Cytokines mediate protective stimulation of glucocorticoid output during autoimmunity: involvement of IL-1

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EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE) has been widely used as a model of human multiple sclerosis (MS). The disease can be induced in different species of laboratory animals by injection of central nervous tissue antigens emulsified in adjuvants. In Lewis rats, a susceptible strain, EAE is manifested by a paralytic attack that affects the tail and hind limbs 11–14 days after injection of guinea pig myelin basic protein (MBP) as encephalitogenic antigen. T helper 1 lymphocytes mediate the autoimmune component of the disease, and cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are implicated in the pathogenesis of EAE (for review see Ref. 22). During the experimentally induced disease in Lewis rats, the endogenous levels of glucocorticoids are elevated (13, 14). Recovery from the disease, which occurs spontaneously 4–5 days after the onset of the paralytic signs, is clearly dependent on this endocrine change because adrenalectomy results in death of the animals (14). Remission can be obtained in adrenalectomized animals only when glucocorticoids are administered in doses that result in an elevation of the plasma level of this hormone comparable to that observed in intact rats with EAE (14).

Despite the crucial role played by the increased glucocorticoid output in the recovery from EAE, the mechanism that triggers this endocrine response is not known. It is reasonable to assume that the hypothalamic-pituitary-adrenal (HPA) axis is stimulated by the stress caused by the paralytic attack that characterizes this disease (13, 18). Alternatively, such an increase in blood glucocorticoid levels could be, at least in part, immunologically mediated. This is hypothesized on the basis of previous work showing that the immune response to innocuous antigens results in stimulation of the HPA axis (7, 26, 27). Furthermore, products derived from activated immune cells, including IL-1β, IL-6, and TNF-α, can increase glucocorticoid output when they are administered to normal animals (Refs. 3–5; for review see Ref. 2). On this basis, we have investigated the possibility that an immunoregulatory circuit, involving immune-derived products and glucocorticoid hormones, operates during the course of EAE. For this purpose, we have first studied whether injection of MBP also results in an increased glucocorticoid output in PVG rats, which develop an immune response to this encephalitogenic antigen but are resistant to the induction of EAE (1). We have also studied whether in vitro stimulation of immune cells with MBP results in the production of factor(s) capable of inducing an increase in glucocorticoid levels on injection into normal animals. Finally, because IL-1β is probably the most potent cytokine that can stimulate the HPA axis (3), we have explored whether endogenous IL-1 contributes to the increase in glucocorticoid levels observed during EAE.

MATERIAL AND METHODS

Animals. Lewis male rats (6–8 wk old, 200 g body wt) were purchased from Ifa Credo and PVG male rats (6–8 wk old, 150 g body wt) from Harlan Olac. Rats were caged individually for 7 days before starting the experiments and kept isolated throughout. Animals were housed in temperature- and light (12:12 h light-dark cycles)-controlled rooms and were fed ad libitum.

Inoculation of MBP, clinical signs, and blood sampling. For minimization of the stress of bleeding, a silicone cannula was chronically implanted into one of the jugular veins of Lewis and PVG rats according to standard procedures (3). Five to seven days after the operation, 100 µl of an emulsion contain-
ing 25 µg guinea pig MBP in complete Freund's adjuvant (CFA)-incomplete Freund's adjuvant (IFA) containing 4 mg/ml killed Mycobacterium tuberculosis, H37 RA (Difco) were inoculated subcutaneously in each hind foot pad. Groups of rats receiving CFA, IFA, or physiological saline (0.9% NaCl) were included, simultaneously, as controls to exclude any effect of the CFA alone. All animals were weighed and scored daily for clinical signs of disease, based on a conventional scale from 0 to 5 depending on severity: 0, normal; 1, limp tail; 2, hind limb paresis; 3, unilateral hind limb paralysis; 4, bilateral hind limb paralysis; 5, bilateral hind limb paralysis and incontinence. Blood samples were withdrawn via the cannula between 9:30 and 10:30 AM 1 day before inoculation and once a day at the same time from day 6 to day 20 postinoculation. Blood samples were collected in chilled EDTA-coated tubes, immediately centrifuged, and aliquoted for hormone determinations.

Spleen cell supernatants. Spleen cells were obtained from Lewis rats that received MBP in CFA 11 days earlier. Then, 1 × 10^7 cells/ml were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 5 × 10^-3 M 2-ME, 1% penicillin-streptomycin, 1% glutamine, and 1% HEPES, with or without addition of 10 µg/ml MBP. Supernatants were collected after 22 h of incubation at 37°C. The culture medium containing 10 µg/ml MBP and incubated for the same time was used as control. Between 9:30 and 10:30 AM, a basal blood sample was obtained from chronically cannulated normal Lewis rats. Immediately after this, 1 ml of each supernatant or of the control medium was injected via the implanted cannula. Additional blood samples were obtained at the time points indicated in the figures and processed as previously described.

Administration of IL-1ra. EAE was induced in chronically cannulated Lewis rats as previously described, and animals were scored daily for clinical signs of disease. Other groups of cannulated rats received only PBS inoculated subcutaneously in each hind foot pad. One day before inoculation and every day from day 5 postinoculation, blood samples were withdrawn via the cannula between 9:30 and 10:30 AM. Immediately after the sample corresponding to day 13 postinoculation was obtained, IL-1 receptor antagonist (IL-1ra; 100 mg/kg body wt; kindly provided by Dr. J. Relton, Synergen, Boulder, Colorado) was administered subcutaneously as a single bolus injection to a group of rats that were inoculated with MBP. The other group of MBP-injected rats and the PBS-injected animals received PBS also injected subcutaneously. Additional blood samples were collected on this day after 2, 4, and 6 h. Thereafter, a blood sample was obtained between 9:30 and 10:30 AM every 24 h until day 20.

Corticosterone determinations. Plasma corticosterone levels were determined by RIA as described previously (3).

Statistical analysis. Results are expressed as means ± SE. Data were analyzed using one-way ANOVA followed by Fisher's test for multiple comparisons. Differences were considered significant at P < 0.05.

RESULTS

Clinical signs and blood corticosterone levels in Lewis rats during EAE. The levels of corticosterone in blood of rats from the three control groups (CFA, IFA, and saline) remained within the range of basal values from day 6 to day 20 postinoculation (Fig. 1A). The only exception was observed on days 17 and 18 postinoculation, when blood glucocorticoid levels of rats that received CFA tended to increase. In contrast, the levels of corticosterone in the group of rats that received MBP started to increase on day 11, peaked on day 13, and remained elevated until day 19 postinoculation. By day 20 postinoculation, the levels of corticosterone in blood of MBP-injected rats returned to the normal range. Only those animals that received MBP lost weight, and this started on day 12 after immunization (data not shown). Mild signs of disease (limp tail) became apparent in some animals on this day (Fig. 1B). The disease was maximally expressed in all MBP-injected rats on days 15–16, and thereafter the clinical signs decreased progressively. It is noteworthy that the levels of glucocorticoids remained elevated until day 19 postinoculation, when the clinical signs had already disappeared in most of the animals.

Changes in blood glucocorticoid levels in PVG rats after immunization with MBP. As in the case of Lewis rats, different groups of chronically cannulated PVG rats received either MBP emulsified in CFA, CFA alone, IFA, or physiological saline. Animals of the four experimental groups gained weight during the 20 days after immunization (data not shown). None of the MBP-injected rats developed symptoms of EAE. However, as in the case of Lewis rats, changes in corticosterone levels were noted in the group of rats that received MBP.
Fig. 2. Blood corticosterone levels after MBP administration to PVG rats. PVG rats received either MBP in CFA (n = 10), CFA (n = 8), IFA (n = 7), or saline (n = 6). Same procedures and statistical analysis as described in Fig. 1 were used. The following statistically significant differences (P < 0.05) were detected: CFA vs. IFA and vs. saline on day 7; MBP vs. IFA and vs. saline on day 8; MBP vs. all other groups on days 9, 11, 12, 14, and 15. None of the MBP-injected PVG rats showed any clinical sign of experimental autoimmune encephalomyelitis (EAE). Each point in curves represents mean ± SE.

Blood levels were noticed in these animals (Fig. 2). With the exception of a transient increase on day 7 in blood corticosterone levels of CFA-injected rats, only the MBP-injected animals showed a sustained elevation in the levels of the hormone. The increase in blood corticosterone levels in this group of animals became evident on day 9 after immunization with MBP and fluctuated at high levels until day 17 postinoculation. Thereafter, corticosterone concentrations in blood returned to basal levels.

Products of MBP-stimulated immune cells, obtained from rats with EAE, increase blood corticosterone levels. Supernatants of spleen cells from MBP-primed rats further stimulated in vitro with the antigen, induced a clear increase in blood corticosterone levels 8 h after injection into naive Lewis rats (Fig. 3). No comparable changes were observed in animals that received supernatants from non-restimulated spleen cells or the culture medium incubated with MBP.

Blockade of IL-1 receptors interferes with increase in corticosterone levels during EAE. A single injection of the specific IL-1ra was injected into Lewis rats 13 days after inoculation of MBP, when the disease was already manifested and glucocorticoid levels were clearly elevated. This treatment did not significantly affect the clinical signs of EAE (data not shown). Two hours after administration of IL-1ra, a 60% decrease in corticosterone levels was observed compared with those of rats with EAE that received PBS (Fig. 4). The levels attained at 4 h were comparable to those of normal rats. When corticosterone blood levels increased in normal rats, in accord with the circadian variation (see controls Fig. 4), the difference between control rats and those rats given MBP, with or without IL-1ra treatment, was no longer apparent. Surprisingly, given the short half-life of IL-1ra (17), decreased corticosterone levels in blood of rats with EAE that received IL-1ra were still observed in the morning of the next day (24 h after IL-1ra injection), and were even more markedly de-

Fig. 3. Products released by MBP-stimulated immune cells from rats with EAE increase blood corticosterone levels. Lewis rats received either supernatants of spleen cells obtained from Lewis rats with EAE and restimulated in vitro with MBP (MBP + MBP), or from nonrestimulated cells (MBP + med) or culture medium containing MBP (med + MBP). Blood corticosterone levels were determined at times indicated in figure. Each point in curves represents mean ± SE of corticosterone values from 5 animals per group. Statistical analysis was performed as described in Fig. 1. *P < 0.05 vs. MBP + med and vs. med + MBP.

Increased on the following day (15 days after injection of MBP, 48 h after injection of IL-1ra, Fig. 5). This was followed by an increase in glucocorticoid levels on day 16, which did not significantly differ from those of MBP-injected rats that received PBS.

Fig. 4. Blockade of interleukin-1 (IL-1) receptors interferes with increase in corticosterone levels during EAE. Lewis rats received MBP or PBS. Blood samples were obtained 1 day before inoculation and once daily from day 5 to day 20 postinoculation. Levels of corticosterone determined on day 13 postinoculation are shown, when all MBP-injected rats already showed clinical signs of EAE. Immediately after obtaining blood sample corresponding to this day (time 0), we administered IL-1 receptor antagonist (IL-1ra) to a group of MBP-injected rats (MBP + IL-1ra, n = 8). Another group of MBP-injected rats (n = 12) and PBS-injected animals (n = 8) received the vehicle alone (MBP + PBS and PBS + PBS, respectively). Additional blood samples were collected on this day after 2, 4, and 6 h. Each bar represents mean ± SE of corticosterone values. Statistical analysis was performed as described in Fig. 1. *P < 0.05 vs. MBP + PBS.
PBS injection, the following statistical significant differences ($P < 0.05$) in corticosterone levels were detected: MBP vs. MBP after injection of IL-1ra into rats with EAE was observed. *$P < 0.05$ vs. MBP + IL-1ra and vs. PBS + PBS.

**DISCUSSION**

The studies reported here tested the possibility that an immune regulatory pathway, involving endogenous cytokines and glucocorticoids, operates during EAE in Lewis rats. We first demonstrate that the elevation of glucocorticoid levels observed in this strain in parallel to the clinical signs was induced by the encephalitogenic antigen and not by the adjuvant. MBP inoculation also triggered an increase in corticosterone levels in PVG rats, which respond immunologically to this antigen but do not develop the disease. These results indicate that the endocrine change observed during EAE can be induced by the immune response to MBP and it is not necessarily a consequence of the stress of the paralytic attack that characterizes the disease.

After in vitro restimulation, spleen cells obtained from MBP-primed Lewis rats released products with glucocorticoid-stimulatory activity. These results support the possibility that cytokines mediate the activation of the HPA axis during EAE. We and others have previously used mitogens to prepare cytokine-contain-

ing supernatants that increase glucocorticoid output when administered to naive recipients (4, 6, 8). These supernatants, as well as purified or recombinant cytokines, induce an increase in glucocorticoid levels within minutes after injection (3–6, 8). In the case of the MBP supernatants, this effect was observed 8 h after administration, which is a delayed response compared with that obtained with supernatants from mitogen-stimulated spleen cells. One main explanation for this is that the supernatants derived from an antigen-specific immune response would be expected to contain a mixture of cytokines that is either qualitatively or quantitatively different from that obtained after polyclonal stimulation of mononuclear cells by mitogens. It seems that the MBP supernatant increased blood glucocorticoid levels indirectly, via secondarily produced endogenous factors. This is supported by the finding that a similar delay in the increase in glucocorticoid levels is observed after administration of conditioned medium from a T cell lymphoma (21). This response requires the presence of T cells in the host, indicating that the material injected triggers the production of the products that finally cause the endocrine effect.

Finally, we show here that endogenous IL-1 contributed to the increased glucocorticoid output during EAE. Administration of IL-1ra close to the peak of EAE caused a reduction in corticosterone levels within 2 h. This effect was most likely due to an acute blockade of the action of IL-1 on the HPA axis. However, the levels of corticosterone were still reduced 24 h later, and the decrease was even more pronounced after 48 h. It is known that IL-1 can stimulate its own production (9, 16) and that of other cytokines produced during EAE (for review see Ref. 22), such as TNF-α and IL-6 (9), which can also increase glucocorticoid levels (3). Thus the prolonged reduction in the levels of corticosterone caused by the receptor antagonist could be the result of interfering transiently with a cytokine cascade that stimulates glucocorticoid output. Administration of a single injection of IL-1ra did not significantly affect the clinical signs of EAE. This is expected because it is known that IL-1ra needs to be injected repeatedly over several days to moderate or prevent EAE (17).

The data reported here, together with previous publications, allow some speculation on how immune-HPA axis interactions may contribute in determining susceptibility to EAE. In Lewis rats that are genetically predisposed to the induction of different autoimmune diseases, the immune-mediated stimulation of the HPA axis results in moderation of EAE. Such stimulation is, however, not sufficient to prevent the development of the disease in this strain. Although the concentration of corticosterone in the blood of rats with EAE was increased in the morning, afternoon levels did not differ from those of the simultaneously injected control rats (Fig. 4). These results suggest that the circadian pattern of plasma corticosterone is abolished in MBP-immunized Lewis rats and that corticosterone levels are stabilized at higher levels of the circadian rhythm. It is also conceivable that the endocrine response that...
parallels the disease in Lewis rats is less intense than what could be expected, especially because several proinflammatory cytokines produced in the brain during EAE (for review see Ref. 2) can stimulate the HPA axis at central levels (for review see Ref. 22). This is further supported by the observations that Lewis rats exhibit a decreased activation of the HPA axis in response to bacterial wall products (28), and hypothalamic corticotrophin-releasing hormone-producing neurons in this strain show a blunted response to the stimulatory action of IL-1 (29). Defects in the response of the HPA axis to immune-derived products have also been reported in animal models of spontaneous autoimmune thyroiditis (26) and lupus (11). In PVG rats, endogenous glucocorticoids seem to be effective enough to impede the occurrence of the disease, because animals of this strain develop EAE when they are adrenalectomized (19). The increase in corticosterone output triggered by the immune response to MBP is a mechanism that may at least partly underlie the resistance of PVG rats to EAE induction. Ultradian variations of plasma corticosterone may also play a protective role. In fact, we have observed that although at the nadir of the circadian cycle plasma corticosterone levels are comparable in normal Lewis and PVG rats, there are major differences in the amplitude of the circadian pattern, being several fold higher in PVG than in Lewis rats (del Rey, Klusman, and Besedovsky, unpublished data). Other authors have reported significant differences in the circadian rhythm of plasma corticosterone between Lewis and Fisher rats, a strain that is resistant to EAE induction (10).

As already mentioned, EAE is considered a model of MS. Some indirect evidence indicates that in humans interactions between immune-derived cytokines and endogenous glucocorticoids may also operate during the course of MS. After allogeneic and mitogen stimulation, human peripheral blood mononuclear cells can produce factors capable of stimulating the HPA axis (4, 6). Also the administration to human volunteers of a low dose of endotoxin, which induces the production of IL-6, and TNF-α, results in activation of the HPA axis (20, 25). More specifically it has been shown that peripheral blood monocytes from MS patients in the acute phase produce more IL-1α, IL-6, and TNF than those from healthy subjects (12) and that there is an excessive production of IL-1β when monocytes from patients with chronic progressive MS are stimulated with T cell-derived lymphokines (15). Taken together, these data indicate that cytokines that can stimulate the HPA axis are produced during the acute phase of MS. On the other hand, it has been shown that the activity of the HPA axis is enhanced in a considerably large proportion of MS patients (23). Also, an increase in the size of the adrenal gland has been found at autopsy, indicating that these glands are being hyper-stimulated in the course of MS (24). Although fragmentary, the evidence suggests that interactions between endogenous glucocorticoids and cytokines also exist during the course of MS.

In conclusion, the results shown here, together with the reported protective effect of endogenous glucocorticoids during EAE (13, 14), illustrate the relevant immunoregulatory role of a feedback mechanism integrated by immune cell products and the HPA axis. These data provide the first example of a specific immune response that triggers an autoimmune process and simultaneously activates an endocrine mechanism that either prevents or moderates this process. In the particular case of EAE, our results indicate the existence of a subtle balance between opposing effects of IL-1 that on one hand contributes to the inflammatory component of the disease (for review see Ref. 22), while on the other induces the release of glucocorticoids, which are powerful anti-inflammatory hormones (for review see Ref. 2).

Perspectives

The knowledge that endogenous cytokines induce the stimulation of corticosterone output during EAE requires a more detailed definition of the cytokine cascade involved in this process. This type of study needs to be extended to other models of autoimmune diseases. The data presented here also provide the rationale to explore the cytokine-HPA axis circuit in patients with MS and its potential relevance for the course of the disease in humans. Furthermore, these studies emphasize the need of simultaneous evaluations of cytokine production and the function of the HPA axis in patients with MS, particularly when cytokine antagonists are used as a therapeutic approach. The possibility exists that inappropriate doses of an antagonist could interfere with the protective glucocorticoid response more effectively than with the autoimmune response, causing paradoxical and undesirable effects.

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