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Protective Effect of Prior Acute Immune Challenge, but Not Footshock, on Inflammation in the Rat

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Previous studies have revealed that a single exposure to an acute stress or acute immune stimulus can produce long-lasting changes in the activity and responsiveness of the hypothalamo-pituitary–adrenal (HPA) axis. The HPA axis is believed to be an important component in determining the susceptibility and severity of inflammation in autoimmune disease models such as adjuvant-induced arthritis (AA). In the present study we have tested the hypothesis that a single exposure to either footshock or lipopolysaccharide (LPS) 3 weeks prior to adjuvant injection can alter susceptibility to AA. Changes in HPA axis parameters were also determined. The results demonstrated that prior exposure to LPS conferred resistance to inflammation in AA, which was not related to a delay in onset of inflammation but rather an alteration in susceptibility. In contrast, prior exposure to the acute stress of footshock did not alter susceptibility. HPA axis parameters were increased in adjuvant-injected rats whether inflammation was present or not. These data suggest that prior exposure to acute immune stimuli, but not to acute footshock stress, may alter susceptibility to inflammation in the rat AA model. These changes in susceptibility do not appear to be solely mediated by increases in HPA axis activity, which were apparent in all AA groups irrespective of the presence of inflammation.

INTRODUCTION

Susceptibility to autoimmune disease is influenced in part by genetic factors. However, environmental and hormonal factors can strongly influence the onset and the subsequent severity of a wide range of inflammatory conditions. One important factor in determining disease severity is activation of the hypothalamo-pituitary–adrenal (HPA) axis. The end point of HPA axis activation is the release of potent anti-inflammatory glucocorticoids (corticosterone in rodents, cortisol in humans) from the adrenal cortex into the blood (Harbuz and Lightman, 1997). Glucocorticoids are crucial for survival since, following adrenalectomy, injections of interleukin (IL)-1 or lipopolysaccharide (LPS) at doses well tolerated in adrenal-intact animals are fatal (Bertini et al., 1988; Ramachandra et al., 1992). Similarly, induction of disease models such as adjuvant-induced arthritis (AA) and experimental allergic encephalomyelitis (EAE) results in earlier onset and increased severity of symptoms in adrenalectomized rats which rapidly results in death (MacPhee et al., 1989; Harbuz et al., 1993). These fatal effects can be prevented with corticosteroid replacement, confirming the important role played by glucocorticoids and the HPA axis.

Activation of the HPA axis in response to an acute stress is generally considered a finite event with a return to the homeostatic setpoint achieved within hours of the stress. In rodents, for example, in response to stress there is a transient increase in plasma concentrations of ACTH and corticosterone. These elevated levels return to baseline within approximately 1 h of the termination of the stress. Changes in mRNA

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levels for the releasing factors involved in the HPA axis, corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP), are subsequently increased and return to baseline within 24 h (Lightman and Young, 1989). Repeated stress results in an increase in the homeostatic setpoint, a blunted response to the same stressor, but a marked hyperactivity of the HPA axis to a novel stressor (Aguilera, 1994). There is evidence that with repeated or chronic stress there is a shift to a predominantly AVP drive to the HPA axis (Harbuz et al., 1993; Chowdrey et al., 1995; Aubry et al., 1999). Previous studies have suggested that exposure to a single, short stress episode can exert long-lasting changes in the HPA axis responsivity and behavior (Van Dijken et al., 1992, 1993). Furthermore, these studies have demonstrated that a single exposure to an acute stimulus can also result in a shift in the CRF/AVP balance in favor of AVP. Of particular interest is the observation that these effects are noted days to weeks after exposure to the stimulus (Schmidt et al., 1995, 1996, 1997). This appears to be a non-stress-specific effect which is independent of the hormonal changes following the stimulus and is independent of the nature of the stimulus itself. This effect has been observed following acute LPS and IL-1 challenge (Schmidt et al., 1996). We have recently observed profound changes in the stress responsiveness of animals previously exposed to acute immobilization a number of weeks prior to reexposure (Marti et al., 2001).

We have noted the importance of the HPA axis in influencing the onset and severity of AA in the rat (Harbuz et al., 1993). In the present study we have extended previous investigations into long-term effects of a single acute stress exposure on subsequent acute stress responsiveness, to consider the effects of a single challenge on the subsequent development of inflammation. We have investigated the effect of prior exposure to an acute injection of LPS or to a single session of footshock on the subsequent development of AA in the rat. To assess the long-term rather than the short-term effects of HPA axis activation on the development of inflammation in this model, animals were exposed to the acute stimuli 3 weeks prior to the injection of adjuvant. Variables in HPA axis activity are also reported.

METHODS

Male Wistar rats (200–220 g) were singly housed under standard lighting conditions with free access to food and water. AA was induced by a single intradermal injection (0.1 ml) of a suspension of ground, heat-killed *Mycobacterium butyricum* in paraffin oil (10 mg/ml) into the tail base. Controls were injected with vehicle (paraffin oil). To assess the development of inflammation, hind paw volume was measured by plethysmometry at the time of adjuvant injection and at various times postinjection (Harbuz et al., 1992). All studies were approved by the Ethical Committee for animal experimentation of the School of Medicine of the University of Cadiz (Licence No. 07-9604).

i. The rats were given an intraperitoneal (ip) injection (0.5 ml) of vehicle or lipopolysaccharide (LPS; Sigma, serotype *Escherichia coli* 055:B5; 250 μg/rat (Conde et al., 1998)) or exposed to footshock (60 shocks; intensity 0.8 mA; duration 15 s; intershock interval 60 + 20 s) and returned to their home cage. Different batches of LPS, even of the same serotype, can have widely differing inflammatory and behavioral effects. Previous studies have demonstrated that a batch of LPS of the 055:B5 serotype, although of a different lot number than that used here, had no apparent effects on animal behavior (locomotion, fur appearance, exploratory activity) at doses

below 1 mg/100 g body wt. We have previously demonstrated that the dose of LPS in the present study (of the same serotype and lot number) was sufficient to stimulate the HPA axis with minimal behavioral effects, whereas a fivefold lower dose of this serotype was unable to stimulate CRF mRNA in the PVN (Conde et al., 1998). Three weeks later the animals were injected with adjuvant or vehicle. Hind paw volume was determined at days 0, 12, and 14. On day 14 the experiment was terminated, the animals were decapitated, and trunk blood was collected into heparinized tubes and centrifuged, and plasma stored at −20°C prior to RIA. The brains and pituitaries were quickly removed, frozen on dry ice, and stored at −70°C prior to *in situ* hybridization histochemistry.

ii. An extended time course was studied to determine whether prior injection of LPS had prevented the development or delayed the onset of hind paw inflammation. Further groups of rats were given an ip injection of LPS or vehicle. Three weeks later the animals were injected with adjuvant. Paw volumes were determined on days 0, 14, and 21.

In situ hybridization (ISH) was performed using 48-mer oligonucleotides complementary to part of the exonic mRNA sequence coding for AVP, CRF, and POMC as described previously (Harbuz and Lightman, 1989). The specific activities of the probes were approximately 1×10^{18} dpm/mol. All control and experimental sections were hybridized in the same reaction. The results are presented as the mean percentage of change from controls with a standard error of the mean. AVP mRNA was determined using the threshold method described by Kinoshita and co-workers (2000).

Total plasma corticosterone was measured directly, by radioimmunoassay, in plasma (1 μl diluted in 100 μl of buffer) using antiserum kindly supplied by G. Makara (Institute of Experimental Medicine, Budapest, Hungary). The tracer was ^{125}I-corticosterone (ICN Biomedicals, CA, U.S.A.) with a specific activity of 2 to 3 mCi/μg. ACTH was measured by in-house radioimmunoassay (Jessop et al., 1989).

Statistical comparisons were made using the Fisher PLSD test following one-way analysis of variance ($n = 5$–8 per group). $P < 0.05$ was considered significant.

RESULTS

Exposure to footshock 3 weeks prior to adjuvant injection had no effect on the time of onset or the severity of inflammation which was evident in both footshock and control animals by day 14 (Fig. 1a). In contrast, exposure to LPS 3 weeks prior to adjuvant resulted in complete absence of inflammation at day 14 compared with that in saline-injected AA rats (Fig. 1b).

Further groups of saline-injected and LPS-treated rats were left for 3 weeks prior to injection of adjuvant. Paw volumes were assessed at day 0. Fourteen days after adjuvant injection the saline-injected rats showed a significant increase in hind paw volume, whereas the LPS-treated rats showed no increase compared to day 0 measurements (Fig. 2). By day 21, there was a further significant increase in paw volume in the saline-injected rats. The LPS-treated rats still showed no change in hind paw volume, confirming that prior injection of LPS had prevented the onset of inflammation.

Exposure to either footshock or LPS 3 weeks prior to adjuvant injection did not alter basal concentrations of ACTH or corticosterone 14 days following adjuvant injection compared with their respective controls (Figs. 3a and 3b). Plasma concentra-

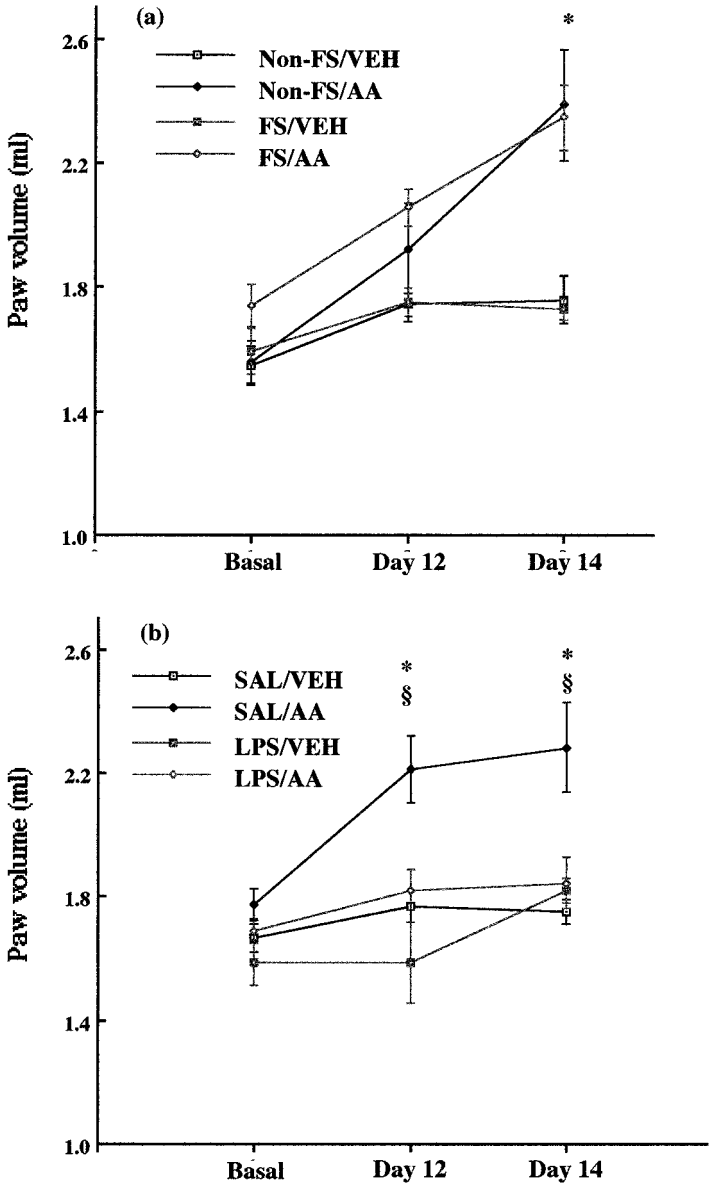

FIG. 1. Paw volume (ml) of rats on days 0, 12, and 14 after injection of adjuvant (AA) or vehicle (VEH). The rats had previously been exposed to (a) footshock (FS) or were non-footshock controls (non-FS); (b) LPS or saline (SAL) 3 weeks prior to injection of adjuvant. Data represent means ± SEM for $n = 8–10$ animals. *$P < 0.05$ compared to respective controls; §$P < 0.05$ compared to LPS-injected AA rats.

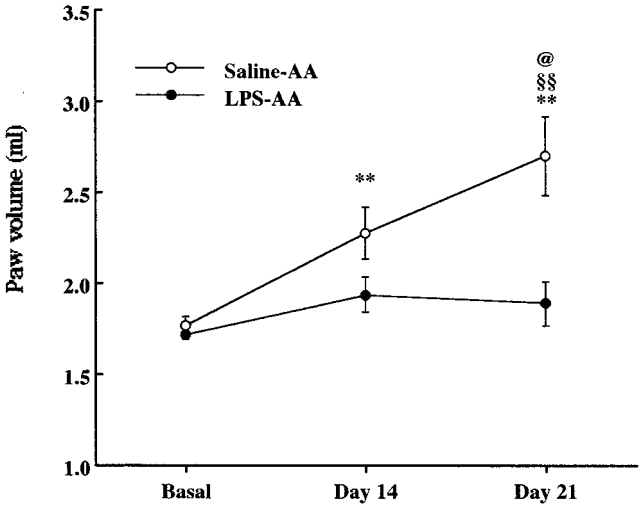

FIG. 2. Paw volume (ml) of rats on day 0 (basal), day 14, and day 21 after injection of adjuvant (AA). The rats had previously been exposed to either saline or LPS 3 weeks prior to injection of adjuvant. Data represent means ± SEM for $n = 8$ animals. $^{**}P < 0.01$ compared to basal levels. §§$P < 0.01$ saline-AA compared to LPS-AA at day 21. @$P < 0.05$ saline-AA at day 21 compared to saline-AA at day 14.

tions of ACTH ($P < 0.05$) were elevated in the AA animals which had previously been exposed to footshock compared to non-AA animals, although the rise in corticosterone did not achieve statistical significance, which was probably due to the relatively elevated levels seen in the footshock control group. Plasma ACTH ($P < 0.05$) and corticosterone ($P < 0.05$) were significantly elevated in plasma of AA rats exposed to LPS or saline injection 3 weeks prior to adjuvant injection despite the lack of inflammation in the LPS-treated rats.

POMC mRNA in the anterior pituitary was significantly ($P < 0.05$) elevated in the adjuvant-injected rats compared with their respective controls in both the LPS and footshock studies (Table 1), despite the lack of inflammation in the LPS-treated animals (LPS/AA). At the hypothalamic level, AVP mRNA was significantly ($P < 0.05$) increased in the parvocellular cells of the PVN of adjuvant-injected rats compared to their respective non-AA controls (Table 1). In addition, a significant ($P < 0.05$) increase in AVP mRNA was observed in the LPS-treated vehicle-injected rats (LPS/VEH) compared to saline controls (SAL/VEH). CRF mRNA levels were not significantly altered in the adjuvant-injected animals compared with their respective controls irrespective of treatment prior to adjuvant injection.

DISCUSSION

These data demonstrate that a single exposure to LPS, 3 weeks prior to injection of adjuvant, can reverse the susceptibility of rats to AA. This effect was not simply a delay in the time of onset of inflammation, usually seen at day 14, as no hind paw inflammation was apparent 21 days after adjuvant injection, which is the time of peak inflammation in this model (Harbuz et al., 1992). This effect does not appear to be related to long-term alterations in the HPA axis induced by the stress-related

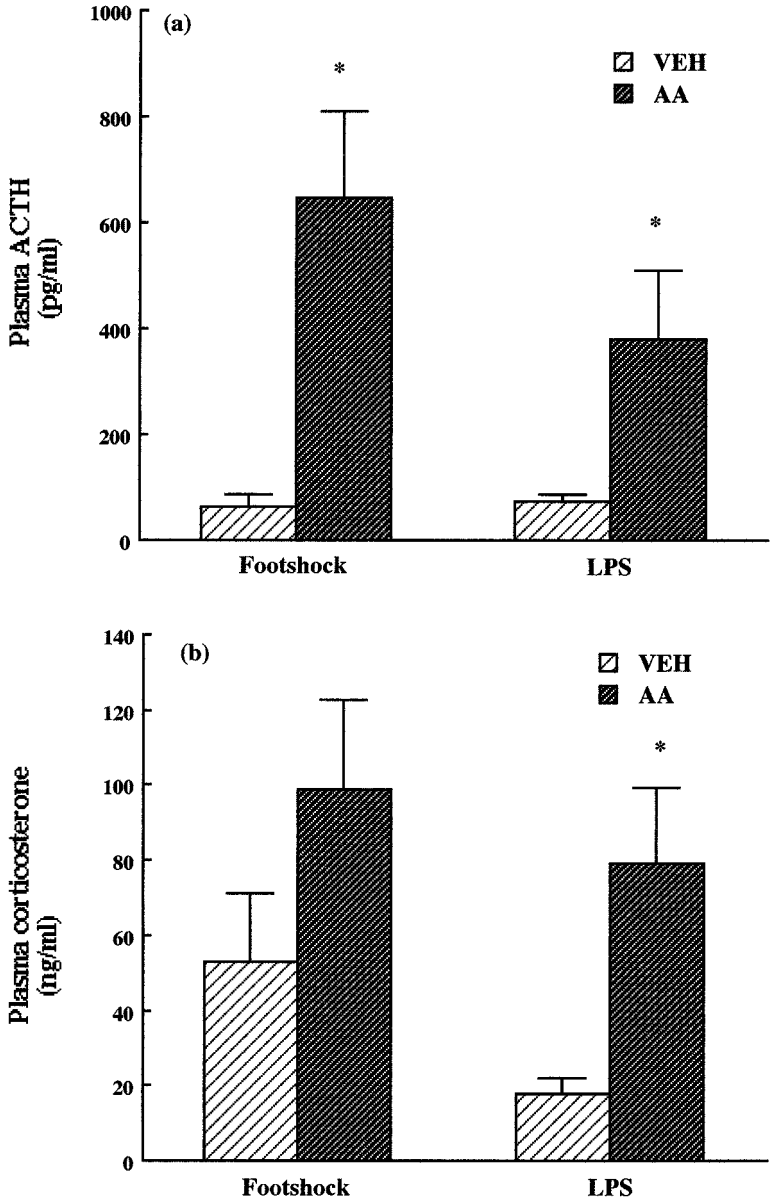

FIG. 3. Plasma concentrations of (a) ACTH and (b) corticosterone 14 days after injection of adjuvant (AA) or vehicle (VEH). The rats had previously been exposed to (a) footshock or (b) LPS 3 weeks prior to injection of adjuvant. Data represent means ± SEM for $n = 8–10$ animals. *$P < 0.05$ compared to respective controls.

TABLE 1
CRF and AVP mRNAs in the Parvocellular Cells of the PVN and POMC mRNA in the Anterior Pituitary Expressed as the Percentage of Change from the Respective Controls

	CRF mRNA	AVP mRNA	POMC mRNA
Non-FS/VEH	100.0 + 7.8	100.0 + 12	100.0 + 4.3
Non-FS/AA	90 + 14.7	141.8 + 11**	130.0 + 4.6**
FS/VEH	113.3 + 11.8	126.3 + 8	111.3 + 2.5
FS/AA	96.1 + 15.3	146.6 + 9.5**	132.1 + 4.3**
SAL/VEH	100.0 + 18.1	100.0 + 3.7	100.0 + 3.1
SAL/AA	80.3 + 6.7	132.8 + 8.6**	120.3 + 3.7**
LPS/VEH	106.9 + 10.6	127.4 + 6.8*	108.2 + 5.1
LPS/AA	80.8 + 9.4	130.1 + 9.9*	120.2 + 5.8**

Note. Rats were exposed to control footshock, i.e., placed in the shock chamber, but no current was applied, and 3 weeks later were injected with vehicle (Non-FS/VEH) or adjuvant (Non-FS/AA). Further groups received footshock (see text for parameters) and 3 weeks later received either vehicle (FS/VEH) or adjuvant (FS/AA). In a second study, rats were given an ip injection of saline and 3 weeks later injected with vehicle (SAL/VEH) or adjuvant (SAL/AA). Further groups received LPS and 3 weeks later were injected with vehicle (LPS/VEH) or adjuvant (LPS/AA). Values represent means ± SEM for $n = 8$–10 animals. *$P < 0.05$, **$P < 0.01$ compared to the respective controls.

elements of LPS injection, as exposure to footshock 3 weeks prior to adjuvant had no effect on the time course of development of inflammation. It is likely, however, that alternative central signaling pathways are differentially activated in response to the physical stress of footshock and to the immune-mediated stress of LPS injection. This has been reported previously in this model comparing stress and LPS-mediated responses where AA rats are unable to mount a response to acute stressors, such as noise, restraint, and ip hypertonic saline injection, but are able to respond to LPS (Harbuz et al., 1993; Aguilera et al., 1997; Harbuz et al., 1999a, 1999b). AVP mRNA levels in the parvocellular cells of the PVN of LPS-treated non-AA (LPS/VEH) animals were significantly elevated above those in SAL/VEH rats, whereas levels of AVP mRNA in the footshock-treated non-AA rats did not achieve a statistically significant difference from controls. These data suggest that prior exposure to LPS resulted in long-term changes in AVP mRNA which were still observed 5 weeks after the LPS challenge. Alterations in the CRF/AVP ratio days after a single exposure to acute stress and acute immune challenges have been reported previously (Van Dijken et al., 1992, 1993; Schmidt et al., 1995, 1997). Following repeated restraint stress CRF mRNA and CRF heteronuclear RNA responses are diminished with increasing frequency of restraint episodes, while AVP mRNA responses are increased (Ma and Lightman, 1998). A similar shift in balance toward increased AVP has been reported in chronic stress situations (Harbuz et al., 1993; Chowdrey et al., 1995; Aubry et al., 1999). One might speculate that this increase in AVP mRNA might contribute to the protective effects of the LPS challenge on the subsequent development of inflammation in AA, although the similar but not significant increase following footshock was not protective.

LPS is a potent activator of B cells, granulocytes, and monocytes. The pyrogenic

and immune activating properties of LPS reside in the lipid A moiety, which binds to specific receptors on monocytes/macrophages to induce release of cytokines such as IL-1 and TNFα (Rietschel et al., 1993). It is generally accepted that acute injection of cytokines such as IL-1 exerts a proinflammatory effect in arthritis models (Hom et al., 1988; Killar and Dunn, 1989). Similarly, there is evidence that LPS, from a variety of different bacterial sources, is able to induce or reactivate experimental arthritis (Stimpson et al., 1987; Noyori et al., 1994; Yoshino et al., 1999). In the present study we were not treating animals with established disease, nor did we see any evidence of inflammation in the LPS-treated rats during the 3 weeks prior to adjuvant injection. In contrast to these reports, one study has demonstrated a decrease in footpad swelling when the animals were treated with LPS 3 or 24 h before adjuvant injection. However, these effects were transient, and injection of LPS at earlier time points prior to adjuvant injection did not modify inflammation (Rosenbaum and Mandell, 1983).

Most studies concerned with questions of susceptibility to inflammation have concentrated on comparing different strains of rat, e.g., susceptibility of the Lewis strain relative to the resistance of the Fischer strain. However, comparison of germ-free (susceptible) and conventionally housed (resistant) Fischer rats has suggested that there is no inherent resistance in this strain of rat. Indeed, the Fischer strain is equally as susceptible to AA as the Lewis rat if paraffin oil is used as the vehicle to inject the mycobacterial adjuvant rather than mineral oil. The apparent resistance of the conventionally housed Fischer strain appears to be generated following bacterial challenge in the novel environment (van de Langerijt et al., 1993). Lewis rats can also be made resistant to AA by preexposure of neonatal rats to *Mycobacterium*, suggesting that immunization can prevent later exposure (Esaguy and Aguas, 1996). These data therefore suggest that resistance or susceptibility is not inherent in a given strain, but can be manipulated by either environmental pathogens or exposure to acute immune stimuli. These observations have recently been extended by treating neonates with LPS (Shanks et al., 2000). This nonspecific immune challenge, with LPS derived from *Salmonella enteriditis*, confered a resistance to AA when the adult animals were challenged. In contrast to the protective effect of LPS, neonatal handling did not influence the progression of inflammation in this study, suggesting that handling of the animals was not a major factor in determining resistance. The present data extend these observations to suggest that this effect is not necessarily due to neonatal plasticity, but that acute immune stimulation in adults is also able to influence disease activity in AA.

AA is a T-cell-mediated disease (Chang et al., 1980; Taurog et al., 1983a, 1983b). Although the antigenic specificities of these T cells remains to be determined it is evident that Th1 cells and Th1-type cytokines mediate AA (Rocken et al., 1996; Ohta et al., 1997). The balance of the Th1 to Th2 cytokine profile in determining susceptibility and resistance to immune-mediated diseases has received increased attention in recent years (Rook et al., 1994). Th1 cytokines, such as interferon-γ and interleukin-2, are involved in the development of autoimmune diseases, e.g., rheumatoid arthritis, multiple sclerosis, and their animal models AA and EAE. In contrast, Th2 cytokines such as IL-4, IL-10, and IL-13 are associated with other types of immune diseases, e.g., systemic lupus erythematosus and progressive systemic sclerosis. One might speculate that the protective effects of LPS which we have observed are the result of subtle shifts in the Th1/Th2 balance. Thus, acute immune activation

with LPS either in neonates (Shanks et al., 2000) or in adults, as in this study, can shift the Th1/Th2 balance to a protective Th2 profile. This may be facilitated by corticosterone, which is increased in AA rats previously exposed to LPS, since corticosterone can shift the balance of Th2 cytokine secretion (Rook, 1999) in response to increased AVP mRNA in the PVN. Confirmation of changes in the Th1/Th2 cytokine balance remain to be established.

In summary, we have demonstrated that rats exposed to acute immune stimulation with LPS, several weeks prior to induction of AA, exhibit a profound alteration in their susceptibility to inflammation. This is not a delay in the time of onset of inflammation, but represents the development of resistance to AA. HPA axis activity was increased to an equal extent in footshock-exposed rats, which did develop AA, and in adjuvant-injected LPS-treated animals, which did not exhibit inflammation. The importance of a responsive HPA axis for suppressing inflammatory flares in disease models and in humans with RA has been firmly established. However, the present data suggest that the protection against inflammation following LPS is not solely due to elevated corticosterone secretion through increased HPA axis activity and that other mechanisms are likely to be involved. These observations are a caveat for the effect that prior stress history might have on animals used in experimental models of inflammation. Furthermore, the data provide novel insights into the long-term modification of physiological systems following a single exposure to stress.

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