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Duchenne Muscular Dystrophy: Functional Ischemia Reproduces Its Characteristic Lesions

Abstract. *The highly characteristic early and midstage histological lesions of Duchenne dystrophy were reproduced experimentally in the rat by the combination of a vascular abnormality, aortic ligation, which does not affect the structure of the intramuscular blood vessels, and the humoral vasoactive substance 5-hydroxytryptamine. Neither ligation nor injection of 5-hydroxytryptamine alone causes changes in the muscle fibers. This result establishes the possibility of a similar combined mechanism for a nonstructural ischemia pathogenesis in Duchenne dystrophy. The proposed pathogenesis is contrary to the generally held idea that the cause is an intrinsic abnormality of muscle fiber metabolism.*

Pseudohypertrophic muscular dystrophy of childhood (Duchenne dystrophy) is a progressive myopathy inherited as an X-linked recessive trait. Its pathogenesis remains unknown. Most investigators consider that it is due to an intrinsic biochemical defect of muscle fibers. A different pathogenesis has recently been hypothesized on the basis of histologic evidence in muscle biopsies of patients with Duchenne dystrophy. It is proposed that the damage to muscle fibers may be secondary to ischemia caused by abnormality of small vessels of the arterial tree (*1*). The early lesion highly characteristic of Duchenne dystrophy, but not of the other muscle diseases bearing the name "dystrophy," is a small group of muscle fibers all in about the same stage of necrosis or regeneration surrounded by a field of normal fibers. In the least affected regions, abnormal fibers occur singly or grouped two or three together, as they do in clinically normal carriers of the trait. The midstage lesion is characterized by a disproportionately large increase of endomysial connective tissue between muscle fibers that are small, enlarged, and sometimes with internal nuclei; often there is but sparse evidence of active muscle fiber necrosis or regeneration in the region. Lesions morphologically identical to those of early and midstage Duchenne dystrophy have been produced in rabbits after embolization of intramuscular arteries and arterioles by injection of 20- to 80-μm dextran beads once or at intervals (*2, 3*). That experimental structural blockage demonstrated the possibility that Duchenne dystrophy may be due to an occlusive vascular abnormality. However, the major shortcoming of that model is that such structural vascular occlusions of intramuscular blood vessels are not a consistent feature of Duchenne dystrophy.

We have now overcome that objection by experimentally reproducing the characteristic pathology of early and middle stages of Duchenne dystrophy by combining a subthreshold vascular abnormality, which does not affect the structure of intramuscular blood vessels, with a subthreshold quantity of the humoral vasoactive substance 5-hydroxytryptamine (5-HT, serotonin).

Seventy male Osborne-Mendel rats were used for these studies. Fresh frozen sections of soleus, gastrocnemius, and quadriceps were stained by modified trichrome, reduced nicotinamide-adenine dinucleotide–tetrazolium reductase, myofibrillar adenosine triphosphatase, thionine, crystal violet, acridine orange, and alkaline phosphatase methods (*4, 5*). All slides were read without knowledge of the experimental history of the animals.

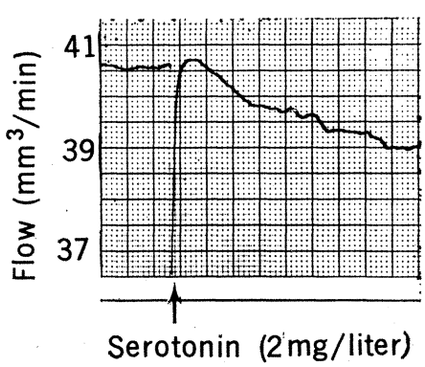

Fig. 1. Effect of 5-HT on the flow rate of perfusate in isolated rat limb. Large baseline deflection represents change in infusion from the control balanced salt solution to one containing 2 mg of 5-HT per liter. Time, 20 seconds between heavy vertical bars.

In animals of the first group, after amputation and removal of the skin, preparations of normal hind limb were perfused with 5-HT creatinine sulfate (2 to 50 mg/liter in a balanced salt solution) or with balanced salt solution alone by the method of Macri and Brown (*6*). The 5-HT caused a decrease in the flow rate of the perfusate in the femoral artery, the onset of action being immediate and well sustained until use of the drug was discontinued (Fig. 1). Balanced salt solution alone had no effect.

To the second group of normal rats daily intraperitoneal injections of 5-HT in doses varying from 20 to 75 mg/kg were given for periods of 3 weeks to 3 months. No weakness developed. No significant histopathological changes were found in these animals. Specifically, the muscle fibers remained normal in size; there was no tendency for central placement of nuclei, and the sarcoplasm showed no abnormalities.

The third group of animals, anesthetized with pentobarbital, underwent ligation of the abdominal aorta below the origin of the renal arteries at the level of the right iliolumbar artery. They were killed 5 to 30 days after ligation. No weakness developed. Normal muscle predominated. Only very rarely was a necrotic fiber found, and occasional fibers had increased internal nuclei.

The fourth group of animals underwent the same aortic ligation and then received a single intraperitoneal injection of 5-HT (20 mg/kg) on the fifth postoperative day. A number of these animals were killed 7 and 30 days after injection. The remaining animals in the group received multiple injections of 5-HT (20 mg/kg) at 5-day intervals and were killed 30 days after the initial injection. All of these animals had weak hind limbs. The skeletal muscle pathology in this group was striking. In the animals killed 7 days after a single injection of 5-HT there were discrete groups of 3 to 20 muscle fibers either undergoing necrosis and phagocytosis

(Fig. 2a) or regeneration (Fig. 2, b and c) surrounded by histologically normal muscle fibers. This pattern was identical to that characteristic of early Duchenne dystrophy (*1, 2*). Sometimes there were larger focal lesions of 20 to 100 fibers or more, in which the interior fibers had preferential loss of mitochondrial oxidative enzymes and nuclei with preservation of myofibrils and myofibrillar adenosine triphosphatase; at the periphery the fibers were being phagocytosed and regeneration was beginning. This is the characteristic picture of ischemic infarction of muscle (*7*). In minimally affected regions the abnormal fibers were often single or grouped in twos or threes among normal fibers (Fig. 2c). This is identical to what is seen in muscle from minimally affected regions of patients with Duchenne dystrophy and in carriers of the disease (*1, 2*). In animals that received multiple injections and were killed after 30 days, there was proliferation of endomysial connective tissue around individual muscle fibers, many of the fibers being smaller than normal, some being enlarged, and some containing internal nuclei (Fig. 2d). This is identical to the midstage change in Duchenne dystrophic muscle (*1, 2*).

Staining of the capillaries and small arterioles for alkaline phosphatase was absent in the areas of focal muscle necrosis and regeneration, in contrast to the normal staining of these vessels in the surrounding uninvolved areas. Neither normal nor abnormal muscle fibers were stained by the alkaline phosphatase method. The identical loss of alkaline phosphatase from capillaries and small arterioles was noted in the focal abnormal regions of embolized rabbit muscle (*2*), and similar loss was reported to precede degeneration of muscle fibers in chickens with hereditary muscular dystrophy (*5*).

The effect of 5-HT varies depending on the site of the vasculature tested as well as the species (*8*). In our study, the fact that a single intraarterial injection of 5-HT markedly and rapidly diminished the flow rate in the isolated rat limb preparation suggests a vasoconstrictor action. Nevertheless, repeated intraperitoneal injections of 5-HT into normal rats did not produce morphologic abnormalities of muscle. This confirms the findings of Selye (*9*) and raises a question about another study said to show minimal muscle abnormalities after the intraperitoneal injection of 5-HT into mice (*10*). Selye demonstrated that ligation of the abdominal aorta in rats is well tolerated but predisposes the muscle to develop lesions from several agents such as cold injury or 5-HT. Our animals with ligated aortas did not develop focal muscle necroses and subsequent fibrosis until they were given 5-HT. These lesions were exactly like those of rabbits whose intramuscular arteries and arterioles were embolized (*2*), which suggests causation by ischemia in both models. The morphological lesions in the muscle of both models were identical to the early and midstage lesions in patients with Duchenne muscular dystrophy and to lesions in carriers of the disease.

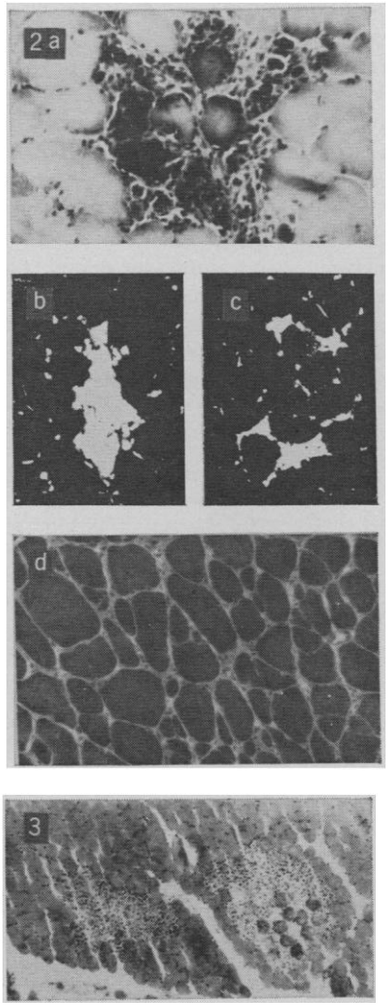

Fig. 2. Changes in skeletal muscle produced by aortic ligation and injection of 5-HT (20 mg/kg), seen 7 days after one injection (a–c) and 30 days after repeated injections (d) of 5-HT. (a) Focal area of necrosis and phagocytosis of muscle fibers (dark) surrounded by normal fibers (modified trichrome, X195). (b) Positive orange fluorescent staining (central white area) of the cytoplasm indicates increased RNA content of regenerating muscle fibers located in a focal group; surrounding muscle fibers are unstained (black) except for the green fluorescent staining of their nuclei (small white dots) (acridine orange, X190). (c) Positive orange fluorescent staining of the cytoplasm of four individual regenerating muscle fibers (two upper right, two lower left) (acridine orange, X190). (d) Moderate increase of endomysial connective tissue surrounding small and large muscle fibers of rounded contour (modified trichrome, X145).

Fig. 3. Two focal lesions of skeletal muscle produced by aortic ligation and norepinephrine (3.25 mg/kg) and seen 7 days after a single injection (X75).

No histologic changes were detected in the intramuscular blood vessels of the second, third, or fourth groups of our experimental animals, which indicates that the ischemia was caused by a functional rather than a structural blockage of the intramuscular blood vessels. Apparently only a portion of the intramuscular blood vessels were functionally inadequate in the present model (group four) because the necrotic muscle fibers were grouped focally in fields of normal fibers, as they are in Duchenne dystrophy (*1–3*). From a completely different approach, especially related to studies of limb blood flow, Demos has also favored an abnormality of the microcirculation as the pathogenesis of Duchenne dystrophy (*11*).

Our study has established that in patients with Duchenne muscular dystrophy and in carriers, as in our experimental model with the identical pattern of abnormality in the muscles, there could be a minimal nonstructural vascular abnormality which only becomes apparent in the presence of a humoral vasomotor agent, such as 5-HT. The humoral agent itself could be normal or be minimally abnormal in amount or type but detrimental only in the presence of abnormal skeletal muscle blood vessels. In patients with Duchenne dystrophy and in carriers such vascular and humoral abnormalities would be genetically determined and would preferentially involve skeletal muscle of certain regions.

After submitting this report, we were able to experimentally produce the identical clinical weakness and histological lesions with another vasoactive amine, norepinephrine (Fig. 3). As in experiments with 5-HT, the rats with ligated aortas received intraperitoneal injections of 3.25 to 3.5 mg of nor-

epinephrine per kilogram once or on succeeding days. In rats without ligatures, that dose caused no adverse effects.

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Cucumber Beetle Resistance and Mite Susceptibility Controlled by the Bitter Gene in Cucumis sativus L.

Abstract. *Antibiotic and nonpreference mechanisms are related in cucumber through the action of the* bi *gene and the absence of cucurbitacins. Cucurbitacins attract cucumber beetles and cause feeding whereas they have an antibiotic effect on two-spotted mites.*

Insects cause tremendous economic losses to agriculture, both directly and indirectly from the cost of control measures. Chemical insecticides cause additional concern because they contribute to environmental pollution. The development of cultivars that are resistant to insect attack would seem to be an ideal method of control. Three mechanisms of insect resistance are tolerance, antibiosis, and nonpreference (*1*).

Nonpreference is due to the absence of an attractant or the presence of a repellant. Antibiosis refers to adverse effects on the biology of the insect. These mechanisms are generally assumed to be independent, but we find they are related and that resistance to specialized insect pests (cucumber beetles) is related to susceptibility to general pests (two-spotted mites) in cucumber, *Cucumis sativus* L.

A class of tetracyclic triterpenoids called cucurbitacins found in the Cucurbitaceae are specific feeding attractants for cucumber beetles of the Crysolimadeae (*2*), and a quantitative relationship exists between these substances and damage by these insects. Cucumbers of the *bibi* genotype completely lack cucurbitacins (*3*) and would seem to be useful as types resistant to cucumber beetles. However, we wondered what was the role of cucurbitacins in the economy of the plant (*4*).

In order to study the effects of cucurbitacins in vivo on the biology of nonspecific pests of cucurbits, "isogenic populations" (*5*) were created by crossing 'Marketer' and 'Eversweet' cultivars of *Cucumis sativus* L. and backcrossing the F_1 hybrid to 'Eversweet.' The resulting populations segregated one bitter plant (*Bibi*) for each nonbitter plant (*bibi*). The two phenotypes were identified by tasting the cotyledons or by a chemical test (*3*) and were transplanted to the field or to pots in the greenhouse.

Cucumber field plots of 50 bitter (*Bibi*) and 50 nonbitter (*bibi*) plants were artificially infested with two-spotted mites, *Tetranychus urticae* Koch. After 1 month, the nonbitter plants were nearly dead and showed signs of much mite feeding whereas the bitter plants were relatively free of mites. It was obvious that nonbitter plants had many more mites and had suffered much more damage from mite feeding than the bitter plants. Differential oviposition does not seem to be a factor in these results since equal numbers of eggs were laid on each phenotype.

Many more mites of all ages were able to develop on nonbitter than on bitter cucumber plants in greenhouse experiments (Table 1). The largest differences, however, were between larvae and young adult mites or the nymphal stages. Feeding on bitter plants seemed to have a deleterious effect on the development and growth of the early larval stages.

The length of feeding period had an effect on the mortality of mite larvae. Larvae were reciprocally transferred after various feeding periods on either bitter or nonbitter hosts. Those designated "long feeding period" were larger and darker due to ingested plant pigments as compared to the smaller, lighter colored "short feeding period" mites. These larval classes differed in age by about 2 days, depending on the temperature. The handling of the young

Table 1. Larval mortality of two-spotted mites on three genotypes of cucumber. Data pooled from oviposition of five females during 2 days; three replications of five plants per replication.

Host	Genotype	Larvae (No.)	Nymphs (No.)	Larval mortality (%)
Bitter	*BiBi*	1072	13	98.79
Bitter	*Bibi*	896	34	96.21
Nonbitter	*bibi*	1592	1512	5.03

Table 2. The influence of feeding duration on bitter or nonbitter plants on larval mortality of two-spotted mites. In the first experiment below, the larvae fed on primary and secondary hosts of the same genotype. In the second and third experiments below, the larvae fed on primary and secondary hosts that differed in genotype. In addition, the duration of feeding was varied before the mites were transferred from one host to the other in these two experiments.

Secondary larval feeding host	Primary larval feeding host	Larvae* (No.)	Nymphs (No.)	Larval mortality (%)
Nonbitter	Nonbitter	150	127	15
Bean	Bean	100	76	24
Bitter	Bitter	150	34	77
	Long feeding period			
Bitter	Nonbitter	150	113	25
Nonbitter	Bitter	200	32	84
Bean	Bitter	350	92	74
	Short feeding period			
Bitter	Nonbitter	100	14	86
Nonbitter	Bitter	150	100	33
Bean	Bitter	100	78	22

* Each host plant received 50 larvae.