Coxsackievirus B-3 Myocarditis in Balb/c Mice

Evidence for Autoimmunity to Myocyte Antigens

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Male Balb/c mice inoculated with a heart-adapted variant of Coxsackievirus, group B, type 3 (Nancy) (CVB3M), develop extensive myocarditis and cytolytic activity to primary cultures of uninfected and infected myocytes. To elucidate the mechanisms of myocyte injury in myocarditis, two distinct cytolytic T-lymphocyte (CTL) populations were isolated by immunoadsorption of lymph node cells to glutaraldehyde-fixed uninfected and infected myocyte monolayers. One population preferentially adsorbed to and lysed uninfected myocytes (autoreactive CTLs), while the other adsorbed to and lysed CVB3M-infected myocytes (virus-specific CTL). Neither CTL population adsorbed to monolayers of HeLa, L929, or umbilical cord endothelial cells, or to myocytes infected with a related but nonmyocarditic Coxsackievirus B-3 variant ($CVB3_0$). While both autoreactive and virus-specific CTLs induced myocarditis *in vivo*, the lesions caused by autoreactive CTLs were more extensive and necrotizing than those of virus-specific cells. These results support the hypothesis that CVB3-induced myocarditis results, in part, from autoimmunity to myocyte antigens. (Am J Pathol 1984, 116:21–29)

COXSACKIE B viruses are commonly associated factors in human myocarditis.[1–4] However, whether damage results primarily from direct virus-mediated injury or from the mononuclear cells infiltrating the heart during the disease is not known. The evidence for immune-mediated injury is circumstantial but compelling. Virus is rarely isolated from patients with myocarditis, although compatible clinical histories and rising antibody titers suggest recent infection.[4–7] In addition, cardiac function in many myocarditis patients will improve with immunosuppressive therapy.[8,9]

A murine model of myocarditis using male Balb/c mice and a highly myocarditic variant of Coxsackievirus B-3 (CVB3) induces lesions histologically similar to the human disease.[10–22] The lesions are characterized by extensive focal inflammation composed predominantly of mononuclear cells.[1–10] In this model, immune rather than viral mechanisms appear to produce most of the cardiac injury. For example, inoculation of T-lymphocyte-deficient mice with the virus fails to induce significant myocarditis even though equivalent concentrations of infectious virus are isolated from the hearts of T-cell-deficient and intact animals.[17]

Cytolytic T-lymphocytes (CTLs) from CVB3-inoculated mice lyse primary cultures of both virus-infected and uninfected myocytes.[18–22] Lysis of the uninfected myocytes could result from autoimmune CTLs recognizing cardiocyte-specific antigens, from virus-specific CTLs recognizing virus-modified cardiocyte antigens and cross-reactively lysing uninfected targets, and from natural killer cells. In this report, we show that the cytolytic activity to the uninfected myocyte targets actually is mediated by a population of autoreactive T cells. These autoreactive T cells are highly specific for cardiocytes and appear to be major mediators of cardiac injury in CVB3 myocarditis.

Supported by PHS Grants-in-Aid HL 27976 and HL/AI 28480 from the National Institutes of Health, American Heart Association Grant-in-Aid 81-1158, and an American Heart Association Established Investigatorship awarded to Dr. Huber.

Accepted for publication January 26, 1984.

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Materials and Methods

Mice

Balb/c mice were originally purchased from Cumberland Farms, Clinton, Tennessee. Neonates and 6–8-week-old adult males were obtained from colonies of these mice maintained at the University of Vermont.

Virus Preparation and Purification

A "myocarditic" variant of CVB3 (Nancy) virus (CVB3M) was obtained from J. F. Woodruff (Cornell University Medical College, New York, NY), and an "amyocarditic" variant of the same virus ($CVB3_0$) was originally kindly supplied by Richard Crowell, Hahnemann Medical College, Philadelphia, Pennsylvania. Both virus variants were grown in HeLa cell suspensions according to the procedure described in detail earlier.[19] The titers of the purified viruses ranged from 9×10^{10} to 10×10^{10} plaque-forming units (PFU) per milliliter. The viruses were stored at −70 C in phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+}.

Virus Titration

The virus preparations were titered by the plaque-forming assay described previously.[20]

Infection of Mice

Each animal was infected by an intraperitoneal injection of 3×10^4 PFU of virus in 0.5 ml PBS.

Preparation, Culture, and Characterization of Myocytes, Endothelial Cells, and Skin Fibroblasts

Myocytes

Hearts were removed aseptically from neonatal mice within 72 hours of birth. Single cell suspensions of myocytes were prepared with the use of a modification of the method of Bollon et al.[23] Briefly, the hearts were minced finely and subjected to stepwise enzymatic digestion with 0.25% pancreatin (GIBCO, Grand Island, NY) or 0.4% collagenase (Worthington Biochemical Corp., Freehold, NJ). The dissociated cells were washed with complete basal medium Eagle's (BME) (medium containing penicillin, streptomycin, 5% fetal calf serum [FCS], 10% horse serum, 0.2 mg crystalline insulin, and 20 mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid] buffer per 100 ml medium) and depleted of endothelial cells and fibroblasts by two sequential 1-hour adsorptions to plastic flasks (Falcon Plastics Inc., Oxnard, Calif) at 37 C. The nonadherent myocytes were removed, washed once, resuspended in complete BME and dispensed into tissue culture wells 1-mm in diameter (Falcon) or into flasks. After 48 hours, the time required by the myocytes to firmly attach to the plastic, the cells were used as described below. Myocytes beat rythmically (50–80 beats/min) and demonstrated sarcomeres in approximately 80% of the cells when stained with phosphotungstic acid–hematoxylin.

Endothelial Cells

Endothelial cells were obtained from two sources. Cardiac endothelial cells were obtained by trypsinizing the cells attached to the plastic flasks during the preparation of the myocytes. Umbilical cord endothelial cells were obtained by removing the umbilical cords from 15–18-day-old embryos, mincing the tissue finely, and subjecting it to stepwise digestion with 0.4% collagenase. The dissociated cells were attached to plastic flasks by incubation in complete BME for 1 hour at 37 C. The attached cells were washed three times for removal of cellular debris. Endothelial cell preparations were checked for purity by immunofluorescent staining with anti-human Factor VIII.[24] Preparations generally consisted of 70% or more cells positive for Factor VIII, compared with fewer than 20% of myocytes; and none of the skin fibroblasts stained.

Skin Fibroblasts

Skin of the neonates used in cardiocyte preparations was washed with soap and with 70% alcohol prior to aseptic removal from the animals. The skin fragments were minced finely, and the fibroblasts dissociated by incubation in a 0.4% collagenase solution (Worthington Biochemical Corp., Freehold, NJ) for 15–25 minutes at 37 C. The dissociated cells were attached to plastic flasks in complete BME.

Preparation of Lymphocytes

Inguinal and mesenteric lymph nodes from normal and CVB3-infected mice were removed 7 days after infection of the animals. The lymphocytes were obtained by pressing the nodes through a fine mesh screen and washing the cells in BME containing 5% FCS. The viability of the lymphocyte preparations was determined by trypan blue exclusion.

Immunoadsorption

Myocyte, HeLa (American Tissue Culture Collection, Bethesda, Md), L929 (ATCC), endothelial cell,

and fibroblast monolayers in plastic tissue culture flasks were washed with BME without serum and incubated with 3×10^7 PFU virus for 6 hours at 37 C. This infection protocol has previously been shown to result in infection of 90–100% of the cells.[19-21] The extraneous virus was removed by washing the monolayers, and the infected and uninfected monolayers were fixed by incubation for 30 seconds with a 0.3% solution of glutaraldehyde (Sigma) in PBS buffered to pH 6.2. The fixed monolayers were then washed four times with BME. To each of the flasks 5×10^7 lymphocytes were added in BME–2% FCS and incubated at room temperature for 1.5 hours. The nonadherent cells were thoroughly removed by washing the monolayers, and the adherent cells were recovered by incubating the monolayers at 37 C for 15 minutes in PBS containing 20 mg/100 ml ethylene diaminetetraacetic acid (EDTA). The number of cells recovered from each flask of uninfected myocytes ranged from 5×10^5 to 1.3×10^6 cells (1–2.6% of initial cell population), and the number of cells recovered from each infected myocyte flask ranged from 8×10^5 to 2.6×10^6 (1.6–5.2% of the initial cell population). Generally, the pooled cells from 5–10 flasks were required for each experiment.

Cytotoxicity Assay

The technique for the cell-mediated cytotoxicity assay has been reported previously.[18-22] Briefly, 3×10^3 myocytes were incubated in tissue culture wells 1 mm in diameter with 100 virus PFU/ myocyte for 4–6 hours at 37 C in a 6% CO_2 humidified incubator. The target cells were washed, and infected and uninfected cells were incubated with 1 μCi ^{51}Cr ($Na^{51}CrO_4$, New England Nuclear Co., Boston, Mass) for 1 hour at 37 C. After washing three times, the monolayers were overlaid with 3×10^5 lymphocytes in BME–5% FCS and the cultures were incubated for 18 hours at 37 C. After the incubation, radioactivity in the supernatant and cell pellet was determined with the use of an Intertechnique CG4000 gamma counter. ^{51}Cr release was calculated by using the following expression: ([counts per minute in supernatant]/[counts per minute in supernatant + counts per minute in cells]) $\times$ 100.

Cytotoxicity was expressed as the percentage of lysis, as calculated by the following expression: ([average percentage of ^{51}Cr released from test group] – [average percentage of ^{51}Cr released from medium group])/([maximum average percentage releasable ^{51}Cr] – [average percentage released from medium group]) $\times$ 100.

The percentage of specific lysis represented the percentage of lysis by sensitized lymphocytes minus the percentage of lysis by nonimmune lymphocytes.

Anti-Thy 1.2 Treatment of Lymphocytes

Monoclonal anti-Thy 1.2 serum (New England Nuclear) was titrated as described previously.[19] Lysis of Thy 1.2-positive cells was performed by incubation of 2×10^7 lymphocytes with 0.5 ml of a 1:1000 dilution of the antibody for 30 minutes followed by incubation in 0.5 ml BME containing 20% adsorbed guinea pig complement (GIBCO) for 45 minutes at 37 C. The complement had been adsorbed with equal washed, packed volumes of Balb/c thymocytes and spleen cells. This procedure decreased the percentage of Thy 1.2-positive cells to less than 3%.

T-Lymphocyte–Deficient Animals

T-lymphocyte–deficient mice were animals which had been thymectomized, lethally irradiated, and reconstituted with syngeneic bone marrow cells (TXBM).[17] Three- to four-week-old mice were anesthetized, and the thymus was removed by opening the anterior mediastinum through an incision in the neck and sternum and aspirating the organ with a pipette. The animals were rested for 2 weeks, then given 850 R whole-body irradiation using a Theratron Jr. gamma irradiator. The same day, $3–5 \times 10^6$ syngeneic sex-matched bone marrow cells were injected into the animals intravenously through the tail vein. The bone marrow cells were obtained by flushing the tibial and femoral bones of donor mice, dissociating the cells by repeated aspiration with a pipette and treatment of the cells with anti-Thy 1.2 serum and complement for removal of any residual T-lymphocytes. After reconstitution with bone marrow cells, the animals were rested for 5 weeks prior to use in the experiments. Animals were determined to be T-cell–deficient by removal of the inguinal lymph node under anesthesia 1 week before the experiment. The lymphocytes (5×10^5 cells/ 6 mm tissue culture well; Falcon) were incubated with 1 μl phytohemagglutinin (PHA, GIBCO) in a total volume of 200 μl BME–2% FCS at 37 C in a 6% humidified CO_2 incubator. Approximately 20 hours prior to harvest, 1 μl ^3H-thymidine (Amersham Searle, Arlington Heights, Ill) was added to each culture. The cultures were harvested onto glass fiber strips at 72 hours with the use of a multiple automatic sample harvester (MASH, Bellco Glass Inc, Vineland, NJ); the strips were air-dried, and the sections containing radioactivity were placed in vials with 5 ml Econofluor liquid scintillation fluid (New England Nuclear) and counted in a Packard

Liquid Scintillation counter. Animals were considered T-cell-deficient if the ratio of radioactivity in the cultures with PHA to cultures without PHA was less than 2.

Adoptive Transfer of Lymphocytes

Two days after infection of the mice, 1×10^6 lymphocytes were injected in 0.1 ml PBS into TXBM mice intravenously through the tail vein. The animals were sacrificed 5 days later.

Histology

The hearts were fixed in 10% buffered formalin and sectioned laterally approximately midway between the apex and atria, resulting in cross-sections of both ventricles. The sections were stained with hematoxylin and eosin (H&E), and projected onto paper, where the total area of the myocardium and the areas of inflammation were outlined. The percent area of myocardium undergoing inflammation and necrosis was determined by image analysis using an Apple computer and the formula (total area of inflammation)/(total area of myocardium) $\times$ 100.[25]

Statistical Analysis

The Student t test was used to analyze the significance of differences between groups in the cytotoxicity assays. The Wilcoxon ranked score test was used for evaluation of histologic features.

Results

Specific Immunoadsorption of Cytotoxic Lymphocytes to Myocyte Monolayers

Lymphocytes obtained from Balb/c mice inoculated 7 days earlier with CVB3M were adsorbed to CVB3M infected and uninfected monolayers of Balb/c myocytes, HeLa cells, and L929 cells. The eluted cells were assayed for cytotoxicity to uninfected and CVB3M infected myocytes (Table 1). The unfractionated lymph node cell population lysed both targets. Cells eluted from either uninfected or infected myocyte monolayers lysed only one target, respectively, either uninfected or infected myocytes. Therefore, two distinct CTL populations were isolated from lymph node cells of CVB3M-infected mice. The cell population recognizing uninfected myocytes was designated "autoreactive CTLs," and the cells eluted from the CVB3M-infected myocytes were designated "virus-specific CTLs." Lymphocytes eluted from either uninfected or infected HeLa and L cell monolayers were not lytic to uninfected myocytes but lysed infected targets minimally. While it is possible that the eluted cytolytic cells recognize shared antigens between the tissue culture cell lines and primary myocyte cultures, it is more probable that the minimal

Table 1—Immunoadsorption of Cytolytic Lymphocytes to Myocytes, HeLa Cells, and L929 Cells*

Adsorbing monolayer‡	Infected with CVB3M(+) or (−)	% Specific lysis† Uninfected myocytes	% Specific lysis† CVB3M myocytes
None		18.8 ± 1.3§	28.6 ± 1.2§
Balb/c			
Myocytes	−	30.2 ± 4.4§	0.2 ± 0.7
	+	3.0 ± 1.9	35.8 ± 3.5§
HeLa cells	−	2.7 ± 1.1	7.1 ± 3.9
	+	2.5 ± 0.5	12.8 ± 2.3§
L929 cells	−	−4.3 ± 2.2	10.6 ± 1.5§
	+	−4.2 ± 0.9	6.8 ± 0.8§

* Lymph node cells were obtained 7 days after inoculation of Balb/c mice with 3×10^4 PFU CVB3M.

† Five $\times$ 10^7 lymphocytes were incubated on glutaraldehyde-fixed uninfected and CVB3M-infected monolayers for 1½ hours at room temperature. The adherent cells were eluted from the monolayers with PBS-EDTA.

‡ Effector/target cell ratio of 100:1 and an incubation time of 18 hours was used. Spontaneous ^{51}Cr release was 24.2% and 27.9%, respectively, for uninfected and infected targets. The percent lysis by nonimmune lymphocytes was 0.4% and 1.6%, respectively. The results represent three experiments.

§ The percent specific lysis is significantly greater than 0 at the $P \leq 0.05$ level.

Table 2—Organ Specificity of Immunoadsorption*

Immune lymphocytes adsorbed-eluted from	% Specific lysis† Uninfected myocyte	% Specific lysis† CVB3M-infected myocyte
None	26.3 ± 4.5‡	44.6 ± 6.7‡
Uninfected monolayers		
Cardiocytes	47.6 ± 5.1‡	12.7 ± 6.2
Cardiac endothelial cells	32.5 ± 6.6‡	9.4 ± 6.1
Umbilical endothelial cells	16.6 ± 8.6	1.8 ± 10.0
Skin fibroblasts	12.3 ± 5.8	7.1 ± 3.1
CVB3M infected monolayers		
Cardiocytes	6.1 ± 3.6	45.0 ± 5.8‡
Cardiac endothelial cells	5.3 ± 4.4	24.0 ± 4.2‡
Umbilical endothelial cells	8.1 ± 4.2	18.5 ± 5.2‡
Skin fibroblasts	15.5 ± 5.6	13.1 ± 3.2

* Lymphocytes from Balb/c mice obtained 7 days after intraperitoneal inoculation with 3×10^4 PFU CVB3M were incubated on monolayers of glutaraldehyde-fixed Balb/c neonatal or embryonic tissue. The adherent cells were eluted with PBS-EDTA and assayed for cytolytic activity in the CMC assay.

† An effector/target cell ratio of 100:1 and an incubation of 18 hours were used. Spontaneous ^{51}Cr release and percent lysis by nonimmune lymphocytes on uninfected and infected myocytes were 27.5% and −2.0% and 31.0% and −0.4%, respectively.

‡ The percent specific lysis was significantly greater than 0 at $P \leq 0.05$. The results represent the mean percent specific lysis ± SEM of five experiments.

cytotoxicity represents non-T-cell effectors such as natural killer cells.

To confirm that the CTL populations were identifying heart-related antigens specifically, the immunoadsorption procedures were repeated with the use of glutaraldehyde fixed monolayers of CVB3M-infected and uninfected myocytes, cardiac endothelial cells, umbilical cord endothelial cells, and skin fibroblasts (Table 2). Autoreactive lymphocytes adsorbed to both uninfected myocyte and cardiac endothelial cell monolayers and were preferentially cytolytic to uninfected myocyte targets (45.6% and 30.5% specific lysis to uninfected cells, respectively, compared with 12.7% and 9.4% on infected targets). Similarly, virus-specific CTLs adsorbed to CVB3M-infected myocytes and cardiac endothelial cells and preferentially lysed infected myocytes. Neither virus-specific nor autoreactive CTL populations appeared to adsorb well to umbilical cord endothelial cell and skin fibroblast monolayers.

Table 3—Sensitivity of Cytolytic Cell Populations to Treatment With Anti-Thy 1.2 and Complement (C)

Lymphocyte population†	Anti-Thy 1.2 + C	% Specific lysis† Uninfected myocytes	% Specific lysis† CVB3M-infected myocytes
Lymph node cells (unfractionated)	–	51.3 ± 5.6	66.3 ± 6.2
	+	15.8 ± 8.2‡	0.4 ± 7.3‡
Autoreactive	–	62.0 ± 3.4	3.5 ± 7.4
	+	13.1 ± 5.3‡	5.0 ± 8.1
Virus-specific	–	19.5 ± 5.8	56.6 ± 4.7
	+	17.0 ± 3.1	21.2 ± 6.4‡

* Lymph node cells were obtained from mice 7 days after CVB3 inoculation and separated into autoreactive and virus-specific CTLs as described before. Half of each cell population was treated with monoclonal anti-Thy 1.2 and C.

† An effector/target cell ratio of 100:1 and an incubation time of 18 hours was used. Spontaneous ^{51}Cr release and percent lysis by nonimmune lymphocytes were 32.1% and −0.4% on uninfected myocytes and 28.9% and −9.4% on infected myocytes.

‡ Cytotoxic activity after treatment with anti-Thy 1.2 and C less than the untreated population at $P \leq 0.05$.

Cytolytic Activity Eluted From Monolayers Is Mediated by Thy 1.2-Positive Cells

As stated above, natural killer cells may be present in the immune lymph node cell populations. It is necessary, therefore, to determine whether the autoreactive and virus-specific CTL populations are susceptible to treatment with anti-Thy 1.2 and complement. Lymphocytes obtained from CVB3M-inoculated mice were divided into unfractionated cells, and autoreactive and virus-specific CTL populations. Half of the cells in each group were treated with monoclonal anti-Thy 1.2 antibody and complement for removal of T cells. The untreated and T-cell-depleted cell populations were assayed for cytotoxicity to uninfected and CVB3M-infected myocytes (Table 3). Anti-Thy 1.2 treatment of all cell groups reduced cytolytic activity. Nonetheless, significant cytolytic activity persisted in several groups, especially in the virus-specific CTL population. This anti-Thy 1.2 resistant cytolytic activity probably represents natural killer cells, which are prevalent during CVB3M infection and might be expected to bind preferentially to infected targets.[20]

Virus Specificity of Immunoadsorption

Two variants of CVB3 infect Balb/c myocytes, but ony one variant will induce myocarditis *in vivo*. The virus-specific antigens induced by the myocarditic (CVB3M) and nonmyocarditic ($CVB3_o$) variants on the cells are apparently non-cross-reactive.[21] We did the following experiment to determine whether CVB3M immune virus-specific CTLs are capable of recognizing and adsorbing to myocytes infected with one or both CVB3 variants. Lymph node cells were obtained from Balb/c mice 7 days after inoculation with either CVB3M or $CVB3_o$. The cells were adsorbed to uninfected Balb/c myocyte monolayers and to monolayers infected with either variant. The eluted cells were assayed for cytolytic activity to uninfected and to CVB3M- and $CVB3_o$-infected myocyte targets (Table 4). As shown previously, lymphocytes from CVB3M-inoculated animals lysed both uninfected and CVB3M-infected targets but were not cytolytic to $CVB3_o$-infected cells. Adsorption of the CVB3M immune lymph node cells to uninfected and CVB3M-infected myocyte monolayers isolated the autoreactive and virus-specific CTL populations described above. None of the CTL populations from CVB3M-inoculated mice adsorbed to or lysed $CVB3_o$-infected targets, emphasizing the unique antigens expressed by cells infected with this variant. Immune cells from $CVB3_o$-inoculated animals lysed $CVB3_o$-infected myocytes but lacked cytolytic activity to either uninfected or CVB3M-infected targets. $CVB3_o$ immune lymph node cells adsorbed exclusively to $CVB3_o$-infected monolayers. Thus, the animals inoculated with $CVB3_o$ in which myocarditis did not develop also lack autoreactive CTLs.

Adoptive Transfer of Immunoadsorbed Cell Populations Into T-Cell-Deficient Mice

Unfractioned lymph node cells from CVB3M-inoculated mice and the autoreactive and virus-specific CTLs derived from this cell population were adop-

Table 4—Virus Specificity Immunoadsorption of Cytolytic Lymphocytes to Infected and Uninfected Cardiocyte Monolayers*

Immunizing virus	Immunoadsorption	% Specific lysis† Uninfected cardiocytes	CVB3M-infected cardiocytes	$CVB3_O$-infected cardiocytes
CVB3M	None	16.7 ± 1.40‡	26.4 ± 2.60‡	−1.2 ± 3.30
	Uninfected cardiocytes	28.6 ± 4.6‡	6.4 ± 5.80	1.2 ± 2.4
	CVB3M-infected cardiocytes	1.30 ± 0.8	34.7 ± 4.7‡	3.8 ± 2.3
	$CVB3_O$-infected cardiocytes	−0.6 ± 5.5	0.3 ± 3.7	3.7 ± 2.9
$CVB3_O$	None	−5.4 ± 2.3	6.4 ± 5.6	19.7 ± 6.9‡
	Uninfected cardiocytes	2.4 ± 1.0	1.7 ± 5.1	2.9 ± 2.1
	CVB3M-infected cardiocytes	4.2 ± 2.5	7.8 ± 6.0	8.9 ± 5.0
	$CVB3_O$-infected cardiocytes	3.1 ± 1.9	13.3 ± 2.3‡	30.5 ± 2.7‡

* Lymph nodes cells were obtained from mice 7 days after inoculation with CVB3M or $CVB3_O$ inoculation and incubated on uninfected and virus-infected glutaraldehyde-fixed myocyte monolayers for 1.5 hours. Adherent cells were recovered by incubation of the monolayers in PBS-EDTA and assayed for cytotoxicity to uninfected and virus-infected myocyte target cells.

† The effector/target cell ratio (E/T) was 100:1, and the incubation time was 18 hours. Spontaneous ^{51}Cr release for uninfected, CVB3M-infected, and $CVB3_O$-infected targets was 28.1%, 32.0%, and 30.9%, respectively. Percent lysis of the targets by nonimmune lymphocytes was −5.6%, 1.5%, and −3.2%, respectively. The results represent the mean percent specific lysis ± SEM of four experiments.

‡ The percent specific lysis is significantly greater than 0 at the $P \leq 0.05$ level.

tively transferred into TXBM mice which had been infected with CVB3M 2 days earlier. We transferred immune cells after virus inoculation to prevent abrogation of the infection. Control animals were infected and immunologically intact. The experimental and control mice were sacrificed 7 days after virus inoculation, and the hearts were examined for myocarditis. The percentage of the total area of the myocardium which was affected by inflammation and/or necrosis was determined by image analysis (Table 5). Immunologically intact animals showed extensive inflammation in the ventricles (mean area involved, 9.3%). The amount of cardiac inflammation was reduced significantly in TXBM mice (1.1%) but increased when either unfractionated immune lymph node cells or autoreactive and virus-specific CTLs were transferred (8.9%, 22.7%, and 6.4%, respectively). Transfer of nonimmune lymph node cells did not significantly enhance cardiac inflammation in the recipients. Interestingly, the greatest cardiac injury occurred with the autoreactive CTLs. The lesions were characterized by extensive focal coagulative necrosis of the myocardium with significant calcification (Figure 1A and C). Less necrosis and calcification were observed in virus-specific CTL recipients (Figure 1B and D).

Discussion

Considerable evidence indicates that cardiac injury in clinical and experimental CVB3-induced myocarditis is mediated by the immune system. Cardiac inflammation and necrosis usually occur in the absence of detectable virus in the heart, body fluids, and feces of the patients. Frequently, identification of an infectious etiologic agent depends upon a compatible clinical history and identification of rising serum antibody titers to a specific pathogen.[1] In the murine model, virus is also rarely observed in the hearts when inflammation and necrosis are maximum.[1,17,22] In addition, inoculation of T-lymphocyte-deficient mice with the virus does not result in significant myocarditis, even though infectious virus is isolated from the heart.[17,22] Both of these observations suggest that the virus, while initiating the immune responses

Table 5—Adoptive Transfer of Autoreactive and Virus-Specific CTLs Into CVB3M-Infected T-Cell-Deficient Mice

Lymphocytes transferred*	Recipient TXBM	Mean % area of myocardium affected
None	−	9.3 ± 1.9†
None	+	1.1 ± 0.5
Nonimmune lymph node cells	+	2.4 ± 1.0
Immune lymph node cells	+	8.9 ± 2.3†
Autoreactive CTL	+	22.7 ± 4.5†‡
Virus-specific CTL	+	6.4 ± 2.7†

* Lymph node cells were obtained from uninfected mice and from mice 7 days after CVB3M inoculation. The immune lymph node cells were separated into autoreactive and virus-specific CTLs by immunoadsorption to uninfected and CVB3M-infected myocyte monolayers. TXBM recipient mice were inoculated intraperitoneally with 3×10^4 PFU CVB3M, and 1×10^6 lymphocytes were injected into TXBM recipients intravenously through the tail vein. The animals were sacrificed 7 days after virus inoculation.

† Inflammation and necrosis is significantly greater than in TXBM mice at $P \leq 0.05$.

‡ Inflammation/necrosis exceeds all other groups at $P \leq 0.05$.

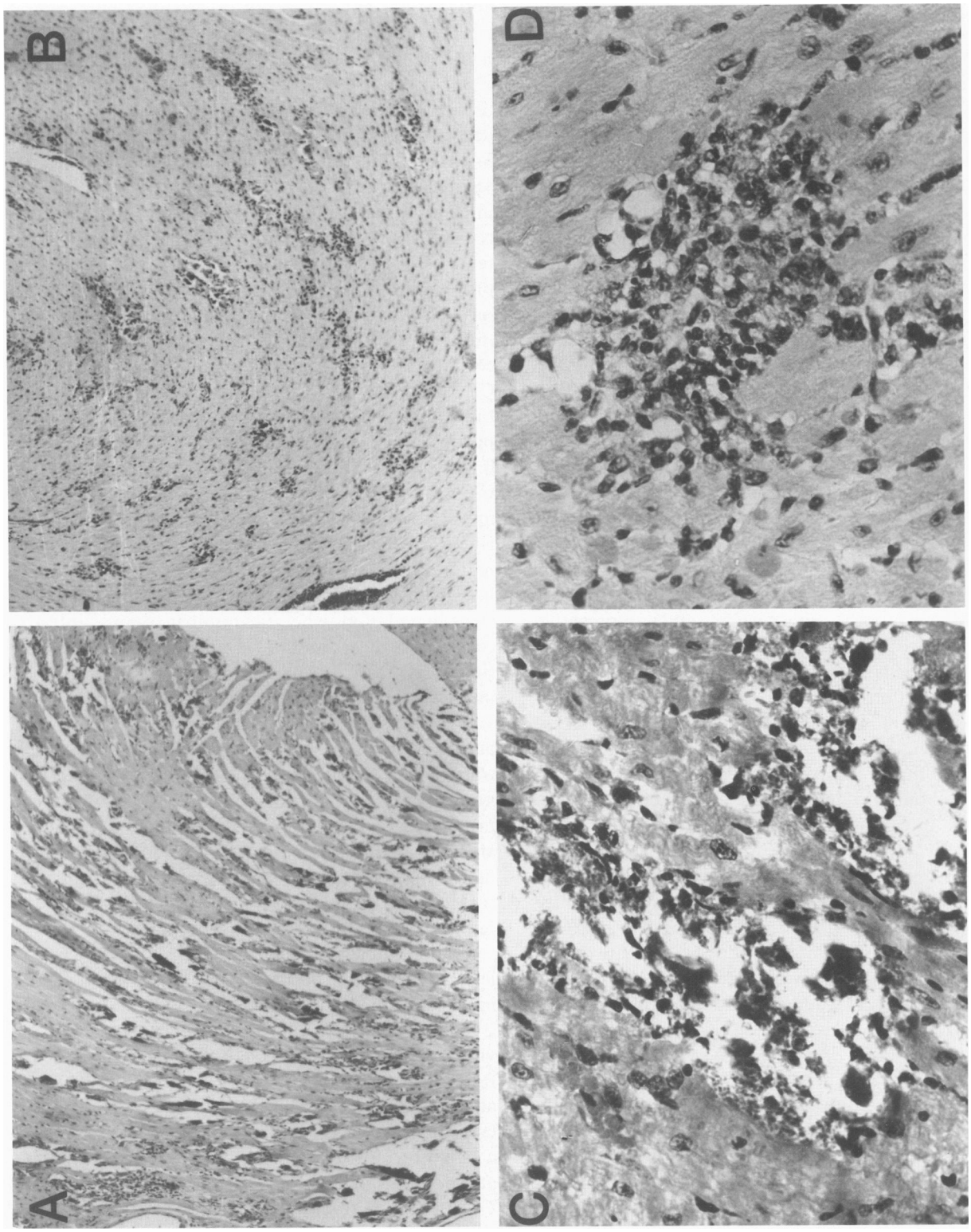

Figure 1—Histologic study of myocardial lesions in TXBM mice 7 days after inoculation with 3 × 10^4 PFU CVB3 and 4 days after transfusion with 1 × 10^6 autoreactive CTLs (**A** and **C**) or virus-specific (**B** and **D**) CTLs. (H&E, **A** and **B**, ×85; **C** and **D**, ×380)

which produce the myocardial inflammatory and necrosis, is of limited importance in directly inducing cardiac injury. If cardiac damage in this disease is immune-mediated, what antigens are the lymphocytes recognizing?

Infected myocytes should express virus-specific and myocyte-specific antigens on the cell surface. Previous work has shown that CTLs from CVB3M-infected animals lyse both uninfected and CVB3M-infected myocardial cells *in vitro*.[21] Lysis of both targets could

result either from a single CTL population primarily directed to virus antigens on the myocyte cell surface but also recognizing myocyte antigens or from two CTL populations recognizing virus-specific antigens and myocyte antigens separately. Whether one or two CTL populations are involved is important in understanding the basic mechanism of cardiac injury in this disease. CTLs primarily directed to virus antigens may decrease in number and activity as virus is eliminated from the heart. Thus, myocarditis induced by these cells could be self-limiting. When the CTLs inducing cardiac damage primarily recognize myocyte-specific antigens, myocarditis becomes an autoimmune disease. Autoimmune injury may continue after virus is eliminated from the heart, because the CTLs do not depend upon virus antigens for either stimulation or recognition of target cells. The present study has shown that the cytolytic activity to uninfected and CVB3M infected myocytes can be separated by selective adsorption of the CTLs to uninfected and infected cardiocyte monolayers. The adsorption is specific in that little cytolytic activity adsorbs either to cardiocytes infected with $CVB3_o$ or to uninfected and CVB3M-infected Hela and L cells. In addition, both virus-specific and autoreactive CTLs adsorb preferentially to myocytes and cardiac endothelial cells but not to umbilical cord endothelial cells or skin fibroblasts. The apparent organ specificity of the immune cells suggest that even the virus-specific CTLs must recognize cardiocyte-specific antigens in addition to virus antigens.

Our inability to demonstrate autoreactive CTLs in animals infected with the nonmyocarditic $CVB3_o$ variant would favor an autoimmune process in CVB3M myocarditis. Most of the other characteristics of $CVB3_o$ and CVB3M infection of Balb/c mice are similar. Both viruses replicate in the heart to approximately equal concentrations and are eliminated during the first week after inoculation. Both viruses induce CTL in inoculated animals which lyse infected myocyte targets. Thus, if cardiac injury resulted either from virus infection of the myocytes or from immune cell-mediated lysis of infected heart cells, it would be difficult to explain why myocarditis fails to develop in $CVB3_o$-inoculated mice. The postulation that autoimmune CTLs lyse uninfected cells and are present only in CVB3M-inoculated animals would explain most of the experimental and clinical observations in myocarditis.

The failure of autoreactive CTLs to lyse infected myocytes suggests that the antigen recognized by these cells is no longer expressed in the infected cell. How this could be is both perplexing and interesting. It is possible that during infection, myocyte-specific antigens are either masked or modified by the viral antigens. Alternately, viruses can efficiently inhibit host cell protein synthesis within a few hours of infection. Any host-specified proteins in the plasma membrane which may have a relatively rapid turnover may decrease significantly in concentration after infection. Without specific antibodies to the myocyte and virus antigens on uninfected and infected cells it will be difficult to determine which of these hypotheses is correct. While identification and separation of autoreactive and virus-specific CTLs *in vitro* does not prove that autoreactive CTLs predominantly cause myocarditis *in vivo*, this was shown by adoptively transferring the separated cell populations into T-cell-deficient virus-infected animals. Autoreactive CTLs induced severe myocarditis, more severe than that observed in any other group. These results support the hypothesis that autoimmunity plays an important role in this disease, although virus-specific CTLs may also be involved. However, the extent and importance of cardiac damage caused by virus-specific CTLs during normal CVB3M infections will require further study. The virus-specific CTLs were injected into the TXBM mice on the second day after virus inoculation, when the number of virus infected cells in the heart would be maximum. During this time it is possible that virus-specific CTLs may be important mediators of cardiac injury. In normal *in vivo* infections, however, neither virus-specific or autoreactive CTLs are detected until the virus concentrations are diminishing. It is therefore presently unknown whether either CTL population will induce myocarditis when virus concentrations in the heart are reduced or absent. Future studies must be done to determine the long-term effects of cardiac injury by these two CTL populations several weeks to several months after administration of cells.

While CTLs are important in inducing cardiac lesions, other nonspecific mediators activated during infection may also cause heart damage. Natural killer (NK) cells and macrophages are both activated during viral infections and are capable of lysing both uninfected and virus-infected target cells. NK cells probably account for the nonspecific anti-Thy 1.2 resistant cytolytic effector cells adsorbing to the HeLa and L cells and to infected umbilical cord endothelial cells and fibroblasts. It is not surprising that the suspected NK cells contaminate the virus-specific CTL population to a greater extent than the autoreactive cells. NK cells usually react more efficiently to transformed and infected cells than to normal ones.[20,26] Whether NK cells have any significant role in CVB3 myocarditis is not known. Whatever their role in this disease, however, it must be directed by the T cells.

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Acknowledgments

We thank Lisa Joyce and Marianne Feran for technical assistance and Mrs. Jackie Marceau for help in preparing the manuscript.