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インターフェロン-α, 及び -γ のラット横隔膜筋への影響

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Although it is well-known that the cytokine TNF-α is involved in endotoxin-induced diaphragm muscle dysfunction, the role of interferons, a class of cytokines, in such dysfunction is unclear. Using 64 Wistar rats, we therefore examined whether two interferons, IFN-α and -γ, affect diaphragm muscle contraction and muscle fiber. In the *in vivo* study, the IFN-α group was injected with 20,000 IU (3 times per week) intramuscularly, the IFN-γ group with 5,000 IU (daily) intravenously, and the control group with 0.2 ml saline (daily) intravenously. Force-frequency curves, twitch kinetics and fatigability were measured at 1, 2, and 3 weeks (W) in each group. The entire force-frequency curve of IFN-α at 3W was significantly lower than that of the control as shown by ANOVA ($p<0.05$), whereas all the force-frequency curves of IFN-γ at 1W, 2W and 3W showed significant decreases from that of the control as shown by ANOVA ($p<0.001$, $p<0.01$, $p<0.001$, respectively). The diaphragm muscle became more fatigue resistant with IFN-γ than with IFN-α. In addition, the cross-sectional areas of type IIb muscle fibers were more decreased by IFN-γ than by IFN-α at 3W according to ATPase staining. In the *in vitro* study, IFN-α and IFN-γ ($n=6$ each, both 10,000 IU/ml) were added to an organ bath solution, and force-frequency curves were measured before the addition and after 4 hours of incubation. IFN-γ decreased the tension of the 20-100 Hz force-frequency curves (peak 64.7%) more than did IFN-α (peak 75.8%). In summary, it can be concluded that both INFs seem to have direct effects on diaphragm muscle, but IFN-γ has a more deleterious effect than IFN-α and induces atrophic changes in type IIb muscle fibers in the case of clinical dosages.

Introduction

Interferons (IFNs) are a group of cytokines produced by leukocytes, fibroblasts or lymphocytes stimulated by viruses, endotoxins and lectins, and not only confer protection against viruses but also inhibit cellular growth and influence a number of processes involved in the immune response. IFNs are grouped into three major classes: IFN-α (leukocyte der-

ived, type I), IFN-β (fibroblast derived, type I), and IFN-γ (CD4 T lymphocyte derived, type II) based on cell origin and chemical properties[1]. The DNA sequences of IFN-α and IFN-γ have been determined by Henco[2] and Gray[3]. Because IFN-α and IFN-γ are distinctly different in terms of molecular weight, antigenic specificity and stability at low pH, it is conceivable that the biological activities of IFN-α and IFN-γ are different not only in their antiviral activities but also in their antitumor activities, as demonstrated by the ability of IFN-γ to inhibit the growth of renal cell carcinoma cells[4] and the brain metastasis of malignant melanoma[5]. Accordingly, IFN-α and IFN-γ have been used to treat viral hepatitis and kidney tumors, respectively. Moreover, a previous study showed that the synergistic effect of IFN-γ and tumor necrosis factor-α (TNF-α) resulted in decreased DNA synthesis in human lung cancer cells[6].

The present authors recently reported that TNF-α mRNA expression and production can be found in endotoxin administered rat diaphragm muscle tissue, and that this TNF-α mRNA induction was closely correlated with the decrease in diaphragm muscle contraction[7]. As it is known that endotoxin induces the production of several cytokines of IFNs and interleukin-1 (IL-1)[8], there is a possibility that these cytokines may be involved in the respiratory muscle dysfunction induced by endotoxin administration. There is a report that capillary myofibroblasts were inhibited by TNF-α and IFN-γ[9], but, to our knowledge, there is no report concerning the effects of IFNs on skeletal muscles including diaphragm muscle.

Since the effects of IFNs on diaphragm muscle have not been sufficiently clarified, we examined those of IFN-α and IFN-γ on contractile properties, using an *in vivo* study for systemic assessment and an *in vitro* study for more direct assessment of the biochemical pathways. In the *in vivo* study, the dose of IFN-α was adjusted according to the body weight of the animal and that of IFN-γ was adjusted according to its body surface area so as to be equivalent to the doses clinically used in adult humans. Additionally, we performed a histological analysis of type I (slow-twitch) and type II (fast-twitch) subtypes IIa, IIb and IIc muscle fiber areas by ATPase staining using the diaphragm muscles of rats.

Materials and Methods

Animal preparation

Experiments were performed using 64 Wistar rats weighing 250-320 g (Charles River Japan, Kanagawa, Japan). I) *In vivo* study: the IFN-α group was injected with 20,000 international units (IU) of recombinant human IFN-α suspended in 0.2 ml saline intramuscularly 3 days per week ($n=18$) (kindly provided by NIPPON Roche K.K.), and the IFN-γ group was injected with 5,000 IU of recombinant human IFN-γ suspended in 0.2 ml saline intravenously via a tail vein daily ($n=18$) (kindly provided by Shionogi Pharmaceutical Co.); the control group ($n=6$) was injected with 0.2 ml saline only intravenously via the tail vein daily. Both the IFN-α and IFN-γ groups were measured for contractile properties and muscle fiber cross-sectional area at 1, 2, and 3 weeks (W) after each injection, and the control group was measured at the starting point. In addition, we measured the serum concentrations of IFN-α and IFN-γ at 30 min, 1 and 4 hours after each injection ($n=2$ each). II) *In vitro* study: The IFN-α and IFN-γ ($n=6$ each) groups, as well as the control group without IFNs ($n=6$), were examined in an organ bath solution (both 10,000 IU/ml, respec-

tively) with measurement of force-frequency curves being conducted at the time of pre-addition and 4 hours after each administration. The studies were reviewed and approved by the Tohoku University Animal Care and Use Committee.

Protocol of measurements

In the *in vivo* study, diaphragm contractile properties were measured in an organ bath (i.e., *in vitro* manner). The animals of both the IFN-α and IFN-γ groups at 1, 2, and 3 weeks (W) after each injection, and those of the control group at the starting point were anesthetized with diethyl ether in a glass chamber, and decapitated. Immediately after decapitation, the diaphragm muscles were dissected and a muscle strip (3-4 mm wide) was made from the costal part of the hemidiaphragm and mounted in an organ bath containing Krebs-Henseleit solution oxygenated with a 95% O_2-5% CO_2 gas mixture (23.5±0.5°C, pH 7.40±0.05). The composition of the aerated Krebs-Henseleit solution was as follows: Na^+, 153.8 mEq/l; K^+, 5.0 mEq/l; Ca^{2+}, 5.0 mEq/l; Mg^{2+}, 2.0 mEq/l; Cl^-, 145.0 mEq/l; HCO_3^-, 15.0 mEq/l; HPO_4^{2-}, 1.9 mEq/l; SO_4^{2-}, 2.0 mEq/l; glucose, 110 mg%; 0.01 mM d-tubocurarine; regular crystalline zinc insulin, 50 U/L. The muscle strip was stimulated with supramaximal currents (i.e., 1.2 to 1.5 times the current required to elicit maximal twitch tension, 200-250 mA, 0.2 msec duration in pulses) by a constant current stimulus isolation unit (SS-302J, Nihon Kohden, Japan) driven by a stimulator (SEN-3201, Nihon Kohden, Japan), and the elicited tensions were measured by a force transducer (UL-100GR, Minebea Co., Japan). The muscle strip length was altered by moving the position of the force transducer with a micrometer-controlled rack and pinion gear (accuracy of displacement, 0.05 mm), and measured with a micrometer in close proximity to the muscle. The optimal length of the muscle (Lo) was defined as the muscle length at which twitch tension development was maximal, and this predetermined Lo was maintained in the following measurements. The elicited tensions of both muscle strips were recorded by a hot-pen recorder (RECTI-HORIZ-8K, San-ei, Japan).

For the *in vivo* study group, force-frequency curves, twitch kinetics and fatigability were measured at 1, 2, and 3 weeks (W) as muscle contractile parameters.

The force-frequency curves in the *in vivo* study were measured by sequentially stimulating the diaphragm muscles at 1, 10, 20, 30, 50, 70 and 100 Hz. Each stimulus train was applied for approximately 1 second, and adjacent trains were applied at approximately 10-seccond intervals. The force-frequency curves obtained from the *in vivo* study group were displayed as elicited tensions (kg/cm^2) on the Y-axis and stimulating frequencies on the X-axis.

Twitch contraction was elicited by single pulse stimulation (200 μsec), and the trace of the twitch contraction was recorded at high speed (10 cm/sec). The twitch contraction was assessed by (1) twitch tension (peak tension of twitch contraction, kg/cm^2), (2) contraction time (the time required to develop peak tension during twitch muscle contraction, msec) and (3) half relaxation time (the time required for peak tension to fall by 50% during the twitch muscle contraction, msec).

Muscle fatigability was assessed as the remaining tension after a repetitive stimulation as follows. Rhythmic contraction was induced by applying trains of 20-Hz stimuli (train duration, 0.3 sec; rest duration, 0.7 sec) at a 60 train/min rate, and was assessed by examining

the rate of fall of tension over 5 min of rhythmic contraction. For muscle fatigability, the final remaining tension was expressed as a percentage (%) of the initial tension. After completion of this protocol, which was completed in around 30 minutes, the muscle strip was removed from the bath and weighed. We also measured the diaphragm wet weight in each group.

In the *in vitro* study, only the force-frequency curves were measured at pre-addition, and at 1, 2 and 4 hours after incubation. The muscle strips were incubated in an organ bath in which the bath solution contained IFN-α, IFN-γ (both 10,000 IU/ml) or the control (no IFNs), and maintained at 37°C in a heated water chamber.

The force-frequency curves in the *in vitro* study were measured by sequentially stimulating the diaphragm muscles at 1, 10, 20, 30, 50, 70 and 100 Hz. Each stimulus train was applied for approximately 1 second, and adjacent trains were applied at approximately 10-second intervals. The force-frequency curves were expressed at 100% peak tension at pre-addition, and the following changes at 4 hours were expressed as percentages of that at pre-addition in each group. After completion of this measurement, the muscle strip was removed from the bath and weighed.

Measurements of serum concentration of IFNs

In the *in vivo* study, the serum concentrations of IFN-α of IFN-γ ($n=2$ each) were measured at 30 minutes, 1 hour and 4 hours after IFN-α (20,000 IU suspended with 0.2 ml saline, intramuscularly) or IFN-γ (5,000 IU suspended with 0.2 ml saline, intravenously) injection. The amount serum taken from each animal was 0.5 or 0.2 ml (IFN-α or IFN-γ, respectively), and measured by a bioassay method. The minimum concentration values to detect IFN-α or IFN-γ were 25.6 pg/ml or 6 IU/ml, respectively.

Adenosine triphosphatase (ATPase) stain

In the *in vivo* study, myofibrillar adenosine triphosphatase (ATPase) staining was performed according to the method of Dubowitz and Brooke[10]. The muscle strips in each group were adjusted to Lo with pins on a cork plate, immersed in isopentane (Wako Pure Chemical Industries Ltd., Osaka, Japan) that had been cooled in liquid nitrogen, and embedded in mounting medium (OCT compound, Miles Inc., Elkhart, IN, USA). Diaphragm sample tissues were sectioned at 10 μm in sequential slices with a cryostat (BRIGHT Instrument, Huntingdon, UK) kept at −20°C. On the basis of their staining reactions for myofibrillar ATPase after alkaline (pH 10.4) and acid (pH 4.2 and 4.6) preincubation, muscle fibers were classified as either type I (slow-twitch and oxidative, SO) or type II subtypes IIa (fast-twitch, oxidative and glycolytic, FOG), IIb (fast-twitch and glycolytic, FG) and IIc. This nomenclature is as described by Dubowitz and Brooke[10]. With preincubation at a pH of 10.4, light stained fibers were classified as type I, and dark stained fibers were classified as type II (IIa, IIb and IIc) muscle fiber. With preincubation at a pH of 4.2, light-stained fibers were classified as types IIa and IIb, and dark stained fibers were classified as type I and IIc (weak) muscle fiber ; at a pH of 4.6, light-stained fibers were classified as type IIa, and dark stained fibers were classified as type I, IIb (weak) and IIc muscle fiber. The cross-sectional areas (CSAs) of muscle fibers were measured by digitalization with a computerized Personal Image Analyze System (LA-500, PIAS Co.,

Tokyo, Japan). The sample pictures (each picture shows about 100-150 muscle fibers) were input using a microscope (OPTIPHOT, Nikon, Tokyo, Japan) and a TV camera, and presented on a color display TV (CPV-14CD2, Sony, Tokyo, Japan). One fiber CSA was determined from the number of pixels within the outlined borders, each pixel having an area of 0.67 μm^2 at $\times 20$ magnification. The measurements of CSA of type I, IIa and IIb muscle fibers were performed on about 100 muscle fibers. However, only 10-20 type IIc muscle fibers were examined because of their small number.

The diaphragm thickness was measured from the number of pixels, which was linearly drawn between the edges of pleural and abdominal surfaces of diaphragm muscles on the low magnification sample pictures ($\times 10$).

Data analysis

The strip cross-sectional area was calculated by dividing the muscle mass by the product of strip muscle length and muscle density (1.06 g/cm^3)[11], and tension was defined as force per unit area (kg/cm^2). To compare the mean tension at each frequency of the force frequency curves, the mean values of twitch kinetics, fatigability, muscle weight, thickness and CSAs in the experimented group, Student's t test was used. To compare all force frequency curves among groups, analysis of variance (ANOVA) with Fisher's PLSD *post hoc* test was used. In the comparison of the force frequency curves, if there were significant differences between tensions at each frequency, the entire differences of the force frequency curves were considered to be possibly meaningful. Data are presented as means±SEM (standard error of means). Comparisons with a p value of less than 0.05 were considered statistically significant.

Results

In the *in vivo* study, the body weight of the IFN-α group was gradually increased at 1W (213.3±17.6 g), 2W (253.3±12.0 g), and 3W (280±11.5 g, $p<0.05$ compared to 1W), and that of the IFN-γ group was more slowly increased at 1W (213.3±12.0 g), 2W (217.5±15.4 g) and 3W (247.0±19.5 g, not significant compared with both 1W and 2W of IFN-γ). With normal feeding at the Tohoku University Animal Facility, the body weight of control animals (6 weeks after birth) increased as 1W (232.8±10.5 g), 2W (275.5±6.9 g), and 3W (309.8±12.1 g). Therefore, the body weight gain was not suppressed by IFN-α, but was significantly suppressed by IFN-γ at 2W and 3W ($p<0.05$ compared to control, respectively).

Table 1 summarizes the serum concentrations of IFN-α or IFN-γ ($n=2$ each) after one injection in the *in vivo* study. The serum concentrations of IFN-α were less than 25.6 pg/ml at 30 min, 1 hour and 4 hours, and the transfer of IFN-α to serum seemed to be very low after intramuscularly injection. The serum concentrations of IFN-γ were 17 or 15 IU/ml at 30 min, then decreased below 6 IU/ml at 1 and 4 hours, and the serum concentrations of IFN-γ seemed to decrease rapidly.

Table 2 summarizes the diaphragm wet

Table 1. Changes of serum conentration of IFN-α and IFN-γ after each injection *in vivo*.

	30 min	1 hour	4 hour
IFN-α ($n=2$)	<25.6 pg/ml	<25.6 pg/ml	<25.6 pg/ml
	<25.6 pg/ml	<25.6 pg/ml	<25.6 pg/ml
INF-γ ($n=2$)	17 IU/ml	<6 IU/ml	<6 IU/ml
	15 IU/ml	<6 IU/ml	<6 IU/ml

(The concentrations of 25.6 pg/ml for IFN-α and 6 IU/ml for IFN-γ are minimum values for detection.)

Table 2. Changes of wet weiht and thickness of diaphragm muscles.

	wet weight (g)	thickness (mm)
1W : Control	0.72±0.02	0.78±0.04
IFN-α	0.76±0.04	0.79±0.03
IFN-γ	0.55±0.03***††	0.54±0.05**††
2W : Control	0.83±0.02	0.86±0.07
IFN-α	0.77±0.03	0.86±0.03
IFN-γ	0.56±0.05***††	0.51±0.05**††
3W : Control	0.97±0.03	0.94±0.07
IFN-α	0.92±0.07	0.77±0.08
IFN-γ	0.57±0.04***††	0.56±0.03***†

Values are means±SEM.
*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with control at each week, and †$p<0.05$, ††$p<0.01$ compared with IFN-α at each week.

weight and thickness in the *in vivo* groups. The wet weight and thickness of the IFN-γ groups were significantly decreased compared with both the control and IFN-α groups through 3 weeks.

For the *in vitro* study, the both weights of animals in the control, IFN-α and IFN-γ groups were 210±13.4 g, 215±12.6 g and 217±12.8 g respectively, with were not significantly different.

Changes of force-frequency curves in the in vivo study

Figure 1A shows the mean force-frequency curves induced by IFN-α intramuscular injection from 1 to 3 weeks. The effect of this injection was observed at lower frequencies from 1 to 30 Hz, but there was no significant decrease in tension from 50 to 100 Hz in the control and three IFN-α groups. As for tension at corresponding frequencies, asterisks indicate significant changes compared with the control. The entire force-frequency curve of IFN-α in the 3W group was significantly decreased from that of the control as shown by ANOVA ($p<0.05$).

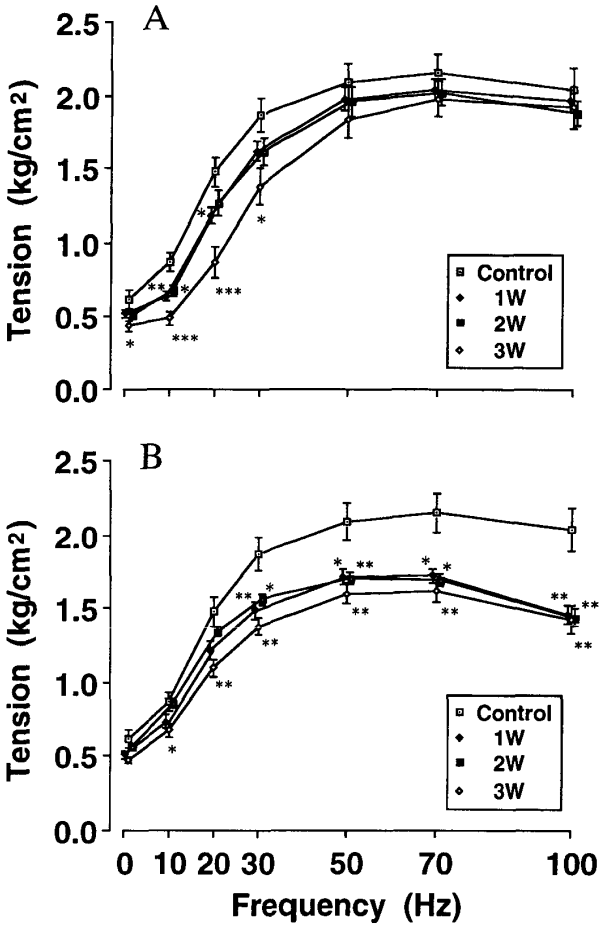

Fig. 1 A : Force-frequency curves of control and IFN-α at 1 week (1 W), 2 weeks (2 W) and 3 weeks (3 W). B : Force-frequency curves of control and IFN-γ at 1 week (1 W), 2 weeks (2 W) and 3 weeks (3 W). Symbols indicate significant differences at given frequencies compared with control diaphragm (*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with control).

Figure 1B shows the mean force-frequency curves induced by IFN-γ intravenous injection for 1 to 3 weeks. The effects of this injection were observed at higher frequencies, but there were no significant changes at 1 Hz in any of the 4 groups. As for tension of corresponding frequencies, asterisks indicate significant changes compared with the control. The

entire force-frequency curves of IFN-γ in the 1W, 2W and 3W groups showed significant decreases from that of the control as shown by ANOVA ($p<0.001$, $p<0.01$, $p<0.001$, respectively).

The force-frequency curves of the IFN-γ groups were more decreased compared with those of the IFN-α groups at 1W, 2W (both $p<0.01$) and 3W ($p<0.05$) as shown by ANOVA.

Changes of twitch kinetics and fatigability in the in vivo study

Figure 2 shows the mean data of twitch tension, contraction time and half relaxation time for each group. As the twitch tension (A), the IFN-α group showed a significant decrease (0.44 ± 0.04 kg/cm^2) at 3W. Regarding contraction time (B), the IFN-α group showed significant decreases at 1W (43.5 ± 1.3 msec, $p<0.01$) and at 3W (41.8 ± 0.7 msec, $p<0.001$). With regard to half relaxation time (C), the IFN-α group showed a significant decrease at 3W (43.8 ± 2.5 msec, $p<0.05$) compared with those of the control. We expected some elongation of contraction and half relaxation times in the IFN-γ group in light of the muscle fiber changes described below; however, the IFN-γ group did not show any significant changes in these three parameters.

Figure 2D shows the changes of fatigability, in which the final tension is expressed as a percentage of the initial tension in the 5-min repetitive electrical stimulation. The fatigability of the IFN-α group showed a significant but small increase from that of the control at 1W ($p<0.05$), but it did not show any significant changes at 2W and 3W. On the other hand, the IFN-γ group showed significant increases in final tension at 1W ($p<0.001$), 2W ($p<0.05$) and 3W ($p<0.001$) compared with the

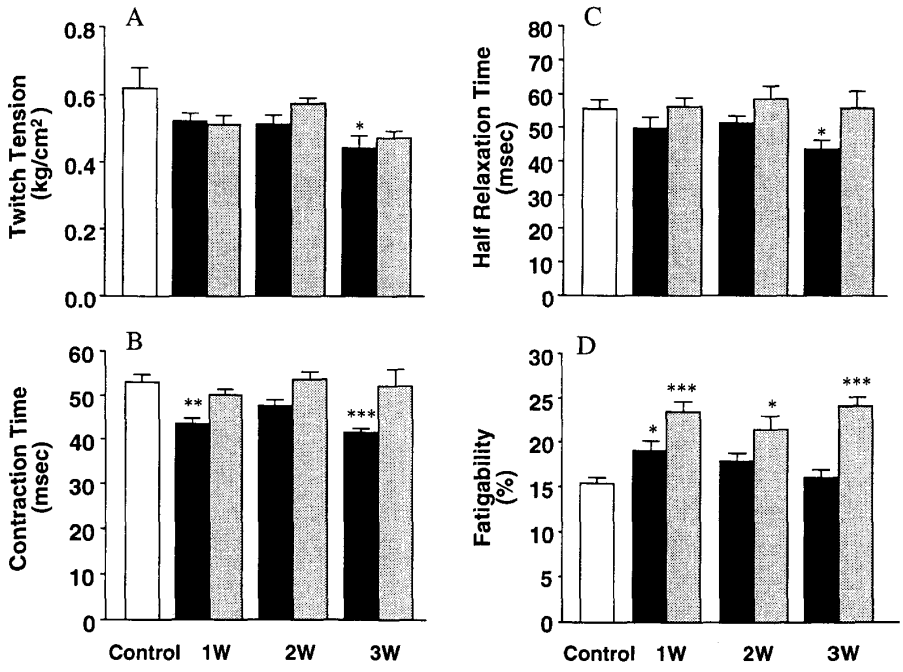

Fig. 2 Mean changes of twitch tension (A), contraction time (B), half relaxation time (C), and fatigability (D) from the diaphragm of control (open column), IFN-α (closed column) and IFN-γ (shaded column) injections at 1 W, 2 W, and 3 W, respectively. Symbols indicate significant differences compared with control diaphragm (*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with control).

control. The diaphragm muscle became more fatigue resistant in the IFN-γ groups than in the IFN-α groups.

Changes of fiber areas in the in vivo study

Figure 3 shows representative photographs of each group at 3W in the ATPase staining, and Table 3 summarizes the mean muscle fiber areas of type I and type II subtypes IIa, IIb and

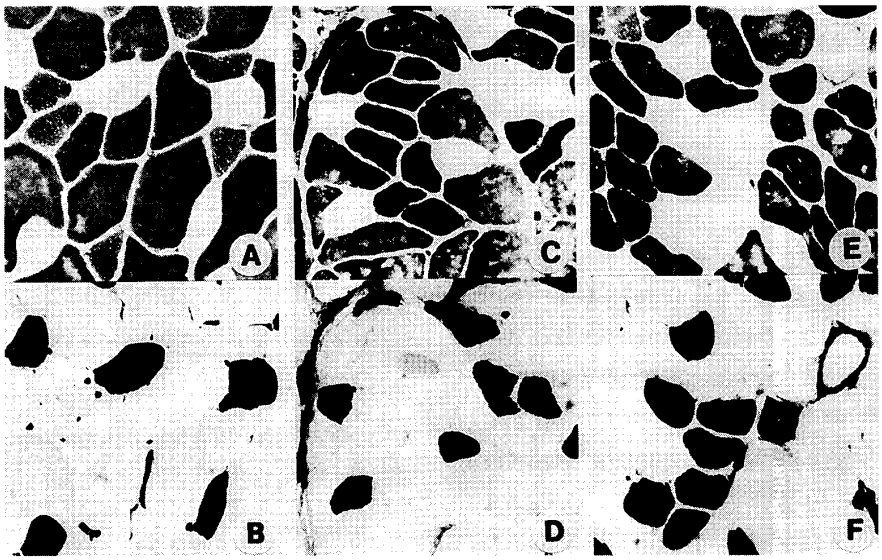

Fig. 3 Representative photographs of control at 3W (A: pH 10.4, B: pH 4.2), IFN-α at 3 W (C: pH 10.4, D: pH 4.2), and IFN-γ at 3 W (E: pH 10.4, F: pH 4.2). At a pH of 10.4 (A, C and E), the light-stained fibers indicate type I and dark-stained fibers indicate types IIa, IIb and IIc. At a pH of 4.2 (B, D and F), the light-stained fibers indicate types IIa, IIb and IIc, and the dark-stainded fibers indicate types I and IIc (weak). Magnification in ×200.

Table 3. Changes of mean cross-sectional areas of type I and II (subtypes: IIa, IIb and IIc) muscle fibers during a 3-week period.

		type II		
	type I (μm^2)	IIa (μm^2)	IIb (μm^2)	IIc (μm^2)
1W: Control	936.2±127.4	967.4±147.5	2,104.9±157.9	624.1±147.1
IFN-α	842.5±186.4	832.1±180.1	2,028.4±131.6	676.1±173.5
IFN-γ	1,061.0±174.2	917.3±130.0	1,530.6±103.2**††	832.1±147.1
2W: Control	1,326.3±196.4	921.3±163.6	2,472.5±213.2	845.1±168.0
IFN-α	1,086.4±173.3	1,229.1±224.9	2,275.0±152	797.5±160.0
IFN-γ	748.9±176.7*	717.7±173.0	1,315.1±144.0***††	817.5±171.5
3W: Control	1,456.3±216.8	1,425.6±194.4	3,160.9±219.6	1,048.3±113.2
IFN-α	958.6±136.6	1,029.4±146.9	2,089.6±198.2*	733.9±104.8
IFN-γ	986.9±148.0	1,014.9±198.7	1,542.4±150.7**†	909.7±159.9

Values are means±SEM.
*$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with control at each week, and †$p<0.05$, ††$p<0.01$ compared with IFN-α at each week.

IIc after IFN-α and IFN-γ injection. In type I, the CSA of IFN-γ at 2W was significantly decreased compared with that of the control at 2W ($p<0.05$); the other CSAs of the IFN-α and IFN-γ groups, however, did not show any significant changes. In type IIb, the CSA of IFN-α at 3W was significantly decreased compared with that of the control ($p<0.01$), and the CSA of the IFN-γ groups was also significantly decreased compared with that of the control (1W: $p<0.01$, and both 2W and 3W: $p<0.001$) and IFN-α groups (both 1W and 2W: $p<0.01$, and 3W: $p<0.05$). Both types IIa and IIc showed no significant changes at each week in any of the three groups.

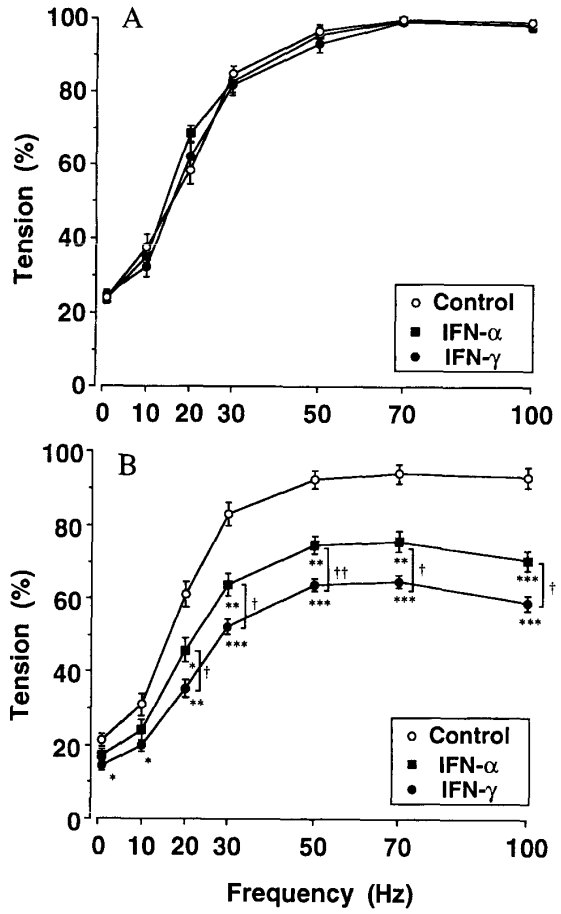

Fig. 4 A: Force-frequency curves were measured in control (open circles) and before the addition of IFN-α (closed squares) or IFN-γ (closed circles). B: Force-frequency curves were measured at 4 hours after the addition of each interferon to the organ bath. With IFN-γ they were more significantly decreased than with IFN-α as compared with the control at 4 hours. ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with control, and $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ compared between IFN-α and IFN-γ at each frequency)

Changes of force-frequency curves in the in vitro study

Figure 4 shows the force-frequency curves obtained from the *in vitro* study. Figure 4A shows the force-frequency curves measured in the control group and in the experimental groups before the addition of IFN-α or IFN-γ; these curves were not significantly different at any frequency. Figure 4B shows the force-frequency curves measured at 4 hours after incubation with IFN-α or IFN-γ in each organ bath at 37°C. As for comparisons of tension at corresponding frequencies, the asterisks and dagger marks indicate significant changes compared with the control and IFN-α groups, respectively. The entire force-frequency curves of IFN-α and IFN-γ were significantly decreased as compared with that of the control as shown by ANOVA ($p<0.05$, $p<0.001$, respectively); however, there was no significant difference between those of IFN-α and IFN-γ.

Discussion

In the present study, there were considerable differences in the effects of IFN-α and -γ on the diaphragm muscle. That is, (I) IFN-α caused decrements in the lower frequencies of the force-frequency curves *in vivo*, and showed a decrement of twitch tension with shortening of contraction time and half relaxation time. (II) IFN-γ caused decrements in the higher frequencies of the force-frequency curves both *in vivo* and *in vitro*, but did not show any

significant changes in twitch kinetics. The diaphragm muscle became more fatigue resistant with IFN-γ. (III) The CSAs of type IIb were more significantly decreased with IFN-γ than with IFN-α. Although the type I CSA decreased significantly at 2W with IFN-γ, CSAs of type I at other weeks and those of types IIa and IIc did not show significant changes in any of the three groups.

According to the changes of the diaphragm wet weight and thickness, and the histologic changes in the present study, it can be concluded that IFN-γ caused greater atrophy of type IIb (FG) muscle fibers, resulting in an increase in the relative precentage of types I (SO) and IIa (FOG) muscle fibers. However, IFN-α caused relatively small changes in both contractile properties and histologic findings compared with IFN-γ. Because IFN-γ has been reported to have a greater suppressive effect on the cell growth of L-1210R cells than IFN-α[12], it is suggested that IFN-γ might also have a stronger effect on cell growth of diaphragm muscle, and induce more atrophic changes than does IFN-α.

Moreover, in the IFN-γ groups, fatigue resistance was increased compared with that in the control and IFN-α groups. In general, type I muscle fiber is more fatigue resistant than type II subtypes IIa (fast-twitch and fatigue resistant) and IIb (fast-twitch and fatigue sensitive) muscle fibers. Therefore, it may be that the increased fatigue resistance by IFN-γ could be caused by the relatively increased ratio of type I and IIa muscle fibers due to type IIb atrophy. Furthermore, based on the diaphragm thickness of the IFN-γ groups, which became thinner than the control and IFN-α groups, there is a possibility that the change of thickness reduced the diffusion distance for oxygen to reach central muscle fibers, thereby promoting aerobic metabolism of the muscle fibers and inhibiting fatigue.

It is possible that the different responses may have been caused by the differences of the dosage and routes of IFN-α (60,000 IU/week, intramuscularly) and IFN-γ (35,000 IU/week, intravenously) in the *in vivo* study. The recommended clinical dose of IFN-α is 3,000,000 IU (3 times per week, intramuscularly) for a patient with hepatitis C[13]. If an adult human's body weight is 60 kg, and an animal's body weight is 0.3 kg, the corresponding dose of IFN-α would be 15,000 IU per animal. The recommended clinical dose of IFN-γ is 2,000,000 IU/m^2 per day for a patient with cancer of the kidney[14]. If an adult human's body surface area is 1.7 m^2 and an animal's body surface area is 0.03 m^2, the corresponding dose of IFN-γ would be 35,000 IU per day. However, we used about one seventh of that dose due to the limitation of IFN-γ storage. Although we selected these dosages of IFNs based on clinical usage, the serum concentrations of IFN-α and IFN-γ by each injection (as shown in Table 1) indicated that the transfer to serum of IFN-α (intramuscular injection) was very low compared with that of IFN-γ (intravenous injection). Therefore, we should consider these different serum concentrations carefully to assess the present results. Although the giwen dosage of IFN-α was 1.7 times larger than that of IFN-γ, the effects of IFN-γ were larger than those of IFN-α in force-frequency curves and fiber areas. Still, we cannot deny the possibility that the differences in serum concentrations between IFN-α and IFN-γ may be related to the present results.

There may be a criticism that the doses of IFN-α and IFN-γ (both 10,000 IU/ml) used in the *in vitro* study were much larger than their concentrations in serum measured in the *in vivo*

study. The previous *in vitro* study used a dose of 10 to 100 IU/ml IFN-γ mixed with lymphocyte cultures to detect its cytotoxic activity[15], and a dose of 10^3 to 10^4 IU/ml incubated with various types of cancer cells to detect its cytocidal effects[16]. These levels are related to a recommendation that a clinical dose of 2×10^6 IU/m^2 of IFNs be used to treat patients with various types of cancer[14]. In the present *in vitro* study, since we suggested that the IFNs in the organ bath, in which the IFNs could be transferred by diffusion not via blood vessels, might require a larger dose, and since we simply wanted to test whether IFNs have or do not have a direct effect on the diaphragm muscle, we selected a dose of IFN-γ (5,000 IU) twice as large as that employed in the *in vivo* study.

In the *in vitro* study, it is interesting that IFN-α and IFN-γ decreased the force frequency curves of the diaphragm muscles only at 4 hours, and that IFN-γ showed a greater reduction of the force-frequency curves than IFN-α at similar doses. Weigent *et al.* showed that IFN-γ has 50 times more cytotoxity against natural killer cells than IFN-α, and also more rapidly acts on natural killer cells than does IFN-α[15]. Because IFN-γ decreased the force-frequency curves more rapidly than IFN-α at 4 hours incubation, these different speeds in the effectiveness of IFNs can account for the fact that IFN-γ showed a significant decrease in the force frequency curves at 1W in the *in vivo* study. Although it is well known that IFNs bind to specific receptors on the cell surface and elicit a variety of cellular responses[17,18], the differences in responsiveness may be attributed to post-receptor signaling of the diaphragm muscle cells.

If IFN-γ induces a deterioration in the diaphragm muscle, it is possible that synergistic effects of IFN-γ and TNF-α can occur in endotoxin-induced diaphragm muscle dysfunction. Recently, we have reported that TNF-α mRNA is expressed in diaphragm tissue after endotoxin administration and suggested that TNF-α contributes to the deterioration of diaphragm muscle contraction[7]. Moreover, TNF-α at a low concentration (2×10^2 U/ml) in combination with IFN-γ (10^3 U/ml) showed a synergistic effect on the inhibition of cell proliferation on the 35th day of treatment[6]. Because there is a consensus sequence (TTATTTAT) present in the 3′-untranslated region of both human and mouse TNF mRNAs as well as in the mRNAs encoding human and mouse IFNs[19], it appears that IFN-γ and TNF-α are simultaneously triggered by endotoxin. If so, these cytokines may play an important role in endotoxin-induced diaphragm dysfunction.

In summary, both IFN-α and IFN-γ seem to have direct effects on diaphragm muscle; furthermore, IFN-γ has a more deleterious effect on diaphragm muscle than does IFN-α and induces atrophic changes in type IIb muscle fibers in the case of clinical dosages.

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