Inhibitory effects of anti-inflammatory drugs on type II collagen induced arthritis in rats

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SUMMARY The effects of steroidal and non-steroidal anti-inflammatory drugs on the established lesion of type II collagen induced arthritis in rats were evaluated by measuring the hind paw oedema and anti-type II collagen antibody titre. Dexamethasone, a steroidal anti-inflammatory drug, reduced the anti-type II collagen antibody titre and markedly suppressed the established lesion of type II collagen induced arthritis in rats. A rebound of the arthritis, i.e., a rapid recovery of the hind paw swelling took place after withdrawal of the treatment with steroidal anti-inflammatory drugs, including dexamethasone, prednisolone, and hydrocortisone. On the other hand, indomethacin, benoxaprofen, piroxicam, and tiolfamizole, which are cyclo-oxygenase inhibitors in prostaglandin synthesis, had no effect on anti-type II collagen antibody titre, but suppressed the established lesion of the arthritis without causing an apparent rebound of the arthritis after withdrawal of the drug treatment. These results suggest that the level of anti-type II collagen antibodies has no relation to the intensity of hind paw swelling in the established lesion of the arthritis, though the antibodies contribute to the incidence of the arthritis. It also indicates that non-steroidal anti-inflammatory drugs having inhibitory action on cyclo-oxygenase are useful antiarthritic drugs without causing the rebound phenomenon, an untoward side effect after withdrawal of steroidal anti-inflammatory drugs.

Key words: experimental arthritis, rebound phenomenon.

In rheumatoid arthritis it has been suggested that prostaglandins may be important mediators of many inflammatory processes.1 Prostaglandins of the E series were found to be stimulators of bone resorption in vitro,2 suggesting that they contribute to the destruction of joints in rheumatoid arthritis. Type II collagen induced arthritis in rats is an experimental animal model of rheumatoid arthritis.3–5 In this model it is speculated that anti-type II collagen antibodies contribute to the incidence of arthritis, and prostaglandins as chemical mediators are associated with acute vascular permeability and bone resorption in the inflammatory processes of joints.

Kalindiphadke et al reported that steroidal and non-steroidal anti-inflammatory drugs were effective for treatment of type II collagen induced arthritis in rats.4 There are scarcely any reports, however, that investigate not only anti-inflammatory effects but also side effects, including reactivation of the arthritis when corticoid therapy is withdrawn.6 Clinically, this rebound phenomenon is an untoward side effect of corticoid therapy. In a previous paper we reported that rebound of the granulomatous inflammation occurs after withdrawal of corticoid therapy.7

New non-steroidal anti-inflammatory drugs, such as piroxicam, benoxaprofen, and tiolfamizole, are known as selective inhibitors of cyclo-oxygenase.8–10 In this paper we discuss the possibility that anti-type II collagen antibodies may have a relationship with the incidence of arthritis, and describe new anti-inflammatory agents having inhibitory action on cyclo-oxygenase, which are effective on type II collagen induced arthritis in rats without the apparent rebound phenomenon usually found after treatment with steroidal anti-inflammatory drugs.
Materials and methods

RATS
Outbred female Sprague-Dawley rats (150–250 g, Charles River Japan Inc.) were freely given standard laboratory food and water.

COLLAGEN PREPARATION
Type II collagen was prepared by limited pepsin digestion of pulverised bovine cartilage according to the method of Trentham et al. Lyophilised collagen was dissolved in 0.01 M acetic acid overnight at a concentration of 2 mg/ml. The solution was emulsified in an equal volume of incomplete Freund’s adjuvant (IFA; Difco Laboratories, Detroit, Mich. USA).

IMMUNISATION
Rats, 6 weeks old, were immunised by intradermal injection of approximately 0.5 ml of native bovine type II collagen emulsified in IFA at five sites (0.1 ml/site) of dorsal skin. Seven days later rats were reimmunised by intradermal injection of the basal site of the tail with 0.1 ml of the emulsified collagen solution.

EVALUATION OF ARTHRITIS
The hind paw diameter was determined by measuring the thickness of the ankle joint with a dial thickness gauge. To minimise the differences among rats the value of the increase in hind paw diameter of each rat was represented as a percentage of the hind paw diameter on day 16 after the first immunisation, because the hind paw diameter was usually maximum on day 16, the first day of drug treatment.

TREATMENT WITH DRUGS
On day 16 after immunisation rats with hind paw diameters of 8.0 mm or greater were pooled as the rats having established polyarthritis and randomly divided into groups. A catheter was transorally introduced into the stomach, and drugs were given through the catheter. The effect of various drugs on the established lesion was evaluated by the oral administration of drugs from day 16 to day 24 after immunisation. The dose of tiflamizole on the first day was 0.16 mg/kg (high dose group) or 0.016 mg/kg (low dose group) and doses on and after the second day were 0.02 mg/kg/day (high dose group) or 0.002 mg/kg/day (low dose group), in an attempt to keep a constant blood level of tiflamizole. Daily doses of the other drugs were fixed during the administration period. The drugs, except dexamethasone, were administered as suspensions in aqueous solution of 0.5% sodium carboxymethylcellulose. Dexamethasone was dissolved in ethanol and diluted with distilled water (ethanol concentration 5%, v/v).

EVALUATION OF DRUGS
Drugs were orally administered from day 16 to day 24 after immunisation, and their effects were evaluated by the area under the time course curve between days 16 and 25. The rebound phenomenon was estimated by the area under the time course curve between days 25 and 33.

MEASUREMENT OF ANTI-TYPE II COLLAGEN ANTIBODIES (1gG)
Plasma anti-type II collagen antibodies were determined by an enzyme linked immunosorbent microassay (ELISA). Briefly, flat bottomed microtitration plates were coated with bovine type II collagen by incubation for six days at 4°C with 0.1 ml/well of type II collagen solution (0.1 mg/ml of 0.1 M NaHCO3 buffer, pH 8.3, containing 0.5 M NaCl, and 0.02% NaN3). The wells were washed three times with phosphate buffered saline (PBS; 0.12 M NaCl, 0.01 M Na2HPO4, 0.004 M KH2PO4, pH 7.8), and the plates were stored over P2O5 at 4°C. Serial dilutions of plasma were made using PBS. An aliquot (0.1 ml) of each plasma dilution, containing

Fig. 1 The time course curves of anti-type II collagen antibody titre and the increase in hind paw diameter after immunisation with type II collagen. Each point represents the mean (SEM) (vertical bars) obtained from 11 rats. Vertical bars smaller than the range covered by the symbols are omitted. The increase in hind paw diameter of each rat was obtained by subtracting the value of the hind paw diameter measured on day 0 (just before the first immunisation) from that measured on each day shown in Fig. 1. The hind paw diameters measured on day 0 and day 17 after the immunisation were 5.72 (0.04) mm and 9.03 (0.53) mm (mean (SEM) of 11 rats) respectively.
1% bovine serum albumin (BSA), was transferred to the collagen coated wells. The plates were agitated for 45 min at room temperature. After washing three times with PBS, 0-1 ml of conjugate (horseradish peroxidase conjugated rabbit antirat IgG antibodies; Miles-Yead Ltd) diluted with PBS and containing 1% BSA was added. The microplates were again incubated for 45 min at room temperature and washed three times with PBS. Bound peroxidase was estimated by adding 0-1 ml of substrate solution. After one hour the reaction was stopped by adding 0-1 ml of 1 N NaOH. One hundred microlitres from each well was transferred into microcuvettes containing 0-9 ml of distilled water. The absorbance of each sample was measured at 450 nm (A450) with distilled water as the blank. Plasma titres are expressed as the reciprocal of interpolated dilution giving a midpoint reading (A450)=0.14 approximately) in the peroxidase titration curve. As substrate solution, 80 mg of 5-aminosalicylic acid was dissolved in 100 ml distilled water at 70°C, and the solution was cooled to room temperature. Before use the pH of the substrate solution was adjusted to 6-0 by adding 1 N NaOH, and 9 ml of this solution was mixed with 1 ml of 0-05% H2O2.

Results and discussion

Changes in the level of anti-type II collagen IgG antibodies

Anti-type II collagen IgG antibodies in plasma were measured by an ELISA at intervals of three days, and the hind paw diameter of each rat was determined by measuring the thickness of the ankle joint as well. The antibody titre increased rapidly after day 5 and reached a plateau on day 11 after primary immunisation, and then the maximum value was maintained for two weeks (Fig. 1). The hind paw swelling increased about four days later than the anti-type II collagen antibodies, reached a maximum value on day 17, and then declined gradually. On day 11 there was positive correlation between hind paw diameter and anti-type II collagen antibody titre in the serum (r=0.82, p<0.01). The results, therefore, suggest that a significant rise of the anti-type II collagen antibodies in plasma may be essential to the incidence of arthritis.

Antibodies to type II collagen in plasma were significantly reduced on day 3 after daily oral administration of dexamethasone (0-3 mg/kg/day) (the antibody titres (–log2) before and after the steroid treatment were 11-84 (0.25) and 10-92 (0.34) (the mean (SEM), n=7) respectively), but no change was found after treatment with indomethacin (3-0 mg/kg/day) (data not shown). Similarly, non-steroidal anti-inflammatory drugs, benoxaprofen (10 mg/kg/day), tiflamizole (0-3 mg/kg on first day, 0-04 mg/kg/day from second to fourth day), and piroxicam (5-0 mg/kg/day) did not reduce anti-type II collagen antibodies on day 3 or day 4 after daily oral administration of these drugs (data not shown).

Effect of anti-inflammatory drugs on the hind paw oedema

Typical inhibitory patterns of dexamethasone and indomethacin on the type II collagen induced arthritis in rats are shown in Fig. 2, and the inhibitory effects of steroidal and non-steroidal anti-inflammatory drugs on the experimental arthritis are summarised in Table 1. After withdrawal of dexamethasone a marked rebound phenomenon occurred, i.e., the hind paw oedema rapidly increased and slightly exceeded the control level, and a weak rebound phenomenon was found after withdrawal of indomethacin (Fig. 2a). Similarly, steroidal anti-inflammatory drugs such as prednisolone (10 mg/kg/day) and hydrocortisone (30 mg/kg/day) suppressed the hind paw oedema but were followed by a rebound phenomenon after their withdrawal (Table 1; data not shown in Fig. 2). On the other hand, tiflamizole and the new non-steroidal anti-inflammatory drugs, piroxicam and benoxaprofen, which are known as cyclo-oxygenase inhibitors, significantly suppressed the hind paw oedema without rebound phenomenon after withdrawal of the drugs (Fig. 2b and Table 1). If the hind paw diameter of the control group remains constant during the course of experiments (from day 16 to day 33) and the suppressed hind paw diameter rebounds to the original value after withdrawal of a drug, the ratio (B) the area after withdrawal of a drug to (A) the area during the drug treatment is close to 0.89 in the rebound group (8 days after withdrawal of drug)/9 (days of drug treatment)). In the present experiments, however, the paw diameter of the control group gradually declined from day 16 to day 33, suggesting that a minimum value of the ratio in the rebound group is less than 0.89, but probably higher than 0.80 in the present experimental conditions. Table 1 indicates that the area ratio (B)/(A) is close to 0.5 (usually less than 0.8) in the non-rebound group, whereas the ratio is usually higher than 0.8 in the rebound group, which includes the steroidal anti-inflammatory drugs. As shown in Table 1 steroidal anti-inflammatory drugs cause the rebound phenomenon, which may occur owing to a reduction of the secretion of endogenous glucocorticoids as a result of administration of exogenous steroids. On the other hand, the area ratio, (B)/(A), is lower than 0.8 in the groups.
Fig. 2  Effects of anti-inflammatory drugs on type II collagen induced arthritis in rats. Drugs were orally administered from day 16 to day 24 after immunisation. Each point represents the mean (SEM) (vertical bars) obtained from four to seven rats. Experimental conditions are described in detail under 'Materials and methods'. (a) ●●●●●●= control (seven rats); □□□□□□= indomethacin (3·0 mg/kg/day; four rats), ○○○○○○= dexamethasone (0·3 mg/kg/day; seven rats). (b) ●●●●●●= control (five rats); □□□□□□= piroxicam (0·1 mg/kg/day; six rats), ○○○○○○= piroxicam (1·0 mg/kg/day; six rats).
treated with non-steroidal anti-inflammatory drugs, except indomethacin for which the values are variable (Table 1). The new non-steroidal anti-inflammatory drugs, piroxicam and benoxaprofen, are known to have a long plasma half life. After withdrawal of the drugs the plasma concentrations of these drugs are still high and, therefore, rebound phenomenon may not occur. In fact, on day 8 after withdrawal of piroxicam (5.0 mg/kg) its serum concentration was 1.1 µg/ml, which was approximately five times as high as IC_{50} (cyclo-oxygenase inhibition). Similarly, serum concentrations of benoxaprofen and tiflamizole were higher than their IC_{50} (data not shown). This long plasma half life of the drugs may account for the fact that rebound phenomenon does not occur after withdrawal of piroxicam, benoxaprofen, and tiflamizole.

Dose-response curves are shown in Fig. 3 based on the area under the time-course curve during drug treatment (Table 1). The relative potencies of the anti-inflammatory effect of the drugs on the type II collagen induced arthritis in rats were dexamethasone > piroxicam, tiflamizole > indomethacin > benoxaprofen (Fig. 3). It has been reported that there are some similarities between type II collagen induced arthritis in rats and rheumatoid arthritis. Type II collagen induced arthritis in rats, therefore, may be a relevant animal model for detection of antiarthritic agents and for the investigation of rebound phenomenon after withdrawal of anti-rheumatic drugs.

We are grateful to Ms C Yamamoto for her participation in the preliminary experiments of this work. We thank Sankyo Co Ltd for supplying tiflamizole and for measuring its concentration in the serum.

### Table 1 Antiarthritic effects of anti-inflammatory drugs on the type II collagen induced arthritis in rats

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Doses (mg/kg/day)</th>
<th>No of rats</th>
<th>Area* (day)×(%)</th>
<th>Ratio (B)/(A)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(A) During treatment†</td>
<td>(B) After treatment‡</td>
</tr>
<tr>
<td>Control</td>
<td>---</td>
<td>7</td>
<td>907.8 (29.6)</td>
<td>618.6 (32.7)</td>
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<td>Dexamethasone</td>
<td>0.01</td>
<td>6</td>
<td>830.5 (61.7)</td>
<td>524.3 (59.5)</td>
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<tr>
<td></td>
<td>0.03</td>
<td>5</td>
<td>662.2 (47.2)</td>
<td>660.3 (80.8)</td>
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<tr>
<td></td>
<td>0.3</td>
<td>7</td>
<td>432.7 (18.0)</td>
<td>603.4 (73.2)</td>
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<tr>
<td>Prednisolone</td>
<td>10</td>
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<td>585.3 (27.0)</td>
<td>479.6 (27.4)</td>
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<td>Hydrocortisone</td>
<td>30</td>
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<td>594.7 (18.1)</td>
<td>530.3 (76.1)</td>
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<td>Indomethacin</td>
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<td>539.8 (77.7)</td>
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<td>1.0</td>
<td>4</td>
<td>687.3 (61.4)</td>
<td>485.8 (86.2)</td>
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<td>2.0</td>
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<td>617.7 (18.4)</td>
<td>376.2 (26.6)</td>
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<td>3.0</td>
<td>4</td>
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<td>30</td>
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<td>665.5 (18.1)</td>
<td>481.7 (40.2)</td>
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<tr>
<td>Piroxicam</td>
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<td>766.4 (75.9)</td>
<td>448.2 (58.4)</td>
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<td>1.0</td>
<td>6</td>
<td>682.8 (22.6)</td>
<td>376.0 (28.4)</td>
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<tr>
<td>Benoxaprofen</td>
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<td>813.4 (23.7)</td>
<td>455.8 (31.4)</td>
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<td>10</td>
<td>7</td>
<td>704.6 (13.4)</td>
<td>389.2 (14.8)</td>
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<td>Tiflamizole</td>
<td>0.016 (1st)§</td>
<td>7</td>
<td>853.1 (10.1)</td>
<td>672.1 (71.7)</td>
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<tr>
<td></td>
<td>0.16 (1st)§</td>
<td>8</td>
<td>727.5 (103.8)</td>
<td>497.6 (72.3)</td>
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</table>

*Values are means (SEM).
†Area during treatment: effect of drugs on the arthritis is represented by the area under the time course curve from day 16 to day 25 after immunisation.
‡Area after treatment: degree of rebound phenomenon of arthritis is represented by the area under the time course curve after withdrawal of drugs from day 25 to day 33 after immunisation.
§Details are given in 'Materials and methods'.
References


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