Expression of major histocompatibility complex class I antigens in rat muscle cultures: The possible developmental role in myogenesis
(Interferon γ /monoclonal antibodies/myoblasts)

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ABSTRACT The expression of major histocompatibility complex class I antigens was demonstrated on aneurally cultured rat muscle cells. Myoblasts showed constitutive expression of class I antigens on their cell surfaces. The presence of the antigens was transitory, disappearing as myoblasts fused and differentiated into multinucleate myotubes. Furthermore, antibody against rat class I antigens showed an inhibitory effect on the generation of myotubes during muscle development. Although mature myotubes did not show any detectable levels of class I antigens on their cell surface, soluble factors from concanavalin A-activated spleen cells or interferon γ could induce the expression of class I antigens on muscle fibers. These results suggest that the expression of class I antigens on muscle cells is not only immunologically modulated but also developmentally regulated and that the antigens may play a role in cell recognition and interactions during the fusion process of myogenesis.

Major histocompatibility complex (MHC) class I antigens are polymorphic membrane glycoproteins and are expressed on cells of most tissues. The immunological importance of class I antigens for recognition of target cells by cytotoxic T lymphocytes has progressively emerged (1, 2). Recently, however, some investigators have postulated that the MHC class I molecule is involved in a general process of cell recognition and interactions not limited to the immune response (3, 4).

In normal muscle tissue, neither MHC class I nor class II (IIa) antigens have been demonstrated on muscle fibers (5, 6). In polymyositis and inclusion body myositis, the strong expression of MHC class I antigens has been seen in muscle fibers invaded by T cells (6, 7), but factors capable of triggering the expression of MHC antigens in muscle fibers have not been identified. This expression on muscle fibers potentially allows immunocytes to recognize and interact with muscle cells and may play a role in initiating the immune-mediated muscle fiber injury in inflammatory myopathy. The recent observation that the class I antigens are displayed also on the cell surface of regenerating muscle suggests a possible role of the class I molecule in the process of muscle regeneration (7). Although myoblast fusion is not a prerequisite for differentiation (8), cell fusion is indispensable for the generation of multinucleate myotubes, and the class I molecule might be involved in the fusion process of myogenic cells during muscle regeneration. In support of this hypothesis is the finding that the H-2 gene complex products are functional in modulating fibroblast–fibroblast intercellular adhesion (9).

In this paper we present evidence for the transitory expression of class I antigens on myoblasts in embryonic rat muscle cultures. This observation bears a striking similarity to the expression of Thy-1 antigen on myoblasts in developing muscle (10). Antibodies against rat class I and, to a lesser extent, Thy-1 antigens showed inhibitory effects on the generation of myotubes during myogenesis. In addition, we show that soluble factors from activated spleen cells or interferon γ (IFN-γ) can induce the expression of class I antigens on mature myotubes. These observations suggest that the MHC class I antigens expressed on muscle cells are involved in cell recognition and interactions in myogenesis and are not merely linked to immunity.

MATERIALS AND METHODS

Rats. Lewis rats were obtained from Charles River Breeding Laboratories.

Antibodies. Mouse monoclonal antibodies against rat MHC class I antigens, anti-class I (monomorphic, MRC OX18) (11), anti-C haplotype of class I (MRC O27) (12) against rat Ia antigens, anti-Ia (monomorphic, MRC OX6) (13) against rat Thy-1 antigen, and anti-Thy-1 (monomorphic, MRC OX7) (14) were obtained from Serotec. Mouse monoclonal anti-skeletal muscle myosin antibody (MY-32, Sigma) was used to identify muscle cells in the cultures. Fluorescein isothiocyanate (FITC)- and rhodamine-conjugated, affinity-purified goat anti-mouse IgG, F(ab′)2 fragments (Cappel Laboratories) were used for indirect immunofluorescence.

Preparation and Culture of Rat Muscle. Hindlimb muscles were removed aseptically from 18-day Lewis rat embryos. Muscles were dissected free of bone and skin under a dissecting microscope, minced into small pieces, and digested with collagenase (2.4 mg/ml; CLS, grade II, Worthington) and DNase (0.01 mg/ml; Sigma) in Wyles medium for 2 hr at 37°C. After digestion, the cells were passed through a 20-μm nylon mesh, washed three times with Wyles medium, and suspended in Dulbecco's modified Eagle medium (DMEM, GIBCO) containing 10% fetal calf serum (Hazleton, Lenexa, KS), 50 units of penicillin per ml, and 50 μg of streptomycin per ml. To obtain myogenic cell-enriched suspensions the selective plating procedure (15) was employed. Briefly, 5 × 10^6 cells were plated in a volume of 10 ml of complete medium in 30-cm² plastic tissue culture dishes and incubated at 37°C for 30 min. The unattached cells were collected by gentle aspiration of the medium. Muscle cultures were prepared on glass coverslips (13 mm in diameter) precoated with poly L-lysine (Sigma) at a cell density of 1 × 10^6 per ml and incubated at 37°C in a 5% CO₂ in air humidified atmosphere. The cultures were maintained and fed every 4–5 days with DMEM supplemented with 2.5% fetal calf serum and antibiotics.

Lymphokines. Soluble factors secreted by activated spleen cells were prepared as described elsewhere (16). Briefly, spleen cells from normal adult Lewis rats were filtered

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through a nylon wool column to obtain a T-cell-enriched fraction. The cells were cultured with concanavalin A (Con A, Sigma) at a final concentration of 5 μg/ml for 3 days in DMEM supplemented with 10% fetal calf serum, 2 mM l-glutamine, 1 mM pyruvate, 0.05 mM 2-mercaptoethanol, and antibiotics. The culture supernatant was collected and filtered through a 0.22-μm filter (Con A supernatant) and was used immediately or stored at −70°C.

Rat recombinant IFN-γ was purchased from Genzyme.

**Induction of MHC Antigen on Muscle Culture.** In early culture, 48 hr after plating, cells were examined for spontaneous expression of MHC antigens in developing muscle. At 7–8 days after culture, when numerous multinucleate myotubes undergoing spontaneous contractions were observed, Con A supernatant (2%, 10%, or 25% final concentration) or IFN-γ (1, 10, or 100 units/ml) was added to each culture. A set of three culture coverslips was prepared in each experiment. Control cultures included those in which no lymphokines or only Con A (concentration same as test culture) was added. One, 3, 5, and 11 days after incubation, the cultures were examined by indirect immunofluorescence for cell surface expression of MHC antigens.

**Indirect Immunofluorescence.** Double immunofluorescence staining was performed in two steps to detect class I and Ia antigens and myosin on muscle cells. In step 1, to detect the class I and Ia antigens on cell surfaces, immunofluorescent staining was carried out on living cells grown on coverslips. Cultures were washed with warm Hanks' balanced salt solution (pH 7.4) and incubated with heat-inactivated normal goat serum (1:20 dilution) at room temperature for 20 min to block nonspecific binding of secondary antibody. After washing, the coverslips were incubated with monoclonal antibody OX18 (1:100 dilution) or OX6 (1:100 dilution) at room temperature for 45 min. The optimal dilutions of these antibodies were determined by their ability to stain Lewis rat lymphocytes obtained from spleen cells stimulated with Con A (5 μg/ml) for 3 days. After washing, coverslips were incubated with FITC-conjugated goat antimouse IgG, F(ab')2 fragment at a 1:50 dilution for 30 min at room temperature. The cells were washed and fixed in ethanol with 5% acetic acid for 1 hr at −20°C. The coverslips were mounted on glass slides in glycerol, examined, and photographed with an Olympus Vanox microscope with epi-illumination and optics for FITC and phase contrast.

In step 2, the coverslip was carefully removed from its glass slide, rinsed with cold distilled water for 30 min, again fixed with 10% formalin for 2 hr at room temperature, and then air-dried at 4°C overnight. The culture was treated with anti-skeletal myosin antibody (1:200 dilution) for 2 hr at room temperature. After washing, the coverslip was incubated with rhodamine-labeled goat anti-mouse IgG at a 1:50 dilution for 1 hr at room temperature, rinsed, and remounted in glycerol. The previously photographed fields were rephotographed with rhodamine optics to identify muscle cells labeled with the anti-myosin antibody.

A set of three culture coverslips was stained in each experiment. Control slides stained with secondary antibody alone showed no significant level of fluorescence.

**Blocking Assay.** We sought to determine the effect of anti-class I and anti-Thy-1 antibodies on the fusion process in myogenesis. OX18, OX7, and OX27, which recognizes only the C haplotype of rat class I antigens, were used for this assay. Fifty microliters of monoclonal antibody solution (ascites fluid) was dialyzed extensively against 0.15 M phosphate-buffered saline (pH 7.4) at 4°C and heat-inactivated at 56°C for 30 min before use.

![FIG. 1. Immunofluorescent staining of embryonic rat muscle cultures at different stages during myogenesis with rhodamine-conjugated anti-skeletal muscle myosin antibody (MY-32) and FITC-conjugated antirat class I antibody (OX18). (a–c) Cluster of myoblasts aligned for fusion, 2-day culture. (a) Phase-contrast. (b and c) Stained with MY-32 (b) and OX18 (c), viewed with rhodamine and FITC optics, respectively. (d–f) Myoblasts (arrows) apposed to a young myotube, 3-day culture. (d) Phase-contrast. (e and f) Stained with MY-32 (e) and OX18 (f). Class I antigens were expressed on the myoblasts but were undetectable on the surface of a newly formed myotube. (g and h) Multinucleate myotube, 10-day culture. (g) Phase-contrast. (h) Negative OX18 staining. (Bars = 30 μm.)
Muscle cultures were prepared as described above. One hundred microliters of the myogenic cell-enriched suspension was plated onto a glass coverslip (13 mm in diameter) at a density of $1 \times 10^5$ per ml in DMEM supplemented with 10% heat-inactivated fetal calf serum and antibiotics. At 12 hr after plating, the medium was replaced by 0.5 ml of medium containing OX18 (at a dilution of 1:100, 1:500, or 1:1000), OX27 (1:100 or 1:500), or OX7 (1:100 or 1:500). The cultures were refed once with freshly prepared test medium after 48 hr. Control cultures included those in which either no antibodies were added to contained only normal mouse IgG (2 $\mu$g/ml, Serotec). In addition, parallel cultures were established by adding Con A (20 $\mu$g/ml), which has been reported to be an inhibitor of myoblast fusion (17). After incubation for 5 days, the cells were fixed in ethanol with 5% acetic acid for 1 hr at $-20^\circ$C and were stained trichromatically. Some cultures were double-stained with Gomori's trichrome and anti-skeletal muscle myosin antibody as described above. The double staining showed that the cytoplasm of multinucleate myotubes and some mononucleate cells stained purple with Gomori's trichrome, and these were the same cells labeled with anti-myosin antibody (Fig. 3a and b). Thus, we used Gomori's trichrome staining to identify and count the number of nuclei and muscle cells, especially multinucleate myotubes that stained purple-green, in the cultures. Cell nuclei and myotubes with three or more nuclei were counted in four randomly selected fields at $\times 200$ (0.25 mm$^2$ per field). Degree of fusion (fusion index) was determined by counting nuclei within the myotubes of three or more nuclei and dividing by the total number of nuclei in each field. Data obtained from triplicate samples in three separate experiments ($n = 9$) were analyzed statistically by Student's $t$ test.

RESULTS

Constitutive Expression of MHC Antigens. In early cultures 24–48 hr after plating, a large number of myoblasts labeled with anti-skeletal myosin antibody were observed (Fig. 1b). At this time, myoblasts, particularly the cells aligned for fusion, were stained with anti-MHC class I antibody (Fig. 1a–c). However, as fusion progressed and myotubes developed, the level of class I antigen expression decreased on newly formed myotubes, with just a few nuclei exhibiting a few speckles of fluorescence on their cell surfaces (Fig. 1d–f). At a later stage in muscle differentiation, 7–8 days after plating, cultures displayed large multinucleate myotubes (Fig. 1g) that did not show any detectable levels of class I antigens on cell surfaces (Fig. 1h). Ia antigens were not detected on muscle cells, and nonmyogenic cells, presumably fibroblasts, also remained negative for either class I or Ia antigens throughout the 3-week culture period.

FIG. 2. In vitro induction of MHC antigen expression on embryonic rat muscle cultures incubated with supernatant from Con A-activated spleen cells or rat recombinant IFN-γ. (a) Phase-contrast view of the culture treated with 25% Con A supernatant for 3 days. (b) The same field stained with anti-class I antibody OX18. (c and d) Phase-contrast and OX18 staining with fluorescein optics, respectively. The cells were incubated with IFN-γ at 100 units/ml for 24 hr. (e and f) Phase-contrast and fluorescein (anti-rat Ia antibody, OX6) optics, respectively. The culture was incubated with IFN-γ at 100 units/ml for 5 days. Note negative staining on the multinucleate myotube. Flat, irregular-shaped cells (presumably fibroblasts) were stained with OX18 (b and d) and OX6 (f) antibodies. (Bars = 20 $\mu$m.)
**Induction of MHC Antigens by Lymphokines.** Con A supernatant and rat IFN-γ induced class I antigens on matured myotubes (Fig. 2 a–d) but did not induce detectable levels of Ia antigens on myotubes (Fig. 2 e and f) throughout the culture period. The minimum requirement for induction of class I antigens on myotubes was 25% Con A supernatant or IFN-γ at 10 units/ml for 24 hr. On the other hand, neither Con A supernatant nor IFN-γ, at any concentrations tested, could induce the expression of Ia antigens on myotubes for up to 11 days' incubation period.

These lymphokines also induced both class I and Ia antigen expression of nonmyogenic cells (presumably fibroblasts). Class I antigens were detectable on nonmyogenic cells after 2 hr of stimulation with these lymphokines, whereas longer exposure (>3 days) and a higher concentration of IFN-γ (100 units/ml) were required to induce Ia antigens on cell surfaces of nonmyogenic cells (presumably fibroblasts).

**Effect of Anti-Class I and Anti-Thy-1 Antibodies on Myogenesis.** OX18 and OX7 antibodies were used to determine the role, if any, of class I or Thy-1 antigen in modulating cell fusion during myogenesis. As shown in Fig. 3 and Table 1, these antibodies had an inhibitory effect on formation of multinucleate myotubes. Cultures treated with OX18 or OX7 displayed a significantly lower degree of fusion (fusion index) than did control cultures. The inhibitory effect of anti-class I antibody on myotube formation appeared to be stronger than that of anti-Thy-1 antibody. Indeed, OX8 antibody treatment at a concentration of 1:100 resulted in a marked decrease in total number of myotubes, whereas the inhibitory effect was relatively mild in cultures incubated with OX7 at the same concentration (Table 1). Furthermore, at a lower concentration (1:500), OX8 inhibited myotube formation to approximately the same degree as Con A (20 μg/ml), which has been reported to inhibit myoblast fusion (17). There was no significant difference in fusion index between the cultures incubated with normal medium and normal mouse IgG (2 μg/ml). In addition, OX27 antibody, which recognizes the C haplotype of rat class I antigens, did not inhibit the generation of myotubes in cultures of Lewis (RT-11 haplotype) origin.

**DISCUSSION**

The present study demonstrated the constitutive expression of class I antigens on myoblasts in rat embryonic muscle cultures. The presence of the antigen was transitory, being expressed on cell surfaces of myoblasts but disappearing as myoblasts fused and differentiated into multinucleate myotubes. This observation seems to be analogous to the presence of MHC class I antigens on regenerating muscle cells in human skeletal muscle (7) and their absence in normal adult muscle (5, 6). In normal muscle development, it has been reported that cytoplasmic fusion is not a prerequisite for muscle differentiation (8). However, cell fusion is indispensable for generation of multinucleate myotubes. Thus, the transient appearance of class I antigens on myoblasts and its absence of multinucleate myotubes raise the possibility that the antigens are functional in normal muscle development, especially at the time of myogenic cell fusion. Lesley and Lennon (10) have shown that there is a transitory expression of Thy-1 antigen on myoblasts of rat myogenic cell line (L6) as well as in embryonic rat muscle cultures. They suggested that Thy-1 antigen plays a role in cell differentiation. Indeed, there is a striking similarity in the transitory expression of class I and Thy-1 antigens on developing muscle, and it may be that in myoblasts the expression of class I and/or Thy-1 antigens serves to modify or modulate cell–cell interaction in the process of myogenesis.

![Fig. 3. Effect of Con A and OX18 and OX7 antibodies on myotube formation. Cultures were derived from hindlimb muscle of 18-day rat embryos. Culture conditions were the same as those cited in the legend of Table 1. Cultures were fixed 5 days after plating and stained with Gomorri's trichrome. Cells with their cytoplasm stained purple-green with trichrome stain (A) were the same cells as those labeled with anti-skeletal muscle myosin antibody MY-32 (B). (C) Control culture containing several multinucleate myotubes. (D) Culture treated with Con A (20 μg/ml) showing closely packed mononucleate cells but no apparent myotube formation. (E) OX18 (1:100 dilution) strongly inhibited the formation of multinucleate myotubes. (F) Culture treated with OX7 (1:100). The inhibitory effect on myotube formation appeared to be less than that of OX18. (Bars = 50 μm.)](https://example.com/fig3.png)
Table 1. Effect of anti-class I and anti-Thy-1 antibodies on myogenesis

<table>
<thead>
<tr>
<th>Culture treatment</th>
<th>No. of myotubes*</th>
<th>Fusion index†</th>
<th>% inhibition‡</th>
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<tr>
<td>None</td>
<td>25.3 ± 4.6</td>
<td>17.9 ± 1.7</td>
<td>0</td>
</tr>
<tr>
<td>Normal mouse IgG</td>
<td>22.4 ± 3.2</td>
<td>16.3 ± 2.3</td>
<td>8.9</td>
</tr>
<tr>
<td>Con A</td>
<td>10.0 ± 3.6</td>
<td>7.7 ± 1.4</td>
<td>56.9</td>
</tr>
<tr>
<td>(20 µg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OX18 dilution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100</td>
<td>4.6 ± 2.6</td>
<td>5.5 ± 0.6</td>
<td>69.3</td>
</tr>
<tr>
<td>1:500</td>
<td>11.4 ± 3.4</td>
<td>6.4 ± 0.8</td>
<td>64.2</td>
</tr>
<tr>
<td>1:1000</td>
<td>19.6 ± 4.1</td>
<td>11.1 ± 0.9</td>
<td>70.8</td>
</tr>
<tr>
<td>OX27 dilution</td>
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<td></td>
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</tr>
<tr>
<td>1:100</td>
<td>26.9 ± 5.4</td>
<td>16.6 ± 1.0</td>
<td>7.2</td>
</tr>
<tr>
<td>1:500</td>
<td>27.1 ± 4.7</td>
<td>17.6 ± 0.8</td>
<td>1.7</td>
</tr>
<tr>
<td>OX7 dilution</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1:100</td>
<td>14.1 ± 2.7</td>
<td>8.7 ± 1.6</td>
<td>51.4</td>
</tr>
<tr>
<td>1:500</td>
<td>22.6 ± 4.3</td>
<td>13.2 ± 0.9</td>
<td>62.3</td>
</tr>
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</table>

*Number of myotubes with three or more nuclei per mm².
†Data are presented as the mean ± SD of triplicate samples in three separate experiments (n = 9).
‡Fusion index (% of fusion) was determined by counting the nuclei within myotubes of three or more nuclei and dividing by the total number of nuclei.
§Degree (%) of inhibition was calculated by following formula: % inhibition = [(fusion index in control (mean) – fusion index in test sample (mean))/(fusion index in control (mean))] × 100.

In this respect, our study showed that antibodies directed against rat class I and Thy-1 antigens had inhibitory effects on formation of multinucleate myotubes in embryonic muscle cultures. Cell recognition (specific adhesion) and interactions during the cytoplasmic fusion process of myoblasts are believed to be mediated by specific macromolecular components such as glycoproteins on the cell membrane (18, 19). Thus, the inhibition observed with anti-class I and, to a lesser extent, anti-Thy-1 antibodies appears to be due to the binding of these antibodies to cell surface glycoproteins. It is not clear whether this inhibition is due to the specific blocking of glycoprotein(s) essential for the fusion process or to the ability of the antibody to cause gross changes in the distribution of cell surface molecules, such as capping (20), which prevent sufficient intercellular contact for cell fusion. In fact, many extra- and intracellular factors influence myogenic cell differentiation and formation of myotubes (8, 21), and several agents have been known to inhibit myoblast fusion (17, 22–24). However, the observations that class I and Thy-1 antigens seem to be transiently expressed on the cell surfaces of myoblasts and that the antibodies against these antigens showed an inhibitory effect on myoblast fusion suggest that these antigens are developmentally regulated and may play a role in myoblast fusion and myotube formation.

Although mature myotubes did not show any detectable levels of class I antigens on their cell surfaces throughout the 3-week culture period, soluble factors from activated lymphocytes or IFN-γ alone did induce the expression of class I, but not La, antigens on muscle fiber in vitro. The consistent expression of MHC class I antigens on muscle fibers has been observed in some inflammatory myopathies (6, 7). In these diseases, immunocytochemical studies have shown that the damaged muscles display the heavy infiltration of activated T lymphocytes (25). Thus, on the basis of our findings, it seems likely that the secretion of soluble factors or lymphokines from activated T cells results in the induction of class I antigens on muscle fibers, rendering them susceptible to lysis by cytotoxic T cells in inflammatory myopathy.

The present study has focused on the expression of MHC class I antigens on muscle cells in vitro. Our findings indicate that the expression of class I antigens on muscle cells is not only immunologically modulated but also developmentally regulated. The results also suggest that in muscle development the class I antigens might play a role in modulating intercellular adhesion during the cytoplasmic fusion process of myoblasts. The transitory expression of class I molecules on myoblasts warrants further investigation of the mechanism that involves class I antigens during myogenesis.

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