Stress and Immune Responses

III. Effect of Restraint Stress on Delayed Type Hypersensitivity (DTH) Response, Natural Killer (NK) Activity and Phagocytosis in Mice

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Abstract—Several experiments were conducted to evaluate the influences of restraint stress on cell-mediated immune events in mice. Delayed type hypersensitivity response to sheep red blood cells was inhibited by the stress, regardless of the timing of restraint stress loading. The activity of phagocytosis of macrophages in vitro and in vivo were measured by using the zymosan-particle uptake method and the carbon clearance test, respectively. Both activities were decreased in restraint-stressed mice. The suppressed carbon clearance rate in stressed mice, however, was recovered by the transfusion of serum from normal mice. Natural killer activity in spleen cells was decreased to 30–50% of the control in stressed mice. However, no suppressor cells which could inhibit NK activity existed in the spleen from stressed mice. These results show that the restraint stress suppresses various kinds of cell-mediated immune events, which might play an important role in antitumor immunity.

In recent years, there has been a growing concern regarding the role of environmental and psychological factors in the induction and growth of malignant tumors (1–3). A considerable number of experimental studies using animals have been reported (4–8). Many investigators have reported stress-induced exacerbation of tumorigenicity. Riley found that moderate chronic or intermittent stress might predispose C3H/He mice to an increased risk of mammary carcinoma (4). Sklar and Anisman have reported that a single session of inescapable shock resulted in earlier tumor appearance, exaggeration of tumor size, and decreased survival time in recipient animals (6). Recently, general remarks about “Stress and Cancer” was published (9). However, these data only showed about the correlation between stress and tumor growth, not the mechanisms of these phenomenon.

It has been pointed out that reduced cell-mediated immunity may facilitate growth of tumors or metastasis (10–12). So in order to clarify the mechanisms of facilitation of tumor growth and metastasis by stress loading, we investigated the activity of cell-mediated immunity such as delayed type hypersensitivity (DTH) response, natural killer (NK) activity and phagocytic functions in stressed mice. In the present paper, we described the changes of cell-mediated immunity by restraint stress, and the mechanisms of stress-induced growth of tumors were discussed.

Materials and Methods

Animals: Female BALB/c mice and C57BL/6 mice, all 8 weeks old, were obtained from Japan Charles River Breeding Laboratories.
Chemicals, culture media and antigens: Chemicals used in the present work were purchased from the following sources: carbon particles (Pelikan C11/1432a), zymosan (Sigma Chemical Company, St. Louis, MO), and \(^{51}\)Cr (RCC Amersham, Amersham, England). Sheep red blood cells (SRBC) were obtained from Nishinippon Sheep Farm (Fukuyama, Japan) as a suspension in Alserver's solution. Foetal calf serum (FCS) was the product of GIBCO (Gland Island, NY). RPMI-1640 medium was purchased from Nissui Pharmaceutical Company (Tokyo, Japan).

DTH response: The estimation of DTH response to SRBC was carried out following the method described by Lagrange et al. (13). Mice were immunized by injection of \(1 \times 10^8\) SRBC in 0.05 ml of saline into the back. Four days thereafter, the reaction was elicited by injection of \(1 \times 10^8\) SRBC into the hind footpad, and the increase of the thickness of the footpad was measured at a loading weight of 100–110 g with a dial thickness gauge G (Ozaki MFG. Co., Ltd.) 24 hr later.

In vivo phagocytosis of macrophages: Phagocytic activity in vivo was estimated by using the carbon-clearance test. The carbon suspension was centrifuged at 5,000 r.p.m. for 15 min, and the supernatant was diluted 3-fold with sterile 1.5% gelatin saline to bring the carbon concentration to about 30 mg/ml. Diluted carbon suspension was injected at 0.1 ml/10 g body weight into the tail vein of mice. A blood sample was obtained 0.5 and 10 min after the injection of carbon by puncturing the retro-orbital venous plexus. The blood (0.05 ml) was hemolyzed by the addition of 1 ml 0.1% \(\text{Na}_2\text{CO}_3\) solution, followed by measurement of its optical density at 600 nm. The phagocytic index, \(K\), was derived from the following equation:

\[
K = \frac{1}{t_{10} - t_{0.5}} \log \frac{C_{0.5}}{C_{10}},
\]

where \(C_{0.5}\) and \(C_{10}\) express the carbon concentration at time \(t_{0.5}\) and \(t_{10}\), respectively.

In vitro phagocytosis of macrophages: Macrophages were collected by washing the peritoneal cavity with ice-cold PBS (0.1 M, pH 7.3). The cells were washed once and then resuspended in an adequate volume of RPMI-1640 medium supplemented with 10% heat-inactivated FCS to give a concentration of \(1 \times 10^6\) nucleated cells per ml. Aliquots (1 ml) of this suspension were seeded in 35 mm petri dishes (Falcon) and cultivated at 37°C in the humidified atmosphere of 5% \(\text{CO}_2\) and 95% air. Three hours later, non-adherent cells were washed off, and 1 ml fresh medium, i.e., RPMI-1640 containing 10% FCS, was added. Zymosan was added to give \(5 \times 10^6\) particles/ml and incubation was continued. Thirty minutes later, particle uptake was measured by light microscopy. Cells containing three or more zymosan particles were counted as phagocytic.

NK activity: Various concentrations of effector cells (5–20 \(\times 10^5\)/well, spleen cells from C57BL/6 mice) were incubated with \(1 \times 10^4\) \(^{51}\)Cr labeled YAC-1 target cells for 4 hr at 37°C in a round bottomed 96-well microtiter plate (Nunc) according to the method of Reynolds et al. (14). After incubation, the supernatant from each well was carefully removed and counted in a gamma counter (Aloka). Supernatants from microwells containing target cells alone served as controls for the baseline release of \(^{51}\)Cr. \(^{51}\)Cr-labeled target cells (\(1 \times 10^4\)) were also counted to determine total \(^{51}\)Cr incorporated into the target cells. Percent lysis was calculated as follows:

\[
\% \text{ lysis} = \frac{\text{Test cpm} - \text{medium control cpm}}{\text{Total cpm} - \text{background cpm}} \times 100
\]

Stressing procedure: Experimental mice were fixed in the restraint cages for 12 hr a day at night (21:00–9:00) and placed in home cages for the remaining 12 hr with food and water ad libitum. The restraint cages were prepared according to the literature (15). Usually, mice were restrained for 2 consecutive days. The control mice were allowed to remain in their home cages from which food and water were removed during the stress period of the counterparts.

Statistics: Statistical significance was calculated by Student's \(t\)-test. Differences were considered to be significant when the probability (\(P\)) value was <0.05.
Results

Effect of restraint stress on the DTH response: Mice were stressed prior to immunization with SRBC. In restrained mice, the DTH response to SRBC was significantly reduced at 24 hr post challenge (Fig. 1A). Similar results have been obtained when the mice were stressed after immunization (Fig. 1B). These results indicate that the DTH response, not antibody response to SRBC, can be suppressed by restraint stress, irrespective of timing of stress application.

NK activity in spleen cells from restrained mice: NK activity in spleen cells from restrained mice was compared with that from non-stressed control mice. As shown in Fig. 2, NK activity of the stressed mice was significantly reduced to 25–50% of the control within the ratios of 1:50–1:200 as target cells: effector cells. Uchida et al. have reported that operative stress inhibited NK activity in humans and suggested the existence of suppressor monocytes in this suppression (16). To ascertain the possible role of this type of suppressor cells, keeping a constant cell density of $2 \times 10^6$ cells/well, spleen cells from control and restrained mice were mixed in various ratios and the NK activities were measured. As shown in Fig.

![Graph](image1.png)

![Graph](image2.png)

Fig. 1. Suppression of delayed-type hypersensitivity reaction to SRBC in restrained mice. (A): BALB/c mice were fixed in the restraint cage for 12 hr a day for 2 consecutive days and then subcutaneously immunized with SRBC as described under Materials and Methods. (B): Mice were immunized with SRBC and then fixed for 2 consecutive days. Four days after immunization, DTH reaction was elicited. The results represent the mean±S.D. of 4 animals. Significant difference from each control group: *P < 0.05.
3, the observed NK activity was very close to the expected value. The expected values were calculated from the 100% values for the control cells. At no ratio was significant suppression observed, indicating that spleen cells from restrained mice contain few, if any, cells that can suppress the normal NK activity.

Effect of restraint stress on the function of phagocytosis of macrophages: At first, we investigated in vitro phagocytic activity of peritoneal macrophages. Peritoneal macrophages obtained from control and stressed mice were cultured with zymosan particles

Table 1. Phagocytosis by peritoneal macrophages obtained from control and restraint-stressed mice

<table>
<thead>
<tr>
<th>Peritoneal macrophages from</th>
<th>% particle (zymosan) uptake of 100 cells</th>
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<tbody>
<tr>
<td>Control mice</td>
<td>46.0±10.2</td>
</tr>
<tr>
<td>Stressed mice</td>
<td>22.3±5.4**</td>
</tr>
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BALB/c mice were fixed in the restraint cage for 12 hr a day for 2 consecutive days. Macrophages were collected by washing the peritoneal cavity of these mice and cultured in RPMI-1640 medium containing 10% FCS with 5×10⁶ zymosan particles for 30 min. Particle uptake was measured as described under Materials and Methods. The results represent the mean±S.D. of 4 animals. Significant difference from the control group: **P<0.01.
for 30 min. The result are shown in Table 1. The same number of macrophages were obtained from the peritoneal cavity of control and stressed mice. However, the number of macrophages containing zymosan particles were reduced to 50% of the control in stressed mice. About 100% of the macrophages from control mice ingested zymosan by further incubation for 30 min.

In the next experiment, the carbon clearance test was carried out for measuring in vivo phagocytic activity. Two days stressed mice were injected with carbon particles immediately after the finish of the stress loading. As shown in Fig. 4, the clearance rate of stressed mice was significantly decreased at 30% of the control.

The rate of carbon clearance is known to be influenced by some serum factors (17). So we infused 0.3 ml of serum from normal syngeneic mice to stressed ones and measured the carbon clearance rate after 30 min of injection. These data are shown in Fig. 5. The suppressed rate was recovered to 70% of the control by the infusion of normal serum.

Discussion

In the present study, it was clarified that the DTH reaction, NK activity and phagocytic functions were suppressed in restraint-stressed mice. DTH response is a typical model for the type IV allergic reaction, and Lyt-1+ T_{DTH} cells have been known as an
effector cell in this response. In recent years, a close correlation between the activity of the DTH reaction and that of anti-tumor immunity has been found in many laboratories; that is, Lyt-1+ T\text{\textsuperscript{L}}/T cells rather than Lyt-2,3+ T cells have been shown to play a major role in anti-tumor immunity (18, 19). So the suppression of the DTH response by restraint stress, which was observed in this study, might be one of the mechanisms of facilitation of tumor growth and metastasis by stress loading. Both NK activity and phagocytic activity were also suppressed in restrained mice. These cells have been recognized to play an important role in anti-tumor immunity and suggested to be activated by Lyt-1+ T cells (17, 20, 21). So anti-tumor immunity may be influenced by the restraint stress through the various pathways of the suppression.

On the other hand, the suppression of in vivo phagocytic activity was recovered by the infusion of the serum from normal mice. We suspect that depression of phagocytic activity was caused by the changes of some serum components in restrained mice. However, in vitro phagocytic activity of peritoneal macrophages was also significantly suppressed in stressed mice. These results indicate that depression of phagocytic activity was due to both the impairment of the functions of macrophages and the changes in serum components of stressed mice.

We observed the inhibition of NK activity of spleen cells in restraint-stressed mice. However, it is not clear whether this suppression is attributable to the inhibition of the function or the decrease in the number of NK cells. Recently, it has been demonstrated that there is a close association between large granular lymphocytes (LGL) and NK cells in humans and rats (22–24). We preliminary examined the LGL contents in stressed spleen cells and observed the decrease in the number of LGL (data not shown).

In recent years, there has been a growing concern regarding the role of environmental and psychologic factors in the induction and growth of malignant tumors. A considerable number of investigators have found that stress exacerbates tumor development in experimental studies using animals. Therefore, the influences of stress on the development of cancer must be considered in the course of human therapy.

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References


