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NEUROENDOCRINE AND IMMUNOLOGICAL MECHANISMS IN STRESS-INDUCED IMMUNOMODULATION

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Summary—Here, we report that emotional stressors (restraint, footshock) can affect humoral immune responses as well as the capacity of immune and accessory cells to secrete interleukins. Acute restraint stress (5 min) caused a 4- to 6-fold enhancement of splenic antibody responses to sheep red blood cells. In an attempt to study endocrine mechanisms, we administered antibodies raised in rats to corticotropin releasing factor (CRF). Intravenous administration of these antibodies prior to stress-exposure and immunization prevented the stress-induced increase in the humoral response. In a parallel experiment, we observed that CRF-immunoneutralization prevented the restraint stress-induced increase in plasma ACTH concentrations, but was without effect on plasma prolactin, melanocyte stimulating hormone, adrenaline and noradrenaline responses. These data suggest the presence of an indirect pathway involving ACTH and related peptides by which CRF controls humoral responses to stress. A pathway involving a direct mechanism of CRF at the level of the immune cells will be discussed. In a set of other experiments, we addressed the question of whether interleukin-1 and interleukin-6 plasma levels induced by injection of endotoxin could be modulated by emotional stress. Exposure to prolonged footshock stress (20 min) prior to endotoxin injection resulted in a blunted plasma ACTH and interleukin-1 response, without affecting the endotoxin-induced plasma interleukin-6 respose. These data suggest that at least one level at which emotional stress may influence immune function is by changing the capacity of immune cells to produce and/or secrete immune regulatory interleukins.

INTRODUCTION

Both severe and mild stress stimuli have been shown to modulate immune responses [1]. Over the past decade, a variety of studies have addressed questions related to the humoral and neural pathways by which stress may influence immune responses. Immunocytochemical studies at the light and electron microscopic level have demonstrated that lymphoid organs are innervated with nerve fibres containing classical neurotransmitter and neuropeptides [2–4] and corresponding receptors with binding characteristics, and second messenger pathways similar to those in nerves and endocrine cells have been identified on lymphoid cells and macrophages [5–8]. In addition, receptors and/or *in vitro* effects of a variety of humoral stress factors have been described which supports the view of a complementary involvement of endocrine pathways in stress-induced immunomodulation [9–13]. Furthermore, neuroendocrine markers and neuropeptide genes have been demonstrated in organs and cells of the immune system [14]. Effects of stress on immune function cannot be disconnected from the effects of the activated immune system on the brain. In fact, the immune system can be looked upon as a diffuse sensory organ, signalling the brain on the presence of a pathogenic agent, that can not be detected by primary sensory organs. For instance, interleukin-1, a macrophage product that promotes as first mediator the growth and differentation of lymphocytes by inducing other interleukins such as interleukin-2 and interleukin-6 [15], in addition, functions as a signal to activate neuroendocrine systems during infections, leading to increased plasma levels of stress hormones such as ACTH and adrenal glucocorticoids [16]. Glucocorticoids in turn down regulate immune responses at several levels, including limiting production of interleukin-1 [17]. Moreover, interleukins play an important role in coordination of behavioural (inactivity, sleepiness, loss of appetite) and physiological (fever) changes to

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infections [18–22]. These considerations point to the importance of understanding in what way emotional stress modulates production of interleukins. In this study, we present data on the role of an endocrine pathway involving corticotropin releasing factor (CRF) in stress-induced modulation of humoral response. Moreover, data are presented showing effects of emotional stress on induction of circulating interleukin-1 and interleukin-6 in response to a pyrogenic dose of endotoxin.

EXPERIMENTAL

Experimental animals

Male wistar rats were housed two per cage in a quiet room under a 12-h light–12 h dark regimen in which the light period was from 7.00 a.m. to 7.00 p.m. Food and water were available *ad libitum*. The animals were adapted to experimental conditions as described elsewhere in detail [23].

Stress exposure and immunization

To expose rats to restraint stress, they were placed in front of a translucent restraint box (inner size: 14.5 × 6.4 × 5.4 cm). After spontaneous entry, the boxes were closed for 5 min. After removal of the boxes, the rats were injected i.p. with 1 ml of saline containing 5×10^8 sheep red blood cells (SRBC). Rats injected with SRBC without prior stress exposure served as controls. After the immunization the rats were kept undisturbed as described above for 7 days, whereafter they were decapitated to collect the spleen.

To expose rats to unescapable footshock stress, they were placed on steel grids in a closed shock box. Footshocks of 0.5 mA lasting 4 s were delivered in a frequency of 4 shock/min for a period of 20 min. After footshock exposure, the rats were injected i.v. with endotoxin (B55, Westfall, Difco, U.S.A.) in a dose of 2.5 mg/kg; 2 h after endotoxin administration, the rats were decapitated and trunk blood was collected.

CRF antiserum and immunoneutralization protocol

To obtain a homologous antiserum to CRF, 4 female rats (180–240 g) were immunized by repeated i.p. administration (4-week intervals) of 25 μg rat CRF (Peninsula Labs, U.S.A.) conjugated to bovine thyroglobulin by using gluteraldehyde. The first dose was emulsified in Freund's adjuvant (1:1 v/v), 4 subsequent boosts given in incomplete Freund's adjuvant. After the third boost rats were screened for their plasma corticosterone (B) response to ether stress. Moreover, CRF binding capacity of the plasma using radiolabelled CRF was determined. All animals developed antibodies to CRF and showed reduced plasma corticosterone responses to ether stress. The rat with the highest antibody titre and lowest plasma corticosterone response to ether was decapitated 10 days after the fourth boost and serum was stored at −20°C. Rat immunized with Freund's complete and incomplete adjuvant according to the same schedule were used as a source of control serum (NRS). To verify the potency and selectivity of the antisera, 0.2 ml of antiserum or control serum, respectively was administered i.v. to male Wistar rats 90 min prior to exposure to restraint stress; 5 min after restraint stress, the animals were decapitated for collection of trunk and head blood. Trunk blood was used to determine concentrations of B, ACTH, prolactin (Prl) and melanocyte stimulating hormone (MSH). Head blood was used for the determination of adrenaline (A) and noradrenaline (NA).

In order to study the effects of CRF-immunoneutralization on the plaque forming response to SRBC in control and restraint stressed animals, rats were (0.2 ml) injected i.v. with CRF antiserum or NRS. Ninety minutes later rats were exposed to 5 min restraint stress, followed by immunization with SRBC as described above.

Blood samples

Trunk or head blood was collected in heparinized tubes on ice and was centrifuged (1000 *g* for 15 min at 4°C). Plasma was aliquoted and stored at −20°C until assayed.

Radioimmunoassays (RIAs)

Plasma ACTH and MSH concentrations were measured as described elsewhere [23]. Plasma Prl concentrations were measured by RIA using reagents supplied by NIADDK and are expressed in ng RP-3/ml.

Plasma interleukin-1 β concentrations were determined by a newly developed RIA specific for interleukin-1 β (R. Derijk and F. Berkenbosch, in preparation). Briefly, rat recombinant interleukin-1 β was used as a tracer and standard. Interleukin-1 β was labelled using Bolton and Hunter reagent (500 μCi; Amersham, England).

The antiserum was a gift from Dr J. McKearn (Searle, U.S.A.), and was raised in goat to human recombinant interleukin-1 β. The assay was performed under non-equilibrium conditions. Serially diluted standard, in assay buffer (range: 100–10,000 pg interleukin-1 β/200 μl buffer; buffer was composed of 0.1 M phosphate buffer containing 0.1% gelatin) was incubated with diluted antiserum (final dilution 1:30,000; 30% binding of the tracer) for 24 h at 4°C. Then, the tracer (10,000 cpm) was added followed by a second incubation of 24 h at 4°C. Separation of bound and free was performed by a solid phase second antibody precipitation (Saccel, Welcome Reagents, England). Displacement curves of serial diluted plasma obtained from endotoxin injected rats were parallel to that of rat interleukin-1 β used as standard. Human recombinant interleukin-6, human recombinant tumor necrosis factor and endotoxin did not cross react in concentrations up to 100 μg. Sensitivity of the assay was 250 pg/ml.

Catecholamine determinations

Plasma catecholamines (A and NA) were determined by using HPLC followed by electrochemical detection as described [24].

Corticosterone determinations

The concentrations of B in plasma samples were determined by a fluorimetric assay as described elsewhere [25].

Antibody forming cells to SRBC

Five days after immunization the number of antibody-forming cells (PFCs) in the spleen was determined by using a plaque forming cell assay as described in detail elsewhere [26].

Interleukin-6 bioassay

The interleukin-6 concentrations in plasma samples were measured by the use of a B9-cell line as described in detail elsewhere [27]. Half maximal stimulation of the proliferation of B9-cells was defined as 1 U of biological activity.

Statistics

Data are expressed as mean and SEM and are evaluated by ANOVA followed by a Duncan range test for multiple comparisons or by two-way ANOVA followed by Scheffé F-test or independent t-test. Significance was defined at the 0.05 level.

Table 1. Effect of i.v. administration of CRF antiserum on splenic PFC numbers to SRBC in control and restraint-stressed rats

Treatment	Control	Restraint
—	308 ± 26	1620 ± 96*
NRS	520 ± 30	1510 ± 96*
Anti-CRF	480 ± 20	430 ± 30

Rats were injected i.v. with NRS or antiserum to CRF (anti-CRF) 90 min prior to exposure to restraint stress (5 min). Immediately after exposure they were immunized with SRBC. Control animals were only subjected to immunization. The numbers of splenic PFCs were determined 5 days after immunization and are expressed as PFC/10^6 lymphocytes.
*$P < 0.05$ (Scheffé F-test).

RESULTS

Endocrine Mechanisms Involved in Modulation of the Primary Humoral Response to Stress

Modulation of a primary antibody response by restraint stress

Table 1 shows that subjection of rats to a restraint stress of 5 min duration resulted in an increase in the number of plaque forming cells (PFCs) as compared with that of animals which had remained in their home cage. This effect varied from a 2- to 6-fold enhancement, and appeared independent of the amount of SRBC injected (range: 4×10^8–5×10^9). In a further series of experiments, we investigated whether the increased PFCs in restraint-stressed animals was due to an acceleration or delay of the onset of the primary antibody response. Non-stressed rats and rats subjected to restraint stress were immunized at 3 subsequent days, and the number

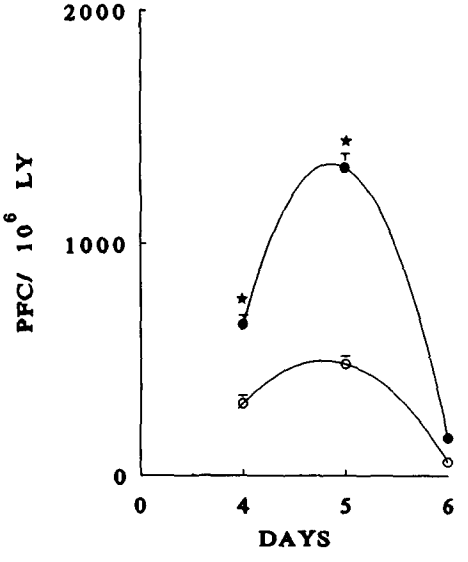

Fig. 1. Time course of the number of splenic anti-SRBC PFCs in control rats (○—○) and rats exposed to restraint stress (●—●). The spleen was removed 4, 5 and 6 days after immunization. ★ $P < 0.05$ (independent t-test).

of PFCs was determined at a fixed time-interval. As shown in Fig. 1, the difference in the number of PFCs on days 4 and 5 after immunization were comparable. At day 6 after immunization the antibody response in all groups returned to basal levels.

Effect of CRF-immunoneutralization on the primary antibody response in restraint-stressed rats

As shown in Table 1, i.v. administration of CRF antibodies or NRS did not affect the primary antibody response in non-stressed rats. Moreover, NRS administration did not modify the increment of the number of PFCs in rats subjected to restraint stress. However, the administration of CRF antibodies clearly prevented the increased PFC response of rats exposed to restraint stress.

Effect of CRF-immunoneutralization on endocrine responses to restraint stress

Figure 2 illustrates some of the endocrine responses in rats after an i.v. injection of NRS and exposure to restraint stress. Plasma catecholamine concentrations were measured in head blood in order to limit interference of reflex-activation of sympathetic–adrenomedullary system in response to decapitation. Restraint stress resulted within 5 min in increased plasma concentrations of ACTH, Prl, MSH and A. No significant increases in plasma concentrations of NA and B were observed as compared with control values. Intravenous administration of CRF antibodies did not affect plasma concentrations of any of the endocrine parameters in non-stressed rats. However, the antibodies fully neutralized the ACTH response to restraint stress, without affecting the other hormonal responses.

Modulation of Interleukin Responses to Endotoxin by Emotional Stress

Effect of endotoxin on plasma ACTH, interleukin-1 and interleukin-6 concentrations

Table 2 shows the effect of increasing doses of endotoxin on plasma ACTH, interleukin-1 β and interleukin-6 concentrations. The effect of endotoxin on these parameters was dependent on the dose administered. Plasma ACTH concentrations were significantly increased at endotoxin doses as low as 0.025 mg/kg. A 20- to 25-fold increase was noted at the highest doses used. Plasma interleukin-6 concentrations were below detection limit (< 10 U/ml) of the interleukin-6 assay in animals injected with saline, but drastically increased even at the lowest doses of endotoxin used. Maximal interleukin-6 responses of 6–8 $\times$ 10^5 U/ml were obtained at doses of endotoxin ranging from 0.5 to 2.5 mg/kg. Plasma interleukin-1 concentrations were below detection limit of the interleukin-1 assay (250 pg/ml) at doses of endotoxin up to 0.5 mg/kg. Detectable interleukin-1 concentrations were observed in plasma after administration of endotoxin at doses of 0.5 mg/kg and higher. Higher doses resulted in higher levels of interleukin-1 in the circulation although variation in responses between animals was large, especially at the highest doses of endotoxin used.

Table 2. Effect of increasing doses of endotoxin (LPS) on plasma concentrations of ACTH, interleukin-1 and interleukin-6

LPS (mg/kg)	ACTH (ng/ml)	Interleukin-1 (ng/ml)	Interleukin-6 (U/ml $\times$ 10^3)
Saline	0.06 ± 0.005	< 0.25	< 10
0.025	0.52 ± 0.04*	< 0.25	23.8 ± 6.8*
0.1	0.67 ± 0.03	< 0.25	46.5 ± 13.7
0.5	0.7 ± 0.08	< 0.25	80.6 ± 6.3
0.25	0.93 ± 0.16	0.29 ± 0.006*	600.1 ± 112.3
1.25	1.44 ± 0.15	0.43 ± 0.07	ND
2.5	1.73 ± 0.18	0.84 ± 0.2	834.3 ± 235.3
5	1.67 ± 0.2	1.2 ± 0.5	ND

Groups of rats ($n = 6$) were injected i.v. with increasing doses of endotoxin and were decapitated 2 h later for collection of trunk blood. *$P < 0.05$ different from saline-injected rats (Scheffé F-test). ND, not determined.

Effect of footshock stress on ACTH, interleukin-1 and interleukin-6 concentrations induced by injection of endotoxin

Figure 3 shows the effect of exposure of rats to footshocks (20 min duration) on plasma ACTH, interleukin-1 and interleukin-6 responses to administration of endotoxin (2.5 mg/kg i.v.). Endotoxin was administered directly after exposure of the rats to footshock, and plasma concentrations of ACTH and of both interleukins were determined 2 h after endotoxin administration. Footshock stress significantly attenuated the ACTH response to endotoxin. Even more pronounced was the reduction of the plasma interleukin-1 response to endotoxin in rats that were exposed to footshocks. In contrast, footshock stress did not affect plasma interleukin-6 responses to endotoxin as compared with that in non-stressed rats.

DISCUSSION

The aim of the present study was 2-fold: (1) to investigate whether emotional stress could modulate the reactivity of the immune system

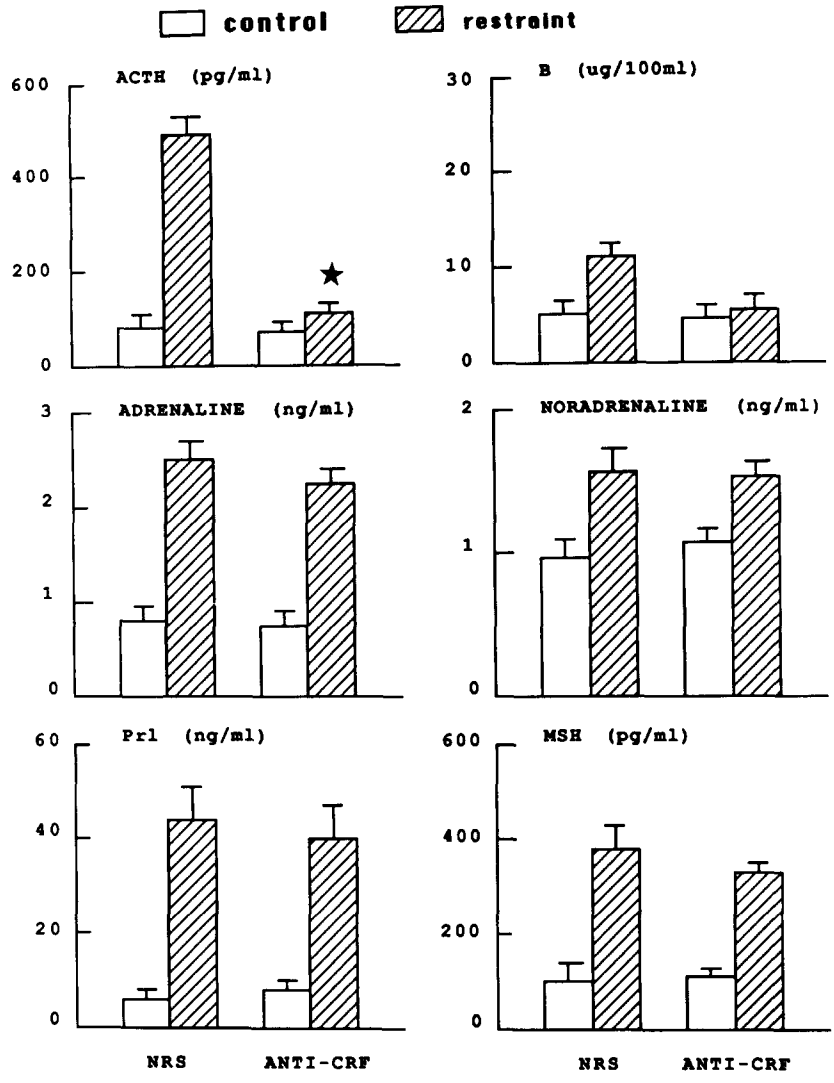

Fig. 2. Effect of i.v. administration of NRS or antiserum to CRF (anti-CRF) on plasma ACTH, B, A, NA, Prl and MSH concentrations in peripheral blood of non-stressed rats and rats exposed to restraint stress. Antiserum and control serum were injected i.v. to groups of Wistar rats 90 min prior to stress-exposure. Five minutes after restraint stress, the animals were decapitated for collection of trunk and head blood. Control rats injected with antiserum or NRS were decapitated within 10 s after transfer from an adjacent room. ★ $P < 0.05$ (Scheffé F-test) stressed rats injected with NRS vs stressed rats injected with CRF antiserum.

as measured by a complex *in vitro* immune response, the generation of primary antibodies, to SRBC and subsequently to define a possible endocrine pathway; and (2) to investigate whether emotional stress modifies the concentrations of endotoxin-induced circulating interleukins, substances which play an important role in immunoregulation and coordination of the acute phase response.

Humoral immune responses are modulated by acute emotional stress

As illustrated in Table 1, restraint stress (5 min duration) clearly increases primary antibody response in comparison with the response of animals that remained in the home cage. From the data presented in Fig. 1, it can be concluded that the enhanced response reflects an absolute increase in the number of antibody forming cells in the spleen rather than a change in the time course of the response.

It is intriguing that acute restraint stress may cause long-lasting effects in the immune system. Our data indicate that antigen presentation immediately after restraint stress will result in an increased efficacious immune response 5 days later. Recently, Groiset *et al.* [28] have shown that the ability of a stressor to influence the immune response is largely dependent on the time-interval between stress exposure and

antigen presentation. Apparently, the activity of the lymphocytes are changed by the stressor within a defined time-interval leading to a change in the functional capacity to respond to antigen when presented within that time-interval. This view is supported by our recent observations that proliferation of T-cells obtained from the spleen of acutely stressed rats is clearly changed in response to ionomycin and phorbol ester (unpublished observations). Moreover, the data presented in Fig. 2, clearly demonstrates that interleukin-1 secretion to endotoxin can be modified by prior stress-exposure.

Not only restraint stress, but also acute foot-shocks (shock periods of 2–5 min duration) enhance immune responses, including the primary antibody responses [26, 29]. Therefore, it can be argued that the increase in immune responses may be a reflection of general arousal of the animal. However, recent studies have shown that acute aversive situations may down regulate immune responses [26, 29]. This suggests that the immune system can react differentially to acute environmental stimuli, and that the direction of the immunomodulation is largely determined by the emotional "colour" of the acute stressor.

CRF is involved in stress-induced modulation of the humoral response

From the data presented in Table 1, we can conclude that CRF is involved in the enhanced primary antibody response to restraint stress. The observation that the CRF antibody used selectively prevents the restraint stress-induced release of ACTH from the pituitary gland, suggests that ACTH and related peptides, whose release is controlled by hypothalamic CRF [30], are involved in immunomodulation in response to acute restraint stress. Immunomodulatory effects of ACTH and endorphins on immune responses *in vitro* have been described previously [31, 32]. In our studies, β-endorphin concentration dependently augmented humoral responses to ovalbumine *in vitro* [29]. Thus, ACTH and related peptides may be signalling molecules by which the brain controls the immune system during restraint stress. However, there are alternative explanations. CRF receptors have been detected in the mouse spleen, particularly in the marginal zones where macrophages are concentrated [33]. Moreover, CRF administration to rats in doses leading to plasma CRF concentrations that circulate during stress causes endorphin production in B-lymphocytes by mechanisms involving increased interleukin-1 secretion from macrophages [34]. Circulating CRF in response have been shown to originate from the hypothalamus [35]. Moreover, CRF with similar characteristics to hypothalamic CRF has been demonstrated in the adrenal medulla and detailed experiments have shown that this CRF is cosecreted with A in response to stress [36]. Therefore, the possibility has to be considered that the endocrine mechanisms involved in effects of restraint stress on primary antibody responses may bypass the pituitary gland, and may involve direct effects of CRF originating from the hypothalamus and/or adrenal medulla.

Circulating interleukin-1 and interleukin-6 levels are induced by endotoxin

Immunological mechanisms involved in the effects of stress on immune function are only poorly understood. Recent advances in immunology have demonstrated that interleukins, such as interleukin-1 and interleukin-6 play a crucial role in the regulation of the immune response to invading pathogens (for reviews see Refs [15, 37]). For instance, antigen induced T-cell activation requires interleukin-1 as an accessory signal and interleukin-1 induced T-cell costimulation appears to be mediated by interleukin-2 production and expression of interleukin-2 receptors in T-cells. Moreover, interleukin-6 is one of the prime interleukins involved in B-cell differentiation. The relation between interleukins and immune responses encouraged us to study the effects of emotional stress on concentrations of circulating interleukin-1 and interleukin-6. Preliminary observations showed that only minimal plasma interleukin-1 and interleukin-6 responses could be induced by immunization with SRBC, limiting the possibility of studying the effects of stress-exposure using this experimental protocol. In contrast, preparations of bacterial cell walls (lipopolysaccharide or endotoxin) have been shown to be powerful agents in raising plasma levels of various interleukins, including interleukin-2 and interleukin-6 [38, 39]. Table 2 shows that in normal pathogen free rats, concentrations of interleukin-1 and interleukin-6 in the circulation are lower than the detection limit of our assays being 250 pg/ml and 10 U/ml, respectively. Moreover, the data in Table 2 clearly show that injection of endotoxin rapidly increases plasma levels of these interleukins. Therefore, we designed experiments to study the effect of emotional stress on endotoxin-induced interleukin-1

and interleukin-6 concentrations. In order to maximize the possible effects of emotional stress on interleukin responses to endotoxin, rats were subjected to prolonged footshocks rather than to acute restraint stress prior to endotoxin administration. It is worth noting that prolonged footshock stress suppresses rather than enhances immune function (preliminary observations).

Footshock stress attenuates the ACTH and interleukin-1 response but not the interleukin-6 response to endotoxin

As shown in Fig. 3, footshock stress for a period of 20 min differentially affected the endotoxin-induced plasma levels of interleukin-1 and interleukin-6. The increased plasma interleukin-1 concentrations were largely inhibited to levels around the detection limit of the interleukin assay, whereas the endotoxin-stimulated plasma interleukin-6 concentrations were not affected.

The mechanisms by which footshock stress attenuates plasma interleukin-1 response to endotoxin is not clear. The main factors known to inhibit interleukin-1 production and secretion are prostaglandins and glucocorticoids [17,40]. Whether these factors are involved in the reduced interleukin-1 response in stressed rats needs to be elucidated.

The differential effect of footshock stress on plasma interleukin-1 and interleukin-6 concentrations may be related to the cellular origin of both interleukins in the circulation. Although *in vitro* studies have indicated that macrophages can secrete interleukin-6 [37], our *in vivo* macrophages depletion studies show that these cells are not the predominant source of interleukin-6 in the circulation after injection of endotoxin (R. Derijk *et al.*, in press). In fact, interleukin-6 in the circulation may originate from fibroblasts and endothelial cells by mediation of interleukin-1 [6]. Hence, the data shown in Table 2 allow us to conclude that circulating concentrations of interleukin-1 around the 250 pg/ml (detection limit of the interleukin-1 assay) are sufficient to induce a maximal interleukin-6 response. Moreover, the data suggests that the emotional stress experienced by footshocks does not interfere with the mechanisms of interleukin-1 to induce interleukin-6 production and secretion from fibroblasts, endothelial cells and/or other interleukin-6 producing cells.

At least two interrelated explanations are possible for the attenuated ACTH response to endotoxin in rats exposed to footshocks (Fig. 3). Since endotoxin was injected 20 min after footshocks, a time-period at which plasma glucocorticoids are maximally increased, the attenuated ACTH response may be the result of increased glucocorticoid feedback inhibition at the level of the hypothalamo–pituitary–adrenal axis [41]. Moreover, a complementary mechanism may involve a reduced immunoregulatory signal from the immune system. Besedovsky *et al.* [42] have present evidence for the existence of an immunoregulatory feedback circuit involving interleukins and the pituitary–adrenal system.

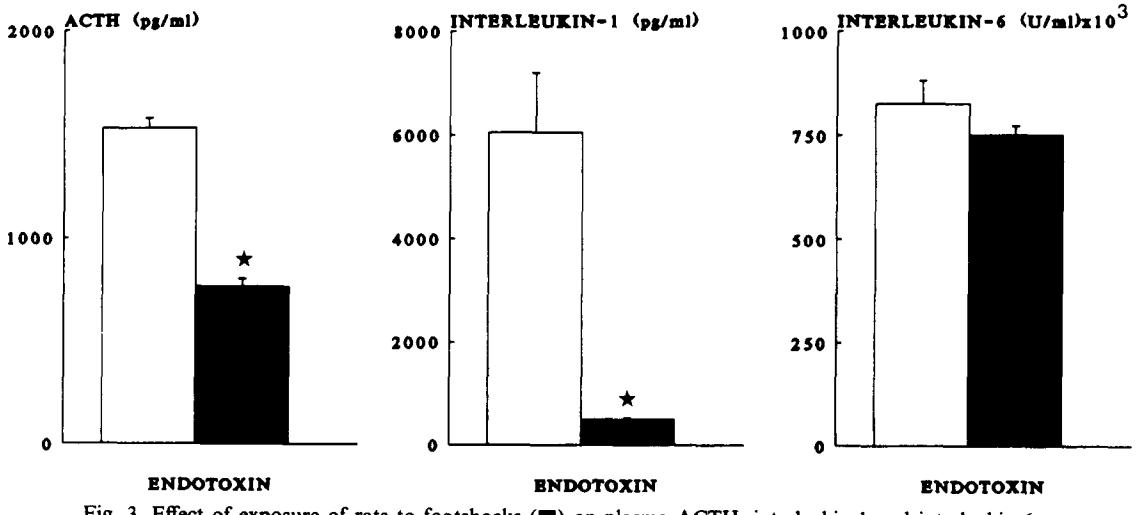

Fig. 3. Effect of exposure of rats to footshocks (■) on plasma ACTH, interleukin-1 and interleukin-6 responses to injection of endotoxin. Rats were exposed to footshocks for 20 min (4 shock/min, shock duration 5 s, shock amplitude 0.75 mA). Directly after ending the shock procedure, they received an i.v. injection of endotoxin (2.5 mg/kg). Non-stressed animals injected with endotoxin served as control (□). Animals were decapitated 2 h after endotoxin administration to collect trunk blood. ★ $P < 0.05$, stressed groups vs non-stressed groups (Scheffé F-test).

A recent study using blocking interleukin-1 receptor antibodies has supported this hypothesis and has indicated that interleukin-1 is one of the prime signals involved in the ACTH response to endotoxin [43]. Therefore, it is possible that the attenuated ACTH response in rats exposed to footshocks may be the result of a reduced interleukin-1 drive. This explanation finds support from the data in Table 2 which compare favourably with data in Fig. 3, and which show that plasma interleukin-1 levels relate to the amplitude of the ACTH response.

In summary, the data presented in this report show that emotional stress can modify splenic humoral responses by mechanisms involving direct and/or indirect actions of CRF. Moreover, our data suggest that changes in immune responses in stressed animals may involve altered functions of immune cells such as for instance their capacity to produce and secrete interleukins. Since interleukins also coordinate other aspects of the defence to pathogens such as behaviour (sleep and eating) and metabolism (fever and acute phase proteins), it can be anticipated that these adaptive responses may also be modified by stress-exposure.

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