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The Role of CD8$^+$ T Lymphocytes in Coxsackievirus B3-Induced Myocarditis†

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Coxsackievirus infections have previously been shown to cause acute or chronic myocarditis in humans, and several mouse models have been established to study the pathology of this disease. Myocardial injury may result from direct viral effects and/or may be immune mediated. To determine the relative roles of these processes in pathogenesis, we have compared coxsackievirus B3 (CVB3) infections of normal and immunocompromised transgenic knockout (ko) mice. CVB3 was able to infect all strains used (C57BL/6, CD4ko, and β-microglobulin ko [β2Mko]), and following intraperitoneal injection, two disease processes could be distinguished. First, the virus caused early (3 to 7 days postinfection) death in a viral dose-dependent manner. Immunocompetent C57BL/6 mice were highly susceptible (50% lethal dose = 70 PFU), while immunodeficient transgenic ko mice were less susceptible, showing 10- and 180-fold increases in the 50% lethal dose (for CD4ko and β2Mko mice, respectively). Second, a histologic examination of surviving CD4ko mice at 7 days postinfection revealed severe myocarditis; the inflammatory infiltrate comprised 40 to 50% macrophages, 30 to 40% NK cells, and 10 to 20% CD8$^+$ T lymphocytes. The infiltration resolved over the following 2 to 3 weeks, with resultant myocardial fibrosis. In vivo depletion of CD8$^+$ T lymphocytes from these CD4ko mice led to a marked reduction in myocarditis and an increase in myocardial virus titers. β2Mko mice, which lack antiviral CD8$^+$ T cells, are much less susceptible to early death and to the development of myocarditis. We conclude that our data support a strong immunopathologic component in CVB3-induced disease and implicate both CD4$^+$ and CD8$^+$ T cells. Compared with immunocompetent animals, (i) mice lacking CD4$^+$ T cells (CD4ko) were more resistant to virus challenge, and (ii) mice lacking CD8$^+$ T cells (β2Mko and in vivo-depleted CD4ko) showed enhanced survival and a reduced incidence of the later myocarditis. Nevertheless, the picture is complex, since (iii) removal of the CD4$^+$ component, while protecting against early death, greatly magnified the severity of myocarditis, and (iv) removal of the CD8$^+$ cells from CD4ko mice, although protecting against early death and later myocarditis, led to markedly increased virus titers in the heart. These data underscore the complex balance between the costs and benefits of effective antiviral immune responses.

Coxsackieviruses, which are members of the picornavirus family, are important human pathogens. Although many infections are subclinical or mild, acute neonatal infections are often severe and may be lethal. Coxsackievirus B3 (CVB3) is a common associated factor in human subacute, acute, or chronic myocarditis (36, 46). In young adults CVB3 infections may cause cardiac arrhythmias and acute heart failure, and chronic disease may supervene, leading to dilated cardiomyopathy, requiring transplantation, or to death. To better understand the pathogenesis of this disease, several mouse model systems have been established, which suggest that the outcome of infection is determined by complex interactions among several variables. The virus genotype (7, 28) and mouse strain (4, 42) are critical, and as to the sex (13, 17), age (24), and immune status (46, 47) of the host, each plays a role.

Depending on the virus and host strains, CVB3 infection of mice may result in a marked myocarditis, with extensive myocardial destruction and a severe, predominantly mononuclear, infiltrate; infectious CVB3 is easily detected in the heart tissue. It is uncertain whether the myocardial damage is directly virus mediated or whether the inflammatory response is responsible, and evidence for both possibilities abounds. The availability of transgenic knockout (ko) mice affords the opportunity to dissect the roles of different immunological compartments in protecting against, or causing, this disease. In this study we have focused on mice lacking T-cell functions, and we compare the outcomes of infection of CD4ko and β2-microglobulin ko (β2Mko) mice with that of infection of normal immunocompetent mice. CD4ko mice lack the CD4 protein, a cell surface molecule which greatly enhances the ability of T cells to interact with antigen presented by major histocompatibility complex (MHC) class II molecules. Such mice effectively lack T-helper responses (25). In contrast, β2Mko mice are unable to efficiently present antigen via the MHC class I pathway and consequently have a greatly diminished population of CD8$^+$ T cells, which normally interact with these molecules; these mice have a greatly reduced ability to mount classical cytotoxic T-cell (CTL) responses (31).

In this study we show the following. (i) The three mouse strains show markedly different susceptibilities to CVB3, as measured by 50% lethal dose (LD_{50}) analyses. The immunocompromised mice show diminished susceptibility, indicating an immunopathologic factor in normal mice. (ii) In C57BL/6 mice which survive the early phase, myocarditis cannot be detected. (iii) In CD4ko mice which survive the early phase, myocarditis is readily apparent 7 days postinfection (p.i.). (iv) Development of myocarditis in CD4ko mice requires CD8$^+$ T lymphocytes, and (v) β2Mko mice, which lack CD8$^+$ T cells,

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are resistant to early death and show minimal myocarditis, underscoring the immunopathologic role played by this T-cell component in CVB3-induced diseases.

MATERIALS AND METHODS

Mice. Inbred C57BL/6 mice ($H2^{b/b}$) were obtained from the Scripps Research Institute breeding colony. CD4ko mice (25) (obtained from M. B. A. Oldstone) and β2Mko mice (31) (obtained from J. Frelinger) were bred by our group. Both transgenic lines have the $H2^{b/b}$ background. Adult male mice 7 to 10 weeks of age were used in these experiments. Experimental groups consisted of a minimum of four mice, and experiments were repeated at least twice and usually three or four times.

Virus. The CVB3 (Nancy) used is a cardiopathogenic strain (designated H3) of the original stock of CVB3 obtained from J. F. Woodruff and was isolated by S. Huber. The virus was propagated in HeLa cells. Six to eight hours after infection, the culture medium was removed, and the monolayer of infected cells was frozen at −20°C. Thereafter, the cells were disrupted by three cycles of freezing and thawing. Cell debris was removed by centrifugation, and aliquots were stored at −70°C. The titer of infectious virus particles was determined by a standard plaque assay on HeLa cell monolayers.

Infection protocols. Mice were infected by intraperitoneal injection of 0.2 ml of saline containing the stated amounts of CVB3 and were monitored daily for morbidity and mortality (LD_{50}) or sacrificed at different time points p.i. For calculation of LD_{50}s, serial (10-fold) dilutions of CVB3 were inoculated into groups of mice (one dilution per group of eight mice), and mortality was followed over a 14-day period. No excess mortality was noted beyond this time point. LD_{50}s were calculated by the method of Reed and Muench (35).

Virus titer in organs. Organs were aseptically removed, washed with sterile saline, and homogenized with RPMI 1640 medium (Irvine Scientific, Santa Ana, Calif.) containing 50 U of penicillin per ml, 50 mM of streptomycin (Gibco BRL, Grand Island, N.Y.) per ml, 2 mM glutamine (Gibco BRL), and 10% fetal calf serum (Gibco BRL). The cellular debris was removed by centrifugation, the supernatant was subjected to sequential 10-fold dilutions in RPMI 1640, and the virus titer was determined by plaque formation assays on HeLa cell monolayers. The statistical comparison of virus titers was carried out with Microsoft Excel by using Student's *t* test.

Virus-neutralizing antibody titers. Murine blood obtained by heart puncture at the same time as the animals were killed was allowed to clot, and serum was recovered. All sera were heat inactivated at 56°C for 30 min and serially diluted in RPMI 1640 medium (with 10% fetal calf serum), and 125 μl of each dilution was combined with an equal volume of RPMI 1640 medium containing 100 PFU of CVB3. The mixture was incubated at 37°C for 1 h. Fifty microliters of each dilution was added in quadruplicate to 96-well plates containing confluent Vero cell monolayers. After a 3-day incubation at 37°C, the cytopathic effect in each well was determined. The neutralizing antibody titer for each serum was the dilution of antibody required to reduce the cytopathic effect by 50%.

Preparation and staining for routine histology. Aseptically removed hearts were fixed in Bouins fixative and mounted in paraffin, and 5-μm sections were cut and stained with hematoxylin-eosin or with Gomori trichrome.

Immunohistochemistry with paraffin sections. For detection of CVB3 antigen in murine heart tissue, 4-μm paraffin sections were obtained and rehydrated by sequential incubation with xylene and 100, 95, 80, and 50% ethanol for 5 min each. The slides were incubated with a 3% bovine serum albumin (BSA) solution for 10 min at room temperature (RT) to block nonspecific binding. The primary antibody (rabbit anti-CVB3 in 3% BSA solution) was applied for 1 h at RT. After three washing procedures with TBST (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20) for 10 min each, all slides were incubated with the secondary antibody (anti-rabbit immunoglobulin G alkaline phosphatase conjugate in 3% BSA solution; ProtoBlot II AP-System kit [Promega, Madison, Wis.]) for 1 h at RT. The samples were washed three times with TBST (10 min each) and once with TBS (TBST without Tween 20). A color reaction was obtained with Western blue-stabilized substrate (ProtoBlot II AP-System kit). Some sections were counterstained with hematoxylin-eosin.

Immunohistochemistry with frozen sections. Immunochemical studies were carried out with cryomicrotome sections. Aseptically removed heart tissues were quickly frozen in optimal cutting temperature compound (Miles Inc., Elkhart, Ind.). For characterization of inflammatory cells and cytokine expression, 8-μm cryostat sections were obtained, air dried overnight, fixed for 1 min with −20°C acetone-methanol (50:50), washed in saline, and incubated separately with avidin-biotin solutions (blocking kit; Vector Laboratories, Burlingham, Calif.) for 15 min each to block nonspecific binding. Primary antibodies were applied for 1 h at RT. These consisted of rat anti-CD8a (clone 53-6.7; PharMingen, San Diego, Calif.), rat anti-Mac I (rat ascites fluid; kindly supplied by I. Campbell, the Scripps Research Institute), rat anti-Ia (clone M5/114; Boehringer Mannheim Inc., Indianapolis, Ind.), mouse anti-NK1.1 (clone PK136; PharMingen), and rat anti-tumor necrosis factor alpha (anti-TNF-α) (clone MP6-XT3; PharMingen) antibodies. After a 15-min washing procedure with saline, secondary antibodies (biotinylated horse anti-rat or rabbit anti-mouse immunoglobulin G; Vector) were applied for an additional hour at RT. A color reaction was obtained after washing of the slides for 15 min (RT) and sequential treatment with avidin-horseradish peroxidase conjugate (Boehringer Mannheim) and diaminobenzidine-hydrogen peroxidase (DAB tablets with peroxidase substrate; Sigma, St. Louis, Mo.). The slides were counterstained with hematoxylin-eosin.

Depletion of CD8+ cells. CD4ko mice were treated twice, 24 and 48 h prior to infection, with 0.5 ml of an ascites fluid (diluted 1:5 with phosphate-buffered saline) containing 5 mg of anti-CD8 monoclonal antibodies (MAbs) (clone 2.43) by intraperitoneal injection. The effectiveness of the in vivo depletion was confirmed by fluorescence-activated cell sorter (FACS) analysis of spleen cells at 7 days p.i.

Detection of apoptosis. Apoptotic cells in heart tissues of CVB3-infected CD4ko mice were detected by using the ApopTag *In Situ* Apoptosis Detection Kit (Oncor, Inc., Gaithersburg, Md.) as described in the Oncor manual. Briefly, frozen heart and spleen sections (8 μm) were incubated with the enzyme terminal deoxynucleotidyl transferase and digoxigenin-labeled nucleotides. Single positive cells were detected after a color reaction with peroxidase-labeled antidigoxigenin antibodies and diaminobenzidine-hydrogen peroxidase (DAB tablets with peroxidase substrate; Sigma). The slides were counterstained with hematoxylin-eosin.

RESULTS

Immunocompromised mice are less susceptible to CVB3-induced disease. The LD_{50} of the pathogenic CVB3 strain H3 was determined for each mouse strain as described in Materials and Methods, and the results are shown in Fig. 1A. Normal C57BL/6 mice were highly susceptible to CVB3 (LD_{50} = 70 PFU), while immunodeficient animals were 10- to 178-fold less susceptible (LD_{50}s = 700 and 12,455 PFU for CD4ko and β2Mko mice, respectively). Furthermore, even when the outcome was fatal, a difference was seen in immunodeficient mice; as shown in Fig. 1B, after injection of 1 LD_{50}, C57BL/6 mice died at 3 to 4 days p.i., whereas transgenic mice receiving 1 LD_{50} died at 5 to 7 days p.i. An additional difference was noted when myocardial virus titers were measured at days 0, 4, 7, 14, and 21 p.i. following intraperitoneal infection with 1 LD_{50} of CVB3 (Fig. 1C). Infectious particles were detected 24 to 48 h p.i. in the hearts of all infected mice (data not shown) and reached maximal titers after 3 to 4 days. Statistical analyses of cardiac CVB3 titers at day 4 p.i. showed significant differences among all three mouse strains (C57 > β2Mko [$P < 0.002$], C57 > CD4ko [$P < 0.0002$], and β2Mko > CD4ko [$P < 0.008$]). As measured by plaque assay, all surviving animals were able to clear the viral infection from the heart by 2 to 3 weeks p.i.

In addition, we investigated the induction of virus-specific neutralization antibodies in sera of mice infected with 1 LD_{50} (calculated for each mouse strain separately). Virus-specific antibodies were found in the sera of all CVB3-infected mice starting at 4 to 5 days p.i. and with maximal concentrations at 7 to 9 days p.i. (not shown). The specificity of the neutralizing reaction was demonstrated by incubation of sera from CVB3-infected mice with vaccinia virus or vesicular stomatitis virus. The cytopathic effects of these viruses were not decreased by sera obtained from CVB3-infected mice.

Only CD4ko mice develop severe myocarditis following CVB3 infection. Histologic examination of the hearts of mice which died following the administration of >1 LD_{50} of CVB3 revealed no abnormality (data not shown), but since all mice died in the acute stage, we were unable to extend our observations beyond this point. Therefore, mice were given 1 LD_{50} (allowing 50% survival), and the hearts of survivors were examined at various time points. Under these conditions, severe myocarditis developed by day 7 p.i. in the CD4ko mice, as shown in Fig. 2C; in contrast to the CD4ko pathology, the hearts of the immunocompetent C57BL/6 mice and the CD8-deficient β2Mko mice showed no, or very limited, infiltration at this (or any) time point. Infiltration was maximal in CD4ko mice at 7 to 8 days p.i. and declined over a 2-week period to undetectable levels; this recovery correlated with fibrosis, which was detected at 14 days p.i., was extensive by day 30 (Fig.

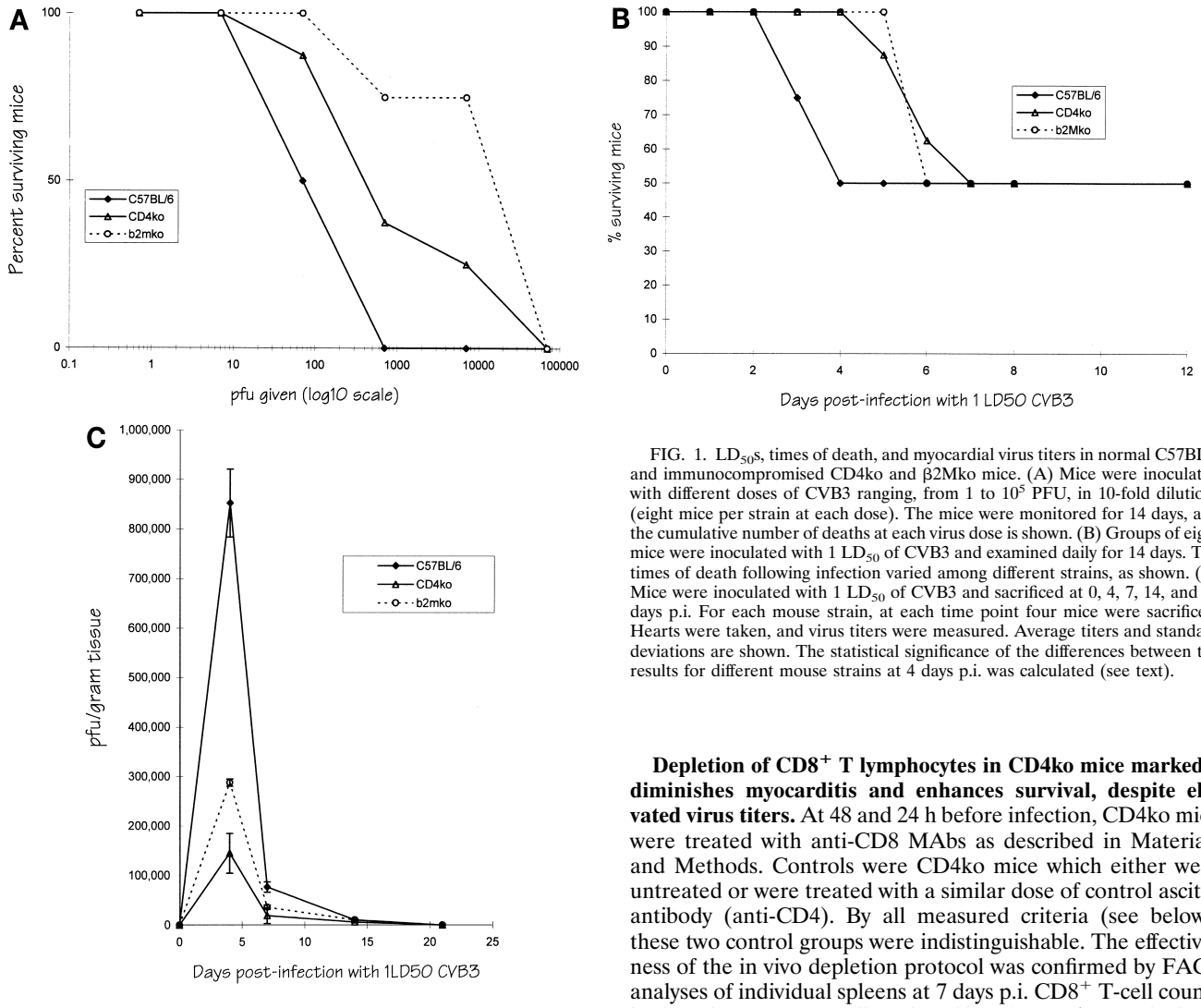

FIG. 1. LD_{50}s, times of death, and myocardial virus titers in normal C57BL/6 and immunocompromised CD4ko and β2Mko mice. (A) Mice were inoculated with different doses of CVB3 ranging, from 1 to 10^5 PFU, in 10-fold dilutions (eight mice per strain at each dose). The mice were monitored for 14 days, and the cumulative number of deaths at each virus dose is shown. (B) Groups of eight mice were inoculated with 1 LD_{50} of CVB3 and examined daily for 14 days. The times of death following infection varied among different strains, as shown. (C) Mice were inoculated with 1 LD_{50} of CVB3 and sacrificed at 0, 4, 7, 14, and 21 days p.i. For each mouse strain, at each time point four mice were sacrificed. Hearts were taken, and virus titers were measured. Average titers and standard deviations are shown. The statistical significance of the differences between the results for different mouse strains at 4 days p.i. was calculated (see text).

2E), and was at a similar level after 8 weeks (not shown). No obvious fibrosis was noted in the surviving mice of other strains.

Characterization the mononuclear myocardial infiltrate in CD4ko mice. Morphological examination of the infiltrating cells suggested that most were macrophages or lymphocytes; few polymorphic nuclei were apparent. MAbs to CD8a ($CD8^+$ T cells), Mac I (macrophages, NK cells, and polymorphs), Ia (macrophages, B cells, and specialized antigen-presenting cells), and NK1.1 (NK cells) were employed to better determine the cell types involved in the infiltrate. Each stained slide carried spleen tissue as a positive control, and negative controls were included by omitting the specific first antibody during the staining reaction. Figure 3A demonstrates that many of the infiltrating cell were Mac I^+, consistent with their being macrophages or NK cells. Approximately 40 to 50% of the cells were positive for MHC class II (Fig. 3B), consistent with activated macrophages, and 30 to 40% were positive for NK 1.1 antigen (Fig. 3C). Finally, 10 to 20% of the infiltrating cells were positive for CD8a surface antigen (Fig. 3D). In summary, the cardiac infiltrate in CD4ko mice 7 days after CVB3 infection consisted of 40 to 50% macrophages, 30 to 40% NK cells, and 10 to 20% $CD8^+$ lymphocytes.

Depletion of $CD8^+$ T lymphocytes in CD4ko mice markedly diminishes myocarditis and enhances survival, despite elevated virus titers. At 48 and 24 h before infection, CD4ko mice were treated with anti-CD8 MAbs as described in Materials and Methods. Controls were CD4ko mice which either were untreated or were treated with a similar dose of control ascites antibody (anti-CD4). By all measured criteria (see below), these two control groups were indistinguishable. The effectiveness of the in vivo depletion protocol was confirmed by FACS analyses of individual spleens at 7 days p.i. $CD8^+$ T-cell counts in the spleens were usually reduced by 95 to 99% from the level in untreated CD4ko mice, indicating effective depletion by the MAb treatments.

The biological effects of $CD8^+$ T-cell depletion were determined in the following three ways. (i) Myocarditis, which occurs in undepleted CD4ko mice following injection of 1 LD_{50} of CVB3 (Fig. 2), was markedly reduced in $CD8^+$-depleted mice receiving this virus dose. As shown in Fig. 4B to D, only infrequent and small areas of infiltration were observed, compared with the widespread process seen in mice with a competent $CD8^+$ T-cell compartment (Fig. 4A). In addition, $CD8^+$ depletion was occasionally less effective as judged by FACS analysis, and in these mice the levels of myocarditis were less dramatically reduced (data not shown). These data suggest that although $CD8^+$ cells constitute only 10 to 20% of the infiltrate, they appear to be critical to its inception, since in their absence the recruitment of NK cells and macrophages is greatly reduced. (ii) The reduced inflammation could result from a reduced viral load in the hearts of $CD8^+$-depleted animals. We therefore measured cardiac CVB3 titers 7 days after infection with 1 LD_{50} of CVB3. $CD8^+$ depletion resulted in a 17-fold elevation of cardiac virus titers ($P < 0.004$) (Fig. 5A), despite which myocarditis was markedly reduced (Fig. 4B to D). (iii) Injection of 3 LD_{50}s of CVB3 led to early death (days 3 to 5) in all CD4ko mice, while

FIG. 2. Myocardial lesions in CVB3-infected mice. (A to D) Paraffin sections of heart tissue from C57BL/6 (A), β2Mko (B), and CD4ko (C) mice 7 days following infection with 1 LD_{50} of CVB3 or from noninfected CD4ko mice (D) were stained with hematoxylin-eosin (magnification, ×100). (E and F) Paraffin sections of heart tissue from CD4ko mice 30 days p.i. with 1 LD_{50} of CVB3 (E) or from uninfected CD4ko mice (F) stained with Gomori trichrome, with which connective tissue is stained turquoise (magnification, ×400).

depletion of $CD8^+$ T cells allowed 75% survival (Fig. 5B). Furthermore, in $CD8^+$-depleted mice which did succumb, death was delayed (Fig. 5B). Since these mice were not subjected to FACS analyses, it is possible that the dying mice reflected less-efficient eradication of $CD8^+$ T cells. Thus, removal of $CD8^+$ T cells, while benefiting the mice (increased survival and reduced myocarditis), also allowed higher levels of virus replication.

FIG. 3. Immunohistochemical characterization of infiltrates in myocardial lesions. Frozen sections of heart tissue from CD4ko mice (7 days p.i. with 1 LD_{50} of CVB3) were stained for Mac I (A), MHC class II (B), NK1.1 (C), and CD8 (D) surface antigens. The inset in the right lower corner of each panel demonstrates areas of inflammation stained without the specific first antibody (magnification, ×400; sections counterstained with hematoxylin-eosin). The percentage of positive cells was determined by visual inspection.

FIG. 4. Depletion of $CD8^+$ T lymphocytes in CD4ko mice leads to a diminished amount of myocardial infiltrate. CD4ko mice were treated twice with anti-CD8 MAbs and were subsequently infected with 1 LD_{50} of CVB3, as described in Materials and Methods. At 7 days p.i., hearts were harvested, and paraffin sections from three individual mice are shown (B to D). (A) Section from a non-CD8-depleted CD4ko mouse. All sections were stained with hematoxylin-eosin (magnification, ×200).

Virus detection by immunohistochemistry. At several time points p.i., paraffin sections of murine heart tissue were stained for CVB3 antigen by using virus-specific polyclonal antibodies. Many areas of viral antigen were detectable in sections from all infected mice, and their frequency correlated with the highest concentration of infectious CVB3 (measured by plaque assay). Figure 6A to C shows typical CVB3-positive areas in paraffin sections from C57BL/6, CD4ko, and β2Mko mice at 4 days p.i. Note the absence of inflammation at this time point. Investigation at later times, when infiltrates were detected in CD4ko mice, showed no clear correlation between CVB3-positive areas and the presence of infiltrating cells (Fig. 6D). Although in a few instances we could identify CVB3-positive foci with surrounding inflammatory cells, in many cases virus antigens were undetectable in severely inflamed tracts. The absence of viral antigen from areas showing severe inflammation may reflect the rapid cell death and proteolysis resulting from the immune infiltrate. Conversely, several CVB3-positive areas lacked detectable inflammatory responses (Fig. 6D). This apparent discordance between antigen and inflammation may reflect the kinetics of virus dissemination and the consequent immune reaction; a more detailed analysis would be required to dissect this issue. Viral antigen was no longer detectable by immunostaining on or after day 14.

Apoptosis in heart tissue of CVB3-infected CD4ko mice. Apoptosis can occur under a variety of conditions. Relevant to this study is that apoptosis may be induced virus infection (38) or by CTL action on certain virus-infected target cells (37, 49). Therefore we analyzed frozen sections of heart tissue from CVB3-infected CD4ko mice for the presence of apoptotic cells. As shown in Fig. 7A, apoptotic cells were occasionally identified, but only within areas of inflammation. No apoptosis was detected in noninflamed regions, although, as shown in Fig. 6, virus was readily detected in these areas.

Detection of TNF-α in myocardial lesions. The presence of infiltrating immune cells in the myocardia of CVB3-infected CD4ko mice implied the probable local production of cytokines. We used MAbs directed against TNF-α and detected positive cells only in inflamed regions (Fig. 7C). As a positive control for this staining we used the murine monocyte/macrophage cell line J774A.1 (American Type Culture Collection [TIB 67], Rockville, Md.) (not shown), which, after CVB3 infection, releases large amounts of TNF-α (16).

DISCUSSION

Most coxsackievirus infections in humans are mild, and sufferers manifest only nonspecific symptoms. However, coxsackieviruses also are implicated in serious diseases such as encephalitis, meningitis, diabetes mellitus type I, and myocarditis (1). The correlation between coxsackievirus infection and acute myocarditis or chronic disease with end-state dilated cardiomyopathy has been established by serologic conversion (29, 36, 41), isolation of virus from the myocardium (27, 40), and in situ cDNA-RNA hybridization methods (2, 20–22). Serologic evidence of coxsackievirus infection was found in 75% of patients suffering from idiopathic dilated cardiomyopathy but in none of the control patients (41). During the acute phase of infection, massive infiltrates of mononuclear cells have been found in various murine models of CVB3 infection and in

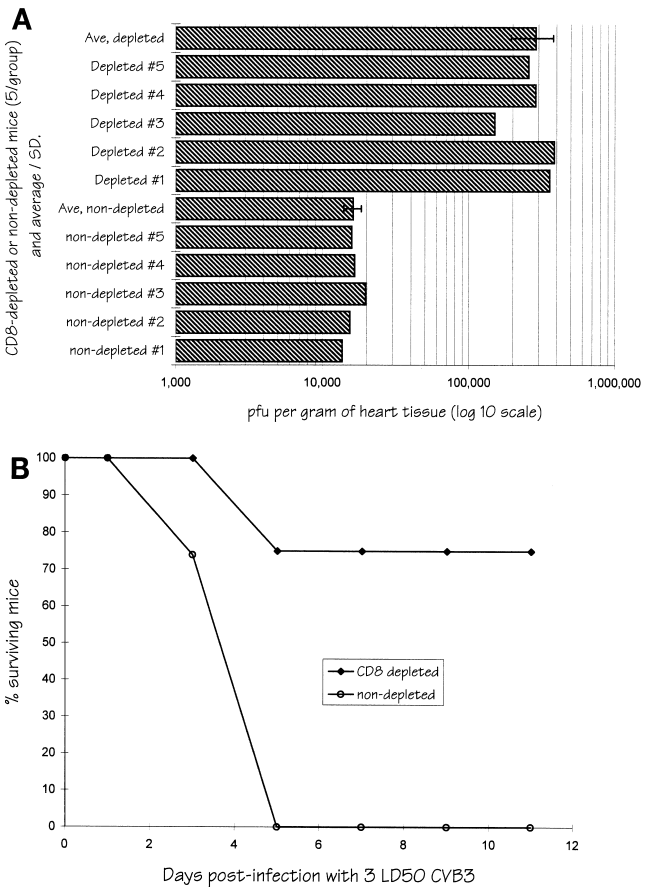

FIG. 5. Depletion of $CD8^+$ T lymphocytes in CD4ko mice leads to elevated virus titers and enhanced survival. (A) CD4ko mice (two groups of five, with one group CD8 depleted and the other nondepleted) were infected with 1 LD_{50} of CVB3 (as calculated for the nondepleted CD4ko mice). At 7 days p.i., hearts were harvested and virus titers were analyzed by standard plaque assay on HeLa cell monolayers. Ave, average; SD, standard deviation. (B) Groups of eight CD4ko mice (one group CD8 depleted and the other nondepleted) were infected with 3 LD_{50}s of CVB3 (as calculated for nondepleted CD4ko mice), and mortality was monitored over an 11-day period.

CVB3-infected patients (23), with consequent myocardial cell death. Whether myocardial damage is directly virus mediated or whether it results from immunopathologic damage is controversial. Histopathologic observations in various CVB3-infected mouse strains support the possibility that cytolytic and necrotizing heart damage is dependent mainly on viral replication (30). Chow et al. (3) have also shown in their model of homozygote CVB3-infected SCID mice that these immunodeficient animals develop severe and mainly virus-induced lysis of cardiocytes. In contrast, others have found that activated T lymphocytes, rather than direct viral mechanisms, are primarily responsible for cell damage. Mice made T-cell deficient by either thymectomy, irradiation and bone marrow reconstitution, or injection of antithymocyte serum did not develop cardiac inflammation and myocardial necrosis (39, 48).

Although many laboratories espouse the immunopathologic explanation, the relative roles attributed to different immune compartments have varied; CTLs (14, 39, 44, 48), autoantibody-producing B cells (15, 32, 43), and NK cells (10) all have been associated with the immunopathogenesis of CVB3-induced myocarditis. To evaluate the roles of $CD4^+$ and $CD8^+$ T cells in this disease, we have evaluated two transgenic ko mouse lines, each deficient in a different aspect of T-cell-mediated immunity. Such immunocompromised animals represent valuable tools in such analyses and have, for instance, lent clarity to our understanding the role of CTL antiviral mechanisms (19) and the redundancy of the immune system (31).

In all three mouse strains used, i.e., C57BL/6, CD4ko, and β2Mko, CVB3 is lethal if sufficient virus is given. The immune status of the host appears to play a critical role, as the removal of T-cell function confers enhanced resistance to virus challenge. Furthermore, even when the outcome is lethal, the disease course appears to differ depending on the host's immune status, as death from 1 LD_{50} occurs at days 3 to 4 in immunocompetent mice and at days 5 to 7 in immunocompromised mice. Thus, the immune system appears to play a mostly detrimental role at this stage of infection. Direct viral effects cannot be completely discounted, however. There is a correlation between cardiac virus titer and susceptibility; C57BL/6 mice, which are highly susceptible to lethal infection, carry approximately 8×10^5 PFU/g of heart tissue, while the virus replicates to three- to fivefold-lower levels in the hearts of the less-susceptible immunocompromised hosts. The reason for this reduction in titer despite immunosuppression is unclear. Redistribution of virus to other organs in the immunodeficient hosts is an unlikely explanation, as virus was not detected in several other sites (spleen, liver, kidney, brain, and serum [data not shown]). These results indicate that the cardiac virus load may play a role in determining whether early death will ensue. However, CD4ko mice which are depleted of $CD8^+$ T cells and subsequently are given 3 LD_{50}s of CVB3 show a 17-fold increase in virus titers but a diminished susceptibility to early death (Fig. 5). Thus, there is no simple relationship between CVB3 titers in the heart and early death, but the presence of a normal immune system is deleterious in this regard.

Reasoning that the early death would prevent the development of myocarditis, we reduced the dose of virus to 1 LD_{50} and evaluated virus titers and cardiac histology in surviving mice at several time points. At all time points myocarditis was absent, or very mild, in surviving β2Mko mice and in C57BL/6 animals, and the hearts of survivors bore no long-term histologic changes. In contrast, in CD4ko mice extensive myocarditis was present at 7 days p.i., with a marked mononuclear infiltrate comprising macrophages, NK cells, and $CD8^+$ T cells. This infiltrate peaked at days 7 to 8, was resolving by day 14, and was cleared by day 21 p.i. As the infiltrate resolved, fibrosis developed, and it appeared to plateau by day 31 p.i.

What cells are important for the development of myocarditis, and why does this disease not appear in the β2Mko and C57BL/6 mice? NK cells (10) and CTLs (6, 14, 45, 48) have been associated with immune pathogenesis of CVB3-induced myocarditis, and we were interested in characterizing the role of these lymphocytes in our model. $CD8^+$ T cells are present as a minor component (10 to 20%) of the myocardial infiltrate, but they appear to be vital to the development of this inflammatory process, since CD4ko mice depleted (by MAb treatment) of $CD8^+$ T cells prior to CVB3 infection have a greatly reduced incidence and severity of myocarditis (Fig. 4). The requirement for these cells in the development of myocarditis is consistent with the diminution of myocarditis in β2Mko mice. Why, however, do normal mice, which have an intact $CD8^+$ T-cell compartment and high levels of virus in the myocardium, not develop myocarditis? The $CD8^+$ response may be suppressed by $CD4^+$ cells induced by this CVB3 strain; the virus used in this study can (by an as-yet-unexplained mechanism) induce a $CD4^+$ response skewed towards the Th2 subclass, which may elaborate cytokines able to suppress $CD8^+$

FIG. 6. Immunochemical detection of CVB3-infected foci in myocardium. Paraffin sections (5 μm) of heart tissue from C57BL/6 (A), β2Mko (B), and CD4ko (C) mice at 4 days p.i. with 1 LD_{50} of CVB3 were stained for CVB3 antigen by using virus-specific polyclonal antibodies; stained areas were visualized with alkaline phosphatase-labeled secondary antibodies and an appropriate substrate. In the inset to panel A, specificity of staining is demonstrated by omission of the first (anti-CVB3) antibody. (D) Paraffin section of heart tissue from a CD4ko mouse at 7 days p.i., stained for CVB3 and counterstained with hematoxylin-eosin. Magnification, ×200.

antiviral responses and thereby forestall myocarditis (18, 28). In CD4ko mice the absence of this Th2 response would allow the development of an elevated antiviral $CD8^+$ response and hence myocarditis. The hypothesis is consistent with other findings in this study. Normal mice have high cardiac virus titers, but the titers in CD4ko mice are reduced threefold (Fig. 1C), perhaps because of the antiviral activity of the $CD8^+$ T cells which develop as a result of the release from $CD4^+$-mediated suppression. That the antiviral effect in CD4ko mice is $CD8^+$ T-cell mediated is confirmed by $CD8^+$ depletion, which results in a 17-fold increase in virus titers (Fig. 5A). If the absence of $CD8^+$ T cells results in high virus titers, then one might expect that titers in the β2Mko mice would approach or exceed those in C57BL/6 mice. This is not the case; titers in this mouse strain are intermediate between those in C57BL/6 and CD4ko mice (Fig. 1C), and some myocarditis is detectable (Fig. 2B), suggesting that some $CD8^+$ T cells may be present. This is consistent with a previous demonstration of low levels of effective $CD8^+$ T cells in this mouse strain (9). Thus, $CD8^+$ T cells are important both in the control of virus titers and in the development of myocarditis, and the $CD4^+$ response in normal mice serves to prevent myocarditis but simultaneously renders these hosts more susceptible to early death. These data underscore the delicate balance which exists between beneficial and detrimental antiviral immune responses.

The above-described results confirm the importance of the host genetic background in determining the outcome of coxsackievirus infection. Immunocompetent $H2^{b/b}$ mice, which can mount $CD4^+$ responses, were less capable of controlling viral replication in the myocardium and died in the acute phase, 3 to 4 days p.i. In contrast, mice unable to mount a $CD4^+$ T-cell response to the agent controlled the infection, but in so doing developed severe myocarditis with consequent long-term myocardial damage (extensive fibrosis). Interestingly, both positive and negative correlations between the MHC class II type (which will determine the pattern of antigen presentation to $CD4^+$ T cells) and the incidence of idiopathic dilated cardiomyopathy have been found in humans (41).

The mechanism by which $CD8^+$ T cells can instigate myocarditis, recruiting other cell types to the lesions, presumably involves cytokine release. CVB infection can induce cytokine production in vitro. Following CVB3 infection, human monocyte cultures responded with a high level of release of TNF-α, interleukin-1β (IL-1β), and IL-6 (11), and the murine monocyte/macrophage cell line J774A.1 produced TNF-α (16) and alpha/beta interferon as well as IL-1 (18). The critical part played by cytokines in CVB3 infection has recently been demonstrated in vivo. Exogenous administration either of TNF-α or IL-1β to virus-infected myocarditis-resistant mice (26, 33) or of IL-1 or IL-2 to susceptible mice infected with a nonmyocarditic CVB3-variant (18) results in a substantial increase in myocarditis. In the present study we showed the presence of TNF-α in cells of the myocardial infiltrate (Fig. 7C). These observations are consistent with the possibility that cytokine patterns differ in different mouse strains (or with different virus strains) and that these patterns determine the overall nature of the antiviral T-cell response and dictate whether myocarditis will supervene. The role of apoptosis in myocardial damage is uncertain. We were unable to detect apoptotic cells in noninflamed myocardium (Fig. 7A) despite the presence of readily detected CVB3 (Fig. 6), suggesting that apoptosis is not induced in myocardiocytes by CVB3 infection. The nature of the cells detected in the infiltrate has not been determined; it is possible that they represent apoptotic immune effector cells (34).

FIG. 7. Presence of apoptotic cells and of TNF-α in myocardial lesions. (A and B) Frozen sections of myocardium from CD4ko mice (7 days p.i. with 1 LD_{50} of CVB3) were stained to detect apoptotic cells as described in Materials and Methods. (A) Some apoptotic cells (arrows) were detectable, only in areas of cellular infiltrates. (B) Specificity of staining demonstrated by leaving out the terminal deoxynucleotidyl transferase enzyme. Slides were counterstained with hematoxylin-eosin (magnification, ×400). (C and D) Similar samples were stained for the presence of TNF-α. (C) Positive cells were found only in infiltrated areas. (D) Specificity of staining demonstrated by leaving out the first antibody.

Thus, both T-cell compartments may be immunopathologic during CVB3 infection. $CD4^+$ T cells are implicated in early death, as are $CD8^+$ cells, and the latter also are required for the development of myocarditis. The antigen specificity of these $CD8^+$ cells has not been determined in our study. It has been suggested that this cell population comprises three different groups of CTLs (39): (i) classical virus-specific effectors which recognize viral antigen presented by infected cells (8, 12), (ii) CTLs which recognize cellular neoantigen in cells infected with CVB3 (39), and (iii) autoimmune or autoreactive CTLs which lyse uninfected cardiocytes (5). We have cloned and expressed several CVB3 proteins in recombinant vaccinia viruses (11a), and these reagents should help clarify the antigen specificity of these immunopathologic $CD8^+$ T cells.

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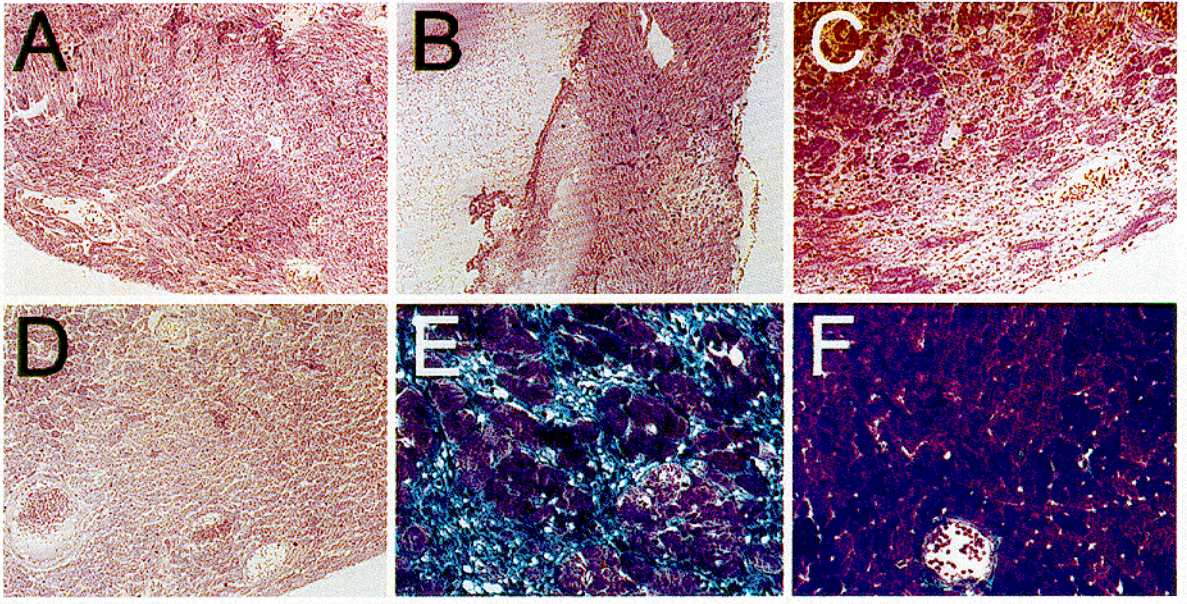
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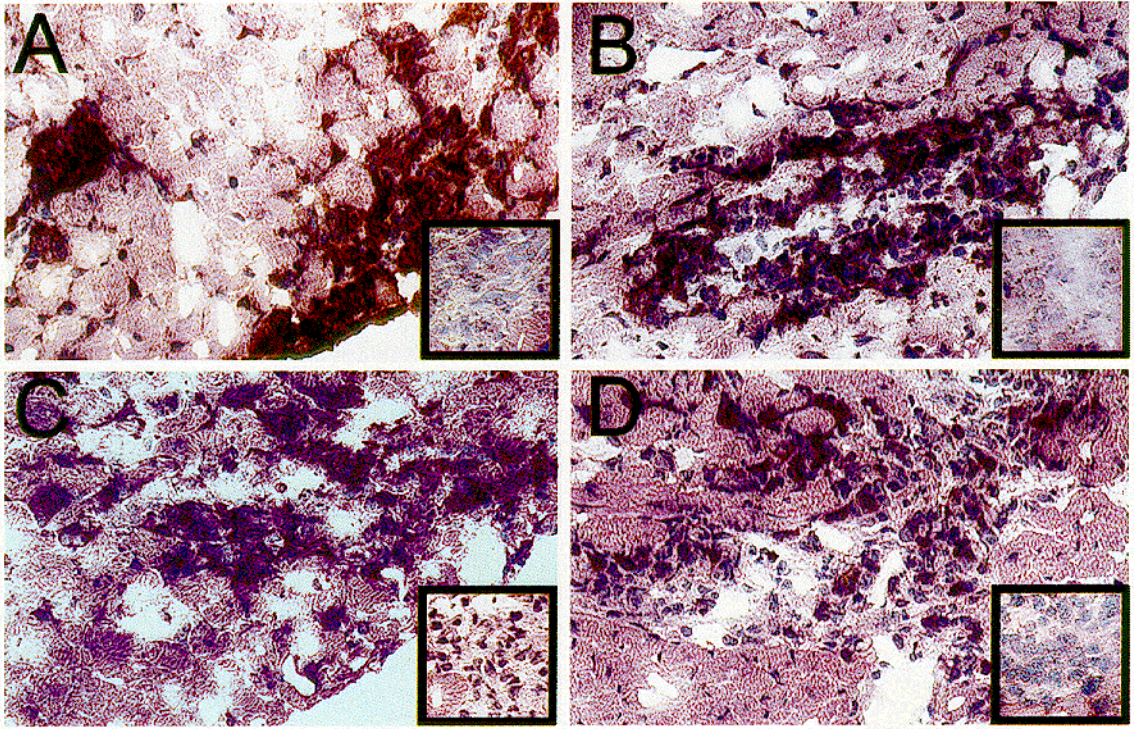
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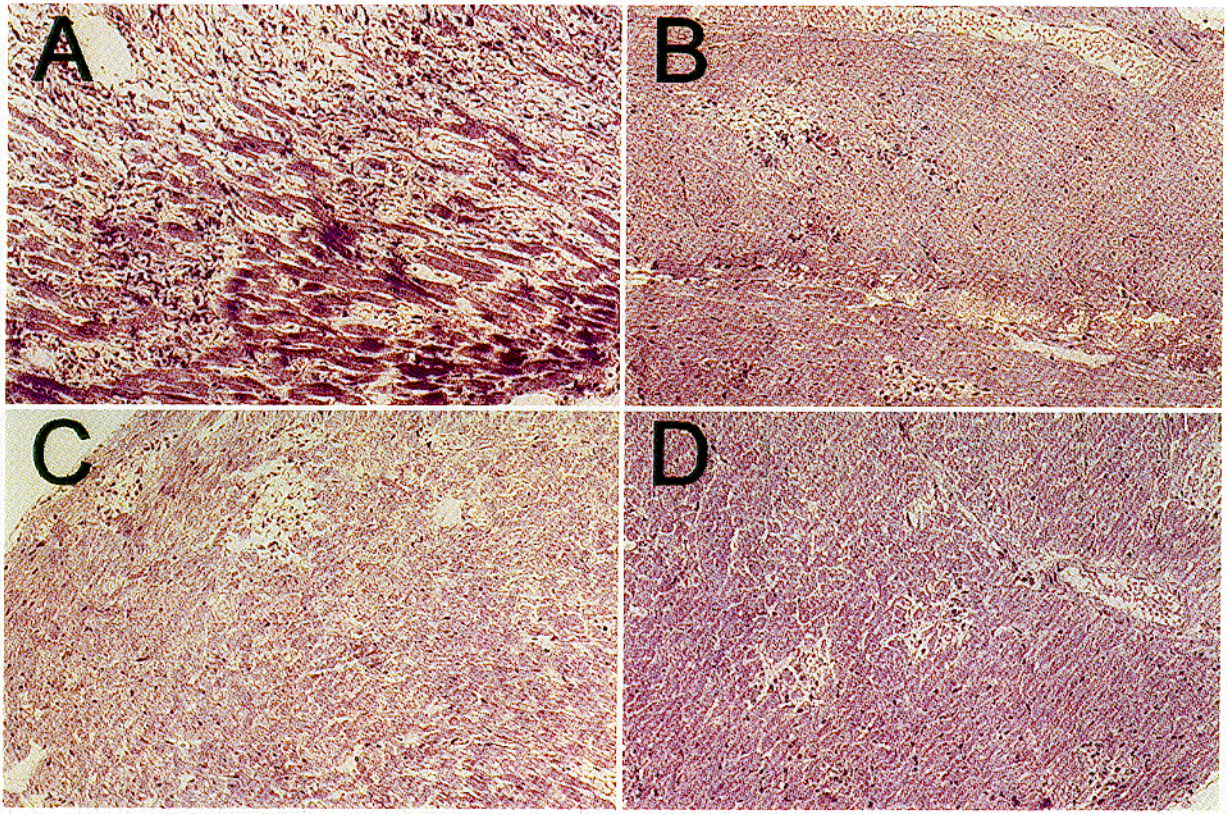
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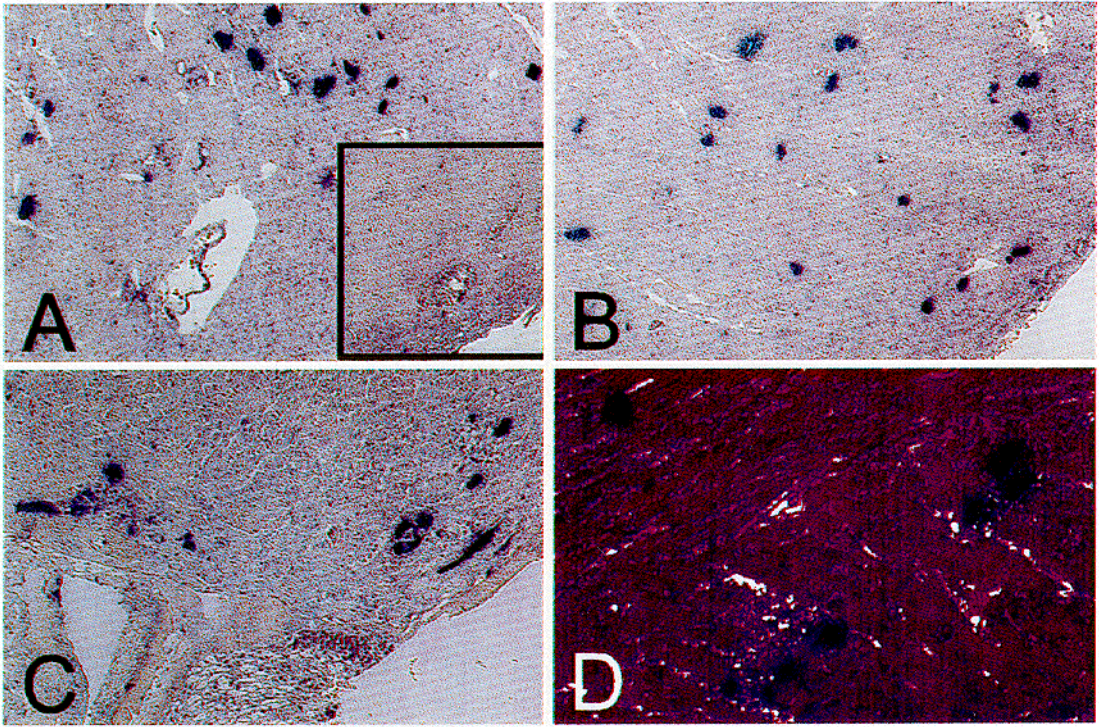
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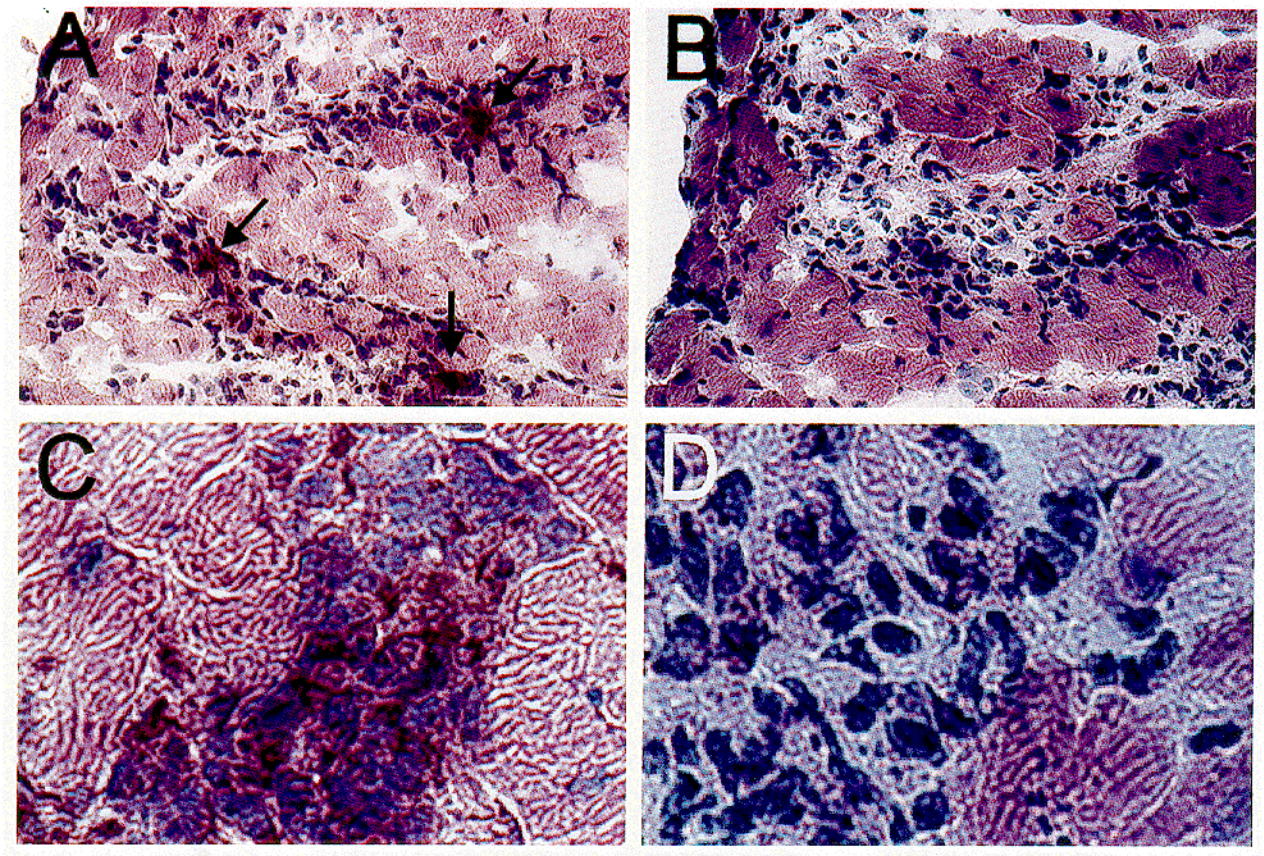
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