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Linomide, a new immunomodulatory drug, shows different effects on homologous *versus* heterologous collagen-induced arthritis in rats

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SUMMARY

The effects of the immunomodulatory drug Linomide (LS-2616) have been investigated on two variants of collagen-induced arthritis (CIA) in Lewis rats, i.e. arthritis induced either with heterologous (bovine) or with homologous (rat) collagen type II (CII). Treatment with Linomide from the day of immunization (prophylactic) had a mild ameliorative effect on the severity of arthritis in the heterologous CIA, while the homologous CIA was strongly augmented. In both models, Linomide treatment caused a more severe arthritis when given from onset of clinical signs of disease and onwards (therapeutic). Serum antibody levels to CII were significantly decreased by prophylactic Linomide treatment in rats immunized with heterologous CII, while elevated levels of anti-rat CII antibodies were seen in the homologous model. No effect on antibody levels was seen with the therapeutic treatment regime. The opposing effects of prophylactic treatment with Linomide in heterologous *versus* homologous CIA indicate that the immune response to an autoantigen may be regulated differently from that to a foreign antigen. These results further strengthen the view that heterologous and homologous CIA should be regarded as separate experimental models, and that the studies on homologous CIA may represent a novel approach for future studies of autoimmune responses and evaluation of anti-rheumatic drugs.

Keywords Linomide bovine collagen II rat collagen II arthritis

INTRODUCTION

Various experimental animal models have been used in the search for immunomodulating drugs and for the understanding of autoimmune diseases, such as rheumatoid arthritis. One experimental animal model of polyarthritis, with certain similarities to rheumatoid arthritis, is the type II collagen-induced arthritis (CIA) (Trentham, 1982). This experimental disease can be induced in certain strains of rats (Trentham *et al.*, 1977) or mice (Courtenay *et al.*, 1980) by immunization with heterologous or homologous native type II collagen (CII) (Trentham *et al.*, 1977; Holmdahl *et al.*, 1986). The development of arthritic lesions appears to be the result of an autoimmune response, dependent on a combined action of autoreactive T cells and autoantibody production against CII (Trentham *et al.*, 1978; Holmdahl *et al.*, 1988a).

Clinical and histological symptoms of the disease are often more easily induced with heterologous CII than with the homologous self antigen (Holmdahl *et al.*, 1985). Immunogenetic studies indicate a concordantly higher degree of responsiveness to the heterologous antigen as compared with the homologous protein (Holmdahl *et al.*, 1988b). It is not known how the introduction of 'foreign' determinants on the CII molecules influences the autoimmune reactions.

The recently described compound Linomide (LS-2616), a quinoline-3-carboxamide compound, has been shown in several experimental models to have immunomodulatory properties (Tarkowski, Gunnarson & Stålhandske, 1986a; Tarkowski *et al.*, 1986b). In the present study the effects of Linomide have been evaluated both on heterologous and homologous CIA in rats. The drug was administered by three different treatment regimens (pretreatment, prophylaxis, and therapy) and its effects on arthritis incidence and severity as well as on antibody production against CII was studied.

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MATERIALS AND METHODS

Animals

Female Lewis rats were used from an inbred colony at the animal unit at the Biomedical Centre in Uppsala. The Lewis rats were originally obtained from Möllegaard Laboratories (Roskilde, Denmark). The animals were used at 2–4 months of age.

Collagens

Bovine CII was obtained from the nasal cartilage of calf, and rat CII was obtained from a rat chondrosarcoma. Both types of collagens were prepared by pepsin digestion and purified as described by Miller (1977), except that the rat CII preparation was not subjected to ion-exchange chromatography during purification. The lyophilized collagens were stored at −20°C prior to use.

Induction and evaluation of arthritis

Native CII dissolved in 0·1 M acetic acid at a concentration of 1 mg/ml was emulsified in an equal volume of Freund's incomplete adjuvant at 4°C, and 500 μl of the emulsion were injected intradermally around the root of the tail. Rats were randomly selected for inclusion in the groups given Linomide and water, respectively. The animals were observed every second day for development of arthritis. Clinical severity of arthritis was quantified according to the following grading scale: 1, detectable swelling in one joint; 2, swelling in two or more joints; 3, severe swelling of the entire paw and/or ankylosis. The maximum possible score was 12.

Drug treatment

Linomide (LS-2616, *N*-methyl-*N*-phenyl-1,2-dihydro-4-hydroxy-1-methyl-2-oxo-quinoline-3-carboxamide, sodium salt hydrate; Pharmacia LEO Therapeutics AB, Helsingborg, Sweden) provided in lyophilized form was dissolved in the drinking water to a final concentration of 0·38 mg/ml and was prepared fresh every 3 days. The average water consumption of a rat weighing 180 g was found to be 19 ml/day, thus resulting in a daily dose of Linomide of 40 mg/kg body weight per day. The addition of Linomide to the drinking water was found not to influence the water consumption of the rats; control animals received tap water only. Toxicological studies, performed *in vitro* and *in vivo* in several animals species including rodents and monkeys have shown that Linomide is non-mutagenic and non-cytotoxic in the concentration used in the present study (T. Stålhandske, unpublished results).

Collection of sera

Rats were bled under ether anaesthesia by retro-orbital puncture at days 16 and 35 post immunization. Sera was collected individually and stored at −20°C until assayed.

ELISA

For quantification of anti-CII reactive antibodies in sera, an ELISA technique was used. Microtitre plates (Dynatech, Plochingen, FRG) were coated overnight at 4°C with 50 μl/well of 10 μg/ml bovine or rat CII in phosphate-buffered saline (PBS) (pH 7·4). All subsequent incubations were made in a volume of 50 μl/well. Dilutions of sera and washings were done with PBS containing 0·05% Tween 20. All tests were carried out in duplicate.

The amount of antibody in a sample was estimated after incubation with goat anti-rat IgG antibodies conjugated to alkaline phosphatase (Jackson Laboratories, West Grove, PA). The subsequent quantification of bound enzyme was performed with a para-nitrophenol-containing substrate buffer in a Titertek multiscan spectrophotometer (Flow Laboratories, Irvine, UK).

In estimating anti-CII reactive antibodies in serum, purified anti-CII antibodies were used as the standard and were titrated in 10-fold dilution steps in parallel with the unknown serum samples. Affinity-purified antibodies from Lewis rats immunized with bovine CII were used as a standard; anti-CII antibodies were purified from serum by chromatography on sepharose-bound bovine CII. The purified antibodies were quantified spectrophotometrically at 280 nm. For each serum sample, the dilution that gave 50% absorbance compared with the maximum value obtained for the standard antibodies was related to the concentration (mg/ml) of standard antibody to give the same 50% absorbance value. By this method all calculations were performed with data from the steep portion of the slope, where the sample titration curves were parallel to the standard titration curves.

Statistical analysis

Mean day of onset and antibody levels were analysed by Student's *t*-test, and Wilcoxon's rank sum test was used for arthritic scores. The χ^2 test was used to analyse the incidence of arthritis. P values $<0·05$ were considered to be statistically significant.

RESULTS

Effects of prophylactic and therapeutic treatment with Linomide on arthritis induced with heterologous (bovine) CII

Rats immunized with bovine CII were given Linomide either from the day of immunization (prophylactic) or from the day of onset of clinically evident arthritis (therapeutic). Rats immunized simultaneously and given only tap water were used as controls. Administration of Linomide prophylactically had only a minor effect on the development of arthritis (Table 1, Fig. 1); all rats within both the Linomide and the control groups developed arthritis and the mean day of onset was similar. However, the development of arthritis measured by arthritic scores may have occurred a little more slowly in the Linomide treated group, but the scores were the same at the termination of the experiment.

When the rats were treated with Linomide therapeutically, the effect of the drug was very different. Significant aggravation of the arthritis was apparent throughout the experimental period in the group of animals given Linomide when compared with the control group (Fig. 2).

Table 1. Incidence and day of onset of arthritis in rats immunized with heterologous (bovine) CII with or without administration of Linomide

	Treatment (days)	
	Linomide (0–35)	Control
Incidence*	6/6	10/10
Mean day of onset	16·7	14·6

* Number of rats with arthritis per number of rats tested.

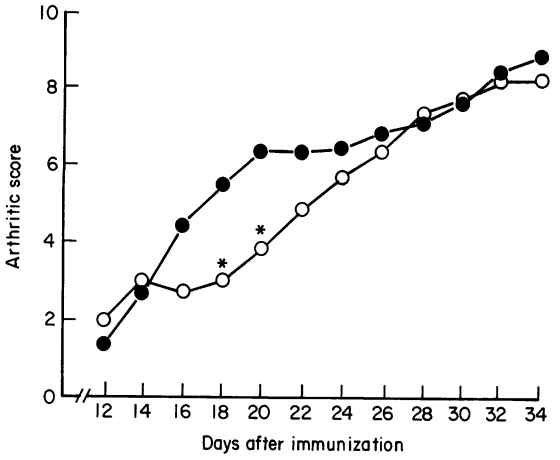

Fig. 1. Effects of prophylactic administration of Linomide on mean arthritic scores in arthritic rats immunized with heterologous CII. (○) Linomide-treated group; (●) control group, given water only. * $P<0.05$.

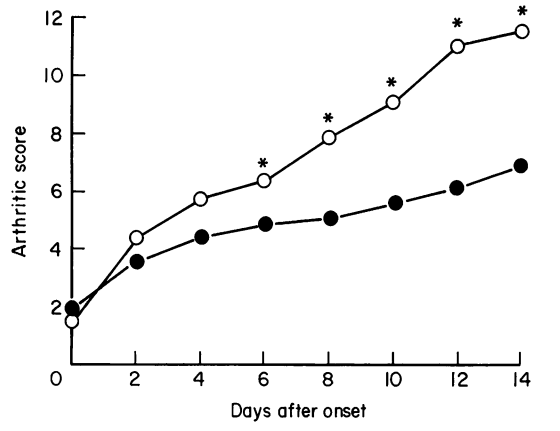

Fig. 2. Effects of therapeutic administration of linomide on mean arthritic scores in arthritic rats immunized with heterologous CII. (○) Linomide-treated group; (●) control group, given water only. * $P<0.05$.

Effects of prophylactic and therapeutic treatment with Linomide on arthritis induced with homologous (rat) CII

Rats immunized with native rat CII were given Linomide either by the prophylactic or the therapeutic treatment regime. In a separate experiment we also investigated the effect on arthritis when Linomide was administered to the rats for 2 weeks before CII immunization but not after immunization.

In contrast to heterologous CIA, Linomide administration made the disease more severe both when the drug was given prophylactically and therapeutically (Figs 3 and 4). In addition, prophylactic treatment resulted in a moderate but significant increased incidence and an earlier onset of disease (Table 2). Pretreatment with Linomide before immunization had no effect on disease development (Fig. 3).

Effects of Linomide administration on antibody production to CII in heterologous and homologous CIA

Tables 3 and 4 show the development of serum antibody titres to bovine and/or rat CII from the experiments described above.

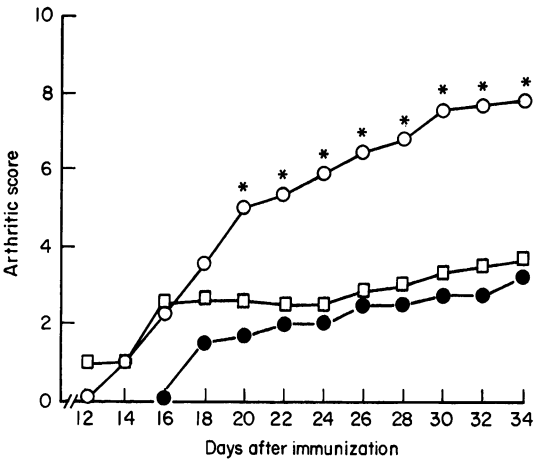

Fig. 3. Effects of prophylactic administration of Linomide on mean arthritic scores in arthritic rats immunized with homologous CII. (○) linomide-treated group; (●) control group, given water only; and (□) group of rats that received Linomide for 2 weeks before CII immunization but not thereafter. * $P<0.05$.

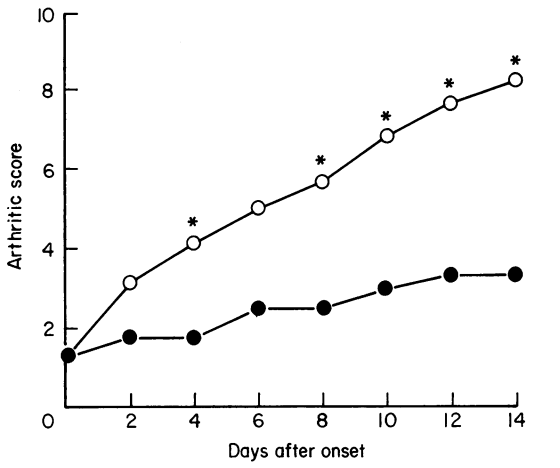

Fig. 4. Effects of therapeutic administration of Linomide on mean arthritic scores in arthritic rats immunized with homologous CII. (○) Linomide-treated group; (●) control group, given water only. * $P<0.05$.

Table 2. Incidence and day of onset of arthritis in rats immunized with homologous (rat) CII with or without administration of Linomide

	Treatment (days)		
	Linomide (0–35)	Linomide (−14–0)	Control
Incidence*	9/9†	6/10	4/10
Mean day of onset	16·2†	15·7	20

* Number of rats with arthritis per number of rats tested.

† $P<0.05$ compared with the control group.

Table 3. Serum levels of anti-CII antibodies (IgG) (mg/ml) in rats immunized with bovine CII with or without administration of Linomide (mean ± s.d.)

Treatment (days)	Number of rats	Day 16 post immunization		Day 35 post immunization	
		Bovine CII	Rat CII	Bovine CII	Rat CII
Prophylactic					
Control	10	1·15 ± 0·28	0·39 ± 0·16	3·03 ± 1·24	0·60 ± 0·25
Linomide (0–35)	6	0·66 ± 0·20*	0·21 ± 0·14*	1·12 ± 0·49*	0·53 ± 0·21
Therapeutic					
Control	9	0·51 ± 0·10	0·33 ± 0·10	2·00 ± 0·79	0·39 ± 0·18
Linomide (onset–35)	8	0·51 ± 0·21	0·35 ± 0·19	2·79 ± 1·17	0·67 ± 0·34

* $P < 0·05$ compared with the control group.

Table 4. Serum levels of anti-CII antibodies (IgG) (mg/ml) in rats immunized with rat CII with or without administration of Linomide (mean ± s.d.)

Treatment (days)	Number of rats	Day post immunization	
		16	35
		Rat CII	Rat CII
Prophylactic			
Control	10	0·14 ± 0·09	0·22 ± 0·08
Linomide (0–35)	9	0·21 ± 0·14	0·38 ± 0·22*
Linomide (−14–0)	10	0·24 ± 0·09*	0·29 ± 0·11
Therapeutic			
Control	4	ND	0·24 ± 0·10
Linomide (onset–35)	7	ND	0·57 ± 0·53

* $P < 0·05$ compared with the control group.
ND not done.

The antibody titres were significantly decreased by prophylactic Linomide treatment in rats immunized with heterologous CII, and elevated levels of anti-rat CII antibodies were seen in rats immunized with the homologous CII.

DISCUSSION

In this study we were concerned with two different issues. The first was the effect of the immunomodulatory drug Linomide on various experimental immunological conditions. This question is of considerable principal and practical interest, as this drug has been shown to have profound ameliorative effects on experimental lupus in NZB/W mice and on survival and kidney disease in the MRL lpr/lpr mice (Tarkowski *et al.*, 1986a; b). Investigations of other types of experimental autoimmune diseases with this drug, however, are particularly urgent as the effects of this drug may be two-edged inasmuch as it not only ameliorates certain autoimmune conditions, but may also enhance other immune reactions such as delayed-type hypersensitivity (DTH) reactions in the lungs of rats (Stålhandske & Kalland, 1986).

The second issue of interest was the use of different variants of the CIA both for basic studies of autoimmunity and for the evaluation of different immunomodulatory regimens. We have previously observed certain differences between the clinical course of arthritis induced by homologous and heterologous CII respectively in both mice (Holmdahl *et al.*, 1986) and rats (Larsson *et al.*, manuscript submitted). Immunogenetic studies in mice suggest the existence of two different T cells of importance for development of CIA; one recognizing heterologous CII and one also reacting with homologous CII (Holmdahl *et al.*, 1988b). T cells recognizing 'foreign' determinants on heterologous CII are likely to provide help to potentially pathogenic auto-anti-CII producing B cells and such T cells may be regulated differently from those auto-reactive T cells that only recognize the 'self' CII molecule. In this perspective it was most interesting to investigate whether the effects of an immunomodulatory drug are different in these two models.

Finally, in both the heterologous and homologous CIA models we had to make a distinction between prophylactic and therapeutic treatment; the latter situation is the one that would eventually lend itself to comparisons with a human situation.

As to the first of the raised issues, all the experiments except the one in which rats immunized with heterologous CII were given Linomide prophylactically showed an increased incidence and/or severity of arthritic disease, which is in line with the previous experiments on the enhancement of DTH reactions to pertussis in the rat lung (Stålhandske & Kalland, 1986). These results are thus in contrast with those obtained in the above-mentioned mouse lupus models. We do not yet know the mechanism to explain this discrepancy, since the basic mode of action of Linomide is not yet known. However, an explanation of the different effects of prophylactic Linomide treatment in heterologous *versus* homologous CIA on disease development and CII antibody titres may be that Linomide exhibits different effects on pre-activated and previously not activated T cells, which in turn may influence B cell autoantibody production differently. Thus, we have preliminary evidence that T cells reactive with homologous CII may be activated in the unimmunized Lewis rat, whereas no signs of such pre-activation have been observed for heterologous CII. This postulated effect of Linomide on T cell reactivity may also have bearings on the mouse lupus models that exhibit a decreased response to mature T cells (Dixon, 1979).

The basis for the discrepancy between the effects in lupus on one hand and CIA on the other hand, as well as the discrepancy of the results of the different collagen models remains unre-

solved; perhaps the present experimental systems may be of help in the study of cellular and molecular basis for the Linomide effects. The main conclusion as to the drug action is that the drug is two-edged also in autoimmune situations and, therefore, that further applications of the drug, e.g. in clinical trials, should be performed and monitored with great caution.

As to the second principal issue—the different results of the administration of an immunomodulatory drug in heterologous *versus* homologous CIA—the present data further strengthen the idea that basic differences in immunoregulation exist in these two models. As discussed in detail above, we do not at present have an explanation for the divergent effects of prophylactic treatment with Linomide in the two models, but the results further indicate that homologous and heterologous CIA should be considered as two separate models both during basic studies of autoimmunity and in further testing of drugs. The latter point may be of particularly importance since pharmaceutical industries are increasingly using heterologous CIA as a standard model in the examination of new immunomodulatory drugs with potential applications on humans.

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