Chronic Intermittent Psychosocial Stress (Social Defeat/Overcrowding) in Mice Increases the Severity of an Acute DSS-Induced Colitis and Impairs Regeneration

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Ulcerative colitis is a multifactorial disease, with immunological, genetic, and environmental factors playing an important role in its pathogenesis. Here we investigated the consequences of exposure to chronic psychosocial stress on the severity of a dextran sulfate sodium (DSS)-induced colitis in male C57BL/6 mice. Chronic stress was induced by repeated exposure to social defeat (SD, 2 h) and overcrowding (OC, 24 h) during 19 consecutive days. SD/OC mice showed a diminished body weight gain, thymus-atrophy, and adrenal hypertrophy, but similar light-phase plasma corticosterone concentrations, compared with unstressed mice. In contrast, the rise in dark-phase corticosterone concentration was significantly attenuated in SD/OC mice, whereas plasma ACTH concentrations and hypothalamic CRH mRNA expression did not differ between stressed and nonstressed groups. Additionally, adrenal cells from SD/OC mice showed a decreased in vitro response to ACTH stimulation. Subsequent treatment with 1% DSS for 7 d resulted in a more severe intestinal inflammation in SD/OC mice, as reflected by an increase in body weight loss, histological damage scores, and secretion of IL-6, TNFα, and interferon-γ from mesenteric lymph node cells and by decreased colon length. The impaired health status of stressed mice was also reflected by a significantly lower survival rate after termination of the DSS treatment. In conclusion, the present findings demonstrate that chronic intermittent exposure to a psychosocial stressor before the induction of acute DSS-colitis results in adrenal insufficiency, increases in the severity of the acute inflammation, and impairs the healing phase. (Endocrinology 147: 4968–4976, 2006)

Inflammatory bowel disease (IBD) can be classified as a chronic relapsing inflammatory condition of the small and large intestine that appears either as ulcerative colitis or Crohn’s disease (1, 2). The pathogenesis of IBD is still unknown, and it has a complex, multifactorial etiology comprising genetic (3) and environmental factors (4, 5), which are associated with dysregulation of the mucosal immune system. For example, IBD is predominantly associated with industrialized temperate regions and is rare in tropical countries with poor sanitation and a low level of overcrowding (6). Migration to developed countries leads to an increased risk of developing IBD (7, 8), which indicates that genetic factors are not solely responsible for the disease. Various environmental factors have been proposed to contribute to the enhanced risk of IBD in industrialized countries, including infection rate with nematodes (9–11) and the level of stress experienced (12–17). However, the role of stress in the pathogenesis of IBD remains controversial. Exposure to a variety of life stressors have been shown to exacerbate colitis (12–14). For example, Salem and Shubair (14) showed that Arab bedouins frequently developed a spontaneous colitis after they were forced to leave their familiar environment. However, in retrospective human literature, which must be interpreted with caution, there are studies showing contrary findings (15–17).

In animal studies, captivity stress and readjustment to a novel social environment cause spontaneous colitis in cotton-topped tamarins (Sanguinus oedipus) (18, 19). This is supported by other studies demonstrating that repeated exposure to various stressors over a relatively short time period (4–5 d), including restraint (20, 21), or a combination of cold and restraint stress (22), applied before or immediately after induction of an experimental colitis, exacerbated the colonic inflammation in rats. In addition, reactivation of a completely resolved acute colitis has been described after a combination of restraint and sonic stress and a subthreshold dose of dinitrobenzenesulfonic acid but not by a subthreshold dose of dinitrobenzenesulfonic acid alone (23). In agreement with the above, stress has been described to affect the pathogenesis of diseases in further immunological animal models (24–27).

However, the influence of a chronic psychosocial, and therefore clinically relevant, stressor on the development and severity of experimental colitis has not previously been examined. Therefore, in this study, we aimed to establish, and extensively characterize, an ethologically relevant model of chronic intermittent psychosocial stress in male mice and investigated whether exposure to this stress procedure before the induction of an acute dextran sulfate sodium (DSS)-induced colitis influences the severity, and subsequent regeneration process, of the colonic inflammation. In par-
ticular, we aimed to reveal stress-induced alterations of hypothalamo-pituitary-adrenal (HPA) axis functions, which could be at least partially responsible for mediating the effects of chronic stress on the chemically induced colitis.

Materials and Methods

Animals

Male C57BL/6 mice (Charles River, Sulzfeld, Germany) weighing 19–22 g (experimental mice) or 30–35 g (used as residents during the stress procedure) were individually housed in standard polycarbonate mouse cages (16 × 22 × 14 cm; experimental mice) or polycarbonate observation cages (38 × 22 × 35 cm; residents) for at least 1 wk before the start of the experiment. All mice were kept under standard laboratory conditions (12-h light, 12-h dark cycle, lights on at 0600 h, 22 ± 2°C, 60 ± 5% humidity) and had free access to tap water and standard mouse diet. All experimental protocols were approved by the Committee on Animal Health and Care of the local government and conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

Experimental procedure

Mice were exposed to 19 d of a social defeat (SD)/overcrowding (OC) stress paradigm (Fig. 1; control: n = 49; SD/OC: n = 48). Some of the stressed and unstressed mice (control: n = 5; SD/OC: n = 5) were killed on d 20, at the beginning of the light phase (0700 h), to study the effect of the chronic stress on body weight, thymus weight, adrenal weight, and basal plasma corticosterone concentrations during light phase. To investigate basal plasma corticosterone and ACTH concentrations at the beginning of the dark phase, i.e. under stimulated conditions, further animals (control: n = 8; SD/OC: n = 8) were killed on d 20 at the beginning of the dark phase (2000 h). Adrenals of these mice were also taken and examined for their ability to respond to an acute ACTH challenge in vitro. Additionally, brains were removed to analyze the effect of the SD/OC stress on hypothalamic CRH mRNA expression.

The effect of prior exposure to SD/OC stress on the severity of an acute 1% DSS-induced colitis (for 7 d starting on d 20; Fig. 1) was assessed in stressed (n = 9) and control mice (n = 10). Respective controls continued to drink tap water (SD/OC, n = 8; control, n = 8). On d 27, i.e. after 7 d of DSS treatment, all mice were killed for quantification of colon length and histological assessment of the colon (histological score, measured on a scale of 1–8), and plasma corticosterone and ACTH concentrations. Additionally, cytokine secretion by mesenteric lymph node cells of all DSS-treated mice was assessed as described before (28).

To determine whether prior chronic stress exposure influences the regeneration process after acute DSS-induced colitis, nonstressed (n = 18) and SD/OC-stressed (n = 18) animals were given 1% DSS for 7 d, and 4 d after the termination of DSS treatment, the survival rate was monitored (see Fig. 1).

SD/OC stress paradigm

Mice were randomly assigned to the control or the SD/OC group. Control mice were singly housed and remained undisturbed except change of bedding once a week. The SD/OC group was exposed to unpredictable SD and OC during the 19-d stress period (Fig. 1). SD consisted of repeatedly placing mice into a male resident’s home cage for 2 h either once or twice a day according to the protocol (see Fig. 1 for details). To avoid physical injuries, the two opponents were separated by a perforated partition wall immediately after the first attack of the resident, allowing visual, olfactory, and auditory contact. The intruder was confronted with different residents to avoid habituation to the resident. During OC, performed on d 3, 10, 13, 14, and 18 (Fig. 1), large groups (n = 16) of experimental mice were housed together in one observation cage (38 × 22 × 35 cm) for 24 h with free access to water and food.

Induction of acute colitis

The induction of an acute colitis was achieved by administering 1% DSS (36–50 kDa; ICN, Eschwege, Germany) in the drinking water ad libitum for 7 d, as previously described (28).

Blood sampling and RIA for corticosterone and ACTH

Immediately after the conclusion of the experimental phase, mice were rapidly killed by decapitation under CO2 anesthesia, and approximately 200 µl trunk blood were collected on ice in EDTA-coated tubes (Sarstedt, Nümbrecht, Germany) containing 10 µl aprotinin (Trasylol; Bayer Corp. AG, Leverkusen, Germany). The tubes were then centrifuged at 4°C (5000 rpm, 10 min) and finally stored at −20°C until assayed using a commercially available RIA for corticosterone and ACTH (ICN Biomedicals, Inc., Costa Mesa, CA) with a detection limit of 10 ng/ml and 4 pg/ml, respectively.

In situ hybridization of hypothalamic CRH mRNA

After decapitation, brains were removed, snap frozen in isopentane cooled on dry ice and stored at −80°C for subsequent in situ hybridization. A series, with six 16-µm cryocut hypothalamic sections per mouse, was thaw mounted onto slides in a cryostat at −20°C and afterward used to assess hypothalamic CRH mRNA. The hybridization protocol was adapted from Bosch et al. (29). Briefly, hybridization to CRH mRNA was performed by using a 48-base 35S-labeled oligonucleotide probe complementary to bases 64–111 (probe sequence: 5'-ggc cgg cgg ccc gca gca gca gac gcc cgg cgt cca gga gac gcc cgg cgc tcc agg gac gga gga tcc cct gct gca). Using the National Center for Biotechnology Information BLAST search the sequence was demonstrated to be specific for the mouse CRH transcript. Hybridized slices were exposed to BioMax MR film (Eastman Kodak, Rochester, NY) under safe red light conditions for 21 d. The region of the hypothalamic paraventricular nucleus of four out of six slices per slide was measured bilaterally for each subject to provide individual means. Expression of CRH mRNA was measured as OD on a Macintosh computer with a

FIG. 1. Schematic illustrating the experimental design of the SD/OC stress paradigm. Single-housed male C57BL/6 mice were exposed to SD (2 h) and OC (24 h) as indicated during a 19-d period. On d 20, mice were given either 1% DSS or tap water the next for 7 d before they were killed on d 27.

SD SD SD SD SD SD SD SD SD OC OC OC OC OC OC OC OC OC OC

time [d]

day 4: after DSS

DSS
Determination of colonic length and histological score

As described before (30, 31), the reduction of colonic length was used as a parameter to assess colonic inflammation. The colon was removed, mechanically cleaned, and measured to 0.1 cm precision. Afterward, 1 cm of the distal third of the colon was cut longitudinally, laid on a filter paper, and fixed in 10% formalin overnight. The next day, the fixed tissue was embedded in paraffin and cut longitudinally. Three 3-µm hematoxylin-eosin stained sections taken 100 µm apart were evaluated by histological scoring performed by an investigator blind to treatment. For statistics, each individual score represented the mean of the three sections. Histology was scored as follows based on reports published previously (32, 33): 1) epithelium (0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas); and infiltration (0, no infiltration; 1, infiltrate around crypt basis; 2, infiltrate reaching to lamina muscularis mucosae; 3, extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant edema; 4, infiltration of the lamina submucosa).

The total histological score represents the sum of the epithelium and infiltration score and ranges from 0 to 8.

Isolation and incubation of mesenteric lymph node cells

Mesenteric lymph nodes (pooled from each experimental group) were harvested under sterile conditions and collected on ice in cell culture medium [RPMI 1640 supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco-BRL, Eggenstein, Germany) and 3 × 10^−5 M β-mercaptoethanol (Sigma, Deisenhofen, Germany)]. Lymph nodes were mechanically disrupted and filtered through a cell strainer (70-µm nylon, Falcon; Becton Dickinson, Heidelberg, Germany). Afterward cells were washed three times in cell culture medium and adjusted to a concentration of 10^6 cells/ml. Then 2 × 10^7 (200 µl) lymph node cells were transferred to wells of a 96-well plate and stimulated by precoating wells with 200 µl of 2.5 µg/ml anti-CD3 antibody in the presence of IL-2 (final concentration 100 U/ml). Eight wells were transferred with the respective number of cells of each experimental group. After incubation for 24 h (37°C, 5% CO2), cytokine concentrations were measured in the supernatants by ELISA (all from Endogen, Woburn, MA) using four wells per experimental group.

ACTH stimulation of isolated adrenal cells in vitro

To reveal the consequences of chronic stress on the capability of adrenal cells to respond to an ACTH challenge in vitro, the stimulation protocol was adapted from Bazhan et al. (34). After decapitation, adrenal glands of four mice of the same experimental group (control vs. SD/OC) were pooled and stored in ice-cold Krebs-Ringer bicarbonate-glucose buffer [KRBB (pH 7.4), 0.5% BSA] until they were cleared, cut into small pieces, and incubated in 1.5 ml KRBB (4% BSA, 4% collagenase) for 60 min at 37°C in an atmosphere of 95% O2-5% CO2. Adrenal cells were then dispersed by gentle homogenization (repeated pipetting) and filtered through five layers of gauze bandage. The suspensions were then centrifuged (4°C, 10 min at 300 rpm; 10 min at 800 rpm) and washed three times in 2.5 ml cold KRBB (0.5% BSA). Finally, cells were counted (trypan-blue), and cell viability was assessed (trypan-blue exclusion method) and adjusted to a cell concentration of 4 × 10^5 cells/ml in cold KRBB (0.5% BSA). Two aliquots (200 µl) of each group were incubated for 2 h at 37°C in an atmosphere of 95% O2-5% CO2 in the presence or absence of different doses of ACTH (10^−13, 10^−12, 10^−11, and 10^−10 M). Afterward cells were sedimented by brief centrifugation (5 min at 2000 rpm) and supernatants were stored at −20°C until radioimmunological quantification of corticosterone (triplicates of each sample were analyzed).

Statistics

A Mann-Whitney U test was used to compare SD/OC and non-stressed mice (body weight gain during SD/OC, thymus and adrenal weights, plasma corticosterone and ACTH concentrations, hypothalamic CRH mRNA expression, number of adrenal cells isolated per animal, cytokine secretion). Two-way ANOVA (factor stress, factor ACTH dose) followed by Tukey honestly significant difference (HSD) post hoc testing were appropriate to compare the effect of chronic stress on in vitro stimulation of adrenal cells. For comparison of the effects of chronic stress on DSS-induced colitis, two-way ANOVA (factor stress, factor DSS) and post hoc Tukey HSD tests have been applied. The survival rates were analyzed by Fisher’s exact test. Data represent mean ± SEM. Significance was set at P < 0.05. All data were analyzed using the software package SPSS (version 12; SPSS Inc., Chicago, IL).

Results

Effects of exposure to SD/OC on body, thymus and adrenal weights, plasma corticosterone and ACTH concentrations, and hypothalamic CRH mRNA expression

SD/OC mice gained significantly less body weight during the 19-d chronic stress paradigm, compared with controls (Fig. 2A). In addition, SD/OC mice, killed 1 d after termination of the stress procedure, showed a significant decrease in thymus weight (Fig. 2B) and a significant increase in adrenal weight (Fig. 2C), compared with unstressed controls.

Basal plasma corticosterone concentrations did not differ...
between control and SD/OC mice during the light phase at 0700 h (Table 1). Consequently the light-phase corticosterone to adrenal weight ratio was significantly lower in SD/OC (Table 1) mice, compared with unstressed controls. In the dark phase at 2000 h, a 3-fold rise in plasma corticosterone concentrations was observed in unstressed controls, which was attenuated in SD/OC mice (1.5-fold; Table 1). In contrast, plasma ACTH concentrations, as well as hypothalamic CRH mRNA expression, did not differ between control and SD/OC mice during the dark phase at 2000 h (Table 1).

**Effects of exposure to SD/OC on adrenal corticosterone secretory responses in vitro**

The total number of isolated adrenal cells per animal of SD/OC (5.7 \(\times\) 10³ \(\pm\) 9.9 \(\times\) 10³), compared with control mice (6.8 \(\times\) 10³ \(\pm\) 6.9 \(\times\) 10³), was not statistically different (\(P = 0.667\)). No effect of chronic stress was found for baseline corticosterone secretion from adrenal cells in vitro (Fig. 3). However, exposure of adrenal cells to ACTH, which has a stimulating effect on the release of corticosterone, was dependent on prior SD/OC exposure (factor dose \(\times\) stress: F₄,₈₅ = 5.33; \(P < 0.01\); Fig. 3). Post hoc Tukey HSD analysis showed a significantly diminished corticosterone response of adrenal cells from SD/OC mice at all ACTH doses tested (Fig. 3).

**Effects of exposure to SD/OC on the severity of a DSS-induced colitis and on plasma corticosterone and ACTH concentrations after 7 d of DSS treatment**

Prior exposure to SD/OC significantly increased the severity of an acute DSS-induced colitis, as indicated by greater body weight loss, shorter colon length, higher histological damage score of the colon, and increased secretion of proinflammatory cytokines by draining mesenteric lymph node cells.

**Body weight** (Fig. 4A). The body weight gain of mice between experimental d 20 and 27 was found to be dependent on prior stressor exposure and DSS treatment (factor stress \(\times\) DSS: F₁,₃₁ = 24.03; \(P < 0.01\)). DSS treatment resulted in a decrease in body weight in both stressed and unstressed groups, which was found to be more pronounced in SD/OC mice, compared with unstressed controls. When performing a Mann-Whitney U comparison between the body weight gain of SD/OC and control mice that did not receive DSS, stressed mice gained significantly more body weight during the 7 d after SD/OC exposure (\(P = 0.003\)).

**Histological score** (Fig. 4B). The histological score of colon tissue was found to depend on prior stressor exposure and DSS treatment (factor stress \(\times\) DSS: F₁,₃₀ = 53.5; \(P < 0.01\)). The histological score of stressed or unstressed mice receiving no DSS indicated no colonic inflammation (Fig. 4D). In contrast, DSS treatment increased the histological score in both stressed and unstressed mice. Importantly, chronically stressed mice (Fig. 4F) receiving DSS showed a significantly higher histological score than respective nonstressed controls (Fig. 4E), reflecting more severe inflammatory infiltration and increased epithelial damage, i.e. focal disappearance of mucosal crypts.

**Colon length** (Fig. 4C). Statistical analysis revealed a main interaction of SD/OC exposure and DSS treatment (factor stress \(\times\) DSS: F₁,₃₁ = 23.32; \(P < 0.01\)) on the length of the colon. More specifically, colon length was significantly reduced by DSS application in both unstressed and stressed mice, compared with respective mice treated with tap water. However, the effect of DSS was more severe in SD/OC compared with unstressed mice.

**Secretion of proinflammatory cytokines from draining mesenteric lymph node cells** (Fig. 5). In DSS-treated mice, the secretion of interferon (IFN)-\(\gamma\) and IL-6 as well as TNF\(\alpha\) from mesenteric lymph node cells was found to be significantly increased in SD/OC compared with control mice.

**Plasma corticosterone and ACTH concentrations** (Table 2). Both plasma corticosterone and ACTH concentrations were dependent on prior stressor exposure and DSS treatment (corticosterone: factor stress \(\times\) DSS: F₁,₃₁ = 30.6; \(P < 0.001\); ACTH: factor stress \(\times\) DSS: F₁,₃₀ = 11.0; \(P = 0.002\)). Hormone concentrations of stressed or unstressed mice, which did not receive DSS, were not statistically different. However, DSS treatment significantly increased plasma corticosterone and ACTH concentrations only in chronically stressed mice.

**Effects of chronic exposure to SD/OC on the regeneration process after DSS-induced colitis**

The regeneration process during a 4-d recovery period after termination of DSS treatment was found to be impaired in mice exposed to SD/OC before DSS treatment. This was reflected by a significantly reduced survival rate in SD/OC compared with nonstressed control mice (Fig. 6).

### TABLE 1.

|                          | Controls                  | SD/OC                   |
|--------------------------|---------------------------|-------------------------|
| Light-phase corticosterone (ng/ml) | 24.2 ± 3.0 (n = 5)         | 24.9 ± 3.9 (n = 5)     |
| Dark-phase corticosterone (ng/ml) | 76.0 ± 15.4 (n = 7)        | 37.5 ± 6.7 (n = 5)     |
| lpC to A (1:1000 ml)      | 7.41 ± 0.81 (n = 5)        | 4.84 ± 0.76 (n = 5)    |
| Dark-phase ACTH (pg/ml)   | 41.9 ± 5.1 (n = 7)         | 52.4 ± 7.1 (n = 8)     |
| Hypothalamic CRH mRNA expression (gray density) | 22.2 ± 1.75 (n = 6)       | 21.9 ± 1.84 (n = 8)    |

Mice were exposed to the stress procedure for 19 d as described in the text or were kept individually (control). Data represent mean ± SEM. lpC to A, Light-phase corticosterone (nanograms per milliliter) to adrenal weight (milligrams) ratio.

\(a\) \(P < 0.05\) vs. controls.
controls.

trations of CSC mice were found to be unchanged during the stressor exposure, plasma light-phase corticosterone concen-

chosocial stressor. Apart from a rise within the first days of results parallel our findings from a comparable study using found to be significantly lower in stressed animals. These Therefore, the corticosterone to adrenal weight ratio was the corresponding unstressed mice during the light phase.

plasma corticosterone concentrations were not different from

mean

a reduced response to ACTH at all doses tested. Data represent corticosterone RIA in triplicates. Adrenals of four control and four SD/OC, respec-

vitro release of corticosterone from isolated adrenal cells after stim-

FIG. 3. Effects of exposure to the SD/OC stress paradigm on the in vitro release of corticosterone from isolated adrenal cells after stim-

ulation with ACTH. Adrenals of four control and four SD/OC, respec-

tively, mice were pooled (n = 8 pooled samples), and two aliquots (a 200 μl) of each group were stimulated with ACTH over a concentration range of 10^{-13} to 10^{-10} M. Supernatants were analyzed by a corticosterone RIA in triplicates. Adrenal cells of SD/OC mice showed a reduced response to ACTH at all doses tested. Data represent mean ± SEM. ***, P < 0.001; ****, P < 0.001 vs. respective untried stress controls.

Discussion

This study was designed to evaluate the impact of prior chronic intermittent psychosocial stress on the severity of a DSS-induced colonic inflammation and the subsequent regeneration period. We could demonstrate that: 1) SD/OC is a relevant chronic psychosocial stressor in male mice, 2) prior exposure to SD/OC increases the severity of the DSS-induced colitis, and 3) prior SD/OC impairs the regeneration of the colitis as indicated by a reduced survival rate.

The present data demonstrate that the SD/OC paradigm is a valid and relevant mouse model for chronic psychosocial stress. Various physiological markers are well-known indicators of chronic stress, including reduction in body weight gain, thymus atrophy, and adrenal hypertrophy (35–38). Indeed, the mice that underwent the 19-d stress period gained less body weight than individually housed control mice. Additionally, SD/OC-stressed mice showed severe thymus atrophy and adrenal hypertrophy as further indicators of chronic stress and activation of the HPA axis (38–41).

Despite the elevated adrenal weights of SD/OC mice, plasma corticosterone concentrations were not different from the corresponding unstressed mice during the light phase. Therefore, the corticosterone to adrenal weight ratio was found to be significantly lower in stressed animals. These results parallel our findings from a comparable study using chronic subordinate colony (CSC) housing as a chronic psychosocial stressor. Apart from a rise within the first days of stressor exposure, plasma light-phase corticosterone concentrations of CSC mice were found to be unchanged during the rest of the stress procedure (Reber, S., unpublished data). Importantly, and in agreement with the present SD/OC mice, these subordinate mice also showed thymus atrophy and adrenal hypertrophy within 24 h of the commencement of CSC procedure. In contrast, Zelena et al. (41) demonstrated that repeated restraint stress resulted in increased adrenal weights as well as higher levels of plasma corticosterone. This discrepancy may be explained by the different stress procedures used; as in their study, animals were stressed for a shorter time frame (7–8 consecutive days, compared with 19 consecutive days in the present study) and a shorter daily duration (1 h, restraint, compared with 2 h SD or 24 h OC) (41). It has also been described that basal plasma corticosterone levels are increased during the first weeks of chronic stress but return to baseline afterward despite continued stressor exposure (42–44). Therefore, the finding that basal corticosterone is similar in stressed and nonstressed mice might be due to adaptive processes of the HPA axis during a long-lasting stressor exposure, protecting the body from an immunosuppressive, and therefore deleterious chronic exposure to increased glucocorticoid concentrations (45, 46).

Another possible explanation for comparable plasma corticosterone levels during the light phase could be that the adrenal cells of SD/OC mice were simply insufficient to produce appropriate amounts of glucocorticoids. Indeed, this is supported by our finding that plasma corticosterone concentrations during the dark phase were significantly lower in SD/OC mice, compared with nonstressed controls. Thus, it is possible that their adrenal glands became insensitive and lost their synthetic and/or secretory capability to appropriately respond to the diurnal rhythm (47). In support, hypothalamic expression of CRH mRNA, as well as plasma ACTH concentrations, during the dark phase were found to be similar in control and SD/OC mice, indicative of unchanged reactivity of hypothalamic and adrenohypophysial corticotroph cells. Thus, the observed corticosterone deficiency reflects an adrenal rather than a hypothalamic/ad-

enohypophysial dysfunction at the end of the stress proce-

dure. Moreover, the finding of an attenuated corticosterone secretion of SD/OC mice adrenal cells in response to an acute ACTH challenge in vitro supports the hypothesis that chronic stress results in a loss of functional responsiveness of adrenal cells, which is necessary to mount an appropriate cortico-

sterone response.

Based on the effects of SD/OC on body weight, thymus and adrenal weights, and adrenal functions, the chronic stress paradigm characterized in this study is highly suitable for studying the effects of a chronic stressor on various aspects of the immune system. Furthermore, because this paradigm includes significant components of psychosocial stress, it is clinically relevant for studying psychosocial stress effects on the outcome, severity, and regeneration of IBD (8, 12). The establishment of such a paradigm is of potential relevance for studying numerous diseases, besides colitis, which are believed to be exacerbated by chronic stress, such as major depression (48, 49).

Although the effects of stress on the severity of an experimentally induced colitis is controversial (20, 50–52), there is evidence that a blunted responsiveness of the HPA axis as a result of chronic stressor exposure makes the animals more prone to a chemically induced inflammation (20, 50–52). However, our study is the first that describes the effects of
a chronic psychosocial stressor on the severity of the DSS-induced colitis. Thus, chronically stressed mice showed a significant increase in colonic inflammation with respect to all the major relevant physiological, histological, and immunological parameters investigated. For example, body weight loss was more pronounced and the histological score of the colonic tissue was higher in SD/OC mice, compared with nonstressed, DSS-treated mice. Moreover, the secretion of proinflammatory cytokines like IFNγ, IL-6, and TNFα from stimulated mesenteric lymph node cells was found to be increased in stressed mice after DSS treatment, indicating an increased activation of the intestinal immune system.

Concerning the increased body weight gain observed in SD/OC vehicle-treated mice, compared with unstressed mice, after the completion of the 19-d stress protocol, it is likely that stressed animals compensated for their reduced body weight gain during the 19 d of stressor exposure in the subsequent recovery period.

Adrenal insufficiency and consequent attenuation of corticosterone secretion in response to an inflammatory stimulus (DSS), as mentioned above, may underlie the increased severity of the DSS-induced colitis. Interestingly, in chronically stressed CSC mice, plasma corticosterone concentrations were not even elevated after 4 d of DSS treatment, compared with control mice, despite the immunological parameters indicating signs of colonic inflammation (Reber, S., unpublished data). However, after 7 d of DSS treatment and termination of the stress procedure, SD/OC DSS mice showed increased plasma corticosterone and ACTH concentrations, but this delayed rise in immunosuppressive glucocorticoids did not efficiently counteract the overcompensatory production of proinflammatory cytokines in SD/OC mice. It is im-

FIG. 4. Effects of exposure to the SD/OC stress paradigm on the severity of an acute DSS-induced colitis. Stress exposure before DSS treatment exaggerated the severity of the induced acute colitis, indicated by an enhanced body weight loss (A), a higher histological score of the colon (B) and a more significant reduction of colonic length (C). Induction of an acute colitis was achieved by administration of 1% DSS in drinking water for 7 d. Numbers in parentheses indicate group sizes. Data represent mean ± SEM; *** P < 0.001 vs. respective unstressed controls; ### P < 0.001 vs. respective group without DSS; P < 0.05 vs. respective group without DSS. Furthermore, three representative colonic hematoxylin-eosin sections [a, lamina mucosa; b, lamina muscularis mucosae; c, lamina submucosae; d, lamina muscularis (circular muscle); e, lamina muscularis (longitudinal muscle)] from mice receiving tap water without DSS (D; normal colon histology), nonstressed DSS controls (E; goblet cell loss and crypt loss in locally restricted areas; infiltration reaching the lamina muscularis mucosae), and SD/OC DSS mice (F; crypt loss in large areas; thickening of the mucosa with abundant edema; infiltration reaching the lamina submucosa) are shown.
Fig. 5. Effects of exposure to the SD/OC stress paradigm before DSS treatment on cytokine secretion by mesenteric lymph node cells. Both stressed and unstressed mice were given 1% DSS in drinking water for 7 d. Stress exposure resulted in increased IFNγ, IL-6, and TNFα secretion, compared with single-housed control mice. Data represent mean ± SEM; *, P < 0.05 vs. respective unstressed, DSS-treated controls.

TABLE 2. Effects of chronic exposure to SD/OC prior to DSS treatment on plasma light-phase corticosterone and ACTH concentrations in male mice after 7 d of DSS treatment

|                     | Controls, no DSS | SD/OC, no DSS | Controls, DSS | SD/OC, DSS |
|---------------------|------------------|---------------|---------------|------------|
| Corticosterone (ng/ml) | 32.4 ± 5.6 (n = 8) | 27.9 ± 2.7 (n = 8) | 21.6 ± 12.0 (n = 10) | 414.8 ± 47.6 (n = 9)<sup>a,b</sup> |
| ACTH (pg/ml)         | 41.4 ± 2.1 (n = 8) | 37.4 ± 3.7 (n = 8) | 65.3 ± 12.4 (n = 10) | 187.4 ± 37.0 (n = 9)<sup>a,b</sup> |

Mice were exposed to the stress procedure for 19 d as described in Fig. 1 or were housed individually (control). Following the termination of the stress procedure a subpopulation of mice from each group (chosen randomly) were treated with 1% DSS in drinking water or tap water for 7 consecutive days. Data represent mean ± SEM.

<sup>a</sup> P < 0.001 vs. respective unstressed controls.
<sup>b</sup> P < 0.001 vs. respective group without DSS.

Important to mention that the rise in plasma corticosterone was absent in nonstressed DSS-treated animals because probably the degree of inflammation was too low to cause HPA axis activation.

The finding of increased severity of a DSS-induced colitis by exposure to chronic psychosocial stress over 3 wk is in agreement with other studies. Thus, Salem and Shubair (14) and Drossman (18) demonstrated that chronic stress increases the outcome of a spontaneous colitis in human and nonhuman primates, respectively. In our study, estimation of the survival rate 4 d after termination of DSS treatment revealed the same trend. At this time point, 72% of the unstressed mice survived the treatment, whereas only 20% of stressed mice survived. Therefore, chronic exposure to stress before DSS treatment is likely to increase the severity of an acute inflammation and consequently to impair regeneration. Detailed mechanisms underlying these differences, with respect to the activation of the HPA axis and the sympathetic nervous system among others, need to be elucidated in future studies. However, we hypothesize that in chronically stressed mice, maladaptations of the HPA axis, including a deficiency of the adrenal gland secretory cells, occur.

This is substantiated by the finding of a reduced corticosterone: adrenal weight ratio, lower plasma corticosterone concentrations in the dark phase, i.e., during the diurnal rise in corticosterone secretion in mice, and diminished reactivity to ACTH stimulation in vitro. Additionally, unchanged plasma ACTH concentrations and hypothalamic CRH mRNA expression provide evidence that this phenomenon reflects an adrenal instead of a hypothalamic dysregulation. Consequently, adrenal cortical cells are, at least initially, incapable of producing and secreting glucocorticoids in appropriate concentrations necessary for controlling the DSS-induced inflammatory reaction at the level of the colon. In support, throughout an inflammatory episode in humans, a rise in plasma cortisol, epinephrine, and norepinephrine was observed (53, 54), and these endocrine mechanisms were shown to prevent an overreaction of the immune system via distinct feedback mechanisms (55). Consequently, the delayed increase in glucocorticoid concentrations found in SD/OC mice after 7 d of DSS treatment is insufficient to counteract the development of the inflammation.

Future experiments are required to reveal the involvement of the sympathetic nervous system in the pathogenesis of an acute colitis after chronic stress. An important role of the sympathetic nervous system, for inducing as well as controlling an acute inflammation, was recently reported in a model of arthritis in mice (56). In further support, after chronic CSC exposure, increased plasma norepinephrine concentrations were found (Reber, S., unpublished data), which may, at least in part, explain the increased severity of colitis found in chronically stressed mice (56, 57). The uncoupling of the HPA axis and sympathetic nervous system may further help to explain the proinflammatory situation after chronic stress because the synergism of steroid hormones and neurotransmitters of the sympathetic nervous system will be attenuated (58).

In summary, our data provide evidence that an acute in-
testinal inflammation induced by DSS is more severe in chronically stressed mice as indicated by physiological, histological, and immunological parameters as well as the subsequent survival rate of the animals. The HPA axis reactivity, at the level of the adrenal glands, was found to be insufficient in the chronically stressed mice. This implicates that a reduced and delayed glucocorticoid response is responsible for the greater severity in stressed mice because this response is essential for down-regulating the inflammatory activation.

These results suggest that impaired glucocorticoid activity after sustained exposure to stressors in humans may be a contributory factor for the development of IBD.

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