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# Susceptibility of Skeletal Muscle to Coxsackie $A_2$ Virus Infection: Effects of Botulinum Toxin and Denervation

Abstract. *Coxsackie A viruses can infect denervated but not innervated mature skeletal muscles. The role of synaptic transmission in preventing susceptibility to Coxsackievirus infection was studied by surgically denervating leg muscles of mice or injecting the muscles with botulinum toxin to block quantal release of acetylcholine. Control muscles were injected with heat-inactivated toxin. Subsequent injection of Coxsackie $A_2$ virus resulted in extensive virus replication and tissue destruction in the denervated and botulinum toxin–treated muscles, while the control muscles showed only minimal changes. This suggests that the susceptibility of skeletal muscle to Coxsackievirus infection is regulated by synaptic transmission.*

Coxsackie A viruses are capable of infecting skeletal muscles, but only under certain circumstances. Immature (*1*) or denervated (*2*) muscles are susceptible to infection, while mature innervated muscles are relatively resistant. At present, neither the factors that determine susceptibility to Coxsackievirus infection nor the role of innervation in regulating muscle resistance to infection are understood. Certain other properties of skeletal muscles are regulated by acetylcholine (ACh) released from nerve terminals (*3*). We therefore wondered whether muscle cell resistance to Coxsackie A infection might also be regulated by synaptic transmission. To investigate this possibility, we used botulinum toxin to block the quantal release of ACh from motor nerve terminals in mouse muscles and then determined their susceptiblity to infection with Coxsackie $A_2$ virus.

A total of 132 adult (30 to 40 g) female Swiss mice (Buckberg) were surgically denervated or injected with botulinum toxin or given control injections of heat-inactivated botulinum toxin. Unilateral denervation of the lower limb was performed in 44 mice by excising a 5- to 6-mm length of the proximal sciatic nerve from the mid-thigh. In 48 mice the gastrocnemius muscles were surgically exposed and 40 μl of type A crystalline botulinum toxin freshly diluted in sterile Ringer solution ($4.5 \times 10^{-6}$ mg/ml) was injected into each muscle through a 30-gauge needle. (Surgical exposure was found to facilitate accuracy of injection in these small muscles and did not produce any artifactual effects in controls.) Another 40 mice received control injections of 40 μl of heat-inactivated (100°C for 30 minutes) botulinum toxin ($4.5 \times 10^{-6}$ mg/ml) into their gastrocnemius muscles. One week later all the muscles were directly injected with $2.6 \times \log_{10}$ $TCID_{50}$ (50 percent tissue culture infective dose) of Coxsackie $A_2$ virus suspended in 40 μl of Ringer solution (*4*). At 1.5, 3.0, 4.5, and 6.0 days after the virus injections, groups of rats were killed and their gastrocnemius muscles were excised. One hundred five muscles were assayed for the content of infectious virus (*5*) and 27 muscles were quick-frozen and prepared for histological examination (*6*).

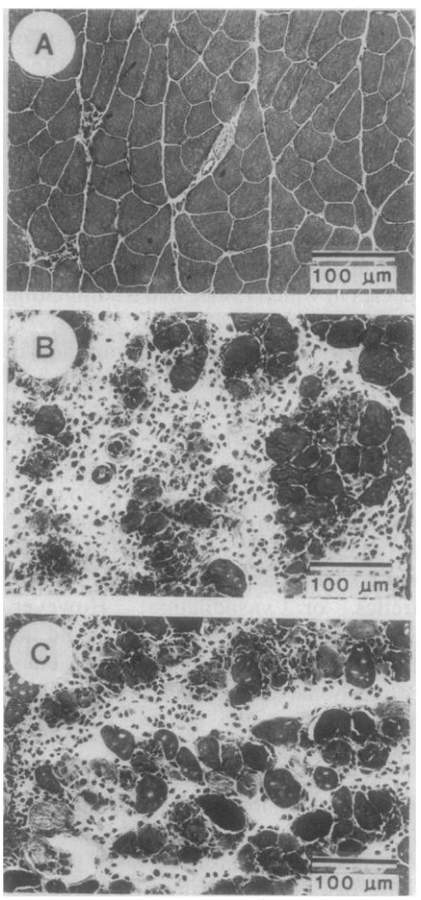


Fig. 1. Effects of various treatments on susceptibility of skeletal muscles to Coxsackie $A_2$ virus infection. Light photomicrographs show transverse sections of gastrocnemius muscles 4.5 days after inoculation. The muscles had been treated 7 days previously with (A) heat-inactivated botulinum toxin, (B) surgical denervation, or (C) botulinum toxin ($0.18 \times 10^{-9}$ g). Some artifact due to freezing is seen in the fibers in (C) and does not represent any effect of toxin or virus on the muscle. The control muscle cells (A) show only minimal changes while the denervated and botulinum-treated muscles show cellular infiltration and fiber necrosis.

In general, both denervated and botulinum toxin–treated muscles developed severe infection, while control muscles showed only mild changes. The progression of infection was slightly but significantly more rapid in denervated muscles than in botulinum-treated muscles (Table 1). At 1.5 days after inoculation, muscles from denervated and botulinum-treated animals had between 10 and 100 times more infectious virus than control muscles. At 3 days the virus concentration in denervated muscles reached its maximum, 1000 times greater than that of the original viral inoculum. By 4.5 days the concentration had decreased slightly. The concentration of virus in botulinum-treated muscles continued to rise through day 4.5, reaching levels nearly 1000 times greater than that of the original inoculum. Control muscles showed less than a tenfold increase in virus throughout the experimental period. Virus titers in all three experimental groups declined after day 4.5.

The histological changes in infected skeletal muscles followed a similar pattern, though lagging somewhat behind the changes in virus titer (Figs. 1 and 2). At 1.5 days denervated and botulinum-treated muscles showed a few focal areas of infiltration, with occasional degenerating fibers, while control muscles had only rare foci of minimal cellular infiltration. At 3 days denervated muscles showed widespread diffuse or multifocal cellular infiltration and fairly extensive

fiber degeneration. Botulinum-treated muscles had multifocal cellular infiltrates and some fiber degeneration of less severe degree than in denervated muscles. Control muscles had only focal infiltrates of restricted extent. At 4.5 and 6 days fiber degeneration had progressed to massive necrosis in both denervated and botulinum-treated muscles. Control muscles showed focal infiltration involving 0 to 15 percent of the muscle cross-sectional area, and necrosis was absent or minimal. Figure 1 shows typical sections of the three groups of muscles 4.5 days after Coxsackievirus inoculation. Figure 2 shows representative higher magnification views of histological changes occurring 1.5 to 6 days after injection of Coxsackievirus into botulinum toxin–treated muscles.

Our results indicate that treatment of skeletal muscle with botulinum toxin greatly increases its susceptibility to infection with Coxsackie $A_2$ virus. The peak virus titers and muscle destruction were similar to those observed in the denervated condition. However, infection progressed somewhat more slowly in botulinum-treated muscles, resulting in virus production and histological changes comparable to those seen 1.5 days earlier in denervated muscles. By contrast, control muscles injected with inactivated toxin remained relatively free of infection, thus excluding the possibility that injection trauma might account for the effect of botulinum toxin.

The simplest interpretation of our findings is that the effect of botulinum toxin on synaptic transmission is responsible for the observed change in muscle properties. The type A toxin used is a pure crystalline protein whose only known action is the highly specific and prolonged blockade of quantal ACh release at cholinergic nerve terminals. Impulse-dependent ACh release is virtually eliminated, and more than 90 percent of spontaneous quantal release of ACh is blocked by botulinum toxin (*7*). Prolonged treatment with botulinum toxin does not alter axonal transport, damage nerve terminals, or have any direct effect on skeletal muscles (*8–10*). Our experiments do not exclude the possibility that botulinum toxin might interfere with some other neurotrophic substance released with ACh, but previous studies on the neural regulation of other properties of skeletal muscles (*11*) make such a mechanism less likely. Because the effect of botulinum toxin on muscle susceptibility to Coxsackievirus infection was somewhat less pronounced than that of denervation ($P < 0.02$ at 3.0 days), it is probable that some neural influence remains incompletely blocked in botulinum-treated nerves. Various factors have been postulated, including substances carried by axonal transport (*12*), persistent quantal and nonquantal ACh release (*11, 13*), and nerve-muscle membrane interaction. Current evidence suggests that the incompleteness of botulinum toxin's denervation effect is due to its failure to block nonquantal ACh release (*14*). This large fraction of ACh transmission has been found to play an important contributory role in the neural regulation of certain other properties of skeletal muscle (*11*).

Our finding adds a new denervation-like change induced by botulinum toxin to those previously documented, includ-

Table 1. Effects of neuromuscular treatment on Coxsackie $A_2$ virus titer. Values (means ± standard errors) are $\log_{10}$ $TCID_{50}$ units generated per milliliter of gastrocnemius muscle. The number of animals per group is shown in parentheses. At 1.5, 3.0, and 4.5 days after inoculation the muscles were assayed for virus content (*8*).

| Treatment | Days after virus inoculation | | |
|---|---|---|---|
| | 1.5 | 3.0 | 4.5 |
| Control | 2.55 ± 0.13 (11) | 3.50 ± 0.18 (15) | 3.50 ± 0.11 (9) |
| Denervation | 4.30 ± 0.40 (9)* | 5.85 ± 0.14 (16)* | 5.16 ± 0.15 (8)* |
| Botulinum toxin | 3.58 ± 0.24 (10)† | 5.20 ± 0.20 (16)* | 5.40 ± 0.19 (11)* |

*Significantly different from corresponding control value at $P < 0.001$ (Student's *t*-test). †$P < 0.002$.

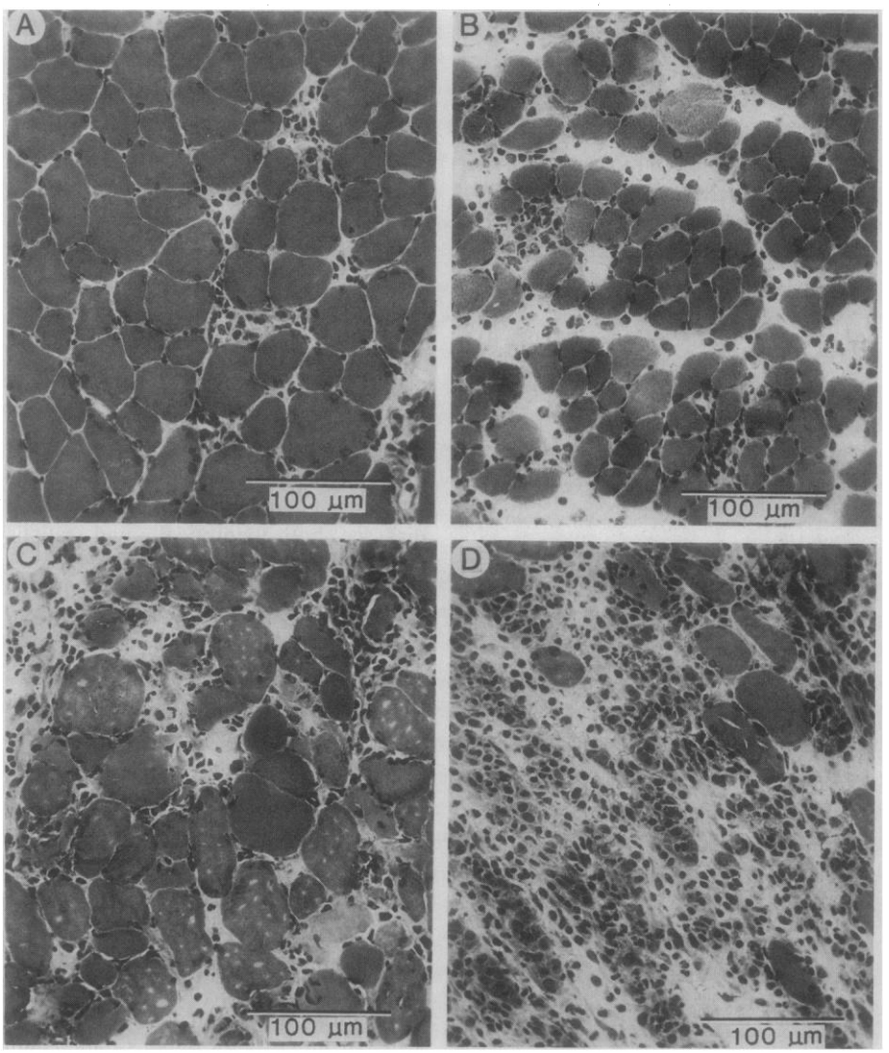


Fig. 2. Development of Coxsackie $A_2$ virus inflammatory myositis in botulinum toxin–treated muscle. Light photomicrographs show transverse sections of adult gastrocnemius muscle taken (A) 1.5, (B) 3.0, (C) 4.5, and (D) 6.0 days after inoculation. Muscle fibers in different animals varied slightly in size; these differences are not due to any effect of the toxin or virus. All muscles had been treated for 7 days with botulinum toxin ($0.18 \times 10^{-9}$ g). Note the increasing severity of infiltration and necrosis with increasing time after infection.

ing skeletal muscle atrophy (*10–15*), fibrillation (*16*), increased numbers of extrajunctional ACh receptors (*17*), and other effects (*9, 18*). Although our studies indicate that surgical and pharmacological denervation have profound effects on the susceptibility of skeletal muscle to Coxsackievirus infection, they do not specify the nature of the changes that permit infection at the muscle cell level. The susceptibility of muscle cells must depend on their ability to support the entire sequence of steps in the virus replication process, including virus binding, entry, uncoating, and synthesis and release of viral components. Studies with poliovirus (another related picornavirus) have demonstrated that host cell susceptibility occurs relatively early in the infectious cycle (before synthesis of viral components) (*19*). Further studies are needed to determine which step in the infection sequence is limiting in innervated muscle and is altered after denervation to permit Coxsackie $A_2$ virus infection.

CLIFFORD G. ANDREW*
DANIEL B. DRACHMAN
ALAN PESTRONK
OPENDRA NARAYAN
*Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

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4. Coxsackie $A_2$ virus stock (American Type Culture Collection lot Cox 82VR ET L1184A 792162) was prepared by two serial passages through Swiss mice less than 24 hours old. The mice were inoculated intraperitoneally and subcutaneously with 75 μl of a 1:10 dilution of the original virus preparation in phosphate-buffered saline. Seven days later the mice were killed and their limb muscles were homogenized with a Potter-Elvehjem tissue grinder and centrifuged for 10 minutes at 2500*g*. The supernatant was inoculated into a second litter of neonatal mice. When moribund (1.5 to 2 days), these animals were killed and their limb muscles were homogenized and centrifuged as above. The supernatant solution was collected, assayed, and stored at −70°C until being used as stock virus for infection of mice.
5. In the Coxsackievirus assay rhabdomyosarcoma cells (supplied by R. L. Crowell) were cultured in Eagle's minimum essential medium containing sodium bicarbonate (*p*H 7.3) and 10 percent fetal bovine serum at 37°C in air with 5 percent $CO_2$. Microtiter plates were seeded with $1 \times 10^4$ cells per well and grown to 90 percent confluence over 24 hours. Each muscle to be assayed was homogenized in 2.0 ml of medium containing 0.2 percent fetal bovine serum in a mortar cooled to −70°C. Serial tenfold dilutions of the homogenate were made and 0.25 ml of each dilution was added to quadruplicate microtiter wells containing rhabdomyosarcoma cells. Plates were examined at 4 to 6 days for a cytopathic effect. The dilution yielding 50 percent positive and 50 percent negative responses was estimated by interpolation of the data, and the resultant virus titer was expressed in multiples of $TCID_{50}$ units per milliliter of muscle homogenate. Statistical analysis by Student's *t*-test for two samples was used to compare results for different groups of animals.
6. After excision the muscles were pinned to a stiff card at resting length and quick-frozen in isopentane cooled with solid $CO_2$. Multiple 6-μm sections were made of each muscle in a cryostat. After staining with hematoxylin and eosin the slides were coded and examined microscopically.
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* To whom requests for reprints should be addressed.

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## Residential Firewood Use

In their report on the use of residential firewood in the United States, Lipfert and Dungan (*1*) used the New England Fuelwood Study (*2*) results in developing an equation to estimate national firewood consumption by air pollution control regions. Although the degree of fuelwood-induced air pollution is partially a function of the concentration of fuelwood use in any given region, I strongly disagree, both theoretically and statistically, with their estimation method. I will limit my discussion to the following two points: (i) the issue of causality is ignored and (ii) the extrapolation of New England household woodburning habits to the rest of the country is questionable.

The Lipfert-Dungan estimation equation states that the amount of residential fuelwood burned per degree day is dependent on population density. They also imply in their reference 5 (*1*) that this variable, since it is important in air quality analysis, is also important in explaining the number of cords of wood a household might consume. While population density may be an appropriate index for air quality analysis, Lipfert and Dungan fail to substantiate their claim that it is important in predicting household fuelwood consumption. By not discussing the causal relations between cords of wood consumed, climatic conditions (degree days), and population density, their extrapolation of New England data to the country as a whole is not defendable.

The New England Fuelwood Survey found a number of socioeconomic variables that were significant in explaining residential fuelwood consumption patterns. These included cost of conventional energy in comparison with wood energy, woodburning apparatus used, percentage of owner-occupied households, and household location in relation to firewood supplies and wood-using industries. These variables were not included by Lipfert and Dungan. Their unsuccessful attempt to explain the divergence of a number of state consumption patterns from their best-fit line as well as a recently completed nationwide residential fuelwood consumption survey (*3*), which estimated that average consumption of fuelwood per household is greater in the southwest region than in the north central region, provide ample evidence that there are serious weaknesses in Lipfert and Dungan's model specifications. The equation is not only inadequate but also inappropriate for estimating national fuelwood consumption rates by region.

MARK R. BAILEY
*Economic Research Service, U.S. Department of Agriculture, Broomall, Pennsylvania 19008*