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Resolution of skeletal muscle inflammation in mdx dystrophic mouse is accompanied by increased immunoglobulin and interferon-γ production

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Summary. Mdx mouse, the animal model of Duchenne muscular dystrophy, develops an X-linked recessive inflammatory myopathy with an apparent sustained capacity for muscle regeneration. We analysed whether changes in the skeletal muscle during myonecrosis and regeneration would correlate with functional alterations in peripheral lymphoid tissues. Here we show that during the height of myonecrosis, mdx mice display marked atrophy of peripheral lymph nodes and extensive muscle inflammation. In contrast, enlargement of draining lymph nodes with accumulation of $CD4^+$ $CD44^+$, $CD4^+$ $CD25^+$, $CD8^+$ $CD44^+$ T lymphocytes and type-2 B cells was consistently observed during amelioration of the muscle lesion. In addition, regeneration of the muscular tissue was accompanied by concomitant increase of immunoglobulin-secreting cells in regional lymph nodes and bone marrow. Double immunolabelling analysis revealed intense B cell proliferation and formation of germinal centre in the follicles of dystrophic regional lymph nodes. Furthermore, lymph node cells produced large amounts of IFN-γ but not IL-4, IL-6 or IL-10 after *in vitro* mitogen stimulation with Concanavalin A. As these alterations occurred mainly during the recovery period, we suggested that local activation of the immune system could be an influence which mitigates the myonecrosis of muscular tissue in the mdx dystrophic mouse.

Keywords: immune system, inflammation, mdx, muscular dystrophy

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The mdx mouse, an animal model of Duchenne muscular dystrophy (DMD), similar to the human disease, develops an X-linked recessive inflammatory myopathy (Nonaka 1998). Both mdx mice and DMD patients have a deficit in the gene coding for dystrophin, a subplasmalemmal protein that binds to actin and associates with a complex of cell surface proteins which in turn interacts with the extracellular matrix (ECM) (Sicinski *et al*. 1989; Brown *et al*. 1999). Dystrophin deficiency impairs intracellular Ca^{2+} homeostasis and disrupts multiple protein interactions important for signalling from the ECM to the nucleus (Mehler 2000). Despite sharing the same genetic and molecular defect, the disease varies markedly among mice and humans. MDX mice have a more benign course and show an apparent sustained capacity for regeneration, whereas muscles of young male DMD patients are replaced by fat and connective tissue, with death occurring around the second decade of life (Allamand & Campbell 2000). Unfortunately, most therapeutic strategies have been palliative rather than curative, with a focus on ameliorating or restraining the disease process. This is partly due to a lack of understanding, not only of the precise molecular function of dystrophin, but also of the dystrophic process itself and the epistatic factors that influence the ultimate clinical phenotype (Infante & Huszagh 1999).

Muscular dystrophy in mdx mouse is characterized by abrupt onset of muscle fibre degeneration with intense inflammatory infiltrate present since weaning (Nonaka 1998). The height of myonecrosis occurs near adulthood and is followed by effective muscular regeneration. Yet, persistent fibrosis and accumulation of connective tissue affect almost exclusively the diaphragm (Boland *et al*. 1995), though limb muscles show fibrosis towards the end of the second year (Pastoret & Sebille 1995). In this sense, mdx mice may be considered a valuable model for studying the process of muscle regeneration in Duchenne-type muscular dystrophy (DiMario *et al*. 1989).

Myonecrosis in mdx mice is often preceded by breakdown and detachment of sarcolemma from the basement membrane and subsequent degeneration of muscle fibres associated with extended inflammatory process. Macrophages, $CD4^+$ and $CD8^+$ T lymphocytes represent the main constituents of the inflammatory cell population surrounding the degenerating myofibres (McDowall *et al*. 1990; Spencer *et al*. 2001). Several findings support the hypothesis that the immune system might be playing a relevant role in the pathophysiology of dystrophin-deficient muscular dystrophy. For instance, T lymphocytes may contribute during early stages of the disease**,** by inducing apoptosis through perforin dependent and independent mechanisms (Spencer *et al*. 1997) and also later, by the onset of fibrotic events that undermine the ability of muscle to regenerate (Morrison *et al*. 2000). However, a recent study in mdx mice using adenovirus vectors expressing β-galactosidase or green fluorescent protein pointed out a potential role for cytokines in promoting up-regulation of utrophin, a compensatory structural muscle protein (Yamamoto *et al*. 2000). Interestingly, a patient with Becker muscular dystrophy, developing active arthritis with great production of inflammatory cytokines, showed amelioration of muscular dystrophy with normalization of creatine kinase levels (Maegaki *et al*. 1999). A better understanding of the participation of the immune system in Duchenne-type muscular dystrophy may therefore provide valuable information for designing new strategies of treatment.

Previous data from our group showed that major changes in the cytoarchitecture and microenvironment of lymphoid organs, such as atrophy and ECM overdeposition, paralleled the process of myonecrosis and extensive inflammatory reaction in the muscular tissue of mdx mice (Quirico-Santos *et al*. 1995; Seixas *et al*. 1997). In contrast, enhanced cellularity of draining lymph nodes with significant increase of $CD4^+$ and $CD8^+$ cells expressing ECM receptors was consistently observed during regeneration of muscular tissue and clinical amelioration of the disease in mdx mice (Lagrota-Candido *et al*. 1999). Here we report that during the recovery phase of muscular dystrophy, decline in myonecrosis was accompanied by increased immunoglobulin and interferon-γ production. The results are discussed in terms on the possible role of the immune system in the mitigation of the muscular lesion in mdx mouse.

Materials and methods

Animals

Mdx dystrophic and age-matched C57BL/10J control nondystrophic mice were maintained in the animal house of the Department of Cellular and Molecular Biology at Fluminense Federal University. Mice were kept at a constant temperature (20 °C) with a light cycle of 12 : 12 h, received acidified water and enriched diet supplemented with vitamins *ad libitum.* In an attempt to minimize distress and avoid starvation due to muscular dystrophy, water was provided in a bottle with longer sip and pelleted food in a recipient placed on the floor of the cage. Male animals were selected at ages corresponding to main phases of the disease: height of myonecrosis (4 weeks), amelioration of myonecrosis (12 weeks) and early fibrosis (24 weeks).

Histological staining and quantitative analysis

Triceps brachii muscle from 4, 12 and 24 week mdx mice were carefully removed and fixed in formalin-buffered (pH 7.2) Millonig fixative. Five-μm sections of paraffin-embedded tissue were stained with haematoxylin–eosin (HE). Degenerating/necrotic fibres were identified by an homogeneous pale eosinophilic sarcoplasm, whereas regenerating fibres by their strong sarcoplasmic basophilia and centrally located nuclei. The total surface and that occupied by myonecrosis, regenerating fibres or fibrosis was determined with the SCION Program (National Institutes of Health Image Program, USA). The difference between these two measurements was expressed as the percentage of pathological area in the cross-section. Images from the entire cross section using a 20× objective were acquired with a Sound Vision Micro (USA) digital camera.

Immunohistochemistry

Triceps brachii skeletal muscle with draining axillary and brachial lymph nodes was carefully removed and fixed for 3 h in Carnoy's fixative. Sections placed on poly-L-lysine (Sigma Chemical Company, MO, USA) precoated slides were blocked for endogenous peroxidase activity with 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 5 min and washed with PBS containing 0.1% BSA (fraction V) and 0.01% Triton X-100 (Sigma). Optimal dilutions of biotinylated anti-PCNA clone PC10 (cell proliferation-associated marker) and purified anti-B220 clone RA3-6B-2 (B cell marker) both purchased from Pharmingen (San Diego, CA, USA) were applied and sections incubated at room temperature in a moist chamber for 60 min. Sections were then subjected for 40 min to goat anti-rat IgG peroxidase conjugate (Sigma) and streptavidin-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL, USA). Peroxidase was revealed with aminoethylcarbazole (Sigma) in the presence of H_2O_2 and phosphatase with 5-bromo 4-chloro 3-indolyl phosphate and nitro blue tetrazolium (Zymed Laboratories Inc., USA). All sections were lightly counterstained with Mayer's haematoxylin. For histological studies at least five animals per group were examined.

For assessment of $CD4^+$ and $CD8^+$ T cell subsets in the inflammatory infiltrate, *Triceps brachii* was embedded in OCT (Tissue-Tek, Miles Inc, Elkhart, USA) and subsequently frozen in liquid nitrogen. Five-μm thick sections were fixed in acetone and incubated with anti-CD4 (GK1.5) and anti-CD8 (5367) hybridoma supernatants. Three mdx mice were included per age and three entire cross-sections of skeletal muscle were analysed with a 20× objective for each mouse.

Analysis of cell surface markers by flow cytometry

Double or three-colour staining were performed with fluorescein–isothyocianate (FITC), R-phycoerythrin(R-PE), tricolour (TC) or Quantum red conjugates, using the following monoclonal antibodies (mAbs): CD4/R-PE clone L3T4 (Becton Dickinson, San Jose, CA, USA); CD8/biotin clone 53-6.7 (Southern Biotechnology Associates, Birmingham, AL, USA); CD5/biotin clone Lyt-1 (Becton Dickinson); CD25/FITC (clone AMT-13), TCRαβ/FITC (clone H57-597) from Sigma. Anti-B220/R-PE (clone RA3–6B2), CD44/FITC clone IM7, CD62L/FITC (clone Mel-14) and anti-IgM/FITC (clone R6-60.2) were purchased from Pharmingen.

Axillary and brachial lymph nodes, spleen and bone marrow were carefully removed and cleaned from adipose tissue. Bone marrow cells were collected from thigh and shin bones. Cell suspensions prepared by fine mincing the organs with needles in PBS pH 7.2, were passed through a nylon sieve and suspended in ice-cold PBS supplemented with 2% heat-inactivated fetal calf serum (FCS) (Gibco BRL, Gaithersburg, USA). Red blood cells were removed following incubation with 0.165 M Tris-ammonium chloride for 2 min. Cells were washed with PBS−2% FCS and viability determined by the trypan blue exclusion test.

Three hundred thousand cells were incubated for 10 min at 4 °C with PBS – 2% mixture (v : v) fetal calf serum and 1/10 diluted normal mouse serum. For three-colour staining, cells were incubated with optimal concentration of specific antibodies for 20 min at 4 °C. Binding of biotinylated antibodies was determined following stepwise incubation for 30 min with streptavidin–Quantum red complex (Sigma). Cells were fixed with PBS−1% formaldehyde containing 0.05% azide. A living gate excluding cell debris and nonviable cells was determined using forward vs. side scatter parameters and 20 000 events were acquired using a FACSCalibur cytometer using CELLquest software (Becton Dickinson, San Diego, USA).

Detection of immunoglobulin-secreting cells

For *ex vivo* experiments, total numbers of IgM, IgG and IgA plasma cells were determined by a modification of the Sedgwick and Holt ELISA-Spot-Assay technique (ESA) (Sedgwick & Holt 1983; Gradien *et al.* 1994). Briefly, microtitre plates (Luxlon M29 LSE, CEB, France) were coated for 16 h at 4 °C with anti-IgM, anti-IgG or anti-IgA antibodies (Southern Biotech., Birmingham, AL, USA) diluted in coating buffer (0.05 M K_2HPO_4) and blocked for 1 h with PBS containing 1% gelatin (Sigma).

Serial dilutions of cell suspensions in RPMI 1640 with 2% FCS obtained from individual lymph nodes, spleen or bone marrow were seeded and incubated for 6 h at 37 °C in a humidified 5% CO_2 incubator. After washing out the cells, plates were incubated with the corresponding goat anti-mouse IgM, IgG or IgA alkaline-phosphatase conjugate (Southern Biotech.) for 90 min at 37 °C in a humidified 5% CO_2 incubator. Spots generated by specific binding of secreted Ig, were revealed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate diluted in 2-amino-2-methyl-1-propranolol alkaline buffer solution. The number of plasma cells per seeded cell was determined by counting the spots with a magnifier lens. The absolute number of naturally occurring plasma cells in each organ was calculated from the total number of cells.

B lymphocyte stimulation for in vitro *antibody production*

Bone marrow, axillary and brachial lymph node cells obtained from C57BL/10 or mdx mice, were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 5×10^{-5} M β-mercaptoethanol in a 24-well tissue culture plate (Corning, NY, USA) at a concentration of 2×10^6 cells/mL. Bone marrow cell suspensions were centrifuged through a high density Ficoll–Paque solution (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA) to obtain mononuclear cell suspensions. B cells were enriched by panning with 100 μg/mL of purified hamster anti-mouse anti-TCRα clone H57-597 (Southern Biotech.). This procedure was carried out twice and yielded more than 80% B220 positive cells, as confirmed by immunocytochemistry and flow cytometry. For induction of *in vitro* antibody production, 10 μg/mL of lypopolysaccharide (LPS) from *Escherichia coli* serotype 055:B5 was added (Sigma). After incubation for 5 days at 37 °C in a humidified 5% CO_2 incubator, culture supernatants were harvested and stored at −20 °C until use.

Detection of antigen-specific immunoglobulin secretion in B cell supernatants

Microplates (Luxlon M29 LSE, CEB, France) were coated with 5 μg/mL of anti-IgM, anti-IgG or anti-IgA antibodies diluted in coating buffer (0.05 M K_2HPO_4) for 16 h at 4 °C. Additional ELISA plates were coated with 10 mg/mL of rabbit muscle myosin, myelin basic protein (MBP) or type II-A histone from calf thymus (Sigma) for *in vitro* antigen-specific antibody production. Non-specific binding sites were saturated with PBS containing 1% gelatin for 1 h and washed subsequently with PBS containing 0.05% Tween-20 (PBS-T).

Serial dilutions of culture supernatant were incubated at 37 °C for 90 min with the corresponding specific antigen coated in 96-well microplates. Immunoglobulin (Ig) binding was determined following incubation with goat anti-mouse IgM, IgG or IgA peroxidase-conjugate (Southern Biotech.). Enzyme activity was then revealed with O-phenylenediamine (Sigma) in the presence of H_2O_2 and the reaction stopped with 3 N H_2SO_4. Absorbance at 492 nm was measured using an immunoreader (Anthos 2010, Austria). Antibody concentrations were calculated by using the linear ranges of the dilution and standard curves generated with purified specific mouse monoclonal IgM, IgA or IgG antibody (Southern Biotech.).

Lymphocyte stimulation for in vitro *cytokine production and quantification*

Two million cells/mL, obtained from mdx or control axillary and brachial lymph nodes were seeded in 24-well tissue culture plates (Corning, NY, USA) in the presence of 5 μg/mL Concanavalin A (Sigma) and incubated at 37 °C in a 5% CO_2 atmosphere. Culture supernatants were collected after 24 or 48 h for cytokine quantification by ELISA (Pharmingen, San Diego, CA). Microtitre plates (Luxlon M29 LSE, CEB, France) were coated overnight at 4 °C with 2 μg/well of purified capturing mAb to mouse IFN-γ, IL-4, IL-6 or IL-10 (Pharmingen) in 100 mM carbonate buffer, pH 9.5. Plates were washed five times with PBS-Tween. Non-specific binding sites were saturated with 10% FCS in PBS for 1 h and washed with PBS-Tween. Thereafter supernatants and corresponding cytokine standard diluted in PBS/FCS were added. Plates were incubated overnight at 4 °C, subsequently washed four times with PBS-Tween and incubated at room temperature for 40 min with the corresponding biotinylated anti-IFN-γ, IL-4, IL-6 or IL-10 antibody (Pharmingen). After washing the plates, avidin-conjugated horseradish peroxidase was added and incubated for 30 min. Enzyme activity was revealed with 3,3′,5,5′-tetramethylbenzidine and 1.2 mM H_2O_2 in citrate buffer (pH 5.0). The reaction was stopped by adding 1 N HCl. Absorbance was measured at 450 nm using the Anthos 2010 ELISA device (Austria) and cytokine concentrations were quantified using a double eight-point standard curve.

Statistical analysis

Microsoft Excel 97 software (Microsoft, Seattle, WA), was used to calculate mean and standard deviations. Unpaired Student's *t*-test was applied to assess the level of statistical significance.

Results

Histological and morphometric analysis of dystrophic muscle

During the height of myonecrosis (at 4 weeks) skeletal muscles of dystrophic mice (Table 1) were undergoing a similar extent of necrosis and regeneration with prominent inflammation (Fig. 1a,d). Thereafter (at 12 weeks) a marked reduction of regenerating basophilic fibres and a prevalence of regenerated hypertrophied myofibres with centrally located nuclei were observed (Fig. 1b,e). In contrast, older (24 week) mdx mice showed extensive

Table 1. Frequency of distinct pathological lesions in mdx skeletal muscle. Results are expressed as mean ± SD (standard deviation) in percentage/unit area of inflammatory infiltrate, regenerating myofibre and fibrosis. A 20x objective was used for the analysis of three entire cross section of *Triceps brachii* skeletal muscle from three animals per group at ages 4 weeks, 12 weeks and 24 weeks.

Age (weeks)	Inflammatory infiltrate	Regenerating myofibre	Fibrosis
4	19.0 ± 9.2	11.7 ± 2.8	0.2 ± 0.1
12	5.7 ± 0.6	0.3 ± 0.3	1.1 ± 0.7
24	5.3 ± 3.2	0.3 ± 0.6	10.3 ± 1.9

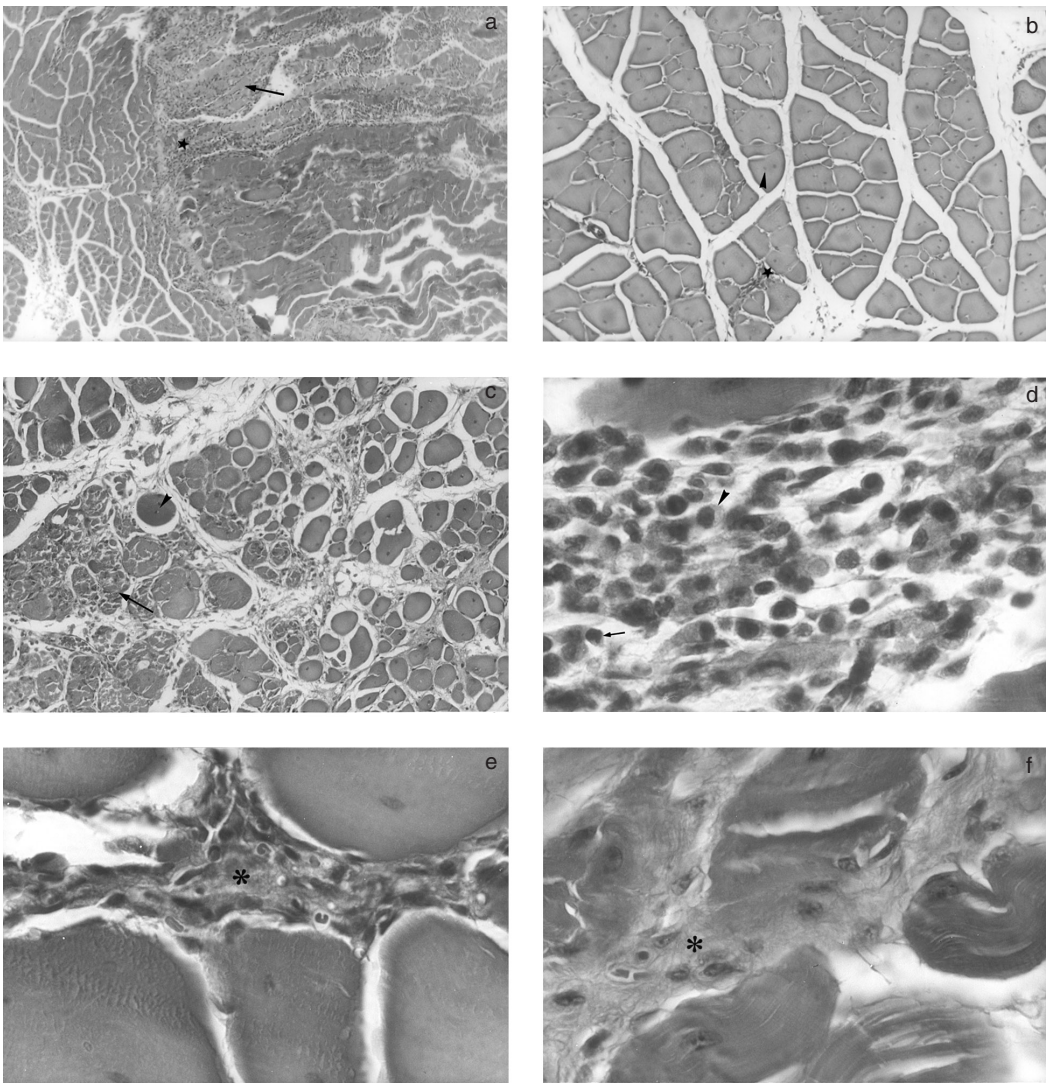

Figure 1. Haematoxylin-eosin staining of a 5 μm wax-embedded section of *Triceps brachii* muscle fragments of 4 week (a, d), 12 week (b, e) and 24 week (c, f) dystrophic mice. (a–d) Extensive myonecrosis with hyaline fibres (large arrow) and inflammatory cell infiltration (star) with many polymorphonuclear (arrow) and mononuclear cells (arrow head) at the height of myonecrosis; (b–e) numerous myofibres with central nuclei (arrow head), scarce inflammatory focus (star) and connective tissue (asterisk); (c–f) hypertrophied (arrow head) and necrotic (arrow) myofibres and overdeposition of connective tissue (asterisk) at early fibrosis period. Original magnification ×100 (a, b), ×200 (c), ×1000 (d, e, f).

fibrosis with dense deposition of connective tissue, numerous degenerating/necrotic fibres and minimal inflammation (Fig. 1c,f).

Immunohistochemical analysis showed that during the height of myonecrosis $CD4^+$ (510 ± 40 cells/mm^3), $CD8^+$ (289 ± 20 cells/mm^3) and some B lymphocytes (52 ± 8 cells/mm^3) were present in the skeletal muscle of dystrophic mice. In contrast, rare T and B lymphocytes cells were found in the skeletal muscle of dystrophic mice at 12 and 24 weeks.

Accumulation of B lymphocytes in dystrophic lymph nodes and bone marrow during skeletal muscle regeneration

During the height of myonecrosis, regional lymph nodes of 4-week-old mdx mice showed a marked reduction of T cells in comparison to control C57BL/10, although a more significant ($P < 0.0001$) effect was observed for B lymphocytes (Fig. 2).

Regeneration of myonecrosis (at 12 weeks) was accompanied by reduction of $CD4^+$ and $CD8^+$ cells in the skeletal muscle of mdx mice (data not shown). However, enlargement of regional lymph nodes with significant ($P < 0.0001$) increase of B lymphocytes was consistently observed concomitant to regeneration of myofibres and mitigation of muscular inflammation. This feature was more prominent in 24 week mdx mice. A mild increase in the absolute numbers of $CD4^+$ and $CD8^+$ T cells were also observed in a 12 week mdx mouse, although a significant augmentation was consistently observed in 24 week-old mice. Further analysis to evaluate the percentage of $CD5^+$ cells within the $B220^+IgM^+$ population showed that the majority belonged to a conventional type B-2 lymphocyte subset (Fig. 3). In addition, a significant increase of $CD44^+$ and $CD62L^+$ subsets within $CD4^+$ and $CD8^+$ T lymphocytes and also $CD25^+CD4^+$ cells were observed in 24 week

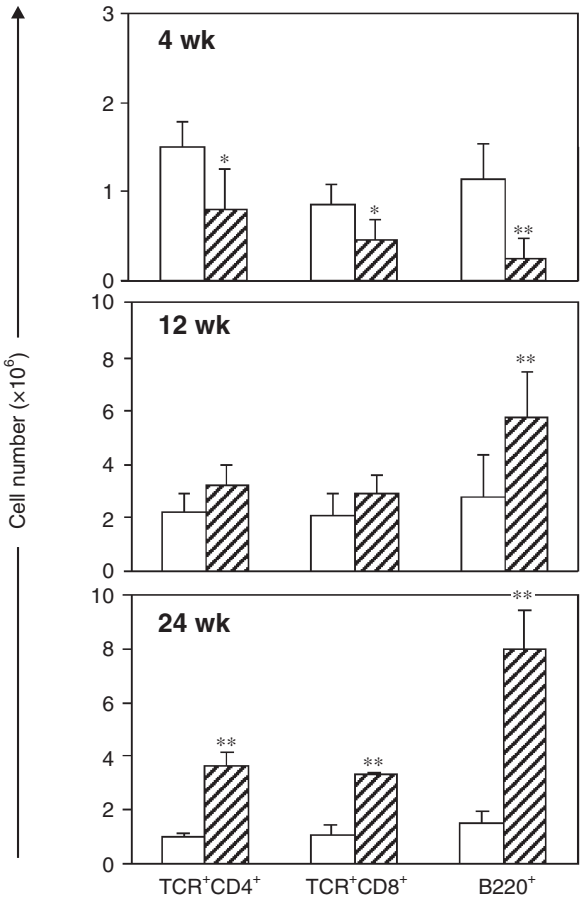

Figure 2. Flow cytometry analysis of mononuclear cells present in axillary and brachial lymph nodes of mice at 4 weeks, 12 weeks and 24 weeks. One representative experiment is presented for each age. Results are expressed as mean ± SD of four animals. This experiment was performed four times. ☐ C57BL/10, ▨ mdx. *$P < 0.05$ and **$P < 0.0001$.

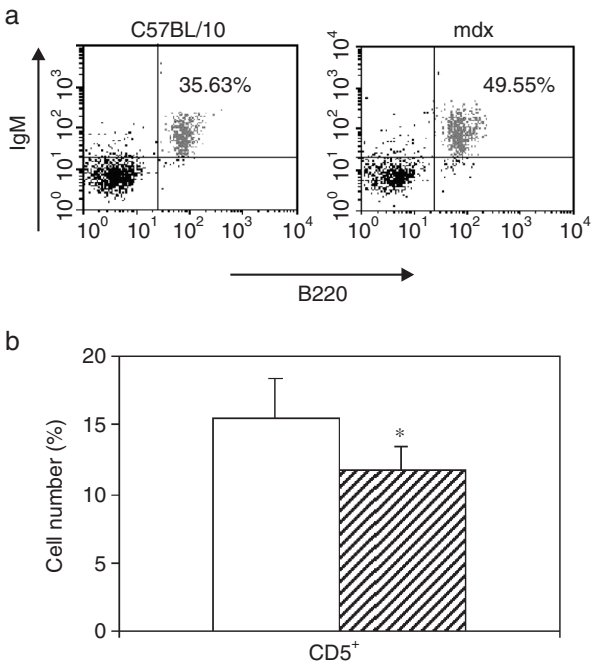

Figure 3. Flow cytometry analysis with anti-B220/R-PE, anti-IgM/FITC and anti-CD5 biotin/streptoavidin-quantum red of single cell suspensions from axillary and brachial lymph nodes of control and mdx mice at 24 weeks. (a) Expression of B220 and IgM; (b) percentage of $CD5^+$ cells in the $B220^+IgM^+$ population. One representative experiment is presented. Results are expressed as mean ± SD of four animals. Similar results were seen in four additional experiments. ☐ C57BL/10, ▨ mdx. *$P < 0.05$.

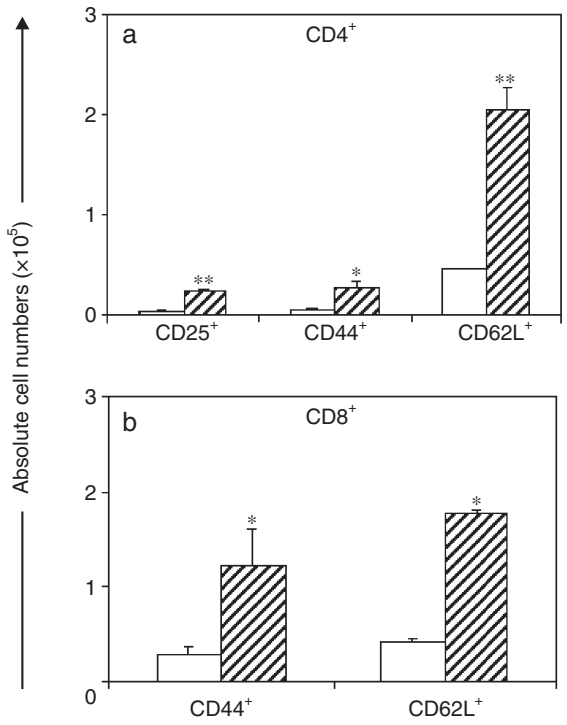

Figure 4. Expression of CD25, CD44 and CD62L within $CD4^+$ (panel a) and $CD8^+$ (panel b) T cells from 24 week mdx lymph nodes. Results are expressed as mean ± SD of four animals. One representative experiment is presented. Similar results were seen in three additional experiments. □ C57BL/10, ▨ mdx. $*P < 0.05$, $**P < 0.0001$.

mdx mice (Figs 4a.b). Nonetheless expression of $CD25^+$ cells within the $CD8^+$ population was very low in lymph nodes from both strains of nondystrophic C57BL/10 and dystrophic mice (data not shown).

Immunohistochemical analysis of the *Triceps brachii* muscle with anti-PCNA and anti-B220 monoclonal antibodies confirmed that after extensive myonecrosis, reduction of muscular inflammation correlated with accumulation of B cells ($B220^+$) in the regional lymph nodes of mdx mice. Indeed, 24-week-old mdx mice showed enlargement of cortical areas (Fig. 5a,b). Moreover, it was possible to observe B cell proliferation and formation of germinal centres in the follicles of mdx mice (Fig. 5c,d).

Bone marrow of mdx dystrophic mice (Fig. 6) showed a marked reduction of $B220^{lo}$ cells during the height of myonecrosis (at 4 weeks). In contrast, a consistent increase of $B220^{hi}$ cell numbers was observed in older animals (24 weeks), although no difference was observed between control and mdx mice at 12 weeks.

A slight decrease in the absolute number of T and B cells was seen in the spleen of mdx mice, but only at the height of myonecrosis (at 4 weeks). Yet there was no difference in relative numbers of T and B cells between control C57BL/10 and mdx dystrophic mice at all ages studied (data not shown).

Immunoglobulin-secreting cells in lymphoid organs

Inasmuch as B lymphocytes were increased in peripheral lymph nodes and bone marrow of mdx mice during amelioration of myonecrosis (at 12 weeks), it appeared relevant to assess whether those cells differentiated into plasma cells. Indeed a consistent increase of Ig-secreting cells in regional lymphoid organs during muscle regeneration was found **(**Fig. 7).

In *ex vivo* experiments, IgM-secreting cells were significantly increased, especially in draining lymph nodes of mdx mice at 12 weeks ($P < 0.05$, Fig. 7a) and 24 weeks ($P < 0.0001$, Fig. 7d). Presence of IgG-secreting cells ($P < 0.01$) was consistently observed in lymph nodes of mdx mice at 24 weeks. We also found an increment of IgM-secreting cells ($P < 0.01$) in the spleen of mdx mice at 12 weeks (Fig. 7b) and of IgA-secreting cells ($P < 0.01$, Fig. 7f) in the bone marrow of 24 week mdx mice during the period of increased connective tissue deposition in the skeletal muscle.

Because of atrophy, it was not possible to detect by ELISA spot assay the presence of Ig-secreting cells in regional lymph nodes during the height of myonecrosis. Nonetheless, no difference was observed concerning the presence of Ig-producing cells in the bone marrow and spleen of mdx mice at this age.

In vitro *immunoglobulin production*

B lymphocytes from brachial and axillary lymph nodes of 12 and 24 week mdx mice were stimulated *in vitro* with 10 μg LPS, a polyclonal B-cell activator, to ascertain the levels of Ig production and the pattern of antigenic recognition. Both control and mdx mice produced comparable levels of Ig in the bone marrow supernatants (data not shown). Rather surprisingly, B lymphocytes from lymph nodes of 12 week and 24 week mdx mice produced lower levels of IgM and IgG (Fig. 8a). Lymphocytes from dystrophic mice also produced IgM antibody with lower reactivity (Fig. 8b) for antigens (histone, myosin) known to be released from the lesioned tissue rather than for unrelated antigens (MBP).

In vitro *cytokine production*

Lymphocytes derived from draining lymph nodes of dystrophic and age-matched control mice were stimulated *in vitro* with Concanavalin A (Con A). Culture

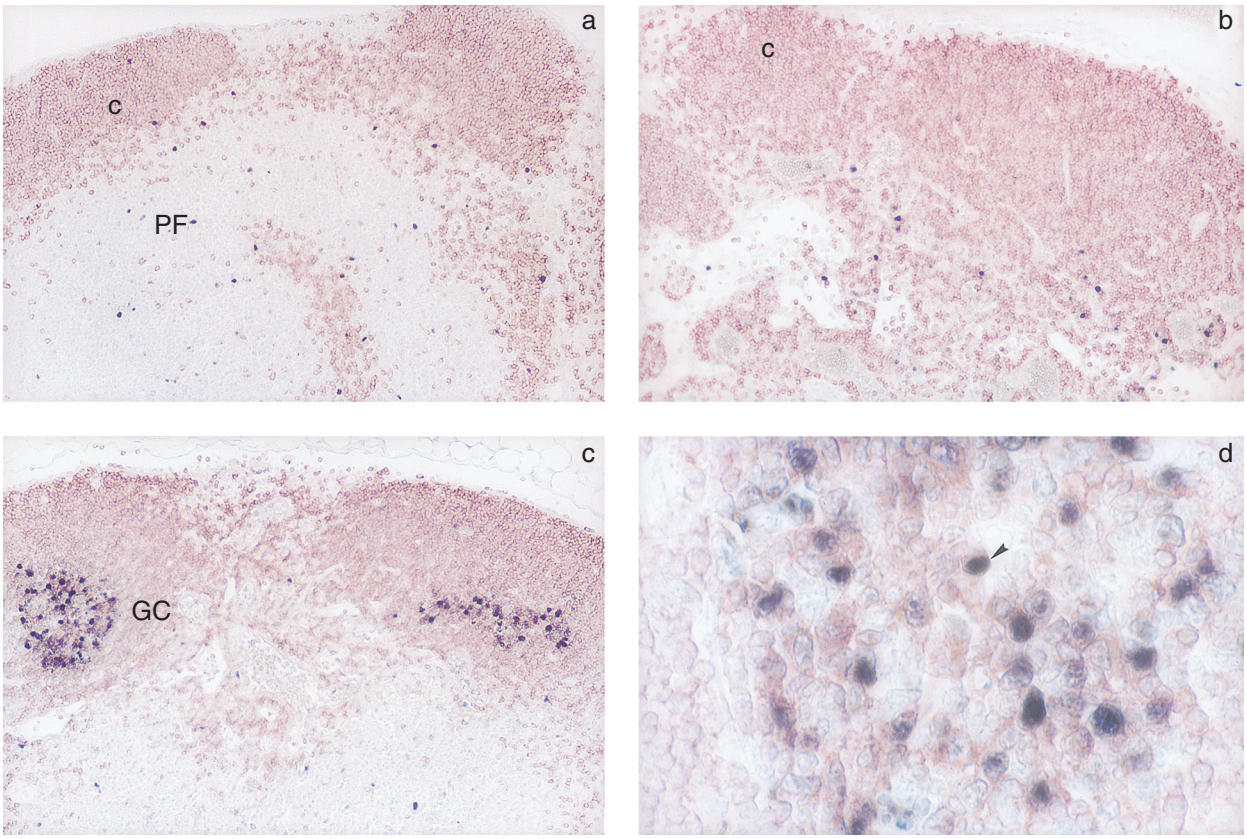

Figure 5. Photomicrographs showing characteristic staining of B220 (red) and PCNA (black) expression in 24 week mdx lymph node (b) compared with control C57BL/10 (a). Note the presence of germinal centre formation in mdx lymph node (c). $B220^+$ $PCNA^+$ lymphocytes in the germinal centre are indicated by arrow head (d). Original magnification $\times 200$ (a, b, c) and $\times 1000$ (d).
c, cortical; PF, parafollicular; GC, germinal centre.

supernatants were collected 24 or 48 h after and examined for IFN-γ, IL-4, IL-6 and IL-10 cytokine production by ELISA. As shown in Fig. 9, dystrophic lymphocytes from regional lymph nodes stimulated with Con A produced more IFN-γ than those generated from control mice at 4 weeks ($P < 0.005$), 12 weeks ($P < 0.05$) and 24 weeks ($P < 0.005$). Similar results were obtained with supernatants harvested from 24 and 48 h cell cultures. Control cultures with cells growing in absence of Con A produced undetectable levels of IFN-γ. Likewise, cell culture supernatants harvested after 24 and 48 h produced undetectable levels of IL-4, IL-6 and IL-10 (data not shown).

Discussion

In the present paper we report that during the height of myonecrosis, when skeletal muscles of mdx mice show extensive inflammatory reaction and degeneration, there is a general atrophy of lymphoid organs. This contrasts with accumulation of conventional type-2 B lymphocytes and marked immunoglobulin production in both regional lymph nodes and bone marrow during clinical amelioration and regeneration of the muscular lesion. We further observed that replacement of myofibres by connective tissue in the limb muscles of mdx mouse occurred several weeks earlier than previously reported (Pastoret & Sebille 1995). Interestingly, increased deposition of connective tissue in 24-week mdx dystrophic mice was also accompanied by prominent enlargement of draining lymph nodes and increased accumulation of T and B cells.

T cells are reported to contribute significantly to apoptosis of skeletal muscle and progressive fibrinogenesis evident during repeated cycles of mdx muscular tissue degeneration (Morrison *et al*. 2000; Spencer *et al*. 2001). Implication for the participation of T cells in the process of fibrosis was recently demonstrated in experiments carried out in mdx mice with the nu/nu background.

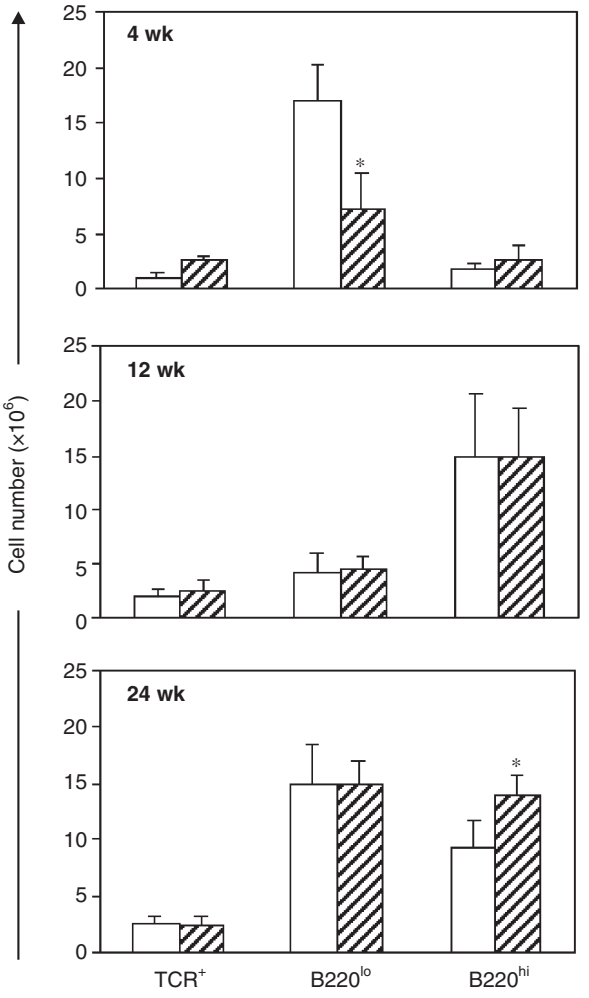

Figure 6. TCR^+ and $B220^+$ cell numbers in the bone marrow of mice at ages 4 weeks, 12 weeks and 24 weeks. One representative experiment is shown. Results are expressed as mean ± SD of four animals. Similar results were seen in three additional experiments. □ C57BL/10, ▨ mdx. $*P < 0.05$.

In this model, transplantation of normal thymic tissue into mdx-nu/nu mice replenished deposition of altered collagen in the muscular tissue comparable to wild-type mdx dystrophic mice (Morrison *et al*. 2000). However, the frequency of activated T cells was not elevated in mdx draining lymph nodes during the height of myonecrosis, as recently reported (Spencer *et al*. 2001). The high expression of CD62L in mdx lymph nodes at 24 weeks suggests the existence of strong recruitment from the circulating T cell pool during amelioration of myonecrosis in mdx mice. This notion is supported by a previous observation showing increased α6 integrin and ECM expression in the postcapillary high endothelial venules (HEVs) of mdx draining lymph nodes (Lagrota-Candido *et al*. 1999). Furthermore, we also observed a significant increase of $CD25^+$ and $CD44^+$ T cells in regional lymph nodes of mdx mice at 24 weeks, during mitigation of inflammation and early fibrosis of skeletal muscle. Interestingly, there are reports indicating that regulatory T cells express the phenotype $CD4^+CD25^+$ (Roncarolo & Levings 2000), thus suggesting that regulatory T cells might be playing a critical role in the control of inflammation also in the mdx muscular dystrophy. In this sense, the immune regulatory activity of the $CD4^+CD25^+$ T population is partly mediated through TGF-β secretion (Sakaguchi 2000), a cytokine involved in development of fibrosis in muscular dystrophy (Bernasconi *et al*. 1995). Experiments are now under way in order to establish *in vivo*, the precise function of such putative immunoregulatory cells in the control of inflammatory lesion in mdx dystrophic mice.

Mdx lymph node cells produced large amounts of IFN-γ but not IL-4, IL-6 or IL-10 after *in vitro* mitogen stimulation with Concanavalin A, especially during regeneration of muscular dystrophy. Several growth factors (e.g. IFN-γ) are reported to participate as regulators of connective tissue deposition partly by influencing the production of ECM components (Billiau 1996; Lagrota-Candido *et al*. 1996). Proliferation and differentiation of myogenic cells are events dependent on the expression of certain genes, which are greatly influenced by cell interactions with the ECM (Foster *et al*. 1987; Menko & Boettinger 1987). Members of the interferon-inducible protein p200 family have been reported to be expressed during myoblast differentiation (Liu *et al*. 2000).

IFN-γ is a cytokine that also up-regulates the expression of adhesion molecules, major histocompatibility complex gene products and chemokines, which can ultimately stimulate an inflammatory reaction occurring in the muscular tissue (Michaelis *et al*. 1993; Reyes-Reyna & Krolick 2000). Paradoxically, there are reports showing that IFN-γ may also exert a protective effect in models where macrophages are considered as major effector cells in tissue damage (Matthys *et al*. 1999; Tran *et al*. 2000; Matthys *et al*. 2001). Interestingly, hepatic granuloma of *Schistosoma mansoni*-infected mice with deficient interferon-gamma receptor (IFN-γ $R^{o/o}$) had an enhanced fibrotic reaction and an increased ratio of T over B cells (Oliveira *et al*. 2000). Since in mdx inflammation, $Mac\text{-}1^+$ cells are more abundant during the height of myonecrosis, it is possible that IFN-γ is playing a protective role in the control of skeletal muscle inflammation and fibrosis in the mdx mice.

In regard to B lymphocyte development, IFN-γ participates as a direct B cell-maturing cytokine, driving normal B cell to active Ig secretion (Zhang *et al*. 1999). In this

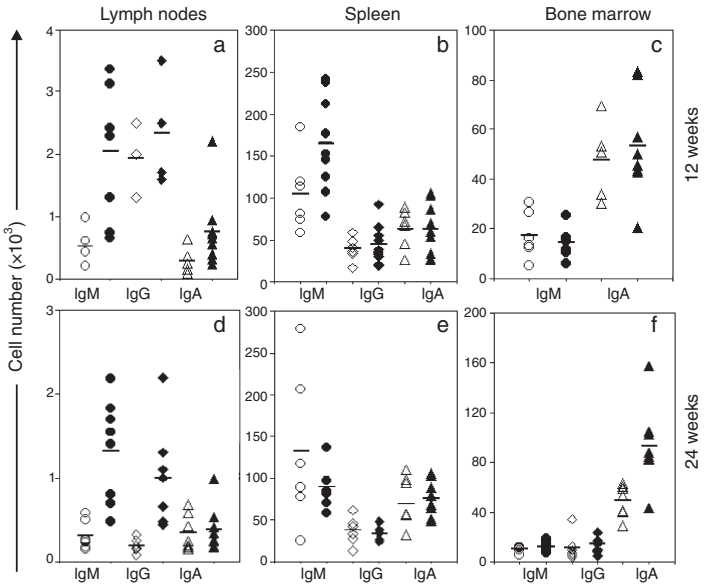

Figure 7. Numbers of Ig-secreting cells in draining lymph nodes (a, d), spleen (b, e) and bone marrow (c, f) of control and mdx mice at 12 weeks (a, b, c) and 24 weeks (d, e, f). Each symbol represents one animal. ● IgM, ◆ IgG and ▲ IgA for mdx and corresponding open symbols for control mice. Bars depict mean values.

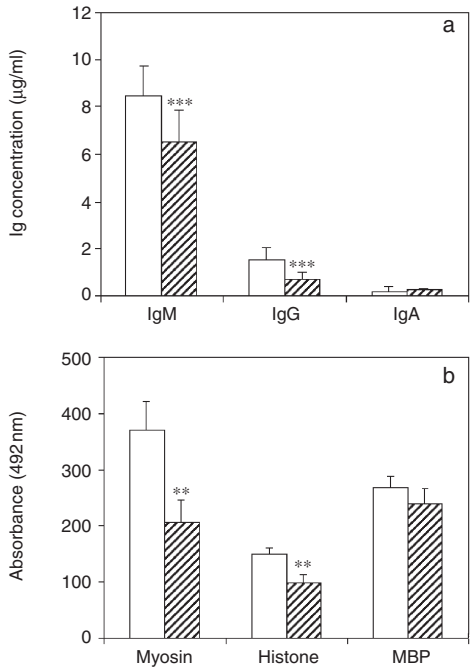

Figure 8. *In vitro* immunoglobulin secretion in the supernatants of lymphocytes derived from axillary and brachial lymph nodes of 12 week mdx mice cultivated in presence of LPS (10 μg/mL). Panel (a) shows IgM, IgG and IgA secretion in C57BL/10 or mdx supernatants. Panel (b) shows antigen reactivity of lymphocyte supernatants. One-hundred μL of protein antigens (10 μg/mL) were used for coating wells of microtitre plates. Supernatant reactivity was assessed with anti-IgM antibodies by direct ELISA. Representative experiments are presented. Results are expressed as mean ± SD of five animals. Similar results were obtained in three separate experiments. □ C57BL/10, ▨ mdx. $^{**}P < 0.001$, $^{***}P < 0.0001$.

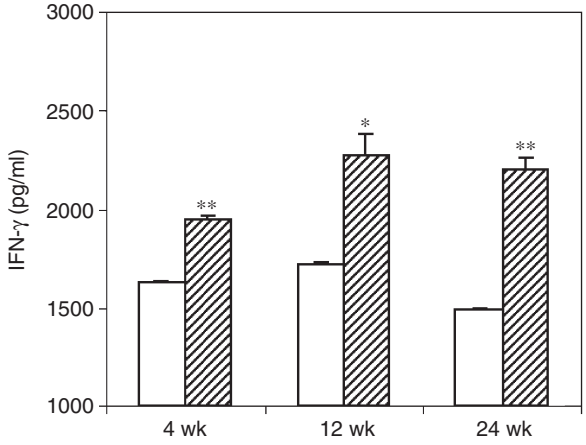

Figure 9. Increased production of IFN-γ by lymphocyte pool from 5 mdx or C57BL/10 mice at ages of 4, 12 and 24 weeks. Lymphocytes were stimulated or not with Con A (5 μg/mL) for 24 h. Results are expressed as mean ± SD of wells. One representative experiment is shown. Similar results were obtained in three separate experiments. □ C57BL/10, ▨ mdx. $^{*}P < 0.05$, $^{**}P < 0.005$.

sense, *ex vivo* experiments showed that regeneration of myonecrosis was accompanied by increased numbers of Ig-secreting cells in the regional lymph nodes and bone marrow of mdx mice after myonecrosis. In keeping with these findings, we observed increased B cell proliferation and formation of germinal centres in the follicles of mdx mice after the period of myonecrosis. The results showing increased numbers of $B220^{+}$ B cells and IgA-secreting cells in the bone marrow of 24 week mdx mice suggest

that activated B cells are migrating from the draining lymph node towards the bone marrow for antibody secretion. Conversely, the poor response of *in vitro* B lymphocyte stimulation might be due to maximal *in vivo* activation of these cells thereby limiting further Ig production. This suggestion is supported by similar *in vitro* studies with cerebrospinal fluid lymphocytes from patients with multiple sclerosis, an inflammatory demyelinating disease of the central nervous system with immune reactivity for myelin antigens (Henriksson *et al*. 1986). Lymphocytes from mdx mice (12 weeks onwards) also produced *in vitro* IgM antibody with lower reactivity for antigens (histone, myosin) known to be released from the lesioned tissue, rather than for unrelated antigens (MBP). During apoptosis, nuclear components (DNA, histone and phosphatidylserine) translocated into membranous blebs may bind to trapped self-antigens and activate complement cascade, which therefore promotes their removal mediated by IgM autoantibodies (Mizoguchi *et al*. 1997). The process of degeneration and removal of necrotic tissue is an importantevent preceding effective muscle regeneration (Pastoret & Sebille 1995). In this regard, the presence of phagocytes and increased IgM production could be promoting removal of apoptotic blebs from myonecrotic tissue and probably blocking activation of autoreactive cells. This would further contribute to the resolution of inflammation and skeletal muscle regeneration. Interestingly, intravenous gamma-globulin therapy of patients with dermatomyositis (IDM), an inflammatory autoimmune muscular disease, attenuates tissue inflammation and improves muscle cytoarchitecture (Amemiya *et al*. 2000).

It can be argued that activation of the immune system in mdx mouse is an epistatic event, secondary to muscular inflammation. However, as opposed to inflammation with accumulation of $CD4^+$, $CD8^+$ T cells in the skeletal muscle in 4-week-old mdx mice, draining lymph nodes showed atrophy during onset and height of myonecrosis. In addition, enlargement of mdx lymph nodes was only evident during mitigation of inflammation. Furthermore, accumulation of T and B lymphocytes persisted for several months. Regardless of whether activation of the immune system should be considered an epiphenomenon or not, the important fact is that increased lymphocyte activation with immunoglobulin and cytokine secretion could be partly responsible for mitigation of inflammation and skeletal muscle regeneration in mdx dystrophic mice. In fact, it is currently accepted that the development of Duchenne-type muscular dystrophy is a net result of interactions between the primary genetic lesion and a set of compensatory responses from a variable genetic–environmental network (Infante & Huszagh 1999).

The study described herein has generated several important findings with particular relevance to a better understanding on the pathophysiology of muscular dystrophy in the mdx mouse. To our knowledge, our results are the first to demonstrate a correlation between lymphocyte activation and resolution of myonecrosis in mdx mice. Taken together, these data suggest that changes occurring within the muscular tissue during distinct phases of Duchenne-type muscular dystrophy are eliciting functional changes in the immune system, which ultimately could be participating in the resolution of inflammation and regeneration of skeletal muscle in the mdx dystrophic mouse.

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