**Antigen-Specific Suppressor Cells Induced by FK506 in Experimental Autoimmune Uveoretinitis in the Rat**

Hidetoshi Kawashimo,* Yujiro Fujino,* and Manabu Mochizuki†

The authors previously reported that FK506 effectively suppressed the induction of experimental autoimmune uveoretinitis (EAU) in rats with much lower doses than cyclosporine A. This study was aimed at analyzing the immune status of the FK506-treated and EAU-suppressed rats and examining the hypothesis whether the agent could induce antigen-specific suppressor T (Ts) cells. It was found that spleens from S-antigen-immunized and FK506-treated rats contained a population of Ts cells inhibiting the proliferative responses of S-antigen-sensitized lymphocytes to S-antigen, yet these cells did not affect the proliferative responses of interphotoreceptor retinoid-binding protein (IRBP)-sensitized lymphocytes to IRBP. The helper T (Th) cells did not exhibit such suppressor activities. Furthermore, transfer of Ts cells from S-antigen-immunized and FK506-treated rats to naive syngenic rats induced partial inhibition of EAU induction or delay of EAU onset after immunizing the recipient rats with S-antigen. Lymphocytes from the EAU-suppressed recipients showed low proliferative response to S-antigen and low levels of antibody to S-antigen. These data thus indicate that FK506 treatment after S-antigen immunization induces an activation of Ts cells specific to S-antigen and that the Ts cells might contribute, at least in part, to the uniquely prolonged and intensive immunosuppression by FK506. Invest Ophthalmol Vis Sci 31:2500–2507, 1990

Experimental autoimmune uveoretinitis (EAU) is an organ-specific autoimmune disease of the eye that can be induced in various experimental animals by immunization with retinal specific antigens, ie, retinal soluble antigen (S-antigen)1–2 or interphotoreceptor retinoid-binding protein (IRBP).3 The immuno-pathogenic mechanism of EAU is still controversial, but recent studies indicate that T lymphocytes play the major role in EAU.4–6 This animal model is useful for testing the efficacy of various immunosuppressive agents in uveitis. We previously reported immunopharmacologic effects of cyclosporine A (CsA)7,8 and cyclosporine G using this model;9 we found that CsA was effective in suppressing EAU induction and inducing immunologic unresponsiveness specific to S-antigen.8 Recently, CsA has been used for the treatment of uveitis patients. Although the agent is effective in many cases,10,11 its adverse effects, in particular, nephrotoxicity are serious problem in some patients.12 Therefore, some other immunosuppressants with more effects on uveitis or less side effects are needed.

FK506 is a new immunosuppressant isolated from the fermentation broth of **Streptomyces tsukubaensis** No. 9993 in the laboratories of Fujisawa (Osaka, Japan).13,14 This agent has similar immunologic activity to CsA: FK506 suppresses mixed lymphocyte reaction, the production of T cell-mediated soluble factors, and the expression of interleukin-2 (IL-2) receptor.14,15 Furthermore, FK506 was shown to have a potent activity in prolonging allograft survival in animal organ transplantation models, and it was more efficacious than CsA.16–22 FK506 was also reported to suppress some experimental autoimmune diseases.23,24 Our previous study showed that FK506 suppressed EAU induction in rats at ten to 30 times lower doses than CsA. In addition, rats immunized with S-antigen and treated with FK506 from days 0–14 postimmunization did not develop EAU at any time even after the secondary immunization with S-antigen on day 30; similarly treated rats, which did not develop EAU after the primary immunization with S-antigen, fully developed EAU after the secondary immunization when another uveitogenic antigen, IRBP, was used as the secondary antigen. However, the immune responses after the secondary immunization were not fully studied in the previous report. Furthermore, the previous findings suggest that FK506 treatment can induce immunologic unresponsiveness specific to S-antigen. Therefore,
this study was aimed at analyzing immunologic status in FK506-treated and EAU-suppressed rats.

Materials and Methods

Animals

Rats of the inbred Lewis strain were purchased from Charles River Japan (Atsugi, Kanagawa, Japan). Male rats between 8–12 weeks of age were used. These investigation confirmed to the ARVO Resolution on the Use of Animals in Research.

Immunization

S-antigen and IRBP were prepared at our laboratory from bovine retinas according to the methods of Dorey et al. and of Fujino et al., respectively. The antigens were emulsified (1:1) in complete Freund’s adjuvant (CFA) containing *Mycobacterium tuberculosis H37Ra* at a concentration of 2.0 mg/ml. A total of 0.1 ml/rat, containing 30 μg of antigen and 100 μg of bacteria, was injected into one hind footpad.

Drug Treatment

FK506 (Fujisawa) was suspended in physiologic saline and administered by intraperitoneal injection. Drug treatment with FK506 (1 mg/kg/day) was done from days 0–14 after S-antigen immunization since our previous study showed that this treatment schedule caused complete and prolonged suppression of EAU induction. In some experiments, a secondary immunization either with S-antigen or with IRBP was done on day 30 postimmunization.

Evaluation of Disease Development

Clinical signs of EAU were monitored daily under an operating microscope. Rats immunized with uveitogenic antigens were killed with CO₂ on 14–18 days after the primary or secondary immunization as indicated, and all eyes were removed and fixed in 2.5% glutaraldehyde 2% formaldehyde solution. The histologic examination was done on paraffin sections and classified into four grades according to the histologic changes, ie, 0, no inflammation; +1, mild infiltration of inflammatory cells in the ocular tissues; +2, moderate cell infiltration and mild destruction of visual cell layer; +3, intense cell infiltration and complete destruction of visual cell layer.

Evaluation of Immune Responses to Uveitogenic Antigens in Immunized Rats

Immediately after the rats were killed, their blood and spleens were collected. Blood samples were used to measure the levels of antibody to uveitogenic antigens using an enzyme-linked immunosorbent assay as described previously. Mitotic responses of lymphocytes to S-antigen, IRBP, and concanavalin A (Miles-Yeda, Rehovot, Israel) were measured using nonadherent spleen cells as described previously. The results were expressed as the arithmetic means of counts per minute (cpm) ± the standard error of the mean (SEM) or the stimulation index, as indicated.

In Vitro Assay for Suppressor T (Ts) Cells

Preceding the in vitro assay of for suppressor activities of Ts cells from drug-treated rats, the influence of FK506 treatment on T-cell subsets was examined by fluorescein-activated cell sorter to eliminate the possibility that the suppressor activities shown in the following experiments could be due to the decreased number of helper T (Th) cells or the increased number of Ts cells. It was confirmed that FK506 treatment did not change the Th:Ts ratio of the unfractonated spleen cells significantly, when the cells were harvested on day 7, 14, or 30 postimmunization. However, the absolute numbers of spleen cells in treated rats collected on day 14 or later were decreased by approximately 30% compared with those of untreated control rats (data not shown).

Inhibition of Proliferative Response of Lymphocytes to Antigens

Splenocytes from S-antigen-immunized or IRBP-immunized rats were used as “responder” cells, and splenocytes obtained from rats immunized with S-antigen and treated with or without FK506 were used as “additional cells.”

Preparation of responder cells: In each experiment, two rats immunized with S-antigen or IRBP were killed with CO₂ on day 16 postimmunization after confirming the development of EAU. The spleens from the rats were gently teased in RPMI-1640 medium with HEPES (GIBCO, Grand Island, NY) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 mM L-glutamine, 5 x 10⁻⁵M 2-mercaptoethanol, and 2% fetal calf serum (FCS; Boehringer-Mannheim, Mannheim, West Germany). The cell suspension was washed twice in the medium and was deprived of red blood cells by treatment with Tris ammonium chloride. The cell suspension was incubated in 100 x 15-mm plastic petri dishes (1 x 10⁶ cells/dish; Fisher, Springfield, NJ) at 4°C for 1 hr. Then the nonadherent cells were collected and used as responder cells.

Preparation of additional cells: In each experiment, ten rats immunized with S-antigen and treated with FK506 for 15 days were killed with CO₂ 14 days after the termination of the FK506 therapy. The splenocytes of the animals were collected as described. Then, aliquots of cell suspension were used as un-
fractionated additional cells. To obtain Ts or Th cells, the cell suspension was fractionated further by the panning method as follows: the cells suspended in RPMI-1640 medium with 5% FCS were placed in 100 × 15-mm plastic petri dishes (1 × 10^5 cells/dish) that had been coated with rabbit anti-mouse immunoglobulin (Ig) G, IgA, and IgM (heavy and light chains) (100 μg/ml; ZYMED, San Francisco, CA). After incubation at 4°C for 1 hr, nonadherent cells were collected and washed twice. Aliquots of these cells were incubated with mouse IgG monoclonal antibody against rat Th cells, clone W3/25 (50 ng/ml; Sera-Lab, Sussex, England), or with that against rat Ts cells, clone OX8 (50 μg/ml; Sera-Lab), in 17 × 120-mm plastic tubes (Falcon, Oxnard, CA). After incubation for 45 min at 4°C, the cell suspension was washed twice and resuspended to a concentration of 1 × 10^7/ml in RPMI-1640 medium with 5% FCS. The cell suspension (10 ml/dish) was incubated in the 100 × 15-mm plastic petri dishes coated with rabbit anti-mouse IgG, IgA, and IgM (heavy and light chains) antibody. A 1-hr incubation at 4°C preceded collection of nonadherent cells; they were washed twice. The nonadherent cells after incubation with W3/25 antibody were used as the enriched Ts cells fraction, and those, after incubation with OX8 antibody, were used as the Th cell fraction. Examination of the enriched Ts cell fraction by indirect immunofluorescence with mouse IgG monoclonal antibody showed approximately 80% of specific staining of OX8. The same examination was done for the enriched Th cell fraction using W3/25 monoclonal antibody, and it was demonstrated that the cell fraction had 75% of specific staining of W3/25 antibody.

As a control for the cells obtained from S-antigen immunized and FK506-treated rats, cells from ten rats immunized with S-antigen but not treated with FK506 or those from rats treated with FK506 without S-antigen immunization were prepared by the same method.

**The assay for suppressor activity:** A mixture of responder cells (2 × 10^5 cells/well) and additional cells (1–5 × 10^5 cells/well) was cultured in triplicate in 96-well flat-bottomed plates (Coster, Cambrige, MA) in 0.2 ml of RPMI-1640 medium with 5% FCS. The cultures were stimulated with either S-antigen (5 μg/ml) or IRBP (5 μg/ml) at 37°C with 100% humidity and 5% CO2 in air for 4 days and pulsed with 1.0 μCi of 3H-thymidine (6.7 Ci; New England Nuclear, Boston, MA) for the last 16 hr of culture. The cells were harvested by an automatic cell harvester (PHD cell harvester; Cambrige Technology, Cambrige, MA) and incorporated radioactivity was measured by a liquid scintillation counter (Packerd Tri-carb B460; Tokyo, Japan). The results were expressed as the arithmetic means of counts per minute (cpm) ± SEM or as the percentage suppression. The percentage suppression was calculated by the following formula:

\[
\text{%suppression} = \frac{\text{cpm (positive control) - cpm (negative control)}}{\text{cpm (experimental) - cpm (negative control)}} \times 100
\]

where experimental indicates the proliferation of responder cells stimulated with antigens in the presence of additional cells, negative control indicates that of the responder cells alone not stimulated with antigens, and positive control indicates that of the responder cells alone stimulated with antigens.

**Adoptive Transfer of Suppression for EAU Induction**

Enriched Ts cells from FK506-treated and EAU-suppressed rats were adoptively transferred intraperitoneally into naive syngenic rats. Immediately after the cell transfer, the animals were immunized with S-antigen. Enriched Ts cells obtained from rats immunized with S-antigen but not treated with FK506 were used as a control. Clinical signs of EAU were monitored daily for up to 18 days after the immunization. All eyes were examined histologically as described before. Immune responses in some rats were examined as described previously.

**Statistical Analysis**

All results in experimental groups were compared with the control group and a statistical analysis was carried out using student t-test or the chi-square test as indicated.

**Results**

**Induction of Antigen-Specific Unresponsiveness in Rats Treated With FK506**

Table 1 summarizes the EAU induction and the immune responses after the secondary immunization. As demonstrated in Group A, all six rats treated with FK506 (1 mg/Kg/day) from days 0–14 after the primary immunization with S-antigen did not develop EAU following the primary immunization with S-antigen nor after the secondary immunization with the same antigen on day 30. Similarly treated rats were susceptible to EAU when another uveitogenic antigen, IRBP, was used as the secondary antigen on day 30 (Group C). The antibody levels and the proliferative responses to the primary antigen, S-antigen, in Groups A and C (FK506-treated groups) were significantly lower than those in respective control groups, Groups B and D. However, when another retinal antigen (IRBP) was used as the secondary antigen, it was found that the FK506 treatment did not affect the immune responses to the secondary antigen.
Table 1. EAU development and immune responses following the secondary immunization

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EAU development following</th>
<th>Immune responses following secondary immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Antibody</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAgn</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IRBP</td>
</tr>
<tr>
<td>Group</td>
<td>Primary immunization (day 0)</td>
<td>Secondary immunization (days 0-14)</td>
</tr>
<tr>
<td>A</td>
<td>SAgn</td>
<td>+ SAgn</td>
</tr>
<tr>
<td>B</td>
<td>SAgn</td>
<td>- SAgn</td>
</tr>
<tr>
<td>C</td>
<td>SAgn</td>
<td>+ IRBP</td>
</tr>
<tr>
<td>D</td>
<td>SAgn</td>
<td>- IRBP</td>
</tr>
</tbody>
</table>

* Background cpm used to derive the stimulation indexes were 1194 ± 968 (Group A), 7767 ± 997 (Group B), 588 ± 24 (Group C), 7524 ± 1059 (Group D), respectively.
† Not determined.
‡ P < 0.05, student t-test.
§ P < 0.001, student t-test.
¶ Photoreceptor layer was burnt out in the process of EAU after the primary immunization and no further active inflammation was observed.
** Not significant, student t-test.

(Groups C and D, Table 1). The FK506 treatment initiated at the time of primary immunization with S-antigen thus caused the specific immunosuppression to the primary antigen but not to the secondary antigen inoculated after the cessation of the drug treatment.

Enriched Ts Cell Fraction From FK506-Treated Rats Inhibits Antigen-Specific Proliferative Responses In Vitro

The possible involvement of Ts cells in the inhibition of EAU by FK506 was examined by testing the suppressive capacity of splenocytes from FK506-treated and EAU-suppressed rats on the antigen-specific proliferative response of lymphocytes. Figure 1 summarizes the results of a typical experiment. Similar findings were obtained in three other experiments and are summarized in Table 2. Addition of unfractionated spleen cells from donors immunized with S-antigen and treated with FK506 had little effect on the proliferative response to S-antigen of lymphocytes (Fig. 1, culture 6 in Table 2). However, the enriched Ts cell fraction of these spleen cells produced a marked level of suppression (Fig. 1, culture 11 in Table 2). The mean percentage of suppression in four experiments was calculated to be 61.9 ± 10.9% with the high dose of enriched Ts cell fraction from rats immunized with S-antigen and treated with FK506. Unlike these cells, cells from control rats immunized with S-antigen but not treated with FK506 were found to increase the level of thymidine incorporation in the test culture system (Fig. 1, cultures 5, 10 in Table 2). Even the enriched Ts cell fraction from these donors had a tendency to enhance the proliferative response to S-antigen in the test cultures. The proliferative response to S-antigen by additional cells alone was tested, and it was found that FK506 treatment did not affect the proliferative response of the additional cells to S-antigen (culture 3 versus 4 or 7 versus 8, Table 2). Therefore, the suppression of the proliferative responses of the mixed
Table 2. Suppressive effects of splenocytes obtained from FK506-treated and EAU-suppressed rats on antigen-specific lymphocyte proliferation

<table>
<thead>
<tr>
<th>Treatment on donors for additional cells</th>
<th>Treatment on donors for additional cells</th>
<th>Responder SAg in culture</th>
<th>[3H]-Thymidine incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture no.</td>
<td>SAg treatment</td>
<td>Additional cells</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>–</td>
<td>T†</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>T</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>–</td>
<td>T</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>T</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>S‡</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>+</td>
<td>S</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>–</td>
<td>S</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>+</td>
<td>S</td>
</tr>
</tbody>
</table>

-206±4% | 72% | 14% | 24% | 134% | -1140±53.9%§ |
-206±4% | 72% | 14% | 24% | 134% | -1140±53.9%§ |
-206±4% | 72% | 14% | 24% | 134% | -1140±53.9%§ |
-206±4% | 72% | 14% | 24% | 134% | -1140±53.9%§ |
-206±4% | 72% | 14% | 24% | 134% | -1140±53.9%§ |
-206±4% | 72% | 14% | 24% | 134% | -1140±53.9%§ |

* The results represent mean ± SEM of triplicate wells.
† Unfractionated total cell.
‡ Number indicates % suppression.
§ P < 0.05, significantly enhanced, student t-test.
** P < 0.01, significantly suppressed, student t-test.

Table 2 shows the suppressive effects of splenocytes obtained from FK506-treated and EAU-suppressed rats on antigen-specific lymphocyte proliferation. The table includes various treatments and their effects on [3H]-Thymidine incorporation, indicating the percentage of suppression. The results show that certain treatments, such as FK506 treatment and antigen immunization, can suppress lymphocyte proliferation, with varying degrees of suppression indicated by the percentage values.

Cell cultures by additional cells were considered to be due to the effects of the Ts cell population from FK506-treated rats on the responder cells. The Ts cells from donors which did not receive the primary immunization with S-antigen but were treated with FK506 did not have suppressor activity (culture 9, Table II; mean % suppression was 0.0 ± 14.5%). Therefore, the Ts cells from donors receiving both S-antigen immunization and FK506 treatment had suppressor activity. The Th cell fraction from either treated rats or untreated control rats did not have any suppressive activity (Fig. 2). On the contrary, the Th cells even from FK506-treated rats showed a tendency to increase thymidine incorporation.

The antigen specificity of the suppressor activity of the Ts cells is shown in Figure 3. The Ts cells obtained from S-antigen-immunized and FK506-treated rats caused significant suppression in the proliferative response of S-antigen-sensitized responder cells to the antigen, and the Ts cells did not suppress the proliferative responses of IRBP-sensitized responder cells to the respective antigen. Therefore, the FK506 treatment initiated on the day of S-antigen immunization induced a cell population (Ts cell) with antigen-specific suppressor activity in the treated animals.

Enriched Ts Cell Fraction From FK506-Treated Rats Inhibits the Induction of EAU In Vivo

The enriched Ts cell fraction from rats immunized with S-antigen and treated with FK506 was examined if it had an inhibitory effect on disease induction in vivo. The activity was tested by injecting enriched Ts cells into naive syngenic rats which were actively immunized with S-antigen immediately thereafter. These results are summarized in Table 3. The EAU development in the recipient (Group C) was partially inhibited, and this was statistically significant compared to the controls.
pared with control (P < 0.05). Furthermore, recipient rats developing EAU showed a significant delay of the disease onset and decrease of disease intensity. On the other hand, the enriched Ts cell fraction from donors immunized with S-antigen but not treated with FK506 had no detectable effect on the development of EAU in recipient rats (Group B). The three recipients in Group C which did not develop EAU showed a marked suppression on the antibody levels to S-antigen and proliferative responses to S-antigen compared with those in control recipient rats (C" versus B, Table 4). Yet, four rats which developed EAU in Group C showed a significantly higher antibody levels to S-antigen than those without EAU (C versus C", Table 4). The proliferative responses of lymphocytes to concanavalin A were not significantly different between the two recipient groups.

**Discussion**

FK506 is a novel immunosuppressive agent, an antibiotic of the macrolide family. This agent has been reported to have strong immunosuppressive activity, ie, the potent activity to suppress (1) mixed lymphocyte reaction; (2) cytotoxic T-cell generation; (3) production of IL-2 and gamma-interferon; and (4) the proliferation of an antigen-specific T-cell clone. Recently, FK506 was demonstrated to suppress selectively the induction of mRNA for IL-2 of T cells but not the expression of mRNA for IL-2 receptor with 100 times lower concentration than CsA. This agent has also been shown to be effective in prolonging allograft survival in cardiac, renal, and skin transplantation in animals. Furthermore, it has been reported that FK506 is effective in suppressing the induction of some autoimmune diseases.

In agreement with our previous report, the current study showed that FK506 treatment induced immunologic unresponsiveness specific to S-antigen. Rats immunized with S-antigen on day 0 and treated with FK506 for 14 days thereafter did not develop EAU even after the reimmunization with the same antigen on day 30; similarly treated rats developed EAU when another retinal antigen, IRBP, was used as a secondary antigen (Table 1). Our study further analyzed immune responses in these animals. The antibody titer and proliferative response to the primary antigen, S-antigen, was markedly suppressed, yet those to the secondary antigen, IRBP, which was inoculated after the cessation of FK506 treatment, were not suppressed at all (Table 1). Therefore, FK506 treatment initiated at the time of S-antigen immunization induces antigen-specific unresponsiveness of both disease induction and immune responses to the antigen.

Furthermore, the data recorded here suggest that the unresponsiveness toward S-antigen in the FK506-treated rats is mediated, at least in part, by S-antigen-specific Ts cells induced by the FK506 treatment. The suppressive activities of Ts cells induced by the FK506 treatment were demonstrated both in vitro and in vivo. In vitro experiments, Ts cells

**Table 3. Transfer of suppression on EAU induction by Ts cells**

<table>
<thead>
<tr>
<th>Group</th>
<th>SAg immunization</th>
<th>FK506 treatment</th>
<th>EAU rats/total</th>
<th>Days of onset</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>-</td>
<td>9/9</td>
<td>10.6 ± 1.0*</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>+</td>
<td>9/9</td>
<td>11.1 ± 0.4</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
<td>6/9†</td>
<td>12.5 ± 0.6‡</td>
<td>1.7 ± 0.3‡</td>
</tr>
</tbody>
</table>

* Mean ± SEM.
† P < 0.05, significantly suppressed as compared to Group A, χ² test.
‡ P < 0.01, significantly suppressed as compared to Group A, student t-test.
Table 4. Immune responses in Ts cells-transferred recipients

<table>
<thead>
<tr>
<th>Donors</th>
<th>Recipients tested</th>
<th>Proliferative response (Stimulation index)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>SAg immunization</td>
<td>FK506 treatment</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C†</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C*</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* Background cpm used to derive the stimulation indexes were 7915 ± 422 (Group B), 6925 ± 413 (Groups C & C†), respectively.
† This group consists of rats that developed EAU among rats of Group C in Table 3.
‡ Not significant as compared with Group B, but significantly high (P < 0.01) as compared with Group C*, student t-test.
§ Not determined.

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