Suppression of experimental autoimmune encephalomyelitis by gallium nitrate

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Summary

We examined the effect of gallium (Ga) nitrate on the development of the development of experimental autoimmune encephalomyelitis (EAE). Weekly subcutaneous injections of 10–30 mg/kg prevented clinical signs as well as histopathological changes of EAE. The optimal timing of a single injection of Ga was 6 days after induction of EAE, with amelioration also apparent following a single injection on day 3 or 9 but not day 12. Ga administered in vivo suppressed myelin basic protein (MBP) and purified protein derivative-specific lymphocyte proliferative responses in vitro. Addition of Ga to MBP-specific T lymphocyte line cultures at various times after initiation of culture revealed that Ga exerts an effect at an early stage of cellular activation.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is a central nervous system (CNS) autoimmune disease which serves as a model for the study of human demyelinating diseases such as multiple sclerosis (MS) (Paterson and Swanborg, 1988). Following a single injection of myelin basic protein (MBP) and complete Freund’s adjuvant (CFA), the prototype Lewis rat develops an ascending paralysis of the hind limbs, together with CNS histopathological changes which can be readily quantified. EAE has been shown to be mediated by CD4+ T lymphocytes which react with defined epitopes on the MBP molecule.

Immunosuppressive regimens for the treatment of EAE have included drugs such as cyclophosphamide (Paterson and Drobish, 1969), and cyclosporine (Bolton et al., 1982). Immunotherapy using non-antigen-specific approaches has been successfully applied to EAE by treatment with monoclonal antibodies directed against the CD4 molecule (Swanborg, 1983; Brostoff and Mason, 1984; Waldor et al., 1985;
Sriram and Roberts, 1986) or monoclonal antibodies directed against major histocompatibility complex class II glycoproteins (Sriram and Steinman, 1983; Sriram et al., 1987). Recent interest in the treatment of EAE has focused on antigen-specific means of immunosuppression utilizing the oral administration of MBP (Bitar and Whitacre, 1988; Higgins and Weiner, 1988) and treatment with T cell receptor peptides (Vandenbark et al., 1989; Howell et al., 1989).

We have recently shown that the group IIIA metal gallium (Ga) is effective in the treatment of Paget’s disease of bone (Matkovic et al., 1990) and hypothesized that this drug inhibits the activity of osteoclasts, cells derived from the myeloid lineage. More recently, Ga was also shown to prevent adjuvant arthritis (AA) in rats and to inhibit the proliferative response of a purified protein derivative (PPD)-specific T cell line in vitro (Matkovic et al., 1991). In the present study, we have extended our investigations to include the effects of Ga on EAE. We report here that Ga exerts a potent inhibitory effect on the induction of EAE, and that Ga exerts an effect at an early phase of cellular activation.

Materials and methods

Rats

Male Lewis rats (8–12 weeks of age) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). They were housed in a controlled environment with a 12-h light/dark cycle and allowed to acclimate for at least one week prior to experimentation.

Antigen

Guinea pig MBP was prepared from spinal cords (Rockland, Inc., Gilbertsville, PA) according to the procedure described by Swanborg et al. (1974).

Induction of EAE

Rats received an intradermal injection of guinea pig MBP (25 μg total) combined with CFA containing 4 mg/ml Mycobacterium tuberculosis Jamaica strain in both hind footpads. Rats also received subcutaneous (s.c.) injections of normal saline or Ga in the form of Ga nitrate (Mr 255,700, 27.3% elemental Ga, Ben Venue Laboratories, Inc., Bedford, OH) in the nape of the neck. On days −1, 6, 13, and 20 relative to MBP injection on day 0, rats randomized into five groups received the following dosing regimen in mg/kg of Ga: (1) 0/0/0/0 (n = 10), (2) 30/10/10/10 (n = 10), (3) 0/30/10/10 (n = 10), (4) 30/30/10/10 (n = 4) or (5) 0/10/10/10 (n = 4). In an additional experiment to examine the effect of timing of treatment, Ga was administered as a single dose of 30 mg/kg on day 3, 6, 9, or 12 after induction of EAE. All rats were monitored daily for clinical neurological signs, scored as follows: no signs = 0, partial limp tail = 0.5, flaccid tail = 1, ataxia = 2, partial hindlimb paralysis = 3, full hindlimb paralysis = 4, death = 5. Histopathological evaluations of the CNS were performed on all rats as previously reported (Bitar and Whitacre, 1988). 7-μm sections of the thalamus, mesencephalon, cerebellum–pons, and longitudinal sections of the entire spinal cord were stained with Hematoxylin and Eosin. Duplicate slides for each animal were assessed for perivascular mononuclear infiltrates, scored as follows: no lesions = 0, 1–10 lesions = 1, 11–30 lesions = 2, and more than 30 lesions = 3.

Lymphocyte proliferative response

Rats were killed on day 13 after induction of EAE, and the lymph nodes draining the sites of footpad injection were obtained (popliteal, inguinal and periarteric). Lymph nodes from individual rats were expressed through a wire mesh screen to produce a single-cell suspension. Lymph node cells (LNC) were suspended in RPMI 1640 (Whittaker M.A. Bioproducts, Walkersville, MD) containing 2% fresh autologous Lewis rat serum, 2 mM glutamine (Whittaker), 50 U/ml penicillin, 50 μg/ml streptomycin (Whittaker), 25 mM Hepes buffer (Gibco, Grand Island, NY), and 5 × 10^-5 M 2-mercaptoethanol (Bio-Rad, Richmond, CA). LNC (4 × 10^5 in 100 μl) were cultured in quadruplicate in round-bottomed 96-well plates (Linbro, Mclean, VA) together with either 20 μg/ml MBP, 20 μg/ml PPD, 2 μg/ml concanavalin A (ConA), or medium alone for 72 h at 37°C in 5% CO₂, including a final 18-h pulse with [³H]thymidine (1 μCi/well) (Amersham, Arling-
ton Heights, IL). Cultures were harvested using a semiautomatic harvester (Skatron, Sterling, VA) and counted by liquid scintillation.

Production of MBP-specific T cell lines
MBP-specific T cell lines were established as previously described (Ben-Nun et al., 1981; Vandenberg et al., 1985; Whitacre et al., 1991). LNC draining the site of footpad injection were obtained from Lewis rats 9 days after MBP-CFA injection. These LNC (8 x 10^6/ml) were cultured for 3 days in complete RPMI 1640 medium in the presence of 40 μg/ml MBP. Lymphoblasts were obtained by centrifugation, using lymphocyte-separating medium (Organon Teknika, Durham, NC) (240 x g, 25 min), and the cells were washed and cultured in medium containing 10% fetal bovine serum (Whittaker M.A. Bioproducts) and 10% (v/v) lectin-free rat T cell growth factor (24-h supernatant from rat spleen cells stimulated with ConA). After 4–8 days in culture, the cells were restimulated for 3 days with MBP (20 μg/ml) in the presence of syngeneic gamma-irradiated (3300 Rad) thymocytes (10^7/ml) as a source of antigen-presenting cells (APC). The T-line cells were alternately expanded in rat growth factor or restimulated with antigen and irradiated thymocytes. Following the second round of antigen restimulation, the T-lymphocyte line proliferated in response to MBP and ConA but not to PPD. After the T-cell line was established, it was re-stimulated with antigen at approximately 2-week intervals. At the time of these experiments, the cell line had been in culture for approximately 8 months.

To measure the effect of Ga on the MBP-specific T cell line, a proliferative assay was performed. T-line cells (2 x 10^4/well) were cultured with 10^6 irradiated (3300 rad) thymocytes and MBP, PPD, or ConA. Cultures, performed in quadruplicate, contained no Ga or Ga nitrate (50 μg/ml) added at the initiation of culture (0 h) or at 16, 24, 40, 48 or 62 h after culture set-up. T-cell line cultures were pulsed with [3H]thymidine during the final 18 h of incubation, and harvested cells were counted by liquid scintillation. Companion cultures were observed for cell viability by exclusion of Trypan blue at the termination of the culture period.

Results
Ga suppresses EAE
In the first series of experiments, Ga was administered using a similar regimen and schedule effective in suppressing AA (Matkovic et al., 1991). Rats received either 30 mg/kg of Ga beginning 6 days after induction of EAE, with maintenance doses of 10 mg/kg on days 13 and 20 (n = 6) or saline (n = 6). Control untreated rats progressed to maximal paralysis by day 13 (Fig. 1), whereas Ga-treated rats exhibited either no signs or minimal disease. Representation of the

<table>
<thead>
<tr>
<th>Group</th>
<th>Ga dose (mg/kg)</th>
<th>Incidence of EAE</th>
<th>Clinical score</th>
<th>Histo score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/0/0/0</td>
<td>10/10</td>
<td>4.0 ± 0.0</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>30/10/10/10</td>
<td>9/10</td>
<td>1.3 ± 0.3 *</td>
<td>0.9 ± 0.3 *</td>
</tr>
<tr>
<td>3</td>
<td>0/30/10/10</td>
<td>4/10</td>
<td>0.2 ± 0.1 *</td>
<td>1.1 ± 0.3 *</td>
</tr>
<tr>
<td>4</td>
<td>30/30/10/10</td>
<td>4/4</td>
<td>0.6 ± 0.2 *</td>
<td>0.3 ± 0.3 *</td>
</tr>
<tr>
<td>5</td>
<td>0/10/10/10</td>
<td>4/4</td>
<td>1.3 ± 0.6 *</td>
<td>1.0 ± 0.7 **</td>
</tr>
</tbody>
</table>

* Rats received injections of Ga or saline as described in Fig. 1 on days -1, 6, 13 and 20 relative to induction of EAE. Clinical signs of EAE and CNS histological evaluations were scored as described in Materials and methods. Rats which received 30 mg/kg Ga on day 6 had the lowest clinical scores, irrespective of dosing on day -1.
* Denotes significantly different (P < 0.001) from Group 1 by analysis of variance with Bonferroni correction of P-values.
** P < 0.01.
entire clinical picture reveals that clinical signs were not merely delayed in Ga-treated animals, but were nearly completely suppressed.

Studies undertaken to determine the effects of dosage and timing of administration of Ga on EAE showed marked suppression of disease in all Ga-treated (30–80 mg/kg) animals (Groups 2–5) (Table 1). Administration of Ga on day −1 did not further suppress EAE (Groups 2 and 4). Maximal inhibition of clinical disease was achieved when 30 mg/kg was given on day 6 (Group 3), and maximal suppression of histopathological changes occurred at the largest total dose of Ga (Group 4). We observed no adverse clinical effects of Ga in any of the treated rats.

Studies to determine the optimal timing for administration of Ga following induction of EAE showed that Ga exerts its maximal suppressive effect when administered on day 6, with a significant inhibitory effect also observable when administered on day 3 or day 9 after induction of EAE (Fig. 2). However, after the onset of clinical signs (day 12), Ga exerted no demonstrable effect. These results suggest that Ga acts during the afferent phase of the autoimmune process, preventing the expansion of autoreactive cells.

In vitro proliferative responses

To assess the extent of immunological unresponsiveness in Ga-treated rats, we performed lymphocyte proliferation assays using LNC from those rats treated with single-dose Ga on day 3, 6, 9, or 12 following induction of EAE. Ga administered in vivo at any time after induction of EAE caused a suppression in the MBP- and PPD-specific proliferative response in vitro (Fig. 3). In contrast, the mitogenic response to ConA was unchanged by Ga. The differential inhibition of the proliferative response between specific antigen and mitogen stimulation in vitro suggests that Ga acts in vivo on proliferating T cells. Because the response to ConA was not reduced, Ga does not appear to exert a generalized toxic effect.

Based on our hypothesis that Ga affects T lymphocytes which are undergoing activation, we designed an experiment to examine the effect of Ga nitrate on an MBP-specific T-lymphocyte line. These cell lines have been shown to bear T-cell markers (i.e., CD4, CD5, and the T-cell receptor) and to be highly reactive (proliferation) in response to either ConA or the antigen used for in vitro selection, when presented together with syngeneic APC (Ben-Nun et al., 1981; Vandenbark et al., 1985; Wekerle and Fierz, 1985; Whitacre et
Fig. 3. Lymphocyte in vitro proliferative response of lymph-node cells from rats treated with Ga. Three rats from each of the groups shown in Fig. 2 were killed on day 13 after induction of EAE and cells were obtained from the lymph nodes draining the site of footpad injection. Proliferative responses to MBP, PPD, and ConA were assessed in quadruplicate from individual rats. * Indicates significantly different (P < 0.05) from the saline-treated control group by analysis of variance with Bonferroni correction of P-values.

al., 1991). The T-cell line used for these studies proliferated vigorously in response to MBP and ConA but not to PPD when presented in the context of syngeneic APC (Fig. 4). When Ga nitrate was present during the entire 72-h culture of T-line cells with MBP or ConA and irradiated thymocytes (as a source of APC), the antigen-specific and mitogen-induced proliferative responses of the T-cell line were completely inhibited. Furthermore, addition of Ga nitrate within 24 h of initiation of culture also completely suppressed the proliferation of the T-line cells to both antigen and mitogen. Partial responses were observed when Ga nitrate was added at 40–48 h, with no effect at 62 h. Results of identical cultures, examined for cell viability by Trypan blue exclusion, paralleled those obtained by [3H]thymidine incorporation. No viable cells were observed in wells containing Ga nitrate added at 0–24 h. Cell viability increased in cultures receiving Ga at 40 h, with a further increase at 48 h. The number of viable cells was equivalent in cultures receiving Ga at 62 h and those receiving no Ga. These studies show that Ga is not simply toxic to lymphocytes and, more importantly, that Ga acts at a relatively early stage of T-cell activation.

Discussion

We have previously reported that Ga exerts a potent suppressive effect on AA, a T-cell-mediated autoimmune process, in Lewis rats (Matkovic et al., 1991). The present studies were undertaken to examine further the immunosuppressive effects of Ga on an autoimmune response directed against CNS antigens in the well-studied animal model, EAE. Like the rats with AA, rats with EAE which had been treated with Ga exhibited markedly suppressed clinical signs and histopathologic changes relative to untreated controls.

Both the in vivo and in vitro results point to a critical time factor for administration of Ga in EAE. A single injection of Ga was shown to be sufficient to inhibit disease when administered 6 days following induction of EAE (Fig. 2). This
corresponds to a time when MBP and adjuvant-specific lymphocytes are concentrated within the regional lymph nodes draining the site of antigen challenge and are in an active phase of proliferation and differentiation. It should be noted that Ga was still quite effective at inhibiting the clinical signs of EAE when administered immediately prior to the appearance of clinical signs (9 days following neuroantigen injection), yet appeared ineffective once clinical disease began.

The in vitro lymphocyte proliferative responses in Ga-treated rats revealed a suppression of antigen-stimulated, but not mitogen-stimulated responses (Fig. 3). The antigen-reactive cell populations theoretically represent only a small percentage of the total lymphocyte repertoire present in the lymph node. The fact that MBP-specific and PPD-specific responses in vitro were both suppressed following administration of Ga in vivo suggests that Ga inhibits T cells undergoing proliferation; however, the lack of inhibition with ConA suggests that Ga is not broadly suppressive for all T lymphocytes.

An MBP-specific T-lymphocyte line was employed to elucidate further the timing of the Ga-mediated suppression of autoreactivity (Fig. 4). These experiments revealed that Ga is not simply toxic to lymphocytes and, more importantly, that Ga affects a relatively early stage of T-cell activation. Once terminal differentiation of lymphocytes has occurred, the immunosuppressive properties of Ga are no longer demonstrable, as noted by the in vivo administration of Ga after the appearance of EAE (Fig. 2) and the late addition of Ga to T-cell line cultures (Fig. 4). In further support of this notion, Ga did not exert any inhibitory effect on the development of EAE when already activated MBP-specific T-line cells were injected into six Ga-pretreated recipients (data not shown).

We have previously shown that Ga exerts a suppressive effect on the clinical course of AA, presumably through its action on T cells as well as macrophages (Matkovic et al., 1991). In those studies examining macrophage function, gamma interferon-treated peritoneal macrophages from mice exhibited a decrease in the expression of I-A glycoproteins during the first 16 h after in vitro exposure to Ga. The possibility exists that Ga is acting similarly in the experiments reported here, viz., inhibiting the expression of I-A on APC in vitro, resulting in an inhibition of T cell proliferation. In addition, similar events may be occurring in the local lymph node in vivo, whereby suppression of I-A or other functions of APC prevents EAE only when Ga is administered prior to appearance of symptoms; Ga administered later, after antigen presentation events have occurred, would have little or no effect on the appearance of disease. The effects of Ga on macrophage function in the EAE model are currently being further investigated.

An increase in the uptake of iron during the S phase of the cell cycle has been observed and has been attributed to the iron-containing enzyme ribonucleotide reductase, required for DNA synthesis and cellular proliferation (Seligman et al., 1992). Ga has been shown to have an inhibitory effect on the proliferation of B lymphocytes, likely resulting from competition with iron for cellular uptake via the transferrin–transferrin receptor pathway (Kovar et al., 1990). A similar mechanism may be operative in T lymphocytes since the expression of transferrin receptors is necessary for T-cell proliferation (Neckers and Cossman, 1983). In studies using the HL60 human leukemic cell line, Ga was shown to impair the intracellular release of iron from transferrin by disrupting intracellular acidification processes, resulting in iron depletion and inhibition of cellular proliferation (Chitambar and Seligman, 1986).

Ga, like orally administered MBP (Bitar and Whitacre, 1988; Higgins and Weiner, 1988; Whitacre et al., 1991), has shown efficacy in the treatment of EAE and may also prove effective in treating other autoimmune diseases. Further work is necessary to elucidate the mechanism of action of Ga on the immune system.

References


