Adrenal Cortical Activation in Murine Colitis

DENIS FRANCHIMONT, GERD BOUMA, JEROME GALON, GERNOT W. WOLKERSDÖRFER, ANDREA HAIDAN, GEORGE P. CHROUSOS, and STEFAN R. BORNSTEIN

Pediatric and Reproductive Endocrinology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland

Background & Aims: Proper adrenal glucocorticoid secretion is crucial in the course of inflammatory diseases. However, the function and structure of the adrenal glands have not been examined in inflammatory bowel diseases. Methods: After induction of trinitrobenzene sulfonic acid (TNBS) colitis in SJL/J mice, plasma hormone and cytokine levels were measured, adrenal structure was analyzed by immunohistochemistry and electron microscopy, and adrenal cytokine/cytokine receptor expression were studied by RNase protection. Results: Adrenals of colitic animals were enlarged and hypervascularized. These animals had a marked increase in plasma corticosterone levels during the course of colitis (270 ± 34 vs. 16 ± 11 ng/mL; P < 0.0001) but only a modest elevation of their concurrent adrenocorticotropin levels (57 ± 13 vs. 29 ± 9 pmol/L; NS). On electron microscopy, adrenocortical cells showed ultrastructural signs of marked stimulation, and intra-adrenal lymphocytes were frequently found in direct contact with these cells. Concurrent plasma levels of interleukin (IL)-6, the major cytokine activating the hypothalamic-pituitary-adrenal axis, were markedly increased (495 ± 131 vs. 20 ± 1.5 pg/mL; P < 0.0001), and this cytokine directly stimulated corticosterone secretion by adrenocortical cells in vitro. Intra-adrenal expression of IL-6 in animals with colitis was increased 80-fold, and the IL-6 receptor subunits IL-6Rα and gp130 were present in the adrenal cells. Treatment of animals with neutralizing anti–IL-6 antibody reduced the TNBS-induced growth and activation of the adrenal cortices. Conclusions: Colitis is associated with a profound stimulation of adrenocortical cell function and glucocorticoid release. Direct immune-adrenal interactions seem to contribute to this activation of the adrenal glands during colitis.

The hypothalamic-pituitary-adrenal (HPA) axis plays an important role in the pathogenesis and course of inflammatory diseases. Cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 modulate the activity of the HPA axis, and the end effectors of this axis, cortisol and corticosterone, restrain the innate and T cell–specific immune responses. A deficient HPA axis has been associated with increased susceptibility to Th1-type inflammatory diseases. For instance, the inflammatory response of inflammatory disease–susceptible Lewis and –resistant Fischer rats to streptococcal cell wall (SCW)-induced arthritis is inversely related to the magnitude of their HPA axis response to inflammatory mediators.

The adrenal glands both influence and may be a direct target of the immune system. Adrenalectomy in rats with experimental allergic encephalomyelitis leads to a chronic active disease, and glucocorticoid replacement promotes recovery from the disease. Similarly, adrenalectomy produces more severe inflammation in experimental models of acute pancreatitis, and hydrocortisone replacement decreases both the severity and mortality of the disease. Furthermore, major human inflammatory and infectious diseases are associated with excess glucocorticoid secretion, and critically ill, septic patients have increased plasma cortisol levels with relatively normal adrenocorticotropic (ACTH) concentrations. Thus, the activation of the adrenal glands in response to inflammation is an important component of the host anti-inflammatory response.

These mechanisms may have relevance for Crohn’s disease (CD), one of the idiopathic chronic inflammatory bowel diseases (IBDs). Although the etiology is unknown, it is well accepted that in patients with CD, an uncontrolled immune reaction to an unknown antigen takes place in the gut in a genetically susceptible host. To address how colitis affects the morphology and function of the adrenal glands, we studied a murine model for this condition. Intrarectal application of trinitrobenzene sulfonic acid (TNBS) in ethanol leads to an IL-12–mediated inflammatory response in the colon and cecum.
of susceptible mice. The disease occurring in these animals resembles, to a certain extent, the histologic and immunologic abnormalities observed in human CD. After induction of TNBS colitis in SJL/J mice, levels of the plasma hormones ACTH and corticosterone as well as cytokine levels were measured. Adrenal morphology was analyzed by immunohistochemistry and electron microscopy, and adrenal cytokine/cytokine receptor messenger RNA (mRNA) expression was studied by RNase protection. To further elucidate the role of IL-6 in adrenal stimulation in the course of TNBS-induced colitis, we studied the effect of blocking antibodies to this cytokine. To ensure that the results were not caused by a generalized stress reaction, a mouse strain resistant to this type of colitis was used as an additional control.

**Materials and Methods**

**Animals and TNBS-Induced Colitis**

Colitis was studied in 5–6-week-old male SJL/J mice obtained from the National Cancer Institute (NCI, National Institutes of Health [NIH], Bethesda, MD). C57Bl/6 animals were obtained from the Jackson Laboratories (Bar Harbor, ME). Mice were maintained under specific pathogen–free conditions in the National Institute of Allergy and Infectious Diseases (NIAID) animal facility with a 12:12 hour light/dark cycle, and all experiments were performed at the same time in the afternoon. For induction of colitis, mice were first lightly anesthetized with metofane (methoxyflurane; Pitman-Moore, Mundelein, IL). To induce colitis, 3.15 mg TNBS (Sigma, St. Louis, MO) was mixed and dissolved with an equal amount of 100% ethanol. A total volume of 150 μL of the TNBS-ethanol mixture was slowly administered per rectum via a 3.5F catheter equipped with a 1-mL syringe. To ensure distribution of the TNBS within the entire colon and cecum, mice were held in a vertical position for 30 seconds after the injection. Control mice were administered placebo-vehicle phosphate-buffered saline (PBS) using the same technique. Three days after induction of colitis, mice were killed. The presence of colitis was established by the presence of clinical signs of colitis (weight loss and diarrhea), as well as macroscopic and microscopic signs of inflammation, which was used as an additional control.

**Histology and Electron Microscopy**

Adrenal glands were removed, dissected, and fixed for 3 hours in 4% formalin (immunohistochemistry) or 2% formaldehyde and 2% glutaraldehyde in 0.1 mol/L phosphate buffer at pH 7.3 (electron microscopy).

**Immunohistochemistry.** Paraffin-embedded sections of the adrenals were stained with anti-mouse CD45 monoclonal antibody or isotype-matched immunoglobulin (Ig) G control and subsequently with a peroxidase-labeled secondary antibody (Pharmingen, San Diego, CA).

**Electron microscopy.** Semithin sections (0.5 μm) were stained with toluidine blue. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate and examined at 80 kV in a Phillips EM 301. Adrenals of 3 different animals in each group were analyzed by electron microscopy.

**Cell Culture**

Y-1 immortalized mouse adrenocortical cells (ATCC, Rockville, MD) were maintained in RPMI 1640 with 10% steroid-free heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 2 mmol/L glutamine at 37°C in 5% CO₂ and stimulated with increasing concentration, from 0.1 to 10 ng/mL, of IL-6 (R&D Systems). The culture supernatant was removed 48 hours later and frozen at −20°C until assay.

**Immunoassays**

Plasma and culture supernatant corticosterone concentrations were measured by the same radioimmunoassay. Once all the plasma samples were collected, ACTH and corticosterone were measured by radioimmunoassay in a single batch (Immunotech, Marseille, France), and IL-6 was measured using a specific enzyme-linked immunoassay (R&D Systems) according to the manufacturer’s instructions.

**RNase Protection**

RNA extraction was performed (RNAgents; Promega, Madison, WI), and mRNA expression was evaluated by RNase protection as follows: 32P-labeled RNA probes were synthesized using SP6 RNA polymerase or T7 RNA polymerase for the multiprobe template set (Riboquant; Pharmingen). DNA was digested with DNase I (Boehringer Mannheim, Indianapolis, IN), and RNA probes were extracted with phenol/chloroform and precipitated with ethanol. Labeled RNA probes were hybridized with target RNA (5 μg) at 56°C overnight and digested with T1 RNase (Life Technology, Gaithersburg, MD). The protected mRNA fragment was extracted with phenol and chloroform, precipitated with ethanol, resolved on a 6% denaturing polyacrylamide gel, and subjected to autoradiography. Gene transcripts were identified by the length of the protected fragments. Equal loading of RNA was estimated from the amounts of protected fragments of 2 housekeeping genes, L32 and glyceraldehyde 3-phosphate dehydrogenase. Band densities were measured using the Computing Densitometer (Molecular Dynamics, Sunnyvale, CA).
Statistics

Statistical analyses were carried out using nonparametric (Mann–Whitney) or parametric (Student \( t \)) tests as appropriate. A \( P \) value of <0.05 was considered statistically significant.

Results

TNBS-Induced Colitis in Resistant and Susceptible Mice

Intrarectal administration of TNBS causes an IL-12–driven Th1 inflammatory reaction in the colon and cecum of susceptible SJL/J mice.\(^{15}\) Severe diarrhea and weight loss characterize the disease. The clinical and immunologic characteristics of this colitis model have been described previously.\(^{15}\) In contrast to the SJL/J mice, C57Bl/6 mice are highly resistant to TNBS-induced colitis.\(^{16}\) After an initial weight loss during the first 24 hours after TNBS administration, C57Bl/6 animals quickly recovered without showing any signs of disease or diarrhea. When mice were killed, the colons appeared normal or had only minimal signs of inflammation (Figure 1A). Therefore, C57Bl/6 mice were used as controls to exclude the potential effect of stress on the HPA axis associated with the experimental procedure. The TNBS-treated C57Bl/6 mice will be referred to as colitis-resistant and the placebo-treated C57Bl/6 as resistant control mice.

As expected, SJL/J mice had severe diarrhea and weight loss after TNBS administration. When mice were killed, the colons showed marked signs of inflammation (Figure 1B).

Increased Corticosterone Secretion in SJL/J Mice During Colitis

To assess the activation of the adrenals in mice with colitis, we analyzed their plasma corticosterone and ACTH levels. Corticosterone levels in SJL/J mice increased within 24 hours and remained highly elevated throughout the study period (270 ± 34 vs. 16 ± 11 ng/mL at day 3; \( P < 0.0001 \); Figure 2A). Compared with the more than 15-fold increase in plasma corticosterone levels, ACTH concentrations were only moderately increased in these animals (57 ± 13 vs. 29 ± 9 pmol/L; NS; Figure 2B). This resulted in an inversion of the corticosterone/ACTH ratio in mice with colitis compared with placebo-treated SJL/J mice (\( P < 0.05 \)). In the colitis-resistant C57Bl/6 mice, plasma corticosterone levels were only modestly increased (Figure 2A), as were ACTH levels (Figure 2B). Although plasma corticosterone levels increased somewhat after the enema (\( P < 0.01 \)), most likely because of the stress of the procedure and chemical irritation of the intestine, there was no major early increase in corticosterone or ACTH levels that could have prevented disease in the C57Bl/6 mice. Moreover, the increase in corticosterone levels in SJL/J
mice was significantly higher than in C57Bl/6 mice (P < 0.0005).

**Morphologic Change of Adrenals During Colitis**

In contrast to those of control animals, the adrenal glands from SJL/J mice with colitis showed dramatic signs of stimulation with hyperplasia of the adrenocortical cells and a marked hypervascularization of the cortex (Figure 3A and B). However, the medulla remained unchanged. No morphologic differences were detected between colitis-resistant mice and placebo-treated resistant mice (data not shown).

**Ultrastructural Changes of the Adrenocortical Cells**

At the ultrastructural level, adrenocortical steroidogenic cells of placebo-treated SJL/J mice showed normal...
smooth endoplasmic reticulum, liposomes, and the characteristic tubulovesicular round mitochondria. In contrast, adrenocortical steroidogenic cells of TNBS colitis SJL/J mice showed marked signs of activation. The cell volume was increased, the size and number of liposomes were greatly diminished, and there was an increased number and size of mitochondria and dilated smooth endoplasmic reticulum. In parallel with an increased steroidogenesis, the mitochondria formed dense vesicular internal membranes. Along with the hypervascularization, there was an increased cell/membrane surface in the form of filopodia (Figure 4A and B). In both placebo-treated mice and mice with colitis, single CD45-positive cells infiltrating the adrenal cortex were observed, but the same level of infiltration was present in normal and diseased mice (data not shown). However, lymphocytes characterized by scarce cytoplasm with few organelles were found in direct contact with adrenocortical cells only in mice with colitis; the adrenocortical cells extended filopodia to the cell membrane of the lymphocytes (Figure 5A and B).

**Increased Plasma and Adrenal IL-6 in Mice With Colitis**

We next examined which cytokines are expressed in the adrenals of normal and colitic mice. TNF-α, IL-1β, and IL-6 are inflammatory cytokines whose levels are increased in patients with CD and are expressed by human adrenocortical cells.7,17 Therefore, we first evaluated the expression of these proinflammatory cytokines. As shown in Figure 6A, IL-1β, TNF-α, and IL-6 were not expressed in the normal adrenal glands obtained from placebo-treated SJL/J and C57Bl/6 mice (lanes 1 and 3). Also, there was no TNF-α expression in the adrenal glands of animals after TNBS administration (lanes 2 and 4). However, significant expression of IL-1β and IL-6 was observed in the adrenal glands of SJL/J mice with colitis (lane 2), although no such expression was seen in colitis-resistant mice (lane 4). Laser densitometric analysis showed that IL-1β mRNA was increased 24-fold, and IL-6 mRNA was increased 80-fold in TNBS colitis compared with controls. There was no expression of IL-1 receptor antagonist, IL-10, IL-12 (p35 and p40), or interferon γ in any of the mice studied (data not shown).

Previous reports have proposed IL-6 as a critical regulator of the adrenal function in inflammatory diseases.18,19 This prompted us to examine the levels of plasma IL-6 in this model. As shown in Figure 6B, plasma IL-6 levels were greatly enhanced in mice with colitis compared with controls (lanes 1 and 2; 495 ± 131 vs. 20 ± 1.5 pg/mL; P < 0.0001). However, there were no increases in circulating IL-6 in the C57Bl/6 mice (lanes 3 and 4).

Because of the hyperplasia of the adrenal glands, the discrepancy between the increases of corticosterone and ACTH levels during colitis, and the adrenal expression of cytokine mRNA, we next examined the hypothesis that
proinflammatory cytokines directly stimulate the adrenals through their own receptor-mediated signaling. Therefore, we evaluated which cytokine-receptor mRNAs were present in the adrenals of normal SJL/J mice. The mRNAs of both the IL-6 receptor subunits, IL-6Rα and gp130, were expressed in the adrenals (Figure 6, lane 2). The mRNA of TNF-αR subunit p75 (but not the p55 subunit) was also present (Figure 6C, lane 2). However, the mRNA of IL-1 RI and RII were not detected (Figure 6C, lane 2). We also assessed the presence of other cytokine-receptor mRNA expression. No mRNA for IL-2Rα, β, and γ chains, IL-4Rα, IL-7Rα, IL-9Rα, IL-10R, IL-12Rβ1β2, or IL-13Rα was found (data not shown). Interestingly, the expression of the mRNAs of the IL-6 receptor subunits, IL-6Rα and gp130, was not affected by the disease in SJL/J mice (Figure 6D, lanes 1 and 2).

To determine a possible direct effect of IL-6 on the stimulation of corticosterone production, we examined the ability of various concentrations of IL-6 to promote the secretion of corticosterone by the mouse adrenocortical cell line Y-1. We used ACTH stimulation as a positive control. As shown in Figure 6E, ACTH (10^{-8} mol/L) stimulated corticosterone secretion (5.6 ± 0.9 vs. 1.24 ± 0.6 ng/mL; P < 0.05; lanes 1 and 2). Similarly, IL-6 stimulated corticosterone secretion dose-dependently at 0.1 ng/mL (3.2 ± 0.3 ng/mL; P < 0.05), 1 ng/mL (5.7 ± 0.3 ng/mL; P < 0.001), and 10 ng/mL (9.4 ± 0.9 ng/mL; P < 0.001; lanes 3–5). Control corticosterone secretion was 1.24 ± 0.6 ng/mL.

Effect of Anti-IL6 Treatment on Plasma Corticosterone Levels and Adrenal Morphology

To investigate the in vivo relevance of IL-6, SJL/J mice were injected with 1 mg of anti–IL-6 monoclonal antibody 6 hours before induction of colitis. A single injection of this antibody at this dose did not affect susceptibility to or severity of colitis. Clinical signs of disease, weight loss, macroscopic abnormalities, and histologic changes did not differ between mice treated with anti–IL-6 and those that received an isotype-matched control antibody. In the adrenals, however, treatment with this antibody led to partial normalization of hyperplasia and hypervascularization (Figure 7A and B). Mean plasma corticosterone decreased 22% (from 225 ± 17 to 176 ± 20 ng/mL; P < 0.05; n = 10 in each group) in anti–IL-6–treated mice compared with control mice that did not receive this antibody; this decrease was statistically significant (Figure 7C).

Discussion

CD is a chronic inflammatory disease of the digestive tract. It is also a multisystemic disorder in which the skin, joints, eyes, and/or lungs may be targets of the ongoing immune response. The HPA axis responsiveness to inflammation determines the susceptibility and course of many inflammatory diseases.1,2 Inflammatory mediators, such as the cytokines TNF-α, IL-1β, and IL-6, stimulate the HPA axis at the suprachiasmatic, hy-
pothalamic, and pituitary levels, enhancing ACTH and, hence, glucocorticoid secretion. The latter suppresses the innate and T cell–specific immune response. This study describes functional and morphologic transformations of the adrenals in an experimental model of CD and shows that these changes are modulated not only by ACTH but also by immune mediators such as IL-6.

The adaptation of the adrenal cortex to activation during colitis was reflected by an increase in adrenal size, hypervascularization, and augmentation of the intracellular apparatus necessary for steroidogenesis. Steroids are synthesized in the smooth endoplasmic reticulum and the mitochondria using cholesterol stored in liposomes. Both the number and size of mitochondria were increased along with thickening of their inner membranes and transformation from a tubulovesicular to a more vesicular membrane pattern. The smooth endoplasmic reticulum was also greatly increased and dilated, while large numbers of filopodia appeared on the surface of adrenocortical cells. The liposomes, on the other hand, were severely depleted by the colitis-induced adrenal activation. All of these changes correlate with the degree of adrenal stimulation.

Intra-adrenal lymphocytes, seen in both colitis and control animals, were mostly found in direct contact with adrenocortical cells in the adrenal glands of the former. We, as well as others, have previously reported massive adrenal lymphocyte infiltration in certain pathologic states, such as in adrenal tumors or 21-hydroxylase deficiency. We also recently demonstrated enhanced cortisol and dihydroergocryptine (DHEA) secretion by human adrenocortical cells after coculture with CD4+ and CD8+ lymphocytes. Although increased lymphocyte infiltration was not observed in the adrenal glands of mice with colitis, the contact elicited between adrenocortical cells and lymphocytes during colitis emphasizes the close interaction between endocrine and immune cells.

Proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, have been implicated in the pathogenesis of CD because these cytokines are elevated in active disease, both in serum and in the lamina propria of the inflamed gut. Among them, the main immune mediator of adrenocortical stimulation in inflammatory diseases appears to be IL-6, a cytokine with both proinflammatory and anti-inflammatory properties. In the model we studied, both plasma IL-6 and adrenal expression of IL-6 were increased in colitis. These increases were specific to colitis because they were not seen in colitis-resistant mice that also received TNBS. Moreover, the subunits of the IL-6 receptor IL-6Rα and gp130, were strongly and constitutionally expressed in the adrenal glands of all animals; however, the level of expression was not modulated by the disease. Finally, treatment of an-
imals with an anti–IL-6 monoclonal antibody resulted in a significant decrease in corticosterone levels and adrenal hyperplasia. Although a single injection of anti–IL-6 induced only a mild decrease in circulating glucocorticoid levels, a decrease in adrenal activation and circulating plasma corticosterone levels (>20%) during the severe stress of a life-threatening disease such as TNBS colitis is relevant. This finding is in accordance with previous studies showing that anti–IL-6 antibodies could diminish the lipopolysaccharide-induced activation of the HPA axis. This treatment regimen was not able to completely normalize corticosterone levels, suggesting that IL-6 is not the only immunologic factor affecting adrenal function. Other factors, such as cytokines or neuropeptides, may also have direct stimulatory effects on the adrenal cortices. IL-1RI and RII were not detected within the adrenal glands, making a role for IL-1 in this model unlikely. Indeed, adrenal IL-1RI expression has been reported only in neoplasia. Although the TNF-αRp75 receptor was also expressed in the adrenals, TNF-α would not be expected to participate in adrenal stimulation because it has been previously shown to inhibit adrenocortical steroidogenesis. We confirmed that IL-6 directly stimulated adrenocortical cells in vitro and increased corticosterone secretion in a dose-dependent fashion. Taken together, this study shows activation of adrenal cortical function during colitis.

In a recent study, a neutralizing antibody against the IL-6 receptor was able to suppress experimental colitis in various models of colitis. It was hypothesized that the IL-6–soluble IL-6R system mediates resistance to T-cell apoptosis as specific neutralization of the soluble IL-6 receptor induced apoptosis in T cells. In our study, looking at the acute phase of colitis, we could not demonstrate a significant effect of anti–IL-6 on severity of the colitis. This may be because the antibody we used was directed against IL-6, and not against the sIL-6R. Also, we did evaluate the acute phase of colitis rather than the chronic phase, in which T cells play a more profound role.

Several studies have reported a hyporeactive HPA axis in inflammatory diseases. In postoperative and critically ill patients, persistently elevated cortisol levels were accompanied by low or normal ACTH levels. So far, such studies have not been performed in CD. Our data indicate that in an inflammatory state, the HPA axis may be activated to a great extent by immune signals rather than by the suprathyroidial, hypothalamic, or pituitary control systems. This may indicate a mechanism in which the body attempts to down-modulate a potential life-threatening inflammatory reaction. This suggests a secondary physiologic response to inflammation rather than the intrinsic defect described by Sternberg et al. in the Lewis rats, in which a defect in the hypothalamic/pituitary/adrenal response is thought to be responsible for the high susceptibility to arthritis in this strain. In fact, these immune signals may gradually replace ACTH in the stimulation of the adrenal glands during the course of these diseases. This is further supported by our previous work, in which long-term treatment of cancer patients with daily human recombinant IL-6 injections resulted in enlarged adrenals with increased cortisol but normal ACTH secretion. Thus, there appears to be a time-dependent shift from neuroendocrine to immune signals regulating adrenal glucocorticoid secretion in inflammatory diseases.

References

13. Stober W, Ludviksson BR, Fuss UJ. The pathogenesis of mucosal


Received October 26, 1999. Accepted July 19, 2000.

Address requests for reprints to: Stefan R. Bornstein, M.D., Pediatric and Reproductive Endocrinology Branch, NICHD, National Institutes of Health, Building 10, Room 9042, 10 Center Drive, MSC 1583, Bethesda, Maryland 20892-1583. e-mail: Bornstes@mail.nih.gov; fax: (301) 402-0574.

Dr. Franchimont is supported by the Belgian National Foundation for Scientific Research (FRS). Dr. Bouma is supported by the “Dr. Saal van Zwanenberg Stichting” and the “Dr. Hendrik Muller’s Vaderlandsch Fonds.”

Drs. Franchimont and Bouma contributed equally to the manuscript.