

T Cells Expressing the $\gamma\delta$ T-Cell Receptor Potentiate Cocksackievirus B3-Induced Myocarditis

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Received 27 January 1992/Accepted 18 August 1992

Initial studies determined whether intraperitoneal (i.p.) injection of BALB/c mice with 0.1, 1.0, and 10 mg of adriamycin (a cardiotoxic anthracycline antibiotic) for times ranging between 1 and 9 weeks prior to i.p. injection of 10^5 PFU of coxsackievirus B3 (CVB3) would alter the severity of virus-induced myocarditis. Prior adriamycin exposure enhanced pathogenicity of a poorly pathogenic CVB3 variant (H310A1) but had no effect on myocarditis produced by the pathogenic variant (H3). Cardiac virus concentrations were equivalent in H3- and H310A1-infected mice irrespective of adriamycin treatment. BALB/c mice treated with either 0.1 ml of complete Freund's adjuvant (CFA), 10 mg of adriamycin, or 10^5 PFU of H3 and H310A1 i.p. developed cytolytic Thy 1.2⁺ lymphocytes (CTL) to H3-infected myocytes 7 days later. CFA-, adriamycin-, and H3-treated mice developed CTL expressing the $\gamma\delta^+$ T-cell receptors, while H310A1-infected animals did not. Only H3- and H310A1-infected mice developed $\alpha\beta^+$ CTL. Treatment of BALB/c mice with 0.1 ml of CFA 5 days prior to H310A1 infection dramatically increased myocarditis. Selective depletion of $\gamma\delta^+$ T cells abrogated this effect. The ability of $\gamma\delta^+$ T cells to augment the pathogenicity of H310A1 infection was confirmed by adoptive transfer of CFA-stimulated T cells depleted of either $\gamma\delta^-$ or $\gamma\delta^+$ cells into H310A1-infected recipients.

T lymphocytes express either $\alpha\beta$ or $\gamma\delta$ T-cell receptors (TcR) (9, 16, 37). $\alpha\beta^+$ cells are recognized as the classical antigen-reactive T cells. That is, these lymphocytes represent the predominant T-cell population, are distributed widely throughout the body and lymphatic system, react to antigenic peptides presented in either class I or class II major histocompatibility complex molecules, and show extensive TcR diversity. In contrast, $\gamma\delta^+$ cells are more selectively distributed, show limited TcR diversity, which might imply that these cells react to a minimal number of antigens, and often recognize antigens outside the context of major histocompatibility molecules. A high percentage of $\gamma\delta^+$ cells may react to either microbial or self heat shock proteins (HSPs) (13, 18). Approximately 10% of $\gamma\delta^+$ T-cell hybridomas obtained from fetal murine thymus react to the 65-kDa HSP of *Mycobacterium tuberculosis*. Most $\gamma\delta^+$ cells are located in epithelial tissue and are especially well represented in the intestinal epithelium, where they may play an important role in host defense against microbial invasions (1, 19). Since many bacteria express HSPs and these molecules are highly conserved in nature, $\gamma\delta^+$ cells could be expected to function effectively against most microbial agents.

T cells expressing the $\gamma\delta$ TcR often accumulate at the sites of chronic inflammation, including after viral infections (influenza pneumonitis) (3, 10). In some cases, $\gamma\delta^+$ cell influx may occur in response to HSP-like antigens in the viruses (14). However, inhibition of cellular metabolism is one mechanism of cellular HSP induction, and many viruses effectively interfere with host cell RNA and protein synthesis. Therefore, these lymphocytes may also infiltrate infected tissues in response to self HSP expression in virus-infected cells. The precise role(s) for $\gamma\delta^+$ T cells in either normal immunity or disease remains unclear. These cells can produce interleukin-2 (IL-2), IL-3, IL-4, gamma interferon and granulocyte-macrophage colony-stimulating factor and

have been reported to reverse anergy of autoreactive $\alpha\beta^+$ T cells (22, 27, 38). Virus infections which induce potent $\gamma\delta^+$ T-cell responses may, in essence, jump start an autoimmune response which might not occur without these T cells.

Cocksackievirus B3 (CVB3) has been extensively studied for its ability to induce inflammatory heart disease (myocarditis) in humans and other animals (15). Many investigators believe that the virus infection triggers an autoimmune response to cardiac antigens (5, 8, 21, 25, 30). This effect occurs either through antigenic mimicry between the virus and self molecules, through induction of anti-idiotypy, or possibly by promotion of release, processing, and presentation of self molecules to autoreactive T cells. Previously, we described two variants of CVB3, one that is highly pathogenic (H3) and one that is not (H310A1) (17, 34). In this report, we provide evidence that lack of $\gamma\delta^+$ cell response in H310A1-infected animals may partially explain the poor pathogenicity of this virus.

MATERIALS AND METHODS

Mice. BALB/c mice were originally purchased from Cumberland Farms, Clinton, Tenn. Male mice 8 weeks of age and neonatal mice less than 72 h old were obtained from colonies of these mice maintained at the University of Vermont.

Viruses. Two variants of CVB3 were isolated by plaque purification as described earlier (34). These viruses were grown in HeLa cells and titered by the plaque-forming assay.

Organ virus titer. Hearts were removed aseptically, weighed, and homogenized in Dulbecco modified Eagle medium (DMEM) containing 2% fetal bovine serum (FBS). Cellular debris was removed by centrifugation at $300 \times g$ for 10 min, and the titers of virus in the supernatants were determined by the plaque-forming assay (17, 34).

Adriamycin. Doxorubicin hydrochloride (Adriamycin RDF; Adria Laboratories, Columbus, Ohio) in 10-mg single-dose vials contained 10 mg of the drug, 50 mg of lactose, and 1 mg of methylparaben in powdered form. Aliquots of the

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drug were measured on the day of use, dissolved in saline to 1 mg/ml, and subsequently diluted to the appropriate concentration as described in the text.

Immunization of mice. Animals were (i) infected intraperitoneally (i.p.) with 10^5 PFU of virus in 0.5 ml of phosphate-buffered saline (PBS), (ii) injected i.p. with 0.1 ml of complete Freund's adjuvant (CFA) containing 1 mg of heat-killed *M. tuberculosis* (GIBCO, Grand Island, N.Y.) per ml, or (iii) injected i.p. with between 0.1 and 10 mg of adriamycin per kg of body weight in 0.5 ml of PBS.

Preparation of myocytes. Hearts were aseptically removed from neonatal mice, minced finely, subjected to stepwise enzymatic digestion with 0.4% collagenase II, and cultured in DMEM (GIBCO) containing 5% FBS, 10% horse serum, 100 U of penicillin, and 100 μ g of streptomycin per ml at a concentration of 3×10^5 viable cells per ml.

Preparation of lymphocytes. Mesenteric lymph nodes from mice were removed and pressed through fine-mesh screens to form single lymphocyte suspensions. The cells were washed in DMEM-5% FBS and incubated on nylon wool for 30 min at 37°C. The nonadherent T-lymphocyte-enriched population was retrieved and washed, and viability was determined by trypan blue exclusion.

Cell-mediated cytotoxicity assay. Approximately 10^3 myocytes were cultured for 2 days in 4.7-mm-diameter tissue culture wells (Bellco Glass Inc., Vineland, N.J.) at 37°C, washed, and overlaid with 10 μ l of medium containing either 1 μ Ci of ^{51}Cr ($\text{Na}_2^{51}\text{CrO}_4$; NEN, Boston, Mass.) alone or with the radioisotope and virus or drug as indicated in the text. Some myocyte targets were subjected to heat shock by incubating the cells at 42°C for 1 h and then incubating them at 37°C for 12 to 18 h prior to chromium labeling. After incubation with the isotope for 2 h at 37°C, the monolayers were washed four times with medium and overlaid with 10 μ l of DMEM-5% FBS containing 3×10^5 nonimmune or sensitized lymphocytes. Controls consisted of wells with medium but no lymphocytes. The assay mixtures were incubated for 18 to 22 h at 37°C, and the radioactivity in the supernatants and cell pellets was determined by using an Intertechnique CG4000 gamma counter (Intertechnique, Lyons, France). Percent ^{51}Cr release was determined by using the formula [(cpm in supernatant)/(cpm in supernatant + cpm in cell pellet)] \times 100. Percent lysis was determined by the formula [(% ^{51}Cr release in experimental - % ^{51}Cr release in medium control)/(% ^{51}Cr release in freeze-thaw - % ^{51}Cr release in medium control)] \times 100.

Infection of macrophage cells. A BALB/c-derived monocyte-macrophage line (J774A.1; American Type Culture Collection [ATCC], Rockville, Md.) was maintained in DMEM-10% FBS. Approximately 10^5 cells per well were dispensed into 96-well tissue culture plates (Bellco) and exposed to 10^6 PFU of either H3 or H310A1 for 1 h at 37°C. The cells were washed, treated with a 1:100 dilution of horse anti-CVB3 (ATCC) to eliminate extracellular virus, washed again, and cultured in 200 μ l of DMEM-10% FBS for times ranging from 2 to 72 h. Supernatants of the triplicate cultures were removed for determination of tumor necrosis factor alpha (TNF- α) concentrations; the cells were retrieved by trypsinization (0.25% trypsin; GIBCO) and frozen and thawed three times, and the titer was determined by assay of 50% tissue culture infectious dose (TCID₅₀) on HeLa cell monolayers in 96-well plates.

TCID₅₀ assay. The TCID₅₀ assay involved making 10-fold dilutions of the cell preparation in medium and adding 100 μ l of the dilutions to the HeLa cells. The cultures were incubated overnight at 37°C, the supernatant was removed, and

the cell monolayer was fixed with 10% buffered formalin and stained with 0.5% crystal violet in 20% ethanol. After the monolayer was washed six times with PBS to remove unincorporated stain, the crystal violet was eluted in 200 μ l of 70% ethanol. Controls consisted of uninfected monolayers and monolayers lysed by distilled H₂O prior to fixation. The crystal violet eluate was measured at 599 nm, using a Biotek enzyme-linked immunosorbent assay plate reader (Biotek, Winooski, Vt.). Controls consisted of monolayers without virus (negative control; results in 0% cell lysis) and monolayers infected with 10^4 PFU of H3 (positive control; results in 100% cell lysis within 20 h). The titer end point was taken as the absorbance half-way between values for the negative and positive controls.

TNF- α assay. WEHI 164, subclone 13 cells (ATCC) were grown in DMEM-10% FBS, centrifuged at $160 \times g$, washed, resuspended to 10^7 cells per ml, and incubated with 100 μ Ci of ^{51}Cr for 2 h at 37°C. The cells were washed four times and dispensed (2×10^4 cells per well) into 96-well tissue culture plates together with an equal volume of supernatant from the macrophage cultures. A standard curve for TNF- α -mediated cell lysis was established by using recombinant TNF- α (kindly supplied by Genentech, South San Francisco, Calif.) ranging in concentration from 1 to 1,000 U/ml. The plates were incubated at 37°C for approximately 15 h in a humidified 5% CO₂ incubator. Percent ^{51}Cr release and percent lysis of WEHI cells were determined as described above.

MAbs. Hybridoma clone 30-H12 (anti-Thy 1.2) was obtained from the ATCC. Hybridoma clones GL3-3A (anti- $\gamma\delta$ TcR) and H57-597 (anti- $\alpha\beta$ TcR) were kindly supplied by Leo LeFrancois (Upjohn Co., Kalamazoo, Mich.) and Ralph Kubo (National Jewish Center, Denver, Colo.), respectively (2, 28). The cells were initially cultured in DMEM-10% FBS. Subsequently, 10^7 cells were injected i.p. into BALB/c mice which had been treated i.p. with 0.5 ml of pristane and 550 R of sublethal irradiation. Ascites fluid containing the MAbs was collected 7 to 10 days later and purified by ammonium sulfate precipitation.

Histology. Hearts were fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin. Sections were graded blindly for cardiac inflammation by one of us, using a scale of 0 to 4 in which 0 represented no myocarditis, 1 represented 1 to 10 lesions, 2 represented 11 to 20 lesions, 3 represented 21 to 40 lesions, and 4 represented widespread and confluent inflammation.

Flow cytometry. Mesenteric lymph node cells, obtained from BALB/c mice given 0.1 ml of CFA 6 days earlier, were stained with a 1:500 dilution of primary fluorescein isothiocyanate (FITC)-conjugated hamster anti-murine $\gamma\delta$ TcR (PharMingen, San Diego, Calif.) for 30 min at 4°C, washed, and resuspended in DMEM-2% FBS at a concentration of 10^7 cells per ml. The lymphocyte population was sorted into $\gamma\delta^+$ and $\gamma\delta^-$ subfractions by using a Coulter Elite flow cytometer (Coulter Co., Hialeah, Fla.).

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

RESULTS

Augmentation of H310A1-induced myocarditis with adriamycin treatment. Adriamycin is a cardiotoxic chemotherapy agent (6, 24, 31, 32). Initially, studies were designed to determine whether prior exposure of animals to adriamycin could augment CVB3-induced myocarditis. BALB/c mice were injected i.p. with a single dose of adriamycin ranging in concentration from 0.1 to 10 mg/kg of body weight. None of

TABLE 1. Enhanced myocarditis and mortality in H310A1-infected mice previously exposed to adriamycin^a

Virus	Adriamycin concn (mg/kg)	Time (wk) before infection	Cumulative mortality (no. dead/total) ^b	Myocarditis score ^c
H310A1	0		2/33 (6)	0.5 ± 0.1
		1	16/20 (80) ^d	1.4 ± .4 ^d
		6	2/10 (20)	0.5 ± 0.1
	1.0	1	4/20 (20)	0.6 ± 0.1
		6	14/22 (64) ^d	1.4 ± 0.2 ^d
		9	2/10 (20)	1.0 ± 0.1 ^d
	10.0	1	0/10 (0)	0.6 ± 0.2
		6	16/18 (89) ^d	1.4 ± 0.1 ^d
		9	7/9 (78) ^d	1.2 ± 0.4 ^d
H3	10	1	9/21 (43) ^d	1.0 ± 0.2 ^d
		6	7/10 (70)	1.5 ± 0.2
		9	8/10 (80)	1.3 ± 0.2

^a BALB/c mice were injected i.p. with different concentrations of adriamycin or with an equal volume of PBS. Between 1 and 9 weeks after receiving the drug, the mice were injected i.p. with 10^5 PFU of virus. Animals were sacrificed 7 days later.

^b Values in parentheses are percentages.

^c Hematoxylin- and eosin-stained heart sections of organs obtained 7 days after virus infection were read blindly by one investigator, using a scale of 0 to 4 as published previously (34).

^d Significantly different at the $P \leq 0.05$ level from the value for the group receiving virus only.

these drug concentrations directly caused significant myocardial inflammation or necrosis. At times ranging from 1 to 9 weeks after drug treatment, the animals were infected with 10^5 PFU of either the highly pathogenic H3 or nonpathogenic H310A1 virus variant. All animals were sacrificed 7 days after receiving virus, and the hearts were evaluated for inflammation and virus titer (Table 1). H3-infected animals developed significant cardiac inflammation and exhibited high mortality. Adriamycin treatment had no effect on H3 pathogenicity. In contrast, H310A1 infection of adriamycin-treated animals resulted in significant increases in both myocarditis and mortality. Generally, the greatest effects were observed when the drug was administered 1 week prior to infection. However, at the highest adriamycin concentration (10 mg/kg), augmentation of heart injury was detected even when infection occurred 9 weeks after drug exposure. Virus titers were $5.3 \times 10^6 \pm 0.8 \times 10^6$ and $4.1 \times 10^6 \pm 1.1 \times 10^6$ PFU/100 mg of tissue for H3 and H3-adriamycin-treated groups, respectively. The titers for the H310A1-only group was 1.1×10^6 to $\pm 0.4 \times 10^6$ PFU/100 mg of tissue, and those for the various adriamycin-H310A1 groups ranged from 0.8×10^6 to 1.2×10^6 PFU/100 mg of tissue. No significant differences in virus concentrations in the heart were noted.

The results presented above indicated that adriamycin could augment the pathogenicity of a relatively nonmyocarditic CVB3 variant while having no effect on the pathogenicity of a highly myocarditic variant. However, the drug's effects may not be mediated by direct alterations in virus infection or replication in the heart. This possibility was confirmed by *in vitro* studies. Myocytes were cultured in medium containing 0, 10^{-8} , 10^{-9} , and 10^{-10} M adriamycin overnight, and then the cells were infected with between 0.1 and 10 PFU of H310A1 per cell for 45 min at 37°C. After being washed, the monolayers were cultured for 20 h. The cells and supernatants were retrieved and frozen and thawed three times, and titers were determined. Again, no significant differences in virus concentration between drug-treated and untreated cultures were detected (data not shown).

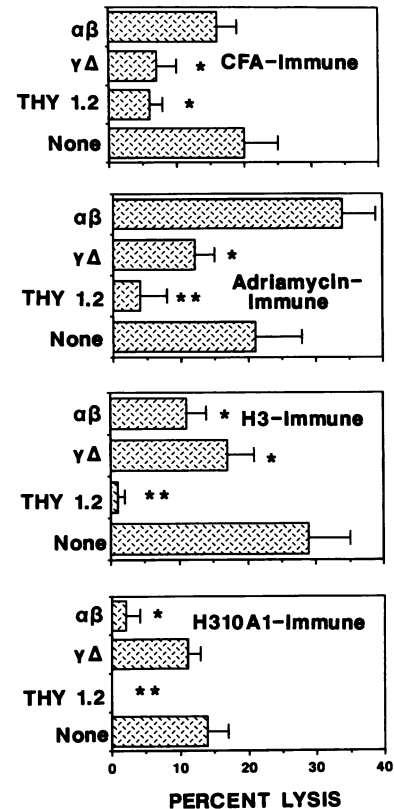


FIG. 1. Cytolysis of myocyte targets by CFA, adriamycin, and virus-stimulated lymphocytes. BALB/c mice were injected with 0.1 ml of CFA, 10 mg of adriamycin, or 10^5 PFU of H3 and H310A1 i.p. and sacrificed 7 days later. Mesenteric lymph node cells from these animals were either untreated or treated with antibodies to Thy 1.2, $\alpha\beta$ TcR, or $\gamma\delta$ TcR and complement and then assayed for cytotoxicity to H3-infected syngeneic myocytes at an effector/target cell ratio of 100:1. Results represent mean percent lysis \pm standard error of the mean of at least triplicate cultures. * and ** indicate values that are significantly different at the $P \leq 0.5$ and $P \leq 0.01$ levels, respectively, from those of the group containing untreated lymphocytes.

Demonstration of $\gamma\delta^+$ T-cell responses in CVB3 infection. BALB/c mice were either infected with 10^5 PFU of H3 and H310A1, injected with 0.1 ml of CFA, or injected with 10 mg of adriamycin per kg and sacrificed 7 days later. Mesenteric lymph node cells were treated with either anti-Thy 1.2, anti- $\alpha\beta$ TcR, or anti- $\gamma\delta$ TcR and assayed for cytotoxicity to H3-infected myocytes (Fig. 1). All four immune lymphocyte populations showed cytotoxicity to targets, and in each case, activity was largely mediated by Thy 1.2⁺ cells. CFA- and adriamycin-immune cytotoxic T lymphocytes predominantly used $\gamma\delta$ TcR, since treatment with this antibody abrogated cytotoxicity. H3-immune effectors belonged to both $\alpha\beta^+$ and $\gamma\delta^+$ cell populations, since treatment with antibodies to either TcR reduced cytotoxicity. However, only anti- $\alpha\beta$ TcR antibody inhibited cytotoxicity in H310A1-immune cells, suggesting that few $\gamma\delta^+$ T cells are induced with this virus variant.

Demonstration of a possible role for $\gamma\delta^+$ T cells in CVB3-induced myocarditis. BALB/c mice were infected with either H3 or H310A1. Some of the mice had received 0.1 ml of CFA i.p. 5 days prior to infection. Additionally, some mice were

TABLE 2. Effect of CFA on CVB3 pathogenicity

Virus ^a	Treatment		Myocarditis score ^b	Cardiac virus titer (log ₁₀ PFU/heart)
	CFA ^c	MAb ^d		
H3-10A1	-	-	0.5 ± .4	4.1 ± 1.2
	+	-	1.8 ± .4 ^e	3.4 ± 0.5
	+	γ/δ	0.6 ± .6	3.6 ± 0.7
None	-	-	0 ± 0	ND ^f
	+	-	0 ± 0	ND
	+	γ/δ	0 ± 0	ND
	-	γ/δ	0 ± 0	ND
H3	-	-	2.1 ± .3	4.7 ± 0.8
	-	γ/δ	2.1 ± .2	3.5 ± 0.7

^a BALB/c mice were injected i.p. with 5×10^4 PFU of virus in 0.5 ml of PBS. Animals were sacrificed 7 days later. Each group contained a minimum of seven animals.

^b Hematoxylin- and eosin-stained heart sections of organ obtained 7 days after virus infection were read blindly by one investigator, using a scale of 0 to 4 as published previously (34).

^c Some animals received 0.1 ml of CFA i.p. 5 days prior to virus inoculation.

^d Indicated groups received 2 mg of MAb to the γδ⁺ TcR i.p. 2 days prior to virus inoculation.

^e Significantly different at the $P \leq 0.05$ level from the value for the group not receiving CFA.

^f ND, not determined.

given 2 mg of anti-γδ TcR i.p. 2 days before infection. All animals were sacrificed 7 days after infection, and the hearts were evaluated for myocarditis and virus titer (Table 2). Uninfected animals, irrespective of whether they were treated with anti-γδ antibody or CFA, failed to develop heart disease. H310A1-infected mice showed minimal disease except in animals given CFA before infection. Administration of anti-TcR antibody into H310A1-infected and CFA-treated mice prevented the augmentation of disease, suggesting that these effectors, presumably induced by CFA, may overcome the poor pathogenicity of this virus variant. Interestingly, anti-γδ treatment of H3-infected mice did not alter the pathogenicity of this variant. This finding suggests that while γδ⁺ T cells may enhance pathogenicity of a poorly myocarditic virus, other factors besides γδ⁺ T cells may additionally contribute to disease caused by the wild-type H3 variant.

One problem with the study described above is demonstrating that antibody to the γδ TcR actually depletes these cells in vivo. The rarity of γδ⁺ T cells in peripheral lymphoid

TABLE 3. Adoptive transfer of γδ⁺ and γδ⁻ into H3-10A1-infected mice^a

Treatment	% Cumulative mortality	Myocarditis score
None	0	0.3 ± 0.3
CFA	60 ^b	1.2 ± 0.4 ^b
T cells		
Unfractionated	100 ^b	1.6 ± 0.5 ^b
γδ ⁺	80 ^b	1.5 ± 0.3 ^b
γδ ⁻	0	0.5 ± 0.3

^a BALB/c mice were injected i.p. with 10^4 PFU of H3-10A1 alone and in combination with either 0.1 ml of CFA, 2×10^5 unfractionated T cells from CFA-stimulated donor mice, 2×10^5 γδ⁻ T cells, or 5×10^3 γδ⁺ T cells. The donated lymphocytes were obtained from the mesenteric lymph nodes of syngeneic mice given 0.1 ml of CFA i.p. 6 days earlier. Infected mice were killed on day 6. Each group consisted of a minimum of five mice.

^b Significantly greater at the $P \leq 0.05$ level than the value for the no-treatment group.

origins makes evaluation of depletion efficiency very difficult. Therefore, T-cell-enriched populations were isolated from the mesenteric lymph nodes of BALB/c mice injected 6 days earlier with CFA. Part of the lymphocyte population was stained with a primary FITC-conjugated anti-γδ TcR and sterilely sorted into positive and negative subsets (Fig. 2). The original (unfractionated) population contained 0.7% γδ⁺ T cells. After sorting, the positively selected preparation contained >90% γδ⁺ cells. No γδ⁺ T cells were obtained in the γδ⁻ population. Next, BALB/c mice were simultaneously infected with 10^4 PFU of H310A1 and adoptively transfused with either 2×10^5 unfractionated T cells, an equal number of γδ⁻ T cells, or 5×10^3 γδ⁺ cells. A separate group of infected mice was injected i.p. with 0.1 ml of CFA along with the virus.

Control animals consisted of animals infected with H310A1 alone (untreated group) (Table 3). The results showed that BALB/c mice given either CFA or unfractionated CFA-immune cells showed significantly greater animal mortality and myocarditis than did untreated, H310A1-infected mice. This increase in myocarditis resulted directly or indirectly from transfer of γδ⁺ cells since adoptive transfer of this population from the CFA-immune cells also enhanced the pathogenic response. Transfer of γδ⁻ T cells had no effect in H310A1-infected mice.

These results indicate that CVB3 infections may require a

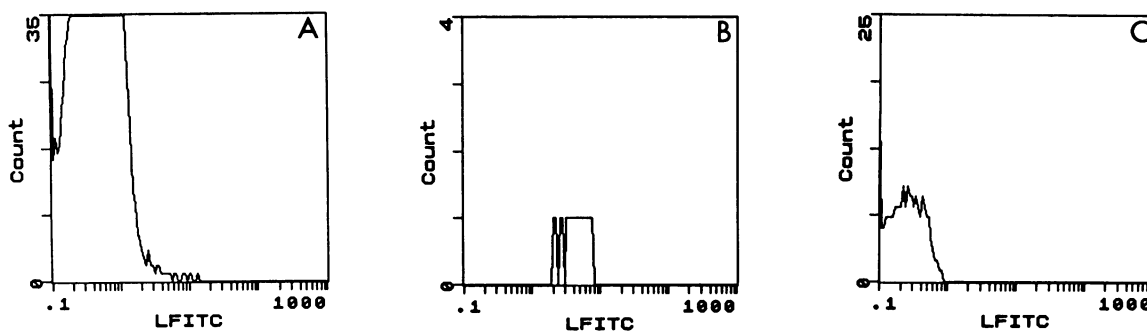


FIG. 2. Histogram of γδ⁺ TcR lymphocytes in mesenteric lymph node cell preparations obtained from BALB/c mice 6 days after injection with 0.1 ml of CFA. Lymphocytes were stained with a 1:500 dilution of primary FITC-conjugated hamster anti-mouse γδ TcR antibody. (A) Unfractionated lymph node population contained 0.7% γδ⁺ T cells. After sterile sorting of the positive and negative populations by flow cytometry, the positively selected cell population (B) consisted of >90% γδ⁺ cells, while the γδ⁻ cell population (C) contained no stained cells.

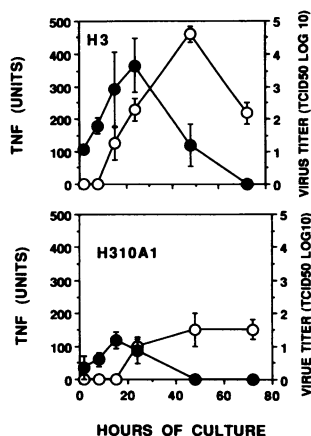


FIG. 3. Differential induction of TNF- α in monocytes infected with H3 and H310A1. Approximately 10^5 BALB/c monocytes (line J774A.1) were exposed to 10 PFU of virus per cell for 1 h, washed, and cultured for times ranging from 2 to 72 h. Supernatants were assayed for TNF- α (○), and the cells were homogenized and titered for virus (●). Results represent mean \pm standard error of the mean of four to six replicate cultures.

complex interaction of immune responses for induction of disease. H310A1 can initiate myocarditis when provided with an activated $\gamma\delta^+$ T-cell response which this particular virus does not induce. H310A1 may additionally lack other characteristics needed for pathogenicity. One such factor might be induction of immunomodulating cytokines during infection. To determine whether H3 and H310A1 differ in the ability to either replicate or induce cytokine production in infected macrophage, a murine monocyte-macrophage line (J774A.1) was exposed to 10 PFU of virus per cell for 1 h at 37°C, washed, and cultured for between 2 and 72 h. Supernatants of triplicate cultures were evaluated for TNF- α concentrations, and the cells were homogenized and titered for virus by using the TCID₅₀ assay (Fig. 3). Maximum virus concentrations were observed in both H3- and H310A1-exposed macrophage at approximately 15 to 20 h, although the concentration of virus was significantly lower in H310A1 cultures than in H3 cultures. TNF- α concentrations peaked at 48 h in H3-exposed cultures. Cytokine release was slightly delayed in H310A1-infected cultures, and only approximately 20% of the concentrations observed in H3 culture supernatants were observed.

DISCUSSION

Woodruff and Woodruff first demonstrated a clear role for immunity in the pathogenesis of murine myocarditis when they showed that T-cell-deficient mice developed few inflammatory lesions after infection, whereas restoration of the T-cell population enhanced disease susceptibility (36). Since the T-cell-deficient mice contained both macrophage and B lymphocytes, these results implied that one or more responding T-cell populations must be required for expression of myocarditis. Furthermore, evidence from several laboratories now implicates autoimmunity following infection as the basis of pathogenicity (5, 8, 21, 25, 30). Several autoantigens have been implicated in CVB3-induced myocarditis. These antigens include cardiac isoforms of myosin, the calcium channel, and the adenine nucleotide translocator protein (ANT) found in mitochondria. In each case, CVB3-infected animals develop either humoral or cellular immunity to these self molecules, and immunization of susceptible

strains of mice with the isolated protein in CFA results in cardiac inflammation resembling the myocarditis obtained after virus infection.

How picornaviruses trigger autoimmune responses is a complex question. Several mechanisms are likely. Antigenic mimicry between the virus and various self proteins, including cardiac myosin and ANT, has been demonstrated and probably plays a major role in pathogenicity (18, 23, 29, 33, 36). If antigenic mimicry is an important aspect of virus-induced autoimmunity, the question remains as to why autoimmunity does not occur in the absence of virus infection. Presumably, the self epitope is continuously present in an individual. However, either normal myocardial cells express little or no MHC proteins, which prevents presentation of the heart self epitopes, or presentation of the self epitope by myocytes may preferentially lead to T-cell anergy rather than clonal stimulation (7, 12, 26). In the latter case, proper antigen presentation not only depends on the antigen-presenting cell but also requires a secondary signal such as IL-1 or surface costimulatory molecules (CD2, CD28, or LFA-1) to activate $\alpha\beta^+$ T lymphocytes (20, 35). Some investigators feel that peptide presentation in the absence of the secondary signal may actually result in T-cell anergy and that this may represent an important immunoregulatory mechanism preventing autoimmunity to peripheral tissue antigens. Presentation of cross-reacting (mimicking) epitopes during microbial infections might circumvent anergy either through the release of necessary cytokines by virus-specific T cells and virus-activated macrophage in the myocardium or by induction of $\gamma\delta^+$ T cells during infection (22, 27).

In previous studies, we reported two distinct CVB3 genetic variants which infect the heart but differ dramatically in pathogenicity (17, 34). The pathogenic variant, H3, triggered autoimmunity to heart antigens, while the nonpathogenic H310A1 variant preferentially stimulated immunosuppression. On the basis of studies by Palmenberg (24a) which indicate that nonpathogenic encephalomyocarditis virus variants showed defective infection and cytokine induction in macrophage, one explanation for the nonpathogenicity of the H310A1 variant may be that antigen-presenting cells exposed to this virus process and present the viral epitopes, but deficient cytokine release prevents T-cell proliferation and may even render the antigen-reactive lymphocytes tolerant. Such a possibility seems highly probable, since both H3 and H310A1 variants contain mimicking epitopes to both ANT and cardiac myosin, the two most likely antigens in the autoimmune response (11). As shown here, H310A1 is less able to infect macrophage, and TNF- α production is lower in these cells than in H3-exposed cells.

T cells expressing the $\gamma\delta^+$ TcR produce a variety of lymphokines which can augment major histocompatibility complex antigen expression on tissues (4) and reverse anergy in autoreactive $\alpha\beta^+$ T cells (22, 27). Virus infections which induce potent $\gamma\delta^+$ T-cell responses may, in essence, jump start an autoimmune response which might not occur without these cells. This effect of $\gamma\delta^+$ T cells might additionally explain the usual practice of immunizing animals with self molecules in CFA to induce autoimmunity. In the present system, the pathogenic H3 virus may simultaneously promote autoimmunization through stimulation of cytokines from virus-exposed macrophage and $\gamma\delta^+$ T cells. In contrast, the H310A1 virus induces neither $\gamma\delta^+$ T cells or good cytokine production from virus-exposed macrophage. Supplying exogenously either of these immunomodulating effects should lead to augmentation of H310A1 pathogenicity, as indeed has been shown by giving H310A1-infected ani-

mals either CFA or the $\gamma\delta^+$ T cells from CFA-immunized donors. Depletion of $\gamma\delta^+$ cells from H3-infected mice fails to abrogate myocarditis. This finding indicates either that depletion of the $\gamma\delta^+$ cells was incomplete in H3-infected mice or that the other immunomodulating characteristics of the virus were adequate to promote autoreactive T-cell responses in the absence of $\gamma\delta^+$ T-cell responses.

ACKNOWLEDGMENTS

We gratefully acknowledge the expert secretarial assistance of Laurie Sabens, who helped organize and prepare the manuscript.

This work was supported by Public Health Service grant-in-aid HL28833 from the National Institutes of Health and by American Heart Association grant-in-aid 90-0999.

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