Myonuclear Apoptosis in Dystrophic mdx Muscle Occurs by Perforin-mediated Cytotoxicity

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Abstract

Myonuclear apoptosis is an early event in the pathology of dystrophin-deficient muscular dystrophy in the mdx mouse. However, events that initiate apoptosis in muscular dystrophy are unknown, and whether elimination of apoptosis can ameliorate subsequent muscle wasting remains a major question. We have tested the hypothesis that cytotoxic T-lymphocytes initiate myonuclear apoptosis in dystrophic muscle, and examined whether perforin-mediated cytotoxicity plays a role in the pathophysiology of muscular dystrophy. Mdx mice showed muscle invasion by cytotoxic T cells and helper T cells at the onset of histologically detectable muscle fiber pathology. At this time, perforin-expressing cells were also present at elevated concentration. Mdx mice depleted of $CD8^+$ cells showed a significant reduction of apoptotic myonuclei concentration and a reduction in necrosis, judged by macrophage invasion of muscle fibers. Double-mutant mice, deficient in dystrophin and perforin, showed nearly complete absence of myonuclear apoptosis, and a significant reduction in the concentration of macrophages in the connective tissue surrounding muscle fibers. However, muscle fiber invasion by macrophages was not reduced significantly in double mutant mice. Thus, cytotoxic T-lymphocytes contribute significantly to apoptosis and necrosis in mdx dystrophy, and perforin-mediated killing is primarily responsible for myonuclear apoptosis. (*J. Clin. Invest.* 1997. 99:2745–2751.)
Key words: Duchenne muscular dystrophy • cytotoxicity • lymphocytes • in vivo depletion • cellular immunity

Introduction

Duchenne muscular dystrophy (DMD)[1] involves severe muscle wasting and the premature death of afflicted individuals attributable to the absence of dystrophin, a cytoskeletal protein (1). Mdx mice are also dystrophin-deficient and experience muscle necrosis that resembles the DMD pathology. In mdx mice, histologically discernible muscle cell death does not appear at the onset of muscle use. Instead, muscle cell death is first apparent at $\sim$ 3–4 wk of age in mdx mice, and DMD humans display mild, histologically discernible muscle pathology at birth but remain asymptomatic until 3–4 yr of age (2, 3). Mdx mice differ from DMD pathology in that they experience functional recovery and display little muscle pathology after a brief episode of muscle cell death (4), while DMD pathology is progressive (5). Thus, mdx muscle provides a valuable model of the early stages of the pathology of dystrophin-deficient human muscle, although important differences exist between muscle regenerative mechanisms in mdx mice and DMD patients.

Although muscle cell death in mdx and DMD tissue involves muscle necrosis at the stages of the disease in which cell death is most prominent, the earliest stages of histologically detectable muscle cell death in mdx tissue show that apoptosis precedes necrosis (6). An increasing number of studies conducted in vivo and in vitro have shown that apoptotic and necrotic cell death are not always distinct forms of pathology. For example, cytotoxic T-lymphocytes (CTLs) can induce death of some target cells via apoptotic mechanisms and other target cells in the same culture via necrotic mechanisms (7). It is possible, therefore, that a common effector can induce both apoptotic and necrotic death of mdx muscle. Alternatively, initiation of apoptotic death could create conditions that give rise to tissue necrosis, although this morbid process has not been demonstrated to occur.

The ability of inflammatory cells to induce target cell death by either necrotic or apoptotic processes drew our interest to the question of whether inflammatory cells could be effectors of dystrophic muscle death. Inflammation is a characteristic common to both DMD and mdx dystrophies (8, 9), although the extent to which it contributes to the pathological process is unknown. Immunohistochemical observations show that macrophages and T-lymphocytes, especially $CD8^+$ CTLs, are major constituents of the inflammatory cell population of dystrophic muscle (9–11), and that many of the invading CTLs are activated (12). The likelihood that these CTLs contribute to the pathology of dystrophin-deficient muscle is supported by clinical observations that steroidal antiinflammatory drugs can ameliorate the pathology (13). One of these drugs, prednisone, provides significant benefit to DMD patients by improving muscle strength (13–15) and pulmonary function (16), and by decreasing the number of lymphocytes invading the muscle (17). The prednisone-mediated improvements were associated with a specific decrease in the number of $CD8^+$ CTLs, without an accompanying decrease in B cells, natural killer cells, $CD4^+$ cells, or macrophages (17).

Identification of specific T cell receptor gene rearrangements in DMD muscles (18) has provided important evidence supporting a role for CTLs in DMD. Muscles from all 12 patients analyzed in that investigation (18) contained Vβ2 family

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1. *Abbreviations used in this paper:* CTLs, cytotoxic T-lymphocytes; DMD, Duchenne muscular dystrophy; DMM, double mutant mice; RT-PCR, reverse transcription PCR; TUNEL, Tdt-mediated dUTP-biotin nick end labeling.

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transcript, and 5 of the patients showed a conserved four–amino acid peptide in a hypermutating area (CDR3) of the T cell receptor. Control patients with no disease or with other muscle diseases did not contain this peptide. The CDR3 area is believed to interact with antigen, and its conservation in T cell receptors from these DMD patients indicates an antigen-driven selection of one T cell population.

In this investigation, we have tested the hypothesis that CTLs contribute to apoptotic or necrotic cell death of mdx muscle by depleting CTLs from mdx mice before the onset of muscle pathology, and assaying for reductions in muscle fiber apoptosis, necrosis, or inflammation. We have also tested the specific mechanism through which CTLs can induce death in dystrophic muscle by generating double mutant mice that do not express dystrophin or perforin, which is a key cytotoxic molecule synthesized and released by CTLs (19, 20). Perforin, which is also known as cytolysin or pore-forming protein (21), is contained in lysosomal-like structures in CTLs, along with a group of serine proteases (granzymes) that are believed to play a role in CTL-induced lysis (22). Upon binding of CTLs with a target cell, perforin and granzymes are secreted onto the cell surface and the target is killed by apoptosis. Double mutant mouse muscle was analyzed for changes in the frequency of occurrence of muscle apoptosis, necrosis, and inflammation, to determine the importance of perforin-mediated processes in the pathology of dystrophin-deficient muscle. In addition, the effects of CTL depletion were compared to the effects of perforin deficiency to determine whether all CTL-induced pathologies were via perforin-mediated events.

Methods

Reagents. *Taq* polymerase was purchased from Promega Corp. (Madison, WI), DNAse, random primers, and restriction enzymes from Pharmacia (Alameda, CA); digoxigenin 11-dUTP and antidigoxigenin antibody from Boehringer Mannheim Biochemicals (Indianapolis, IN); MMLV reverse transcriptase from GIBCO BRL (Gaithersburg, MD); goat anti–rabbit and rat IgG conjugated to biotin and avidin-HRP from Vector Laboratories, Inc. (Burlingame, CA); DME and DEPC from Sigma Chemical Co. (St. Louis, MO); and all other reagents were obtained from Fisher Scientific Co. (Pittsburgh, PA).

Antibodies. Monoclonal antidystrophin was a kind gift of Dr. Louise Anderson (Newcastle General Hospital, Newcastle upon Tyne, United Kingdom). Antivinculin and antilaminin were purchased from Sigma Chemical Co. Anti-CDllb and anti-CD8 were ammonium sulfate–precipitated from hybridoma supernatant according to established procedures (23). All hybridomas were purchased from American Type Culture Collection (Rockville, MD). Antibodies for flow cytometry were obtained from PharMingen (San Diego, CA).

Flow cytometry. Splenocytes were isolated by dissociation of the spleen in PBS on a mesh screen, filtration through nylon mesh, and removal of red blood cells by hypotonic shock with Tris-ammonium chloride. One million cells per antibody stain were used. Cells were stained with either anti-CD8 or anti-CD4 with fluorescent markers FITC or PE. Anti-CD4 was used to demonstrate the specificity of the depletion by anti-CD8. The stained cells were suspended in a solution of PBS containing 0.5 μg/ml propidium iodide. Flow cytometry was performed on a FACScan® analytic flow cytometer (Becton Dickinson) attached to a Hewlett Packard computer for data analysis.

Immunoblots. Immunoblots were performed as described previously (24).

Immunohistochemistry. Tissue preparation and immunohistochemistry were performed as described previously (25, 26). The sections were air dried for 30 min and fixed in ice-cold acetone for 10 min followed by air drying. The slides were developed using 3-amino-9-ethyl carbazole (AEC, red) as substrate and allowed to react for 2 min. Sections were counterstained with hematoxylin to emphasize pathological features of the tissue.

In situ RT-PCR. In situ reverse transcription polymerase chain reaction (RT-PCR) protocol was adapted from a previously published procedure (27), that was modified as described previously (28).

Assay for apoptosis. Myonuclear apoptosis was detected using Tdt-mediated dUTP-biotin nick end labeling (TUNEL) labeling as described by Gavrieli et al. (29) and modified by our laboratory (6). Using this technique, apoptotic nuclei appear red. Proteinase K was omitted from the reaction so that immunohistochemistry could be performed on the same section. After completion of the procedure, the sections were stained immunohistochemically for the basement membrane protein laminin to ensure that positive apoptotic cells were indeed myonuclei located deep to the basement membrane. The secondary antibody used was an alkaline phosphatase–conjugated anti–rabbit IgG and the substrates were NBT and BCIP which produce a purple color. All TUNEL labeling experiments included as a positive control a section of young mouse thymus that was treated in parallel with experimental sections. All thymus sections in every experiment showed a high concentration of apoptotic nuclei. For negative controls, each labeling experiment also included sections treated identically to experimental sections, except TdT was eliminated from the reaction mixture. No false positives were observed in any negative control section. Furthermore, accuracy of detection of the TdT labeling was tested on C2C12 myoblasts and myotubes subjected to serum starvation to induce apoptosis, and then double labeled by TUNEL labeling and Hoechst staining to determine whether TUNEL-labeled nuclei also displayed morphological characteristics of apoptosis. These tests showed a one-to-one correspondence between TUNEL-labeled myonuclei and pycnotic and fragmented nuclei revealed by Hoechst staining.

Generation of perforin-deficient, dystrophin-deficient mice. Mdx mice were crossed with perforin knock-out mice (20) to generate double mutant mice (DMM). In the first cross, perforin-deficient (p−/p−) male mice and mdx female (d−/d−) mice were paired and produced females that are heterozygotes for both deficiencies (p+/p−, d+/d−) and males that are heterozygous for perforin deficiency and hemizygous for dystrophin deficiency (p+/p−, d−/y). The males from this F1 generation were backcrossed with mdx females from the original mdx colony. The p−/p+;d−d− female mice were identified by PCR analysis and were then backcrossed with F1 males to produce F3.

Mice from the F2 and F3 generations above were screened for the desired genotype by PCR analysis. Homozygous mutants for perforin were identified by isolating genomic DNA from tail tissue, and screening for the mutation by PCR. The primers used were a common downstream primer from intron 2 (CGT GAG AGG TCA GCA TCC TTC) and a common upstream primer from exon 2 (TGG CCT AGG GTT CAC ATC CAG). In addition, a primer to the neomycin cassette was included in the reaction. PCR conditions were 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM of each primer, 2.5 U *Taq*, and 1× PCR buffer (94°C denature, 64°C anneal, and 72°C extension). Using these primers, the wild type produces a band of 500 bp while the perforin knock-outs produce bands at 350 and 1,600 bp. All mice obtained in the F2 and F3 generations were dystrophin deficient.

Analysis of pathological features of mdx dystrophy. The concentration of pathological fibers in double mutant mice, CD8-depleted mdx mice, and mice injected with isotype control (IgG2B) for CD8 were blindly assayed in frozen sections of quadriceps muscle samples. Fibers displaying pathological, hyaline cytoplasm or invasion by macrophages were quantified according to the number per cross-sectional area at the mid-belly. The concentration of macrophages per unit volume of tissue was determined using anti-CD11b by indirect immunohistochemistry.

Statistical analysis. All quantitative data were analyzed using statistical analysis. Experiments were analyzed by Mann-Whitney analysis with alpha level set at 0.05.

Results

Quantitation of CD8$^+$ cells in mdx muscles. We first examined mdx muscle through the course of the disease process to determine whether they contained CTLs in their muscles, as has been shown previously for DMD patients. A significantly greater number of CD8$^+$ cells was observed at 4 and 8 wk of age relative to age-matched controls using quantitative immunohistochemistry (Fig. 1). The number of CD8$^+$ cells decreases precipitously by 14 wk of age, at which time the muscles are regenerating.

Quantitation of CD4$^+$ cells in mdx muscles. CTLs are dependent on IL-2 to achieve complete activation. This cytokine is supplied by helper T cells (CD4$^+$) in close proximity to CTLs. Helper T cells have also been implicated in known autoimmune diseases such as multiple sclerosis and rheumatoid arthritis (30, 31). We observed helper T cells in 2-wk mdx muscle and an increase in their number until the mice reached 8 wk of age. Thus, accessory cells necessary for CTL activation are also present in mdx muscle.

CD8 depletions. CD8$^+$ CTLs were depleted from mdx mice by intraperitoneal injections of anti-CD8 beginning at 10–12 d of age, to assess the importance of CTLs in mdx pathology. The mice show normal muscle histology at this age. Successful depletions were confirmed by flow cytometry of splenocytes from anti-CD8 injected mice and mice injected with isotype control (IgG2B) (see Fig. 3 *A*). Blind analysis of depleted and control muscles for the presence of apoptotic myonuclei showed a significant decrease in the number of apoptotic myonuclei in the muscle of CTL-depleted animals (Table I, Fig. 2). All but one of five CTL-depleted animals were completely devoid of detectable apoptosis. Thus, CTLs are important contributors to the presence of apoptosis in dystrophic muscle. Tissue necrosis was also significantly reduced in the CTL-depleted animals, as indicated by a 60% reduction of muscle fiber invasion by macrophages, although the total concentration of macrophages in the tissue was not significantly affected by CD8$^+$ cell depletion. This decrease in fiber invasion without a decrease in macrophage concentration in CD8$^+$ cell depleted animals indicates that CTLs may mediate macrophage invasion of dystrophic muscle fibers (Table I).

Identification of perforin-expressing cells and generation of perforin-deficient, dystrophin-deficient mice. We have found using in situ RT-PCR that perforin-expressing cells invade mdx muscle at the time that increased muscle apoptosis begins (Fig. 3 *B*). We tested whether mdx muscle apoptosis and necrosis may result from perforin-mediated processes by crossing perforin knock-out mice (20) with mdx mice to produce DMM (p−,p−/d−,d−). DMM were screened as described in Methods (Fig. 3 *C*). Successful production of the desired phenotype was confirmed by PCR (32) and Western analysis (24) (Fig. 3 *D*). Mice were also checked for dystrophin expression using immunoblots and the absence of dystrophin was confirmed in all mice (Fig. 3 *D*).

Pathological characteristics of DMM. Blind, quantitative, morphological analysis of muscles from DMM showed that

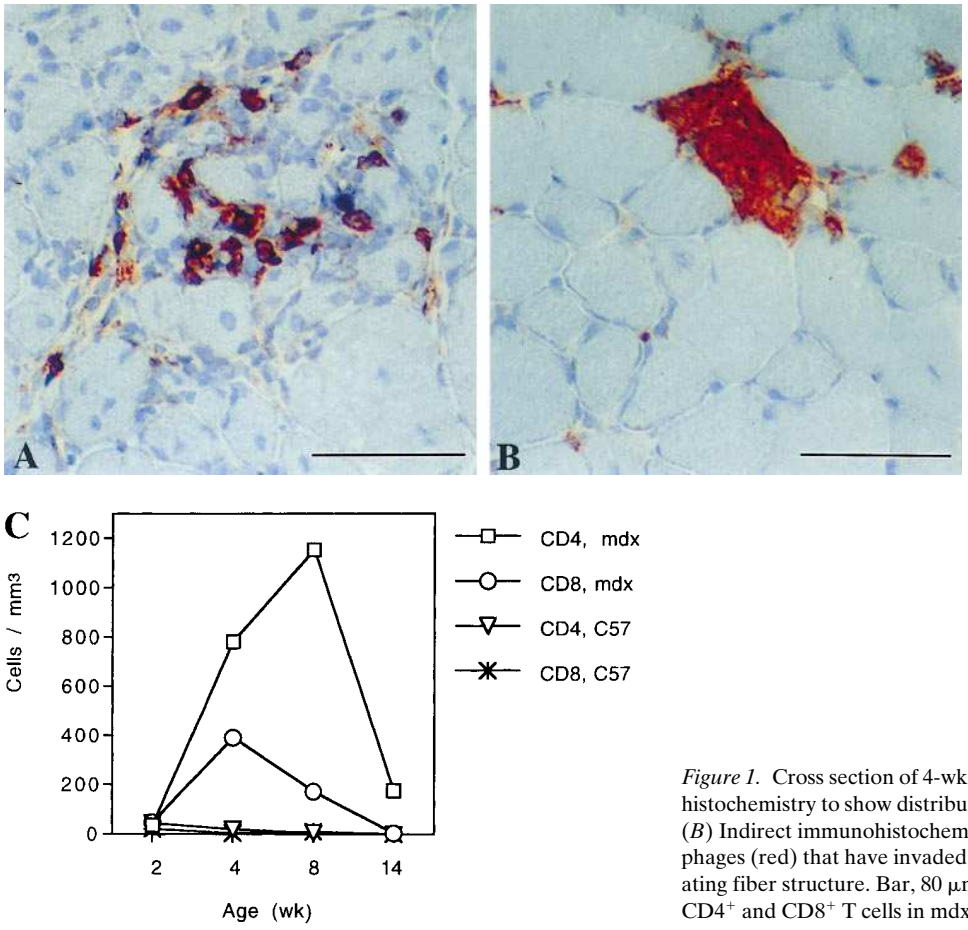

Figure 1. Cross section of 4-wk mdx quadriceps. (*A*) Indirect immunohistochemistry to show distribution of CD8$^+$ CTLs (red). Bar, 100 μm. (*B*) Indirect immunohistochemistry to show anti-CD11b$^+$ macrophages (red) that have invaded a necrotic fiber at high density, obliterating fiber structure. Bar, 80 μm. (*C*) Graph showing invasion of CD4$^+$ and CD8$^+$ T cells in mdx and C57 muscle.

Table I. Pathological Characteristics of CD8-depleted Mdx Mice and Double Mutant Mice

	Macrophages	Invaded fibers	Hyaline fibers	Apoptotic myonuclei
Mdx	73902 (n = 6; SEM = 23337)	25.0 (n = 6; SEM = 7.0)	9.1 (n = 6; SEM = 5)	48.3 (n = 9; SEM = 19) [0; 0; 30; 88; 170; 11; 59; 77; 0]
CD8-depleted mdx	61006 (n = 9; SEM = 8948)	10.1 (n = 9; SEM = 2.1)*	1.7 (n = 9; SEM = 0.8)	36.8 (n = 5; SEM = 37.4)* [0; 0; 0; 0; 184]
Dystrophin−/perforin−	41504 (n = 10; SEM = 14493)*	14.9 (n = 10; SEM = 5.2)	1.9 (n = 10; SEM = 0.5)*	1.6 (n = 8; SEM = 1.6)* [0; 0; 0; 0; 0; 0; 0; 13]

Two sections were analyzed for each animal. The numbers of animals analyzed and standard errors in each group are shown parenthetically. Values for macrophages and apoptotic myonuclei are expressed per mm^3 of muscle. Values for invaded fibers and hyaline fibers are expressed per mm^2 cross-sectional area of muscle. The concentration of apoptotic myonuclei observed for each individual animal is shown in brackets. Note that apoptotic myonuclei were observed in only one CD8-depleted animal. *Value differs significantly from mdx values at $P < 0.05$ (Mann-Whitney).

virtually all myonuclear apoptosis was eliminated by the double mutation (Table I). There was also a significant decrease in tissue inflammation, as indicated by a 44% decrease in the concentration of macrophages in the connective tissue surrounding muscle fibers ($P < 0.05$), and a significant decrease in muscle fiber pathology shown by a 79% decrease in the concentration of hyaline fibers ($P < 0.05$) (Table I).

Discussion

The current view, held by some investigators, that death of dystrophin-deficient cells is not attributable to primary involvement of immune cells, is derived largely from immunohistochemical and electron microscopic observations made over a decade ago, before the identification of fas or perforin-mediated cytotoxic mechanisms (33–35). At that time, it was suspected that CTLs killed muscle fibers by invading the individual fibers. The infrequent observation of CTL-invaded fibers in DMD muscle led those early investigators to conclude that CTLs did not function importantly in killing dystrophin-deficient muscle (33, 34, 36). However, current knowledge shows that CTLs do not kill targets by invasion, but rather by cell surface interactions (22). Also, observations made in those earlier studies were based on tissues obtained from patients who had already experienced clinical onset of the disease, so that the conditions initiating the pathology would have been obscured by extensive tissue morbidity.

The findings of this study show that CTLs contribute significantly to the pathology of dystrophin deficient muscle through both apoptosis and necrosis, and that perforin-mediated events are responsible primarily for apoptosis of mdx myonuclei. However, the finding that CTL-mediated, dystrophic muscle death can also occur by perforin-independent mechanisms indicates that CTLs also kill dystrophic muscle by a second mechanism, for which cytokine-mediated or fas-mediated processes are possible candidates. Our current knowledge of the mechanisms by which CTLs can kill target cells supports cytokine-mediated killing as the more likely possibility, because killing induced by fas occurs by apoptosis (37) in all of the systems examined thus far. Furthermore, cytokine-mediated killing is also consistent with our observation that CD8 depleted animals showed a reduction of macrophage invasion of fibers, although there was not a reduction of macrophage concentration in the muscle. Previous investigations have shown that IL-12 secretion by macrophages is critically dependent on activated T cells (38). Thus, CD8 depletions could yield a reduction in the concentration of cytokines that facilitate macrophage killing of targets via necrotic mechanisms. Future studies will be directed toward identifying the specific, nonperforin mechanisms used by CTLs in killing dystrophic muscle.

CTL killing of target cells requires activation by antigen, and the findings of Gussoni et al. (18) indicate that such an antigen may be present in DMD. Although the identity and

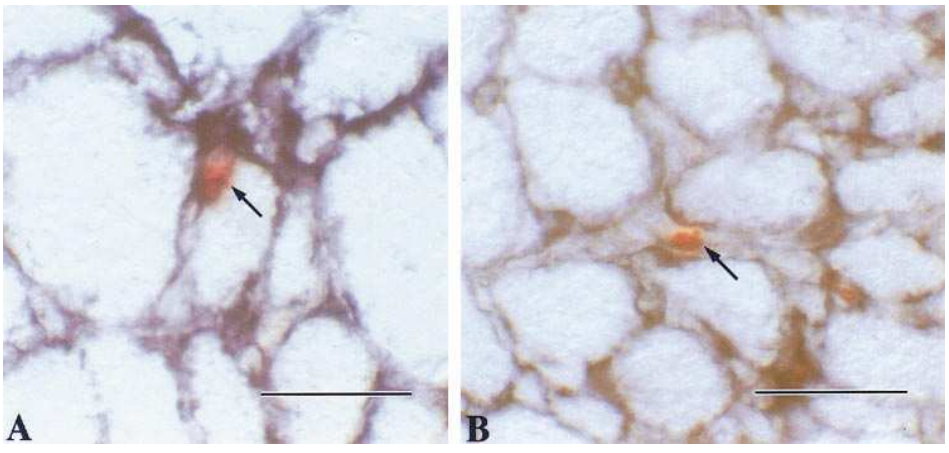

Figure 2. Double-labeled cross section of 4-wk-old mdx quadriceps muscle labeled with antilaminin to show basement membrane distribution (dark purple) and TdT-mediated end labeling of apoptotic DNA fragments (red). (*A*) An apoptotic myonucleus lying deep to the basement membrane is indicated (*arrow*). (*B*) An apoptotic nucleus of a nonmuscle cell lying outside the basement membrane is indicated (*arrow*). Bars, 50 μm.

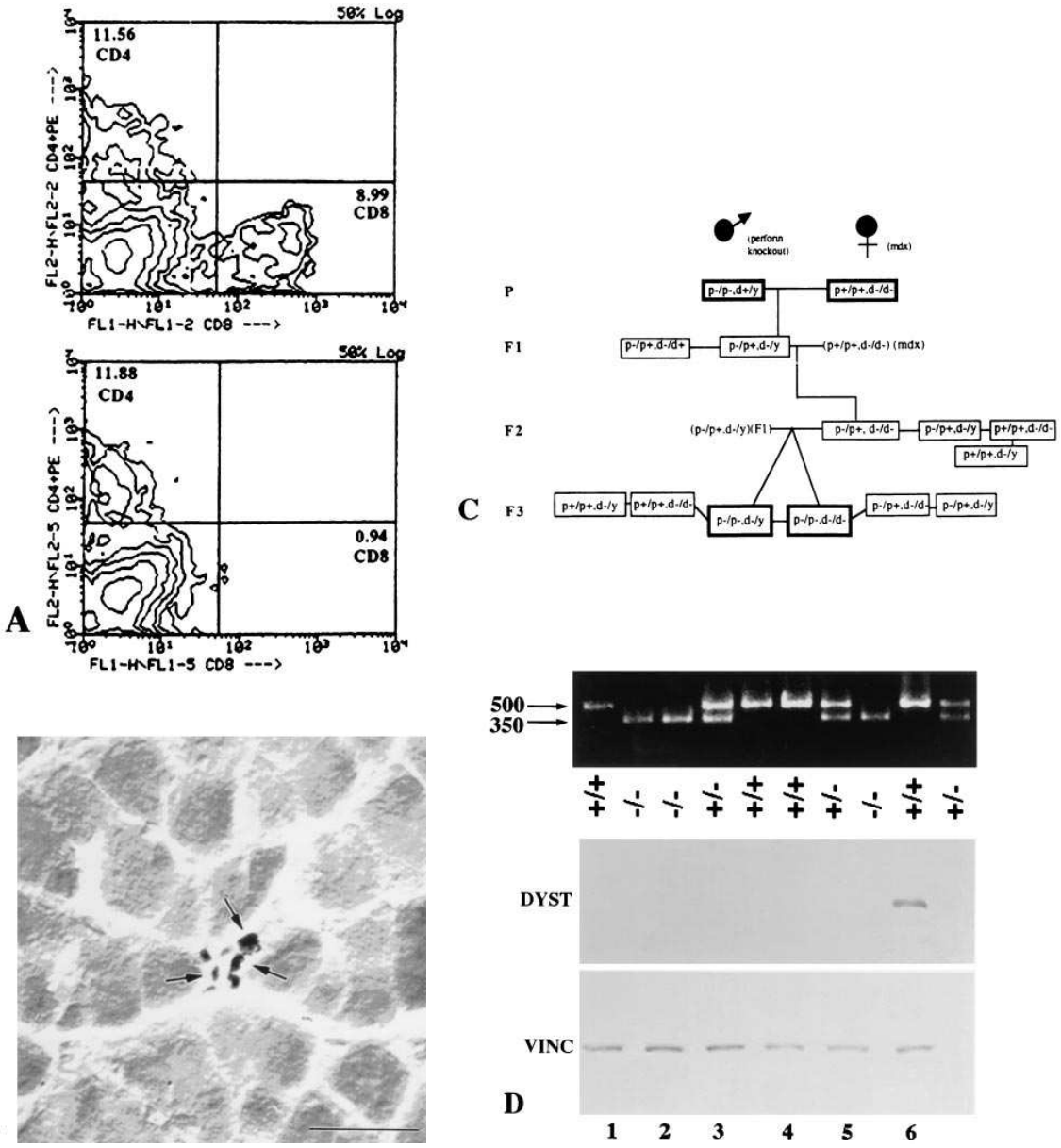

Figure 3. (*A*) Flow cytometry of splenocytes isolated from 4-wk-old mdx mice after 2 wk of intraperitaneal injection of 50 μg of antibody every 4 d. Upper left quadrant in each graph represents $CD4^+$ cells; lower right quadrant represents $CD8^+$ cells. Upper graph shows data from control animal receiving isotype control antibody (IgG2B). Lower graph shows data from $CD8^+$ cell depleted animal, receiving anti-CD8 injections. (*B*) RT-PCR in situ of cross section of paraffin-embedded 4-wk mdx hindlimb muscle using perforin specific primers to show distribution of perforin-expressing cells. A cluster of $perforin^+$ cells is indicated by arrows. Controls (not shown) were a positive control for which DNAse was eliminated from the procedure, yielding dense reaction product on all nuclei, and a negative control for which reverse transcriptase was eliminated, yielding no reaction product on any cell. Bar, 50 μm. (*C*) Diagram of breeding strategy to produce $perforin^-$/$dystrophin^-$ mice. p^+, perforin wild type; p^-, perforin mutant; d^+, dystrophin wild type; d^-, dystrophin mutant; and *y*, y chromosome. (*D*) Screening strategy for double mutant mice. *Top*, PCR products of mouse DNA obtained from tail of F2 mice. PCR of wild type (+/+) DNA yields only the 500-bp band. PCR of homozygous mutant (−/−) yields only the 350-bp band. PCR of heterozygotes (+/−) yield both bands. *Bottom*, immunoblots of muscle samples from population of mice during breeding of $perforin^-$/$dystrophin^-$ crosses. Top blot was probed with antidystrophin; bottom blot was probed with antivinculin as a positive control. Animals *1* and *2* were mdx mice, animals *3–5* were $perforin^-$/$dystrophin^-$. Animal *6* was C57 control.

source of the activating antigen are currently unknown, it is feasible that it arises from nonspecific damage that occurs to muscle in the absence of dystrophin. Several reports have demonstrated the development of autoimmunity after trauma to tissue, such as in vasectomy (39), rheumatic fever (40), myocardial infarction (41), cardiomyopathy (42), or myocarditis (43). In these cases, proteins that are not expressed in the thymus during positive and negative selection, and are normally sequestered from the immune system, are released to the bloodstream and subsequently stimulate an immune response. The lack of the dystrophin at the membrane causes a weakening of the membrane and eventual damage (44) that may result

in the release of cytosolic proteins not normally seen by the immune system, which may be capable of activating T-lymphocytes. In addition, widespread proteolytic degradation is a prominent feature of DMD and mdx dystrophies (45), and this proteolysis may produce conditions in which proteins that are released upon muscle cell death are presented to T cells in the context of MHC-1 after being engulfed by a phagocytic antigen presenting cell.

An alternative, hypothetical source for autoantigens that may activate T-lymphocytes in dystrophin-deficient tissues are proteins that are not normally expressed in healthy muscle, but are expressed in dystrophic muscle. Heat shock proteins have been shown to be upregulated in degenerating fibers from mdx muscle (46) as well as during heat stroke (47), exercise (48), and cardiac hypertrophy (49). In one recent investigation (46), 80% of the patients with cardiomyopathy synthesized autoantibodies against heat shock protein 40, and 42% of patients generated antibodies against heat shock protein 60 (42). Thus, heat shock proteins or another pathologically expressed protein may stimulate an immune response to diseased or injured muscle.

Understanding of the cellular mechanisms involved in the inflammatory aspects of dystrophin deficiency can possibly lead to therapeutic treatment strategies by providing valuable information regarding the interactions between CTLs and muscle cells. Although the missing gene product is known for DMD, no successful therapeutic strategies have been developed to correct for that defect. Thus, it will be useful to provide a scientific basis upon which alternative treatments could be developed. In addition, the actions of CTLs on muscle are believed to interfere with successful myoblast transfer or adenoviral gene delivery (50, 51), both of which are potential therapies for muscular dystrophy. Therefore, a better understanding of the interactions of CTLs and muscle cells is fundamental for the success of gene therapy.

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