8, and B220 on the cells in the PP of MAIDS mice by FACScan. The absolute number of each subset was calculated by multiplying the absolute number of leukocytes by the percentages determined by FACScan. As shown in Table 1, significant decreases in the absolute numbers of $CD3^+$, $TcR\alpha\beta^+$, $Thy1^+$, and $B220^+$ cells were evident in the PP of mice 4 weeks after infection with LP-BM5. Among $CD3^+$ T cells, $CD8^+$ T cells were more decreased than $CD4^+$ T cells during the course of MAIDS. At 10 weeks after infection, the absolute number of $B220^+$ cells in the PP of MAIDS mice was more decreased than those of $CD3^+$, $TcR\alpha\beta^+$, and $Thy1^+$ T cells. Furthermore, the remaining $B220^+$ cells expressed only low surface levels of IgM in PP of MAIDS mice (data not shown).

To determine whether apoptosis was associated with the decrease in the number of lymphocytes in PP during the course of MAIDS, we examined morphological changes by light microscopy. The histological analysis (hematoxylin-and-eosin staining) showed that PP in age-matched control mice had a typical tissue architecture characterized by germinal centers and follicles, whereas the latter were quite fibrous in the PP at 10 weeks after MAIDS induction although the stromal cell net

TABLE 1. Absolute numbers of cells expressing antigenic markers in PP from uninfected and MAIDS-infected mice

Group	Positive cells (10^5)[a]					
	$CD3^+$	$TcR\alpha\beta^+$	$Thy1^+$	$CD4^+$	$CD8^+$	$B220^+$
Uninfected[b]	18.0 ± 4.4	17.9 ± 4.1	17.2 ± 5.1	13.0 ± 5.5	4.5 ± 1.9	82.2 ± 34.4
MAIDS infected[c]	9.6 ± 3.3**	9.3 ± 3.4**	6.4 ± 2.7**	6.9 ± 2.9	0.8 ± 0.3**	16.8 ± 7.2**
MAIDS infected[d]	6.5 ± 2.6**	6.3 ± 2.4***	4.5 ± 2.2***	4.5 ± 2.0*	0.5 ± 0.2**	2.2 ± 1.0***

[a] Number of lymphocytes recovered from PP of each subset as determined by flow cytometry. Results are presented as means ± standard deviations of results for five mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus uninfected controls. The data are representative of three experiments with similar results.
[b] Eight-week-old mice. Results for 14-week-old uninfected mice were almost the same.
[c] Mice with MAIDS were inoculated with LP-BM5 4 weeks previously (8-week-old mice).
[d] Mice with MAIDS were inoculated with LP-BM5 10 weeks previously (14-week-old mice).

work remained intact (Fig. 2). Cells with chromatin condensation were more abundant in PP of mice with MAIDS than in PP of control mice. To further confirm the evidence for apoptosis in PP of MAIDS mice, we investigated apoptosis by in situ direct labelling of degraded DNA. As shown in Fig. 3, cells with DNA fragmentation were more abundant in PP of MAIDS mice than in PP of control mice.

B cells in PP were infected with the defective LP-BM5 MuLV. To confirm whether B cells in PP are infected with LP-BM5 MuLV, DNA was extracted from the sorted $B220^+$ cells (5×10^4) of PP and MLN from B6 mice infected with LP-BM5 MuLV 4 weeks previously and examined for integration of MuLV by PCR. As shown in Fig. 4, we found the genomic integration of defective LP-BM5 MuLV in B cells of PP, comparable to that of MLN B cells. Thus, B cells in PP, as well as those in the other lymphoid tissues, were infected with LP-BM5 MuLV at an early stage after infection.

Increment of CD95 expression on the surface of B cells in PP with the progression of MAIDS. We reported that CD95 expression was increased on T and B cells in the spleen during the course of MAIDS (15). We examined the kinetics of CD95 expression on $CD3^+$ T cells and $B220^+$ cells in PP and MLN during the course of MAIDS. Consistent with our previous report (15), $CD95^+$ B and T cells increased in MLN with the progression of MAIDS (Fig. 5). However, the degree of increased CD95 expression in T cells was weak. With the progression of MAIDS, B cells in PP displayed a marked increase in CD95 expression, but increased CD95 expression was not detected in T cells. The average mean fluorescence intensities of CD95 expression from six mice were as follows: 9.9 ± 1.3 and 29.1 ± 4.1 in MLN T cells, 8.8 ± 2.4 and 40.9 ± 6.7 in MLN B cells, 22.2 ± 2.8 and 14.0 ± 3.3 in PP T cells, and 15.9 ± 1.9 and 70.8 ± 9.1 in PP B cells (0 and 8 weeks after infection, respectively).

Decreased number of B cells in PP in CD95-deficient mice infected with the MAIDS virus. We found that PP T and B cells of MAIDS mice expressed CD95. To assess the contribution of the CD95 system to the apoptosis of T and B cells in PP of MAIDS mice, we examined the absolute numbers and percentage of cells in PP from LP-BM5-infected B6 *lpr* mice in which CD95 is mutant (1). Kanagawa et al. have found that murine AIDS is accelerated in CD95 mutant C57BL/6 *lpr*/*lpr* mice (23). Consistent with their findings, MAIDS was accelerated in CD95-deficient B6 *lpr* mice as assessed by mortality, splenomegaly, and increased number of abnormal $B220^+$ T cells (unpublished observation). As shown in Table 2, we found that

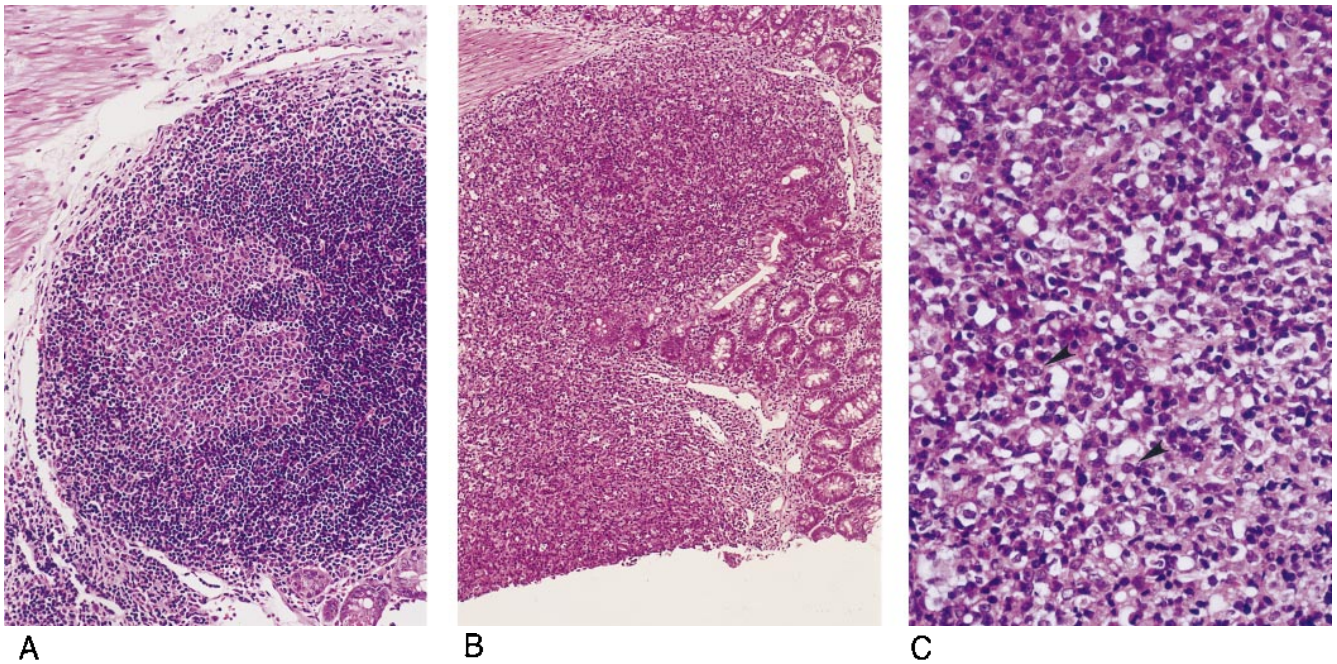

FIG. 2. Hematoxylin-and-eosin-stained tissue sections of PP in uninfected mice (A) and mice infected with MAIDS for 10 weeks (B and C). Arrows represent chromatin condensation. Magnifications: (A and B) ×64; (C) ×256.

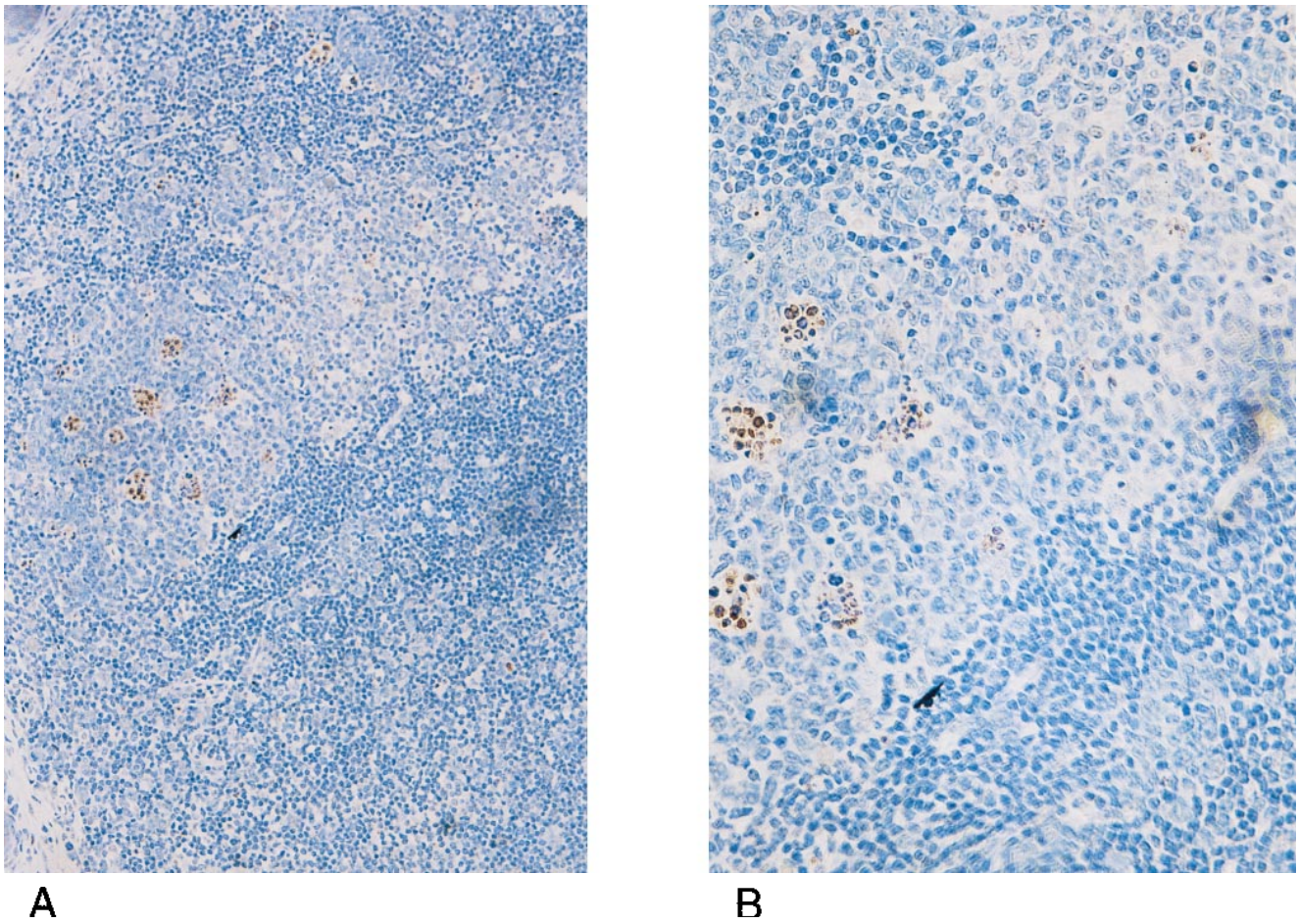

FIG. 3. In situ labelling of apoptotic cells in PP in uninfected mice (A and B) and LP-BM5-infected mice (C and D). Paraffin-embedded tissue pieces of PP from uninfected and 6-week-infected MAIDS mice were stained with peroxidase-labelled antidigoxigenin antibody and diaminobenzidine as described in Materials and Methods. Magnifications: (A and C) ×91; (B and D) ×364.

the number of B cells decreased in the PP of LP-BM5-infected B6 *lpr* mice but that the number of T cells was similar to that in age-matched uninfected controls. These results suggested that the CD95/CD95 L system participates in the induction of apoptosis in T but not B cells of the PP from mice with MAIDS. The decreased number of $CD95^+$ T cells in PP from MAIDS mice may be explained as a result of the apoptosis of $CD95^+$ T cells during the course of MAIDS.

Bcl-2 expression level in PP B cells. Bcl-2 represses the apoptosis of B cells induced by various stimuli (39). It was reported that interleukin (IL-10) prevents spontaneous death of germinal center B cells by induction of the Bcl-2 protein (31). We speculated that IL-10, which is reported to be overexpressed in MAIDS mice (35), might decrease in PP and not be able to inhibit apoptosis of cells. However, our findings indicated that IL-10 mRNA expression in PP cells from MAIDS mice was much greater than that in PP cells from uninfected controls (data not shown). Next, we examined Bcl-2 protein expression in PP cells by using a FACScan. The expression of Bcl-2 in B cells remaining in PP from 6-week-infected mice was similar to that in uninfected B cells (data not shown). Expression in LN B cells from 6-week-infected mice was also similar to that in uninfected LN B cells (data not shown). These results suggest that Bcl-2 protein may not be involved in suppressing the lymphocyte population in PP of MAIDS mice.

In vitro proliferative response of lymphocytes in PP of MAIDS mice. T-cell signals through CD40 rescues B cells from apoptosis induced by antigen receptor cross-linking (46). Therefore, it is possible that the apoptosis of B cells in PP of MAIDS mice is associated with aberrant CD40 signaling. We therefore examined the expression and function of CD40 on B cells in PP of MAIDS mice. The CD40 expression level on B cells in PP in MAIDS mice at 0, 4, 7, or 10 weeks after LP-BM5 infection was similar to that in uninfected mice (data not shown). Furthermore, that on the surface of B cells in MAIDS LN cells was also similar to that on B cells in uninfected LN cells (data not shown). We then examined the proliferation of B cells in PP from control mice and from 5-week-infected mice in response to anti-CD40 MAb. As shown in Fig. 6, B cells from control mice proliferated vigorously in response to anti-IgM and LPS, but the those from 5-week-infected mice had a diminished ability to respond to these stimuli. However, B cells in PP from 5-week-infected MAIDS mice proliferated to a similar extent as those from uninfected mice in response to anti-CD40 MAb. Moreover, intact CD40 signaling was also found in MAIDS LN B cells (data not shown). These results indicated that the signal pathway via CD40 may be intact, although signals through via the B-cell receptor or LPS are impaired in B cells of MAIDS mice. We speculate that aberrantly activated B cells in PP, but not in LN, may be particu-

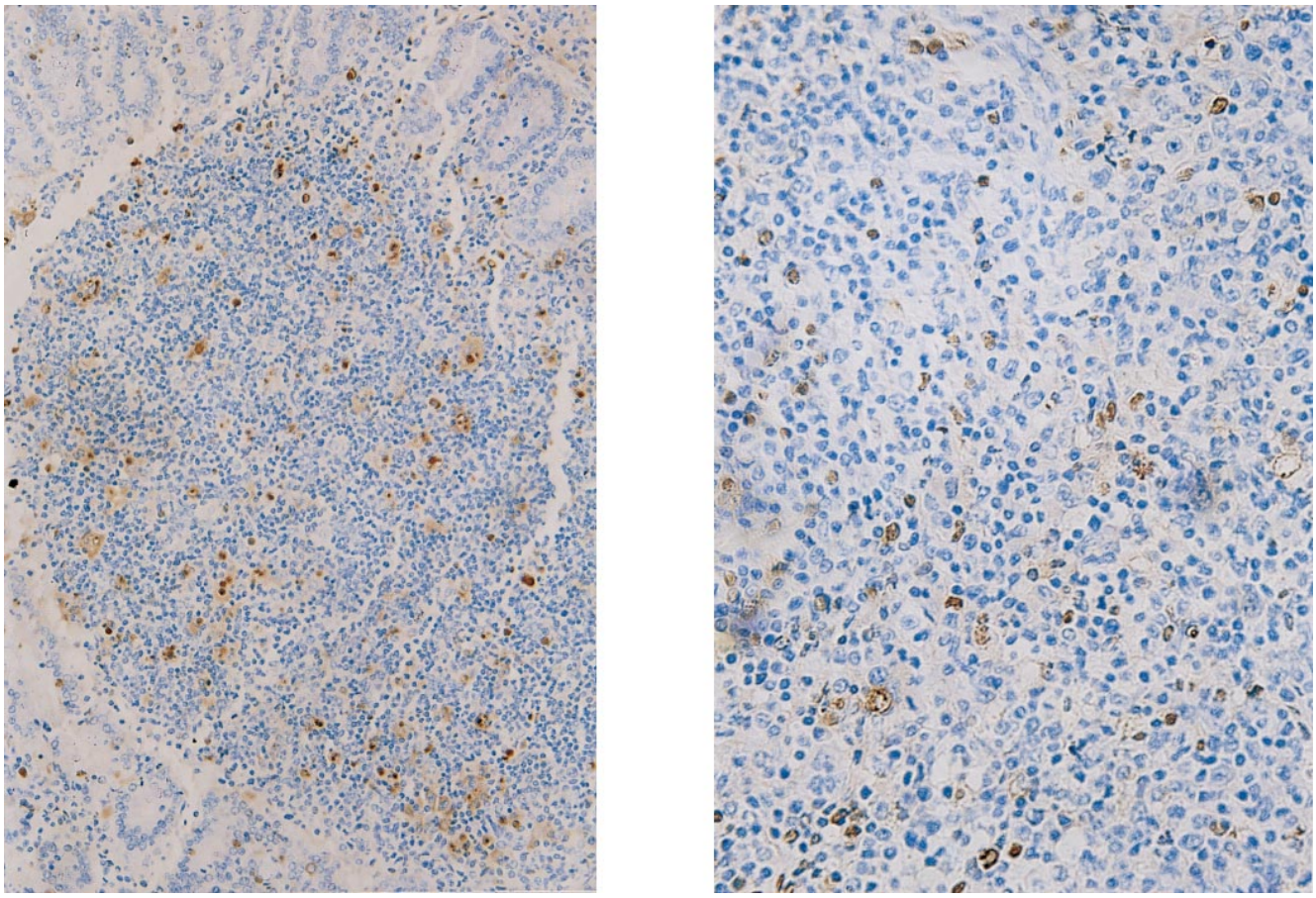

FIG. 3—*Continued.*

larly susceptible to apoptosis during the course of infection with the MAIDS virus.

DISCUSSION

We identified increased CD95 expression on T and B cells in the spleen during the course of MAIDS (15). Kanagawa et al. demonstrated that mice carrying mutations in either CD95 or CD95 L exhibited accelerated progression of the disease upon infection with the MAIDS virus (23). These results suggest that the CD95/CD95 L system plays an important role in protection against the lymphoproliferation induced by MAIDS virus. However, massive lymphoproliferation of both T and B cells is seen in the spleen and LN, and apoptosis in these organs during the progression of MAIDS is not evident. Here, we studied the lymphocytes in PP from MAIDS mice and found evidence of apoptosis. Our data suggested that the CD95/CD95 L system is involved in the apoptosis of T but not B cells in PP from MAIDS mice.

The CD95/CD95 L system is involved in the induction of apoptosis in various cells, including activated T and B cells, and contributes to termination of the immune response after a foreign antigen is eliminated. Several reports have shown that antigen induces apoptosis in peripheral activated T cells through CD95/CD95 L-mediated autocrine suicide (4, 9, 22). Westendorp et al. have reported that CD95-mediated apoptosis contributes to $CD4^+$ T-cell apoptosis in AIDS (48). These findings, together with a recent report showing the accelerated progression of MAIDS in CD95-mutated B6 *lpr* mice, indicated that the CD95/CD95 L system also plays a vital role in the host defense mechanism against retrovirus infection through eliminating virus-infected host cells (23). Kanagawa et al. have shown that the number of $CD4^+$ and $CD8^+$ T cells,

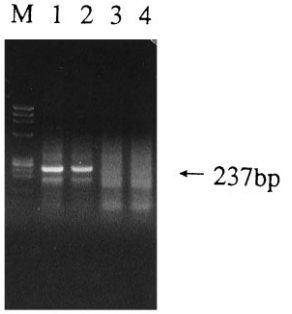

FIG. 4. Detection of the defective LP-BM5 MuLV genome integration by PCR. $B220^+$ B cells (5×10^4) were sorted with an EPICS Elite from MLN and PP cells of uninfected B6 mice or B6 mice infected with LP-BM5 MuLV 4 weeks previously. DNAs were extracted, and PCR was carried out as described in Materials and Methods. Lanes: M, size marker; 1, MLN B cells of infected mice; 2, PP B cells of infected mice; 3, MLN B cells of uninfected mice; 4, PP B cells of uninfected mice.

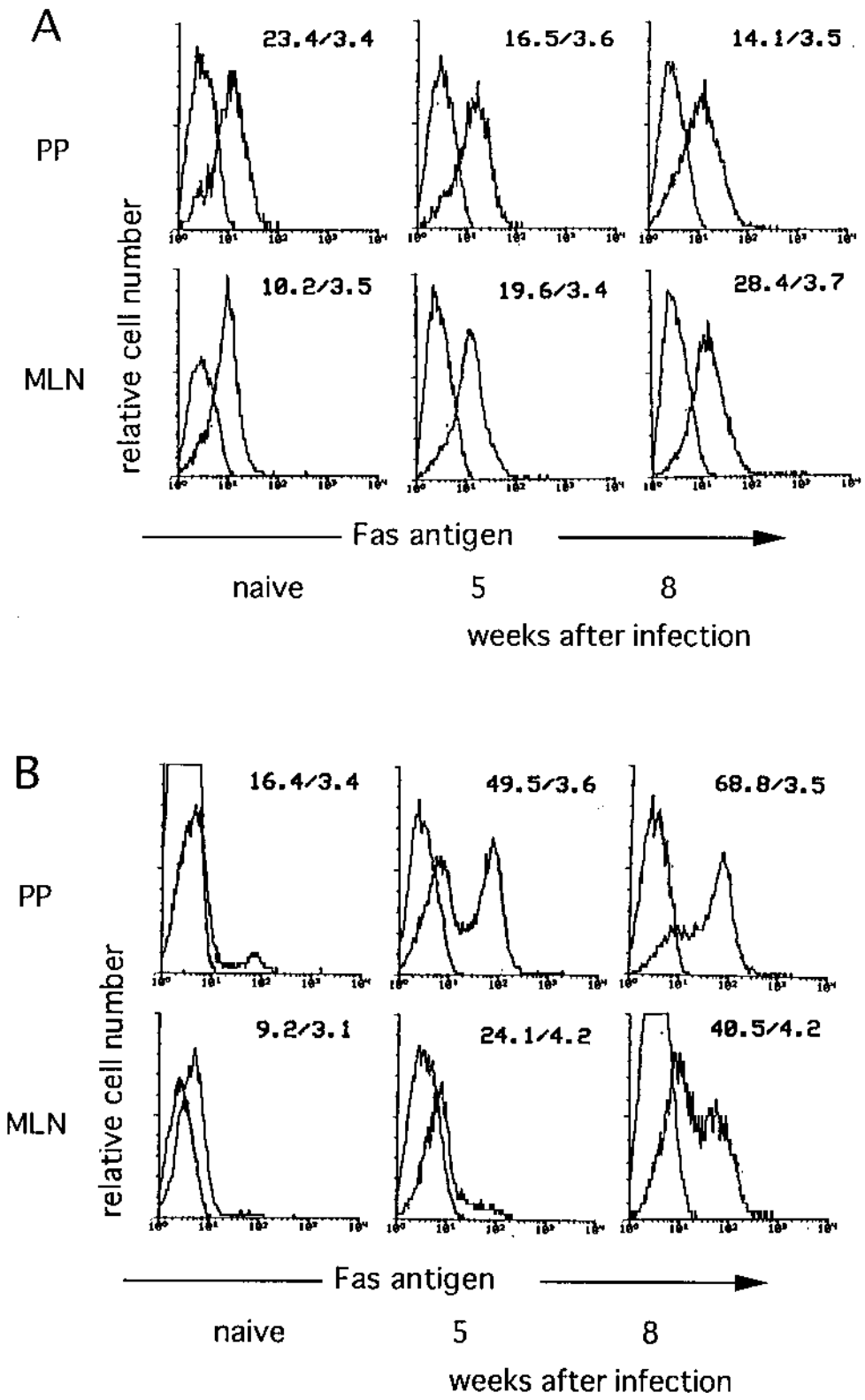

FIG. 5. Kinetics of CD95-positive cells in T cells (A) and B cells (B) during the course of MAIDS. PP and MLN cells from mice inoculated with LP-BM5 MuLV (0, 5, or 8 weeks after infection, respectively) were analyzed by two-color flow cytometry using PE-conjugated anti-CD95 MAb and FITC-conjugated anti-B220 MAb or FITC-conjugated anti-CD3 MAb. CD95 expression is represented by histograms after gating of B220- or CD3-positive cells. Negative controls were similarly stained without PE-conjugated anti-CD95 MAb. The number in each group indicates mean fluorescence intensity compared with the negative control. Results from one representative experiment of three are shown.

FIG. 6. Activation of B cells in PP from uninfected mice and 5-week-infected MAIDS mice with various stimuli. PP cells were plated at 2×10^5. Cells were stimulated with either anti-CD40 MAb (5 μg/ml), anti-IgM MAb (5 μg/ml), LPS (10 μg/ml), or anti-CD40 plus anti-IgM MAbs (each at 5 μg/ml) for 48 h and pulsed with [^{3}H]TdR for the last 8 h. Determinations were performed in quadruplicate, and the results are expressed as the means and standard deviations. Black bars, uninfected mice; striped bars, MAIDS mice. *, $P < 0.001$ versus uninfected controls. The data are representative of three experiments with similar results.

including Thy1$^-$ CD4$^+$ T cells, increases about sixfold, while non-T cells showed only a twofold increase in number in B6 *lpr* mice with MAIDS compared with B6 MAIDS mice (23). Consistent with their results, we found that the number of CD3$^+$ T cells, especially Thy1$^-$ CD4$^+$ TcRαβ T cells which are thought to be infected with the MAIDS virus, significantly increased in B6 *lpr* mice with MAIDS, whereas the number of surface IgM$^+$ B cells, including both IgM^{high} and IgM^{low} B cells, was not changed in LN from B6 *lpr* mice with MAIDS (data not shown). We demonstrated here that the absolute number of T cells and the frequency of CD95$^+$ T cells decreased in PP from B6 mice with the progression of MAIDS and that B6 *lpr* mice infected with MAIDS did not show a decrease in the number of T cells in PP. These results suggested that the CD95/CD95 L system plays an important role in T-cell apoptosis in PP during the course of MAIDS. On the other hand, the number of B cells in PP decreased even in B6 *lpr* mice with MAIDS to the level seen in B6 MAIDS mice. B cells, which were mostly infected with MAIDS virus (19), may die through apoptosis independently of the CD95/CD95 L system.

The mechanism of B-cell apoptosis remain elusive. Several studies have demonstrated that stimulation through CD40, a glycoprotein expressed in both immature and mature B cells, rescued germinal center and mature B cells from apoptotic death (3, 10, 46). B cells in MAIDS mice simulate germinal center B cells expressing low surface IgM. Therefore, we speculate that CD40 signaling on B cells in PP of MAIDS mice is impaired, and then those cells are vulnerable to apoptosis. However, our data provided evidence that the CD40 expression level on B cells of PP in MAIDS mice is similar to that in uninfected control mice and that the proliferative response of PP cells to anti-CD40 MAb occurred normally, in contrast to the response to anti-IgM MAb or LPS. These results exclude the notion of an intrinsic defect in CD40 signaling in B cells in PP of MAIDS mice. Our data also demonstrated that T-cell function appeared to be impaired before B-cell function in PP during the course of MAIDS, indicating that insufficient signaling provided by CD40 L on PP T cells causes apoptosis in B cells in PP from MAIDS mice (data not shown). However, B6 *lpr* mice with MAIDS showed a decrease in the number of PP B cells, although the number of T cells in PP remained normal. Moreover, our preliminary experiment indicated that CD40 L mRNA expression levels in PP T cells from both B6 MAIDS

TABLE 2. Absolute numbers and percentage of cells expressing antigenic markers in PP from uninfected and MAIDS-infected B6 *lpr* mice

Group	No. (10^6) of cells[a]						
	Total	B220$^+$	CD3$^+$	TcRαβ$^+$	CD4$^+$	CD8$^+$	B220$^+$ CD3$^+$
Uninfected[b]	7.2 ± 2.5	5.7 ± 1.8 (79.8 ± 1.4[c])	1.3 ± 0.4 (17.6 ± 1.1)	1.2 ± 0.5 (16.3 ± 0.9)	0.9 ± 0.3 (12.4 ± 1.1)	0.3 ± 0.1 (3.6 ± 1.0)	0.2 ± 0.1 (1.8 ± 0.2)
Infected[d]	2.8 ± 0.3*	1.2 ± 0.1** (44.2 ± 5.3)	1.3 ± 0.1 (46.2 ± 0.2)	1.2 ± 0.2 (39.9 ± 0.1)	1.2 ± 0.1 (42.4 ± 6.6)	0.3 ± 0.1 (10.4 ± 2.1)	0.3 ± 0.1 (9.8 ± 0.8)

[a] Means ± standard deviations of results for five mice. *, $P < 0.01$; **, $P < 0.001$ versus uninfected controls. The data are representative of three experiments with similar results.
[b] Ten-week-old mice.
[c] Percentage of cells in the population.
[d] Mice with MAIDS were inoculated with LP-BM5 6 weeks previously (10-week-old mice).

and B6 *lpr* MAIDS mice were much higher than in uninfected PP T cells (unpublished observations). Therefore, B cells in PP of MAIDS mice may undergo apoptosis independently of T-cell help in MAIDS mice. Selevy et al. have reported that splenic B cells from MAIDS mice reflect defects in early signaling through the antigen-specific IgM receptor as well as a generalized defect in proliferative responsiveness (45). On the other hand, the signal pathway that mediates the biological effects of CD40 remains elusive, although a CD40-binding protein designated CRAF1 has been identified (6). B cells stimulated via only CD40 easily undergo apoptosis induced by CD95 (42, 43). Moreover, it has been demonstrated that CD40 ligation induces apoptosis in transformed cells of mesenchymal and epithelial origin through pathway distinct from CD95 (14). Further analysis of the signal pathway via CD40 is required to understand B-cell apoptosis.

The *bcl-2* gene family, which includes *bcl-2* and *bcl-xL*, is expressed in lymphoid cells, and its members are potent regulators of T- and B-cell apoptosis (39). We compared the expression levels of Bcl-2 protein on B cells in PP in MAIDS and uninfected mice and found that there was no significant difference. Wang et al. have recently reported that CD40 engagement induces *bcl-xL* in immature B-cell lines (47). In contrast, Merino et al. reported that activation through IgM receptors but not CD40 induced up-regulation followed by the rapid down-modulation of *bcl-xL* (33). Our preliminary data suggested that the *bcl-xL* mRNA expression is increased in PP lymphoid cells of MAIDS mice without stimulation (data not shown). The level of *bcl-2* gene family expression may not be involved in the apoptosis of PP B cells during the course of MAIDS.

We do not know at present why apoptosis but not massive lymphoproliferation occurs in PP during the course of MAIDS. PP are exposed to an abundant level of LPS derived from intestinal flora. Therefore, stimulation with LPS derived from intestinal microflora may be involved in accelerating apoptosis in activated B cells, particularly in PP from MAIDS mice. L-selectin antigen (Mel-14) and integrin $\alpha4\beta7$ (LPAM-1) are important as homing receptors for T cells committed to PP (11, 12, 17). Preliminary data obtained by Lopez et al. indicated no changes in the percentage of LPAM-1^+ cells in the thymus but a significant decrease in the percentage of L-selectin-bearing cells in the thymuses of LP-BM5-infected mice (32). Most of the lymphocytes of the LN and spleens of MAIDS mice expressed CD44 but not L-selectin (reference 15 and data not shown). It is alternatively possible that the migration of lymphoid cells to PP is impaired, and consequently apoptosis rather than lymphoproliferation may be evident in PP from MAIDS mice.

PP are GALT and may participate in the host defense at the mucosa by IgA secretion. Lopez et al. (32) have reported that LP-BM5 MuLV infection suppresses lymphocyte populations in PP or in ILP. The decrease in the $CD4^+$ T cells in the PP or ILP from MAIDS mice is in agreement with the observation that patients with AIDS exhibit an inverted $CD4^+/CD8^+$ ratio not only in peripheral blood but also in the duodenal lamina propria (44). It is speculated that suppression of IgA plasma cell populations in ILP is partly due to impaired T-cell differentiation or a decrease in the number of T helper cells, which collaborate in all steps of IgA B-cell differentiation (25–27). Darban et al. (7) have found that resistance to gastrointestinal pathogens such as *C. parvum* is impaired in mice with MAIDS because of decreased IgA plasma cell precursors in the mucosa. IgA produced by B cells binds to intestinal parasites, reducing attachment to mucosal surfaces.

In conclusion, the CD95/CD95 L system is involved in apoptosis of T but not B cells in PP of mice with MAIDS. Aberrant activation of B cells in PP from MAIDS mice may be implicated in the apoptosis. The cellular mechanisms of these events in MAIDS need further investigation. However, our findings provide a clue to understanding the mechanisms of apoptosis in PP cells from retrovirus-infected patients and animals and serve as an experimental basis to approach the intervention to prevent or reverse the resulting immunosuppression.

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