Treatment of chronic-relapsing experimental autoimmune encephalomyelitis with the synthetic immunomodulator linomide (quinoline-3-carboxamide)

(multiple sclerosis/natural killer cells)

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Communicated by Michael Sela, February 9, 1993

ABSTRACT Linomide is a synthetic immunomodulator that enhances natural killer cell activity and significantly activates several lymphocytic cell subpopulations in both experimental animals and humans. In this study we examined the effect of linomide (80 mg per kg per day in drinking water) on mice with chronic-relapsing experimental autoimmune encephalomyelitis (CR-EAE), a T-cell-mediated organ-specific autoimmune disease that resembles human multiple sclerosis. None of the mice (n = 17) that were treated with linomide from day 7 after disease induction developed any clinical or histopathological signs of CR-EAE, as compared to 19 of 20 untreated controls that were severely paralyzed and had extensive demyelinating lesions in the central nervous system. Linomide-treated animals were also resistant to an induced attack by a booster injection with a murine spinal cord homograft. When administered to mice exhibiting severe clinical signs of paralysis, linomide inhibited both spontaneous and induced relapses. Linomide treatment protected mice from passively induced CR-EAE as well, when given from the day of injection with myelin-basic-protein-specific lymphocytes. Lymphocytes obtained from linomide-treated mice had a reduced in vitro proliferative response to the myelin basic protein and to the tuberculin purified protein derivative, whereas the mitogenic response to concanavalin A was not affected. Natural killer cell and lymphokine-activated killer cell activities were enhanced. These results suggest that linomide regulates autoimmunity in the absence of systemic immunosuppression. Since linomide is very well tolerated in experimental animals and humans, it might be used in the treatment of multiple sclerosis.

Experimental autoimmune encephalomyelitis (EAE) is a T-cell-mediated organ-specific autoimmune disease of the central nervous system (CNS). EAE can be induced in genetically susceptible animals by subcutaneous inoculation of a spinal cord homograft emulsified in complete Freund’s adjuvant (CFA). The disease is characterized either by acute monophasic or chronic-relapsing and remitting paralysis, associated with inflammatory and demyelinating lesions in the white matter of the CNS (1–3). Both clinical forms of EAE correspond in many respects to human multiple sclerosis (MS) (4). Chronic-relapsing EAE (CR-EAE) bears even closer resemblance to MS, particularly with regard to its clinical course. Morphologically, CR-EAE has the two characteristic features of MS lesions, namely the presence of recent and old demyelinating plaques and the periventricular distribution of lesions. Moreover, in both CR-EAE and MS, advanced disease is associated with chronic rather than acute lesions (5). CR-EAE can, therefore, be used as a more reliable model to study therapies for CNS-targeted autoimmune disorders.

CR-EAE is induced in SJL/J mice by immunization with two subcutaneous injections of mouse spinal cord homograft (MSCD) emulsified in CFA (2). CR-EAE can also be induced by adoptive transfer of lymphocytes that have been sensitized in vitro to myelin basic protein (6). T cells are crucial for the development of both types of CR-EAE (7). T cells of the Lyt-1 phenotype were observed in perivascular cuffs in the brain and spinal cord of animals with CR-EAE (8). Likewise, T-cell clones that react with the encephalitogenic determinant of basic protein are sufficient to induce the disease (9).

Conventional therapeutic approaches to EAE or MS are based on nonspecific suppression of the immune system. Several immunosuppressive agents such as cyclosporine (10–14), cyclosporine A (15, 16), azathioprine (12), corticosteroids (17–21), and total lymphoid irradiation (22) have been used. However, suppression of the disease by such treatments requires chronic administration of the cytoreductive drug (13), which usually results in cumulative toxic side effects; moreover, discontinuation of therapy is often associated with reappearance of paralytic signs (12, 23). Treatment of ongoing CR-EAE with apparent paralytic signs and histological evidence of brain damage was shown to be more difficult (13). More restricted, but specific, immunomodulations with anti-T-cell receptor variable region Vβ gene products and T-cell receptor binding peptides (24–26) are still considered experimental. Copolymer 1, which has been shown to be effective in suppression of EAE, was recently found to specifically compete with basic protein for binding to the major histocompatibility molecules (27).

Depressed immune functions, including low suppressor and natural killer (NK) cell activities have been reported in MS and other experimental and human autoimmune diseases (28–31). In view of recent data on the role of the “immune networks” (32, 33) in the pathogenesis of autoimmunity, active controlled recognition of “self,” rather than immunosuppression (34), might be needed for activation of the normal immunoregulatory circuits. Linomide (quinoline-3-carboxamide, LS-2616, Kabi Pharmacia Therapeutics AB, Lund, Sweden) is a very well tolerated immunoregulatory

Abbreviations: CFA, complete Freund’s adjuvant; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; CR-EAE, chronic-relapsing EAE; GMBP, guinea pig myelin basic protein; LAK, lymphokine-activated killer; MS, multiple sclerosis; NK, natural killer; PD, purified protein derivative; MSCH, mouse spinal cord homograft; rIL-2, recombinant interleukin 2.

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compound that was shown to stimulate several immune functions including activation of non-major-histocompatibility-restricted cytotoxicity (NK activity) (35-37), with effective prevention of metastatic disease in animal models of cancer (38). Likewise, linomide ameliorated autoantibody production and renal pathology in MRL/lpr and NZB/NZW(F1) mice, which spontaneously develop a systemic lupus erythematosus-like autoimmune syndrome (39, 40).

In the present study we have investigated the efficacy of linomide in modulating CR-EAE. Our results show that linomide very effectively inhibits and even reverses CR-EAE by specifically regulating autoreactive T cells.

**MATERIALS AND METHODS**

Mice. Six- to 12-week-old female SJL/J mice were purchased from The Jackson Laboratory and housed under standard conditions in top-filtered cages. Mice were fed a regular diet and given water ad libitum without antibiotics.

Antigens. **MSCH.** Spinal cords from 3- to 10-month-old mice of various strains were obtained by insufflation. MSCH was homogenized in phosphate-buffered saline [PBS, 1:1 (vol/vol)], lyophilized, reconstituted in PBS to a concentration of 100 mg/ml, and stored at -20°C until used.

**Tuberculin purified protein derivative (PPD).** PPD was obtained from Statens Serum Institut (Copenhagen).

**Guinea pig myelin basic protein (GMBP).** GMBP was prepared from guinea pig spinal cords as described (41).

**Recombinant human interleukin 2 (rIL-2).** Cetus rIL-2 (3.0 x 10^8 units/mg, >97% pure) was kindly supplied by C. R. Franks (Euracetus, Amsterdam).

Active Induction of CR-EAE. CR-EAE was induced according to Brown’s immunization protocol (2), with slight modifications. Briefly, mice were injected subcutaneously at one site over the left flank with a mixture of 1 mg of MSCH in 0.15 ml of PBS and 0.03 mg of Mycobacterium tuberculosis hominis H37Ra in 0.15 ml of incomplete Freund’s adjuvant (Difco). Each mouse received a second inoculation 7 days later into the contralateral flank with the same antigen-adjuvant mixture. First clinical signs of disease were observed 12-14 days after the first immunization; chronic disease with relapses and remissions followed the first attack. A third “rechallenge” injection with the same encephalito-genic inoculum induced a severe relapse after 6-7 days.

Passive Induction of CR-EAE. Donors (SJL/J mice) of T cells were immunized with 400 μg of GMBP in CFA containing 0.03 mg of Mycobacterium tuberculosis at two or three sites subcutaneously over the flanks. Animals were sacrificed 10 days later under ether anesthesia. Draining lymph nodes (popliteal, inguinal, axillary, and paraaortic) were aseptically excised, teased apart in PBS, and passed through a wire mesh to obtain a single-cell suspension. Cells were centrifuged and resuspended at 4 x 10^6 cells/ml in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum, 1 mM sodium pyruvate, 1% nonessential amino acids, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, antibiotics, and optimal concentrations of the following antigens: GMBP (50 μg/ml), PPD (50 μg/ml), and ConA (1 μg/ml). All cultures were incubated in triplicate in 96-well flat-bottom microtiter plates (Costar) for 72 h in a humidified atmosphere of 5% CO2/95% air at 37°C and pulse-labeled for the last 18 h with 1.0 μCi of [3H]thymidine (5 Ci/mm; 1 Ci = 37 GBq; New England Nuclear). Cells were harvested on fiberglass filters using a multiharvester (Dynatech), and the incorporated radioactivity was determined.

Analysis of T Lymphocyte Populations. Surface markers of lymphocytes from pooled spleen cells obtained from naive mice, untreated mice with CR-EAE, and mice with CR-EAE treated with linomide were analyzed. A cell suspension (2 x 10^7 cells per ml; 50 μl) was mixed with 5 μl of fluorescein isothiocyanate-conjugated anti-Thy1.2 or anti-L3T4 (CD4) or phycoerythrin-conjugated anti-Lyt-2 (CD8) (Becton Dickinson) and 45 μl of PBS containing 0.1% sodium azide and 2% (wt/vol) bovine serum albumin. The cell mixture was incubated for 30 min on ice, washed twice, and resuspended in 1 ml of ice-cold medium. Stained cells were counted in a fluorescence-activated cell sorter (Becton Dickinson).

**RESULTS**

Effect of Linomide Treatment on the Clinical Course of CR-EAE. As shown in Fig. 1 and Table 1, continuous oral administration of linomide starting on day 7 after immunization with MSCH (7 days before the expected clinical onset of the disease), completely prevented clinical signs of CR-EAE in all 17 treated mice (two experiments), whereas 19 of 20 control mice developed chronic-relapsing paralysis. Upon termination of linomide treatment, two of the treated mice developed a very mild weakness (grade 1) 2-7 days later. The rest remained disease-free for a period of >60 days. Furthermore, a booster MSCH injection that caused a severe attack with high mortality (60%) in 10 of 10 untreated mice, left all of the linomide-treated animals totally unaffected (with no signs of relapse; Fig. 1 and Table 1).

When linomide treatment was initiated after the appearance of clinical paralytic signs of CR-EAE, it prevented subsequent spontaneous relapses of the disease and rendered mice resistant to a renewed attack induced by a booster MSCH injection (Fig. 2). Only two out of eight mice relapsed mildly, whereas all eight untreated animals suffered a severe relapse with 65% mortality (Table 1 and Fig. 2).

The effect of linomide on an even further advanced stage of CR-EAE (closer homology to the clinical situation in MS) was then examined. Linomide treatment was started individ-

Two weeks on... on day 10/10.

1. Table 1. Incidence of CR-EAE in untreated and linomide-treated SJL/J mice: Effect of a booster MSCH injection

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Linomide from day 7</th>
<th>Linomide from day 14</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Linomide</td>
</tr>
<tr>
<td>Paralysis</td>
<td>19/20</td>
<td>0/17</td>
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<tr>
<td>Relapse*</td>
<td>10/10</td>
<td>2/8</td>
</tr>
<tr>
<td>Mortality*</td>
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SJL/J mice were immunized with MSCH plus CFA into the flanks on day 0 and 7. One group received linomide in the drinking water (50 mg/kg) starting at day 7 after sensitization; the other group started linomide-treatment after the onset of clinical signs (day 14). Two weeks after onset of paralysis, all mice were rechallenged with a booster MSCH plus CFA. The incidence of CR-EAE was measured on day 30.

*After rechallenge.

Fig. 1. Effect of oral administration of linomide on CR-EAE in SJL/J mice. Mice were immunized with MSCH in CFA on day 0 and on day 7. The control group (○) was left untreated and the experimental group (△) received linomide (0.5 mg/ml) in the drinking water, starting at day 7 after immunization. Animals were examined daily for signs of EAE. Results are the mean clinical score of seven or eight mice in each experimental group. Open arrow, initiation of linomide treatment; solid arrow, cessation of linomide treatment.

We then investigated the therapeutic potential of linomide in mice with passively induced CR-EAE. One group of mice was given linomide from the day of lymph-node-cell transfer (day 0) with the control group left untreated. Linomide treatment delayed significantly the onset of CR-EAE and ameliorated its clinical course but was generally less effective than in actively induced CR-EAE (Fig. 3). An active immunization with GMBP in CFA 75 days after injection with GMBP-specific lymphocytes induced a strong reactivation of the GMBP-reacting T cells and a severe paralytic relapse in all untreated animals. This reactivation was effectively inhibited in linomide-treated animals with CR-EAE.

Effect of Linomide on the Histopathological Manifestations of CR-EAE. Since linomide therapy completely inhibited all clinical signs of actively induced CR-EAE, it was important to determine whether it could also block the histopathological manifestations of the disease. Brain and spinal cord sections were obtained from linomide-treated and untreated mice. Brain and spinal cord sections of sick untreated mice showed marked perivascular and leptomeningeal lymphocytic infiltrations during the acute phase of the disease and periventricular demyelinating lesions at later stages. In contrast, immunized mice treated with linomide showed absolutely no evidence of CR-EAE pathology at various times after treatment; no invasion of activated lymphocytes or monocytes and no demyelination into the CNS were noted (data not shown).

Lymphocytic Proliferative Responses. Lymph node cells obtained from untreated and linomide-treated mice on day 10 after immunization for CR-EAE and from naïve SJL/J controls were examined for their in vitro proliferative responses to various antigens and mitogens. Lymphocytes from untreated CR-EAE mice strongly proliferated in response to PPD and GMBP (Table 2). In contrast, linomide-treated

Fig. 2. Effect of orally administered linomide after the clinical onset of CR-EAE. Mice were immunized with MSCH in CFA on day 0 and on day 7. The control group (○) was left untreated, and the experimental group (△) received linomide (0.5 mg/ml) in the drinking water, starting from day 14 after immunization, after the clinical onset of paralysis. Animals were examined daily for signs of EAE. Results are the mean clinical score of seven or eight mice in each experimental group. Arrow, initiation of linomide treatment.

Fig. 3. Effect of linomide treatment on passively induced CR-EAE. SJL/J mice were injected on day 0 with 6 × 10⁶ lymph node cells (LNCs) that were obtained (on day 10 after immunization) from syngeneic donors immunized with GMBP plus CFA and activated for 4 days in vitro in the presence of GMBP. Linomide was continuously administered in the drinking water (0.5 mg/ml) starting at day 0. On day 75 after lymph-node-cell transfer, all mice were challenged with GMBP in CFA. Mean clinical score is the mean of seven or eight mice per experimental group. ○, Untreated mice; △, linomide-treated mice; arrow, challenge with GMBP.
animals displayed a significantly weaker response to the same antigens (P < 0.01: two tail t test). The Con A- and rIL-2-induced proliferations were not significantly affected.

It is noteworthy that there were no differences in the total number of viable lymphocytes recovered from lymph nodes and spleens of limonide-treated animals and their untreated counterparts; it seems thus unlikely that limonide exerts a nonspecific immunosuppressive/cytotoxic effect. A similar reduction in the proliferative responses was found in passively induced CR-EAE (data not shown).

Analysis of Cell Surface Markers in Lymphocytes from Mice Treated with Limonide. Lymph node cells were obtained from untreated and limonide-treated animals with CR-EAE on day 25 after sensitization with MSCH. Flow cytometry analysis of the lymphocyte subpopulations did not reveal any significant differences in the proportions of NK1.1+, CD3+, CD4+, and CD8+ cells between limonide-treated and control animals (Table 3).

NK and Lymphokine-Activated Killer (LAK) Cell Activity in Untreated and Limonide-Treated Mice with CR-EAE. Pooled lymph node cells from untreated and limonide-treated mice were obtained on day 25 after MSCH inoculation and examined for their NK and LAK activity by evaluating cytotoxicity against YAC-1 target cells. Spontaneous NK activity was significantly increased in mice treated with limonide, as compared to untreated controls with CR-EAE (Table 3). In addition, lymph node cells from mice with CR-EAE treated with limonide showed a significant increase in LAK activity.

**DISCUSSION**

In the present study we have shown that limonide administration in the drinking water completely inhibited the development of both actively and passively induced CR-EAE in mice. Moreover, no paralytic signs were observed even after a booster injection with MSCH, which induced a severe relapse in all control animals. When limonide treatment was initiated after the clinical onset of CR-EAE, it totally prevented the spontaneous relapses of the disease and rendered mice resistant to an induced attack after a third MSCH injection. Histopathological analysis of mice treated with limonide showed no evidence of disease in the CNS. Brain and spinal cord sections from treated animals were completely normal without any lymphocyte infiltration. Furthermore, limonide inhibited the sensitization/activation of peripheral lymph node cells to MSCH and to PPD, as shown by the strongly suppressed in vitro proliferative responses of lymphocytes to the above antigens (Table 2); the antigen-independent T-cell-mediated responses, however, remained unaffected.

One of the major features of limonide involves activation of NK cells (36). According to some investigators, NK activity is reduced in several autoimmune diseases including MS (28–31); this reduction may be directly associated with the pathogenic process leading to immune disregulation and CNS damage in MS (28). In our model, limonide treatment strongly enhanced both NK and LAK activity in mice with CR-EAE. NK or NK-like suppressor cells may be important for normal immunoregulation. Some studies have shown that NK cells could downregulate a primary immune response (42) by interfering with antigen-presenting cells (mainly dendritic cells) that have previously interacted with the antigen (43). However, the in vivo relevance of NK cells in autoimmune and in normal immunoregulation has yet to be determined.

Our results indicate that limonide treatment strongly inhibits the sensitization/reactivation of lymphocytes after immunization with MSCH. Limonide prevented a relapse even when given after the first episode of paralytic disease. This could be of major importance for the treatment of MS. It seems that limonide interferes with antigen presentation at the early stages of T-cell activation. When antigen-presenting cells from limonide-treated animals were added to a MSCH-specific T-cell line, a significant reduction in the in vitro proliferation to GMBP was observed (unpublished results). However, limonide was also effective in the inhibition of passively induced CR-EAE (where no active immunization procedures are involved), implying that limonide can also interfere with activated T lymphocytes and prevent their further proliferation/expansion.

Limonide, in comparison to other immunomodulating treatments for MS, has several important advantages: (i) It can be administered orally and is very well tolerated. (ii) It is effective in CR-EAE even when given at advanced stages, after the first paralytic attack of the disease (Fig. 3). (iii) It totally prevents the histological CNS damage. (iv) It acts as immunomodulator rather than as immunosuppressor and as such is not associated with the long-term side effects of conventional immunosuppressive agents.

Limonide may influence immunoregulatory circuits through amplification of naturally existing suppressor/ regulatory networks that could be of the kind involved in the regulation of anti-self reactivity during the ontogeny of the immune system.
Although the mode of action of linomide in the modulation of induced and naturally occurring autoimmunity in genetically susceptible rodents is unknown, it is clear that this compound has a remarkable effect on prevention and treatment of clinical and histopathological manifestations of autoimmunity. The beneficial role of linomide in autoimmune disease is species- and target-antigen-independent; linomide effectively controlled T-cell autoreactivity against several epitopes contained in the homogenate of the spinal cord.

Orally administered linomide is very well tolerated in rodents, primates, and as shown in phase I and phase II clinical trials, also in patients with cancer, giving rise to only minor and acceptable adverse reactions without any cytotoxic or immunosuppressive effects (37). Optimal immunoregulatory effects of linomide occur at dose ranges that are much lower than the maximal tolerated dose. Hence, linomide could become an attractive immunomodulating therapeutic agent in future clinical trials for MS and other autoimmune disorders as well.

We thank Dr. D. Teitelbaum for her help in extracting GMBP. This research was supported in part by the Lena F. Harvey Endowment Fund for Neurological Research and by the Nina Silverman Neurological Endowment Fund.