Original Papers

The effect of emotional stress on the primary humoral immunity of rats

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The effect of exposure to emotional stress on humoral immune function after injection with ovalbumin, a novel antigen, was studied in adult male Wistar rats. Emotional stress was induced by randomly giving empty water bottles to rats trained to drink water at set times. The results showed that emotional stress induced the decrease in spleen weight and specific immunoglobulin G antibody level to ovalbumin, and increased levels of epinephrine, norepinephrine and corticosterone. A decrease of antibody levels correlated negatively with an increase in plasma norepinephrine levels. These findings suggest that emotional stress can modulate immune function, and that sympathetic nervous system may be involved in this immunomodulation.

Key words: antibody, emotional stress, empty water bottles, immunomodulation, sympathetic nervous system

Introduction

The notion that stress can induce alteration of immune function has been supported by increasing evidence from experimental data (Lawrence and Kim, 2000). However, most investigations dealing with the effects of stress on the immune system in animals employed restraint, electric footshock as a stressor (Cunnick *et al*., 1988; Lu *et al*., 1998; Yin *et al*., 2000; Bauer *et al*., 2001). Recently, the impact of psychological stress on immune function has been the subject of extensive research efforts. In human research, psychological stress has been associated with suppression of NK activity, mitogen- and antigen-induced lymphocyte proliferation, in-vitro production of interleukin-2 and interferon (Marshall and Agarwal, 2000) and leukocyte subset distribution (Maes *et al*., 1999). Using animal models, it has been suggested that psychosocial stressors influence the humoral and cellular immunity of rats (Strange *et al*., 2000; Wu *et al*., 2000). Although these studies have suggested that psychological stress has effects on various components of immune responses, the major pathway involved in this immunomodulation has not yet been determined.

It has been well known that stress activates the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS), leading to the release of glucocorticoids and catecholamines. Both catecholamines and glucocorticoids have been shown to affect immune response (McEwen *et al*., 1997), suggesting stress-induced immunomodulation is mediated, possibly in part, through the SNS and HPA axes. Some investigators have found that, using adrenalectomy or chemical blockade of glucocorticoid receptors, certain effects of stressful conditions (e.g. restraint) do appear to be mediated through the action of glucocorticoids (Okimura *et al*., 1986), while others have indicated that administration of catecholamine receptor antagonist nadolol reversed the shock-induced suppression of the response of splenic lymphocytes to mitogen stimulation (Cunnick *et al*., 1990). Dobbs *et al*.

demonstrated that both corticosterone and catecholamine-mediated mechanisms were operative in the restraint stress-induced suppression of anti-viral cellular immunity (Dobbe *et al*., 1993). It is possible that the nature of stressors determines the major pathway involved in the immunomodulation. To date, there has been little data on the relationship between the HPA axis or SNS and the immunoregulation (especially humoral immunity) induced by psychological stress.

The purpose of the present study was to establish a new animal model of emotional stress-mediated effects on the primary humoral immunity and to examine the roles of SNS and HPA axes. In the present study, an empty water bottle was used as an emotional stressor to rats, which were trained to drink water at two set times each day. Ovalbumin (OVA), a novel antigen, was used to induce the humoral immune response. The effect of emotional stress on the formation of anti-OVA immunoglobulin (Ig)G antibody, spleen weights and levels of norepinephrine, epinephrine and corticosterone was investigated.

Materials and methods

Subjects

Male Wistar rats, aged 60 days and weighing 220–250 g, were purchased from the Animal Center of Beijing Medical University. Upon arrival, the rats were weighed, individually caged and maintained on a 12 : 12 h light/dark cycle (lights on 07.00 h) through artificial illumination. The rats were given food and water *ad libitum* except during the testing periods.

Habituation and training

Rats were habituated to the laboratory environment for 1 week and gently handled each day. After the habituation period, the subjects were trained to drink water at 09.00 h to 09.10 h and 21.00 h to

Table 1 Stress procedure

Group	Time	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ES	09.00 h to 09.10 h	N	N	EB	EB	N	EB	EB	N	N	N	EB	N	N	N	EB
	21.00 h to 21.10 h	EB	EB	N	EB	N	N	N	EB	EB	N	EB	EB	EB	N	–
C2	09.00 h to 09.10 h	N	N	NB	NB	N	NB	NB	N	N	N	NB	N	N	N	NB
	21.00 h to 21.10 h	NB	NB	N	NB	N	N	N	NB	NB	N	NB	NB	NB	N	–
C1	09.00 h to 09.10 h	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
	21.00 h to 21.10 h	N	N	N	N	N	N	N	N	N	N	N	N	N	N	–

EB, Empty water bottle; NB, no empty water bottle and water; N, drink water at trained time.

21.10 h by allowing them access to water bottles only during these time periods for 1 week. The fluid intake of the rats was measured for each time period (10 min). After training, all the subjects were weighed and then injected with 100 μg ovalbumin (OVA) (Sigma, St Louis, MO, USA) i.p. in phosphate-buffered saline (PBS) emulsified in an equal volume of Freund's complete adjuvant (Sigma).

Experimental procedure

From the day of immunization (day 0), the rats were randomly assigned to one of three groups (n = 20 for each group): (i) emotional stress (ES); (ii) control 1 (C1) and (iii) control 2 (C2) group. Rats in the ES group were irregularly given empty water bottles during one of the two watering periods for 2 weeks to induce emotional stress. Rats in the C2 group were given neither empty water bottles nor water during the same watering periods as for ES group. This group was used to control the possible effect that an animal, which is trained to receive water at a particular time of day, is also emotionally stressed if it does not receive water at this time. Rats in the C1 group were allowed free access to water during all watering periods (Table 1). Rats in the ES and C1 group were housed in one room and rats in the C2 group were housed in another room. The behaviours of all the rats in the three groups during the experimental session of 10 min were observed throughout, and fluid intake of the rats was also evaluated during 10-min access to water. The behaviours observed included Attacking (biting empty water bottles and cage shed); Exploring (rearing and looking outside from the cage); and Grooming (self-grooming, washing and scratching). According to the intensity of activity, the evaluation of each behaviour item was recorded as a score of 0, 1, 2 or 3, respectively, and a mean of all scores over the 14 days was used for statistical analysis. After the completion of 14 experimental sessions, all subjects were weighed and then decapitated immediately to obtain a blood sample. Their spleens were also removed to measure the spleen indices [spleen weight (mg)/body weight (g)].

Determination of antibody

Blood was centrifuged to separate serum and plasma. Serum levels of IgG antibodies to OVA were determined by enzyme-linked immunosorbent assay (ELISA). The wells of a microtitre plate were coated with OVA (100 μl/well, 1 mg/ml OVA). Plates were incubated overnight at 4 °C. The following day they were washed twice with Tween-phosphate buffered saline (0.05% Tween 20, pH 7.4) and twice with double distilled water. The plates were then blocked for one hour at 37 °C with 10 mg/ml bovine serum albumin (BSA) in PBS (Tween 20 0.005 μl/ml), 100 μl/well and then washed again. 1 : 100 and 1 : 200 dilutions of serum were prepared using BSA containing Tween 20 at a concentration of 0.005 μl/ml. One plate was used for each dilution, three wells per

sample, 100 μl/well. BSA was only added to the wells of the first column of each plate to serve as a blank. The plates were then incubated for one hour at 37 °C. All the wells were washed again and then goat anti-rat IgG antibody (Sigma) diluted 1 : 5000 was added to all the wells of the microtitre plates. The plates were then incubated for 1 h at 37 °C and washed once again. Finally, 100 μl of enzyme substrate was added to each of the wells. Colour was allowed to develop for 10 min and the reaction was stopped by the addition of 50 μl of 2 N H_2SO_4. The optical density (OD) of each well was read on an ELISA plate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 490 nm (Lin *et al*., 1993).

Determination of corticosterone and catecholamine levels

Corticosterone levels in serum were determined by using radioimmunoassay (Sainio *et al*., 1988). Catecholamine levels in plasma were determined using high-performance liquid chromatography (Gerlo and Malfait, 1985).

Statistical analysis

All data are expressed as mean ± SD. For statistical evaluation of these results, a one-way analysis of variance (ANOVA) was used. If a significant main effect of group was identified ($p < 0.05$), post-hoc comparisons between groups were performed using the LSD test. $p < 0.05$ was considered statistically significant.

Results

### The changes in fluid intake and body weight of the rats

During training procedure, the fluid intake of the rats for each day was not significantly different among the three groups ($p > 0.05$). During experimental procedure, the fluid intake of the rats in ES and C2 group for each day was reduced significantly compared to that of the rats in C1 group [ANOVA: $F(2,57)$ = 234.536, $p < 0.001$; post-hoc: $p < 0.001$, $p < 0.001$] but there was no significant difference between that in the ES group and C2 group ($p > 0.05$).

At the time of arrival and after training, there was no significant difference in body weight among the three groups of rats. However, after experimental procedure, the values in body weight of the rats in ES and C2 group reduced significantly compared to that in the C1 group [ANOVA: $F(2,57)$ = 10.202, $p < 0.01$; post-hoc: $p < 0.01$, $p < 0.01$].

The effect of emotional stress on the behaviour of rats

From day 1 to day 14, attacking behaviours were exhibited strongly in the rats exposed to emotional stress, but not in the rats

of C1 and C2 groups, suggesting that the rats in the ES group were significantly behaviourally stressed. Rats of both the ES and C2 groups expressed significant exploring behaviours compared to rats of the C1 group [ANOVA: $F(2,57) = 88.672$, $p < 0.001$; post-hoc: $p < 0.001$, $p < 0.001$] but there was no difference between rats in the ES group and rats in the C2 group. For rats of the C1 group, grooming behaviours were obvious compared to rats of the ES and C2 groups [ANOVA: $F(2,57) = 164.7$, $p < 0.001$; post-hoc: $p < 0.001$, $p < 0.001$] (Fig. 1).
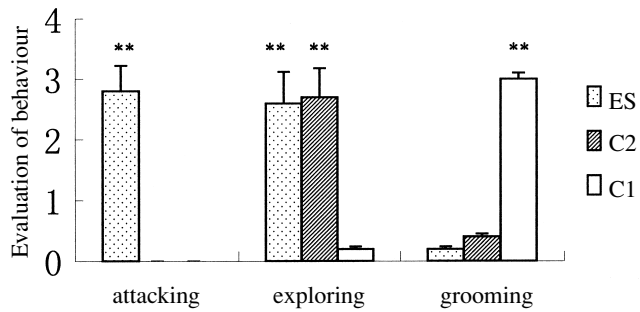
Figure 1 The effect of emotional stress on the behavioural evaluation of rats (mean ± SD). **$p < 0.01$ compared to rats of C2 and C1 groups for attacking; compared to C1 group for exploring; compared to ES and C2 groups for grooming. The score is a mean of all scores over the 14 days

The effect of emotional stress on the humoral immune response
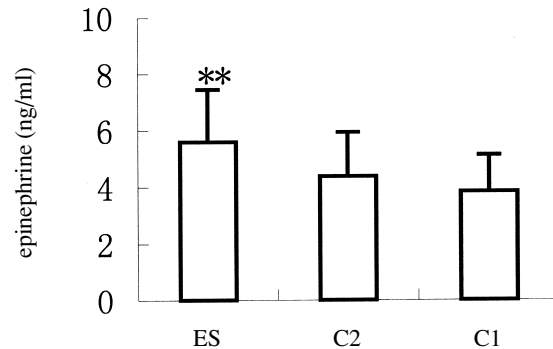
Anti-OVA antibody levels decreased significantly in rats of the ES group compared to that in rats of the C2 group [$F(2,57) = 3.563$, $p < 0.05$; post-hoc: $p < 0.05$] and there was no difference between that of the C2 group and the C1 group (Fig. 2). Spleen indices [spleen weight (mg)/body weight (g)] of the rats in ES group were significantly lower than that of the C2 and C1 groups [$F(2,57) = 10.835$, $p < 0.001$; post-hoc: $p < 0.01$, $p < 0.01$] and there was also no difference between that of the C2 group and C1 groups (Fig. 3).

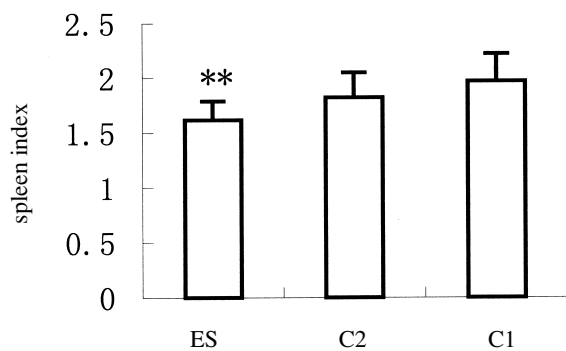## The effect of emotional stress on epinephrine, norepinephrine and corticosterone

Plasma epinephrine levels of the rats in the ES group increased significantly compared to that in the C1 group [$F(2,57) = 4.499$, $p < 0.01$; post-hoc: $p < 0.01$] (Fig. 4), norepinephrine levels of rats in the ES group increased significantly compared to that in the C2 and C1 groups [$F(2,57) = 5.42$, $p < 0.01$; post-hoc: $p < 0.05$, $p < 0.05$] (Fig. 5), there was no difference between that of the C2 group and C1 group. Corticosterone levels of rats in both the ES and C2 groups increased significantly compared to that in the C1 group [$F(2,57) = 8.807$, $p < 0.01$; post-hoc: $p < 0.01$, $p < 0.05$] (Fig. 6).
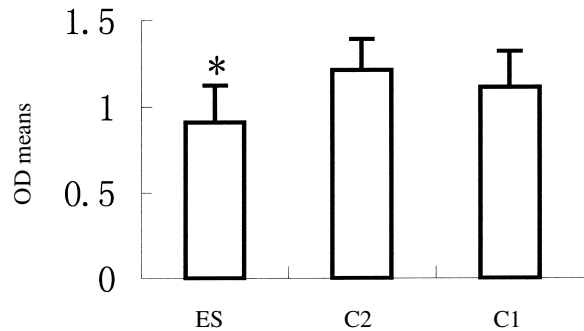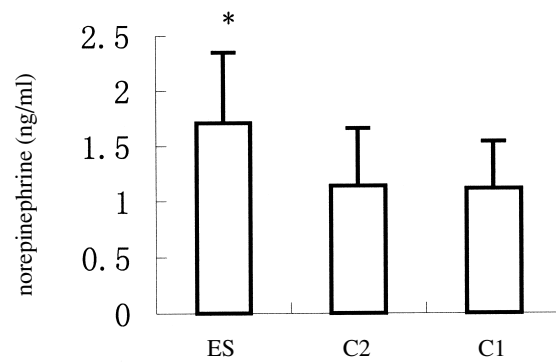

Figure 2 The effect of emotional stress on antibody level (mean ± SD). OD, Optical density. Serum dilution 1 : 100. *$p < 0.05$ compared to C2 group

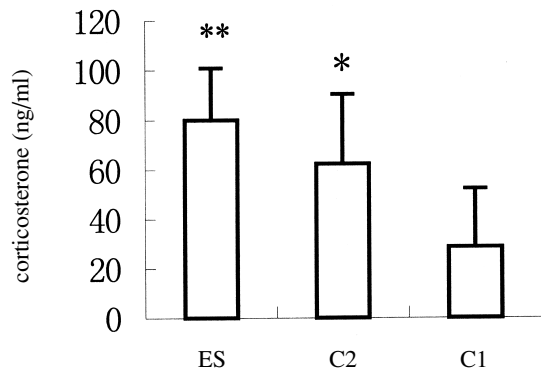**Figure 3** The effect of emotional stress on spleen index (mean ± SD). **$p < 0.01$ compared to C2 and C1 group

Figure 4 The effect of emotional stress on epinephrine level (mean ± SD). **$p < 0.01$ compared to C1 group

Figure 5 The effect of emotional stress on norepinephrine level (mean ± SD). *$p < 0.05$ compared to C2 and C1 group

Figure 6 The effect of emotional stress on corticosterone level (mean ± SD). $**p < 0.01$, $*p < 0.05$ compared to C1 group
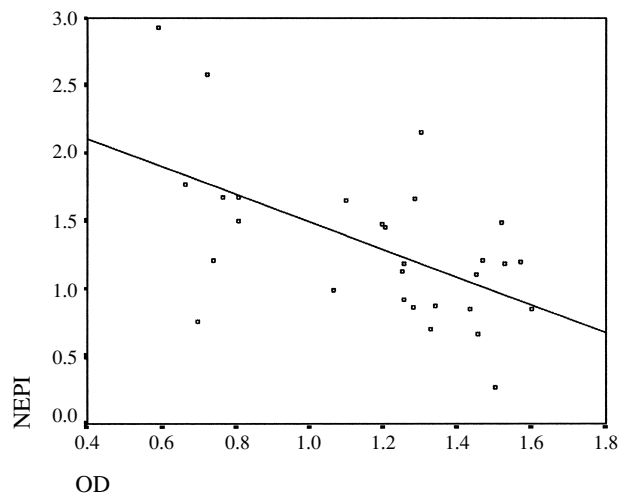
Figure 7 The correlation between optical density (OD) level and norepinephrine level (NEPI)

The correlation between OD level and norepinephrine level

SPSS correlation analysis (SPSS Inc., Chicago, IL, USA) manifested a negative correlation between OD levels and norepinephrine levels ($r = –0.564$, $p < 0.001$) (Fig. 7). No correlation between corticosterone concentrations and OD levels was found ($p > 0.05$), indicating that the sympathetic nervous system may be involved in this immunomodulation.

Discussion

The effect of emotional stress on body weight and fluid intake of rats

According to the results, in the training period, all the rats received a similar fluid intake at two set periods at all times when they had access to water; no group difference appeared. During the experimental period, rats in the ES and C2 groups only received water at one of the two watering periods randomly everyday, so their fluid intake was reduced significantly compared to that of the C1 group, and there was no difference between the ES group and

C2 group. The results suggested that rats in the ES group had the same dehydration as the C2 group.

It is known that fluid intake is associated with food intake, which can directly influence the change of body weight. The results for body weight are in accordance with this. During habituation and training periods, there was no difference in the change of body weight among all the groups. However, in the experimental period, the body weight of rats in the ES and C2 groups increased slower than in rats in the C1 group. These results demonstrated that the change in body weight of rats in the ES group was similar to that in the C2 group.

The induction of emotional stress for rats in the emotionally stressed group

In order to examine whether emotional stress influences the immune system, we must first establish a reliable animal model of emotional stress. According to the present behavioural observation, rats in the ES group that were irregularly given empty water bottles expressed significant attacking behaviours, such as biting bottles and cage shed, whereas rats in the other two groups did not express attacking behaviours. The main behaviours for the C2 group were exploring, and grooming for the C1 group, during the experimental period. These behavioural results showed that rats in the ES group were emotionally stressed significantly during stress procedure, rats in the C2 group were less stressed and rats in the C1 group were not stressed at all.

The effect of emotional stress on the primary humoral immunity

The aim of our study was to investigate whether the emotional stress created by empty bottles could affect the primary humoral immune response determined by the antibody level and spleen weight after immunization with an antigen OVA. The results indicated that antibody levels and spleen weights of rats in the ES group decreased significantly compared to that in the C2 group. According to behavioural, fluid intake and body weight data, the only difference between these two groups was that rats in the ES group were emotional stressed strongly during stress procedure. Thus, the results suggest that emotional stress decreased the levels of specific anti-OVA antibody and spleen weights significantly. The finding of a suppressive effect on immune function is consistent with other research finding showing that exposure to psychological stressors can modulate the primary antibody response. For example, Popovic *et al.* (2000) found that chronic isolation stress decreased significantly the relative spleen weight of adult male Wistar rats immunized with BSA. Croiset *et al.* (1987) demonstrated that psychological stress induced by the conflict situation during one trial learning passive avoidance test could decrease the generation of antibody-forming cells after sensitizing rats *in vivo* with a primary antigen SRBC. Their experiment also found that a short period for only one time of emotional stress, shortly before an immunization with an antigen, could result in immunosuppression.

The involvement of sympathetic nervous system in the immunosuppression of emotional stress

Our study found that emotional stress induced by empty bottles increased norepinephrine levels significantly, and norepinephrine levels were correlated negatively with anti-OVA antibody values.

Although emotional stress also increased corticosterone concentration significantly, no relationship between corticosterone concentration and anti-OVA antibody levels was found. Such an increased level of corticosterone could be regarded as an index of stress in terms of endocrine response. These results suggest that the modulation of emotional stress on humoral immune function in this experiment may be mainly through the SNS.

Such a notion of the involvement of the SNS in immunomodulation was also supported by the finding in which β-receptor blockade (timolol) and surgical denervation of the spleen prevented the modulation of mild emotional stress on the primary antibody response to SRBC (Felten and Felten, 1988). More recently, some brain areas regulating the activation of SNS have been identified to express the c-*fos* gene after stress, including the locus ceruleus and the nucleus tractus solitarius (Herrera and Robertson, 1996). Catecholamines have been shown to be involved in the generation and maturation of lymphocytes, and in antibody formation (Felten *et al*., 1987)**.** However, it is premature to make a definitive statement regarding the involvement of SNS in the immunosuppression of emotional stress which is only based on a correlation study reported in this experiment. However, the correlation found here warrants further investigation into the factors that lead to it.

In summary, the present study demonstrates that emotional stress evoked a behavioural response, such as attacking behaviour, and suppressed the specific primary humoral immune function. The negative relationship between the level of norepinephrine and the level of specific antibody production in this experimental paradigm suggests that the SNS may be involved in mediating the effects of emotional stress on humoral immune function.

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