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Susceptibility to adjuvant arthritis: relative importance of adrenal activity and bacterial flora

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SUMMARY

Previous studies on the regulation of bacterial-induced arthritis in rats have focused on endocrine aspects as well as differences in T cell immunity against bacterial epitopes. We analysed the role of both adrenal activity and bacterial flora in determining susceptibility to bacterial-induced arthritis. Outbred Wistar rats show a low incidence of adjuvant arthritis. Moderate sensitivity to adjuvant arthritis was found in a selected, stress-resistant line of the Wistar rat, whereas no arthritis was found in a stress-susceptible Wistar line. Plasma corticosterone responses after IL-1α exposure were, however, identical in these two lines, excluding a direct correlation between susceptibility and corticosterone levels. In line with previous findings in germ-free (GF) F344 rats, GF Wistars also appeared highly susceptible to arthritis. We further analysed the corticosterone responses in GF and conventional (CV) rats. Administration of IL-1α induced identical corticosterone responses in both CV and GF F344 rats. In addition, plasma corticosterone levels were measured around the time of onset of arthritis. Whereas no rise was seen in the arthritis-resistant CV rats, a significant increase was observed from day 14 in GF rats, at the moment of onset of arthritis. Although this corticosterone response was insufficient to prevent arthritis, it may have ameliorated disease expression in the GF F344 rats. Our data indicate that the bacterial flora, and therefore T cell tolerance, is of prime importance in determining susceptibility, whereas the activity of the hypothalamus–pituitary–adrenal (HPA) axis may modulate disease severity.

Keywords adjuvant arthritis susceptibility corticosterone bacterial flora

INTRODUCTION

Chronic erosive polyarthritis, like adjuvant arthritis (AA) [1] and streptococcal cell wall (SCW)-induced arthritis [2], can be induced in susceptible rat strains. Several investigators have examined mechanisms which determine the susceptibility for this type of joint inflammation. It was found that susceptibility is strain-dependent, and appears to be controlled by an autosomal dominant gene locus linked to the MHC [3,4].

Not only genetic factors but also factors like the bacterial flora seem to regulate susceptibility to AA. For example, Lewis rats are highly susceptible, while the histocompatible F344 rat is resistant, and the germ-free (GF) F344 rat is again susceptible [5,6]. We showed that the ability to generate tolerance determines susceptibility, since only the susceptible Lewis rats were able to mount a specific T cell response against the injected bacteria [6,7]. Furthermore, Wilder and co-workers showed that the Lewis strain has a deficient response of hypothalamic corticotropin-releasing hormone (CRH) to various stressful inflammatory stimuli, while the F344 strain responded in a more robust manner [8]. As a result, the Lewis strain showed a deficient response of plasma corticosterone to these stimuli [9] and it was postulated that this diminished response of the hypothalamus–pituitary–adrenal (HPA) axis plays a role in determining the susceptibility in the Lewis and F344 strains.

In contrast to the Lewis strain, F344 rats display aggressive and stressful behaviour. Recently, two pharmacogenetically selected Wistar lines have been described, which seem to differ in their neurochemical and neurobiological organization/behaviour: the apomorphine susceptible (APO-SUS) and apomorphine unsusceptible (APO-UNSUS) lines [10]. The APO-UNSUS rats exhibit a decreased general locomotor activity and are less reactive to stressful stimuli than the APO-SUS animals [10,11]. Of interest, the APO-UNSUS rat showed enhanced susceptibility to experimental autoimmune encephalomyelitis (EAE) [11].

To examine the relative importance of the neuroendocrine and immune system, we compared the susceptibility to AA in the outbred conventional (CV) and GF Wistar rats, as well as in

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the APO-SUS and APO-UNSUS lines. Moreover, we determined plasma levels of corticosterone after IL-1α injection or during arthritis in the selected Wistar lines and in CV and GF F344 rats.

MATERIALS AND METHODS

Animals

Pharmacogenetically selected F14 offspring female Wistar rats (APO-SUS and APO-UNSUS) were obtained from the Department of Psychoneuropharmacology. Selection was made on their gnawing response to the dopamine receptor agonist apomorphine as described by Cools *et al.* [10]. Rats with gnawing responses < 10/45 min were designated as apomorphine unsusceptible (APO-UNSUS), and rats with gnawing responses > 500/45 min were called apomorphine susceptible (APO-SUS). Retention of genetic selection was tested in rats of the first litter of each generation. At the age of 60 days these rats were injected with apomorphine and tested as described earlier [10]. APO-UNSUS rats with the lowest scores and APO-SUS rats with the highest scores were selected and used for subsequent development of the next generation. Rats of the second and third litter of each generation were used for experiments. Female CV outbred Wistar rats were obtained from Harlan CPB (Zeist, The Netherlands), and female CV inbred F344 rats from the Zentral Institut für Versuchtierzucht (Hannover, Germany). Female Lewis rats were bred in our own facilities and were originally obtained from the Zentral Institut für Versuchtierzucht.

Female GF F344 rats were originally obtained from Jackson Laboratories (Bar Harbor, ME), and female GF Wistar rats from the Zentral Institut für Versuchtierzucht, and both were bred in our own facilities (Central Animal Laboratory) in overpressure isolators. The isolators and faeces were screened once every 2 weeks for bacterial contamination.

The rats weighed between 140 and 180 g at the beginning of each experiment, and were housed in rooms illuminated from 6:00 a.m. to 6:00 p.m. Standard food and tap water were given *ad libitum*.

Induction of adjuvant arthritis

Arthritis was induced by an intradermal injection at the base of the tail with 100 μl of 10 mg/ml *Mycobacterium tuberculosis* (H37RA; Difco Labs, Detroit, MI) suspended in Freund's incomplete adjuvant (FIA; Difco). Arthritis was scored by measuring hind paw thickness with an industrial micrometer. In addition, in some experiments we performed a visual scoring on both hind and fore feet on the basis of: 0 = no signs of redness or swelling; 1 = mild swelling and redness; 2 = severe swelling and redness.

In vivo *cannulation experiments*

In order to determine plasma corticosterone levels in response to administration of IL-1α or during AA, blood was collected from freely moving rats by means of a chronic cannula. Cannulation of the rats was performed according to the technique described by Steffens [12] with some minor modifications as described by Sweep *et al.* [13]. After cannulation, the animals were housed individually and handled daily by the experimenter to diminish stress caused by the experimental procedures.

Animals were allowed to recover from the operation for at least 6 days. At the start of the experiment, a polyethylene cannula was attached to the stainless steel tube on the skull of the head. Two hours later time course experiments were started ($t = 0$ min) by injection of 1 μg of IL-1α intravenously. Recombinant human IL-1α was a generous gift from Dr P. T. Lomedico (Roche Research Centre, Nutley, NJ). Control rats received saline alone. At every time point 300 μl blood were collected for corticosterone measurements, and subsequently 300 μl of saline were returned to the animal. Regarding the time course experiments in AA, blood withdrawal always started around 11:00 a.m., to exclude differences in corticosterone levels due to the circadian rhythm.

In vitro *corticosterone measurements*

Blood samples were collected in dry lithium-heparin tubes and centrifuged at 1500 *g* for 10 min at 4°C. Plasma was separated and stored at −20°C until assayed.

Corticosterone measurements were performed as described by Sweep *et al.* [13]. Briefly, plasma corticosterone was extracted with 7·5 ml of dichloromethane (Baker, Deventer, The Netherlands). The water phase was discarded and the dichloromethane phase was evaporated. The residue was dissolved in 2 ml 0·2% ethylene–glycol–water (EGW), and the concentration of corticosterone in the eluate was measured by a radioimmunoassay using an antiserum raised in sheep against a B-21-hemisuccinate–bovine serum albumin (BSA) conjugate.

The sensitivity of the assay was 25–45 fmol/tube. The intra- and interassay coefficients of variation were 5% and 8%, respectively.

Statistical analysis

In the arthritis susceptibility experiments, the hind paw thickness between the different groups was compared by the Wilcoxon rank sum test. The χ^2 test with Yates' correction was used to analyse the results of the visual scoring.

All corticosterone data are presented as the mean ± s.d. of five to eight animals. Comparisons between the corticosterone levels in the arthritis experiments were made by the ANOVA test with repeated measurements. Only when the ANOVA revealed a significant difference between both groups were values at specific time points further evaluated by Student's *t*-test for unpaired observations.

RESULTS

Susceptibility of the different Wistar rat lines for AA

AA was induced in the APO-SUS and APO-UNSUS Wistar rat lines, and the incidence and severity of AA was compared with observations in CV and GF Wistar rats. All APO-SUS rats were resistant, while 10 out of 16 APO-UNSUS rats appeared to be susceptible for AA ($P = 0{\cdot}0001$, χ^2 test) (Table 1). When we measured the paw thickness, only a moderate increase was noted in the APO-UNSUS rats, with a late onset (Fig. 1).

The susceptibility of the CV Wistar rats, compared with the APO-SUS and APO-UNSUS rats, appeared to be intermediate (3 out of 14). On the other hand, almost all the GF Wistar rats were susceptible for AA, and developed a severe and chronic joint inflammation (Fig. 1, * $P = 0{\cdot}0001$ *versus* CV Wistar rats, Wilcoxon rank sum test).

Table 1. Clinical expression of adjuvant arthritis (AA) in the different Wistar rats after the induction of AA

	0	1	2
APO-SUS	16	–	–
APO-UNSUS	6	6	4
CV Wistar	11	3	–
GF Wistar	1	4	6

The severity of arthritis was scored visually on a scale of 0–2: 0 = no redness or swelling; 1 = mild swelling and redness; 2 = severe swelling and redness. The data shown are the number of animals belonging to each group, 20 days after induction of arthritis. The susceptibility to AA between apomorphine susceptible (APO-SUS) and apomorphine unsusceptible (APO-UNSUS) was significantly different ($P = 0.0001$, χ^2 test).

Corticosterone levels after IL-1α injection in APO-SUS and APO-UNSUS rat lines

We analysed the effect of IL-1α injection on plasma corticosterone levels in both APO-SUS and APO-UNSUS rats. This proinflammatory cytokine is able to stimulate the HPA axis, resulting in an increased plasma concentration of the anti-inflammatory steroid hormone corticosterone.

Intravenous (i.v.) injection of 1 μg IL-1α in cannulated APO-SUS and APO-UNSUS rats resulted in an increase of plasma corticosterone levels at 30 min (Fig. 2). No significant differences could be detected between corticosterone levels in the APO-SUS and APO-UNSUS rats. Corticosterone levels in control animals, injected with saline alone, remained low throughout the entire experiment.

Corticosterone levels after AA induction in Wistar rat lines

We examined corticosterone levels after the induction of AA in cannulated APO-SUS and APO-UNSUS rats. As shown in Fig. 3, no significant differences could be detected in plasma corticosterone between these two different rat lines (ANOVA, days 15, 18, 21).

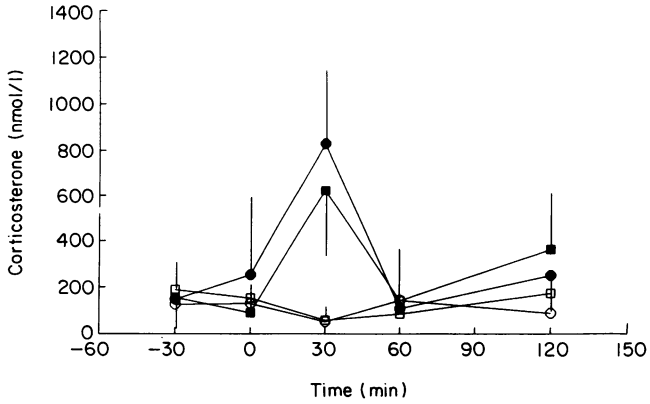

Fig. 2. Plasma corticosterone levels in apomorphine susceptible (APO-SUS) (●) and apomorphine unsusceptible (APO-UNSUS) (■) rats after i.v. injection of 1 μg of recombinant human IL-1α (closed symbols) at $t = 0$ min. Each time point represents the mean ± s.d. of eight animals. The control groups received saline alone (open symbols).

Susceptibility to AA in Lewis, CV F344 and GF F344 rats

It is known from previous studies that GF F344 rats are highly susceptible, whereas CV F344 are resistant to bacterial arthritis. We analysed the corticosterone response in these rats, both after IL-1 exposure and after induction of AA. Figure 4 shows the difference in AA susceptibility between the CV and GF F344 rats in this set of experiments. For comparison, data from Lewis rats are also given.

Corticosterone levels after IL-1α injection in F344 rats

The corticosterone response after i.v. injection of IL-1α was similar in CV and GF F344 rats (Fig. 5). Of interest, the peak levels were twice as high in F344 rats as in Wistar rats (Fig. 2). The control animals, injected with saline alone, showed no increase in plasma corticosterone.

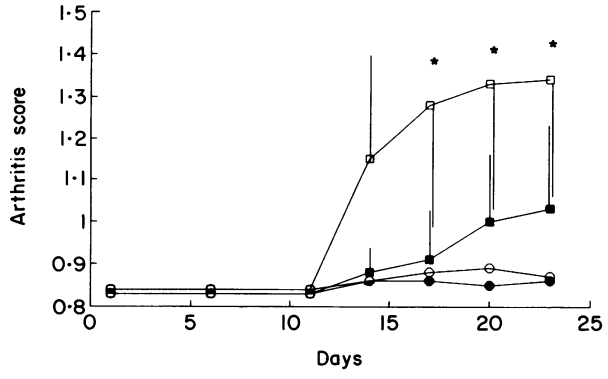

Fig. 1. Susceptibility of the same Wistar rats as shown in Table 1, but now scored by measuring the hind paw thickness with an industrial micrometer. Values represent the mean ± s.d. of 11–16 rats per group. The germfree (GF) Wistar rats (□) were significantly more susceptible than the conventional (CV) Wistar rats (○): * $P = 0.0001$, Wilcoxon rank sum test. ●, Apomorphine susceptible (APO-SUS); ■, apomorphine unsusceptible (APO-UNSUS).

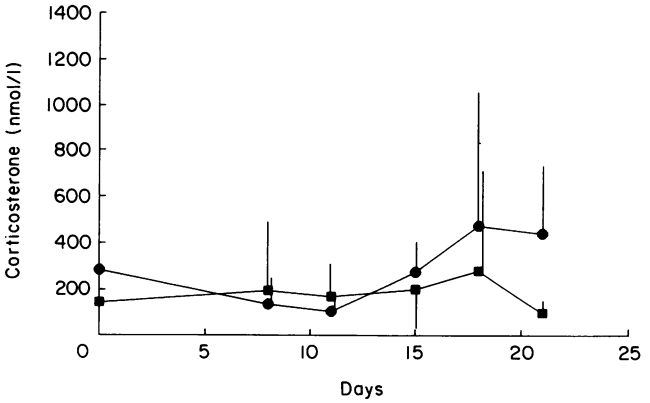

Fig. 3. Plasma corticosterone levels in apomorphine susceptible (APO-SUS) (●) and apomorphine unsusceptible (APO-UNSUS) (■) rats after induction of adjuvant arthritis (AA) at day 0. Blood samples were collected at 11:00 a.m. Values represent the mean ± s.d. of five animals per group. ANOVA (days 15, 18, 21) revealed no significant difference between both groups.

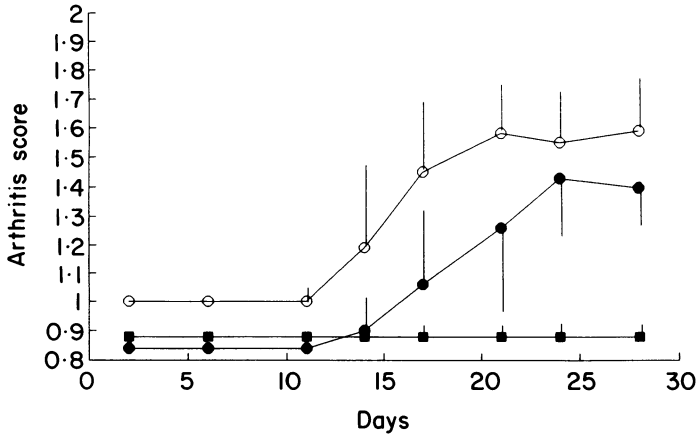

Fig. 4. Different susceptibility of Lewis (○), conventional (CV) F344 (■) and germ-free (GF) F344 (●) rats to the induction of adjuvant arthritis (AA). The arthritis was scored by measuring the hind paw thickness with an industrial micrometer. The values represent the mean ± s.d. of four animals per group.

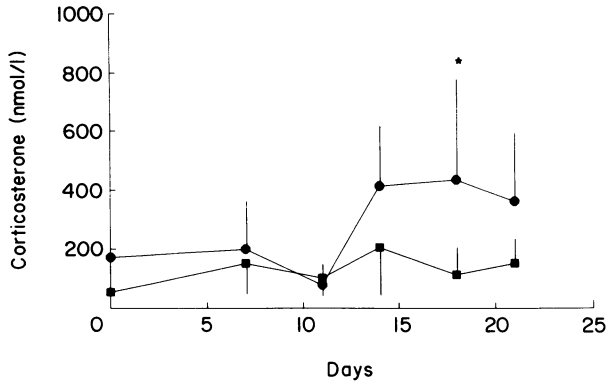

Fig. 6. Plasma corticosterone levels in conventional (CV) (■) and germ-free (GF) F344 (●) rats after the induction of adjuvant arthritis (AA). Blood samples were collected at 11:00 a.m. Values represent the mean ± s.d. of six animals per group. ANOVA revealed a statistically significant difference for both groups (days 14, 18, 21); day 18 * $P < 0.05$, Student's t-test.

Corticosterone levels after induction of AA in F344 rats

Finally, we analysed the course of corticosterone responses after immunization and during the onset of AA in F344 rats. Figure 6 shows that plasma corticosterone levels remained low in the resistant CV F344 rats during the whole period of observation. Compared with the CV F344, a significant increase (ANOVA $P < 0.01$, days 14, 18, 21) in corticosterone levels was observed during the onset of AA in the GF F344 rats (day 18; * $P < 0.05$, Student's t-test).

DISCUSSION

There is now increasing evidence that not only the genetic background, but also the bacterial flora and neuroendocrine system determine the overall immune response of an individual. In this study we analysed the relative importance of the activity of the HPA axis and the bacterial flora in determining susceptibility to AA.

The observation that the stress-susceptible APO-SUS Wistar rats were significantly less susceptible to AA than the stress-resistant APO-UNSUS rats is in agreement with recent findings in the EAE model [11], although the differences appeared to be more pronounced in the latter model. These findings suggest that different types of behavioural and neuroendocrinological responses to stress are correlated to different susceptibility to AA. Although we may not conclude that there is a causal relationship between stress and susceptibility to AA, several other data do indicate that stress can affect susceptibility. For instance, it has been observed that susceptibility to EAE can be partially suppressed by environmental stress [14]. Similarly, we observed that stress, induced by daily injection of a vehicle substance, may result in almost complete prevention of murine type II collagen arthritis ([15], manuscript in preparation). In addition, studies in patients with rheumatoid arthritis (RA) also demonstrated immunosuppressive effects of stress [16,17]. In contrast, other investigators emphasize that stress may selectively affect the T suppressor cell system in RA patients [18].

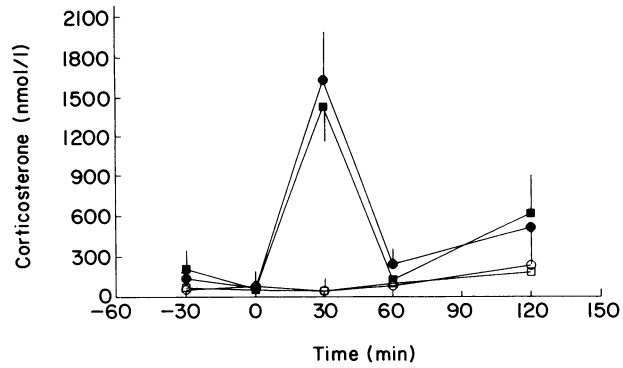

Fig. 5. Plasma corticosterone levels in conventional (CV) (■) and germ-free (GF) F344 (●) rats after the injection of 1 μg IL-1α intravenously at $t = 0$ min (closed symbols). Values represent the mean ± s.d. of six to eight animals per group. The control groups received saline alone (open symbols).

Interestingly, very recently differences were observed in T cell responsiveness between APO-SUS and APO-UNSUS rats (A. Kavelaars *et al.*, manuscript in preparation). It appeared that lymphocytes from APO-SUS rats had an increased mitogen reactivity and a higher number of natural killer (NK) and T suppressor/cytotoxic cells than lymphocytes from APO-UNSUS rats.

The fact that CV outbred Wistar rats show intermediate susceptibility [19], compared with the APO-SUS and APO-UNSUS rats, fits in with the fact that the APO-SUS and APO-UNSUS rats represent extremes in an unselected population of rats [10]. Furthermore, the observation that the GF Wistar rats were highly susceptible suggests that the bacterial flora seem to be extremely important in determining susceptibility to AA. This is in line with several previous studies, in which it was demonstrated that GF F344 rats were susceptible to bacterial-induced arthritis [5,6] and that conventionalization of these GF rats could dramatically moderate disease severity [6,20].

Analysis of corticosterone levels in response to administration of IL-1α showed that the genetic background can determine the activity of the HPA axis, since we observed a twice as high corticosterone response in the F344 rats compared with

the Wistar rats. Interestingly, all the time course experiments with IL-1α showed identical total corticosterone levels between the Wistar rat lines and between the CV and GF F344 strains. This suggests that the activity of the HPA axis does not determine the susceptibility to AA in these different rats. These data support our previous findings [21], but are in contrast with the findings in the susceptible CV Lewis rats [8,9], in which a clear defect of the HPA axis was observed. Furthermore, a recent study showed that RA patients may also have an abnormal HPA axis response to stress [22]. On the other hand, we can not totally exclude that differences in plasma steroid hormone levels may still determine susceptibility, since we did not discriminate between free corticosterone and carrier-bound corticosterone. Regarding this, Rots *et al.* [23] also observed no differences in total corticosterone levels, when the same Wistar rat lines were exposed to a novel environment as stress stimulus. However, when they compared free corticosterone as well as adrenocorticotrophic hormone levels, they were then able to show a small increase in the HPA axis activity of the APO-SUS rats.

Analysis of the corticosterone levels after AA induction showed no significant differences between the APO-SUS and APO-UNSUS rats over a broad time span. This finding parallels the only minor differences in arthritis severity between these two rat lines. In the GF F344 rats, however, we were able to detect a significant increase in corticosterone levels after the onset of arthritis. This increase may be due to the stress of the disease itself. The latter phenomenon has also been observed during the onset of the clinical signs of EAE in the Lewis rat [24,25]. On the other hand, a recent report in AA shows increased levels of plasma corticosterone already at day 7 after immunization in Sprague Dawley rats [26]. At that time point there were still no clinical signs of arthritis. The difference might be explained by the fact that the HPA axis responsiveness during chronic stress is strain-dependent.

Finally, no increase in corticosterone levels was observed in the resistant CV F344 rats, which suggests that resistance of the F344 strain can not be explained by an increased HPA axis activity. This indicates that the arthritogenic responses are probably controlled at another level. A likely candidate is T cell tolerance, especially in the light of our previous findings, in which we were able to detect a correlation between T cell responses and susceptibility [6,7]. On the other hand, we can not exclude that the adrenal response in the CV F344 rats may only increase just after immunization (between days 0 and 7) and in this way directs the immune response to either suppression or reactivity. However, this is not very likely, since an adrenalectomy study in the EAE model showed that corticosterone only had an important regulatory role just before the clinical onset of EAE in the susceptible Lewis strain [24], as well as in the resistant PVG strain [27].

In conclusion, our data indicate that differences in behavioural and neuroendocrinological responses to stressors are correlated to differences in susceptibility to bacterial arthritis. However, the bacterial flora seem far more important in regulating susceptibility. Furthermore, the corticosterone data suggest that the HPA axis activity correlates with disease severity instead of being a mechanism to prevent disease. Analysis of free corticosterone levels in the different susceptible rats and lymphocyte proliferation assays is now in progress. These experiments will provide further insight into the relative importance of steroid hormone levels and T cell tolerance in susceptibility to bacterially induced arthritis.

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