

EFFECTS OF SHAKING AND FOOT-SHOCK ON IMMUNE FUNCTION OF MICE

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Immune responsiveness of DBA/2 mice subjected to chronic treatment with two physical stressors (shaking and foot-shock) was found to be significantly suppressed. Both shaking and foot-shock impaired the humoral but not cell-mediated immune function of the animals. Effector cells in the natural surveillance system for defending the animals against neoplastic cells were also impaired. In the plaque-forming cell assay, depression of immune response induced by foot-shock was significantly greater than that produced by shaking.

INTRODUCTION

Various types of physical (1, 2, 3, 4, 5) and psychological stress (6, 7) in animals have been shown to depress the immune system, in its humoral and cell-mediated functions (4, 8), and consequently to weaken the resistance to viral infections (6) and to neoplastic disorders (9). However, under certain conditions some stressors have been reported to enhance immune response (10), indicating the difficulty in predicting the actual outcome of a stressful event (10, 11).

A graded series of tail-shocks in rats has been found to produce progressively greater suppression of cell-mediated immune response; i.e., phytohemagglutinin-induced lymphocyte proliferation (5). The present stress study was conducted to determine whether or not a similar suppression is observable when an electric shock of lower intensity is daily administered for a longer period (five weeks) by another route (foot-shock) to another species (mice). Moreover, the effects of regular administration of whole-body shaking not only on the cell-mediated immune response but also on the humoral immune function were investigated. Since a subpopulation of lymphocytes known as natural killer (NK) cells has been shown to be one of the effector cells in the natural surveillance system for defending the body against neoplastic cells (12, 13), the effects of the stressors on the splenic NK activities of the animals were also examined.

MATERIALS AND METHODS

Animals

Female DBA/2 mice at the age of five to eight weeks, purchased from Charles River Laboratory, Wilmington, MA, were used in this study.

Stress Treatments

DBA/2 mice were randomly assigned to three groups: (I) shaken group, (II) shocked group, and (III) control group. Group I was put twice daily in a wooden box (65 cm × 48 cm × 13 cm) attached to the top of a shaker (New Brunswick Scientific Co., Model No. R-7). A hardware mesh cover was laid over the box and the animals were subjected to back and forth movements of the shaker for 10 min. The shaker was set to oscillate at a rate of 175 cycles per minute. Group II was placed twice daily in a plastic cylindrical chamber (30 cm high and 30 cm in diameter) with a grid floor. At each treatment session they were exposed to foot-shock (0.8 mA, 70 V dc) for 10 min. The control group was assigned to two subgroups, (a) sham control group that was placed once daily in the shaker and in the shock chamber, but it was neither shaken nor shocked, and (b) naive control group that was left undisturbed in its home cage during the entire experimental period. All mice were subjected to the above treatments for five weeks.

Lymphocyte Collection

Single-cell suspensions were prepared by pressing spleens through a 100-mesh stainless steel screen into sterile Dulbecco's phosphate-buffered saline (DPBS) solution.

Red blood cells were lysed by treating the cell pools with ammonium chloride/tris-(hydroxymethyl) aminomethane - buffered solution (14). Cells were washed three times in DPBS before resuspending in the RPMI 1640 culture medium.

Lymphoproliferative Assay

From four mice in each of the two treated groups and from four animals in each of the two control groups, splenic lymphocytes were collected individually and suspended in DPBS solution. The 16 suspensions were resuspended separately in the RPMI 1640 medium supplemented with 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 5% heat-inactivated fetal bovine serum (FBS, Armour Pharmaceutical Co., Phoenix, AZ) to prepare 16 cultures. The cultures were stimulated with T-cell specific mitogens: namely, concanavalin A (Con A) and phytohemagglutinin (PHA), and with B-cell specific mitogen, i.e., lipopolysaccharide (LPS), in the following manner. From each of the 16 cultures a set of three samples (each 5×10^5 cells) was separately incubated with 0.5 μg Con A in microtiter wells at 37 C for three days. Similarly, from each culture second and third sets of three samples (each 5×10^5 cells) were separately incubated with 0.3 μg PHA, and 5 μg LPS, respectively. All mitogen (Difco Lab., Detroit, MI) concentrations were predetermined to be optimal doses for maximum stimulation. The cells were then pulsed with [^3H] -thymidine solution (0.5 μCi in 10 μl , New England Nuclear, Boston, MA). After an additional 24-hr incubation, the cells were harvested on a MASH II cell harvester (Microbiological Assoc., Walkersville, MD) and counted in a Beckman LS 7000 liquid scintillation counter.

Plaque-forming Cell (PFC) Assay

Splenic PFC levels of stress-treated and control mice were determined by Cunningham's modification of the Jerne-Nordin antibody plaque-forming cells assay for response to sheep erythrocytes (15). Five days prior to the completion of the stress treatments four mice from each of the two treated and each of the two control groups were given an intravenous injection of 0.1 ml of a 20% sheep red blood cell (SRBC) suspension in saline. Upon completion of the treatments, these animals were sacrificed and single-cell suspensions from the spleens were prepared. and adjusted to 4×10^6 cells/ml. A 0.1-ml aliquot of each of the cell suspension was mixed with 0.3 ml of 10% SRBC, 0.1 ml of guinea pig complement (Cappel Laboratories, Inc., Cochranville, PA), and 0.5 ml RPMI 1640 culture medium. A volume of 150 μl of this mixture was transferred to a Cunningham chamber. The chamber was sealed with molten wax and was incubated at 37 C for 1 to 1.5 hr prior to the counting of PFCs with an inverted microscope. Duplicate chambers were made from each splenic cell suspension.

Delayed-type Hypersensitivity (DTH) Reaction

Eight to ten mice from each of the two treated and each of the two control groups were immunized subcutaneously with 1×10^9 SRBC in complete Freund's adjuvant six days prior to completion of the stress treatments. DTH reactions to SRBC were measured by the foot-pad swelling method (16). The thickness of the right hind foot-pad and that of the left were measured prior to injection of 0.03 ml of SRBC (4×10^9 cells/ml) into the right pad and 0.03 ml of sterile physiological saline into the left. Twenty-four hours after injection, the right and left pads were again measured. All measurements were obtained in millimeters with a vernier caliper. The change (Z_{24}) in the foot-pad size at 24 hr after the administration of SRBC was determined by applying the following equation:

$$Z_{24} = (R_{24} - R_0) - (L_{24} - L_0),$$

where R_{24} represents the right foot-pad measurement at 24 hr; R_0 denotes the right foot-pad thickness before injection, and similarly, L_{24} and L_0 stand for the left foot-pad measurements at 24 hr post and zero hour preinjection, respectively.

Chromium-51 Release Assay

A classic *in vitro* 4-hr ^{51}Cr release assay with YAC-1 lymphoma cells as targets (13) was conducted to determine the effects of stress treatments on the tumoricidal activity of NK cells in the spleens of the mice. Target YAC-1 lymphoma cells were grown in the RPMI 1640 medium supplemented with antibiotics and 10% FBS. One $\times 10^7$ cells were incubated with 100 μCi of radioactive sodium chromate (^{51}Cr , New England Nuclear, Boston, MA) for 45 min at

37 C. After three washings to remove excess unbound ^{51}Cr , the labeled target cells were resuspended in the culture medium at 1×10^6 cells/ml.

From four mice in each of the stressed groups and from four animals in the sham control group, effector splenic lymphocyte suspensions were prepared individually. From these suspensions, various amounts of effector cells were added to microtiter wells containing 0.1 ml of target YAC-1 cells in order to achieve final effector-to-target cell ratios of 10:1, 50:1, 100:1, and 200: 1. After incubation for 4 hr at 37 C, the cells were pelleted and 0.08 ml of the supernatant was removed to be assayed in a gamma counter. Percent specific ^{51}Cr release was calculated according to the following formula:

$$\% \text{ } ^{51}\text{Cr} \text{ release} = \frac{T - S}{M - S} \times 100\%$$

where T represents counts per minute (cpm) of ^{51}Cr release in the presence of effector cells, S represents the spontaneous release of ^{51}Cr from target cells incubated with the culture medium alone, and M represents maximum release of ^{51}Cr upon addition of 2N HCl to target cells. All effector-target cultures were prepared in triplicates.

RESULTS

Effects of Stress on Lymphoproliferative Response to Mitogens

The results of the mitogen stimulations of the splenic lymphocytes obtained from the stressed and from the control animals are shown in Table 1. The t-test showed that each of the three means of the sham group was not significantly different from the corresponding means of the naive group. Therefore, each of the three means of the sham group was combined with each corresponding mean of the naive group. The three combined control means were compared with the corresponding means of the stressed groups. In the tests of lymphoproliferative response to Con A and to PHA stimulations the two means of the shaken group and those of the shocked group were not significantly different from the corresponding combined means of the two control groups. Therefore, the stress treatments had no significant effects on the lymphoproliferative response to the T-cell-specific mitogens. However, in the LPS test the shaking and shock treatment produced significant reductions (33% and 37%, respectively) in the lymphoproliferative response to the B-cells-specific mitogen.

Effects of Stress on Plaque Cell Formation

The results of the PFC assay are shown in Table 2. Significant reduction of PFC levels in response to SRBC was observed in mice which had been subjected to shaking (30% reduction) or to foot-shock (50% reduction).

Effects of Stress on DTH Response

The results of the DTH reaction test are summarized in Table 3. Since the t-test indicated that the mean foot-pad swelling of the sham group was not significantly different from that of the naive group, the two means were combined for the main analysis. Further application of the t-test revealed that the mean foot-pad swelling of the shaken group and also that of the shocked group were not significantly different from the combined control mean.

TABLE 1. *Effects of shaking and foot-shock on the in vitro lymphoproliferative response to mitogens*

Treatment	[^3H]-Thymidine incorporation $\times 10^3$ cpm (Mean ^a \pm S.D.)		
	Con A	PHA	LPS
Shaken	61.0 \pm 2.4	6.1 \pm 0.9	37.9 ^b \pm 2.5
Shock	64.7 \pm 3.5	5.8 \pm 1.3	36.0 ^b \pm 2.3
Sham (handled)	58.7 \pm 5.4	6.2 \pm 1.7	57.0 \pm 1.9
Naive (unhandled)	60.4 \pm 1.5	6.5 \pm 1.0	56.6 \pm 2.3

^aMean net cpm of triplicated microcultures from four animals, S.D. = standard deviation.

^b $p < 0.05$; Student's t-test compared with the combined mean of the sham and naive control groups.

Effects of Stress on Splenic NK Activities

The results of the evaluation of the activities of splenic NK cells were graphically summarized by plotting percent specific ^{51}Cr release (in the presence of a graded amount of NK cells and a specific quantity of YAC-1 lymphoma cells) against effector-to-target cell ratio as shown in Fig. 1. The splenic cells from the sham control group exhibited relatively high NK cytotoxicity activities (7 to 12% specific ^{51}Cr release), whereas stressed mice demonstrated substantially lower activities at all effector-to-target ratios examined. Suppression of NK activities was greater in the shocked animals than in the shaken ones at the effector-to-target ratio of 100:1.

DISCUSSION

The humoral immune response, as measured by the enumeration of splenic PFCs, was inhibited in mice after a five-week exposure to shaking or to foot-shock. Inhibition of lymphoproliferative responsiveness to the B-cell mitogen (LPS) in these animals substantiated the detrimental effects of the stressors on the humoral immune function. The cell-mediated immune function, as measured by delayed hypersensitivity response to SRBC and lymphoproliferative responsiveness to the T-cell mitogens (con A and PHA), was not affected. On the other hand, Keller et al. (5) recently reported that a graded series (0.8, 1.0, 1.2, 1.6, 2.4 and 3.0 mA) of tail-shocks produced progressively greater suppression of T lymphocyte function, as measured by PHA stimulation of T lymphocytes in rats. The discrepancy between their *results* and ours might have been due to the difference in the concentration of PHA in the final culture (2.0 and 4.0 $\mu\text{g}/\text{ml}$ in their study and 0.3 $\mu\text{g}/\text{ml}$ in our experiment). The discrepancy might also have been due to the difference (a) in animal species, (b) in the route of stress administration (tail-shock vs foot-shock), (c) in the current intensity, and (d) in the duration of shock treatment (several days vs five weeks).

The results revealed that the activities of splenic NK cells obtained from the shaken mice and those collected from the shocked mice were highly suppressed. In most cases the activities of NK cells of the shaken animals were depressed as much as those of the shocked animals. However, at the target ratio of 100:1 the suppression of NK activities was greater in the shocked than in the shaken group. It will be interesting to

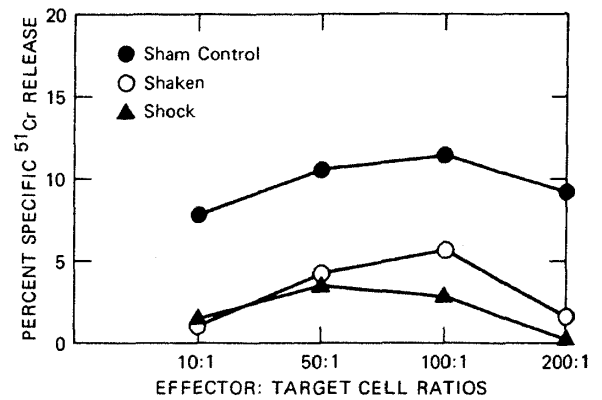


FIGURE 1. Effects of shaking and foot-shock on splenic NK activities

TABLE 2. Effects of shaking and foot-shock on plaque cell formation

Treatment	PFC/spleen Mean ^a	% reduction
Shaken	1400	30 ^b
Shock	1000	50 ^b
Sham (handled)	2003	0
Naive (unhandled)	2025	0

^aAverage PFC obtained from four individual animals.

^b $p < 0.01$; Student's t-test compared with the combined mean of the sham and naive control groups.

TABLE 3. Effects of shaking and foot-shock on DTH response to SRBC

Treatments	Total no. of animals tested	Foot-pad swelling (Z_{24}) Mean \pm S.D. (mm)	t-test ^a	
			t value	Significance
Shaken	8	0.63 \pm 0.26	2.058	No
Shock	10	0.78 \pm 0.32	0.290	No
Sham (handled)	8	0.83 \pm 0.15	—	—
Naive (unhandled)	8	0.81 \pm 0.23	—	—

^aComparison with the combined mean of the sham and naive control groups.

investigate whether or not a similar difference in NK activities will be observed at the target ratio of 100:1 when further experiments are conducted with different intensities of shock and of shaking.

In most assays the immune responses of the shaken mice were depressed as much as those of the shocked animals. However, in the plaque-forming cell assay the reduction was significantly greater in the shocked group than in the shaken group, indicating that in some instances different types of stressors may impose various levels of modulation on the immune system. The mechanism of stress-induced suppression of humoral and NK function is not yet known. It is possible that increased levels of corticosteroids (4, 10, 17), enhanced sensitivity of lymphocytes to inhibition by prostaglandin (18), and stress-induced production of suppressor cells (3, 19) or suppressor factors such as polypeptides (20), may play a role in the immunosuppressions observed in this study. Further investigation is needed to confirm this speculation.

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