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NOTES

The Basal Lamina Is a Physical Barrier to Herpes Simplex Virus-Mediated Gene Delivery to Mature Muscle Fibers

JOHNNY HUARD,[1]† W. GREGORY FEERO,[1] SIMON C. WATKINS,[2] ERIC P. HOFFMAN,[1] DAVID J. ROSENBLATT,[3] AND JOSEPH C. GLORIOSO[1]*

Departments of Molecular Genetics and Biochemistry, Biomedical Science Tower, School of Medicine,[1] and Department of Cell Biology and Physiology,[2] University of Pittsburgh, Pittsburgh, Pennsylvania 15261, and Muscle Cell Biology Group, MRC Clinical Research Centre, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 0NN, United Kingdom[3]

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A major impediment to successful implementation of gene therapy for treatment of muscular dystrophy is the restricted infectivity of mature muscle fibers with viral vectors. This phenomenon has been observed with adenovirus vectors and more recently with herpes simplex virus type 1 (HSV-1)-based vectors. Here we report findings of morphological studies designed to experimentally determine the mechanism underlying the rapid reduction in vector-mediated gene delivery concomitant with the maturation of muscle fibers. Using immunohistochemistry and confocal microscopy, we have colocalized HSV-1 and collagen IV, a major component of the basal lamina, in HSV-1-injected muscles and determined that the virus penetrates and expresses a transgene (*lacZ*) in muscle fibers of newborn animals but cannot efficiently penetrate adult myofibers. This was observed in normal as well as in immunocompromised animals, suggesting that the lack of adult myofiber transduction is not a result of an immune response and clearance of the viral vector. Since heparan sulfate proteoglycan, the initial attachment receptor for HSV-1, was shown to be preserved during the maturation of the myofibers by immunofluorescence assay and HSV-1 was able to infect isolated, viable myofibers in vitro, we suggest that the low-level HSV-1 transduction of mature myofibers is not a consequence of the loss of viral attachment sites on the surfaces of mature muscle fibers. Rather, our results indicate that the mature basal lamina acts as a physical barrier to HSV-1 infection of adult myofibers. This conclusion was further supported by the finding that HSV-1 displayed an intermediate level of transduction in mature *dy*/*dy* muscle which is defective for normal basal lamina formation. Together, these experiments suggest that efficient HSV vector transduction in mature skeletal muscle requires methods to permeabilize the basal lamina.

The muscular dystrophies include some of the most common and debilitating of the heritable metabolic disorders. The underlying genetic defect is now known for most of these, and gene therapy is an intensive area of research for future treatment of these disorders. For example, Duchenne muscular dystrophy (DMD) affects 1 of 3,500 males and is caused by dystrophin deficiency in the membrane cytoskeleton of muscle fibers (5, 10, 22). Patients show progressive muscle weakness and die near the end of the second decade of life unless mechanically ventilated (17). The high mutation rate of the gene precludes genetic carrier screening as an effective method of decreasing disease incidence, making development of novel therapeutics imperative.

Gene therapy via recombinant viral vectors is currently the focus of much DMD research (14). However, the use of viral vectors for gene delivery to skeletal muscle is hindered by a number of technical problems. Current retroviral vectors, for example, do not transduce postmitotic muscle fibers since they require dividing cells for integration and expression (9). Adenovirus vectors have been limited by the virus' small gene-carrying capacity, immune-mediated elimination of vector-transduced cells, and its limited infectivity of myotubes and muscle cells in animals older than 2 weeks (1–3, 12, 15, 16, 21). Recently, we reported that herpes simplex virus type 1 (HSV-1) can efficiently infect and express a reporter gene in myoblasts and myotubes in vitro (11). In addition, a high degree of HSV-1-mediated in vivo gene delivery was observed in newborn muscle, demonstrating that this vector can efficiently infect immature muscle fibers at a lower titer (10^6 to 10^7 PFU) than that necessary for a similar number of transduced muscle fibers using adenovirus vectors (10^8 to 10^9 PFU). However, while intramuscular injection of replication-defective HSV-1 efficiently transduced muscle fibers in newborn mice, only a few muscle fibers in adult animals were infected (11).

The differential transducibility between newborn and adult muscle fibers has been observed for both adenovirus and HSV-1 in several animal species (2, 11, 13). This suggests a common mechanism is responsible for limiting adult fiber infectivity. One possible impediment to account for this loss in viral infectivity might involve the maturation of the basal lamina surrounding muscle fiber and interfiber connective tissue, which thereby acts as a physical barrier to viral penetration. To test this possibility, we used immunofluorescence to visualize the fate of injected HSV-1 particles in newborn and adult

* Corresponding author. Mailing address: Department of Molecular Genetics and Biochemistry, E1246 Biomedical Science Tower, University of Pittsburgh, School of Medicine, Pittsburgh, PA 15261.

† Present address: Musculoskeletal Research Center, Department of Orthopedic Surgery, University of Pittsburgh School of Medicine and Children's Hospital of Pittsburgh, Pittsburgh, PA 15213.

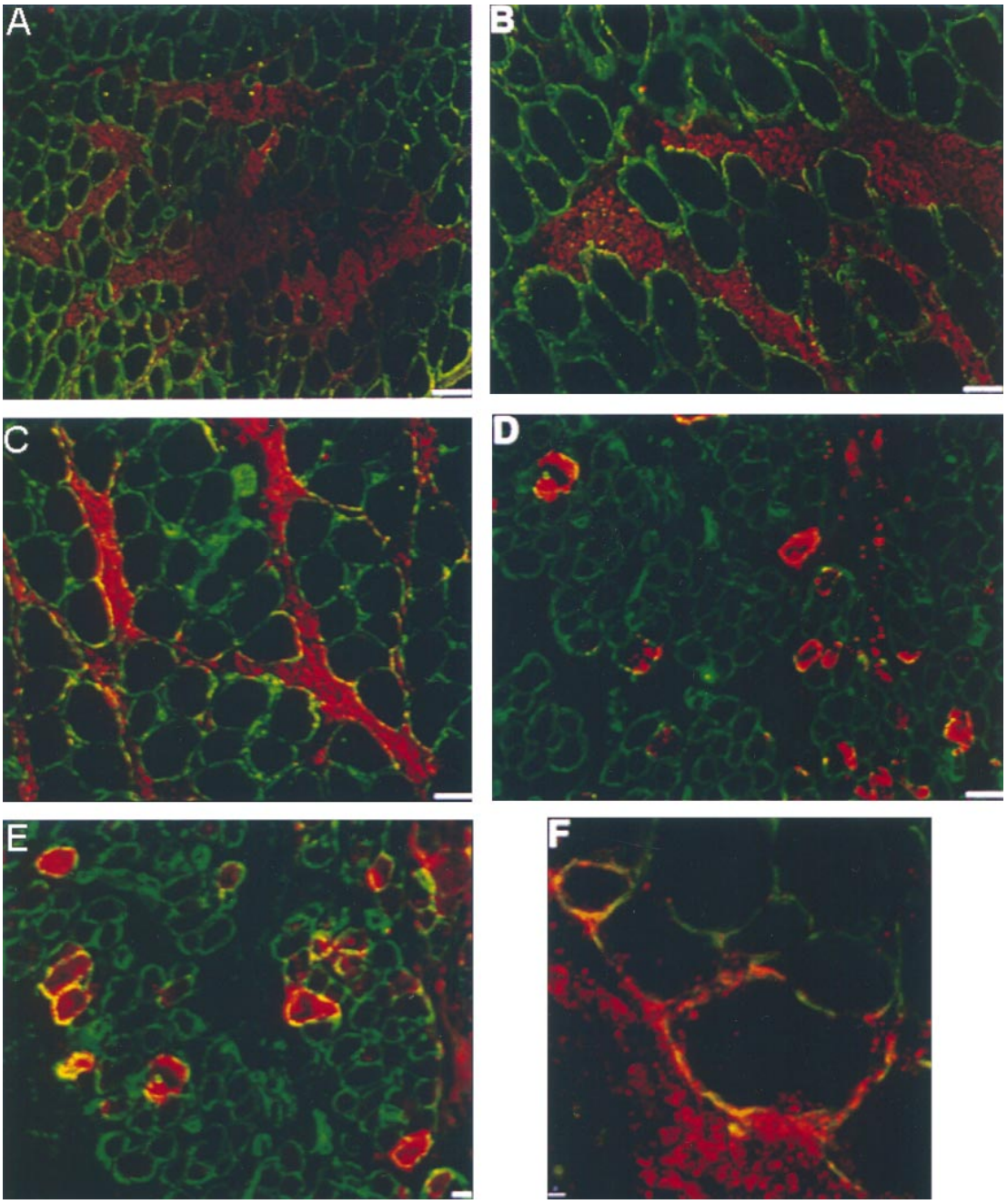

FIG. 1. The colocalization of collagen IV (fluorescein; green) and HSV-1 infiltration (Cy3; red) by immunohistochemistry revealed that HSV-1 remains outside the mature muscle fibers in adult muscles of normal (A and B) and immunodeficient nude mice (C) injected with HSV-1. In contrast, HSV-1-injected newborn muscles resulted in penetration of a high number of muscle fibers by the viral vector (D and E). Moreover, confocal microscopy analysis revealed entry of viral particles through the basement membrane in newborn muscle fibers (F). Bars, 100 μm (A); 50 μm (B and C); 10 μm (D, E, and F).

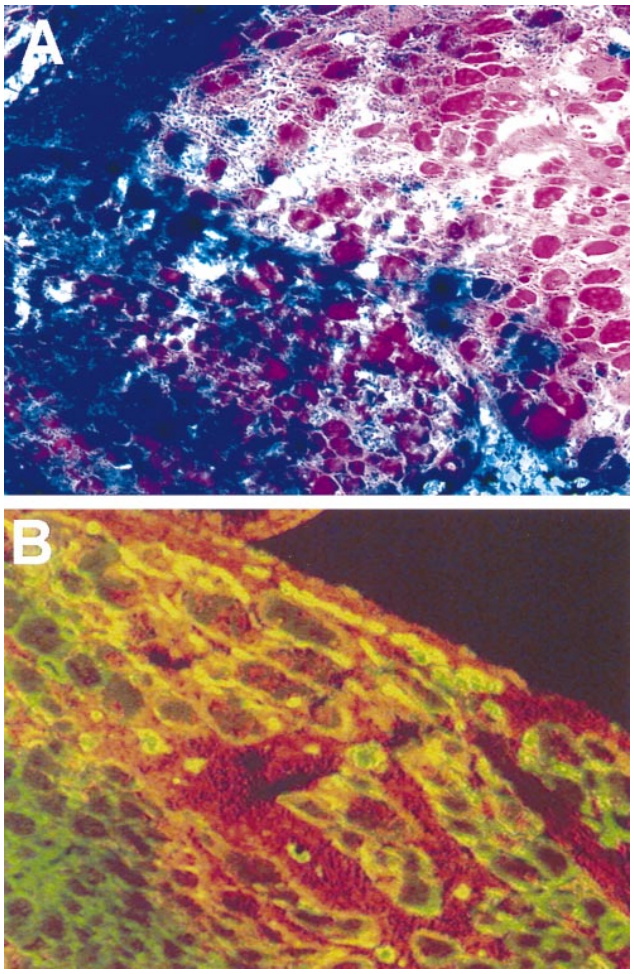

FIG. 2. The colocalization of collagen IV (fluorescein; green) and HSV-1 infiltration (Cy3; red) showed a higher level of HSV-1 penetration in *dy*/*dy* mature myofibers (B) than in control, age-matched normal myofibers (Fig. 1A, B, and C). This resulted in an intermediate level of transduction in *dy*/*dy* mature muscle fibers (A). Original magnification, ×100.

mouse muscles by colocalization of HSV-1 antigens and collagen IV, a major component of the basement membrane.

The techniques to prepare, propagate, and generate high-titer stocks of replication-defective HSV-1 have already been described (11). The *Escherichia coli lacZ* (β-galactosidase) gene under transcriptional control of the human cytolamegalovirus immediate-early (IE) gene promoter was recombined into the *TK* locus of an HSV-1 replication-defective mutant (*d*120) with both copies of the essential IE ICP4 gene deleted (8). The defective viral vector was propagated using an ICP4 complementing (E5) cell line (8). The viral vector titers used in these experiments ranged from 10^7 to 10^9 PFU/ml. Newborn (2 to 4 days postpartum) and adult (2 months old) ICR outbred, nude, and SCID mice (Harlan Sprague-Dawley, Indianapolis, Ind.) anesthetized with metophane (Pittman-Moore, Mundelein, Ill.) were injected percutaneously with 1×10^6 to 5×10^6 PFU of HSV-1 vector into the gastrocnemius muscle at an approximate depth of 2.0 mm, using a Hamilton syringe. The perforated skin was kept closed with forceps for a short period of time to avoid backtracking of the injected virus suspension. At 1 day postinjection, injected muscles were dissected and frozen in isopentane precooled in liquid nitrogen. Cryostat sections (10 μm) were assayed for reporter gene expression (11) or processed for immunohistochemistry for collagen IV and HSV-1. Frozen sections (10 μm) of injected muscles were fixed with 100% cold acetone for 2 min, dried, and incubated with phosphate-buffered saline (PBS)–10% horse serum for 30 min to reduce nonspecific antibody binding. Sections were incubated with goat anti-HSV-1 antibody (1:100; Chemicon International Inc., Temecula, Calif.) and rabbit anti-collagen IV (1:100; Bio-Design Int., Kennebunkport, Maine) overnight at room temperature (RT°C). Sections were washed with PBS and then incubated with biotinylated rabbit anti-goat (1:250; Boehringer-Mannheim Corp., Indianapolis, Ind.) and fluoresceinated horse anti-rabbit (1:100; Boehringer-Mannheim) antibodies for 1 h at RT°C. Sections were rinsed with PBS and incubated with streptavidin-Cy3 (1:1,000; Sigma Chemical Co., St. Louis, Mo.) for 45 min at RT°C. The sections were then mounted in gel mount (Biomeda, Foster City, Calif.) and analyzed by fluorescence microscopy (Microphot-FXA; Nikon, Melville, N.Y.). Confocal analysis was performed using a Multiprobe 2001 confocal microscope (Molecular Dynamics, Sunnyvale, Calif.). By optically sectioning tissue, laser confocal microscopy optimizes the resolution available by light microscopy. This technique was used to visualize the penetration of virus into individual muscle fibers. To ensure maximal X-Y spatial resolution, sections were scanned at 1,024 by 1,024 pixels, 60× objective, 0.2-μm pixel size, using two-color image collection (514-nm laser, 535 primary beam splitter, 564-nm secondary beam splitter, 540 df30-nm final barrier to collect the fluorescein isothiocyanate image and 590-nm final barrier to collect the Cy3 image). Image planes throughout the depth of the specimen were collected at intervals of 0.5 μm, and the midplane section was extracted.

Our data showed that HSV-1 did not efficiently penetrate the mature basal lamina in adult muscle fibers of normal (Fig. 1A and B) and immunodeficient nude mice (Fig. 1C). In contrast, HSV-1 efficiently infected newborn myofibers (Fig. 1D and E) and confocal microscopy analysis of infected tissue revealed the presence of HSV-1 virions that had penetrated immature muscle fibers (Fig. 1F).

Based on the above results, we hypothesized that the basal lamina acts as a barrier to HSV infection of muscle fibers in adult mice and further, that perturbation of this barrier will result in an enhanced ability to infect mature muscle. To test this hypothesis we compared the ability of HSV-1 to infect mature fibers in adult normal and age-matched *dy*/*dy* mice. The *dy*/*dy* mouse is an animal model for autosomal recessive muscular dystrophy that is defective for normal basal lamina synthesis. It has been demonstrated that merosin, a predominant laminin isoform in the basal lamina of striated muscle, is deficient in the dystrophic *dy*/*dy* mouse (homozygous), which causes muscle membrane instability (4, 20, 23, 24). However, other components of the basal lamina, such as laminin B, type V collagen, and fibronectin, are normally expressed in this animal model (*dy*/*dy*). HSV-1 vector injection of the gastrocnemius muscles of *dy*/*dy* mouse at 2 months of age revealed an increased level of transduction (Fig. 2A) relative to age-matched normal myofibers (Fig. 3B). Furthermore, a higher level of penetration of HSV-1 virions in adult myofibers from *dy*/*dy* mice (Fig. 2B) was seen in comparison to that observed in normal adult age-matched control animals (Fig. 1A and B).

In view of these findings, it became important to rule out several other mechanisms that might play a role in the observed differential transducibility of newborn versus adult fibers, namely, the immune-mediated clearance of infectious virus or infected cells and/or the loss of viral receptors on adult muscle fibers. To examine the role of the immune system, HSV-1 carrying the β-galactosidase gene was injected into

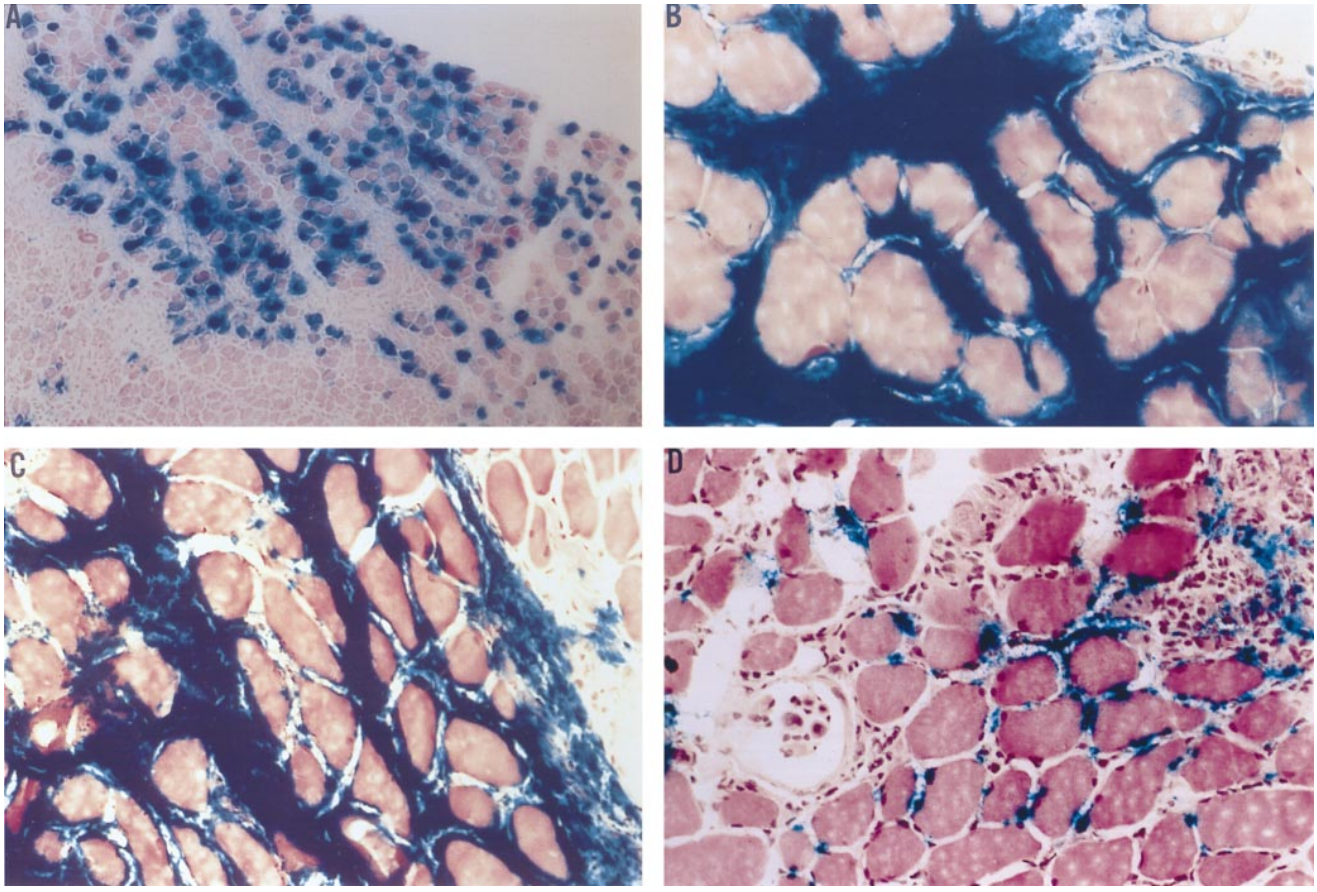

FIG. 3. HSV-1 vector-mediated β-galactosidase gene expression in muscle in vivo. A high number of transduced muscle fibers were observed in newborn injected muscle (A). However, the same viral solution only poorly transduced adult muscle fibers of normal (B), immunodeficient nude (C), and SCID (D) mice. HSV-1 transduced numerous cells in the interstitial space, but adult myofibers remained largely untransduced by the vector. Original magnifications, ×40 (A); ×200 (B); ×100 (C and D).

adult immunodeficient (nude and SCID) mouse muscles. As was the case with immunocompetent adult mice (Fig. 3B), HSV-1 did not transduce adult nude (Fig. 3C) or SCID (Fig. 3D) mouse muscle fibers. This was in marked contrast to the high level of transduction observed in muscles of newborn animals injected with the same viral preparations (Fig. 3A).

To determine whether the HSV-1 receptors are present throughout muscle maturation, we first examined, in vitro, HSV infection of myoblasts, myotubes, and single, viable, isolated muscle fibers prepared by enzymatic dissociation of the soleus muscle from 14-day-old mice. The L6 rat myoblast cell line was grown in proliferation medium (minimum essential medium plus 20% fetal bovine serum) for 5 to 7 days. Differentiated myotubes were produced by using fusion medium (minimum essential medium plus 2% fetal bovine serum) for 5 days. Unfused myoblasts in the differentiated cell culture were removed through treatment with 10^{-5} M cytosine arabinoside (Sigma, St. Louis, Mo.) for 2 days. Myoblast and myotube cell cultures were then rinsed twice in Earl's balanced salt solution (Gibco Lab, New York, N.Y.) and incubated with viral solution for 1 h at a multiplicity of infection of 1. The viral solution was removed and cells were incubated with fresh medium for 48 h and fixed in 1.5% gluteraldehyde (1 min). β-Galactosidase activity was revealed as previously described (11). Single fibers from 14-day-old mice were isolated from the soleus muscle as previously described (18) for studies of HSV infection. Fibers were plated in 24-well plates coated with Matrigel (1 mg/ml; Collaborative Biomedical Products, Bedford, Mass.) in 500 μl of Dulbecco modified Eagle medium containing 10% horse serum, 1% chicken embryo extract, 2% L-glutamine, 1% penicillin, and 1% streptomycin. Viable muscle fibers were infected at 24 or 96 h after plating, using 10^5 or 10^6 PFU added directly to the medium. Fiber viability was assessed by using trypan blue exclusion as well as by observing fiber remodeling over time. Fibers were fixed at 24 h postinfection with 1% glutaraldehyde for 1 min and also stained for β-galactosidase activity (11).

As an alternate approach to detect HSV-1 receptors on mature muscle, newborn and adult myofibers were tested for the presence of heparan sulfate proteoglycan, a major attachment for HSV-1 (19), using immunohistochemistry. Briefly, the frozen sections (10 μm) of injected muscles were fixed with 100% cold acetone and incubated with 10% horse serum for 60 min to reduce nonspecific antibody binding. Sections were incubated with rat anti-heparan sulfate proteoglycan monoclonal antibody (1:100; Chemicon International Inc.) for 60 min at RT°C. Sections were washed three times in PBS and then incubated with biotinylated sheep anti-mouse (1:200; Dimension Lab.) for 1 h. Sections were then rinsed with PBS and incubated with streptavidin-Cy3 (1:1,000; Sigma) for 45 min at RT°C. The sections were then mounted in gel mount (Biomeda) and analyzed by fluorescence microscopy.

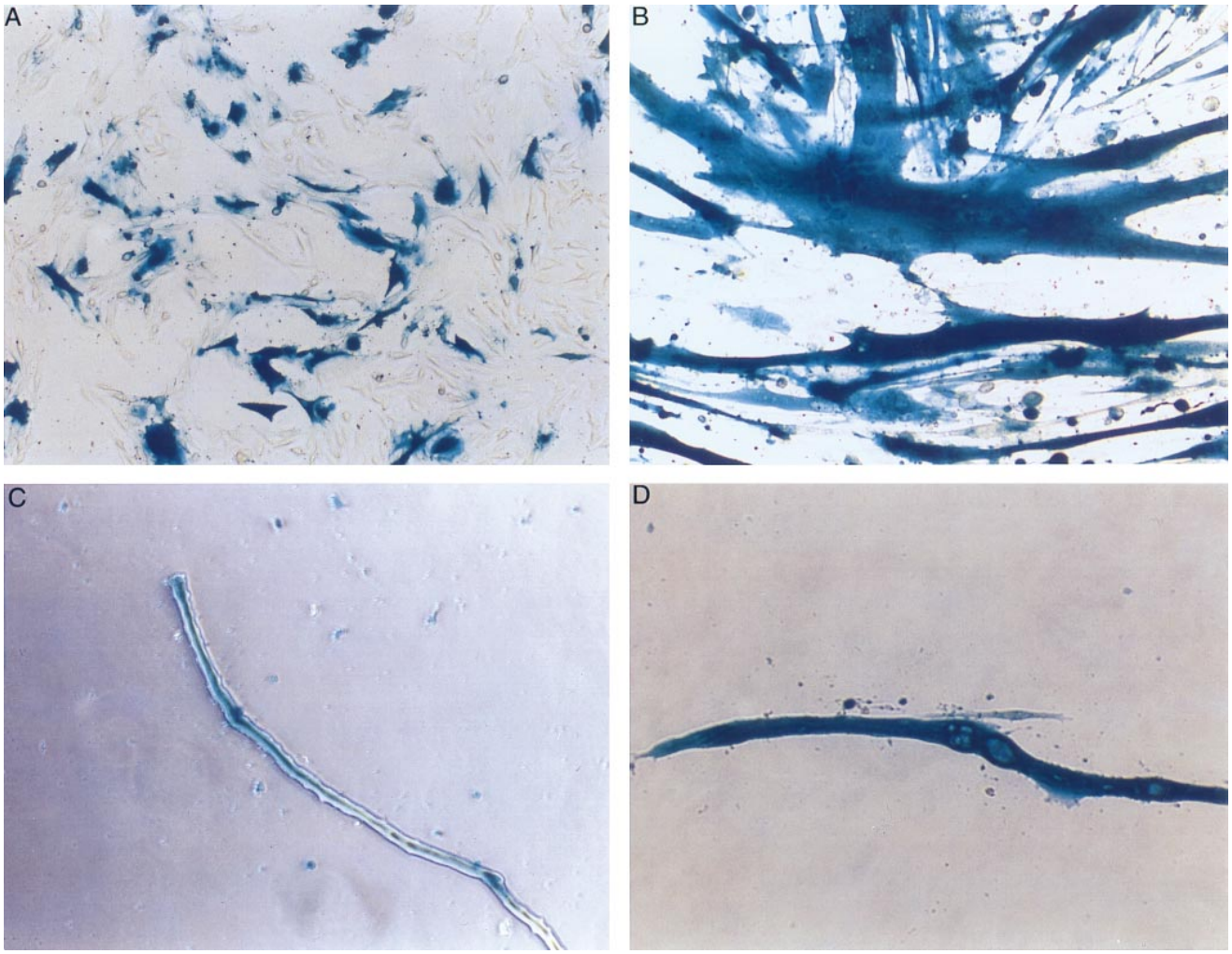

FIG. 4. HSV-1-mediated β-galactosidase expression in cultured muscle cells. Infection of L6 rat myoblasts with recombinant HSV-1 at a multiplicity of infection of 1 showed high transduction efficiency (A). Cultures of myotubes formed by the fusion of the L6 myoblasts were also efficiently transduced by HSV-1 (B). Viable muscle fibers dissociated enzymatically from the mouse soleus of 14-day-old mice, kept in cell culture for 24 h (1/3 of the fibers infected, C) and 96 h (5/6 of the fibers infected, D), were also transduced by HSV-1 vector, demonstrating the presence of viral receptors on 14-day-old muscle fibers. Original magnifications, ×200 (A and D); ×100 (B and C).

The results of these experiments showed that HSV-1 efficiently infected myoblasts and myotubes in vitro (Fig. 4A and B). Further, we were able to infect isolated, viable, single myofibers with HSV-1, demonstrating that the virus was capable of transducing these explanted highly differentiated muscle cells at 24 (Fig. 4C) and 96 (Fig. 4D) h after fiber isolation. Finally, by using a monoclonal antibody against heparan sulfate proteoglycan, the attachment receptor for HSV (19), we observed a uniform distribution of this protein in newborn (Fig. 5A and B) and adult myofibers (Fig. 5C and D). These findings suggest that the inability of HSV-1 to transduce normal adult myofibers in vivo is unlikely to be the result of a lack of viral receptors on fully differentiated myofibers.

In summary, these experiments provided four lines of evidence implicating the mature muscle basal lamina as a physical barrier to viral infection of adult skeletal muscle fibers. First, immunolocalization of viral antigens and active β-galactosidase showed that, in vivo, HSV-1 effectively penetrates and transduces newborn myofibers but is unable to enter adult muscle fibers, appearing to be retained outside of the fiber basal lamina. Second, the lack of entry and transduction of adult muscle is seen in immunocompromised mice (nude and SCID), suggesting that the immune system does not preclude adult muscle fiber transduction with HSV-1. Third, our observation that HSV-1 infects myoblasts, myotubes, and single isolated fibers from 14-day-old mice in vitro suggests that viral receptors are retained during the maturation of myofibers. In addition, the uniform distribution of heparan sulfate proteoglycan observed in newborn and adult myofibers demonstrated that the initial attachment receptor for HSV-1 is present in mature muscle fibers. Furthermore, ultrastructural analysis of developing muscle cell has shown that immature myofibers in vivo as well as young myotubes in vitro, which can be efficiently transduced with HSV-1 (11), have a rudimentary basal lamina which surrounds muscle fibers in vivo (6, 7). Finally, we have observed that HSV-1 can efficiently penetrate and transduce adult muscle fibers of *dy*/*dy* mice in vivo. These animals have a defect in the basal lamina structure due to merosin deficiency (4, 20, 23, 24). Muscle fibers isolated from adult *dy*/*dy* mice were also efficiently transduced with HSV-1 in vitro (data not shown).

In an attempt to achieve efficient transduction of adult nor-

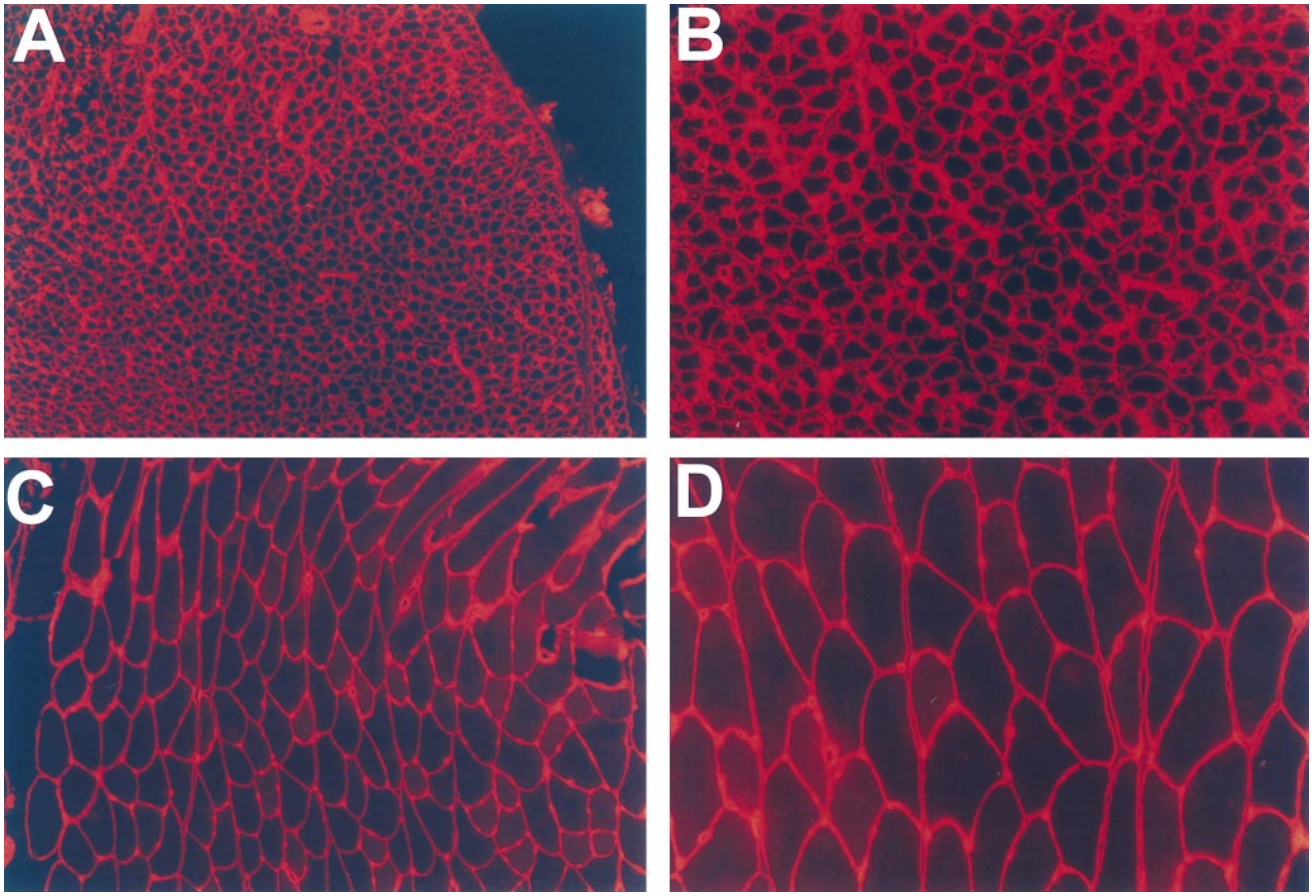

FIG. 5. Localization of heparan sulfate proteoglycan distribution in newborn and adult myofibers using immunohistochemistry. A uniform distribution of heparan sulfate proteoglycan was observed in immature (A and B) as well as in mature muscle fibers (C and D). Original magnifications, ×100 (A and C); ×200 (B and D).

mal skeletal muscle with HSV-1, we are currently exploring biochemical methods of permeabilizing mature muscle basal lamina in order to enhance infection. Success with this approach should make gene transfer to adult muscle feasible and aid the development of gene therapy approaches for the treatment of muscular dystrophies.

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