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Coxsackie B3 Myocarditis in Athymic Mice (41028)[1]

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Abstract. Coxsackie B3 virus produces myofiber necrosis and mononuclear infiltration in the hearts of both BALB/c and athymic mice within 8 days after infection. Viral clearance time from the myocardium of both athymic and BALB/c mice did not differ. Within 2 weeks of infection both strains of mice develop chronic inflammatory infiltrates comprised of cells that were diffusely and intensely acid-α-naphthyl esterase positive and characteristic of monocytes or showed only focal esterase staining characteristic of T-lymphocyte lineage.

The cellular infiltration that occurs in concert with myocardial necrosis during Coxsackie B-3 viral (CVB-3) infection is characterized by mononuclear cells that are presumably lymphocytes and monocytes. There are several lines of circumstantial evidence that the extent of myocardial damage is dependent upon host cellular participation (1). T-Lymphocyte (T-LY)-depleted mice develop CVB-3 myocarditis but with less cellular infiltration and necrosis than similarly infected normal mice. The *in vivo* methods used to effect T-LY deprivation in mice include the use of antithymocyte serum or neonatal thymectomy combined with whole body radiation and subsequent bone marrow repletion (1). Both these methods usually attenuate mature T-LY responses but may not universally inhibit them, and more importantly, they may also delete immature T-LY populations or drastically alter relationships within the immunoregulatory cell network.

A corollary finding during CVB-3 infection in T-LY-deprived mice suggested that T-LY are not needed for termination of CVB-3 myocardial replication during the initial stages of CVB-3 myocarditis (1). The latter observation suggested that athymic mice would also develop viral resistance normally. Thus, CVB-3 infection in this mouse mutant could provide a model for definitively determining whether T-LY-mediated or -amplified host immunopathologic responses during CVB-3 myocarditis are mandatory for full expression of the disease.

Materials and Methods. *Animals.* All animals were received at 4 weeks of age and held a minimum of 4 days before use to exclude diseased specimens. Male/female BALB/c (Lab Supply, Bloomington, Ind.) and nu/nu/BALB/c mice (Sprague–Dawley, Madison, Wisc.) were housed in a single self-contained animal isolation unit. Theta-1.2 antigen is present on less than 5% of lymphocytes from this strain. One to four mice were housed together but segregated by age and sex. All mice, including nu/nu mice, were maintained without special precautions in disposable filter-topped cages. They were handled with gloves by masked and gowned personnel. All injections were done after iodine–alcohol skin preparation. Laminar flow hoods and antibiotics were not used in any experiments. Ages of mice at the time of CVB-3 infection ranged from 4 to 10 weeks.

Virus. CVB-3 (Nancy Strain), VR-30 obtained from American Type Culture Collection, and CVB-3 (Nancy Strain) kindly provided by Dr. J. Woodruff, Cornell University, New York, were grown in VERO or LLC-MK2 cells with Dulbecco's modified Eagle's medium (DME) 10% FCS and gentamicin. A single-seed stock of each was harvested, frozen, and used for all experiments. The ATCC stock contained 9×10^6 PFU virus/ml and CVB-3 (Woodruff) had 4×10^5 PFU virus/ml (2). Viral inoculates

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had been thawed and refrozen once prior to infection.

Animal infection. Mice were infected ip with varying amounts of CVB-3 diluted in 0.5 ml DME or RPMI 1640 and observed daily for lethargy, ruffling, photophobia, or other abnormal behavior. They were killed by cervical dislocation and heart, spleen, kidneys, and striated muscle were removed for histopathologic analysis and detection of tissue virus.

Histopathology. Four coronal sections of heart were done. The initial apical section was snap-frozen for future viral isolation, the second fixed in buffered Formalin for hematoxylin/eosin staining, the third fixed in formal–sucrose for acid α-naphthyl esterase (ANAE) staining, and the last snap-frozen in dry ice-acetone slurry for immunofluorescent studies.

ANAE staining of monocytes and T-LY was done by a modification of the method of Mueller *et al.* (3). Briefly, tissue is fixed in formal–sucrose for 24 hr, then Holt's syrup for 21 hr, and stained with hexazotized pararosaniline and α-naphthyl acetate (Sigma Chemical Co., St. Louis, Mo.) at pH 5.8 for 2.5 hr. Slides were counterstained with 1% toluidine blue, dehydrated in increasing concentrations of ethanol, cleared with Parasol (Scientific Products), and premounted. With this procedure, T-LY stained with a single, or rarely double, red-brown nodule adjacent to a cell membrane, while monocytes stained diffusely and much more intensely.

Immunofluorescence. Four μm cryostat sections of myocardium and kidney were stained for 15 min with goat anti-murine heavy chain specific IgG fluorescein-labeled sera for detection of tissue-bound antibody. All sections were viewed with a Zeiss Universal microscope, halogen lamp, and standard exciter/barrier filters (FITC primary filter, No. 50 barrier filter).

Quantitation of inflammation and necrosis. Quantitation of inflammation (I) and necrosis (N) was done by a modified version of Woodruff (4). All sections were scored at 100× magnification. Necrosis is scored as 0 to 4 with 4+ reflecting widespread necrosis. Inflammatory scoring also ranged from 0 to 4 with the latter number representing 100 or greater mononuclear cells, usually confluent, per low power field.

Viral isolation. Apical coronal sections of myocardium were rapidly thawed and approximately a 2 × 2-mm section suspended in 1.0 ml of RPMI 1640 and homogenized in a ground glass mortar and pestle. Homogenates were clarified at 8000 rpm for 10 min at 4° and the supernatant was inoculated onto 48-hr-old VERO monolayers in 60-mm tissue culture dishes. After viral absorption for 90 min, DME with 10% FCS with gentamicin was added to bring the volume to 5.0 ml and monolayers were incubated at 37°, 9% CO_2 atmosphere. Viral cytopathic effects were assessed every 24 hr until focal monolayer destruction peaked and stabilized. Virus was considered absent if no cytopathic effect was noted within 7 days.

Results. *Coxsackie virus infection in BALB/c mice. Infection.* Virus inocula ranging from 9×10^6 to 4.5×10^4 PFU of CVB-3 (ATCC) consistently produced a myocardial infection in 27 juvenile (4- to 5-week-old) and 9 adult mice (8 to 9 week old) (Table I). CVB-3 (Woodruff), however, when used at 1 to 4×10^5 PFU was lethal in 5 of 6 adult animals. The survivor had 4+ mononuclear infiltrates and necrosis of myocardium after 9 days of infection with CVB-3 (Woodruff). Virus was present in myocardium 7 days after infection, but absent at 9, 15, and 21 days after infection.

Histopathology. By Day 9, there was a spectrum of focal mononuclear infiltrates and myofiber necrosis in the majority of animals (Table I). The histology scoring ranged from 1 to 4+ I and 0 to 4+ N. Early (7-day) lesions occasionally also had neutrophils as part of the infiltrate. At Days 14 and 21, most mice had 2 to 4+ mononuclear infiltrates and moderate necrosis (Fig. 1) and by Day 30, there was still an inflammatory response and myonecrosis present. Two 6-month BALB/c survivors had focal myocardial fibrosis with no cellular infiltrates. No inflammatory lesions or necrosis were found in striated muscle, spleen, or kidney of any of the infected animals. Control (uninfected) mice were histologically normal.

Esterase stains. No definitive ANAE

TABLE 1. COXSACKIE B-3 INFECTION IN NORMAL AND ATHYMIC MICE

Mouse			Virus		Histopathology	
Strain	No.	Age at I[a]	Duration[b]	Infecting dose[c]	Infiltrate	Necrosis
BALB/c	5	4–5	7–9	9×10^6	1–4+	0–1+
	5	4–5	7–9	9×10^5	1–3+	1–2+
	5	4–5	7–9	$4.5\text{-}9 \times 10^5$	1–2+	1–2+
	4	4–5	21–30	9×10^5	1–3+	1+
	2	4–5	21–30	9×10^6	1–3+	1–3+
	4	8	7–14	9×10^6	2–4+	1–2+
	2	9	35	9×10^5	1+	1+
Total	27					
nu/nu/BALB/c	3	5–7	7–9	9×10^6	1–2+	4+
	8	5	7–9	9×10^5	1–4+	1–4+
	4	8	7–9	2×10^5	1–2+	1+
	3	9	14	1×10^5	1–2+	1–2+
	1	9	35	1×10^5	4+	3+
	1	5	35	1×10^5	3+	2–3+
	1	9	60	1×10^5	1+	0
Total	21					

[a] Age in weeks of mouse at time of CVB-3 infection.
[b] Duration in days of CVB-3 infection.
[c] PFU infecting dose of CVB-3 (ATCC).

staining was demonstrable in myocardium of control animals. ANAE staining was intense within myocardial infiltrates of CVB-3-infected animals, and was characterized by approximately 50% of cells demonstrating typical intense uniform cytoplasmic esterase staining characteristic of monocytes while other mononuclears showed focal staining usually described only in thymic-derived lymphocytes (Fig. 2).

Immunofluorescence. Weak but diffuse granular IgG deposition throughout myocardial inflammatory foci was common. Trace amounts of glomerular-bound immunoglobulins were found in all mice, including controls.

Coxsackie virus infection in nu/nu/BALB/c mice. Infection. Mortality rates were higher in athymic mice than in BALB/c mice when comparable, bacterial-free inocula were used. Many of the deaths in athymic mice, however, were due to post ip injection peritonitis and occurred within 3 to 4 days after injection. Hearts of these animals did not show significant cellular infiltration and necrosis, suggesting that nonspecific monocyte activation and myocarditis does not ensue after bacterial infection in these mice. Ranges of CVB-3 (ATCC) infecting doses, comparable to those used in BALB/c mice, consistently produced acute and chronic myocarditis (Table I). Similar amounts of CVB-3 (Woodruff) were usually lethal within 4 to 6 days, but lower doses produced comparable acute disease. Induction of chronic myocarditis was possible only when infecting doses of 1×10^5 PFU virus were used.

Histopathology. When peritonitis did not occur, both CVB-3 strains (Woodruff and ATCC) produced myocarditis with mononuclear infiltrates and necrosis in ten 5-week-old mice (Fig. 3). The scoring range and histology was comparable to that seen in BALB/c mice. Neutrophils were not found in acute lesions. Seven of eight 8-week-old mice developed significant mononuclear infiltrates and necrosis within 14 days after infection (Fig. 4). One nude mouse infected at 5 weeks and another at 9 weeks were long-term survivors. Both showed intense mononuclear infiltration and mild to moderate necrosis 5 weeks after infection (Fig. 5).

The cytoplasm of infiltrating mononuclears in the chronic infection, however,

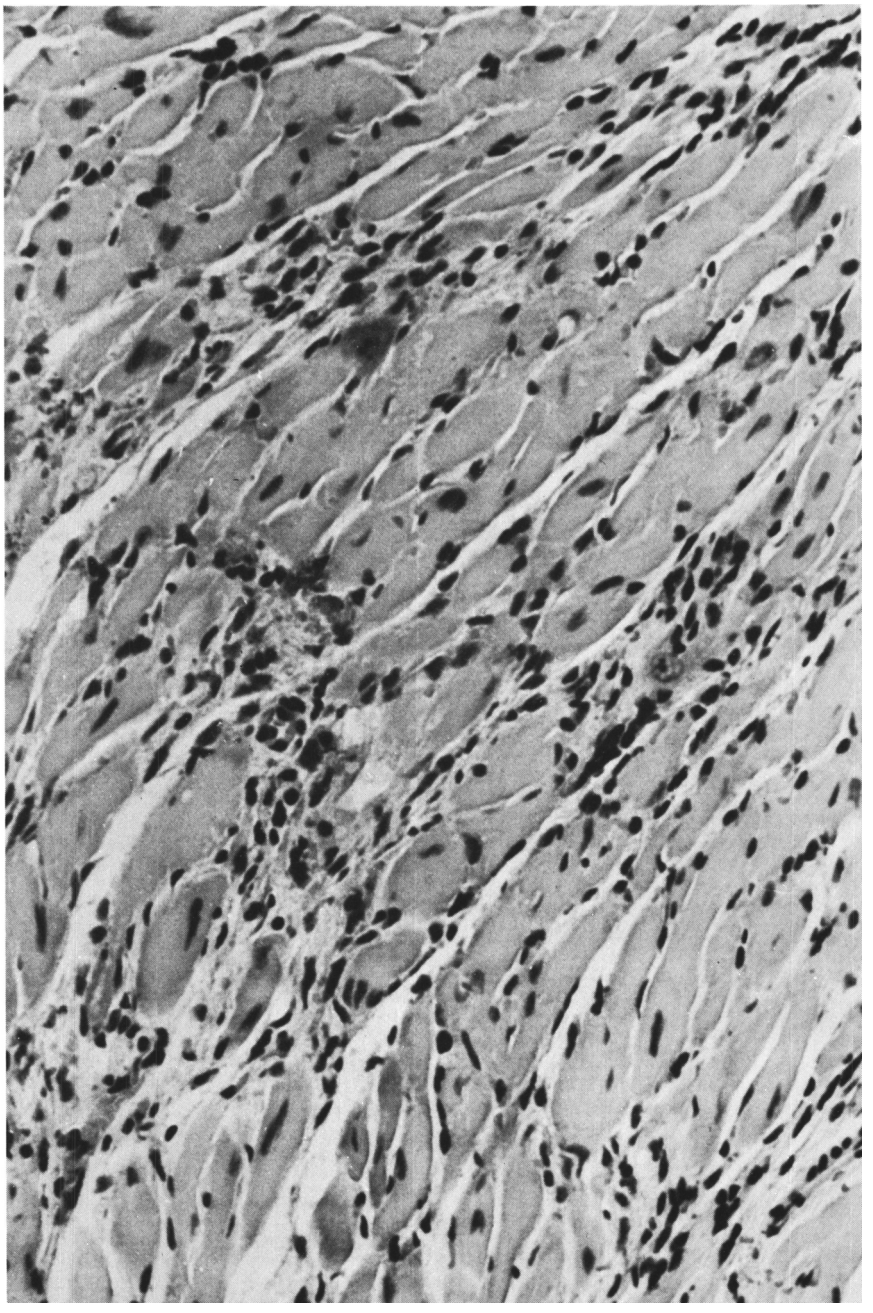

FIG. 1. Myocardium of a BALB/c mouse 9 days after infection with 9×10^5 PFU CVB-3 at 5 weeks of age. Foci of mononuclear cells are mingled with myofiber necrosis (H and E, 125×).

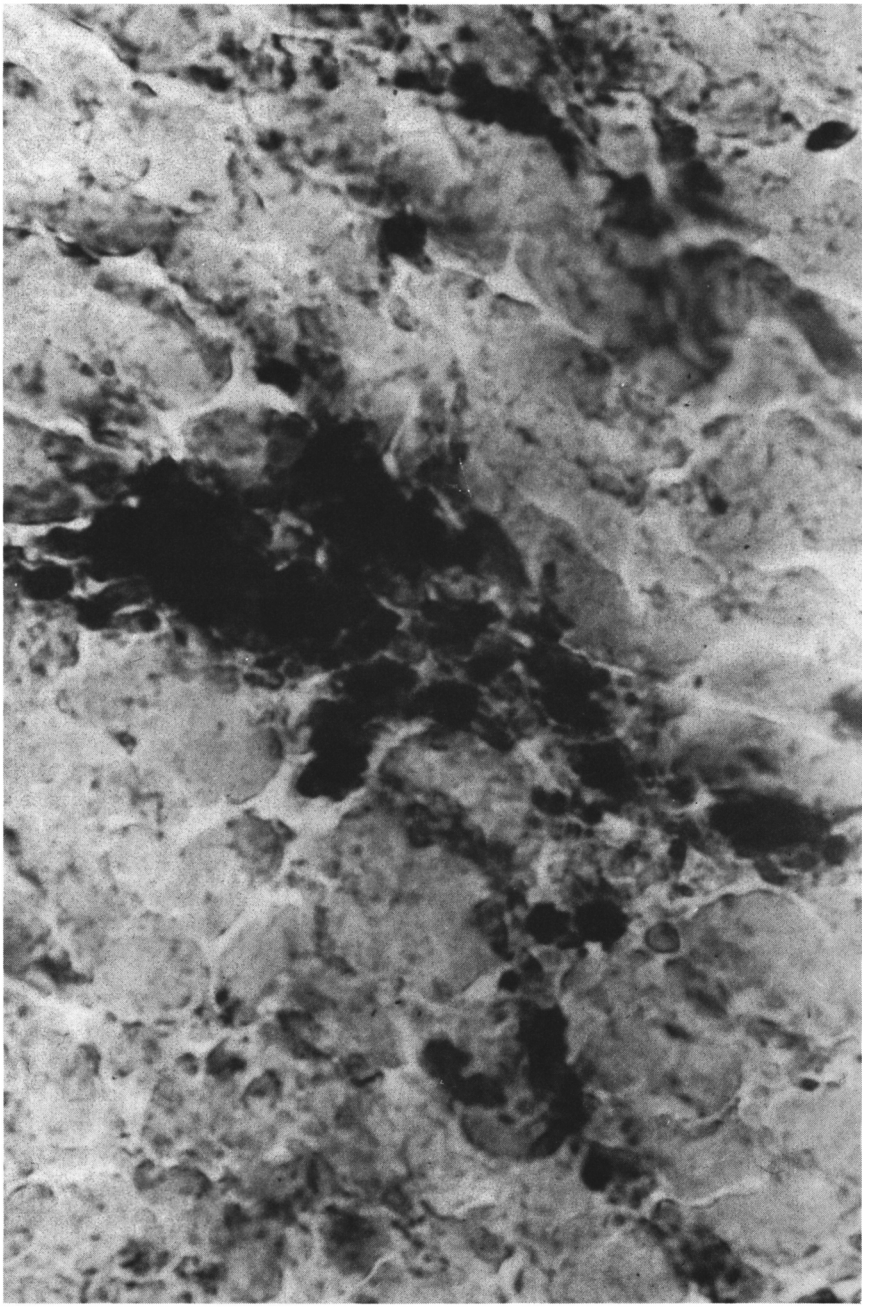

FIG. 2. Myocardium of a BALB/c mouse 9 days after infection with CVB-3 at 5 weeks of age. Both uniform and focal cellular esterase staining is present in inflammatory infiltrates (ANAE, 500×).

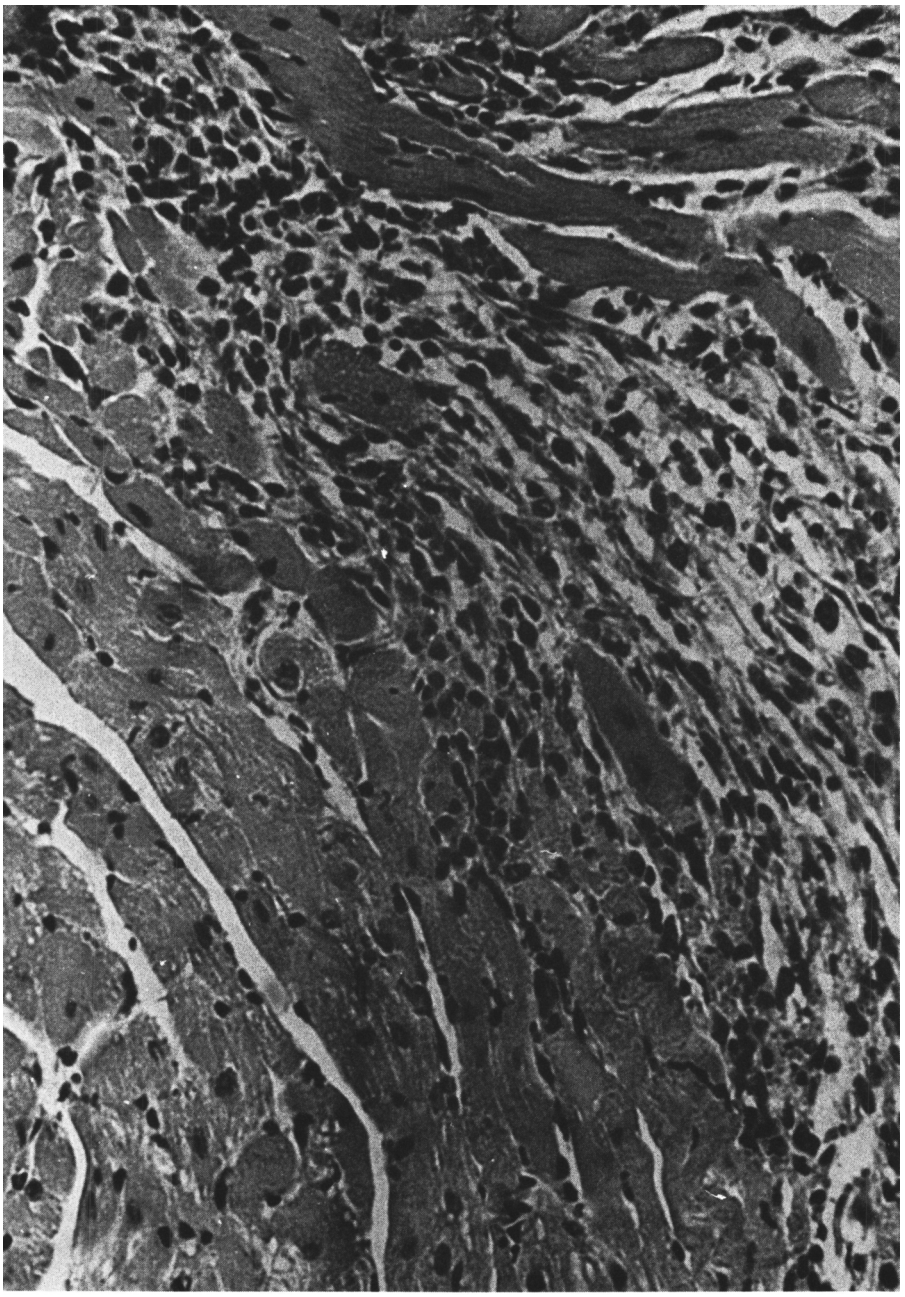

FIG. 3. Extensive myonecrosis and mononuclear cellular infiltrate in the myocardium of a nu/nu/BALB/c mouse 9 days after infection with 1×10^5 PFU CVB-3 at 5 weeks of age (H and E, 125×).

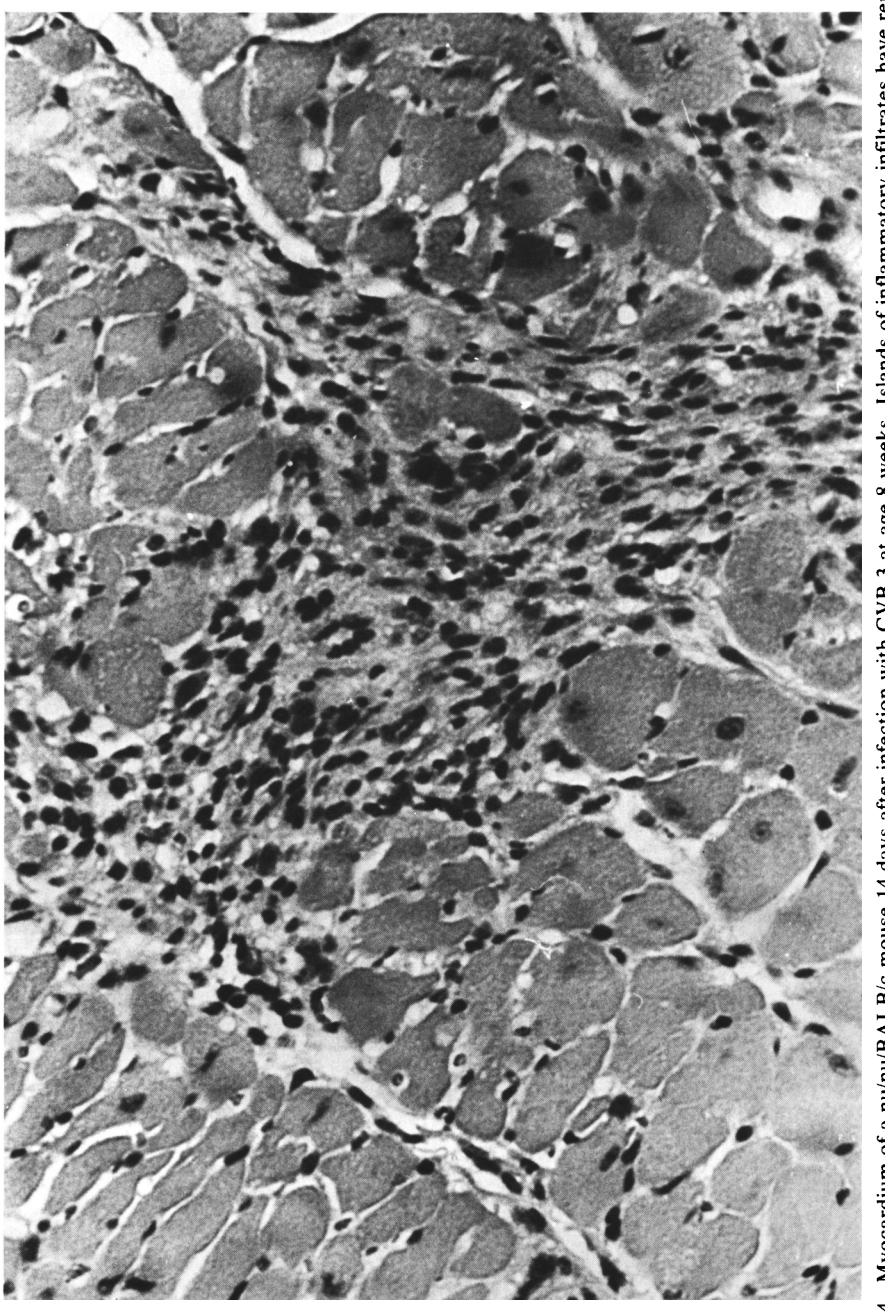

FIG. 4. Myocardium of a nu/nu/BALB/c mouse 14 days after infection with CVB-3 at age 8 weeks. Islands of inflammatory infiltrates have replaced myofibers (H and E, 125×).

FIG. 5. Myocardium of a nu/nu/BALB/c mouse 31 days after infection with CVB-3 at age 9 weeks. Extensive mononuclear cellular infiltration and moderate necrosis is present (H and E, 125×).

was more abundant and nuclei of the inflammatory cells were somewhat larger, more stranded, lacy, and had more nucleoli than those typically described in the mononuclear infiltrates in BALB/c mice (Fig. 6). Virus was present in the heart of athymic mice at Day 7 but absent by Day 14. No involvement of striated muscle, spleen, or kidney could be demonstrated. The hearts of uninfected nu/nu mice, killed at similar time intervals, were negative for myofiber necrosis and any type of cellular infiltration.

Esterase stains. Fifty-five percent of peripheral blood nu/nu/BALB/c lymphocytes partially purified by ficol flotation were ANAE positive. This percentage was comparable to human (56%) and BALB/c (58%) lymphocytes similarly prepared. Spleen suspensions from athymic mice were comprised of 72% ANAE-negative, 14% focal ANAE-positive, and 14% diffusely ANAE-positive cells. There was no definitive ANAE staining demonstrable in hearts from control nu/nu mice. Esterase stains of the mononuclear infiltrates in the athymic mice were similar to that seen in BALB/c mice (Fig. 7). There may have been a slight increase in the number of intensely staining cells that appeared to be monocytes, however, all infiltrates also showed focal esterase staining characteristic of thymic derived cells.

Immunofluorescence. Trace amounts of glomerular IgG in a granular pattern were found in all mice, including controls. Occasional granular deposition of IgG was seen in myocardial infiltrates.

Discussion. Woodruff and co-workers have demonstrated in a systematic investigation that T-LY appear to be important participants in the pathogenesis of CVB-3 heart disease in mice. Mice with modified T-LY functions have reduced severity of myocardial inflammation, necrosis, and mortality during CVB-3 infection (1). T-LY isolated from the spleens of BALB/c mice infected with CVB-3 were cytotoxic for CVB-3-infected myocytes and this activity could not be inhibited by CVB-3 antibody (5). Paque extended these observations by demonstrating that KCl extracts from hearts of CVB-3-infected CD-1 mice contained a novel antigen or antigens that were not CVB-3 virion-related but were capable of inhibiting the migration of peritoneal exudate cells from CVB-3 infected mice (6, 7). Recently, nu/nu/BALB/c mice were infected with CVB-3 and found to have no inflammatory infiltrates within the first 8 days of infection (the table in that report is contradictory, stating that one out of six mice had an inflammatory infiltrate) (8). The latter experiment implied that recruitment and amplification of an inflammatory response in myocardium during CVB-3 infection is T cell dependent.

CVB-3 virus titers in myocardium consistently peak prior to maximal development of inflammatory mononuclear infiltrates and muscle necrosis. Resistance to CVB-3 viral infection does not appear to be strictly dependent on T-cell function since this study and another (1) show that T-LY-depleted BALB/c mice can terminate viral growth in myocardium by 7 to 8 days. CVB-3 titers in myocardium of nude mice were equal to or less than those found in T-cell-competent BALB/c mice at a comparable point in time (8). This also suggests that resistance to Coxsackie virus can occur independent of T-cell function.

Early studies showed that antibody synthesis to CVB-3, at least early during infection, was not impaired in the T-LY-depleted mouse (1), and it was assumed this was the effector of resistance. Subsequent studies, however, showed no correlation between the presence of neutralizing CVB-3 antibody and resistance (9). When mononuclear cells were pharmacologically excluded from host defenses, infected mice had significantly higher virus titers in heart, marked necrosis, and significant reduction of cell infiltrates. The monocyte was thought to be the corticosteroid-inhibited cell (9).

Nude mice lack mature T cells but have normal to increased numbers of natural killer (NK) cells (10). These cells are thought to be of paramount importance for tumor and viral resistance. Hydrocortisone depresses their function indirectly by inducing a non-T-LY phagocytic cell sup-

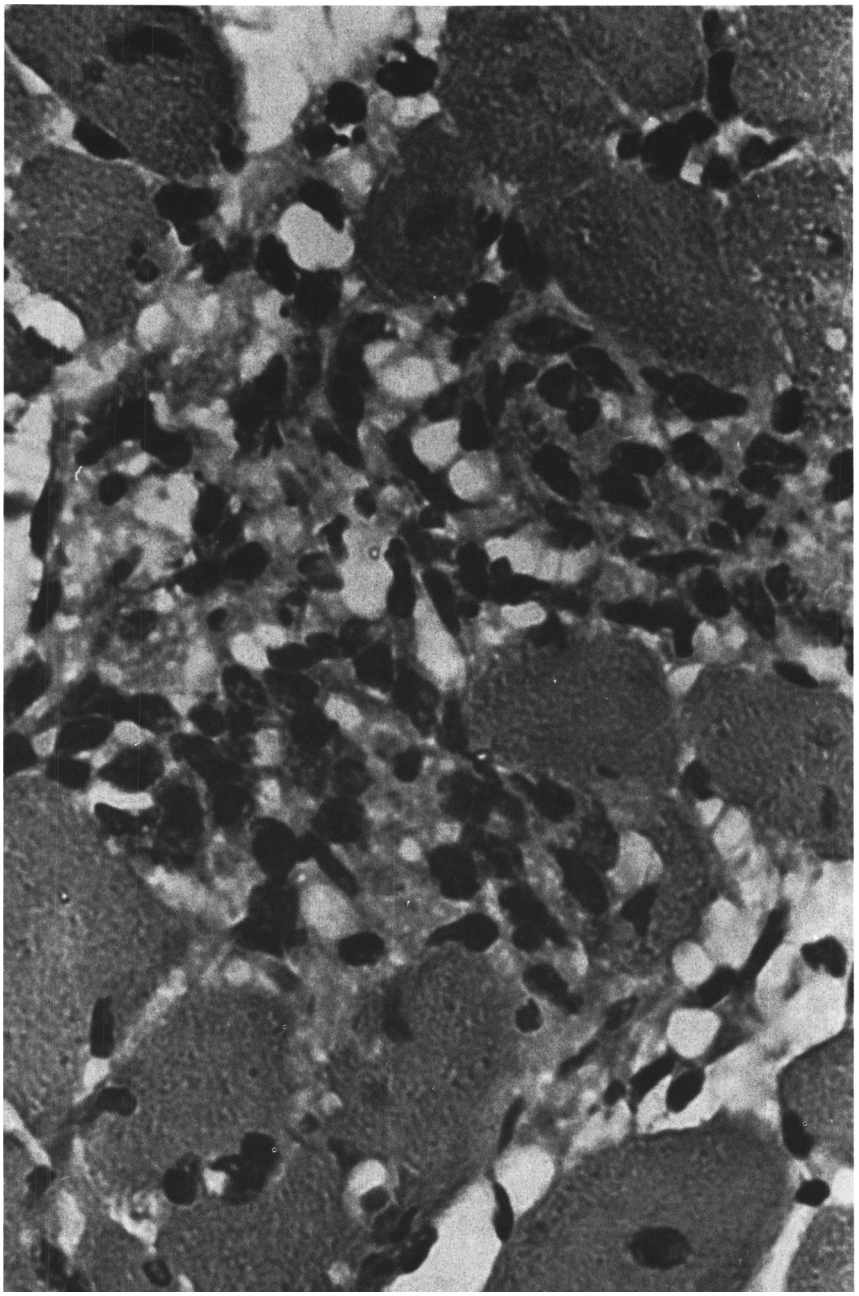

FIG. 6. Infiltrating mononuclear cells in a *nu/nu*/BALB/c mouse 31 days after CVB-3 infection are characterized by nuclei with prominent membranes, increased nucleoli, and reticular appearance and abundant cytoplasm (H and E, 500×).

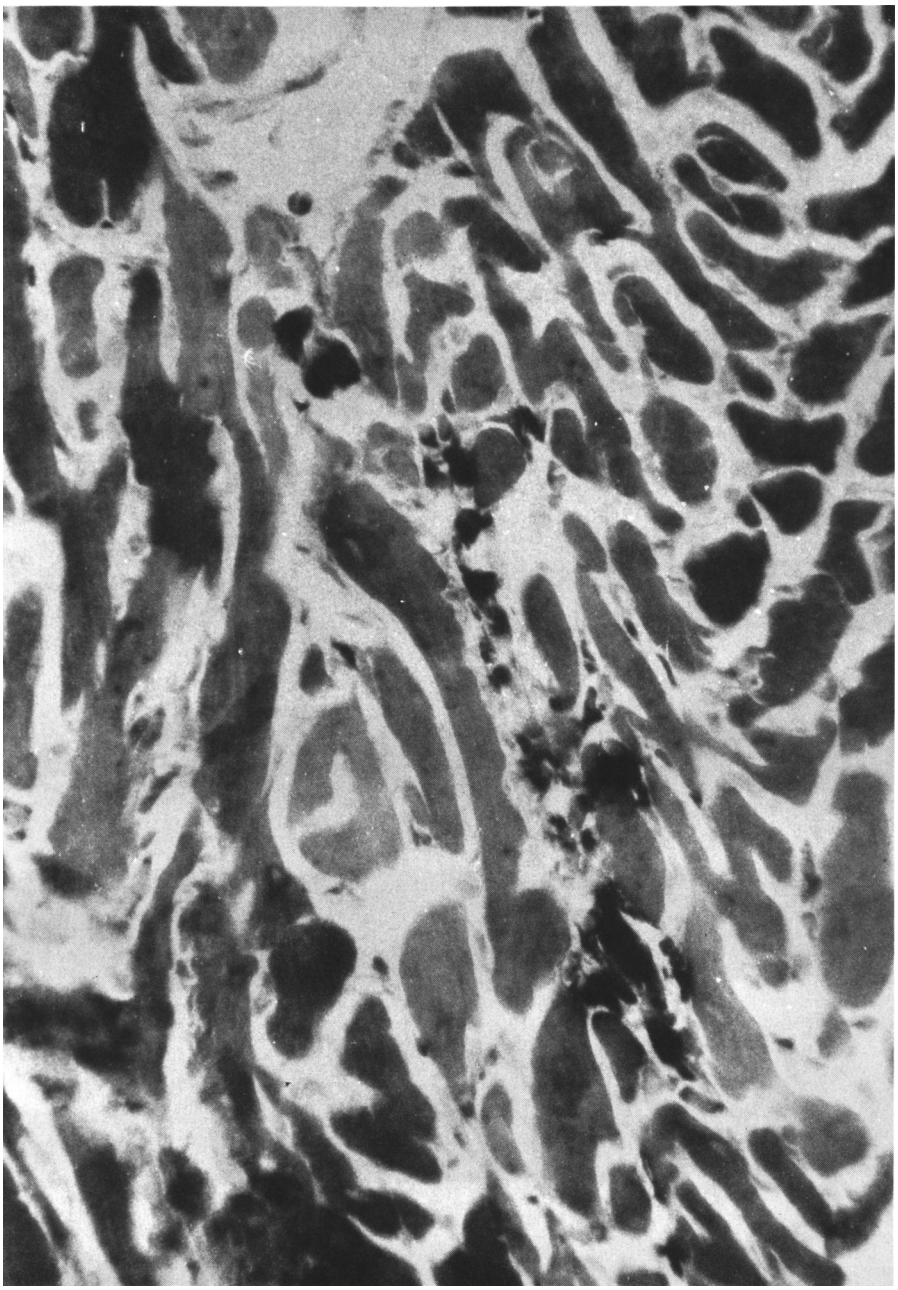

FIG. 7. Inflammatory focus in the myocardium of a nu/nu/BALB/c mouse with chronic myocarditis. Both focal and diffuse esterase staining is present in the mononuclear cells (ANAE, 500×).

pressor of NK activity (11). Thus, steroid treatment in the mouse myocarditis model may actually be inhibiting NK antiviral activity via the monocyte.

An interesting sidelight to this study was the detection of ANAE-positive cells in athymic mice. ANAE staining of splenic cell suspensions from athymic mice reveal 14% of the cells have focal esterase stains characteristic of T-LY in normal mice. Mueller also reported a minority splenic cell population from athymic mice that had similar esterase staining characteristics (3). Analysis of athymic mouse peripheral lymphocytes revealed a distribution of focal ANAE staining cells similar to that seen in BALB/c and human peripheral blood. It is presumed these focal ANAE-positive cells are precursor T-LY since thymic-influenced T cells could not be present to provide this type of staining pattern.

CVB-3 infection in nu/nu/BALB/c mice is characterized by infiltration of the heart with α-napthyl esterase (ANAE)-positive cells, a minority of which showed typical dense monocyte staining characteristics. Nude mice developed cellular infiltrates comprised of typical monocytes and ANAE-positive cells with similar focal staining patterns within the same approximate time sequence as normal BALB/c mice. Presumably, in the nude mouse, these cells must be immature T-LY precursors, NK cells, or another uncharacterized mononuclear cell. BALB/c mice had mononuclear infiltrates, but their cellularity was more typical, with denser nuclei and less cytoplasm present than the athymic infiltrates. Whether the focal staining ANAE-positive cells in BALB/c mice are mature T-LY, NK cells, or T-LY precursors is unclear. It is not apparent why our results differ from the previous report that showed no cellular infiltration during acute CVB-3 disease in athymic mice (8) and an anecdotal comment that athymic mice infected with CVB-3 "did not exhibit the same degree of pathology" (12). These mice also had a BALB/c background, but the infecting strain of CVB-3 may have differed in virulence. Other possibilities for the discrepancies are differences in infecting dosage or analysis of the histopathology at different points in time.

Chronic myocarditis resulted after infection of 5- to 10-week-old nude mice. The histology of the chronic disease was that of extensive infiltration with mononuclear cells that were either densely or focally ANAE positive. Whether NK or other cell populations are reacting to novel myocardial antigens and amplifying the inflammatory response by recruiting monocytes is unknown, but these results strongly suggest that lymphocytes other than mature T-LY can successfully participate in the maintenance of chronic inflammation during viral infection in the athymic mouse and may also be involved in the production of chronic CVB-3 myocarditis in immunologically normal mice.

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