Coxsackievirus B 1-induced Polymyositis

Lack of Disease Expression in nu/nu Mice

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Abstract

Chronic inflammatory myositis similar to human polymyositis occurs in mice after infection with a strain of Coxsackievirus B 1 (CVB 1). To investigate the role of T cells in the pathogenesis of this disorder, we compared disease expression in T cell–deficient athymic nude (nu/nu) mice and heterozygotes (nu/+) with normal T cell function. Acute infectious myositis occurred in nu/nu and nu/+ mice. Chronic (> 21 d postinfection) weakness and myositis, however, developed only in nu/+. Resistance to disease in nu/nu mice was not explained by insusceptibility to infection; the amount of virus lethal for 50% of mice and virus replication were comparable in both groups. Additionally, anti–CVB 1 antibody production was similar in both groups. Reconstitution of infected nu/nu mice with spleen cells from normal mice resulted in disease. These results demonstrate that chronic weakness after infection with this virus is not simply a sequela of acute myonecrosis and suggest that T cells play a pivotal role in the pathogenesis of chronic myositis.

Introduction

Viruses have often been implicated in the pathogenesis of autoimmune disorders in man, but proof of their role in etiology has been obtained in only a few such diseases (1). In human polymyositis (PM)[1] and dermatomyositis (DM), strong evidence implicates immunological mechanisms in disease pathogenesis (2, 3). Suggestive evidence for humoral involvement includes the presence of circulating autoantibodies thought to be unique to PM (4–6), the deposition of immunoglobulin in the muscle of patients with PM (7–9) and perivascular regions of patients with juvenile DM (7), and findings of the membrane attack complex of complement on muscle fibers from patients with PM (10, 11). Stronger evidence supports a role for cellular immune mechanisms in the pathogenesis of PM, especially adult PM. Studies of in vitro cytotoxicity of mononuclear cells from patients with PM/DM for muscle have been conflicting (12–17), but such cells do show increased reactivity to skeletal muscle as assessed by proliferation assays (12, 18) and lymphokine production (13, 19). Additionally, characterization of cells in inflammatory infiltrates of muscle show T cells to be active participants in muscle fiber damage (20–23). Despite this evidence, however, the factor that initiates these mechanisms is poorly understood. Several infectious agents have been proposed in the etiology of PM/DM, among which the group B Coxsackieviruses (CVB) are frequently cited (2, 3, 24, 25).

In 1979, Ray et al. reported studies of a particular strain of type 1 CVB (CVB 1) able to induce chronic weakness in mice involving selectively the proximal muscles of the hindlimbs (26). Other clinical features of the model include histologically chronic inflammatory myositis and myopathic electromyogram changes (27). Thus this CVB 1–induced murine illness bears striking clinical, histological, and electrophysiological similarities to human PM/DM (28, 29). Virus strain specificity has been demonstrated, as not all CVB 1 strains are equally effective in inducing disease (26, 27) and host genetic factors are important in determining disease susceptibility (26). In addition, infection with live virus is required; injection of inactivated virus does not cause myositis (30).

To investigate the role of cellular immunity in the pathogenesis of this chronic virus-induced myositis, we examined disease expression in T cell deficient, athymic nude mice and nude mice reconstituted with normal spleen cells. The results show that T cells in fact play an important role in the production of disease, as T cell–deficient animals do not develop chronic myositis.

Methods

Virus pools. The origins of the virus strain used and methods for production of virus pools have been described (30). Buffalo green monkey kidney cells (BGM) obtained initially from Dr. R. Crowell of Hahnemann University, Philadelphia, PA, were used for growth of CVB pools and for plaque assays. BGM were maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% newborn calf serum (NCS). The CVB 1 strain used is a plaque-purified myotropic strain shown previously to cause selective chronic proximal myositis in CD-1 Swiss mice (27, 30). Virus pools were grown in BGM in DMEM–10% NCS. When 4+ cytopathic effect induced by the virus was visible, monolayers were scraped, frozen and thawed once, and centrifuged to pellet cellular debris. Aliquots of virus were stored at −70°C until use. Control preparations consisted of homogenates of uninfected BGM monolayers.

Mice. Specific pathogen-free random-bred Swiss CD-1 mice were purchased from Charles River Breeding Laboratories, Inc., Wilmington, MA. Normal CD-1 mice as well as CD-1 mice bearing the nude (nu) gene were obtained. Heterozygous (nu/+) females were bred with homozygous nude (nu/nu) males so that each litter provided approximately equal numbers of nude and heterozygous mice for study. Within 48 h

1. *Abbreviations used in this paper:* BGM, buffalo green monkey kidney cell; CVB, Coxsackievirus; DM, dermatomyositis; DMEM, Dulbecco's modification of Eagle's medium; LD_{50}, amount of virus lethal for 50% of mice; NCS, newborn calf serum; OD, optical density; PBS-Tween, phosphate-buffered saline–0.05% Tween 20; pfu, plaque-forming unit; PM, polymyositis.

of birth, entire litters were injected intraperitoneally with 100 plaque-forming units (pfu) of CVB 1 or control preparations in 0.1 ml phosphate buffered saline (PBS). These studies were performed in adherence with the guidelines established in the *Guide for the Care and Use of Laboratory Animals*, published by the United States Department of Health and Human Services.

LD$_{50}$ calculation. To calculate the amount of virus lethal for 50% of mice (LD$_{50}$), two litters of pups (seven to nine pups per litter) were infected with each log-fold dilution of CVB 1 ranging from 2×10^1 to 2×10^4 pfu/animal. Most deaths occurred at 3–8 d after infection, at a time when nu/nu mice could be differentiated from nu/+. For normal CD-1 mice, one litter (8–12 pups per litter) was infected with each dose. LD$_{50}$s were calculated using the method of Reed and Muench (31).

Clinical and histological examination. Mice were examined daily for evidence of clinical weakness, manifest by abnormal posture and gait, with dragging of the hindlimbs. For histologic examination, intact hindlimbs were dissected and placed in buffered formalin for fixation.

Virus titers in muscle. Plaque assays were used to titer virus in proximal muscle from hindquarters (30). Animals were sacrificed by inhalation of methoxyflurane, and proximal hindquarter muscle was removed and weighed aseptically. A 10% homogenate (wt/vol) in PBS was made from each sample, passed through a 0.22-μm filter, and stored at $-70°C$ until titration. Specimens were diluted in PBS and allowed to adhere to confluent monolayers of BGM cells for 1 h at 37°C in a humidified 5% CO$_2$-containing atmosphere. The inoculum was then aspirated and the monolayers were overlaid with agar. After 2 d of incubation, each well was overlaid with neutral red-containing agar and after overnight incubation plaques were counted. Virus titers are expressed in plaque-forming units per gram muscle.

Enzyme-linked immunosorbent assays (ELISA). A standard ELISA technique for assaying antiviral antibody (32) was employed. CVB 1 purified by cesium chloride density gradient centrifugation (33) was used as antigen. Microtiter plates were sensitized with 1×10^6 pfu (1:200 dilution) purified virus in 0.1 ml 0.05 M carbonate-bicarbonate buffer (32) overnight at 4°C. Sera were diluted 1:20 in PBS–0.05% Tween 20 (PBS-Tween), and triplicate 0.1-ml samples were added to microtiter wells and incubated for 4 h at room temperature. After washing three times with PBS-Tween, 0.1 ml of a 1:500 dilution of peroxidase-conjugated goat anti–mouse immunoglobulin serum (Cappel Laboratories, Cochranville, PA) was added for 4 h at room temperature. After three further washes, 0.1 ml of substrate (12.8 mg o-phenylenediamine 2 HCl in 5 ml citrate phosphate buffer containing 0.02% H$_2$O$_2$; Abbott Laboratories, Irving, TX) was added for 45 min. Optical density (OD) was read at 492 nm using a Titertek multichannel spectrophotometer (Flow Laboratories, Inc., McLean, VA). Known negative and positive sera were included with each run as controls.

Transfer of spleen cells. Spleen cells from infected and uninfected 28-d-old normal CD-1 mice were obtained by passage through a wire mesh. After hypotonic lysis and washing three times with PBS, cells were suspended at 10^8/ml in PBS. Aliquots of spleen cell preparations from previously infected mice were screened for infectious virus by plaque assay. No infectious virus was recovered. Litters of pups were infected with 100 pfu at 1 d of age and injected with 10^7 spleen cells in 0.1 ml PBS i.p. 2 d later.

Results

Clinical weakness. After infection with 100 pfu CVB 1, chronic weakness could be produced only in nu/+ mice. Heterozygous (nu/+) mice were easily identified by 3 d of age when hair growth became apparent. By careful observation of posture and gait, we could detect mild acute muscle weakness in both nu/nu and nu/+ mice within 7 d of infection. As shown in Fig. 1, hindquarter weakness was seen in 8 of 42 (19%) nu/nu and 37 of 43 (86%) nu/+ mice at day 7. Thereafter, clinical weakness decreased with time so that by 21 d postinfection, no nu/nu mice
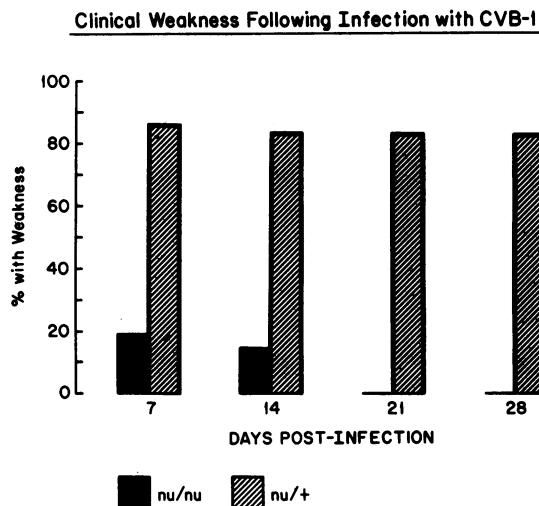
Figure 1. Clinical weakness in CD-1 mice after infection with 100 pfu CVB 1. Weakness was detected by observation of posture and gait. Incidence of weakness in nu/nu and nu/+ mice, respectively, was 8 of 42 and 37 of 43 on day 7, 2 of 14 and 15 of 18 on day 14, 0 of 5 and 5 of 6 on day 21, and 0 of 25 and 18 of 23 on day 28.

were weak. In contrast, the weakness in nu/+ mice persisted. On day 28, 0 of 31 (0%) nu/nu were weak compared with 22 of 27 (81%) nu/+ ($P < 0.001$, chi-square analysis). Animals observed for as long as 7 wk showed continued weakness in nu/+ (6 of 7) and no weakness in nu/nu mice (0 of 5).

Susceptibility to infection. To determine whether nu/nu mice did not develop chronic myositis after infection because they were resistant to infection with the virus, we compared the LD$_{50}$s for nu/nu and nu/+ mice. Nude mice could be infected with CVB 1. As shown in Table I, in normal CD-1 mice (+/+ for the nude gene), the LD$_{50}$ (log$_{10}$ pfu) was 2.7. LD$_{50}$s for nu/+ and nu/nu mice were 2.9 and 2.4, respectively. These data are not significantly different, indicating that the difference in clinical disease between nu/nu and nu/+ was not due to altered susceptibility to viral infection. In addition, increased mortality among nu/nu cannot account for the decreased incidence of mice with chronic myositis.

CVB 1 replication in muscle. To examine whether muscle weakness correlated with ability of virus to replicate in muscle, we examined viral titers in proximal hindquarter muscle. Virus replicated to comparable titers in muscle of both nu/nu and nu/+ mice. As shown in Fig. 2, virus titration curves in nu/nu and nu/+ mice were superimposable, with almost identical values at all time points. Viral titers peaked at day 7 in both groups when titers (log$_{10}$ pfu/g muscle) were 7.97±0.34 (mean±SEM, $n = 10$) for nu/nu and 7.91±0.43 ($n = 10$) for nu/+ ($P = 0.68$, t test). Virus clearance was identical in nu/nu and nu/+ mice; in both groups virus was absent by day 11 and could not be detected when assayed out to 28 d.

Histological examination. In both nu/nu and nu/+ mice 7 d after infection, when virus titers peaked in muscle, acute muscle necrosis was seen in both groups. Fig. 3 shows sections of proximal hindquarter muscle obtained 7 d postinfection from a nu/nu mouse (Fig. 3 *A*) and a nu/+ littermate (Fig. 3 *B*). In both sections extensive myonecrosis is evident, with marked interstitial edema and intense inflammatory cell reaction composed primarily of polymorphonuclear cells.

Table I. Susceptibility to CVB 1 Infection

Mice	LD$_{50}$
	log$_{10}$pfu
+/+	2.7
nu/+	2.9
nu/nu	2.4

Litters of neonatal mice infected with 2×10^2 to 2×10^5 pfu CVB 1 i.p. and observed for mortality for 28 d. LD$_{50}$s calculated by the method of Reed and Muench (31). Normal CD-1 mice (+/+ for nu gene) and heterozygous (nu/+) or homozygous (nu/nu) CD-1 mice were used.

By 14 d after infection, when virus could no longer be detected in muscle of either group, histologic specimens as shown in Fig. 4 demonstrated the regenerative ability of these neonatal animals. The section from a nu/nu mouse (Fig. 4 *A*) demonstrates regenerating myocytes with basophilia and increased cellularity but no inflammatory cells. In contrast, the section from a nu/+ animal (Fig. 4 *B*) similarly shows that muscle has regenerated, but ongoing myocyte degeneration is evident and inflammatory cell infiltration continues.

By 28 d postinfection, sections from the T cell–deficient nu/nu mice showed primarily normal muscle with only rare foci of necrotic muscle fibers, as shown in Fig. 5 *A*. In contrast, sections from immunologically normal nu/+ mice (Fig. 5 *B*) with chronic weakness demonstrated ongoing inflammatory myositis, with continued degeneration and regeneration of muscle fibers, variation of fiber size, and foci of intense inflammatory cell infiltration characteristically adjacent to a blood vessel.

Antibody response to CVB 1. In spite of impaired T cell function in nu/nu mice, humoral function was maintained as determined by ability to make anti–viral antibody detected by ELISA assay (Table II). Control sera from normal CD-1 mice

CVB-I Titers in Hindquarter Muscle

Figure 2. CVB 1 replication in muscle. Neonatal nu/nu and nu/+ mice infected with 100 pfu CVB 1. Virus titers (mean±SEM) in proximal hindquarter muscle. Seven nu/nu and seven nu/+ examined at day 4, 10 of each on day 7, and five each on other days.

(+/+), not infected with virus but injected with virus-free tissue culture homogenate, had a mean optical density of 0.103±0.005 (mean±SEM). Sera from +/+ mice infected with virus yielded a mean OD of 0.602±0.095. 28 d after infection with virus, all nu/+ and nu/nu mice had anti–viral antibody by ELISA. Mean ODs were 0.653±0.032 for nu/+ and 0.556±0.028 for nu/nu. These results are not significantly different by analysis of variance.

Reconstitution of immune responsiveness by transfer of spleen cells. The next set of experiments was designed to determine whether the lack of functional immune cells was responsible for the lack of disease in nu/nu mice. To accomplish this, we reconstituted nu/nu pups with spleen cells from +/+ mice to provide the pups with a full functional complement of lymphocytes. Transfer of spleen cells into infected nu/+ mice did not alter the incidence of chronic myositis normally induced in these animals (3 of 4). In nu/nu mice, which do not develop myositis after infection, disease was present in 6 of 7 (86%) after reconstitution (Table III). No difference was observed if the cell donor was previously infected or uninfected. Spleen cell preparations from previously infected mice were examined for infectious virus by plaque assay. No virus could be recovered from these preparations.

Discussion

The data presented show that this strain of CVB 1 can induce chronic myositis, manifest by clinical weakness and muscle inflammation, in T cell replete nu/+ CD-1 mice but not in nu/nu CD-1 mice with deficient T cell function.

Mutant nude mice lack thymuses and functional T cells, whereas other immunologic functions, including B cell and natural killer cell activities, remain intact (34). Nude mice have been a useful experimental animal for examination of T cell deficiency in immunologic and oncologic research but the age of animals is an important variable in the interpretation of results. Cells bearing T cell antigens can be found in nu/nu mice (35, 36). The origin of these cells has been debated but T cell progenitors are present and can be induced to differentiate into Thy-1$^+$ cells by in vivo administration of thymopoietin (37). More recent investigation (38) has confirmed the presence of T cells in nu/nu mice but only at 20–50% of the numbers in nu/+ mice. Additionally, the predominant cell type is an immature TL$^+$, Lyt-1,2,3$^+$ cell that does not diversify into Lyt-1 and Lyt-2,3 subsets until after 10 wk of age. Although nu/nu mice are generally accepted as lacking normal T cell function, classically demonstrated by inability to reject foreign skin grafts (39–41) and tumors (42, 43), recent studies have demonstrated that older nu/nu do in fact develop some T cell function (44). T cell–enriched spleen cell preparations from 5–7-mo-old nu/nu mice respond to mitogen stimulation with proliferation and interleukin 2 production and develop cytotoxic reactivity to alloantigen stimulation, although responses are milder than those with cells from nu/+ mice. None of these T cell functions are evident in young (< 8 wk old) nu/nu mice. Thus, although T cells and T cell function can be found in older nu/nu mice, the young nu/nu mice used in our studies can be considered T cell deficient.

The availability of CD-1 mice bearing the nude gene permitted examination of the role of T cells in the pathogenesis of CVB 1–induced myositis. CD-1 mice had been shown previously to be susceptible to infection and the development of myositis after infection with this virus strain (27, 30). The incidence of
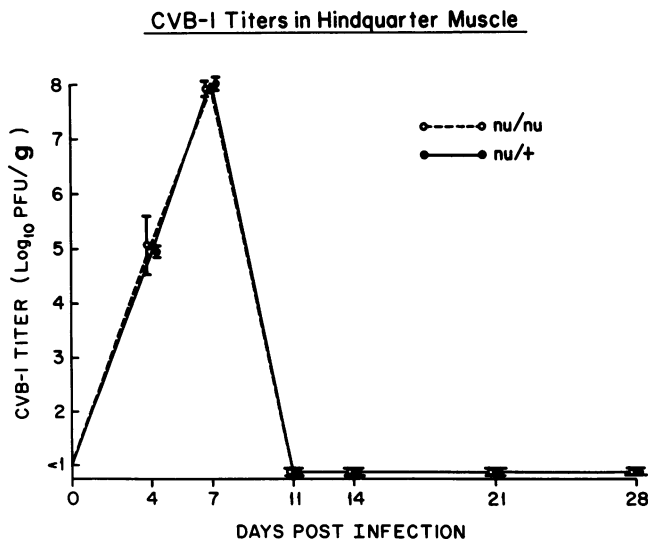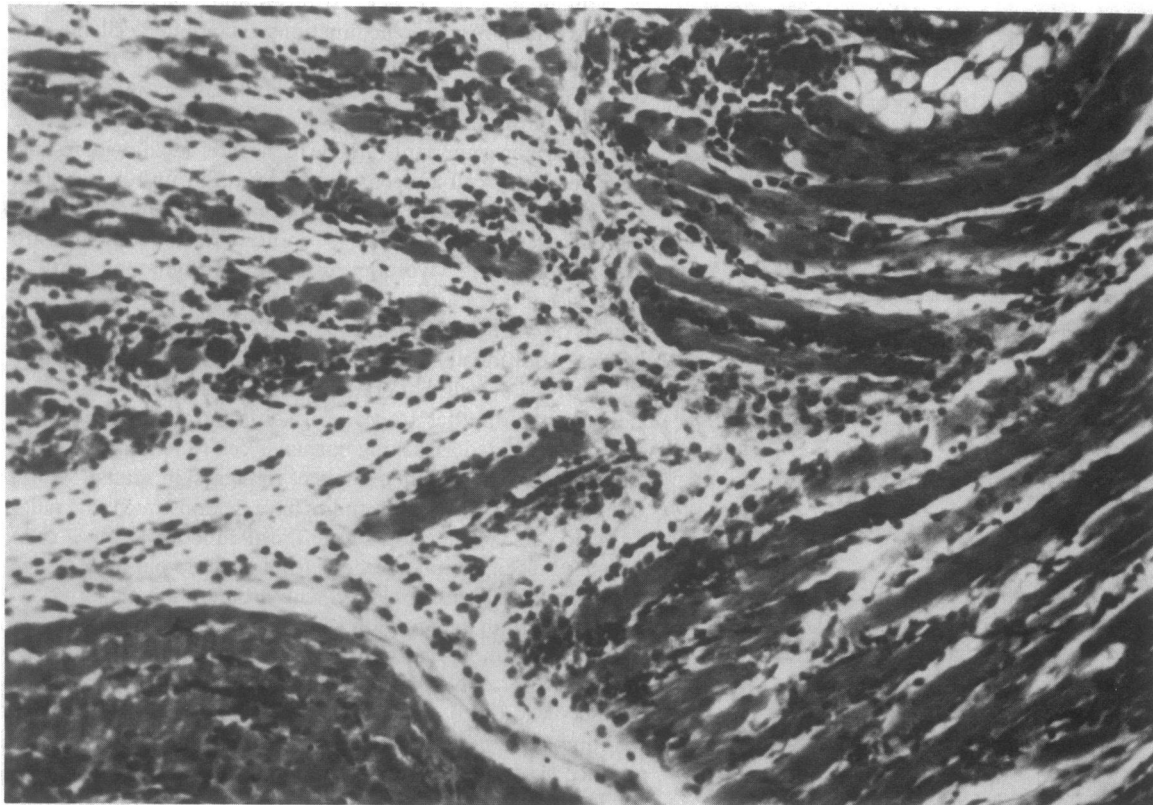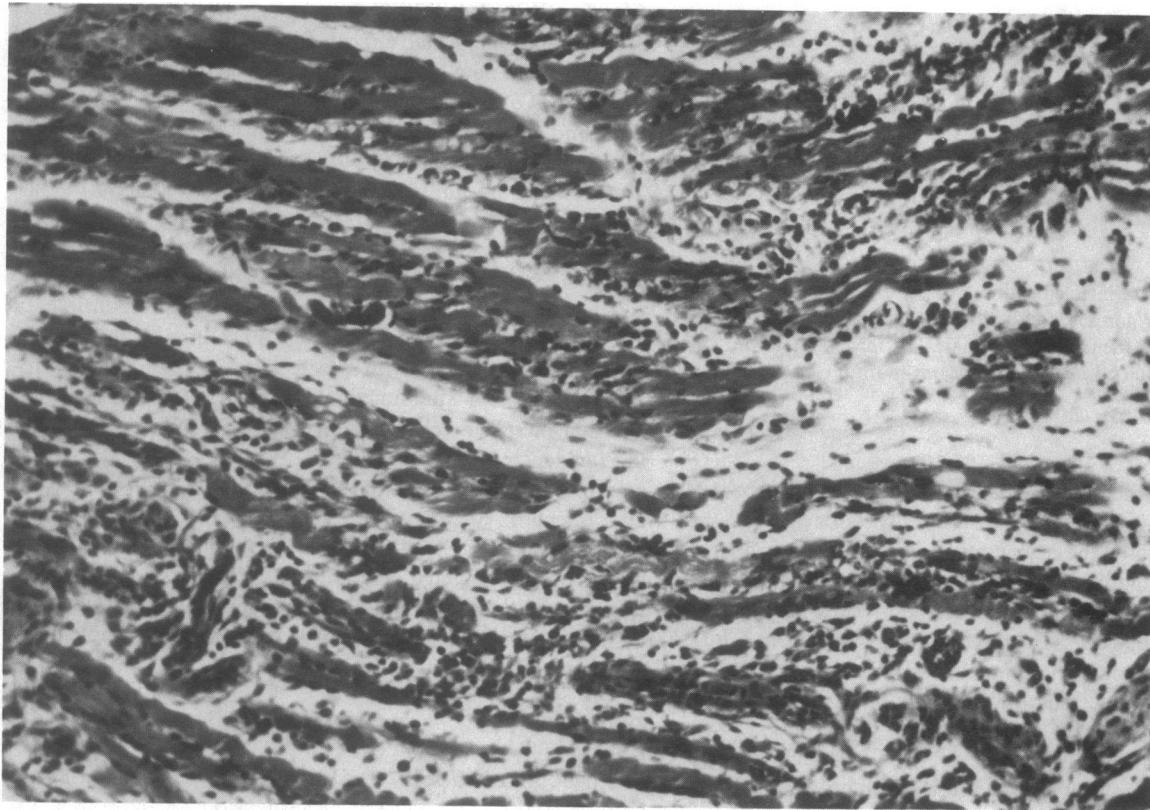
Figure 3. Proximal hindquarter muscle 7 d postinfection. (*Top*) nu/nu. (*Bottom*) nu/+. Both sections show damage caused by acute virus-induced myonecrosis. Hematoxylin and eosin, original magnification, 300.
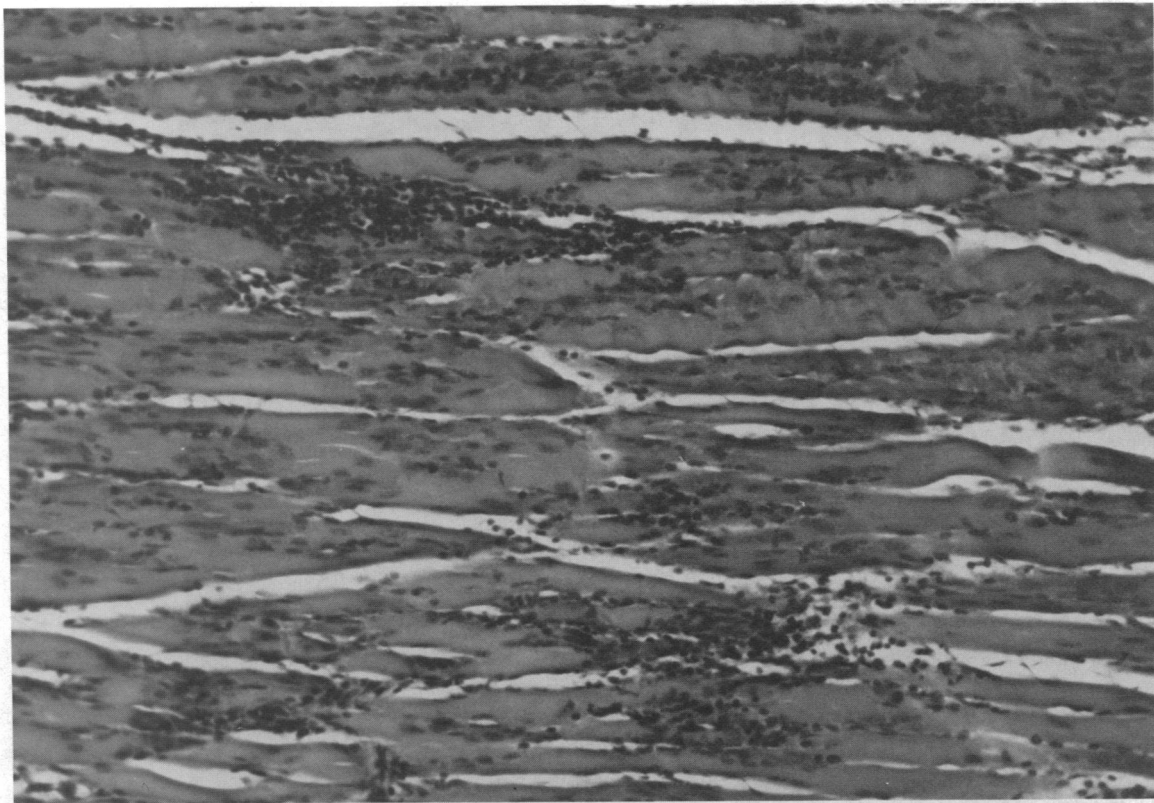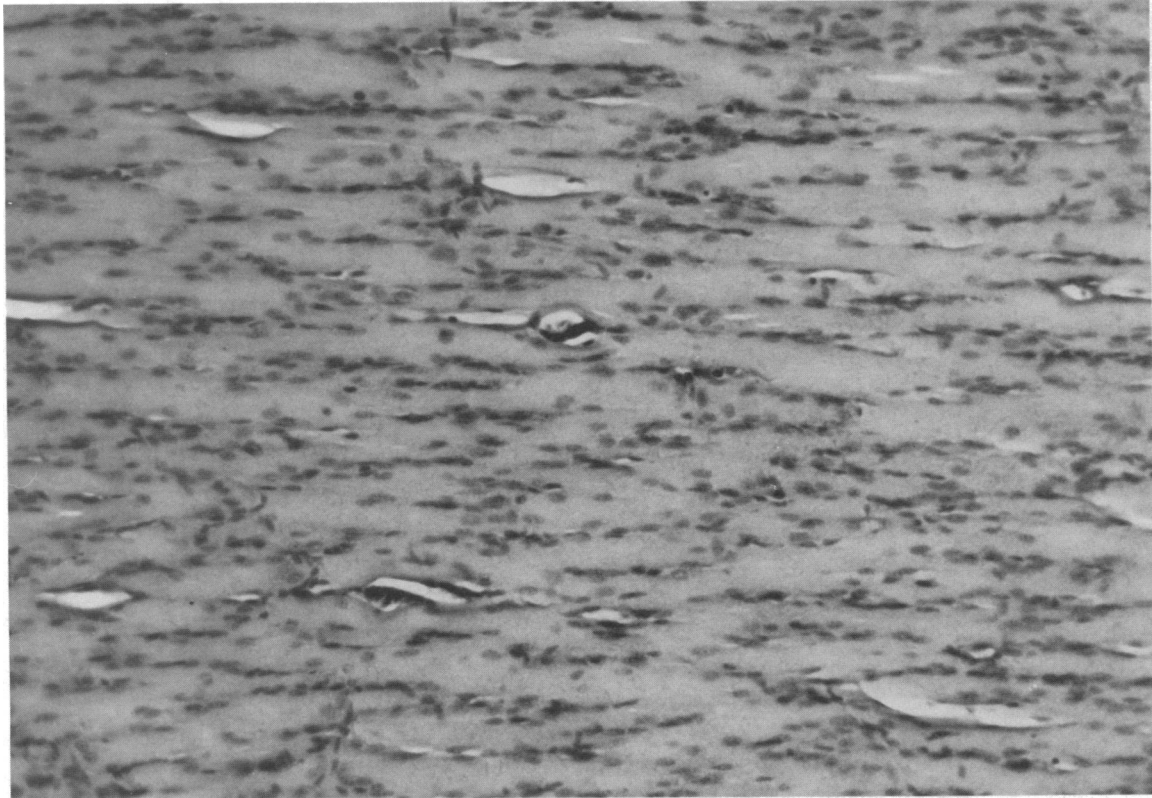
Figure 4. Proximal hindquarter muscle 14 d postinfection. (*Top*) nu/nu, showing basophilia and hypercellularity from regenerating myocytes. (*Bottom*) nu/+, demonstrating regeneration of muscle compared with Fig. 3 *bottom,* but inflammatory cells persist. Hematoxylin and eosin, original magnification, 300.
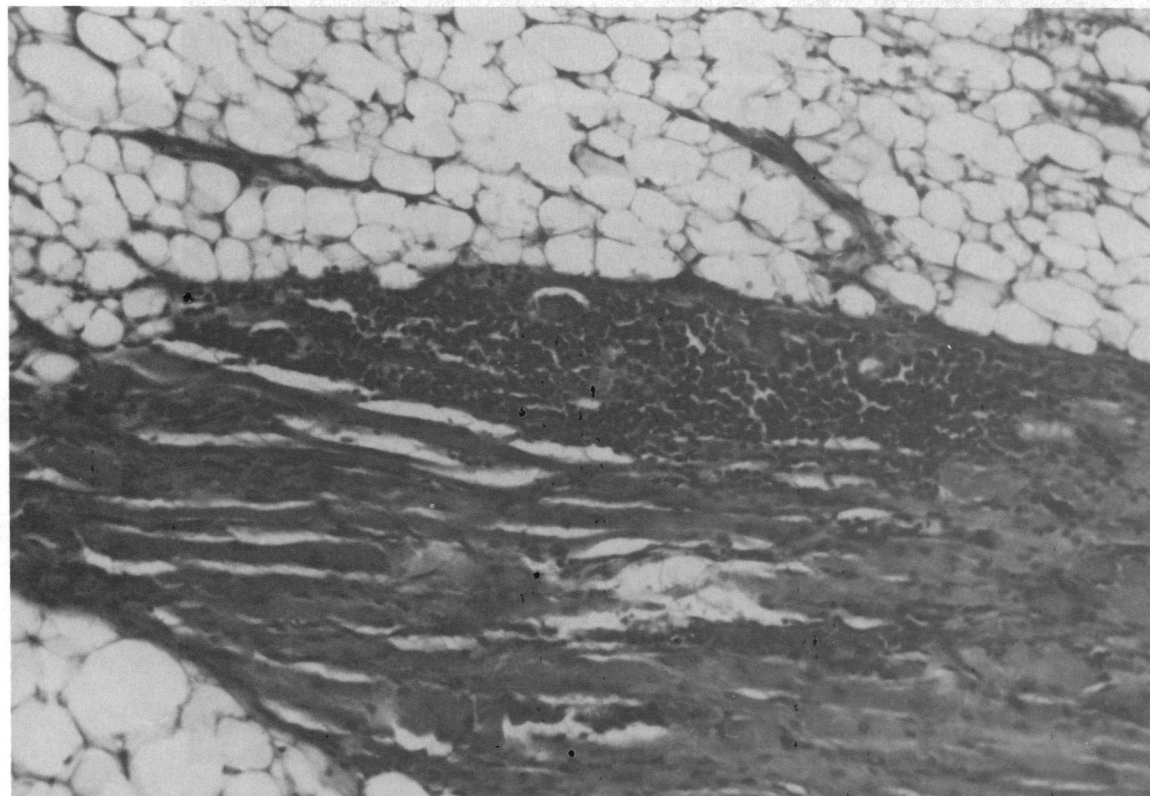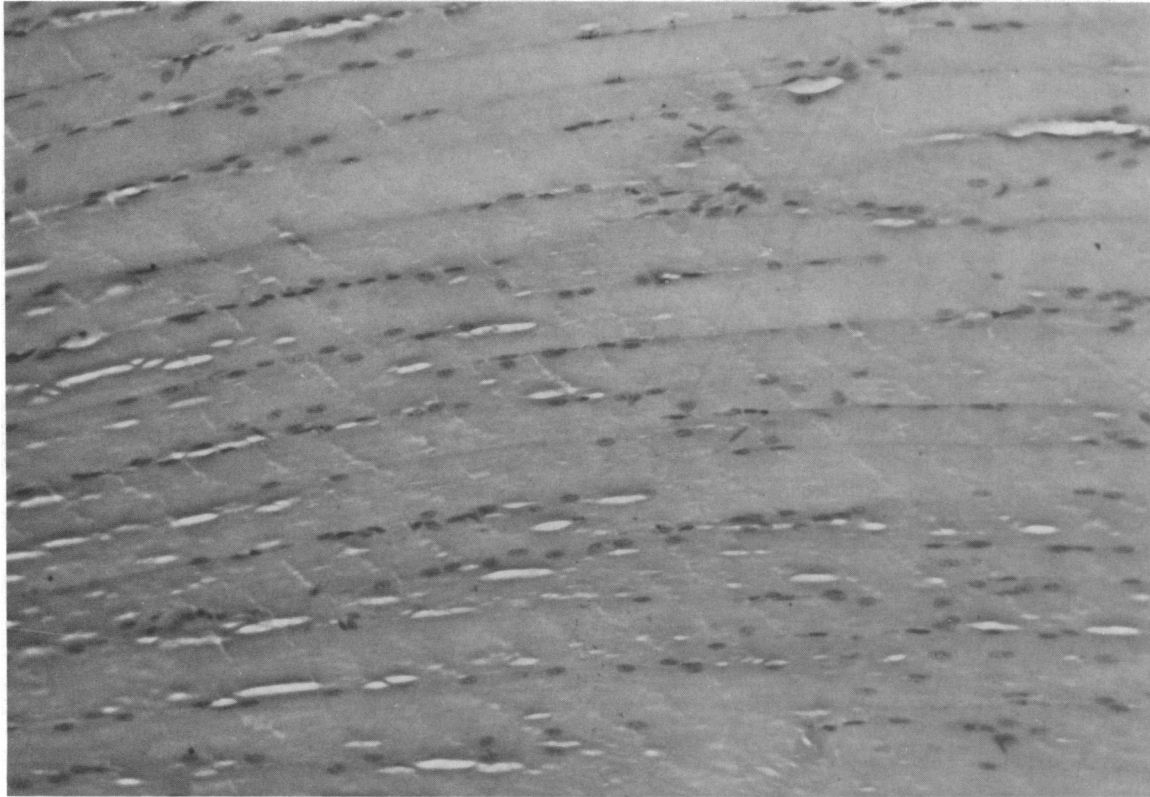
Figure 5. Proximal hindquarter muscle 28 d postinfection. (*Top*) nu/nu, essentially normal muscle. (*Bottom*) nu/+, degeneration and regeneration of muscle fibers, variation of fiber size, and intense foci of inflammatory cells. Hematoxylin and eosin, original magnification, 300.

Table II. Anti–CVB 1 Antibody Production

Mice	Infection	No.	OD$_{492}$
			mean±SEM
+/+	No	8	0.103±0.005
+/+	Yes	10	0.602±0.095
nu/+	Yes	15	0.653±0.032
nu/nu	Yes	16	0.556±0.028

Antibody detected by ELISA assay. Serum obtained from mice 21–28 d postinfection was diluted 1:20 and added to plates coated with purified CVB 1. Bound antibody was detected by addition of peroxidase-conjugated goat anti–mouse immunoglobulin and substrate, with OD read at 492 nm. Titers are not significantly different by analysis of variance. Normal CD-1 (+/+ for nu gene) and heterozygous (nu/+) or homozygous (nu/nu) CD-1 mice were used.

clinical disease seen in nu/+ was comparable with that observed in normal CD-1 mice (> 80%) at the virus inoculum used (30). Although CD-1 mice are not inbred, they are maintained in a closed colony established in 1959. It is possible that other genes coding for disease expression may have been inherited with the nu gene, but the clear distinction between the incidence of clinical disease in nu/nu (0%) vs. nu/+ (83%) at 28 d postinfection indicates that if other susceptibility genes exist they are linked to the nu gene and require homozygosity for expression. To rule out the possibility that other genes coding for disease susceptibility or expression were inherited with the nude gene, we performed cell transfer experiments. Homozygous nu/nu mice reconstituted with immune cells from previously infected or uninfected normal (+/+) CD-1 mice developed disease. Reconstitution with whole spleen cell preparations transferred many different cell types. That the recognized immunologic defect in the nu/nu mice, lack of functional T cells, was overcome by reconstitution with immunocompetent cells provides evidence that T cells are important in disease pathogenesis.

The difference in disease expression between nu/nu and nu/+ was not due to altered susceptibility to viral infection. Both nu/nu and nu/+ mice could be infected by virus and had similar LD$_{50}$s. In addition, virus replicated to equivalent titers in muscle of nu/nu and nu/+ mice and caused acute muscle infection with myonecrosis in both.

The histological acute myonecrosis observed in nu/nu and nu/+ resolved in both groups of mice, but complete recovery

Table III. Disease Expression in nu/nu Mice Reconstituted with Spleen Cells

CVB 1 infection of recipients	Occurrence of myositis	
	Donor infected with myositis	Donor uninfected, without myositis
Yes	3/4	3/3
No	0/6	ND

Litters of neonatal mice were injected with 100 pfu CVB 1 or control prep 1 d after birth and 10^7 spleen cells from previously infected or uninfected normal 28-d-old CD-1 mice (+/+) 2 d later. Disease expression determined by clinical observation.

was apparent only in the T cell–deficient nu/nu mice. In contrast, although muscle regenerated in the nu/+ mice, foci of intense mononuclear cell infiltration often centered around blood vessels, and with continued active muscle degeneration and regeneration persisted for up to 7 wk after infection. The histologic character of this chronic myositis is strikingly similar to the chronic inflammatory myositis seen in human PM/DM (28, 29).

In human PM/DM several different autoantibodies have been demonstrated (4–6) but their relationship to clinical disease is not clear. Antibodies directed toward heart have been reported in mice with autoimmune myocarditis induced by CVB 4 (45, 46) but no data is currently available about anti–muscle antibodies in CVB 1–induced myositis. The finding of equivalent anti–CVB 1 antibody titers in nu/nu and nu/+ mice demonstrated intact B cell function in nu/nu mice and suggests that B cell immune response to virus does not play a pathogenic role in the development of chronic myositis in this model. Although these results suggest that the role of T cells in this disease is mediated through a classic cell-mediated immune mechanism, the possibility that T cells augment autoreactive B cell function and autoantibody production has not been disproved.

Based on these results, three important points about this model of inflammatory myositis can be concluded. First, normal T cell function is not required for mice to clear CVB 1 from muscle or other organs. Some other mechanism that remains intact in nu/nu mice, such as interferon production or natural killer cells, may be the mechanism important in virus clearance. Second, the chronic weakness induced in mice after infection with this strain of CVB 1 is not simply a sequela of the muscle damage caused by acute viral infection. Both nu/nu and nu/+ were equally susceptible to infection, and virus replicated to comparable titers in both groups. Some effect other than the acute muscle damage caused by the virus, presumably an immunologically mediated mechanism, leads to chronic weakness. Finally, because T cell–deficient young nu/nu CD-1 mice did not develop chronic myositis after infection, T cells appear to play a pivotal role in the pathogenesis of CVB 1–induced murine PM.

Acknowledgments

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