Anti-Arthritic and Immunoregulatory Effects of TI-31 on Collagen-Induced Arthritis

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Abstract—TI-31 (TEI-3096, 6-p-chlorobenzyl-5H-2,3,6,7-tetrahydro-5,7-dioxothiazolo[3,2-a]pyrimidine) reduced bovine type II collagen-induced arthritis (CIA) in rats in a time- and dose-dependent manner. Oral TI-31 treatment in doses of 10 and 50 mg/kg daily for 7 days prior to collagen immunization depressed the development of arthritis. However, it had no obvious effect on CIA when administered daily for a 7-day or 28-day period after the immunization. This compound was also ineffective against the established arthritis. On the contrary, cyclophosphamide, dexamethasone, or ibuprofen strongly protected the animals from the development of arthritis and/or cured the established arthritis by these dose regimens. Both humoral and delayed-type hypersensitivity skin responses to bovine type II collagen were decreased in rats treated with TI-31 daily for 7 days before the induction of arthritis. These results suggest that TI-31 depresses CIA by regulating the immune response to collagen through a mechanism different from that of anti-inflammatory drugs or immunosuppressants.

TI-31 (TEI-3096, 6-p-chlorobenzyl-5H-2,3,6,7-tetrahydro-5,7-dioxothiazolo[3,2-a]pyrimidine) is a novel compound which has inhibitory effects on adjuvant-induced arthritis (1) and experimental nephritis (2, 3). TI-31 has immunomodulating properties. It inhibits the elevated antibody response seen in colchicine-treated mice without having any effect on antibody response in untreated mice (1). Furthermore, TI-31 potentiates the induction of suppressor T cells as well as inhibits the induction of helper T cells (4).

Collagen-induced arthritis (CIA), an experimental model of rheumatoid arthritis (5), is induced in rats and mice by immunization with heterologous type II collagen (CII) (6, 7), and it appears to be due to the autoimmune response to collagen (8, 9). This animal model, which is characterized by the development of both cellular and humoral immune responses to CII, can be passively transferred to normal rats by sensitized spleen cells (10) or by immunoglobulin G specific for CII (11, 12).

In this paper, we report that TI-31 inhibited in a time- and dose-dependent manner the development of arthritis through the inhibition of humoral and cellular immune responses to CII.

Materials and Methods

Rats: Male Lewis rats were purchased from Charles River Japan, Inc. (Kanagawa, Japan). They were allowed one week to adapt to their environment and were used when 6 weeks old. All the animals, housed in groups of two or three in metal cages, were kept in an air-conditioned room and given standard chow and water ad libitum for the duration of the study.

Induction and assessment of arthritis: Bovine type II collagen (CII) (Cosmo Bio Co., Ltd., Tokyo, Japan) was dissolved in 0.1 M acetic acid at a concentration of 2.67 mg/ml. The solution was emulsified with an equal volume of incomplete Freund’s adjuvant (IFA) (Difco Laboratories, Detroit, MI). A total volume of 1.5 ml of the emulsion was injected intradermally at several sites on the back of rat on day 0. The volume of the left
hind paw was measured by a plethysmometer (KN-357, Natsume Seisakusho Co., Ltd., Tokyo, Japan) (1). Results represent percent swelling of foot volume calculated from this formula:

\[
\text{% increase in foot volume} = \frac{\text{foot volume (day x)} - \text{foot volume (day 0)}}{\text{foot volume (day 0)}} \times 100
\]

Immune response to CII: Blood was taken from the tail vein under ether anesthesia and diluted twenty times with physiological saline. The diluted blood was centrifuged, and the supernatant was stored at \(-20°C\) until assayed. Antibodies to CII were measured by enzyme-linked immunosorbent assay (ELISA) (13). Briefly, 96-well microtiter plates (Falcon 3912, Becton Dickinson & Co., Oxnard, CA) were coated overnight at 4°C by incubation with 100 \(\mu\)l of CII solution (25 \(\mu\)g/ml in 50 mM bicarbonate buffer, pH 9.6). After washing three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween), the remaining protein-binding sites were blocked by the addition of PBS-Tween containing 1% bovine serum albumin (BSA) and washed as described above. Appropriate dilutions of test sera in 100 \(\mu\)l of PBS containing 1% BSA were added, and the samples were incubated for 2 hr at 37°C. After washing, 100 \(\mu\)l of a 1:1000 dilution of alkaline phosphatase-conjugated rabbit anti-rat IgG (Miles Laboratories, Inc., Elkhart, IN) was added to the wells and incubated for 3 hr at 37°C. The amount of bound enzyme was estimated by the addition of 100 \(\mu\)l of \(p\)-nitrophenyl phosphate (1 mg/ml in 10% diethanolamine buffer, pH 9.8). After 30 min incubation at room temperature, the absorbance was measured at 410 nm using an ELISA analyser SLT210 (SLT Lab instruments, Austria). The standard curve was derived from a commercial rat anti-bovine CII antiserum (Cosmo Bio Co., Ltd., Tokyo, Japan). It was linear between 1:6400 and 1:200 dilution of antiserum. Anti-collagen IgG titer was expressed as a percent of standard anti-bovine CII antiserum (14).

For the estimation of total IgG in the sera of rats by ELISA, the plates were coated with goat anti-rat IgG Fc fragment (Jackson Immunoresearch Laboratories, Inc., Avondale, PA) instead of type II collagen. Procedures for ELISA were the same as described above. Total IgG level was expressed in reference to the standard serum (Miles Laboratories, Inc., Elkhart, IN).

Delayed-type hypersensitivity (DTH) skin tests were performed as described by Phadke et al. (15). In brief, the thickness of the right ear of each rat was measured with a dial thickness gauge (SM112, Teclock Corp., Tokyo, Japan). Then, 75 \(\mu\)g of CII in PBS was subcutaneously injected into the ear under light ether anesthesia. The ear thickness of all the animals was measured before and 24 hr after collagen injection. The results were expressed as the difference between these two measurements.

Drug treatment: All drugs were suspended or dissolved in 5% gum arabic solution and administered at a constant volume of 5 ml/kg body weight, orally once daily by various dose schedules. Control groups received only the dosing vehicle at the same dosage volume.

Statistical analysis: The data were presented as means±S.E.M., and results were statistically evaluated by Student’s \(t\)-test.

Results

Incidence of arthritis

When 2.0 mg of bovine CII in IFA was injected intradermally at several sites on the back, 45 of 52 rats (86.5%) developed arthritis which was indicated by an over 30% increase in foot volume by 3 weeks after the injection.

Effect of TI-31 on CIA in rats

Treatment after immunization: TI-31 and various reference drugs were orally administered once daily for 28 days from the day of immunization (day 0) to day 27.

Table 1 shows TI-31 significantly inhibited the inflammatory response only at a dose of 250 mg/kg 3 weeks after the immunization. However, it showed no reproducible effects
Table 1. Effect of TI-31 and reference drugs on collagen-induced arthritis

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Time of administration* (day)</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>N</th>
<th>% increase in foot volumeb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>29.6±5.6c</td>
<td>8</td>
<td>Day 14</td>
</tr>
<tr>
<td>1</td>
<td>0→27</td>
<td>TI-31</td>
<td>36.8±5.6 (-23.6)</td>
<td>8</td>
<td>53.3±2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>31.3±4.5 (-5.7)</td>
<td>8</td>
<td>40.9±3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>27.1±4.7 (8.4)</td>
<td>8</td>
<td>40.5±5.4*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Levamisole</td>
<td>39.1±6.6 (-32.1)</td>
<td>8</td>
<td>55.1±2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D-Penicillamine</td>
<td>35.7±7.5 (-20.6)</td>
<td>8</td>
<td>45.9±8.2</td>
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<td>6</td>
<td>52.2±2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TI-31</td>
<td>34.6±5.3 (-11.3)</td>
<td>6</td>
<td>48.8±2.8</td>
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<tr>
<td></td>
<td></td>
<td>Ibuprofen</td>
<td>17.5±5.7* (43.7)</td>
<td>6</td>
<td>28.1±5.2*** (51.9)</td>
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<tr>
<td>3</td>
<td>0→27</td>
<td>Control</td>
<td>32.2±5.6</td>
<td>10</td>
<td>41.4±5.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TI-31</td>
<td>21.1±2.0 (34.5)</td>
<td>10</td>
<td>26.5±5.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyclophosphamide</td>
<td>11.8±1.5** (63.4)</td>
<td>10</td>
<td>8.8±1.6*** (78.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dexamethasone</td>
<td>-6.2±0.7*** (78.7)</td>
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<td>-3.0±0.9***</td>
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<tr>
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<td>14→27</td>
<td>Control</td>
<td>37.2±3.9</td>
<td>9</td>
<td>50.5±3.8</td>
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<tr>
<td></td>
<td></td>
<td>TI-31</td>
<td>37.8±4.3 (-1.6)</td>
<td>8</td>
<td>50.9±4.7</td>
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<tr>
<td></td>
<td></td>
<td>Dexamethasone</td>
<td>37.8±4.6 (-1.8)</td>
<td>8</td>
<td>39.7±5.2*** (21.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ibuprofen</td>
<td>38.2±3.1 (-2.7)</td>
<td>8</td>
<td>28.2±3.4*** (44.2)</td>
</tr>
</tbody>
</table>

*Male Lewis rats were injected in the back on day 0 with 2 mg of bovine CII emulsified in IFA. All drugs were administered orally once daily. Paw volume measurements were performed from day 0 and at weekly intervals thereafter, and the percent increase in volume was determined. Percent increase in foot volume (mean±S.E.M.) of nonimmunized rats (N=14) was 11.4±0.9, 11.9±2.8 and 16.0±1.4 on days 14, 21 and 28, respectively. Results are expressed as means±S.E.M. The percent inhibition is shown in parentheses. *P<0.05, **P<0.01, ***P<0.001: significantly different from the control (Student's t-test).
Table 2. Effect of TI-31 and reference drugs on collagen-induced arthritis

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Time of administration (day)</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>N</th>
<th>% increase in foot volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Day 14</td>
</tr>
<tr>
<td>1</td>
<td>-7→-1</td>
<td>Control</td>
<td>8</td>
<td>38.3±6.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52.8±4.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TI-31</td>
<td>10</td>
<td>31.3±6.1 (18.3)</td>
<td>38.2±2.6** (27.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>29.1±5.2 (24.0)</td>
<td>33.5±2.6** (36.6)</td>
</tr>
<tr>
<td>2</td>
<td>-7→-1</td>
<td>Control</td>
<td>8</td>
<td>42.6±4.1</td>
<td>43.7±4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TI-31</td>
<td>50</td>
<td>24.7±5.5* (42.0)</td>
<td>30.8±4.8 (29.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyclophosphamide</td>
<td>5</td>
<td>14.0±3.0*** (67.1)</td>
<td>39.8±10.5 (8.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dexamethasone</td>
<td>0.1</td>
<td>23.5±3.3** (44.8)</td>
<td>49.6±8.8 (-13.5)</td>
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<tr>
<td></td>
<td></td>
<td>Ibuprofen</td>
<td>25</td>
<td>36.3±4.5 (14.8)</td>
<td>47.7±2.0 (-9.2)</td>
</tr>
</tbody>
</table>

*Experimental conditions were as described in the footnotes of Table 1. *Results were expressed as means±S.E.M. The percent inhibition is shown in parentheses. *P<0.05, **P<0.01, ***P<0.001 : significantly different from the control (Student's t-test).

Table 3. Effect of TI-31 on collagen-induced arthritis

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Time of administration (day)</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>N</th>
<th>% increase in foot volume</th>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>Day 14</td>
</tr>
<tr>
<td>1</td>
<td>-7→6</td>
<td>Control</td>
<td>8</td>
<td>38.6±6.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52.1±2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TI-31</td>
<td>10</td>
<td>33.4±4.4 (13.5)</td>
<td>36.3±5.4* (30.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>35.8±4.1 (7.3)</td>
<td>35.6±3.9** (31.7)</td>
</tr>
<tr>
<td>2</td>
<td>0→6</td>
<td>Control</td>
<td>8</td>
<td>40.1±4.5</td>
<td>47.7±1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TI-31</td>
<td>10</td>
<td>27.5±5.8 (31.4)</td>
<td>46.9±3.2 (3.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>35.3±6.9 (12.0)</td>
<td>43.7±2.9 (8.4)</td>
</tr>
</tbody>
</table>

*Experimental conditions were as described in the footnotes of Table 1. *Results were expressed as means±S.E.M. The percent inhibition is shown in parentheses. *P<0.05, **P<0.01 : significantly different from the control (Student's t-test).
at doses of 10 and 50 mg/kg. Immuno-
modulators such as levamisole and D-
penicillamine, at a given dose, showed no
inhibitory effect on CIA. On the contrary, the
rats were markedly protected from the
development of arthritis by treatment with
the nonsteroidal anti-inflammatory drug
ibuprofen (25 mg/kg), the immunosup-
pressant cyclophosphamide (5 mg/kg), and
the anti-inflammatory steroid dexamethasone
(0.1 mg/kg) (Table 1, Exp. 2 and 3).

Further, TI-31 did not show any therapeu-
tic effect on established arthritis in
contrast to dexamethasone and ibuprofen
(Table 1, Exp. 4). These anti-inflammatory
drugs remarkably cured the established
arthritis.

Treatment before immunization: The
experiments just described demonstrate that
TI-31 treatment for 28 days after the immuni-
zation had no obvious effect on the develop-
ment of arthritis, especially when given in
lower doses. This compound was also
ineffective against the established arthritis.

Therefore, we proceeded to see if other
regimens of TI-31 would show suppressive
effects. So the rats were treated with drugs
for a 7-day period before the immunization.
The results are given in Table 2 (Exp. 1). TI-31
prevented the development of arthritis dose-
dependently in doses of 10 and 50 mg/kg.
The anti-arthritic effect of TI-31 was con-
tinuously observed from 2 to 4 weeks after
the immunization with CII. It significantly
reduced CIA by 27.7 and 36.6%, at doses of
10 and 50 mg/kg, respectively, 3 weeks
after the immunization. As shown in Table 2
(Exp. 2), the depressive effects were also
found in the rats treated with cyclophos-
phamide or dexamethasone, but not ibu-
profen. However, the mode of action of these
drugs was different from that of TI-31.
Inhibitory effects of dexamethasone and
cyclophosphamide were observed only at
an early stage of CIA, and they completely
disappeared by 3 weeks after the immuni-
zation.

Treatment before and after immunization:
The anti-arthritic effect of TI-31 on CIA
was also observed when it was adminis-
tered for 14 days before and after the immunization
(Table 3, Exp. 1). TI-31, however, was

Fig. 1. Inhibitory effect of TI-31 on anti-CII IgG antibody production in collagen-induced arthritic rats.
Experimental conditions were as described in the footnotes of Table 1. TI-31 (50 mg/kg) was admin-
istered orally once daily for 7 days before the immunization. Anti-type II collagen IgG antibodies and
total IgG were determined by ELISA. Total IgG level of untreated rats was 0.76±0.07 mg/ml (N=6).
Each column represents the mean±S.E.M. of 10 rats. *P<0.05, **P<0.01, ***P<0.001: significantly
different from the control (Student's t-test).
ineffective when it was given over a 7-day period after the immunization (Table 3, Exp. 2). These results suggest that TI-31 treatment before the immunization with collagen is essential for its inhibitory effect on arthritis induction.

**Effect of TI-31 on immune responses to CII**

Once the anti-arthritic effect of TI-31 had become clear, we next investigated the effect of TI-31 on immune response to CII to clarify the mechanisms of its anti-arthritic effect.

Figure 1 shows the effect of TI-31 on anti-CII IgG antibody production in CII-induced arthritic rats. TI-31 when administered for 7 days before the immunization inhibited the increase in serum anti-CII IgG antibody titer by 63.9, 65.3 and 63.4% relative to the control level on days 14, 21 and 28, respectively, after the immunization. Serum total IgG level was increased probably due to polyclonal B cell activation by IFA, and this increase was not affected by TI-31 treatment during an observation period of 28 days. As shown in Fig. 2, rats having CIA 14 or 21 days after the immunization showed an increase of ear thickness stronger than non-immunized rats, owing to DTH response to CII. TI-31 treatment before the immunization also inhibited the DTH skin reaction to CII by 11.3 and 24.4%, at 14 and 21 days, respectively, after the immunization.

**Discussion**

TI-31 treatment before the induction of arthritis depressed the development of collagen-induced arthritis (CIA), whereas it showed no obvious effect when administered for 28 days, starting on the day of CIA immunization. Also TI-31 had no effect on the established arthritis. In contrast to TI-31, both dexamethasone and ibuprofen showed a therapeutic effect on the established arthritis, and they prevented the development of arthritis by successive treatment after the immunization. The depressive effect of TI-31 on CIA might not be attributable to a general anti-inflammatory action in this regard. TI-31, in fact, showed no inhibitory effect on animal models of inflammation such as carrageenin-induced edema (1) and granuloma pouch (K. Komoriya et al., unpublished data). TI-31 treatment for 7 days before the experiment had no effect on carrageenin-induced edema (K. Komoriya et al., unpublished data). This rejects the possibility that TI-31 showed an anti-inflammatory effect as a result of its accumulation in the hind paw during pre-treatment before the immunization, suggesting that the anti-arthritic effect of TI-31 is due to the modulation of the immune response in collagen-induced arthritic rats. That is to say, both humoral and DTH skin responses to CII were decreased in rats treated with TI-31 for 7 days prior to the collagen immunization. TI-31 treatment significantly reduced the serum level of IgG antibodies to CII without any effect on that of total IgG.

The effect of TI-31 on CIA are similar to that reported for rabbit antithymocyte serum (ATS) (16). ATS treatment on days-1, 1, 3 and 5 and immunization with CII on day 0 reduced the induction of arthritis in comparison to rats injected with ATS on days 5, 7, 9 and 11. Ten days after the immunization, both DTH and antibody responses to CII
were specifically decreased in the rats injected with ATS during the peri-immunization period. These effects, however, were not observed on day 21. They suggested that T cells contribute to the initiation of the disease process occurring in CIA. These findings are similar to the effect of a T cell-specific immunosuppressive drug, cyclosporin A (CS-A), reported by Kaibara et al. (13). CS-A showed paradoxical effects on CIA. CS-A treatment started during the induction phase of immunity suppressed the development of arthritis as well as DTH and humoral immune responses to CII. In contrast, treatment initiated during the preclinical phase of arthritis or at the time of disease onset enhanced the disease. They speculated the paradox might be caused by an altering of the balance between helper and suppressor T cells. Their observations suggest that T cells participate in the induction phase of arthritis in the immediate post-immunization period and play an obligatory role in the pathogenesis of this model.

Recently, Helfgott et al. (17) reported that T cells from collagen-induced arthritic rats generate a CII-specific arthritogenic lymphokine which induces an erosive, proliferative synovitis when injected into the knee joint of normal recipients. Furthermore, CII-specific suppressor T cells are induced in mice injected intravenously with heterologous CII (18). These suppressor T cells depress cell-mediated and humoral immune responses to CII as well as the inflammatory response.

We observed that TI-31 potentiates suppressor T cell induction, and, conversely, inhibits helper T cell induction in BALB/c mice (4). TI-31 suppressed nephritic changes, serum immune complex and anti-double-stranded DNA antibody levels in NZB/NZW F1 mice by its negative influence on polyclonal B cell activation (3). We postulate that pretreatment with TI-31 inhibits CIA through regulation of immune response to CII. Further studies are needed to assess the mechanism of the antiarthritic effect of TI-31.

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