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Effects of Viral Infection on Contraction of the Diaphragm in Mice (42344)

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Abstract. Isometric contractile properties of isolated phrenic nerve–diaphragm muscle preparations were used to study the effects of picornavirus infections on diaphragm muscle function. Properties of muscles from virus-inoculated and control mice were similar during brief contractions. However, when subjected to a series of fatiguing contractions by indirect or direct stimulation, muscles of mice inoculated with a paralytic variant of encephalomyocarditis (EMC) virus showed a greater rate of fatigue and a reduced capacity to recover from fatigue than did muscles from uninoculated control mice or muscles from mice inoculated with a nonparalytic coxsackievirus B3 (CVB3). Mice paralyzed by EMC virus infection had high titers of virus in the brain and similar titers of virus in diaphragm muscle as found in diaphragm muscles of CVB3-inoculated mice. The results indicate that EMC virus infection of mice leads to increased fatigability of the diaphragm muscle and that there are both neural and muscular components of this enhanced fatigue.

Picornavirus infections are often associated with muscular disorders. For example, the respiratory muscles are affected in epidemic pleurodynia caused by coxsackieviruses group B (Bornholm's disease) (1); neonatal mice are susceptible to acute viral myositis of the limb musculature caused by coxsackievirus group B1 (2); and coxsackieviruses groups A and B are associated with cardiac and skeletal muscle disorders in both humans and animals (3, 4). In spite of these associations there have been no systematic studies of the effects of a picornavirus infection of the host on muscle function. The present study arose from the observation that mice inoculated with a variant of encephalomyocarditis (EMC) virus exhibited labored breathing and progressive paralysis. The paralysis occurred within 4 days of inoculation and when death resulted approximately 12 hr later, it seemed to be associated with respiratory failure. When mice were inoculated with coxsackievirus B3, which is known to replicate in cardiac and diaphragm muscle (5, 6), no paralysis or signs of labored breathing occurred. The purpose of the present study was to determine the effects of infection by these two picornaviruses on the contractile properties and fatigability of the diaphragm muscles of inoculated mice. The muscle physiology experiments were conducted *in vitro* so that complications which might arise from virus-induced changes in other physiological systems of the host would be avoided.

Materials and Methods. Semi-inbred male CD-1 mice (age 4–6 weeks, 18–21 g) were purchased from Harlan Sprague–Dawley (Indianapolis, Ind.) and were housed and cared for by this Health Science Center's Laboratory Animal Resources staff. These adolescent mice were fed standard laboratory mouse chow *ad libitum,* with fresh water being provided daily. Two viruses were used, a nonparalytic, myocarditic virus, coxsackievirus B3 (CVB3) (7, 8), and a paralytic variant of EMC virus, isolated from a stock originally obtained from R. Radloff, University of New Mexico School of Medicine, Albuquerque. Stocks of both viruses were prepared in HeLa cells cultured in Eagle's complete minimal essential medium (MEM) as previously described (7). Stocks were titrated by a plaque method (7, 8), and infected tissues were assayed in sextuplicate by a cytopathic effect (CPE) endpoint assay, with the tissue culture infectious dose for 50% of the cultures ($TCID_{50}$) being calculated by the method of Reed and Muench (9). Mice were inoculated intraperitoneally with either virus at 10^5 PFU in 0.2 ml MEM. All mice inoculated with doses of EMC virus at 25 to 10^7 PFU exhibited paralysis, but the time to detection of paralysis varied between 3 and 10 days postinoculation (pi). The dose of EMC virus was therefore

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standardized at 10^5 PFU to induce paralysis by 96 to 108 hr pi. Tissue samples were prepared for assay by homogenization in Dulbecco's phosphate-buffered saline followed by three freeze–thaw cycles. Samples were stored at −70°C until assayed.

Contractile properties. The terminal stages of EMC virus infection are marked by labored breathing, a humped back, and flattened and paralyzed hindquarters. At this stage, it is likely that body homeostasis becomes severely compromised. In order to avoid difficulties in interpretation of data associated with the terminal stage of infection, mice inoculated with EMC virus were sacrificed 96 hr after inoculation when at least one hindlimb had become paralyzed but before the onset of the final stage. Mice inoculated with CVB3 were also sacrificed 96 hr after inoculation. Uninoculated mice served as the control group. All mice were sacrificed by exsanguination after being anesthetized with sodium pentobarbital (3 mg/kg body wt). Phrenic nerve–diaphragm muscle strip preparations were isolated under a high-power dissecting microscope and were mounted horizontally in a bath of Krebs–Ringer solution (pH 7.4, mmole/liter; NaCl, 115.0; KCl, 5.0; $MgSO_4$, 0.65; NaH_2PO_4, 1.20; $NaHCO_3$, 25.0; $CaCl_2$, 2.5; glucose, 11.0; Na acetate, 10.0; osmolarity, 300 mosmole), which was continuously bubbled with a mixture of 95% O_2 and 5% CO_2. The muscle strips were obtained from the costal region of the diaphragm. In Table I, the characteristics of the mice and muscle strips are listed. The EMC virus-inoculated mice weighed significantly less than control mice but the sizes and masses of all muscle preparations in the three groups were similar. The lower body weights of EMC virus-inoculated mice may have resulted from dehydration due to illness, although water was freely available to the mice in the bottom of their cages. For experiments involving direct stimulation of the muscles, *d*-tubocurarine was added (final concentration 2×10^{-6} *M*). The central tendon end of each muscle strip was attached to a Grass FT-03 tension transducer via a stainless steel yoke and the distal end of the preparation was secured to a fixed hook via another stainless steel yoke attached to a fragment of rib. For indirect stimulation, the phrenic nerve was placed across two platinum wire electrodes situated above the chamber and kept moist. Square-wave pulses of supramaximal voltage and 0.2-msec duration were delivered by a Grass S88 stimulator. Massive direct stimulation of the muscle strips (supramaximal voltage for 2 msec) was delivered with bright-platinum plates arranged beside the preparation so as to run the entire length of the muscles. The transducer output was displayed on either a Grass polygraph or a Tektronix 513N storage oscilloscope for measurement of isometric contractile properties. All experiments were conducted at room temperature (24 ± 1°C).

Muscles were allowed to equilibrate in the bath for 30 min, after which the optimum length (L_0; Ref. (11)) for maximum active tetanus tension was determined by indirect stimulation. Maximum force per unit area was calculated by dividing the maximum active tension by the estimated cross-sectional area of the muscle strip at L_0. The cross-sectional

TABLE I. COMPOSITION

	Body weight (g)	Muscle mass (mg)	Dry mass / wet mass	Optimal muscle length, L_0 (cm)
Control mice	31.0 ± 2.0 (7)	8.0 ± 1.0 (9)	0.249 ± 0.003 (9)	0.94 ± 0.04 (9)
CVB3-inoculated mice	30.0 ± 1.0 (6)	9.0 ± 2.0 (6)	0.245 ± 0.006 (6)	0.90 ± 0.05 (6)
EMC-inoculated mice	21.9 ± 1.6* (6)	7.0 ± 0.6 (6)	0.249 ± 0.006 (6)	0.84 ± 0.04 (6)

Note. Values are given as means ± SEM with number of mice in parentheses.
* $P < 0.05$ from control group.

area was estimated by the ratio of wet mass to L_0, assuming a muscle strip density of 1.06 g/ml. Isometric properties were measured at this length and the fatigability of the nerve–muscle preparations was then determined.

Contractile properties and fatigability of the muscles under indirect nerve stimulation were measured first. Then properties under direct stimulation were measured. In three experiments, muscles were directly stimulated immediately after the initial 30-min equilibration. No significant difference was found between these results and those from preparations that had first been stimulated via the phrenic nerve. Muscles were fatigued by performing 10 sustained contractions (each of 8-sec duration) every 30 sec (10). Figure 1 illustrates typical recordings of isometric tension versus time during these sustained contractions. Note that tension decreases much more rapidly during nerve stimulation (Fig. 1A) than during direct stimulation (Fig. 1B). This was found for all muscles, regardless of group. Isometric tension was measured before, during, and after these sustained contractions to provide indices of fatigability. Details of the indices of fatigue are given under Results.

After the fatigability studies were completed, preparations were blotted four times on filter paper and wet weight was measured. Muscles were then dried overnight to constant mass and weighed. Results are presented as mean values ± standard error of the mean (SEM). Data were further analyzed by an analysis of variance (ANOVA). When significant differences were indicated, groups responsible for the significance were identified using a Student–Neuman–Keuls (SNK) test, with significance assumed at $P < 0.05$ (12).

Results. Diaphragm muscle and brain from EMC virus-inoculated mice in paralysis contained high titers of virus. Mean titers from the brain and diaphragm on Day 4 pi were $2.8 \times 10^9 \pm 0.6 \times 10^9$ and $6.7 \times 10^5 \pm 2.0 \times 10^5$ $TCID_{50}$/g tissue (mean ± SEM, $n = 5$), respectively. Titers of CVB3 in the brain and diaphragm of nonparalyzed mice were $<0.0005 \times 10^5$ and $7.5 \times 10^5 \pm 3.5 \times 10^5$ $TCID_{50}$/g tissue (mean ± SEM, $n = 5$), respectively. The data show that diaphragm muscles of EMC virus-inoculated and CVB3-inoculated mice contained approximately the same titers of the respective viruses. However, the brains of EMC virus-inoculated mice contained high titers of virus whereas the brains of CVB3-inoculated mice contained barely detectable levels of virus. In addition, virus titers in the sera of EMC virus-inoculated mice ($n = 6$) were $<0.1 \times 10^2$, $1.7 \times 10^2 \pm 1.1 \times 10^2$, $0.2 \times 10^2 \pm 0.2 \times 10^2$, and $0.4 \times 10^2 \pm 0.4 \times 10^2$ $TCID_{50}$ per milliliter on Days 1, 2, 3, and 4 pi, respectively. Titers of CVB3 in the sera of inoculated mice ($n = 3$) were $1.5 \times 10^5 \pm 1.1 \times 10^5$, $4.1 \times 10^5 \pm 1.8 \times 10^5$, $2.5 \times 10^3 \pm 2 \times 10^3$, and $8.8 \times 10^3 \pm 8.1 \times 10^3$ PFU per milliliter on Days 1, 2, 3, and 4, respectively. These data show that EMC virus induces a minimal viremia in mice, in contrast to that of CVB3.

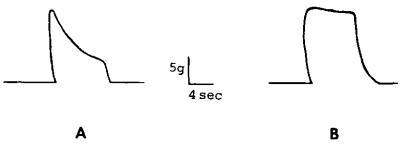

FIG. 1. Isometric tension versus time. Experimental record of 10th tetanus of fatigue series; experiment of 5-3-83. Diaphragm strip from control (noninoculated) mouse. (A) Indirect stimulation at 80 Hz for 8 sec. (B) Direct stimulation at 80 Hz for 8 sec.

The contractile characteristics measured during isometric twitches and during isometric tetanic contractions at the optimal frequency for tension development are illustrated in Table II. These properties did not differ significantly in muscles from all groups subjected to indirect or direct stimulation.

Since diaphragm muscles strongly resist fatigue, a strenuous fatiguing protocol was adopted. This protocol has previously been found to correlate well with resistance to fatigue expected on the basis of known fiber-type distribution (10). First the change in isometric force with frequency of stimulation was established by a series of brief (400 msec) tetanic contractions given at 1-min intervals (initial force–frequency curve FF1). Next, muscles were stimulated continuously at 80 Hz to contract for 8 sec every 30 sec, for a total of 10 tetanic contractions. A second force-frequency curve (FF2) was then determined 5 min after the 10th tetanus. Force–frequency data obtained before fatigue (FF1, open symbols) and 5 min after fatigue (FF2, closed symbols) are shown in Figs. 2 and 3 for indirect and direct stimulation, respectively.

TABLE II. CONTRACTILE PROPERTIES: INDIRECT STIMULATION (IS) AND DIRECT STIMULATION (DS)

	Latent period (msec)		Contraction time (msec)		Half-relaxation time (msec)		Twitch/tetanus		Maximum force per unit area (kg/cm^2)	
	IS	DS	IS	DS	IS	DS	IS	DS	IS	DS
Control mice	5 ± 0 (6)	5 ± 1 (8)	54 ± 3 (6)	47 ± 4 (9)	68 ± 3 (6)	68 ± 4 (9)	0.25 ± 0.03 (6)	0.24 ± 0.02 (8)	1.35 + 0.06 (6)	1.45 ± 0.08 (8)
CVB3-inoculated mice	5 ± 0 (6)	5 ± 0 (6)	53 ± 4 (6)	58 ± 5 (6)	66 ± 8 (6)	77 ± 10 (6)	0.25 ± 0.03 (6)	0.27 ± 0.04 (6)	1.11 ± 0.03 (6)	1.41 ± 0.11 (6)
EMC virus-inoculated mice	6 ± 1 (6)	6 ± 1 (6)	47 ± 3 (6)	49 ± 5 (6)	83 ± 17 (6)	75 ± 13 (6)	0.28 ± 0.05 (6)	0.24 ± 0.03 (6)	0.95 ± 0.20 (6)	1.17 ± 0.23 (6)

Note. Values are given as means ± SEM with number of mice in parentheses.

Resistance to fatigue was assessed from measurements of isometric tension in the fatiguing (8-sec) contractions. The maximum tension recorded in the 10th sustained tetanus expressed as a percentage of the maximum tension obtained in the first sustained tetanus was used as an index of resistance to fatigue (% maximum tension). Using this index, muscles of EMC virus-inoculated mice were significantly less resistant to fatigue than muscles of CVB3-inoculated or control mice. This was found for both indirect and direct stimulation, as illustrated in Table III (% maximum tension).

Differences between direct and indirect stimulation (Fig. 1) suggested a further index of resistance to fatigue. Typical records obtained in the 10th tetanus for indirect and direct tetanic contractions indicated a greater decline in tension during a given contraction for the nerve-stimulated preparations, in spite of the fact that these contractions preceded the directly stimulated contractions. A further index of resistance to fatigue was therefore developed, using the final tension recorded at the end of stimulation: The end tension recorded in the 10th fatiguing tetanus was expressed as a percentage of the end tension

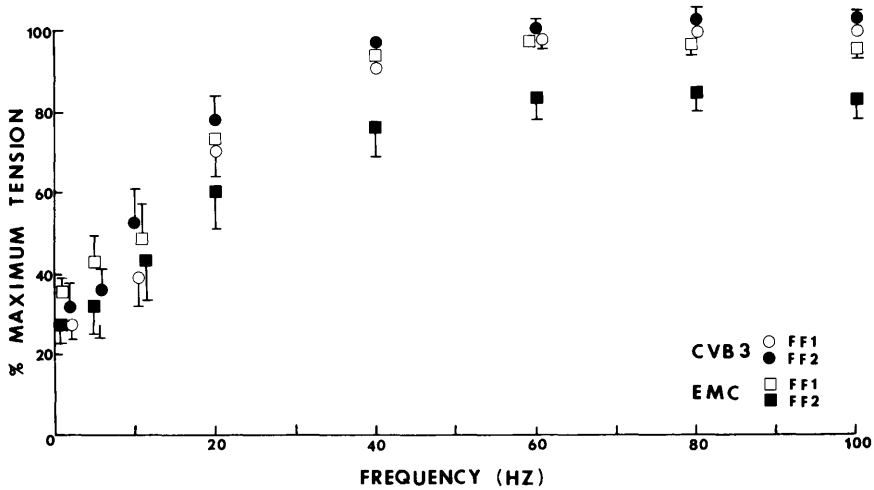

FIG. 2. Variation of isometric tension with frequency of stimulation, measured before (open symbols, FF1) and 5 min after (solid symbols, FF2) the fatiguing protocol. All muscles were *indirectly* stimulated. Circles refer to CVB3-inoculated (nonparalyzed) mice; squares refer to EMC-inoculated (paralyzed) mice. Values are plotted as percentages of the maximum tension obtained during the FF1 series.

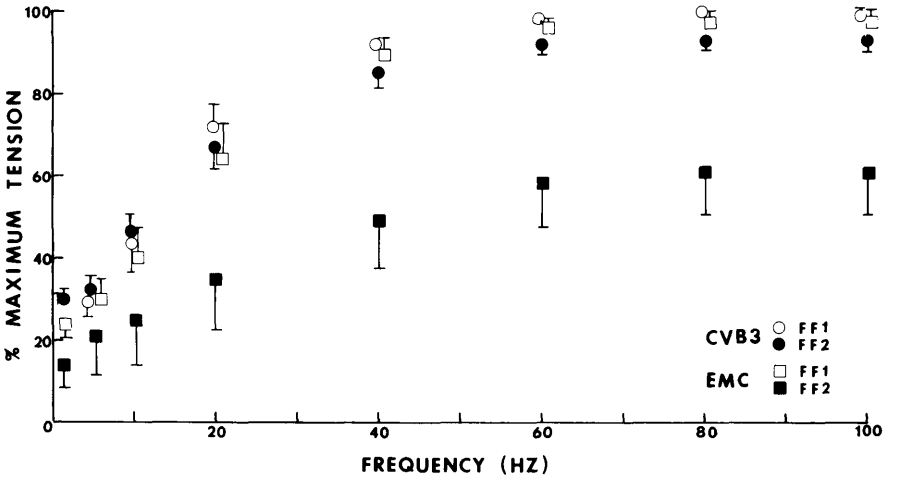

FIG. 3. Variation of isometric tension with frequency of stimulation, measured before (open symbols, FF1) and 5 min after (solid symbols, FF2) the fatiguing protocol. All muscles were *directly* stimulated. Circles refer to CVB3-inoculated (nonparalyzed) mice. Squares refer to EMC-inoculated (paralyzed) mice. Values are plotted as percentages of the maximum tension obtained during the FF1 series.

obtained in the first tetanus (% end tension). Using this measure, muscles of EMC virus-inoculated mice again showed greater fatigue following sustained contraction than did muscles of control mice, for both direct and indirect stimulation. In contrast, the fatigue recorded from muscles of CVB3-inoculated mice were not significantly different from those of control mice. These data are given in Table III (% end tension).

Comparison of the tensions obtained in the two force–frequency curves (FF1 vs FF2) was used as an index of recovery from fatigue and as an indicator of the type of fatigue present (high- or low-frequency fatigue). The different rates of recovery from fatigue are illustrated in Figs. 2 and 3. Muscles of control and CVB3-inoculated mice exhibited a complete recovery of tension at all frequencies of indirect stimulation, whereas there was an approximately 15% decrease in the tension developed at high frequencies (>20 Hz) for muscles from EMC virus-inoculated mice after the fatiguing contractions (Fig. 2). This difference was magnified in the case of direct stimulation: Muscles of control and CVB3-inoculated mice showed only a small (aproximately 8%) decrease in tension after the 5-min rest, whereas muscles

TABLE III. FATIGUE INDEX

	Indirect (nerve) stimulation		Direct stimulation	
	% Maximum tension	% End tension	% Maximum tension	% End tension
Control mice	61 ± 5 (5)	40 ± 4 (5)	61 ± 5 (5)	65 ± 8 (5)
CVB3-inoculated mice	72 ± 5 (6)	46 ± 4 (6)	64 ± 2 (6)	57 ± 3 (6)
EMC virus-inoculated mice	42 ± 6* (6)	20 ± 6* (6)	37 ± 6* (6)	31 ± 7* (6)

Note. Values are given as means ± SEM with number of mice in parentheses.
* $P < 0.05$, from control group.

from paralyzed mice were significantly impaired in their ability to develop tension at high frequencies (>20 Hz) after the fatiguing contractions (a decrease of approximately 40%; solid squares, Fig. 3).

Discussion. Our data on virus titers in inoculated mice confirm previously reported infections of diaphragm muscle by viruses (6). EMC virus variants are also known to cause myocardial lesions and infections of the nervous system in a wide variety of animals, including man (13–16). In our study, EMC virus infection did not affect the acute isometric contractile properties of the diaphragm, but did cause the muscles to fatigue more rapidly and to recover more slowly. The fact that these effects were obtained only for mice in which virus titers were high in the brain (EMC virus-inoculated mice) suggests that a direct effect of the virus on muscle function may be mediated by viral infection of the central nervous system. As noted earlier, EMC virus-infected animals exhibited labored breathing. Infection of the central nervous system could produce altered patterns of innervation of the diaphragm which, combined with enhanced muscle fatigability, could result in the labored breathing seen in the paralyzed mice. The similarity of results obtained for directly and indirectly stimulated muscles indicates that the virus does not affect the neuromuscular junction significantly. However, the combination of altered central nervous system output and enhanced muscle fatigability could act to produce respiratory failure via the neuromuscular junction in EMC virus-inoculated mice. In these mice the observed labored breathing resulted in prolonged periods of inspiration. This in turn would lead to a marked decline in tension-generating ability of the diaphragm, as shown in Fig. 1A, and possibly to eventual respiratory failure.

The absence of any differences in contractile properties (Table II) indicates that the advancing limb paralysis of EMC virus-infected mice is not accompanied by a change in the mechanisms of excitation, contraction, or relaxation of the fibers or in the types of muscle fibers present in the diaphragm. The striking fatigue present at high frequencies of stimulation (Fig. 3), however, suggests that at these high frequencies there is a failure of excitation of action potentials in muscle cell membranes (17).

In summary, the data demonstrate a direct effect of EMC virus on the fatigability of diaphragm muscles of infected mice. This direct effect may be enhanced by virus-induced degenerative changes of the central nervous system, leading to eventual respiratory failure. Understanding of the mechanisms of fatigue induced by this viral infection require a more detailed study. However, the EMC virus-inoculated mouse is a convenient system for *in vitro* as well as for *in vivo* studies of such mechanisms and could well serve as a model system for studying the effects of viral infection on muscle function.

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