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Modulation of Cytokine Expression by CD4$^+$ T Cells during Coxsackievirus B3 Infections of BALB/c Mice Initiated by Cells Expressing the γδ$^+$ T-Cell Receptor

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Two variants of coxsackievirus B3 have been used to investigate the pathogenesis of myocarditis in BALB/c mice. H3 virus induces moderate myocarditis and H310A1 virus induces minimal myocarditis, although both viruses infect and replicate in the heart. Cells expressing the γδ T-cell receptor composed 5 to 13% of the lymphocytes infiltrating the hearts of H3 virus-infected mice and belonged to either the CD4$^-$ CD8$^+$ γδ$^+$- or CD4$^-$ CD8$^-$ γδ$^+$-cell population. Giving 5,000 γδ$^+$ cells isolated from the hearts of H3 virus-infected mice to H310A1 virus-infected recipients restored myocarditis susceptibility in the recipient animals and shifted the pattern of cytokine production in the virus-immune CD4$^+$-cell population from being predominantly interleukin-4 producing to being predominantly gamma interferon producing in the H310A1 virus-infected mice. Apoptosis was evident in the infiltrating lymphocyte population in the myocardia of H3 virus-infected mice by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling assay and in splenic lymphocytes by DNA fragmentation in agarose gel electrophoresis and was confined to the CD4$^+$ population. No apoptosis was observed in H310A1 virus-infected mice, but apoptosis was induced subsequent to γδ$^+$-T-cell transfer. These results are consistent with the hypothesis that γδ$^+$ T cells may help modulate cytokine responses during virus infections in vivo and that apoptosis might be involved in this modulation.

Although γδ$^+$ T cells often accumulate in inflammatory lesions, especially during viral infections (4), their role in the inflammatory process is poorly understood. Because there can be an inverse relationship between the number of γδ$^+$ and CD4$^+$ cells in the inflammatory cell infiltrate (1a) and because γδ$^+$ cells selectively inhibit Th2-like CD4$^+$-cell responses (3, 20), one function of these cells may be to regulate CD4$^+$-cell responses. The mechanism(s) by which this occurs is less clear. These effectors often produce substantial levels of proinflammatory cytokines which may directly suppress Th2-like cell differentiation (26, 33). Alternatively, since γδ$^+$ cells express high levels of FasL mRNA but little Fas, it is possible that γδ$^+$ cells might regulate CD4$^+$-cell responses through Fas-mediated mechanisms of apoptosis (1).

Previously, this laboratory reported that BALB/c mice infected with a myocarditic variant (H3) of coxsackievirus B3 (CVB3) activated γδ$^+$ T cells in vivo, while mice given an amyocarditic CVB3 variant (H310A1) did not (14, 17, 18, 29). Adoptive transfer of as few as 5,000 purified γδ$^+$ cells to H310A1 virus-infected mice restored pathogenicity, demonstrating that these cells may play an important role in the disease process (14). Evidence further suggests that Th2-like cells activated during H310A1 virus infections may prevent the induction of substantial myocarditis (17, 18). Since published studies indicate that γδ$^+$ T cells can selectively inhibit Th2-like cell responses (20), we hypothesize that the γδ$^+$ T cells activated during H3 virus infections might be contributing to pathogenesis either by the selective elimination of the Th2-like CD4$^+$-cell population or through the release of cytokines which preferentially activate Th1-like CD4$^+$ cells.

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MATERIALS AND METHODS

Mice. BALB/c mice were originally purchased from Cumberland Farms, Clinton, Tenn., and a breeding colony of these animals has been maintained at the University of Vermont. Male mice, 6 to 8 weeks of age, were used in these experiments. Mice were injected intraperitoneally with 10^4 PFU of virus in 0.5 ml of phosphate-buffered saline (PBS). Groups contained a minimum of three mice, and at least two replicate experiments were performed for each study.

Viruses. H3 and H310A1 viruses were originally derived from the Woodruff variant of CVB3 (Nancy) and their titers were determined by the plaque-forming assay on HeLa cell monolayers as described previously (29). Infectious cDNA clones of these viruses were produced in the laboratory of Kirk Knowlton (Department of Cardiology, University of California at San Diego, San Diego). The cDNA clones were transfected into HeLa cells by the calcium phosphate coprecipitation method. The virus-containing supernatants were kindly supplied by K. Knowlton; titers were determined by the plaque-forming assay, and viruses were aliquoted and stored at −70°C until used.

Antibodies. Hybridomas making monoclonal antibody (MAb) to CD4 (clone GK 1.5), CD8 (clone 2.43), γδ T-cell receptor (TCR) (clone GL3-3A), and αβ TCR (clone H57-597) were purchased from the American Type Culture Collection (ATCC, Bethesda, Md.) and were grown in ascites fluid as described previously (30). Fluorescein isothiocyanate (FITC)-conjugated anti-γδ TCR, FITC-conjugated anti-hamster immunoglobulin G, hamster anti-murine Fas, phycoerythrin (PE)-conjugated anti-CD4, biotinylated and unlabeled rat anti-mouse interleukin-4 (IL-4) and biotinylated and unlabeled rat anti-mouse gamma interferon (IFN-γ) were purchased from Pharmingen Co., San Diego, Calif. Red 613-conjugated anti-CD8a was purchased from Sigma Immunochemicals, St. Louis, Mo.

Isolation of lymphocyte subpopulations. Spleens were aseptically removed and pressed through fine-mesh screens. The single-cell suspension was centrifuged on Histopaque (Sigma) at 300 × *g* to remove erythrocytes and passaged through nylon wool to enrich for T lymphocytes. Hearts were aseptically removed, minced, and subjected to enzymatic digestion with 200 U of collagenase II (Gibco) per ml and 2.5 g of pancreatin (Gibco, Grand Island, N.Y.) per liter. The single cells were centrifuged on Histopaque to isolate the lymphocyte population. Enriched populations of CD4$^+$ and CD8$^+$ cells were obtained by using Dynabeads M-450 (Dynal, Oslo, Norway). Lymphocytes were incubated with 50 μg of antibody per ml, washed, and then incubated with anti-rat immunoglobulin-coated Dynabeads for 30 min at 4°C with frequent agitation. The beads and associated cells were subsequently recovered by using a magnetic field. This enrichment protocol usually results in a >90% purity of CD4$^+$ or CD8$^+$ cells.

γδ$^+$ lymphocytes were isolated from BALB/c mice infected 7 days earlier with H3 virus. Lymphocytes were isolated from the hearts as described above and then adsorbed to 6-well tissue culture plates (Corning) coated with 100 μg of anti-γδ TCR MAb (clone GL3-3A) per ml in carbonate buffer, pH 9.6. Nonattached cells were removed by washing, and the attached population was retrieved

by trypsinization (0.25% trypsin; Gibco). The cells were washed and counted by trypan blue exclusion. Average recovery was approximately 7×10^3 $\gamma\delta^+$ cells per mouse.

Flow cytometry. Approximately 10^5 lymphocytes were labeled with a 1:100 dilution of commercial primary fluorochrome-conjugated antibodies in blocking buffer (PBS containing 1% bovine serum albumin [Sigma] and 0.01% sodium azide [Sigma]) at 4°C for 20 min. The cells were washed once with blocking buffer and resuspended in PBS containing 1.5% paraformaldehyde and 0.01% sodium azide. For Fas protein analysis, lymphocytes were initially incubated with a 1:100 dilution of hamster anti-Fas for 20 min, washed, and then incubated with a 1:100 dilution of mouse anti-hamster immunoglobulin G and a 1:100 dilution of PE-conjugated rat anti-CD4 antibodies. Lymphocyte analysis was performed with a Coulter Epics Elite (Coulter Co., Miami, Fla.) with a single excitation wavelength (488 nm) and band pass filters for FITC (530 nm), Red-PE (575 nm), and Red 613 (613 nm).

TdT-TUNEL. The transferase-mediated dUTP-biotin nick-end labeling (TUNEL) staining method for tissue sections uses formalin-fixed, paraffin-embedded tissues, as described by Gavrieli et al. (10). Five-micrometer-thick sections were placed onto glass slides, dewaxed, and rehydrated. Nuclear proteins were stripped by digestion with 25 μg of proteinase K (Sigma) per ml for 10 min at 37°C, and endogenous peroxidases were inactivated with 0.6% H_2O_2. The sections were reacted with terminal deoxynucleotidyl transferase (TdT) enzyme (30 U/100 μl; Boehringer Mannheim) and biotin-UTP (0.5 nM/100 μl; Boehringer Mannheim) in Trizma-cacodylate-cobalt buffer (Sigma). The slides were blocked with 2% bovine serum albumin for 30 min at room temperature and reacted with streptavidin-peroxidase reagent (Zymed) for 20 min and 3-amino-9-ethylcarbazole (AEC) chromogen (Zymed) for 20 min to form the brick red product. No counterstain was used.

DNA isolation and gel electrophoresis. A total of 10^6 lymphocytes were resuspended in sample buffer (2 mg of bromphenol blue in 0.1 ml of 1 M Tris buffer added to 9.9 ml of glycerol) containing 100 μg of RNase A per ml and loaded into wells made of 1% agarose in Tris-borate-EDTA buffer containing 2% sodium dodecyl sulfate (SDS) and 100-μg/ml proteinase K. The 1% agarose section consisting of the loading wells was fused with the remainder of the gel consisting of 2% agarose in Tris-borate-EDTA buffer. The gel was run for 16 h at 30 V at room temperature, stained with 2 μg of ethidium bromide per ml for 1 h, washed overnight with distilled water, and photographed under UV light.

Precursor frequency analysis. Precursor frequency analysis of virus-reactive $CD4^+$ T cells was performed as described previously (17). Cytokine release into culture supernatants was determined with capture enzyme-linked immunosorbent assays (ELISAs). Capture and biotinylated detection antibodies and recombinant cytokines were obtained from Pharmingen, and the cytokine ELISA protocol was supplied by this company. Briefly, the capture antibodies (rat anti-mouse IL-4, clone 11B11; rat anti-mouse IFN-γ, clone R4-6A2) were diluted to 2 μg/ml in coating buffer (0.1 M $NaHCO_3$ [pH 8.2]), and 50 μl was added to wells of Immunol-2 microtiter plates (Dynatech Labs, Chantilly, Va.). The plates were incubated at 4°C overnight, washed twice with PBS containing 0.05% Tween-20 (Sigma), incubated for 2 h at room temperature with PBS–10% newborn calf serum (Gibco), and washed again with PBS-Tween. Dilutions of recombinant IL-4 from 2 ng/ml and recombinant IFN-γ from 10 ng/ml were used to establish standard curves. A total of 100 μl of either the standards or the tissue culture supernatants was added to the wells in duplicate, incubated overnight at 4°C, and removed by washing four times with PBS-Tween. A total of 100 μl of the biotinylated detection antibodies (1 μg/ml) (rat anti-mouse IL-4, clone BVD4-1D11; rat anti-mouse IFN-γ, clone XMG1.2) was added to each well. The plates were incubated for 45 min at room temperature, washed six times, incubated with a 1:400 dilution of a 1-mg/ml solution of an avidin-alkaline phosphatase conjugate (Sigma) for 30 min, washed thoroughly, and then incubated with disodium *p*-nitrophenyl phosphate (1 mg/ml) (Sigma) for 30 min. The reaction was stopped by using 3 N NaOH, and the yellow reaction product was read at A_{405} with a Dynatech MicroELISA Minireader MR590. Estimates of precursor frequency were determined by the maximum-likelihood method of Good et al. (11).

Histology. Hearts were fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin. Slides were coded and read blindly according to a scale of 0 to 4, with 0 representing no myocarditis, 1 representing between 1 and 10 foci per section, 2 representing between 11 and 20 foci, 3 representing between 21 and 40 foci, and 4 representing widespread and confluent lesions.

Statistical evaluation. Statistical evaluations were performed by using either the Wilcoxon ranked score or the Student *t* test.

RESULTS

Demonstration of shift in virus-specific $CD4^+$ Th subset responses after adoptive transfer of $\gamma\delta^+$ T cells into H310A1 virus-infected mice. BALB/c mice were infected with the pathogenic H3 virus and killed 7 days later. Hearts were removed and the infiltrating lymphoid cell population was retrieved. Three-color staining of the inflammatory cells for CD4, CD8, and γδ TCR demonstrated that more $CD8^+$ cells (613) were present than $CD4^+$ cells (Red-PE). Approximately 5 to 13% of the recoverable lymphoid cells were $\gamma\delta^+$ (FITC), and approximately two-thirds of these were $CD4^-$ $CD8^-$ γδ and one-third were $CD4^-$ $CD8^+$ $\gamma\delta^+$. This distribution is derived from the range of $\gamma\delta^+$ cells isolated from the hearts of five individual animals. Figure 1 gives the flow diagrams of the lymphocytes isolated from a single representative mouse. Next, BALB/c mice were infected with H310A1 virus and half of the animals were additionally injected intravenously with various amounts of purified $\gamma\delta^+$ T cells on the day of infection. As a control, an additional group of mice were injected with the myocarditic H3 virus. All mice were killed 7 days after infection and evaluated for myocarditis and cardiac virus titers (Table 1). Figure 2 shows representative histological sections for the mice analyzed in Table 1. As reported previously, H3 virus infection induced severe cardiac inflammation, while the amyocarditic H310A1 virus induced minimal heart disease. Giving H310A1 virus-infected mice the $\gamma\delta^+$ T cells resulted in levels of myocarditis similar to those observed in H3 virus-infected mice. Although cardiac virus titers were slightly lower in H310A1 virus-infected mice than in mice given the H3 virus, this difference is unlikely to explain the poor pathogenicity of the H310A1 virus, since animals given H310A1 virus and $\gamma\delta^+$ cells also had low virus titers but greatly increased myocarditis.

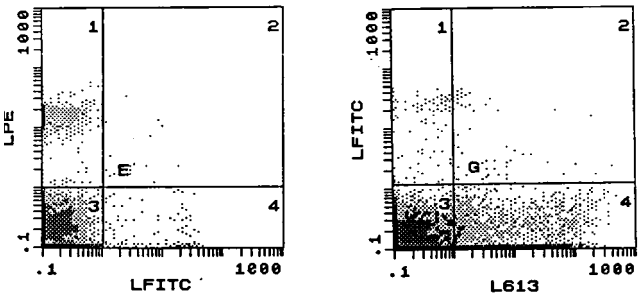

FIG. 1. Analysis of heart-infiltrating lymphocytes by three-color flow cytometry. Lymphocytes were isolated from the hearts of BALB/c mice infected 7 days earlier with H3 virus and were stained with PE-conjugated anti-CD4, R613-conjugated anti-CD8, and FITC-conjugated anti-γδ TCR MAbs. Background fluorescence was less than 3 to 5% as determined with FITC- and PE-conjugated isotypic antibodies. These graphs show the lymphocytes isolated from a single mouse and are representative of the results obtained from the analysis of a total of five individual animals. LPE, lymphocytes stained with PE-conjugated anti-CD4 MAb; LFTIC, lymphocytes stained with FITC-conjugated anti-γδ MAb. L613, lymphocytes stained with Red 613-conjugated anti-CD8 MAb; E, $CD4^+$ $\gamma\delta^+$ cells; G, $CD8^+$ $\gamma\delta^+$ cells.

TABLE 1. Myocarditis and cardiac virus titers in BALB/c mice infected with H310A1 virus and given $\gamma\delta^+$ T lymphocytes[a]

Virus	No. of $\gamma\delta^+$ T cells	Myocarditis score[b]	Cardiac virus titer ($\log_{10}$ PFU)
H3	0	1.8 ± 0.2[c]	6.43 ± 0.16[c]
H310A1	0	0.5 ± 0.2	5.77 ± 0.20
H310A1	5×10^2	0.9 ± 0.1	5.58 ± 0.16
H310A1	5×10^3	1.6 ± 0.2[c]	5.20 ± 0.23[c]
H310A1	1×10^4	2.2 ± 0.1[c]	4.96 ± 0.13[c]

[a] BALB/c mice were infected intraperitoneally with 10^4 PFU of virus. Some animals also received $\gamma\delta^+$ T cells isolated from the hearts of H3 virus-infected mice. $\gamma\delta^+$ cells were injected intravenously into recipient mice on the day of infection. All animals were killed 7 days after infection. Hearts were evaluated for myocarditis and for virus. Values are means ± standard errors of the means for four mice.

[b] Myocarditis scores are reported according to the 0-to-4 scale described in Materials and Methods.

[c] Significantly different from the value for the H310A1 virus-only group at $P \leq 0.05$.

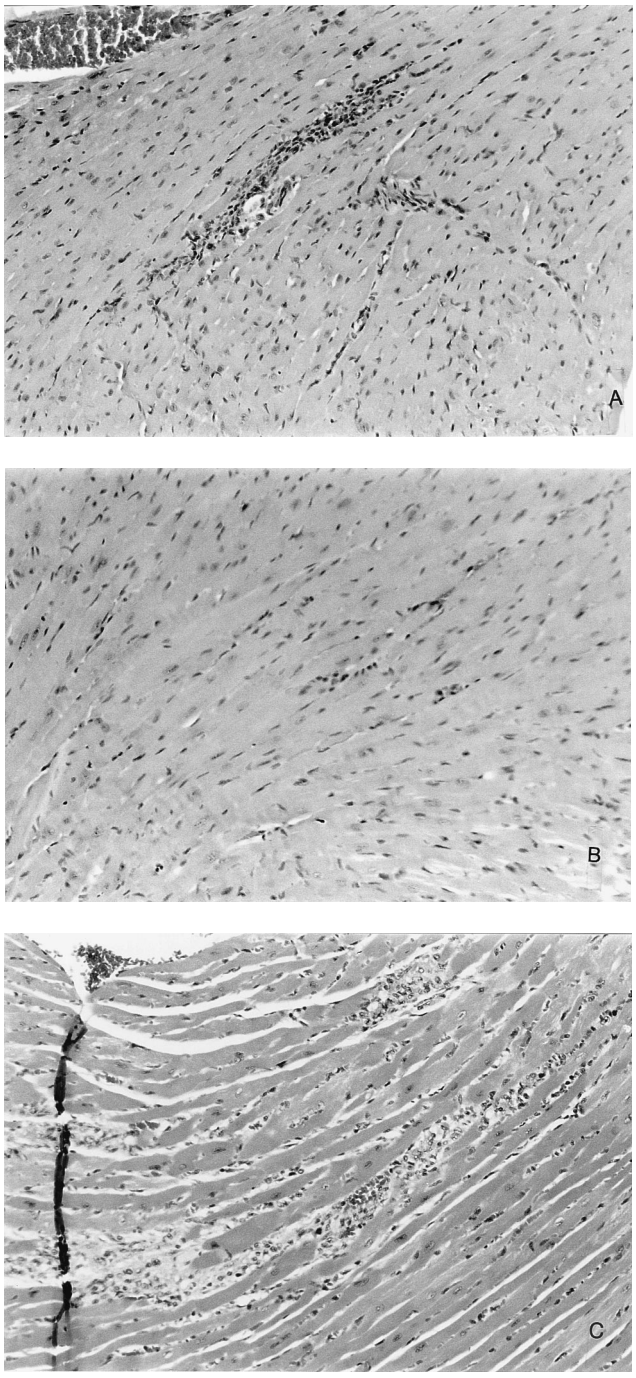

FIG. 2. Cardiac inflammation in BALB/c mice infected 7 days earlier with CVB3. Mice were infected intraperitoneally with 10^4 PFU of either H3 (A) or H310A1 (B and C) virus. Mice represented in panel C received an intravenous injection, on the same day as the H310A1 virus infection, consisting of 5,000 purified γδ+ T lymphocytes derived from the heart inflammatory cells of H3 virus-infected mice. Histological sections were stained with hematoxylin and eosin.

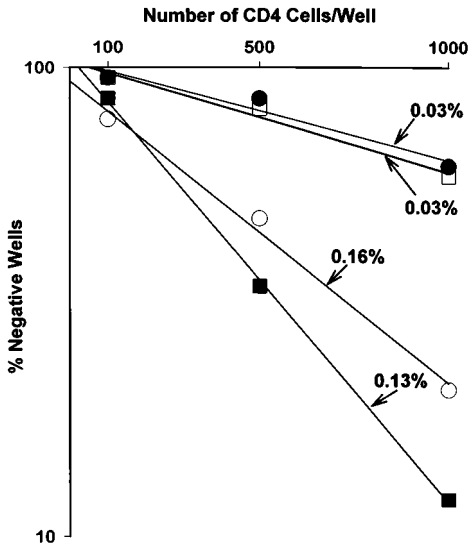

FIG. 3. Precursor frequency analysis of IFN-γ- and IL-4-producing CD4+ T cells. Purified CD4+ T cells were isolated from the spleens of BALB/c mice injected 7 days earlier with H310A1 virus only (solid symbols) or with H310A1 virus and 5,000 purified γδ+ T cells (open symbols). Various concentrations of the CD4+ cells were cocultured with syngeneic antigen-presenting cells and H310A1 virus for 14 days and were washed and restimulated with 5 μg of concanavalin A for 3 days. The supernatants were assayed for IFN-γ (● and ○) and IL-4 (■ and □) by ELISA. Precursor frequencies are given by each line and were determined for each lymphocyte population by using Poisson statistics (14). The data are the results from a single mouse in each group and are representative of three individual animals analyzed per group.

One potential concern is that H3 virus might be carried over with the γδ+-cell population during adoptive transfer. To rule out this possibility, six aliquots of the enriched γδ+ cells (5×10^3 each) were homogenized and virus titers were determined. No PFU were observed in any of the aliquots, indicating that infectious virus could not be detected in these cells. These data are consistent with our previous unpublished observations that while H3 virus infects and replicates in macrophage and B lymphocytes, T lymphocytes appear incapable of harboring the virus.

Next, spleen CD4+ T cells were isolated from H310A1 virus-infected mice with or without γδ+-T-cell treatment. Precursor frequency analysis of IFN-γ- and IL-4-producing virus-specific CD4+ cells was determined as described earlier (17) (Fig. 3). The data show that the frequency of IL-4-producing virus-specific CD4+ cells is fourfold greater than that of IFN-γ-producing cells in H310A1 virus-infected mice. In contrast, adoptive transfer of the activated γδ+ T cells reversed this trend and resulted in a greater-than-fivefold prevalence of IFN-γ-producing, compared with IL-4-producing, virus-specific CD4+ T cells in these animals.

Demonstration of selected apoptosis of CD4+ cells. The above-described studies demonstrate that γδ+ cells constitute a significant portion of the inflammatory cell population in the hearts of H3 virus-infected mice and that adoptive transfer of these γδ+ cells both augments myocarditis in H310A1 virus-infected animals and either directly or indirectly causes a shift in the predominant cytokine produced by virus-specific CD4+ cells. The subsequent experiments investigated how γδ+ cells effect this Th-like population shift. Two explanations seemed most likely. γδ+ cells alter Th subpopulations either through the release of specific cytokines such as IFN-γ or through the induction of selected apoptosis in CD4+ subpopulations. Apoptosis can be observed in the inflammatory cell infiltrates in the hearts of H3 virus-infected mice by using the TdT-TUNEL assay (Fig. 4). Additionally, lymphocytes were isolated from the spleens of H3 and H310A1 virus-infected mice 2, 4, and 7 days after infection. DNA was isolated and evaluated by agarose gel electrophoresis (Fig. 5). DNA from splenocytes of

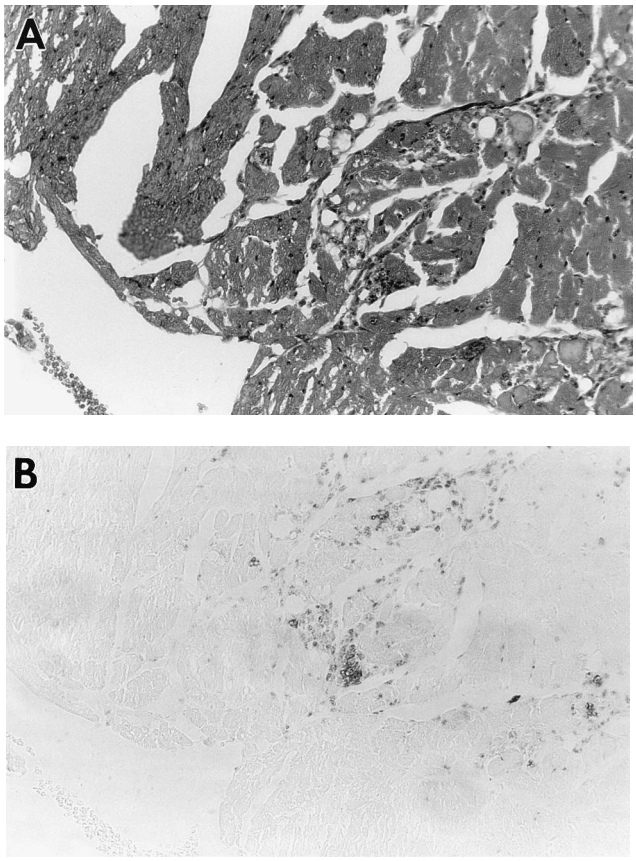

FIG. 4. Apoptosis in H3 virus-infected BALB/c mice. Hearts from BALB/c mice infected 7 days earlier with 10^4 PFU of H3 virus were stained by hematoxylin and eosin (A) or TdT-TUNEL assay (B). Sections are from a single animal out of a total of four mice.

H310A1 virus-infected mice showed no degradation, but DNA from H3 virus-infected mice showed the characteristic laddering pattern associated with apoptosis. Separation of the lymphocytes into $CD4^+$ and $CD8^+$ subpopulations prior to DNA extraction and electrophoresis further indicated that apoptosis is restricted to the $CD4^+$-T-cell population. DNA analysis of splenocytes from mice given H310A1 virus and $\gamma\delta^+$ cells shows a rapid induction of apoptosis (Fig. 6, lane 5). Giving activated $\gamma\delta^+$ cells to uninfected mice did not induce detectable DNA laddering in the splenocyte population, implying that antigen stimulation is necessary for this effect (data not shown). Depletion of $CD8^+$ cells, by intraperitoneally injecting 100 μg of MAb to CD8 per mouse on the same day as the virus, reduces but does not eliminate DNA laddering. DNA degradation in lymphocytes from mice given $\gamma\delta^+$ T cells was primarily localized in the $CD4^+$ T-cell population (data not shown).

DISCUSSION

$CD4^+$ cells can be distinguished by the types of cytokines they produce (5, 6, 22). $CD4^+$ cells start as Th0 cells which produce a range of cytokines and then differentiate subsequent to antigen stimulation into Th1 cells (producing predominantly proinflammatory cytokines, e.g., IFN-γ, IL-2, and tumor necrosis factor) or Th2 cells (producing cytokines involved with T-cell-dependent-B-cell help, e.g., IL-4, IL-6, and IL-10) (25). Conditions prevalent during $CD4^+$-cell differentiation (such as the type of antigen-presenting cell, the presence or absence of

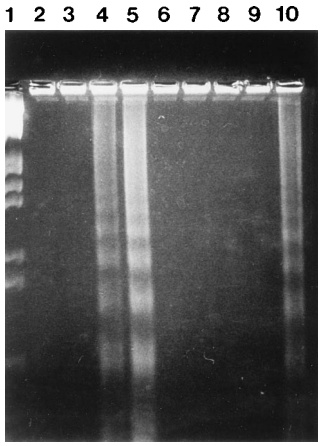

FIG. 5. DNA fragmentation in CVB3-infected mouse spleens. A total of 10^6 spleen cells were treated with 2% SDS, 100-μg/ml RNase A, and 100-μg/ml proteinase K and run on a 2% agarose gel. Lane 1, molecular weight standard; lane 2, normal spleen cells; lanes 3 to 5, H3 virus-immune spleen cells on 2, 4, and 7 days after infection, respectively; lanes 6 to 8, H310A1 virus-immune spleen cells on 2, 4, and 7 days after infection, respectively; lane 9, $CD8^+$-T-cell-enriched population from day 7 H3 virus-immune spleens; lane 10, $CD4^+$-T-cell-enriched population from day 7 H3 virus-immune spleens.

$CD8^+$ cells, and the presence or absence of specific cytokines such as IFN-γ or IL-4) determine which Th cell phenotype occurs (6). Generally, it has been assumed that the presence of these factors acts in a positive manner to promote selective differentiation pathways. However, since $CD8^+$ cytolytic T lymphocytes, NK cells, and $\gamma\delta^+$ T cells, which preferentially promote Th1 cell responses, all express high levels of FasL mRNA (28), it is possible that apoptosis might also modulate $CD4^+$-cell responses.

The type of $CD4^+$ cell response occurring during infection can significantly influence disease pathogenicity. This was shown quite effectively for leishmaniasis, in which Th2 cell

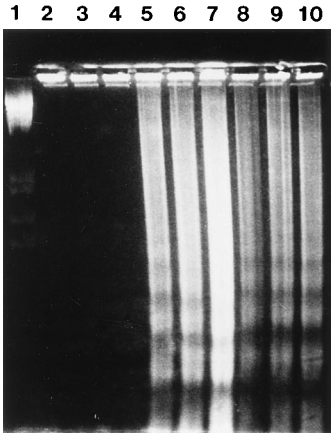

FIG. 6. DNA fragmentation in H310A1 virus-infected mice given $\gamma\delta^+$ T cells. Mice were infected intraperitoneally with 10^4 PFU of H310A1 virus, and some mice additionally received either 5,000 purified $\gamma\delta^+$ T cells intravenously on the day of infection or $\gamma\delta^+$ T cells on the day of infection and 100 μg of anti-$CD8^+$ MAb per mouse on days −2 and −1 relative to virus. Lane 1, molecular weight standard; lanes 2 to 4, spleen cells from H310A1 virus-infected mice on days 2, 4, and 7 after infection, respectively; lanes 5 to 7, spleen cells isolated on days 2, 4, and 7 after infection, respectively, from H310A1 virus-infected mice given $\gamma\delta^+$ T cells on the same day as virus; lanes 8 to 10, spleen cells isolated on days 2, 4, and 7, respectively, after infection from mice given H310A1 virus, $\gamma\delta^+$ T cells, and anti-$CD8^+$ MAb.

responses promote disease susceptibility and resistance correlates to preferential activation of Th1 cells (21). Similarly, encephalitis in Theiler's virus infections involves activation of Th1 cells, while selective stimulation of Th2 cells induces anergy and inhibits neurological injury (24). In CVB3-induced myocarditis, there are three conditions associated with disease resistance. These are the genetic strain of the inbred mouse (15, 23, 31), the genetic variations of the virus (7–9), and the physiological state of the animal (2, 19, 32). In each of these situations, resistance correlates to the preferential activation of suppressor $CD4^+$ or Th2 cells (12, 16, 17). What has not been shown is why certain conditions in the CVB3 myocarditis model promote either Th1 or Th2 cell activation. With the H3 and H310A1 virus variants, $\gamma\delta^+$ cells most likely play a role in determining Th subset responses. H3 virus infections activate peripheral $\gamma\delta^+$-cell responses, while H310A1 virus infections do not (13). Furthermore, adoptive transfer of enriched $\gamma\delta^+$ cells into H310A1 virus-infected mice restores myocarditis susceptibility (14). Since $\gamma\delta^+$ cells can selectively downregulate Th2 cell responses (20), the activation of these cells in H3 virus infections may explain the predominant Th1 cell response in infected mice. Indeed, as shown in Fig. 3, giving $\gamma\delta^+$ cells to H310A1-virus-infected mice caused a shift from a predominantly Th2-like to a predominantly Th1-like cell response.

The question remains whether $\gamma\delta^+$ cells modify Th-cell responses in vivo through the release of proinflammatory cytokines, the selective elimination of Th2 cells through apoptosis, or both mechanisms. The correlation between the shift in Th subset response and the early (day 2) induction of selective apoptosis in the $CD4^+$ T-cell population prior to the appearance of cardiac inflammation lends circumstantial evidence that apoptosis might be involved in Th subset regulation. These data are also consistent with published information by Salmon et al. (27) which demonstrates that progressive activation of human peripheral T cells toward cells producing increased IL-4 but decreased IL-2 correlated to enhanced expression of Fas and decreased expression of Bcl-2, the protein known to inhibit Fas-mediated apoptosis. That study (27) implies that T cells expressing different cytokine-producing profiles might exhibit a distinct susceptibility to Fas-mediated apoptosis. Unpublished studies from this laboratory demonstrate that mice having the *lpr* or *gld* mutation (lacking Fas or Fas ligand, respectively) fail to develop myocarditis and generate more virus-specific Th2-like $CD4^+$ cells than wild-type mice, which develop severe myocarditis and respond with Th1-like cells. These results emphasize that apoptosis may have a direct effect on Th subset regulation.

The nature of the $CD4^+$ cells undergoing apoptosis was not investigated in the present study. Possibly, most of the apoptosing cells may be Th2 cells. Since IL-2 is effective in inhibiting apoptosis (27), and Th1 cells produce this cytokine, it is likely that the higher concentrations of IL-2 in the vicinity of Th1 cells would either prevent or delay the death of this cell type. Most likely, the apoptosing cells have been activated, as published reports suggest that activation augments apoptosis susceptibility in $CD4^+$ cells (27). A further question is whether the $\gamma\delta^+$ cells are directly responsible for the $CD4^+$ cell apoptosis or whether these effectors activate other cell types which then induce the apoptotic response. This question is especially relevant since so few $\gamma\delta^+$ T cells are used in adoptive transfer experiments. While an indirect effect cannot be ruled out, it seems more likely that the $CD4^+$-cell apoptosis is a direct result of the $\gamma\delta^+$ cells. This conclusion is based first on the very rapid appearance of apoptosis in the spleen after $\gamma\delta^+$ cell transfer. Activation of antigen-specific $CD4^+$ or $CD8^+$ mediators would undoubtedly require longer than 2 days, and within this period after the transfer of $\gamma\delta^+$ cells, apoptosis is already evident. Second, of all lymphoid cells, $\gamma\delta^+$ cells express the highest levels of Fas ligand mRNA, suggesting that these are the most potent effector cells in apoptosis (28). In conclusion, the data presented in this communication are consistent with $\gamma\delta^+$ cells regulating myocarditis susceptibility through their modulation of $CD4^+$ Th subset responses, and the studies provide circumstantial evidence that this modulation may involve selective apoptosis of $CD4^+$-cell subpopulations.

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