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

(multiple sclerosis/natural killer cells)

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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 homogenate. 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 homogenate 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 homogenate (MSCH) 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 cyclophosphamide (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

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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; PPD, purified protein derivative; MSCH, mouse spinal cord homogenate; 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(F_1) 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 (3.0×10^6 units/mg, >97% pure) was kindly supplied by C. R. Franks (Eurocetus, 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 encephalitogenic 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 centrifugated and resuspended at 4×10^6 cells per 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, and antibiotics [penicillin G, sodium salt (10^4 international units/ml), and streptomycin (100 μg/ml)]. Cells were then plated in 24-well Costar plates (2 ml per well) and incubated for 4 days at 37°C in 5% CO_2/95% air. GMBP was added to each well at 50 μg/ml. Cultured viable cells ($30–60 \times 10^6$ cells per 0.5 ml) were injected intravenously into the tail vein of naive syngeneic SJL/J recipients. The first clinical signs of CR-EAE usually appeared 7–16 days after injection, and a chronic disease with relapses and remissions followed the first attack.

Clinical signs of CR-EAE were scored as follows: 0, no clinical signs of disease; 1, mild tail weakness (floppy tail); 2, tail paralysis; 3, hind leg paresis; 4, hind leg paralysis or mild forelimb weakness; 5, quadriplegia or moribund state; 6, death. Any deterioration of clinical signs by at least one degree according to the above scale, persisting for >3 consecutive days (after a period of remission), was considered as relapse. "Remission" was defined as a period of clinical improvement of at least one degree (according to our "severity scale") that lasts >7 days.

Histopathological Evaluation. For routine histology, untreated and linomide-treated mice immunized for CR-EAE induction were sacrificed under ether anesthesia and then perfused extensively with PBS-buffered formalin. Brains and spinal cords were removed and processed for paraffin embedding. Tissue sections were stained with hematoxylin/eosin and luxol fast blue. Histological evaluation was done on a blind basis by an independent pathologist.

***In Vitro* Proliferative Responses of Lymphocytes.** Single-cell suspensions of lymph node lymphocytes obtained from untreated and linomide-treated mice were assayed *in vitro* for their response to antigens by a standard proliferative assay ([^{3}H]thymidine incorporation). Approximately 4×10^5 cells (0.2 ml per well) were plated in RPMI 1640 medium with 5% 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% CO_2/95% air at 37°C and pulse-labeled for the last 18 h with 1.0 μCi of [^{3}H]thymidine (5 Ci/mmol; 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 (2×10^7 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-

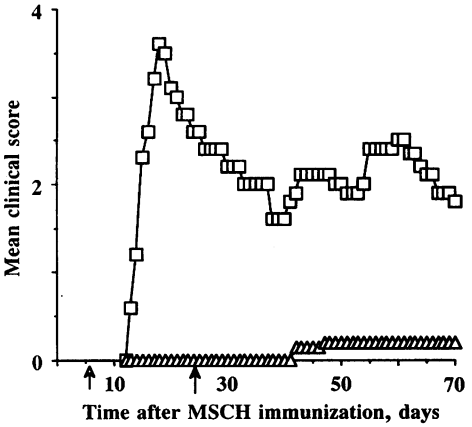

FIG. 1. Effect of oral administration of linomide on CR-EAE in SJL/J mice. Mice were immunized with MSCH in CFA on day 0 and on day 7. The control group (□) was left untreated and the experimental group (△) received linomide (0.5 mg/ml) in the drinking water, starting at day 7 after immunization. Animals 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.

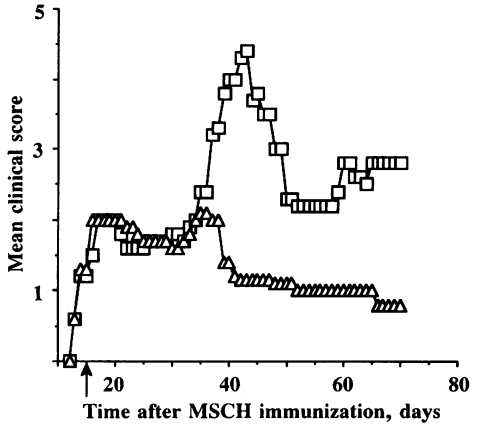

FIG. 2. Effect of orally administered linomide after the clinical onset of CR-EAE. Mice were immunized with MSCH in CFA on day 0 and on day 7. The control group (□) was left untreated, and the experimental group (△) received linomide (0.5 mg/ml) in the drinking water, starting from day 14 after immunization, 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.

ually for each mouse (8–20 days after clinical onset of disease) and its effect was evaluated. A total number of 22 spontaneous relapses of CR-EAE was observed in the control groups (mean relapses per mouse, 1.83 ± 0.3), as compared with 3 relapses in the linomide-treated group (mean relapses per mouse, 0.3 ± 0.2; $P < 0.001$). Treated mice were also more resistant to a renewed attack, induced by rechallenge with MSCH. Almost all untreated controls (seven of eight) relapsed after the rechallenge, compared with only two out of eight linomide-treated mice ($P < 0.01$).

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).

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

	Incidence of CR-EAE, no./total no.			
	Linomide from day 7		Linomide from day 14	
Symptom	Control	Linomide	Control	Linomide
Paralysis	19/20	0/17	6/8	4/8
Relapse*	10/10	2/8	8/8	2/8
Mortality*	6/10	0/8	5/8	0/8

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 (≈80 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.

Lymphocytic Proliferative Responses. Lymph node cells obtained from untreated and linomide-treated mice on day 10 after immunization for CR-EAE and from naive 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

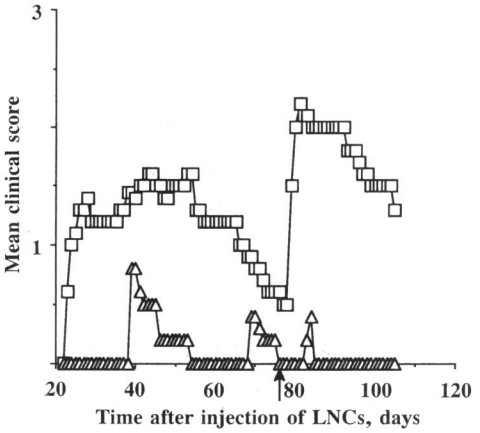

FIG. 3. Effect of linomide treatment on passively induced CR-EAE. SJL/J mice were injected on day 0 with 6×10^7 lymph node cells (LNCs) that were obtained (on day 10 after immunization) from syngeneic donors immunized with GMBP plus CFA and activated for 4 days *in vitro* in the presence of GMBP. Linomide 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.

Table 2. Proliferative responses of lymph node cells obtained from untreated and linomide-treated animals after induction of EAE

Antigen	[^{3}H]Thymidine incorporation, cpm		
	CR-EAE untreated	CR-EAE treated with linomide	Naive SJL/J control
GMBP	15,662 ± 2112	2,240 ± 216*	1,101 ± 214
PPD	21,313 ± 877	3,444 ± 317*	1,998 ± 334
CON A	88,737 ± 7665	56,524 ± 8112	23,431 ± 2219
rIL-2	45,736 ± 3239	44,867 ± 434	10,137 ± 991

All animals were immunized with MSCH for CR-EAE induction and either left untreated or treated with linomide (1.0 mg/ml) in drinking water, starting at day 7 after immunization. On day 10 after immunization, pooled lymph node cells (three animals from each experimental group) from untreated and linomide-treated animals were examined for their proliferative responses to GMBP, PPD, rIL-2, and Con A. All tests were performed in triplicate. Results are presented as mean ± SEM. One representative of three experiments is shown. Baseline cpm (without antigen) was <5000 cpm.
*$P < 0.01$, compared to untreated CR-EAE controls (two tail t test).

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 linomide-treated animals and their untreated counterparts; it seems thus unlikely that linomide 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 Linomide. Lymph node cells were obtained from untreated and linomide-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 linomide-treated and control animals (Table 3).

NK and Lymphokine-Activated Killer (LAK) Cell Activity in Untreated and Linomide-Treated Mice with CR-EAE. Pooled lymph node cells from untreated and linomide-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 linomide, as compared to untreated controls with CR-EAE (Table 3). In addition, lymph node cells from mice with CR-EAE treated with linomide showed a significant increase in LAK activity.

Table 3. Lymphocytic profile and NK activity of lymph node cells obtained from mice with CR-EAE treated with linomide

Group	Frequency, %			% cytotoxicity	
	Thy-1.2	CD4	CD8	NK	LAK
Naive SJL/J	79	48	40	4.26	41.5
CR-EAE control	91	58	28	3.1	31.2
CR-EAE + linomide	89	59	31	16.4*	64.2*

SJL/J mice were immunized with MSCH for induction of CR-EAE. Animals were left untreated or treated with linomide (1.0 mg/ml) in the drinking water, starting from the day of immunization. On day 25 after immunization for CR-EAE, pooled lymph node cells (three animals from each experimental group) from untreated and linomide-treated animals were examined by flow cytometric analysis for the expression of Thy-1.2, CD3, L3T4 (CD4), Lyt-2 (CD8), and NK1.1 surface markers and by means of a cytotoxic assay against YAC-1 target cells for their NK and LAK activities. Cumulative results from three experiments are shown. Statistical analysis was performed by using a two-tail Student's t test (linomide-treated group compared to untreated controls).
*$P < 0.01$, compared to CR-EAE control.

DISCUSSION

In the present study we have shown that linomide 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 linomide 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 linomide showed no evidence of disease in the CNS. Brain and spinal cord sections from treated animals were completely normal without any lymphocyte infiltration. Furthermore, linomide inhibited the sensitization/activation of peripheral lymph node cells to GMBP 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 linomide 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, linomide 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 autoimmunity and in normal immunoregulation has yet to be determined.

Our results indicate that linomide treatment strongly inhibits the sensitization/reactivation of lymphocytes after immunization with MSCH. Linomide 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 linomide interferes with antigen presentation at the early stages of T-cell activation. When antigen-presenting cells from linomide-treated animals were added to a GMBP-specific T-cell line, a significant reduction in the *in vitro* proliferation to GMBP was observed (unpublished results). However, linomide was also effective in the inhibition of passively induced CR-EAE (where no active immunization procedures are involved), implying that linomide can also interfere with activated T lymphocytes and prevent their further proliferation/expansion.

Linomide, 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.

Linomide 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 autoimmunity seems to be 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.

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