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Augmentation of Pathogenesis of Coxsackievirus B3 Infections in Mice by Exogenous Administration of Interleukin-1 and Interleukin-2

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Two variants of coxsackievirus B3 (CVB3) which differ dramatically in the ability to induce myocarditis in BALB/c mice were studied. H3 virus infection of murine monocytes in vitro resulted in release of concentrations of interleukin 1 (IL-1) and alpha/beta interferon that were high compared with those of cells infected with the H310A1 virus variant. In vivo, H3 virus infection caused substantial inflammatory cell infiltration of the myocardium, and lymphocytes from these animals gave predominantly Th_1-cell responses to either whole H3 virus or overlapping peptides of the CVB3 vp1 capsid protein, as determined by IL-2 production. In contrast, H310A1 virus infection produced minimal myocarditis and Th_1-cell responses, but Th_2-cell activation was more pronounced than in H3 virus-infected mice (as determined by IL-4 concentrations). Exogenous treatment of H310A1 virus-infected mice with either IL-1 or IL-2 restored both myocarditis susceptibility and Th_1-cell responses to whole virus and vp1 peptides. Furthermore, H310A1 virus-infected mice given exogenous IL-1 showed substantial in situ IL-2 deposition in the myocardium. These results indicate that CVB3-induced myocarditis may depend upon release of specific cytokines during infection and that activation of Th_1 cells may be an important factor in pathogenesis.

Coxsackievirus B3 (CVB3) induces myocarditis in mice which is characterized by virus replication in the heart and inflammatory cell infiltration of the myocardium. However, not all CVB3 variants are equally pathogenic. In some instances, variants lack tropism for the heart (17). In other cases, variants may infect and replicate in the myocardium to high titers yet still produce minimal or no myocarditis (7, 12). Recently, several studies have focused on factors influencing pathogenicity in this disease. Cytokines, especially interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α), may be essential to the development of myocarditis. CVB3 activates monocytes in vitro, resulting in release of high concentrations of TNF-α, IL-1, and IL-6 (15). While these cytokines may have a direct impact on myocardial cell function, they may additionally affect inflammatory cell responses. Work by Rose and his colleagues has shown that exogenous administration of either TNF-α or IL-1 to myocarditis-resistant inbred mice results in substantial enhancement of cardiac inflammation during CVB3 infection (20). How these specific cytokines might affect inflammatory cell infiltration of the heart has not been determined.

Two subsets of $CD4^+$ T helper cells have been characterized in accordance with their requirements for stimulation, their biological activities, and the cytokines they produce (2, 9, 10, 27, 28, 31, 33). Th_1 cells are major producers of IL-2, gamma interferon (INF-γ), and TNF-β. Th_2 cells primarily activate B lymphocytes, regulate Th_1-cell responses, and produce IL-4, IL-5, IL-6, and IL-10. Th_2-cell activation also requires antigen presentation by class II major histocompatibility complex molecules, but the costimulatory activity is different from that for Th_1 cells. Differential activation of Th_1 and Th_2 cells during infection can substantially influence the course of the resulting disease. An excellent example of this process is provided by the model of murine cutaneous leishmaniasis. BALB/c mice, which develop a progressive disease, give a predominant Th_2-cell response to infection. C57BL/6 mice, which develop localized lesions that ultimately resolve, give a predominant Th_1-cell response (14, 25).

We have isolated two plaque-purified variants from the wild-type CVB3 pool. One of the variants, designated H3 virus, produces both high mortality in infected BALB/c mice and severe inflammatory lesions in the heart (24, 32, 34). The second variant, designated H310A1 virus, infects the myocardium of BALB/c mice but causes minimal mortality and cardiac inflammation. H310A1 virus infection of either cardiocytes or macrophages in vitro does not inhibit cellular RNA or protein synthesis as effectively as infection with the pathogenic H3 virus, and H310A1 virus-infected cells usually produce less progeny virus, although the difference in viral titers between H3 and H310A1 virus infections is not always statistically significant (32). It is tempting to assume that even a minimal reduction in virus replication could explain the poor pathogenicity of the H310A1 variant. Less virus production might mean more restricted virus dissemination and reduced tissue injury. However, our evidence suggests that differences in pathogenicity between these virus variants may partially correlate to the types of Th-cell responses induced during infection.

MATERIALS AND METHODS

Mice. Inbred BALB/c Cum mice ($H\text{-}2^d$) were originally purchased from Cumberland Farms, Clinton, Tenn. Breeding colonies of these animals are maintained at the University of Vermont. MRL +/+ mice ($H\text{-}2^k$) were kindly provided by Ralph Budd (University of Vermont). Adult male mice 6 to 8 weeks of age were used in these experiments. Generally, experimental groups consisted of a minimum of three to five mice and experiments were repeated twice.

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TABLE 1. Amino acid sequences of CVB3 vp1 peptides

Peptide	Sequence	Amino acids	Peptide	Sequence	Amino acids
vp1-1	GPVEDAITAAIGRVAc	1–15	vp1-16	PVPDKVDSYVWQTSTc	149–163
vp1-2	IGRVADTVGTGPNNSc	11–25	vp1-17	WQTSTNPSVFWTEGNc	159–173
vp1-3	GPNNSEAIPALTAAEc	21–35	vp1-18	WTEGNAPPRMSIPFLc	169–183
vp1-4	LTAAETGHTSQVVPGc	31–45	vp1-19	SIPFLSIGNAYSNFYc	179–193
vp1-5	QVVPGDTMOTRHVKNc	41–55	vp1-20	YSNFYDGWSEFSRNGc	189–203
vp1-6	RHVKNYHSRSESTIEc	51–65	vp1-21	FSRNGVYGINTLNNMc	199–213
vp1-7	ESTIENFLCRSAC	61–73	vp1-22	TLNNMGTLYARHVNAc	209–223
vp1-8	CRSACVYFTEYENSGc	69–83	vp1-23	RHVNAGSTGPIKSTIc	219–233
vp1-9	YENSGAKRYAEWVLTc	79–83	vp1-24	IKSTIRIYFKPKHVKc	229–243
vp1-10	EWVLTPRQAAQLRRKc	89–103	vp1-25	PKHVKAWIPRPPRLC	239–253
vp1-11	QLRRKLEFFTYVRFDc	99–123	vp1-27	KNVNFQPSGVTTTRQc	259–273
vp1-12	YVRFDLELTFVITSTc	109–123	vp1-28	TTTRQSITTMTNTGAc	109–123
vp1-13	VITSTQQPSTTQNQDc	119–133	vp1-29	TNTGAIWTTIRGSVC	279–293
vp1-14	TQNQDAQILTHQIMYc	129–143	vp1-30	RGSVCGDYRVVNRHSc	289–303
vp1-15	HQIMYVPPGGPVPDKc	139–153	vp1-31	VNRHSATSADWQNC	299–312

Virus and immunization protocol. The original stock of CVB3 was obtained from J. F. Woodruff and is maintained by passage through HeLa cells. A plaque-purified myocarditic variant of this virus (designated H3) was derived from the stock virus. The nonmyocarditic virus variant, designated H310A1, was isolated as an antibody escape mutant from H3 virus-infected cultures. Characterization and comparisons of H3 and H310A1 virus infections have been published previously (24, 32, 34). All virus stocks were negative for mycoplasm. Mice were infected by intraperitoneal injection of 0.5 ml of phosphate-buffered saline containing approximately 10^4 PFU of virus. All animals were killed 7 days after infection.

Organ virus titer. Hearts were aseptically removed and homogenized with Dulbecco's modified essential medium (DMEM; GIBCO, Grand Island, N.Y.) containing 100 U of penicillin, 100 μg of streptomycin ml^{-1}, and 2% fetal bovine serum (FBS; GIBCO). The cellular debris was removed by centrifugation at 300 × *g* for 10 min, and the supernatant was subjected to sequential 10-fold dilutions in DMEM–2% FBS, and the titer was determined by the plaque-forming assay on HeLa cell monolayers.

Lymphocyte preparation. Mesenteric lymph nodes were aseptically removed and pressed through fine-mesh screens to produce single-cell suspensions. The lymphocyte populations were enriched for T cells by incubating the cells on nylon wool columns at 37°C for 30 min, and the nonadherent T-cell-enriched population was retrieved. The cells were washed in DMEM–5% FBS, and the viability was determined by trypan blue exclusion.

Lymphocyte proliferation. Lymphocytes were suspended in DMEM–10% FBS containing 2×10^5 M 2-mercaptoethanol and 20 U of recombinant IL-2 per ml, after which 5×10^4 cells were dispensed into wells of 96-well tissue culture plates together with 10 μg of antigen per ml. The plates were incubated at 37°C in a humidified 5% CO_2 incubator for 4 days, after which 1 μCi of [^{3}H]thymidine ([*methyl*-^{3}H]thymidine; 50.8 Ci/mmol; ICN Radiochemicals Inc., Irvine, Calif.) was added to each well. The cultures were harvested approximately 20 h later onto glass fiber filter strips. Sections containing radioactivity were placed into vials with Ecolume scintillation fluid and counted in a liquid scintillation counter (Packard Instruments, Downers Grove, Ill.).

Synthetic peptides. Overlapping peptides were synthesized in accordance with the sequence of viral coat protein vp1 of CVB3. The peptides were up to 15 amino acids long with either a naturally occurring or an additional cysteine residue at the carboxy terminus (Table 1). The peptides were prepared by the simultaneous multiple peptide synthesis method of Houghton (16). Briefly, the solid-phase resin was sealed into polypropylene bags and the amino acids were synthesized at the resin. The final peptide was cleaved from the resin by using HF. Purity of the peptides was evaluated by reverse-phase high-pressure liquid chromatography. All of the peptides used in this study had a purity of more than 80%.

Immunoperoxidase staining. Hearts were removed and quickly frozen in O.C.T. compound (Miles Inc., Elkhart, Ind.) by using liquid nitrogen and sectioned on a cryostat into 5-μm-thick sections of the ventricular myocardium. Endogenous peroxidase was blocked by incubation in 0.3% H_2O_2 for 15 min, and then the sections were covered with 25 μl of a 1:500 dilution of the various unlabeled primary antibodies and incubated in a humidified chamber for 30 min. The sections were washed and stained with streptavidin-biotin system kits for rat and mouse primary antibodies (Zymed Histostain-SP kits; Zymed Laboratories Inc., South San Francisco, Calif.). Sections were counterstained with hematoxylin.

T-cell cloning. Lymphocytes were washed and resuspended in RPMI 1640 containing L-glutamine, antibiotics, 10% FBS, 2-mercaptoethanol, 5% concanavalin A (Con A) supernatant, and 10% conditioned medium made with J774A.1 cells (American Type Culture Collection [ATCC], Bethesda, Md.). Feeder cells were syngeneic spleen cells which were centrifuged on Histopaque to remove erythrocytes, subjected to 2,000 R of irradiation, washed, resuspended in 2 ml of medium containing 50 μg of UV-irradiated H3 virus, incubated for 2 h at 37°C, washed to remove unincorporated virus, and resuspended in cloning medium (see above) at a concentration of 5×10^6 cells per ml. Lymphocyte lines were initially made by culturing approximately 10^6 immune lymphocytes with 5×10^4 feeder cells and 1.0 μg of H3 virus in round-bottom 96-well tissue culture plates for 10 to 14 days, and the medium was changed at 3- to 4-day intervals. Plates were incubated in a 7% CO_2–93% air humidified incubator. Viable cells were recovered by centrifugation on Histopaque. To clone cells, 5×10^4 feeder cells, 0.1 μg of H3 virus, and 0.3 cell of the T-cell line were added, in cloning medium, to each well of round-bottom 96-well tissue culture plates. The cultures were incubated and restimulated with fresh feeder cells and antigen at 10- to 14-day intervals.

Flow cytometry. Cells were resuspended to 10^6/ml in phosphate-buffered saline containing 1% bovine serum and stained with a 1:100 dilution (final concentration) of a fluorescein

isothiocyanate (FITC)- or phosphatidylethanolamine (PE)-conjugated primary antibody. After incubation at 0 to 4°C for 30 min, the cells were washed at 4°C. Flow cytometry and cell sorting were performed on an Ortho Diagnostics System 50-H cytometer with an argon laser excitation source tuned to 488 nm and 250 mW and a 2150 data acquisition processor. Fluorescence was measured through a 525-nm bypass filter producing cytograms of forward versus 90° scatter. Histograms were analyzed by setting the threshold on the basis of a negative control so that 1 to 2% of the negative cells fell in the positive range.

Cytokines and reagents. Murine IL-1β was purchased from Sigma Chemical Co., St. Louis, Mo. TNF-α was kindly supplied by Genentech, South San Francisco, Calif. IL-2, IL-4, and IFN-γ were obtained from Pharmingen, San Diego, Calif. Fluorescein isothiocyanate- and PE-conjugated antibodies to T-cell markers and conjugated secondary antibodies were obtained from Coulter Co., Hialeah, Fla. Hybridoma clones making antibodies to CD3 (clone 500A2), L3T4 (clone GK1.5), Lyt-2.2 (clone 2.43), IL-2 (clone S4B6), and IL-4 (clone 11B11) were purchased from the ATCC and grown in DMEM–10% FBS. Supernatants from the hybridoma cell cultures were used as the source of these antibodies.

Infection of macrophages and assay of cytokine production. A BALB/c-derived monocyte-macrophage line (J774A.1; ATCC) was maintained in DMEM–10% FBS. Approximately 10^5 cells per well were dispensed into 96-well tissue culture plates and exposed to 10^6 PFU of either H3 or H310A1 virus for 1 h at 37°C. The cells were washed and cultured in 200 μl of DMEM–10% FBS for 2 to 72 h. Triplicate cultures consisting of cells and supernatant were frozen for determination of IL-1α concentrations with an enzyme-linked immunosorbent assay kit (Genzyme, Cambridge, Mass.) and for virus titer determination by the plaque-forming assay. IFN-α/β concentrations were determined by testing the supernatants for inhibition of encephalomyocarditis virus-induced lysis of L929 cells. Identification of the inhibitory activity as IFN-α/β was achieved by incubating the supernatant with 1,000 U of anti-IFN-α/β.

Assay for IL-2 and IL-4. Lymphokines IL-2 and IL-4 were evaluated by using the specific lymphokine-responsive cell lines CTLL-2 (ATCC) and CT.4S (kindly supplied by S. Sriram, University of Vermont, Burlington), respectively. Twenty-microliter tissue culture samples were added to 5,000 indicator cells in 80 μl of DMEM–10% FBS in wells of 96-well tissue culture plates. Cultures were labeled with 1 μCi of [^{3}H]thymidine for 12 h and harvested as described above. Titrations of recombinant IL-2 and IL-4 were also performed on the indicator cell lines (14).

Limiting-dilution analysis. Limiting-dilution analysis was performed as described by Morris et al. (25). Briefly, mesenteric lymph node cells were enriched for $CD4^+$ T cells by treatment with 100 μg of an anti-CD8 monoclonal antibody per ml and 20% rabbit complement (GIBCO) for 45 min at 37°C. The viable cells were retrieved by centrifugation on Ficoll-Hypaque, and the purity of the resulting $CD4^+$ T-cell population was determined by flow cytometry with a PE-conjugated anti-CD4 antibody. The preparations had greater than 92% $CD4^+$ T cells. Between 0 and 1,000 $CD4^+$ T cells were dispensed into wells of 96-well tissue culture plates together with 5×10^4 irradiated (2,000 R) BALB/c monocytes (cell line J774A.1) as antigen-presenting cells (APC), 20 U of recombinant IL-2 per ml, and 10 μg of CVB3 per ml in RPMI 1640 medium containing 10% FBS, antibiotics, nonessential amino acids, and 2-mercaptoethanol. The wells were cultured for 14 days, washed three times, and restimulated with 0.2 ml of medium containing 5 μg of ConA (GIBCO). After 24 h of incubation at 37°C, wells were scored visually for cell growth and the supernatants were retrieved and assayed for IL-2 and IL-4 with the appropriate indicator cell lines. Groups consisted of 44 wells for each concentration of $CD4^+$ T cells cultured with antigen and eight wells of cells cultured without antigen as controls. Positive wells incorporated more than the mean counts per minute of control wells +3 standard deviations. Estimates of precursor frequency were obtained by the maximum-likelihood method of Good et al. (13).

Thymectomy, irradiation, and bone marrow reconstitution. At 3 weeks of age, mice were thymectomized by opening the anterior mediastinum through an incision in the neck and sternum extending to the second rib. The thymus was aspirated, and the incision was closed with metal sutures. One week later, the animals were exposed to 800 R with a cesium irradiator. On the same day, 5×10^6 syngeneic bone marrow cells obtained from donor mice by flushing the tibial and femoral shafts with medium were injected intravenously into recipient mice through the tail vein. Mice were rested for 3 to 4 weeks and then anesthetized with 60 mg of sodium pentobarbital per kg. An inguinal lymph node was removed from each mouse. Single lymphocyte suspensions were obtained, stained with fluorescein isothiocyanate–anti-Lyt-2.2 (anti-CD8) or PE–anti-L3T4 (anti-CD4) antibodies, and evaluated by flow cytometry. Any mouse showing positive cells was eliminated from the study.

Histology. Hearts were fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin. Sections were blindly evaluated by S.A.H. on a 0-to-4 scale, where 0 represents no inflammation, 1 represents 1 to 10 lesions per section, 2 represents 11 to 20 lesions per section, 3 represents 21 to 40 lesions per section, and 4 represents widespread and confluent lesions. At least two sections per heart per animal were evaluated.

Statistical analysis. Data obtained from animals, such as histology and organ virus titers, were analyzed with the Wilcoxon ranked-score test. Mortality was measured by χ^2 analysis. All other data were evaluated with the Student t test.

RESULTS

Cytokines induced by CVB3 infection determine pathogenicity. The studies described here investigated the potential roles of cytokines in the pathogenicity of CVB3 infections. BALB/c monocyte-macrophage line J774A.1 (ATCC) was infected with either H3 or H310A1 virus, and viral replication was allowed. Production of IFN-α/β and IL-1 were determined at various times during the subsequent 3-day period (Fig. 1). The studies indicated that H3 virus replicated well in this monocyte line and triggered release of high levels of both IFN-α/β and IL-1. In contrast, H310A1 virus replicated poorly in these cells and induced little production of these two cytokines. This study suggested that H310A1 virus is deficient in signals necessary for stimulation of at least some types of cytokines from macrophages, but does the poor IL-1 or IFN-α/β response explain the minimal pathogenicity of this virus variant? In the next experiment, BALB/c mice were infected with either H3 or H310A1 virus and given intraperitoneal injections of either IL-1, recombinant TNF-α, recombinant IL-2, or recombinant murine IFN-γ. Controls received the same amount of cytokine without virus infection. Animals were killed on day 7. Hearts were evaluated for myocarditis (Table 2; Fig. 2) and virus titers (Table 3). The results showed that BALB/c mice given H3 virus developed significant myocarditis, in contrast to animals that received H310A1 virus. Virus titers in H3 virus-infected mice

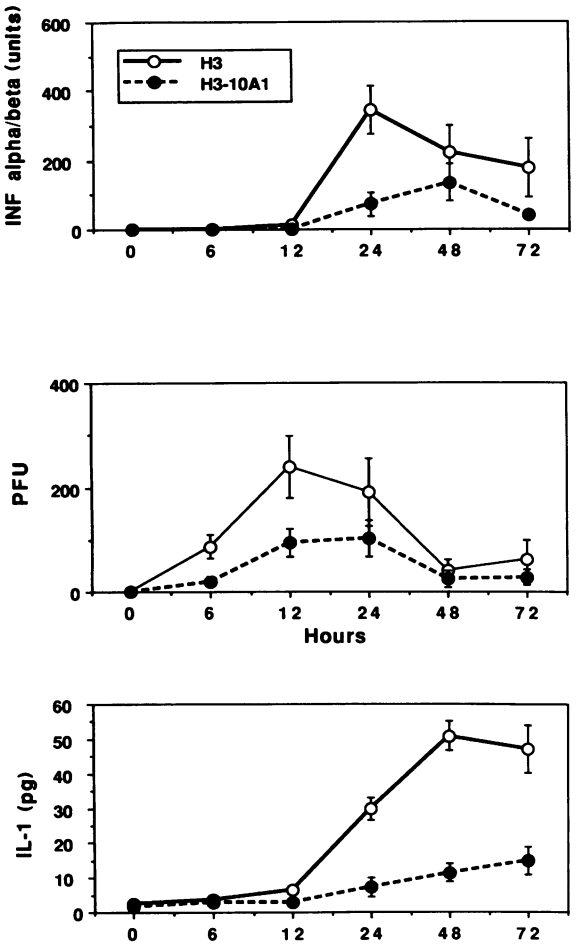

FIG. 1. Differential induction of cytokines in monocytes infected with H3 and H310A1. Approximately 10^5 BALB/c monocytes (line J774A.1) were exposed to 10 PFU of virus per cell for 1 h, washed, and cultured for 2 to 72 h. Supernatants were assayed for IL-1 or IFN-α/β as indicated, replicate cells were homogenized, and virus titers were determined. Results represent means ± standard errors of the means of four to six replicate cultures.

were higher than those in animals given H310A1, but the difference was not significant. Treating H310A1-infected animals with either IL-1, IL-2, or IFN-γ significantly augmented heart pathology, although the effect with IFN-γ was moderate and not always statistically significant in replicate experiments. IL-1 and IL-2 treatment had little effect on H3 virus infections. As expected, IFN-γ administration inhibited virus titers in the heart even though myocarditis was increased. Therefore, poor pathogenicity of the H310A1 virus variants can be reversed by giving a secondary immunostimulatory signal concomitant with infection. The results obtained with IFN-γ further emphasize the fact that myocarditis severity does not necessarily correlate with high levels of virus in the heart.

Th-cell subset differences in myocarditis. The next question was whether the types of T lymphocytes stimulated in H3 and H310A1 virus infections differ and the potential role distinct T-cell subsets may have in pathogenesis. BALB/c mice were injected either with 10^4 PFU of H3 and H310A1 virus or with 10^4 PFU of H310A1 in combination with a single injection of 100 ng of IL-1 or daily injections of 10^4 U of recombinant IL-2. Controls consisted of normal mice with or without cytokine treatment. Five days later, the mice were killed and the mesenteric lymph node cells were cocultured with 10 μg of overlapping peptides of the CVB3 vp1 capsid protein per ml for 5 days. Half of the supernatant from each culture was removed and replaced with fresh medium containing 1 μCi of [^{3}H]thymidine for 18 h. The amount of radioisotope incorporated in the cultures was determined (Fig. 3; peptide sequences are given in Table 1). Lymphocytes from normal mice with or without cytokine treatment showed no proliferation above the medium control in response to any of the peptides (data not shown). Lymphocytes from H3-immune animals responded well to whole H3 virus and to vp1 peptides 1, 3, 6, 13, 14, 17, 21, and 31. In contrast, cells from H310A1-immune mice responded to vp1 peptides 1, 25, 27, and 29 but did not respond well to H3uv virus. Treating H310A1-infected mice with exogenous IL-1, a process which substantially augmented myocarditis in vivo, changed the proliferation pattern of the immune cells. Lymphocytes from the H310A1–IL-1-treated group responded to the whole virus as well as to peptides 1, 13, 14, 17, and 21. When mice were simultaneously given H310A1 and IL-2, their lymphocytes gave a response pattern which was nearly identical to the response to pathogenic H3 virus. The supernatant which was removed from the cultures prior to [^{3}H]thymidine addition was subsequently assayed for IL-2 and IL-4 (Fig. 4). IL-2 was detected predominantly in the supernatants of cultures containing H3, H310A1–IL-1, and H310A1–IL-2 immune lymphocytes. Reduced levels of IL-2 were seen in H310A1-immune cell cultures, but these cultures generally released levels of IL-4 greater than those observed with the other immune lymphocyte populations. All of the responding cells were either T lymphocytes or T cell dependent, since treatment of the lymph node populations with anti-Thy-1.2 and complement prior to coculture with the peptides abrogated the proliferative response (data not shown).

The above-described studies indicate that H3 and H310A1 virus-infected mice give different IL-2 and IL-4 responses and suggest that there are differences between Th_1-cell stimulation and Th_2-cell stimulation. To confirm the latter supposition, precursor frequency analysis was performed. BALB/c mice were infected with 10^4 PFU of either H3 or H310A1 virus. Some H310A1 virus-infected mice were additionally given 100 ng of IL-1 intraperitoneally on the same day as virus. Enriched populations of CD4$^+$ T cells were isolated from the mesenteric

TABLE 2. Effect of cytokine administration on CVB3 pathogenicity[a]

Treatment	Myocarditis score[b]		
	No virus	H3	H310A1
None	0 ± 0	1.0 ± 0.0	0.20 ± 0.10
LPS (10 μg; day 0)	0 ± 0	1.1 ± 0.20	1.13 ± 0.13[c]
IL-1 (100 ng; day 0)	0 ± 0	1.2 ± 0.15	0.90 ± 0.19[c]
TNFα (250 ng; day 0)	0 ± 0	1.1 ± 0.18	0.26 ± 0.11
IFN-γ (10^4 U; days 0, 2, 4)	0 ± 0	1.3 ± 0.20	0.60 ± 0.10[c]
IL-2 (10^4 U; days 0–6)	0 ± 0	1.3 ± 0.30	1.37 ± 0.25[c]

[a] BALB/c mice were infected with 10^4 PFU of the indicated virus intraperitoneally and received lipopolysaccharide (LPS) or the indicated cytokine intraperitoneally at the concentration and on the day(s) relative to infection indicated in parentheses. All animals were killed 7 days after infection, and the hearts were evaluated for myocardial inflammation. Results represent means ± standard errors of the means of three to five mice per group.

[b] On a scale of 0 to 4.

[c] Significantly different from the no-treatment result at $P \leq 0.05$.

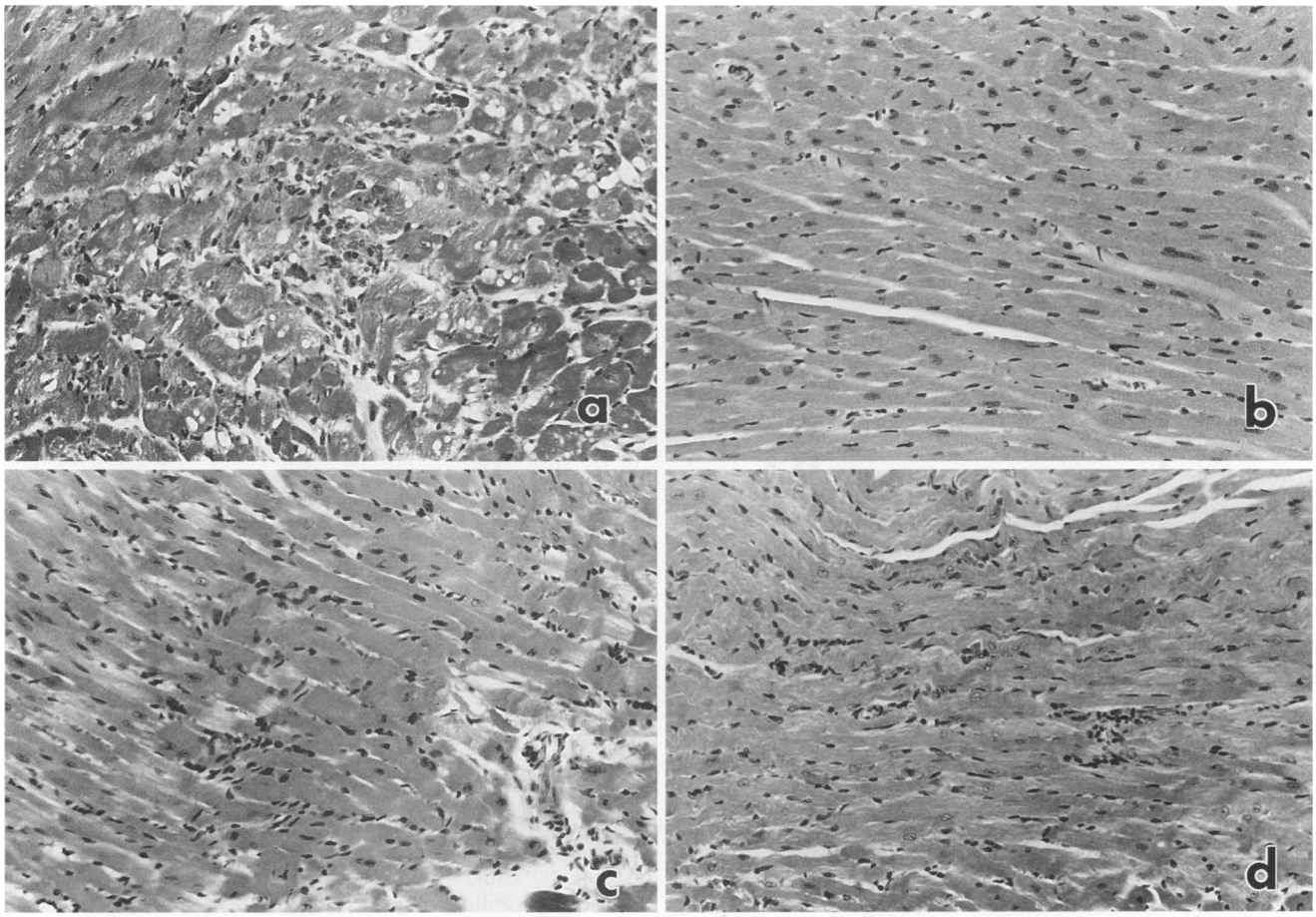

FIG. 2. Hematoxylin-and-eosin-stained hearts from BALB/c mice infected with H3 (a), H310A1 (b), H310A1 plus 100 ng of IL-1 (c), or H310A1 plus 10^4 U of IL-2 (d) for 7 days.

lymph nodes of these animals 7 days after infection and dispensed at 0, 100, 500, or 1,000 cells per well into 96-well tissue culture plates with 5×10^4 irradiated APC and 10 μg of H3 virus per ml. After incubation for 14 days, the cells were washed and restimulated with ConA. Supernatants were evaluated for IL-2 and IL-4 with CTLL and CT.4S cells. Lymphocyte growth was scored visually in the wells. Figure 5 gives the results of this experiment. Precursor frequencies are given by each line. The results demonstrate that H3 virus infection caused substantially greater Th_1-cell activation than did H310A1 virus infection. Conversely, greater frequencies of Th_2 cells were observed in H310A1 virus-infected than in H3 virus-infected animals. The results obtained with H310A1–IL-1-treated mice are less clear. There was a decrease in the Th_2 phenotypic response from 0.16 to 0.07%, but there was no substantial change in Th_1-cell frequencies, at least in lymph nodes.

TABLE 3. Effect of cytokine administration on cardiac CVB3 titers[a]

Treatment	Virus titer (log_{10} PFU)	
	H3	H310A1
None	5.16 ± 1.74	4.33 ± 0.89
LPS (10 μg; day 0)	6.07 ± 2.29	5.62 ± 1.80
IL-1 (100 ng; day 0)	5.10 ± 1.95	4.70 ± 1.42
TNF-α (250 ng; day 0)	5.89 ± 2.70	4.93 ± 1.67
IFN-γ (10^4 U; days 0, 2, 4)	3.75 ± 2.65[b]	3.07 ± 1.16[b]
IL-2 (10^4 U; days 0–6)	5.31 ± 1.59	4.47 ± 1.35

[a] BALB/c mice were infected with 10^4 PFU of the indicated virus intraperitoneally and received lipopolysaccharide (LPS) or the indicated cytokine intraperitoneally at the concentration and on the day(s) relative to infection indicated in parentheses. All animals were killed 7 days after infection, and the hearts were evaluated for virus titer by the plaque-forming assay. Results represent means ± the standard errors of the means of three to five mice per group.

[b] Significantly different from the no-treatment result at $P \leq 0.05$.

The peptide-reactive T cells were successfully cloned from H3 virus-immune animals (Table 4). In nearly every case, the clone was $CD4^+$ and most preferentially produced IL-2. The clones reactive to peptide 1, however, probably represent Th_2 cells, since these preferentially produce IL-4. It should be noted that the relatively high numbers of Th_1 phenotypic clones isolated may, in part, reflect the cloning conditions used. Next, BALB/c mice were thymectomized, irradiated, and bone marrow reconstituted (TXBM mice). These TXBM mice were infected with 10^4 PFU of H3 virus. Some groups of animals also received 10^6 T-cell clones reactive to vp1 peptide 1 (clone 3), 3 (clone 8), 6 (clone 12), 13 (clone 13), 14 (clone 16), or 21 (clone 19). After 7 days, the mice were killed and the hearts were evaluated for myocarditis (Fig. 6). TXBM mice that were infected with virus but did not receive T cells developed minimal myocarditis. Adoptive transfer of T-cell clones reactive to peptide vp1-6, vp1-13, or vp1-21 substantially enhanced

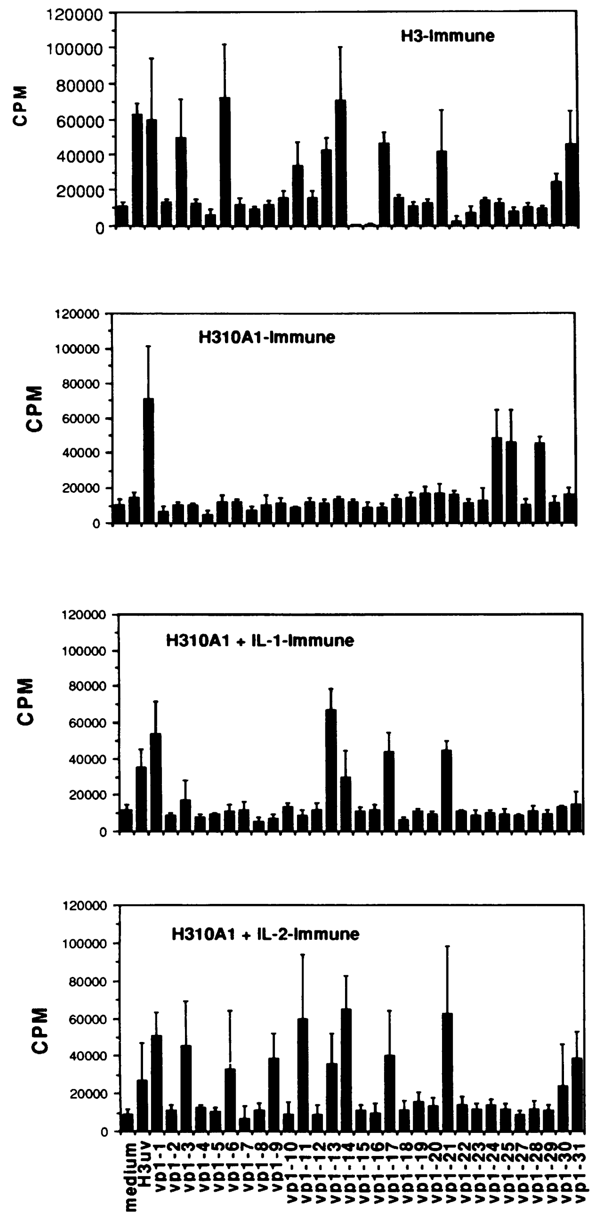

FIG. 3. Lymphocyte proliferation in response to vp1 peptides. BALB/c mice were infected for 5 days with either 10^4 PFU of H3 or H310A1 virus. Some H310A1 virus-infected mice were additionally given either 100 ng of IL-1 or 10^4 U of IL-2 daily. Mesenteric lymph node cells were obtained from normal and infected mice and cultured with 10 μg of the synthetic peptides per ml for 5 days. Results represent mean counts per minute (CPM) of [^{3}H]thymidine incorporated ± the standard deviation of six replicates per group.

pathogenicity in H3 virus-infected mice. None of the other peptide-reactive clones appeared capable of directly restoring disease susceptibility. The reason why these clones are not effective is not known. It may relate to the peptide specificity, the type or concentrations of cytokines made by each clone, or the ability of the clones to migrate to the myocardium. Nonetheless, these results indicate that at least some T-cell clones reactive to vp1 peptides are directly pathogenic in vivo in CVB3-infected recipients.

The apparent cross-reactivity of the T-cell clones with more than one peptide in Table 4 was a concern, since it might indicate that the cell populations were not monoclonal. Therefore, clone 13 (vp1-13 peptide reactive) was subcloned at one cell per 10 wells. Of 480 wells, 14 showed growth. Eight subclones survived expansion and were reassayed by lymphocyte proliferation in response to vp1 peptides 1, 3, 6, 13, 14, and 21. All subclones continued to respond predominantly to peptide vp1-13 but also gave significant reactivity to peptide vp1-21 (data not shown). These results strongly imply that the cell population in the original clone was monoclonal and that the cross-reactivity between peptide responses was real. Confirmation would require T-cell receptor analysis, which was not done. Studies were performed to determine the major histocompatibility complex restriction of T-clonal reconstitution of myocarditis susceptibility in vivo. TXBM and normal BALB/c (*H-2^d*) and MRL +/+ (*H-2^k*) mice were infected with 10^4 PFU of H3 virus. On the same day as infection, some TXBM mice were given either 10^6, 5×10^6, or 10^7 clone 13 (subclone 3) T cells. All animals were killed 7 days later. Hearts were evaluated for myocarditis and virus titer (Table 5). The results demonstrate that BALB/c TXBM mice given any concentration of clone 13 cells developed substantial myocarditis, in contrast to infected TXBM mice not given T cells. Interestingly, the best myocarditis was obtained with 10^6 T cells while higher lymphocyte numbers (5×10^6 and 10^7 cells per mouse) resulted in moderate reductions in both myocarditis and virus titers. One explanation for this finding is that in sufficient numbers, virus-reactive T-cell clones can either directly or indirectly aid virus clearance in the heart, which might have an impact on the resulting severity of cardiac pathology. Interestingly, even though clone 13 T cells could restore some myocarditis, these cells were ineffective in allogeneic MRL +/+ recipients. Thus, the effect of the T-cell clones may be major histocompatibility complex restricted (4).

Immunoperoxidase staining of H3 and H310A1 virus-infected hearts for T cells and cytokines. Hearts from mice infected with either H3 or H310A1 virus alone or from mice given H310A1 virus and IL-1 were frozen, sectioned, and stained with antibody to CD4 and CD8 T cells and to IL-2 (Table 6). H3 virus-infected mice showed greater numbers of T cells and more IL-2 in the myocardium than did mice given H310A1 virus only. However, administration of exogenous IL-1 to H310A1 virus-infected animals resulted in a substantial increase in both in situ T-cell infiltration into the heart and cytokine release into the myocardium (Fig. 7).

DISCUSSION

Myocarditis is a term describing an inflammation of the heart having either an infectious or a noninfectious etiology (8, 21, 23, 36). Although many viruses have been implicated in myocarditis, members of the picornavirus family predominate. Controversy now centers less on whether viruses can cause myocarditis than on the relevant pathogenic mechanism(s) involved in tissue injury. Two pathogenic mechanisms are undergoing extensive study. These are (i) injury due to direct virus infection of and replication in myocardial cells and (ii) injury due to the immunological response to virus infection. Picornaviruses can often produce lytic infections in susceptible cells, and even in nonlytic infection, the virus can interfere with cellular protein and RNA synthesis (19, 29). Interruption of the synthesis of vital cellular components could have an especially devastating effect on individual cell function. When sufficient numbers of cardiocytes are involved, the function of the whole organ could be compromised. Evidence for direct

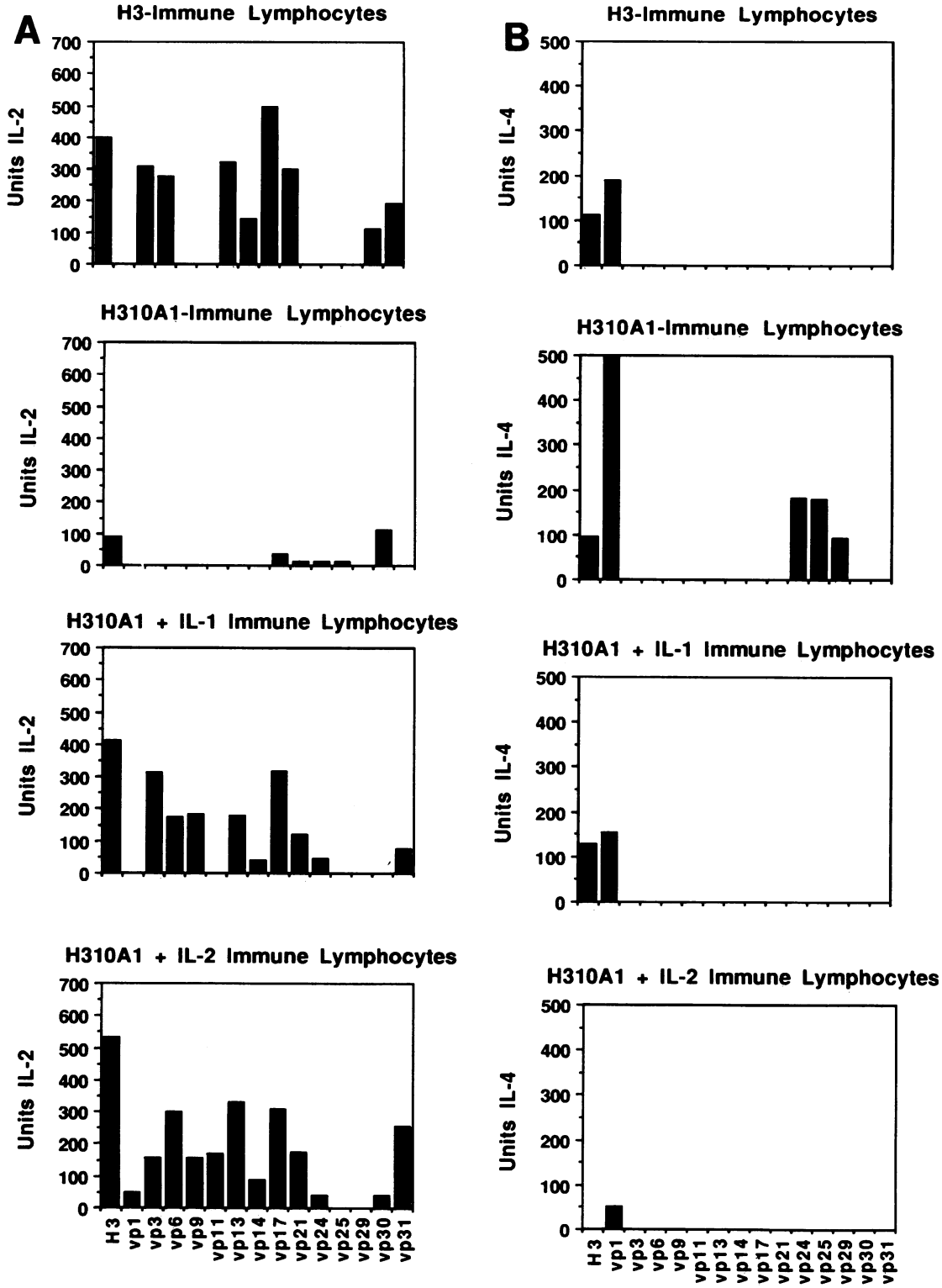

FIG. 4. Measurement of IL-2 in supernatants of T-cell cultures. Supernatants from the cultures shown in Fig. 3 were assayed for IL-2 (A) and IL-4 (B). A standard curve was established by using recombinant IL-2 and IL-4 to determine units of cytokine in the supernatants.

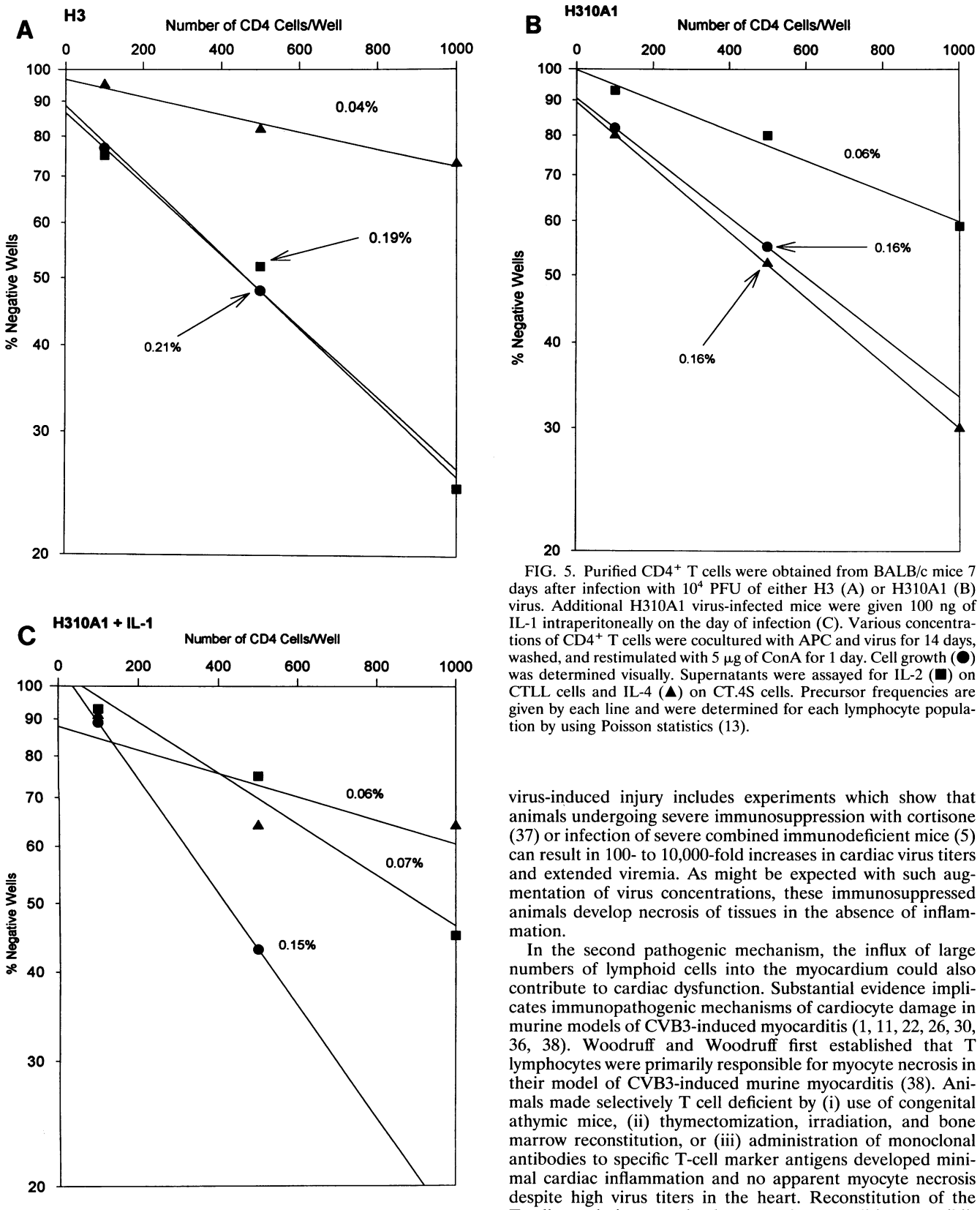

FIG. 5. Purified CD4$^+$ T cells were obtained from BALB/c mice 7 days after infection with 10^4 PFU of either H3 (A) or H310A1 (B) virus. Additional H310A1 virus-infected mice were given 100 ng of IL-1 intraperitoneally on the day of infection (C). Various concentrations of CD4$^+$ T cells were cocultured with APC and virus for 14 days, washed, and restimulated with 5 μg of ConA for 1 day. Cell growth (●) was determined visually. Supernatants were assayed for IL-2 (■) on CTLL cells and IL-4 (▲) on CT.4S cells. Precursor frequencies are given by each line and were determined for each lymphocyte population by using Poisson statistics (13).

virus-induced injury includes experiments which show that animals undergoing severe immunosuppression with cortisone (37) or infection of severe combined immunodeficient mice (5) can result in 100- to 10,000-fold increases in cardiac virus titers and extended viremia. As might be expected with such augmentation of virus concentrations, these immunosuppressed animals develop necrosis of tissues in the absence of inflammation.

In the second pathogenic mechanism, the influx of large numbers of lymphoid cells into the myocardium could also contribute to cardiac dysfunction. Substantial evidence implicates immunopathogenic mechanisms of cardiocyte damage in murine models of CVB3-induced myocarditis (1, 11, 22, 26, 30, 36, 38). Woodruff and Woodruff first established that T lymphocytes were primarily responsible for myocyte necrosis in their model of CVB3-induced murine myocarditis (38). Animals made selectively T cell deficient by (i) use of congenital athymic mice, (ii) thymectomization, irradiation, and bone marrow reconstitution, or (iii) administration of monoclonal antibodies to specific T-cell marker antigens developed minimal cardiac inflammation and no apparent myocyte necrosis despite high virus titers in the heart. Reconstitution of the T-cell population completely restored myocarditis susceptibility in T-cell-deficient animals. Others have identified organ-specific autoantibodies which correlate with susceptibility to

TABLE 4. Cytokine production and stimulationof T-cell clones

Clone[a]	T-cell marker expressed[b]	Cytokine production (U)[c]		Stimulation index[d]						
		IL-2	IL-4	H3 virus	vp1-1	vp1-3	vp1-6	vp1-13	vp1-14	vp1-21
1	CD4	0	350	19	11	—[e]	—	—	—	—
2	CD4	0	259	25	36	7	—	—	—	—
3	CD4	0	275	53	29	9	—	—	—	—
4	CD4	0	287	13	20	3	—	—	—	—
5	CD4	0	400	17	18	6	—	—	—	—
6	CD4	0	133	1	5	21	—	—	—	—
7	CD4	199	0	30	4	15	—	3	—	3
8	CD4	166	0	23	—	30	—	8	—	3
9	CD4	190	0	20	—	17	—	6	—	—
10	CD4	135	0	28	5	24	—	3	—	4
11	CD4	111	0	16	—	—	13	—	—	—
12	CD4	125	0	24	—	—	18	—	—	—
13	CD4	266	81	19	—	—	—	10	—	5
14	CD4	239	95	22	—	—	—	15	—	5
15	CD4	133	47	35	—	3	—	20	—	6
16	CD4	311	85	14	—	—	—	—	10	—
17	CD4	157	98	12	—	—	—	—	7	—
18	CD4	150	40	9	—	—	—	—	5	—
19	CD8	406	40	44	—	—	—	—	—	28
20	CD8	222	105	38	—	—	—	—	—	49
21	CD4	288	105	15	—	4	—	4	—	23
22	CD4	244	37	19	—	—	—	6	—	17
23	CD4	333	111	22	—	3	—	6	—	30

[a] Mesenteric lymph node cells were obtained 7 days after infection of BALB/c mice with 10^4 PFU of H3 virus. Lymphocyte lines were established by culturing the cells with 5×10^4 feeder cells (irradiated at 2,000 R) as APC and 1 μg of H3 virus, and clones were obtained from the lines by limiting dilution with the H3 virus as the antigen. Clones were maintained by restimulation of the cells at 10- to 14-day intervals with fresh APC and antigen.

[b] Cells (10^5) from each clone were dually stained with PE-conjugated anti-L3T4 (anti-CD4) or FITC-conjugated anti-Lyt-2.2 (anti-CD8) antibodies. Stained cells were identified by flow cytometry. Clones were assigned to a specific T-cell subset when >98% of the cells stained with a particular monoclonal antibody.

[c] Lymphocytes (10^5) from each clone were cultured with 5 μg of ConA per ml for 30 h. Supernatants were removed and assayed on CTLL cells for IL-2 and on CT.4S cells for IL-4. Units of activity were established from standard curves of recombinant IL-2 and IL-4 on the respective indicator cell lines.

[d] Cells (10^4) from each clone were cultured with 5×10^4 irradiated APC and 1 μg of the synthetic vp1 peptide per ml in 96-well tissue culture plates. After 4 days of culture at 37°C, 1 μCi of [^{3}H]thymidine was added to each well and the cells were harvested 1 day later. The stimulation index indicates the ratio of the mean counts per minute in cultures with vp1 peptides to the mean counts per minute in cultures with no peptide (medium control). Groups consisted of six replicate cultures.

[e] —, stimulation index of ≤2.

myocarditis in mice and can lyse heart cells in vitro (6, 35). Unlike the experiments using extreme immunosuppression, which affects multiple layers of the immune response, animals undergoing selective immunosuppression of only the T-lymphocyte population show no increase in virus titers in the heart or delays in viral clearance from the blood and tissue (38). Myocardial cell necrosis is not observed in infected T-cell-deficient mice, and animal mortality is substantially lower than that experienced by infected T-cell-competent animals. Thus, immunity in CVB3 infections must encompass both beneficial and injurious responses. Selective depletion of only the T lymphocytes may preferentially eliminate the pathogenic im-

TABLE 5. MHC restriction of T-cell clone reconstitution of myocarditis susceptibility

Mouse strain[a]	TXMB treatment	No. of T cells (clone 13) transferred	Mean myocarditis score[b] ± SEM	Mean cardiac virus titer (log_{10} PFU) ± SEM
BALB/c (*H-2*^{d})	−	0	2.1 ± 0.2 (3)[c]	5.71 ± 0.37
	+	0	0.3 ± 0.2 (3)	6.34 ± 0.12
	+	1×10^6	1.2 ± 0.2 (3)[c]	6.05 ± 0.16
	+	5×10^6	1.0 ± 0.1 (4)[c]	5.52 ± 0.04[c]
	+	1×10^7	0.7 ± 0.1 (5)[c]	5.33 ± 0.32[c]
MRL +/+ (*H-2*^{k})	−	0	1.8 ± 0.6 (3)[c]	6.39 ± 0.16
	+	0	0 ± 0 (3)	5.99 ± 0.10
	+	1×10^6	0.3 ± 0.2 (3)[c]	6.13 ± 0.11

[a] BALB/c and MRL +/+ mice were untreated or thymectomized, irradiated, and bone marrow reconstituted (TXBM treatment). All animals were infected with 10^4 PFU of H3 virus intraperitoneally. On the same day as infection, some animals were given between 0 and 10^7 T lymphocytes of clone 13 (vp1-13 peptide reactive) intravenously. All animals were killed 7 days later. Hearts were evaluated for inflammation and virus titers as described in Materials and Methods.

[b] On a scale of 0 to 4. The results shown are for three to five mice per group. Animal numbers are in parentheses.

[c] Significantly different from result obtained with TXBM mice not given T cells at $P \leq 0.05$.

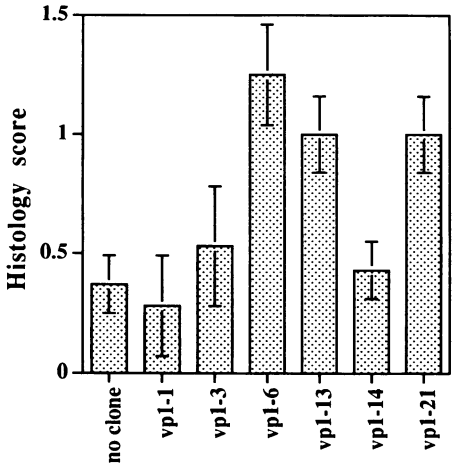

FIG. 6. Adoptive transfer of T-cell clones. Approximately 10^6 cells from the indicated T-cell clone were adoptively transferred intravenously into TXBM mice infected with 10^4 PFU of H3 virus. Control mice were infected TXBM animals not given T-cell clones. Animals were killed 7 days after lymphocyte transfer, and myocarditis was blindly scored on a 0-to-4 scale. The results represent groups consisting of three mice each in one of two replicate experiments.

TABLE 6. Identification of immune cells in CVB3-infected BALB/c mice

Treatment	Staining[a]		
	CD4	CD8	IL-2
H3 virus	10.2 ± 2.4	12.3 ± 4.6	3
H310A1 virus	0.8 ± 1.9	0 ± 0	0
H310A1 virus–IL-1	8.7 ± 1.9	7.6 ± 2.8	2

[a] Comparative immunoperoxidase staining of the myocardium. Numbers of stained cells per high-power field were determined, and 10 randomly selected fields were counted per heart. Evaluation of IL-2 indicates relative intensity of staining on a 0-to-4 scale. The results shown are for two to four mice per group.

munity while leaving adequate antiviral forms in place for virus clearance.

The results presented here demonstrate that T-cell immunity to several peptides of the vp1 capsid protein may be important in myocarditis. Mice infected with the myocarditic H3 virus variant develop sensitized $CD4^+$ lymphocytes reactive to a number of capsid proteins. Nearly all of the reactive cultures produce predominantly IL-2, implying that the responding cells belong to the Th_1 subset of T lymphocytes. Mice infected with the nonmyocarditic H310A1 virus variant fail to elicit detectable proliferative responses to the same peptides as H3 virus-immune animals. Indeed, most of the responsive cultures obtained with H310A1 virus-immune T cells preferentially produced IL-4 rather than IL-2. Preferential production of IL-2 and IL-4 by cells from infected animals was further

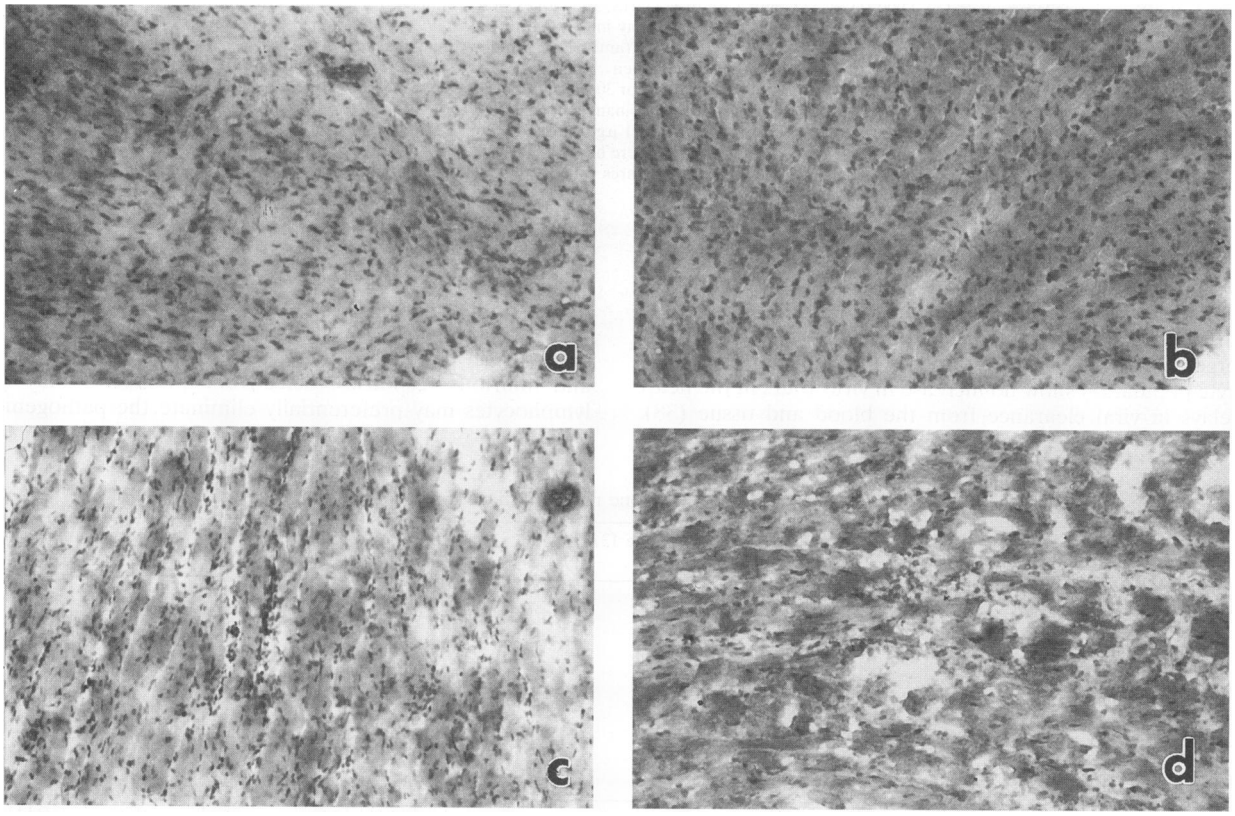

FIG. 7. IL-2 deposition in H310A1- and H310A1–IL-1-treated mice. BALB/c mice were infected for 7 days with 10^4 PFU of H310A1 virus alone (a and b) or infected with 10^4 PFU of H310A1 and additionally given 100 ng of IL-1 intravenously on the same day as the virus (c and d). Hearts were removed, frozen, sectioned, and stained for IL-2 with a monoclonal anti-IL-2 antibody (clone S4 B6) and a peroxidase-conjugated secondary antibody (b and d) or with the secondary-antibody clone (a and c).

confirmed at the clonal level. Thus, the pathogenic and nonpathogenic CVB3 variants demonstrate differential induction of Th_1 and Th_2 cells, respectively. Such differential activation of the Th-cell subsets has been described in murine models of cutaneous leishmaniasis in which preferential stimulation of Th_2 cells occurs in genetically susceptible mice while activation of Th_1 cells correlates with disease resistance (14, 25). Preferential activation of Th_1-cell clones in inflammatory diseases such as myocarditis is logical. Th_1-cell clones synthesize high levels of IL-2, IFN-γ, and TNF-β, lymphokines frequently involved in delayed-type hypersensitivity immune responses. In contrast, Th_2 cells produce IL-4, IL-5, IL-6, and IL-10 (31, 33). Many of these lymphokines function in B-cell help and humoral immunity. Furthermore, lymphokines produced by Th_2 cells (IL-10) suppress Th_1-cell responses. The preferential activation of Th_2 cells during H310A1 virus infection would presumably explain our previous demonstration of $CD4^+$ suppressor cells in these mice. In this case, the Th_2 cells could actively abrogate myocarditis when adoptively transferred into H3 virus-infected recipients (2).

A major question is why these two CVB3 variants initiate such distinct immunological responses in vivo. Several potential explanations are possible. Since H310A1 virus infections may induce less IFN-α/β release from infected cells, lower NK-cell activation might occur in animals given this virus. Since NK cells have been shown to regulate phenotypic T-cell responses, possibly by their production of IFN-γ, a poor NK-cell response might also occur at the macrophage level. Presumably, exogenous administration IL-1 and IL-2 to H310A1 virus-infected mice restores the balance between Th_1-cell activation and Th_2-cell activation to that seen in H3 virus infection. Certainly, lymphocytes from H310A1 virus-infected mice given IL-1 show a decreased Th_2-cell proliferation response similar to that observed in H3 virus-infected animals by precursor frequency analysis. Why the Th_1-cell response in the same animals was not increased is not known, however. H310A1 virus-infected mice given exogenous IL-1 showed substantial in situ IL-2 production in the myocardium in association with exacerbated cardiac inflammation.

In summary, the importance of the above-described studies is that specific T-cell responses which are noticeably absent during nonpathogenic virus infections occur in pathogenic virus infections. The nonpathogenic H310A1 virus must contain the peptide sequences giving rise to pathogenic T-cell responses, or administration of exogenous IL-1 and IL-2 would not result in restoration of the pathogenic T-cell response. The relevant genetic change in H310A1 must be not loss of a pathogenic T-cell epitope but rather failure of the virus to stimulate the pathogenic T-cell response to an existing epitope. The studies presented here and in a previous publication (18) also indicate that if a nonpathogenic infection occurs simultaneously with, or within a specific period of time of, another immunoenhancing event, the cytokines elicited by the immunoenhancing agent might restore the pathogenicity of the virus infection. The outcome of a particular infection depends not only on the virus but on other factors in the host which have an impact on the immune system.

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