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Immunomodulation of rat antigen-induced arthritis by leflunomide alone and in combination with cyclosporin A

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Abstract. The effects of the new immunomodulating isoxazol derivative leflunomide, in comparison with cyclosporin A, on established antigen-induced arthritis in rats as well as serum antibody levels were determined. When treatment with leflunomide, at concentrations from 2.5 to 10 mg/kg/d, was started on day 3 of arthritis, the acute and chronic phases of arthritis were effectively inhibited. This was demonstrated by decreased joint swelling and reduced histopathological arthritis score at the end of experiment (day 26). Furthermore, the treatment resulted in a significantly reduced level of serum antibodies to the matrix components collagen type I, type II and proteoglycans. Neither leflunomide nor cyclosporin A, at doses of 1 mg/kg/d, had an effect on the severity of arthritis and antibody levels. However, when both drugs were used together, at these non-effective doses, the histopathological score of chronic arthritis was significantly reduced. The results of our experiments demonstrate that leflunomide has a strong suppressive effect on both acute and chronic phases of antigen-induced arthritis and formation of autoantibodies in rats. Furthermore, orally administered doses of leflunomide were as effective as doses of cyclosporin A given intraperitoneally. The combination of sub-effective doses of leflunomide and cyclosporin A resulted in significant inhibition of chronic arthritis.

Key words: Antigen-induced arthritis – Leflunomide – Inflammation – Tissue destruction – Autoantibodies

Introduction

Leflunomide (HWA 486) is a novel isoxazol derivative, developed by Hoechst AG, with immunomodulating properties, which is structurally unrelated to any of the known immunosuppressive agents. In the first study, Bartlett and Schleyerbach [1] showed that leflunomide (Lef) prevents the onset of adjuvant arthritis, provided the therapy was started within the first 12 days after induction. The disease-modifying properties in adjuvant arthritic rats were confirmed later by others [2, 3]. Lef treatment of mice with proteoglycan induced arthritis resulted in suppression of acute inflammatory events and blocked the progression of chronic arthritis. Antibody response to proteoglycans declined significantly during the treatment [4]. Lef has been shown to be very efficacious in rheumatoid arthritis (RA) patients, exhibiting good tolerability [5]. The drug is currently being tested in clinical phase III trials for RA.

Antigen-induced arthritis (AIA) is an experimental model displaying several characteristics similar to human RA. A single injection of an antigen into the knee joint cavity of animals preimmunized with the same antigen in complete Freund's adjuvant results in an inflammatory response within the joint. Persistent destructive arthritis can only continue in animals capable of sustaining cellular and humoral immunity to the immunizing antigen. The chronic phase of arthritis, characterized by hyperplasia of synovial lining layer, predominant infiltration of mononuclear cells into the synovium and formation of synovial pannus, which erodes the cartilage and bone, persists for weeks and months. AIA was first described in rabbits by Dumonde and Glynn [6]. Later, it was also established in mice [7] and rats [8, 9]. In rodents, chronic joint inflammation can be induced only by the use of cationic antigens, predominantly methylated bovine serum albumin [10]. Until now, there have been no reports on the influence of Lef on this experimental model of arthritis. The purpose of this study was to examine the immunomodulatory effects of Lef, in comparison to those of cyclosporin A, on the development of arthritis and autoimmunity in rats.

Materials and methods

Induction and assessment of arthritis

Female Wistar rats (Han: WIST; Central Animal Research Facility, Beutenberg-Campus Jena), 10–12 weeks of age, were immunized, subcutaneously, on two occasions, 7 days apart, with 0.5 mg

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methylated bovine serum albumin (mBSA; Serva, Heidelberg) in 0.5 ml saline, emulsified with 0.5 ml complete Freund's adjuvant (CFA; Sigma, Deisenhofen) containing 1.5 mg/ml heat-killed *Mycobacterium tuberculosis* strain H37RA (Difco, Augsburg), and intraperitoneally with 2×10^9 killed *Bordetella pertussis* organisms (SIFIN, Berlin) in 100 µl saline. Two weeks after the second immunisation (day 0), the arthritis was elicited under ether anaesthesia by injection of 0.5 mg mBSA, as a sterile solution in 50 µl saline, into the right knee joint cavity.

Arthritis was monitored by measurement of the lateral joint diameter, using a vernier caliper (Mitutoyo No. 7305) before and at intervals after induction of the arthritis. Knee swelling was expressed as the difference between the two measurements. At the same time points the body weight was estimated. At the end of the experiment (day 26), the animals were bled, under anaesthesia, serum collected and stored at −20 °C until assaying for antibodies. The knee joints were removed, opened, fixed in 4% buffered formaldehyde and decalcified. After paraffin embedding and sectioning, conventional haematoxylin/eosin staining was performed. Slides were evaluated by two independent observers, blinded to the group origin. The extent of changes on lining cells, inflammatory infiltration, pannus formation and cartilage degradation (loss of matrix and necrosis of chondrocytes) were graded separately using a semiquantitative scale from 0–3 (by use of half-points), with higher numbers indicating progressive degrees of inflammation or destruction. Spleen and thymus were weighed.

Drug administration

Drug treatment was started on day 3 after arthritis induction and finished on day 21 (5 times a week with 10 rats/group). Lef (Hoechst, Frankfurt/M) was administered orally at a dose of 2.5, 5, or 10 mg/kg/day suspended in 2 ml of 1% Na-carboxymethylcellulose (Fluka, Buchs/Switzerland). Cyclosporin A (CsA: Sandimmun®; Sandoz, Nürnberg) was injected intraperitoneally at a dose of 5 mg/kg/day in 100 µl saline. The control group received vehicle (Na-carboxymethylcellulose) orally at the same time points.

In a further experiment, rats with AIA (10 animals/group) were treated daily from day 3 to day 22 with Lef or CsA (1 mg/kg/day) alone or with both drugs together in the same concentrations. The control group received saline i.p. The animals were killed on day 23.

Antibody determination

Antibodies were measured with an ELISA assay as described [11]. Briefly, antigen was adsorbed to microtiter plates (Greiner, Nürtingen) at a concentration of 10 µg/ml overnight at 4 °C. All other manipulations were made at room temperature. The sera, inactivated at 56 °C for 30 min, were diluted 1 : 50 (for mBSA antibodies 1 : 250) with wash buffer (PBS, pH 7.2, with 0.5% Tween 20), incubation time 2 h, followed by the detection of bound rat IgG with 1 : 5.000 diluted peroxidase-conjugated anti-rat IgG from goat (Serva). 0.2% o-phenylenediamine with 0.05% H_2O_2 was used as substrate for peroxidase. Collagen type II (CII) and proteoglycans (PG) were prepared from calf articular cartilage [11]. CII from rat chondrosarcoma was a gift from Dr. K. von der Mark (Erlangen), collagen type I (CI) from rat tail was obtained from Sigma.

Statistical analysis

The GraphPad InStat computer program, Version 2.00, was used for all calculations and statistical evaluations. All data are expressed as mean values ± SEM. Differences between the groups were tested for significance by the Mann-Whitney U-test (one-tailed).

Results

Effect of treatment on established arthritis

At the beginning of treatment, 3 days after arthritis induction, the mean value of knee joint swelling, in the various groups, achieved 3.4 mm–4.4 mm. Because of these differences, the swelling for each animal was calculated in % of the swelling at day 3 (Fig. 1). The effect of treatment was recognizable already at day 7 of arthritis, i.e. 4 days after onset of treatment. Only the vehicle-treated control group showed from day 3 to 7 a further weak increase of swelling. After day 7, the swelling in all groups decreased and was clearly lower in drug-treated groups than in controls at any specified time. At day 14 the differences in all treated groups, from the control group, were significant. In animals treated orally with 5 and 10 mg/kg Lef the diminution was stronger than in those treated with 2.5 mg/kg Lef or with 5 mg/kg CsA intraperitoneally.

These results were confirmed by histopathological examination of knee joints at the end of the experiment. Treatment with Lef significantly reduced the arthritis in a dose-dependent manner when compared to the control group (Fig. 2). The severity of arthritis after intraperitoneal treatment with 5 mg/kg CsA was similar to that observed in the group with Lef at an oral dose of 5–10 mg/kg.

The efficacy of treatment could be observed on synovitis as well as cartilage destruction. In this connection, it should be noted that histopathological indices of

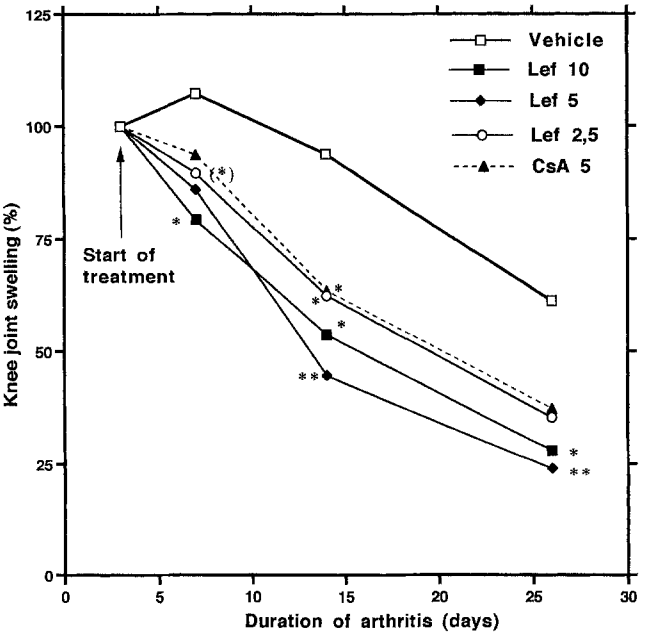

Fig. 1. Effect of leflunomide (Lef), at various concentrations, and cyclosporine A (CsA) on the course of knee joint swelling from rats with AIA. CsA (5 mg/kg/d) was administered i.p., leflunomide (10, 5 or 2.5 mg/kg/d) was given orally from day 3 to 21 (5 times/week). The joint swelling, expressed in % of swelling at the day 3, is decreased in the drug-treated groups compared with vehicle-treated controls. n = 10 (animals per group), (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$.

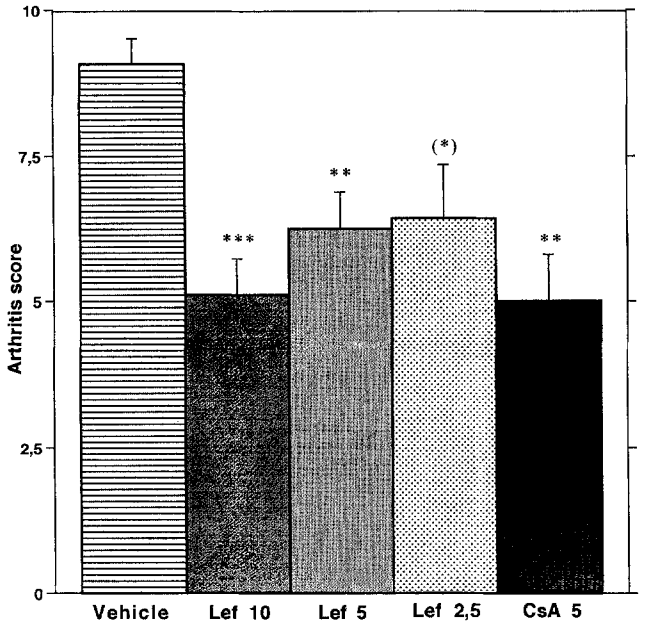

Fig. 2. Histological score of arthritis in the knee joint of rats with AIA at the end of the experiment (day 26). The arthritis in the Lef-treated groups was decreased, dose-dependently, in comparison to vehicle. Intraperitoneal application of 5 mg/kg/d CsA resulted in a similar suppression as Lef at an oral dose of 10 mg/kg/d. (*) $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$.

joint damage were significantly correlated with the joint swelling at the end of the experiment.

In a further experiment, 4 groups of rats with AIA were treated with either saline (control), low concentrations of Lef or CsA (1 mg/kg), or both drugs together.

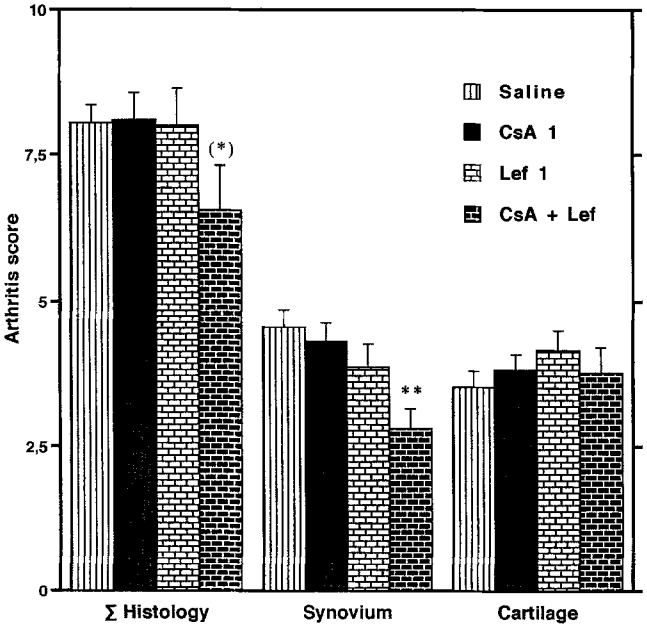

Fig. 3. Histological score of arthritis in the knee joint of rats with AIA after long-term treatment (day 3 to 22) with subeffective doses of Lef (1 mg/kg/d orally) or CsA (1 mg/kg/d i.p.) alone and in combination. Only in the group treated with both drugs together was the arthritis score significantly diminished, and the difference was only marked in synovitis, not in cartilage destruction. (*) $p < 0.1$, ** $p < 0.01$.

The course of knee joint swelling was nearly identical in all groups, i.e. there were no statistically significant differences between the groups at any time point (data not shown). However, the histopathological evaluation showed a significantly diminished arthritis score in animals after treatment with Lef and CsA together (Fig. 3). The reduction of synovitis was highly significant compared with saline control group ($p < 0.01$), but no difference in cartilage damage was seen.

Effect of treatment on serum level of antibodies

As seen in Figure 4, there was a minimal decrease in levels of antibody, to the immunizing antigen mBSA, in drug treated groups when compared to control, but no significant differences were observed. However, the levels of antibodies to matrix components collagen type I (CI), type II (CII) and proteoglycans (PG) were significantly reduced in drug treated groups when compared to control. The antibody levels to CII, from bovine, and CII, from rat, showed scarcely any differences and, thus, were summarized. Lef, at a dose of 10 mg/kg, resulted in the most marked decrease in antibody levels.

Administration of Lef or/and CsA at doses of 1 mg/kg only resulted in a small reduction of antibody levels compared to control without statistically significant differences (data not shown).

Effect of treatment on body, spleen and thymus weight

The body weight of animals treated with different concentrations of Lef was significantly reduced for a few days shortly after the beginning of treatment when compared to either control or CsA group (day 7: $p < 0.01$). By day 14 and day 26, the Lef treated groups had gained comparable weights to both control and CsA groups (Fig. 5).

At nontoxic concentrations of Lef, neither spleen weight nor thymus weight showed significant differences between the groups (data not shown).

Discussion

In the treatment of RA the need for drugs that are able to suppress inflammation and, at the same time, modify the underlying immune response is great. The AIA is a model which shows a striking resemblance to the human disease, especially in the feature of chronic synovitis and cartilage degradation. Furthermore, pharmacological studies have shown that this model responds to antirheumatic agents in a manner similar to RA [12]. Lef has potent activity against a variety of experimental autoimmune disorders in rodents and profoundly suppressed rejection of organ allografts and xenografts [13]. In view of its potent inhibition of T-cell as well as B-cell activities we have examined the immunomodulatory effects of Lef on established AIA and autoantibody formation in rats.

Compared with vehicle-treated controls, we found significant suppression both of arthritis and antibodies to

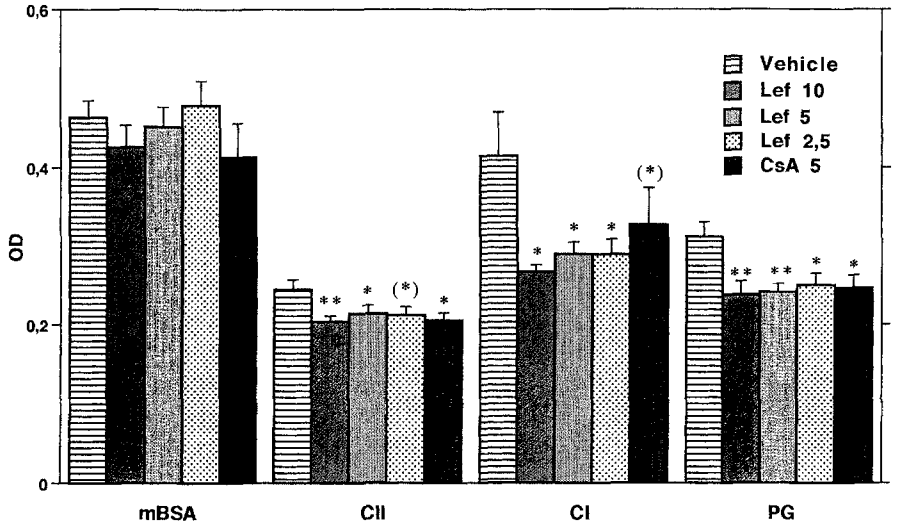

Fig. 4. Level of serum antibodies against mBSA and the matrix components collagen type I (CI), collagen type II (CII) and proteoglycans (PG) in rats with AIA. Antibodies against matrix components were significantly reduced by treatment with Lef (2.5 to 10 mg/kg/d) and CsA (5 mg/kg/d) in comparison with the control (vehicle). (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$.

matrix components CII, CI and PG in rats with established AIA by treatment with Lef in doses from 2.5 to 10 mg/kg/day. The results obtained with intraperitoneal CsA therapy were very similar to those observed after oral treatment with Lef. CsA is a well-known and powerful immunosuppressive drug widely used in the treatment of autoimmune diseases or allograft rejection [14]. Administration of CsA effectively inhibited the chronic phase of AIA in rabbits and rats [15, 16]. Moreover, in arthritic rats, the IL-6 levels in serum and synovial fluid were reduced [16].

Of specific clinical interest are experiments describing the synergistic interactions of Lef and CsA. When Lef and CsA are used in combination and at nontherapeutic doses, significantly prolonged graft survival was observed [17, 18, 19]. Pharmacokinetic studies showed that the combined use of CsA and Lef did not affect the levels of CsA, suggesting that the synergistic effect was not caused by reduced CsA elimination [17]. Until now, there are no reports on a synergy between Lef and CsA in treatment of arthritis. Although Lef or CsA at a dose of 1 mg/kg/day alone were not sufficient to cause a reduction of arthritis in our experiment, the combination of both drugs, at the same concentrations, led to a significant reduction of histological score of synovitis. The ability of Lef to inhibit T- and B-cell proliferation at a later stage in the cell cycle than CsA may account for this additive or synergistic effect with CsA.

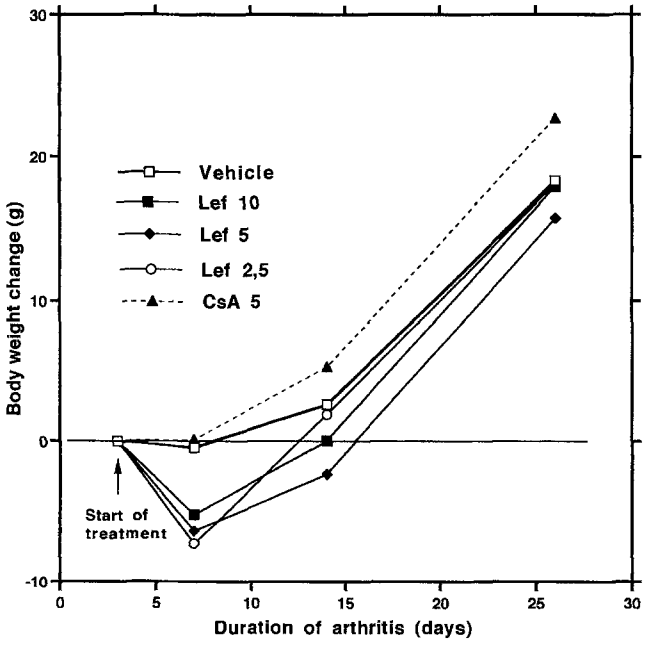

Fig. 5. Change of the body weight in rats with AIA in the course of treatment with Lef or CsA. In the first few days after treatment, a decrease of body weight in all Lef-treated groups was observed. Day 7: Lef 2.5–10 < vehicle, CsA ($p < 0.01$).

In AIA immunization with CFA already leads to the production of antibodies to matrix components. These antibodies, in connection with cell-mediated immunity, are probably involved in the maintenance of chronicity [11, 20]. The significant correlations between serum levels of antibodies to matrix components and severity of arthritis support the hypothesis that autoimmunity plays a role in the pathogenesis of the chronic phase in this arthritis model.

Lef inhibits T-cell as well as B-cell functions. The precise mechanism of its action is still incompletely understood. Inhibition of tyrosine kinases is probably involved in the mechanism of inhibiting T-cell mediated responses [21]. The ability of Lef to block T-cell proliferation stimulated by IL-2 suggests that its site of action in the cell cycle is later than CsA. Another aspect of Lef is its inhibitory effect on the release of histamine from basophils and mast cells [13]. Newer data indicate that Lef may affect de-novo pyrimidine synthesis, and this may account for its anti-proliferative activity [22, 23].

The results described in this report clearly demonstrate the effectiveness of Lef at nontoxic doses in the treatment of established AIA and inhibition of autoantibody formation. Our findings enlarge the number of disorders which have been shown to be suppressed by this new immunoregulatory agent and, furthermore, contribute to clarify the mechanisms involved in the pathogenesis of this chronic joint disease.

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