The Importance of the Hypothalamo-Hypophyseal-Adrenal Axis to the Anti-Inflammatory Actions of the κ-Opioid Agonist PNU-50,488H in Rats with Adjuvant Arthritis[1]

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ABSTRACT

Possible contributions of the hypothalamo-hypophyseal-adrenal axis to the development of adjuvant-induced arthritis and therapeutic actions of the prototypical κ-opioid agonist PNU-50,488H (PNU-50) were studied in DA rats. Paw edema, nociception, histological and radiological joint damage, and tumor necrosis factor-α release by peritoneal macrophages were measured in adrenalectomized (ADX) and sham-operated (SHO) arthritic animals (drug-treated and untreated groups). Disease developed earlier in ADX rats (paw edema was first apparent 11 days postadjuvant compared with day 13 in SHO animals) and remained more severe in that group. Twice-daily PNU-50 treatment completely prevented the development of edema in the SHO group but was effective in the ADX animals only on day 18. PNU-50 substantially reduced the pooled severity index (combined quantitative edema, histological and radiological assessments) at day 18 in both SHO and ADX rats and to an equal extent. During disease development, the paws of SHO, but not ADX, rats became hyperalgesic; paradoxically, ADX animals were hyperalgesic during PNU-50 treatment, but the drug produced analgesia in SHO animals. Compared with cells harvested from healthy animals, macrophages from arthritic rats released about twice as much tumor necrosis factor-α after lipopolysaccharide stimulation. It was concluded that the hypothalamo-hypophyseal-adrenal axis influences the development of adjuvant arthritis and plays a partial role in the therapeutic action of the κ-agonist PNU-50.

The hypothalamo-hypophyseal-adrenal (HPA) axis has anti-inflammatory functions through its production of corticotrophin-releasing hormone (CRH), adrenocorticotrophic hormone (ACTH), β-endorphin, and glucocorticoids. It has been proposed, therefore, that dysfunction of this axis may contribute to the susceptibility to, and even the persistence of, human rheumatoid arthritis (RA) (Hall et al., 1994; Morand et al., 1996). Certainly, in the Lewis rat, which is particularly susceptible to adjuvant-induced arthritis, there is impaired HPA-axis responsiveness to inflammatory stimuli (Sternberg et al., 1989a,b; Crofford et al., 1992; Matta et al., 1995). The finding that patients with RA have low levels of circulating cortisol, which are unaltered by stress, is consistent with this hypothesis (Chikanza et al., 1992; Gudbjörnsson et al., 1996; Templ et al., 1996). Another significant clue is that in RA the HPA system is refractory to the stimulatory actions of inflammatory cytokines (Crofford et al., 1997).

In general, this system is controlled by negative feedback of glucocorticoids, but its sensitivity can be influenced by opioids (through hypothalamic receptors, for instance) as well as by cytokines (Bateman et al., 1989; Blalock, 1989; Perretti et al., 1989). Recently, our group has shown, for the first time, that κ-opioid agonists, which have the advantage of fewer side effects than other opioid receptor agonists, are powerfully therapeutic in adjuvant-induced arthritis in rats. The current study, therefore, used adrenalectomized (ADX) animals to determine 1) the influence of corticosteroids on the development of adjuvant arthritis in DA rats, 2) the importance of these hormones for the antiarthritic action of the prototypical κ-receptor agonist [PNU-50,488H (PNU-50)], and 3) their influence on the production of tumor necrosis factor-α (TNF-α) by macrophages from normal and arthritic rats.

Materials and Methods

Animals

We used male DA rats, the strain that we have found to show the most consistent development of polyarthritis after induction with Freund's complete adjuvant (80–100% success). The animals were

ABBREVIATIONS: HPA, hypothalamo-hypophyseal-adrenal; ADX, adrenalectomized; CRH, corticotrophin-releasing hormone; ACTH, adrenocorticotrophic hormone; RA, rheumatoid arthritis; PNU-50, PNU-50,488H; TNF-α, tumor necrosis factor-α; SHO, sham-operated; AA, adjuvant-induced arthritis; PSI, pooled severity index.

obtained from the University of Queensland (St. Lucia, Australia) and weighed 150 to 210 g at the beginning of the experiment. During the experiments and for the preceding week, they were held in a temperature-controlled room (22 $\pm$ 1°C; 12-h alternating light/dark cycle, with lights on at 6:00 AM), housed in cages lined with cellulose bedding (Fibercycle Pty. Ltd., Mudgeeraba, Queensland) and shredded paper, and provided rat chow (Gordon's Specialty Stockfeeds, Yanderra, New South Wales, Australia) and water ad libitum; ADX animals were provided with 0.9% NaCl solution. The study was approved by the Animal Care and Ethics Committee of the University of New South Wales.

Adrenalectomy

Rats were anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) i.p. A dorsal midline skin incision extending from the last rib nearly to the pelvis and, on each side, a small transverse incision through the muscle caudal to the costal margin allowed access. To ensure a total adrenalectomy, the gland was removed by cutting through the surrounding fat, leaving a generous margin attached to the gland. Control sham-operated (SHO) rats underwent the same operation except that the adrenal gland was left intact in situ. To avoid stress-induced postoperative death, each rat (both SHO and ADX) received a covering dose of dexamethasone (0.1 mg/kg s.c.) and the wounds were infiltrated with local anesthetic (5 mg/ml bupivicaine). An identical dose of dexamethasone was administered to every animal 24 h later.

Induction of Arthritis

Immediately after the surgery and while still anesthetized (day 0), each rat was administered an intradermal injection (100 μl into the base of the tail) of complete Freund's adjuvant, viz. heat-killed and dried *Mycobacterium butyricum* suspension (10 mg/ml) in paraffin oil, and mannide mono-oleate (Difco Laboratories, Detroit, MI).

As part of their husbandry, each rat was handled every 2 to 3 days for 1 week before and throughout the study. During the latter period, body weight, paw volume (plethysmometry; Ugo Basile, Comerio, Italy), and nociceptive sensitivity (pressure analgesymetry; Ugo Basile) of both inflamed hindlimbs were measured on day 0 and days 5, 11, 13, and 18 postadjuvant; the same trained observer performed every measurement. All experiments were terminated on day 18, and the rats were sacrificed with CO_2 to permit the determination of two additional indices of inflammatory arthritic damage: quantitative radiography and histology performed on the right paw from every animal using scoring methodology developed in our laboratory (Binder and Walker, 1998). At the same time peritoneal macrophages were obtained for culture (see later).

Drugs

The κ-opioid agonist ($\pm$)-PNU-50 (kindly supplied by Dr. M. Piercey, Upjohn, Kalamazoo, MI) was dissolved in a mixture of 33% polyethylene glycol 400 (Aldrich Chemical Co., Milwaukee, WI) and 0.9% NaCl solution (20 mg/ml). It was administered by i.p. injection over the entire experimental period (dosage, 20 mg/kg/day). This regimen was chosen on the basis of our previous work with this drug (Wilson et al., 1996).

Corticosterone Assay

Blood was obtained from anesthetized rats through the tail vein on days 0, 5, 11, 13, and 18 in SHO rats but only on days 0 and 18 in the ADX rats lest they became hypersensitive to anesthesia. Plasma corticosterone levels were determined using a radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA). On day 18, plasma corticosteroid levels in the ADX rats were below the detectable limit of this assay, indicating successful adrenalectomy.

Culture of Macrophages

Macrophages were obtained by peritoneal lavage from all four groups of experimental animals on day 18, after euthanasia; five normal (nonarthritic) rats were also used to provide control cells. The skin and the abdominal musculature were cut, and the peritoneal cavity was washed twice with sterile 0.38% sodium citrate in Hanks' buffer (Sigma Chemical Co., St. Louis, MO) using a mixing cannula (Indoplas; Maersk Indoplas, Sydney, New South Wales, Australia). The individual lavage samples were centrifuged, and the supernatant solutions were removed, after which the pellets were resuspended in 1 ml Hanks' solution and pooled ($n = 5–8$). Cells were plated onto 24-well assemblies to produce a final concentration of 1.5×10^4 cells/well in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1000 I.U. of penicillin and 1 μg/ml streptomycin, respectively (Trace Biosciences, Castle Hill, New South Wales, Australia). The cells were stimulated with lipopolysaccharide (0.1 μg/ml; Sigma Chemical Co.) and incubated for 18 h, after which the supernatant solutions were harvested and centrifuged to remove cellular debris. These samples were frozen (-70°C) until assayed. The cytokine TNF-α was measured in 100-μl samples of medium with a murine TNF-α enzyme-linked immunosorbent assay (ELISA; Genzyme, Cambridge, MA).

Experimental Design

To determine the possible influence of the adrenal hormones on the development of adjuvant arthritis and on the efficacy of the κ-opioid agonist, the experiment consisted of four principal groups. The first consisted of ADX rats treated with PNU-50 (a total of 20 mg/kg/day in twice-daily i.p. doses throughout the entire experiment; $n = 12$). The second consisted of ADX rats treated with the polyethylene glycol vehicle solution (2 ml/kg/day twice daily i.p.; $n = 12$). The third consisted of SHO rats receiving treatment with PNU-50 (20 mg/kg/day twice daily i.p.; $n = 8$). The fourth group consisted of SHO animals treated only with vehicle (2 ml/kg/day twice daily i.p.; $n = 8$).

Data Treatment

All measurements (except radiology and histology) were made over the entire 18-day observation of the time course of the adjuvant-induced arthritis (AA), and every measurement was normalized relative to its value on day 0. These normalized data were then time-averaged over the complete experimental period, as described previously (Binder et al., 2000). All graph plots show mean with S.E. values. Multiple comparisons for each variable were made first by performing a repeated measures ANOVA (the factors groups and days being considered fixed) and then (where the F ratios indicated significant heterogeneity, $P < .05$) with post hoc t tests using Fisher's multiple-comparison test (NCSS, Kaysville, UT).

Inflammation. The three indices of arthritic damage were used. Successive paw volumes (edema) were averaged from bilateral measures; radiology and histology scores (made postmortem at 18 days) were unilateral. These were summed to produce a "pooled severity index" (PSI), which is analogous to clinicians' global assessments in human patients (Wilson et al., 1996; Binder and Walker, 1998) with the aggregated scores adjusted to the values of SHO animals (set at 100%).

Nociception. Sensitivity to noxious pressure was also measured over the time course of the AA and averaged for each paw. As detailed above, these data (the applied mass that produced the desired withdrawal reflex) were normalized against each animal's own predisease scores. The plots of nociception used the reciprocals of these values to produce an index of pain sensitivity, revealing either hyperalgesia or hypoalgesia.

Corticosterone and TNF-α. The plasma concentrations of corticosterone were likewise normalized. TNF-α levels were analyzed and plotted as concentration/cell number.

Results

There were 24 ADX animals and 16 SHO animals (the higher number in the former category was because greater mortality rates was likely in that group). One of the SHO rats failed to recover from the initial surgery and anesthesia; one ADX-PNU-50-treated animal died on day 11 and one died on day 15; and two rats from each ADX group died during blood sampling near the conclusion of the experiment. Otherwise, the animals remained in typical condition for arthritis; by day 18, mean body weight in the SHO rats was in effect the same as on day 0 and had risen by about 10% in the other groups.

Corticosteroid Levels

On day 18, there was no detectable corticosterone in the plasma of ADX rats, indicating that the adrenalectomy was successful. Plasma corticosterone levels in arthritic SHO rats had fallen to approximately 50% of normal by day 12 (the point at which disease development is just beginning: Figs. 1 and 2) and had increased above the baseline concentration when the experiment was terminated (Fig. 1). No such decrease in corticosteroid levels during the latent period of the disease was apparent when the animals were treated with PNU-50; by contrast, corticosterone levels increased initially and essentially remained constant thereafter.

Effect of Adrenalectomy on Adjuvant Arthritis

Paw Edema. In the ADX rats, edema of the hindpaws was observed 2 days earlier and was more severe than in the SHO animals (Fig. 2). By day 18, however, the severity of the edema was the same in both groups of animals.

Aggregated Severity. All three quantitative indices of severity (paw volume, radiography, and histology) had greater magnitudes in the ADX rats than in the arthritic SHO rats (Table 1), but neither the individual results nor the pooled values (PSI; Fig. 3) reached the 5% significance level (PSI: ADX, $145 \pm 28\%$; SHO, $100 \pm 6\%$; $P < .12$). (Statistical calculations showed that under these experimental conditions, the variability and values obtained would require sample sizes of about 24 for 5% significance to be obtained.)
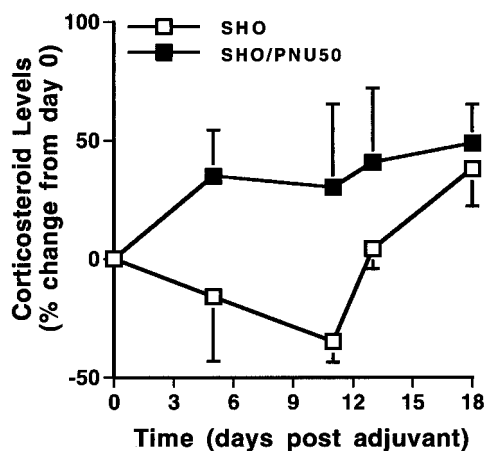

Fig. 1. Time courses (normalized values shown) of the plasma levels of corticosterone in sham-operated rats after the induction of arthritis; levels were below detection in ADX animals. PNU50 indicates drug treatment with PNU-50488H; otherwise the animals are vehicle-treated.
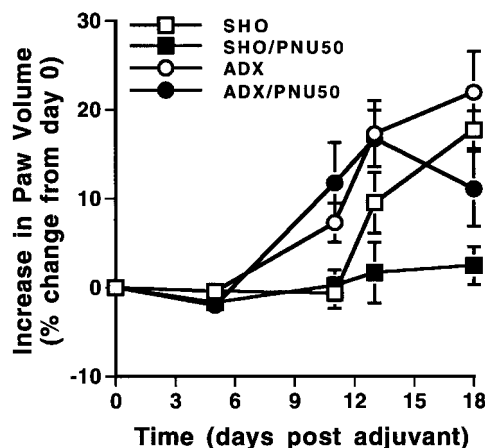

Fig. 2. Plots of the time courses of changes in paw volume in the four experimental groups. Overt disease occurs earlier and remains more severe in the ADX rats than in the SHO rats.

TABLE 1
Effect of adrenalectomy on the severity of adjuvant arthritis
The radiology and histology data consist of right and left paw combined. Thus the maximum score for radiography would be 24 and for histology 36 (see *Materials and Methods* for further detail).

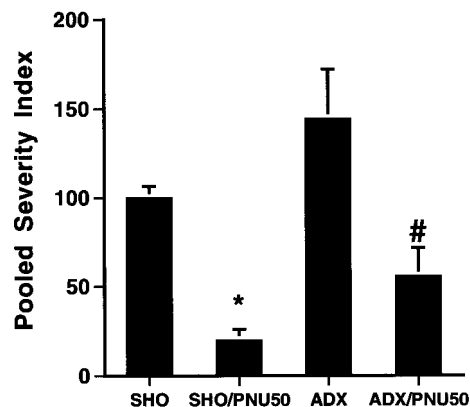|	Paw Volume	Radiography Score	Histology Score
	% increase from day 0		
SHO animals (*n* = 8)	28.0 ± 4.7	9.9 ± 1.8	19.0 ± 2.8
ADX animals (*n* = 8–12)	39.3 ± 7.7	12.5 ± 1.9	24.5 ± 2

Fig. 3. PSI (calculated using day 18 data) in SHO and ADX adjuvant arthritic animals both with and without treatment with PNU-50. Significant differences (#$P < .02$, *$P < .01$) compared with their respective controls.

Effect of Treatment with PNU-50

The time course of paw edema in the experimental groups is complex. The SHO animals that received PNU-50 showed no edema at all throughout the 18 days (Fig. 2, filled squares), whereas in the ADX animals, the therapeutic action of the drug was not apparent before day 18. Before that time, the disease was the same in all of the ADX animals (PNU-50 treated or not; Fig. 2) with marked edema as early as day 11, a time when disease was not yet manifest in normal (SHO) animals.

Overall, however, PNU-50 significantly attenuated the aggregated PSI (Fig. 3) in both ADX (from 100% to $56 \pm 16\%$,

$P < .02$) and SHO (to $20 \pm 6\%$, $P < .01$) animals. There was no difference in the magnitude of the improvement produced by PNU-50 in either treatment group (SHO versus ADX: 60 ± 14 versus 77 ± 9; $P > .05$).

Nociception

As the arthritic signs develop, the rats' nocisensitivity altered as a function of their treatment (Fig. 4). After day 11, the untreated SHO animals showed persistent hyperalgesia; the drug-treated animals showed a similar pattern, at a lower level of nociception. By contrast, at day 11 the ADX rats displayed substantially reduced nociceptive sensitivity (hypoalgesia); thereafter, pain sensitivity returned to normal levels (ADX) or to hyperalgesia (ADX/U50 animals). It is noteworthy that the nociceptive pattern does not parallel the development of the edema (compare Figs. 2 and 4).

TNF-α Release from Peritoneal Macrophages

When stimulated by lipopolysaccharide, the cells that had been harvested from arthritic animals released more TNF-α than those obtained from healthy animals (Fig. 5). Overall, the release of TNF-α was greater when the macrophages were collected from ADX rats (by 22%; $P < .05$). Furthermore, the cells obtained from rats that had been treated (in vivo) with PNU-50 released about 20% less TNF-α than the animals that had received only vehicle ($P < .01$).

Discussion

Our group has shown that κ-opioid agonists are powerfully therapeutic in adjuvant arthritis in rats (Wilson et al., 1996; Binder and Walker, 1998). To address the possible mechanisms involved, in this study we used ADX DA rats 1) to confirm that adrenalectomy aggravates disease in our model, 2) to determine the importance of these hormones for the antiarthritic action of the prototypical κ-receptor agonist PNU-50, and 3) to examine their influence on the production of TNF-α by macrophages.

There has been speculation that immunoinflammatory dis-

Fig. 5. Concentrations of TNF-α measured in the supernatant solutions of 18-h cultures of peritoneal macrophages that had been harvested from normal healthy animals (no arthritis; control, $n = 4$) SHO and ADX animals. The arthritic animals were treated either with vehicle (SHO, $n = 9$; ADX, $n = 6$) or with PNU-50 (20 mg/kg/day) for 18 days (SHO, $n = 5$; ADX, $n = 6$). The final therapy was on day 17; the animals were sacrificed, and the peritoneal macrophages were obtained 24 h after that last treatment (day 18: see *Materials and Methods*).

eases, such as RA (and, analogously, AA), are found in patients whose HPA axis is somehow compromised (Masi and Chrousos, 1996). This idea arose not only from the early recognition of the anti-inflammatory actions of adrenocorticoids (Hench et al., 1949) but also from work that showed that although increased hypothalamic neural activity can be detected in immunized rats that produce antibodies, there is no such activity if the animals do not produce any immune response (Saphier et al., 1991). If those hypothalamic neurons were the ones that induce ACTH release, then glucocorticoid secretion would result. Thus, in animals with uncompromised antibody production, the severity of immunopathological reactions is contained; otherwise, disease develops.

As an example, when Sternberg et al. (1989a,b) injected rats with preparations of streptococcal cell walls, they found a greater susceptibility to arthritis in animals with "defective inflammatory and stress-mediator induced activation of the HPA." Similarly, Neeck et al. (1990) obtained results from RA patients that confirmed this idea: severe disease was seen in patients with lower plasma levels of cortisol. Like Harbuz et al. (1993) and Yang et al. (1997), we found that the AA was worse in the ADX rats. Stephanou et al. (1992) also reported worse paw edema in ADX rats given adjuvant, but their claim of "chronic activation of the HPA" in AA was not supported by their own finding that AA did not further increase the ACTH levels seen in control ADX rats. Nevertheless, the HPA axis must be involved in the animals' response to the agents that provoke AA; we have found that ADX animals develop earlier and more severe disease (see also Stephanou et al., 1992).

Our study of the time course of corticosteroid levels revealed that rather than the HPA being "chronically activated" in AA (Stephanou et al., 1992), it is at first depressed and activated only late in the time course (Fig. 2). This disparity could, perhaps, be rationalized by our knowledge (Brodkin et al., 1999) that DA rats may be atypical in some respects, in particular, in lacking a circadian rhythm in plasma corticosterone concentrations (implying that the release of hypothalamic CRF and, hence, of ACTH is obtunded
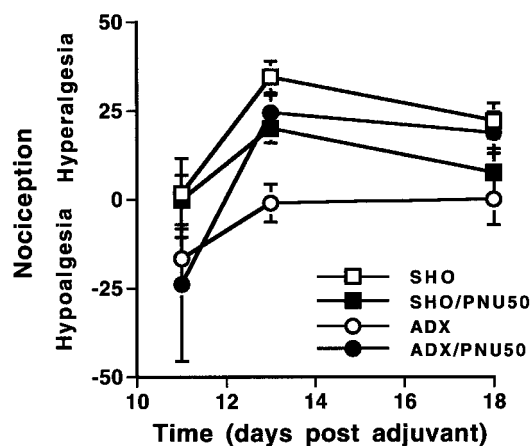

Fig. 4. Time courses of altered mechanical nociceptive thresholds (Nociception) in SHO and ADX adjuvant-arthritic animals with and without treatment with PNU-50. The measurements are of the changes in the smallest force necessary to produce a paw withdrawal response compared with the magnitudes of the stimuli required on day 0 (predisease); hyperalgesia (a reduction in this force) is depicted in the positive direction (increased nociception), whereas hypoalgesia (reduced nociception) is indicated in the negative direction (increased force necessary to elicit the test response).
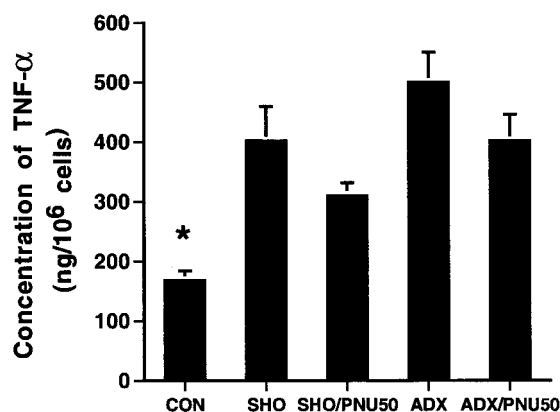
in DA animals). Even so, our result is consistent with the finding by van de Langerijt et al. (1994) of a rise from day 14 of the disease. By contrast, others have found oscillations in corticosterone concentrations in AA (Tanaka et al. 1996). It could be that we potentially ameliorated the disease by the administration of dexamethasone on the first 2 days of the experiment. This is unlikely 1) because all animals (SHO as well as ADX) received dexamethasone and 2) because the half-life of this drug is of the order of only 3 to 4 h (Kazzem and Schulte, 1981).

In view of the anti-inflammatory action of the corticosteroid hormones, our observation of depressed plasma levels during the latent period of AA would substantiate our finding of worse disease in ADX animals (levels are also low in RA patients) just as the elevated levels during PNU-50 treatment may be considered to be a contributor to the markedly reduced disease in those rats. Furthermore, in the ADX rats, we would expect augmented release of β-endorphin from the anterior pituitary (together with ACTH; Guillemin et al., 1977), and from that, one would postulate hypoalgesia in those animals. Our results give support to this view, in that the paws of the SHO rats are more hyperalgesic than those of the ADX animals (Fig. 4).

Considering the edema in more detail, it is striking that there is none in the PNU-50-treated SHO animals (i.e., at the dose used, this drug is powerfully anti-inflammatory). In contrast to its action in SHO animals, the PNU-50 has only a late antiedemal action in the ADX animals (Fig. 2). It may be concluded that although a significant component of the therapeutic action of PNU-50 is independent of the adrenal gland (cortex and medulla), some of its action does appear to require an intact HPA axis. As considered above, this is suggested by the fact that throughout the disease, PNU-50 increases the output of corticosterone in the SHO rats, a result that is consistent with the work of Kapas et al. (1995). Given the anti-inflammatory action of these steroids, it is likely that they contribute to the action of PNU-50 on paw swelling (Fig. 2).

Like edema, pain has long been recognized as a characteristic of inflammation. Quantitative correlation between them is not simple. We have previously found quite distinct temporal patterns of nociceptive sensitivity as AA develops, depending on the type of test that is used (Binder et al., 2000), and the present experiments reinforce that result. A comparison of Figs. 2 and 4 shows that in untreated animals, for example, edema has appeared by day 11 but nociception has not changed; furthermore, the patterns of edema and nociception are quite distinct. In the SHO animals, PNU-50 completely prevented the appearance of edema but did not entirely suppress hyperalgesia. Additionally, in the ADX rats, there was clear edema by day 11 (whether or not drug was administered), yet the animals were hypoalgesic at that time; thereafter, there were paradoxical relationships between these two variables. It is therefore possible that in those ADX animals, elevated levels of β-endorphin and possibly CRH (resulting from the lack of negative corticosteroid feedback onto the hypothalamus and anterior pituitary) consistently inhibit nociception (Guillemin et al., 1977; Schäfer et al., 1994). The finding (Ratka et al., 1988) that adrenalectomy sensitizes animals to β-endorphin would support that hypothesis; such a mechanism would be sensitized as AA develops by the actions of circulating cytokines on hypothalamic secretion of CRH and hence of β-endorphin from the hypophysis (Chikanza and Grossman, 1998). Careful examination of Fig. 2 reveals that the shape is consistent in all plots (i.e., whether the drug has been administered or not); clearly, tolerance cannot be an important issue in such circumstances. Thus, we do not have data to support the contention by Wei et al. (1973) that adrenalectomy potentiates opiate tolerance.

Finally, the in vitro studies of TNF-α release from macrophages offer an explanation for the greater severity of AA in ADX animals in which this important pathogenic peptide (Brennan et al., 1992) is produced in greater amounts (Fig. 5). From our results, we can reasonably presume that PNU-50 is therapeutic, at least in part through reduction of TNF-α production, although (unlike its effect on edema), the drug did not return this secretion to normal (predisease) levels (compare Figs. 5 and 2).

In summary, our current experiments support the hypotheses 1) that the development of AA and the adequacy of function of the HPA are interdependent and 2) that part, although certainly not all, of the therapeutic action of the opioid PNU-50 involves this HPA axis.

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