Neurointermediate pituitary lobectomy decreases the incidence and severity of experimental autoimmune encephalomyelitis in Lewis rats

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Abstract

Acute experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease of the central nervous system, mediated by T lymphocytes. Immunization of Lewis rats with myelin antigens suspended in complete Freund’s adjuvant induces EAE. In a previous study on rats we have found that neurointermediate pituitary lobectomy (NIL) decreased both the humoral and cell-mediated immune responses. Here we investigated the effect of NIL on the incidence and severity of EAE and on the function of the hypothalamic-pituitary-adrenal axis in Lewis rats. NIL, hypophysectomized (Hypox) and sham-operated (Sham) rats were immunized s.c. with guinea-pig brain extract suspended in complete Freund’s adjuvant. Untreated rats were used as controls. Water intake, body weight gain, clinical and histopathologic incidence and severity of EAE were evaluated in the operated groups. On killing, plasma adrenocorticotropin and corticosterone levels were measured and adrenals, thymuses and spleens were weighed. Histopathologic lesions were counted in the brain and spinal cord. Water intake and body weight gain were significantly decreased in Sham and Hypox animals with EAE whereas higher intakes persisted in the NIL group. Plasma levels of adrenocorticotropin were within the normal range whereas corticosterone levels increased in Sham and occasionally in NIL animals. Thymus weights were decreased in NIL and Hypox groups. The clinical and histopathologic incidence and severity of EAE were significantly decreased in NIL animals as compared with Sham and Hypox rats. We concluded that NIL affects the cell-mediated immune response and plays a role in the development and progression of EAE in the Lewis rat.

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Introduction

Acute experimental autoimmune encephalomyelitis (EAE) is an inflammatory disease of the brain and spinal cord induced by T lymphocytes. EAE is widely used as an experimental model for human multiple sclerosis (Martin & McFarland 1995). The Lewis strain of rats are very susceptible to the induction of EAE and to other forms of autoimmune disease. Acute EAE may be induced by injecting guinea-pig brain homogenate or purified myelin basic protein from brain and spinal cord tissue suspended in complete Freund’s adjuvant. The clinical symptoms become manifest after 2 weeks, and include limp tail and muscle weakness and leg paralysis followed by partial or complete recovery within 20 days (Simmons & Mason 1997). The histopathologic findings depend on the severity of the disease. Histologically, the inflammatory reaction is almost invariably localized in the lumbosacral segment of the spinal cord; in addition patchy inflammatory changes can be observed along the thoracic and cervical spinal cord, brainstem and cerebellum, and, in more severe cases, in the cerebral hemispheres. Evidence of meningitis is usually found even in the mildest cases (Al-Sabbagh & Weiner 1994). The inflammatory infiltration has a predilection for perivascular areas (perivascular cuffs) in the white matter. The typical infiltrate consists of T lymphocytes, plasma cells, macrophages, polymorphonuclear neutrophils and eosinophilic leucocytes. Tissue demyelination is almost completely absent in the acute form of the disease (Al-Sabbagh & Weiner 1994).

In chronic stress and in various inflammatory diseases the secretion of vasopressin (VP), adrenocorticotropin
(ACTH) and corticosterone (CORT) is increased without increased release of corticotropin-releasing hormone (Harbuz et al. 1992, 1997a,b, 2003). It has been shown that chronic stress is associated with thymic involution (Karst & Jöels 2003) and alterations of humoral and cellular immune responses in laboratory animals and humans (Shavit 1991). Neurointermediate pituitary lobectomy (NIL) or pituitary-stalk compression (PSC) in rats result in a mild but significant basal increase of ACTH and CORT plasma levels (Fagin et al. 1985, Makara et al. 1996). In rats with PSC, VP and oxytocin are transported via the newly formed neurohumoral connections from the remnants of the neural lobe axons to the long portal vessels (Makara et al. 1995, 1996). We have found that humoral (agglutinin, hemolysin and IgG titers) and cell-mediated (delayed hypersensitivity to dinitrochlorobenzene) immune responses are reduced in NIL rats (Organista-Esparza et al. 2003, Quintanar-Stephano et al. 2004). The aim of the present study was to investigate the effects of NIL on the incidence and severity of acute EAE and the activity of the hypothalamic-pituitary-adrenal (HPA) axis.

Materials and Methods

Animals

Male Lewis rats of 230–270 g body weight from our colony were used. Animals were housed under controlled temperature (22–24 °C) and light/dark conditions (lights on between 0700 and 1900 h). The diet consisted of Purina rat chow and tap water ad libitum. The diets of hypophysectomized rats were supplemented with sugar cookies and lettuce. The animals were habituated to our housing conditions for at least 7 days before surgery. Animals were treated according to the Institutional Normative Welfare Standards (University of Aguasalientes).

Groups and surgery

The following experimental groups (14–15 Lewis rats each, seven or eight per cage) were used: (1) NIL, (2) hypophysectomized (Hypox) and (3) Sham-operated (Sham), as well as (4) 10 untreated rats as normal controls.

For the NIL operation, rats were anesthetized with methyl ether, and the trachea was cannulated per os. 15 min before anesthesia 0·06 mg atropine was administered s.c. to each rat to prevent excessive secretion in the respiratory tract. Removal of the neurointermediate lobe (neural and intermediate lobes) of the pituitary was performed under a dissecting microscope through the parapharyngeal-trans-sphenoidal approach by gentle aspiration via a bent needle after an undisturbed view had been achieved. The method employed was the same as described by Ben-Jonathan & Peters (1982) and Mena et al. (1996). The total time of anesthesia did not exceed more than 15 min, and full recovery occurred within 30–60 min. After surgery all operated animals were injected with penicillin (Penprocilina; 5000 IU i.m.; Lakeside de México, DF, México) once daily for 3 days.

The procedure for Hypox was the same as for NIL except that the entire pituitary gland was removed, whereas in Sham animals the procedure was terminated when the pituitary capsule was opened surgically and the pituitary gland was visualized directly.

Water intake and body weight

Water intake was measured by daily weighing of water bottles (1 ml weighs approximately 1 g) and expressed as weekly mean of water (ml) consumed daily/100 g body weight.

Rats were weighed before surgery and weekly afterwards. The weight changes were expressed as weekly body weight gain (BWG), which was determined by subtracting the initial body weight measured before surgery from the body weight measured on each subsequent week (Groesbeck & Parlow 1987).

EAE induction

Three weeks after surgery the animals were immunized by s.c. injections at the base of the tail, with 100 µl of an emulsion composed of 10% guinea-pig brain homogenate suspended in complete Freund’s adjuvant (1 ml brain homogenate plus 1 ml incomplete Freund’s adjuvant (Sigma), plus 2 mg pulverized Mycobacterium tuberculosis (H37Ra; Difco, Detroit, MI, USA; Simmons & Mason 1997). Three extra animals of each group were injected only with incomplete Freund’s adjuvant. Clinical scoring of EAE was carried out by an experienced researcher according to a conventional scale as follows: 0, no signs of the disease; 1, paralysis of the tail; 2, ataxia in one or both hind legs; 3, paralysis of one hind leg; 4, complete paralysis of both hind legs; 5, urinary incontinence. The daily clinical score of EAE was expressed as the mean ± S.E.M. from the individual values in each group.

Killing

The animals were killed 1 day after the clinical scores had reached their highest level. The rats with lower scores were killed 1 day after no increases in the scores were noted. Animals that did not develop signs of the disease were killed 16 days after immunization. The animals were killed with sodium pentobarbital anesthesia (40 mg/100 g body weight, s.p.), and blood was taken from the abdominal aorta. Subsequently, the animals were perfused through the heart, with saline solution (0-9% NaCl), which was followed by the infusion of 10% buffered formalin. This allowed us to compare and correlate the clinical severity of EAE with histopathology and with...
ACTH and CORT plasma levels. The sella turcica was examined under a dissecting microscope for pituitary or neurointermediate-lobe remnants. Only successfully Hypox rats and NIL rats with no damaged anterior lobe and complete removal of neural and intermediate lobes were included in the study. In NIL and Hypox rats a pituitary-stalk stump, measuring 0.2–0.5 mm, was usually found. The adrenals, thymuses and spleens were dissected and weighed on an analytical balance. Brains and spinal cords were removed and four coronal brain sections were prepared at A 8.25 (frontal), A 5.25 (preoptic), A 1.25 (hypothalamic) and P 2.75 (brainstem–cerebellum) anterior–posterior coordinates (Skinner 1972). In addition four spinal cord sections were cut at levels: cervical 4, thoracic 5, lumbar 3 and sacrum 3 (Hebel & Stromberg 1976). All sections were cut by the same person.

Tissues were fixed in 10% buffered formalin, embedded in paraffin, cut into 6 µm sections and stained with hematoxylin–eosin or immunostained with anti-CD3 antibodies for T lymphocytes (polyclonal rabbit anti-human T-cell CD3; Dako, Carpinteria, CA, USA) using the streptavidin–biotin–peroxidase complex method (Dako). The incidence and severity of EAE were assessed by histology. The number of perivascular cuffed lesions were counted on slides from each level using a Nikon light microscope (Optiphot-2) with magnification of ×40. The presence of CD3-immunopositive T lymphocytes and their distribution was also examined. Blood plasmas were separated and stored at −20 °C until ACTH and CORT levels were measured by RIA (Diagnostic Products Co., Los Angeles, CA, USA). Inter- and intra-assay RIA coefficients of variance were 10.54 and 9.78% for ACTH, and 7.96 and 8.95% for CORT respectively.

Statistical analysis

Data are presented as means ± S.E.M. Statistical significance of the differences between experimental groups was determined by two-way analysis of variance, followed by the Tukey–Kramer test. Where appropriate, multiple group comparisons and contingency tables of χ² and the Fisher exact test were applied as well.

Results

Daily water intake

As compared with the Sham group, in the Hypox group a significant increase (66%) in daily water intake (P<0.05) was noted. In the NIL group a higher water intake was observed (150% over the Sham group and 47% over the Hypox group; Table 1). When clinical signs of EAE developed, a significant diminution in water intake occurred in Hypox and Sham groups. In the NIL group, a mild but non-significant decrease in water intake was apparent.

<table>
<thead>
<tr>
<th>Group</th>
<th>Daily water intake (ml/100 g BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>43.4 ± 3.4³</td>
</tr>
<tr>
<td>Hypox</td>
<td>72.3 ± 10³</td>
</tr>
<tr>
<td>NIL</td>
<td>106.2 ± 6.8³</td>
</tr>
</tbody>
</table>

Superscript with different letters show significant differences. BW, body weight.

BWG

Figure 1 shows that the Sham and NIL groups had similar increases in BWG during the first 4 weeks after surgery. During the fifth week, when the clinical signs of EAE became manifest the Sham group lost 80% of BWG that had accumulated by week 4 (P<0.001). In NIL animals BWG in the fifth week was similar to that in week 4. After surgery, Hypox animals lost weight, which was accelerated during the fifth week.

EAE clinical incidence

EAE incidence in Sham, Hypox and NIL groups was 71.4% (10 of 14 inoculated animals had signs of EAE), 60% (9 of 15) and 21.4% (3 of 14) respectively. No significant differences between Sham and Hypox groups were observed, whereas in the NIL group the incidence was significantly lower (P<0.001 versus Sham and Hypox groups). In animals injected with incomplete Freund’s adjuvant no clinical signs of EAE were observed.

EAE clinical development

As shown in Fig. 2, in the Sham group clinical manifestations of EAE started 9 days after encephalitogenic
antigen inoculation. Maximum score was observed on day 14, with a slight decrease on day 15. The animals from all groups were killed on the next day after a maximum clinical score of EAE was observed. This allowed us to compare and correlate the clinical severity of EAE with histopathology and with ACTH and CORT plasma levels (see the Materials and Methods section). In the Hypox group clinical signs of EAE appeared during the tenth day post-immunization and reached maximum score during the thirteenth day. This group showed a faster development and more severity of EAE in comparison with the Sham and NIL groups. On the fourteenth day the clinical score decreased and the animals were killed. In NIL animals the clinical signs of EAE were delayed until day 13 post-immunization, and exhibited very low means of clinical scores (0·14 ± 0·09; P<0·001 as compared with Sham and Hypox groups). EAE subsided completely by day 15. These results indicate that NIL has a significant protective effect against EAE.

Histopathologic incidence and severity of EAE

Histopathologic alterations in EAE have been described previously (Sobel et al. 1984, Al-Sabbaggh & Weiner 1994). In our study similar lesions were observed; perivascular infiltration with T lymphocytes (about 40% of cells were CD3-immunopositive in perivascular infiltrates), macrophages, polymorphonuclear neutrophils and eosinophilic neutrophils were also evident. Lesions were localized in both white and gray substances of the brain and spinal cord. CD3-immunopositive T lymphocytes invaded the interstitial spaces of the brain and spinal cord. In Sham and Hypox rats histopathologic signs of meningitis were demonstrated together with EAE in the brain and spinal cord. Occasionally mild demyelination was observed in the lower levels of spinal cord of Sham and Hypox groups. In the NIL rats no meningitis and no demyelination were found. Table 2 shows the incidence of animals per group with histopathological lesions of EAE and the severity of the disease (e.g. the mean number of inflammatory perivascular cuffs) in brain and spinal cord. It was observed that all immunized Sham animals (14 of 14) presented histopathological lesions of EAE. In the Hypox group 80% (12 of 15) of the animals showed EAE lesions (not significant as compared with the Sham group), whereas in the NIL group the incidence was significantly less (60%; 9 of 14 inoculated animals, P<0·01) as compared with the Sham group. When the Sham and Hypox groups were compared, no significant difference in severity of

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence (%)</th>
<th>Brain</th>
<th>Spinal cord</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>100% (14 of 14)</td>
<td>49·5 ± 15</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>Hypox</td>
<td>80% (12 of 15)</td>
<td>41·3 ± 13</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>NIL</td>
<td>60% (9 of 14)</td>
<td>16·5 ± 6</td>
<td>6·7 ± 2</td>
</tr>
</tbody>
</table>

Values with statistical identity have the same superscript, whereas values labeled with different superscripts are significantly different (P<0·05). Values with more than one superscript share statistical identity with more than one group.
histopathological lesions was observed (Table 2). However, a significant diminution in perivascular inflammation occurred in NIL animals ($P<0.01$ as compared with the Sham group; Table 2).

**Organ weights**

Table 3 shows that no differences were found between untreated, Sham and NIL groups in adrenal and spleen weights. In Hypox animals, adrenal, thymus and spleen weights were significantly decreased. In Sham rats no changes occurred in thymus weight, whereas a significant reduction was observed in the thymus of Hypox and NIL rats (~37 and ~38% respectively versus untreated and Sham groups respectively, $P<0.001$).

**ACTH and CORT plasma levels**

Table 4 shows that in comparison with the untreated group, CORT but not ACTH plasma levels were significantly increased in Sham and NIL groups. In NIL animals, CORT levels were increased in two additional experiments (not included), but the increase was not significant statistically. As expected in Hypox animals both hormones were significantly decreased ($P<0.001$) as compared with the untreated, Sham and NIL groups.

**Discussion**

The results show that the clinical and histopathologic incidence and severity of EAE were significantly decreased by NIL. Since the EAE is T-cell-mediated, these findings support the concept that NIL affects T cell-mediated immune responses (Quintanar-Stephano et al. 2004). It is assumed that immune–neuroendocrine interactions affect the development of autoimmune diseases. In these interactions the HPA axis plays a crucial role via the immunosuppressive/anti-inflammatory effects of glucocorticoids (CORT in rats; Derijk & Sternberg 1994, Buckingham et al. 1997, Harbuz et al. 1997a, b, Webster et al. 2002, Berzci & Szentivanyi 2003a). In the present experiment plasma CORT levels were increased in Sham and NIL rats developing EAE. The incidence and severity of EAE were significantly decreased in NIL animals only, indicating that NIL exerts a suppressive effect on autoimmune responses.

The effects of NIL on organ weights have been described previously; some investigators reported increases in adrenal weights of NIL rats (Nowell 1959, Smelik 1960, De Wied 1961; discussed in Miller et al. 1974), whereas others found no changes (Miller et al. 1974, Makara et al. 1996). In the present study thymus weights in NIL animals were significantly decreased. No significant changes were observed in adrenal and spleen weights.


In the present study plasma CORT levels were increased in Sham and NIL rats developing EAE, as illustrated in Table 4. The incidence and severity of EAE were significantly decreased in NIL animals only, indicating that NIL exerts a suppressive effect on EAE. Fagin et al. (1985) and Makara et al. (1996) found in rats a mild but persistent

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**Table 3** Effects of EAE on adrenal, thymus and spleen weights (mg of wet weight/100 g body weight) in SHAM, Hypox and NIL Lewis rats. An untreated group was included as a control. Values are expressed as the means ± S.E.M.

<table>
<thead>
<tr>
<th>Group</th>
<th>Adrenal Weight (mg/100 g body weight)</th>
<th>Thymus Weight (mg/100 g body weight)</th>
<th>Spleen Weight (mg/100 g body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>29.4 ± 0.6 a</td>
<td>147.8 ± 5.2 a</td>
<td>285.8 ± 10  a</td>
</tr>
<tr>
<td>SHAM</td>
<td>30.3 ± 1.7 a</td>
<td>135.8 ± 8.5 a</td>
<td>257.8 ± 13  a</td>
</tr>
<tr>
<td>Hypox</td>
<td>14.1 ± 0.9 b</td>
<td>98.1 ± 7.7 b</td>
<td>209.2 ± 13 b</td>
</tr>
<tr>
<td>NIL</td>
<td>32.8 ± 1.4 a</td>
<td>91.3 ± 5.0 b</td>
<td>279.5 ± 6  b</td>
</tr>
</tbody>
</table>

Different letters within the same columns show significant differences. Untreated group, n=10 animals; n=14–15 animals in the remaining groups.

**Table 4** Effects of EAE on ACTH and CORT plasma levels in SHAM, Hypox and NIL animals. An untreated group was included as a control. Values are expressed as the means ± S.E.M.

<table>
<thead>
<tr>
<th>Group</th>
<th>ACTH (pg/ml)</th>
<th>CORT (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>52.4 ± 9 a</td>
<td>123.6 ± 9 a</td>
</tr>
<tr>
<td>SHAM</td>
<td>69.1 ± 10 a</td>
<td>212.9 ± 32 b</td>
</tr>
<tr>
<td>Hypox</td>
<td>15.7 ± 4 b</td>
<td>5.3 ± 0.8 b</td>
</tr>
<tr>
<td>NIL</td>
<td>45.9 ± 9 a</td>
<td>195.1 ± 27 b</td>
</tr>
</tbody>
</table>

Different letters within the same columns show significant differences. n=10–14 animals per group.
increase in basal CORT plasma levels after NIL or PSC respectively. We could not confirm this observation in NIL animals. However, when EAE was induced after NIL, ACTH levels remained in the normal range, and CORT levels were increased, which was significant in one out of three experiments (Table 4). Therefore, the elevation is consistent, but may not reach significant levels in all experiments. The mechanism of this elevation is not known at this time.

Sham-operated animals showed the highest CORT levels at the time of full-blown EAE. Hypox rats had very low CORT levels and showed an aggravated clinical course, but with less-severe histopathological changes. Hypox induced a significant decrease not only in ACTH and CORT, but also of growth hormone and prolactin plasma levels. Long-term Hypox rats have been used, which have significant residual serum prolactin (44.2% of normal; Quintanar-Stephano & Organista-Esparza, unpublished observations). Such animals are immunocompetent (Nagy & Berczi 1991). Therefore, these Hypox animals developed EAE as there was sufficient prolactin available. The fact that aggravated disease was observed with fewer histopathological lesions may indicate a decreased immunocompetence due to growth hormone and prolactin deficiency, coupled with an increased susceptibility to develop clinical symptoms in Hypox rats. This interpretation is fully in accord with numerous previous investigations on the role of pituitary hormones in immune and inflammatory reactions (Berczi & Szentivanyi 2003b).

Our results suggest that the deficiency in VP secretion in NIL rats plays a major role in the decreased incidence and severity of EAE. All NIL rats developed diabetes insipidus, indicating a decrease of VP secretion. Although NIL did not affect pregnancy and parturition (Quintanar-Stephano & Espino-Lopez, unpublished observations), oxytocin deficiency is indicated by the inability of the lactating NIL rats to eject milk (Mena et al. 1996). Based on our studies it is possible that VP is necessary for the direct stimulation of the immune responses. This is suggested by the known effects of VP on several types of immune cell (Johnson et al. 1982, Torres & Johnson 1988, Bell et al. 1992, Martens et al. 1998, Hu et al. 2003). Additional experiments are required to clarify these questions.

It is known that Hypox animals have decreased humoral, cell-mediated and autoimmune responses (Nagy & Berczi 1978, Bercezi et al. 1981, 1984, Nagy et al. 1983, Neidhart & Fluckiger 1992, Cruz et al. 1996). The present results show that Hypox rats respond to immunization for EAE. Several studies indicate that decreased immune responses in Hypox rats are time dependent. It was demonstrated that short-term hypophysectomy (less than 4 weeks after an operation) induces a significant decrease in immune responses (Nagy & Berczi 1978, Bercezi et al. 1981, 1984, Nagy et al. 1983, Quintanar-Stephanco et al. 2000a) whereas long term hypophysectomy results in only a slight or no decrease in immune responses (Quintanar-Stephanco et al. 2000b). Since Hypox rats increase their serum prolactin levels to about 50% of normal within 6 weeks of the operation this may explain the restoration of their immunocompetence (Nagy & Berczi 1991).

Immune homeostasis does not rely exclusively on internal regulatory mechanisms of the immune system. Immunocompetence is dependent on, and adaptive and natural immune responses and inflammation are regulated by, neuroendocrine mechanisms. By definition, autoimmune disease develops upon the loss of self-tolerance. An autoimmune reaction is due to de-regulated lymphocyte proliferation and maturation into effector cells directed towards self-antigens. Current evidence indicates that de-regulation at the cellular level as well as an altered neuroendocrine milieu are necessary for autoimmune disease to develop. Abnormalities of the hypothalamus (growth hormone and prolactin)–insulin-like growth factor axis and of the HPA axis are frequently observed in autoimmune disease. An imbalance of these major immunoregulatory hormones of the pituitary is very likely to play a major role in the pathogenesis of autoimmune disease. It is also becoming apparent that defective regulation by other hormones, neurotransmitters and neuropeptides contribute significantly to the pathogenesis of autoimmune and inflammatory conditions (Berczi & Szentivanyi 2003b).

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