Observations in clinical and experimental ocular autoimmunity

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Chapter 11

Treatment of Autoimmune Uveoretinitis in the Rat with Rapamycin, an Inhibitor of Lymphocyte Growth Factor Signal Transduction

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Marc D. de Smet, Robert B. Nussenblat, Huifang Chen

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ABSTRACT

Rapamycin (RAPA) is a macrolide antibiotic with unique immunosuppressive properties. RAPA inhibits T-cell function by interfering with IL-2 and IL-4 signal transduction. It does not prevent IL-2 production or IL-2R expression. The efficacy of RAPA in the treatment of autoimmune diseases was evaluated using the experimental autoimmune uveoretinitis (EAU) model. EAU was actively induced in Lewis rats by immunization with S-antigen in Hunter’s adjuvant. RAPA and control vehicle were administered by continuous intravenous infusion over a 14 day period by miniosmotic pump. RAPA treatment initiated on the day of immunization or 7 days later was found to efficiently inhibit EAU induction. The minimal effective dose was 0.1 mg/kg/d. EAU inhibition was correlated with reduced number of cells in the immunization site draining lymph nodes, as well as with a shift and lowering of the peak of the lymphocyte proliferative response curve. The anti-S-antigen antibody response was delayed by 3 days under RAPA treatment and the serum levels lowered in a dose dependent manner. An initial body weight loss was observed during the first week of drug administration, but there was a normal weight gain afterward.

INTRODUCTION

Rapamycin (RAPA) is a macrolide antibiotic originally studied for its antifungal properties. It has more recently attracted attention as an immunosuppressive agent (1). It was purified from isolates of Streptomyces hygroscopicus collected on Easter Island (2). RAPA was shown to have a strong anti-rejection activity in several animal models of organ transplantation. Heart and skin allografts were prolonged in the mouse by intraperitoneal (i.p.) injection and oral treatment (3, 4). In rats, early treatment prevented the rejection of heart, kidney, and pancreaticoduodenal grafts, but delayed treatment could reverse the process of ongoing rejection (5-8). Over the past two years the mechanism of action of RAPA has begun to be understood. RAPA was found to inhibit T cell proliferative signals of both Ca\(^{2+}\) dependent and Ca\(^{2+}\) independent pathways (9-12). The inhibition could not be overcome by the addition of IL-2 or IL-4. In fact the production of these lymphokines as well as the expression of the IL-2 receptor were not suppressed by RAPA as they were by cyclosporin A (CsA) and FK 506. On B cells the effect of RAPA varied with the stimulus tested. Mouse B cell proliferation stimulated by 8-Mercaptoguanine was inhibited in the presence of RAPA but only delayed when stimulated by anti-IgM or lipopolysaccharide (13). On the other hand the pokeweed mitogen driven proliferation and antibody production of human B cells was profoundly suppressed by RAPA (14).

These observations and an early report by Martel et al indicated that RAPA could be useful in the treatment of autoimmune diseases (15). One of the best described models of these diseases is experimental autoimmune uveoretinitis (EAU). EAU is induced in various species by immunization with purified retinal antigens (16-20). It is better characterized in the Lewis rat in which retinal lesions are caused by a mononuclear infiltration occurring 12 to 15 days after immunization with S-antigen (S-Ag) (21). A critical role was ascribed to T cells because athymic nude rats are resistant to EAU induction unless adoptively transferred with heterozygous syngeneic T cells (22). In the Lewis rat, the T cell responsible for transfer was shown to be phenotypically contained within the CD4\(^{+}\) subpopulation (23). EAU was also inhibited by treatment with CsA (24), and by selective killing of activated T cells with a recombinant IL-2-toxin fusion protein (25). In this first study on the activity of RAPA in EAU, we report the inhibition of actively
Chapter 11
Current Eye Research

induced disease with low dose intravenous (i.v.) infusion and characterize its effect on the immune response.

MATERIALS AND METHODS
Immunization and treatment protocol
Male Lewis rats between 8 to 12 weeks old (Charles-River, Montreal, Que.) were immunized in one hind footpad with 20 μg S-Ag (26) in PBS, emulsified 1:1 v:v in Hunter's adjuvant (TiterMax™, CytRx, Norcross, Ga.) for a total volume of 40 μl per rat (27). RAPA (provided by Wyeth-Ayerst Research, Princeton, NJ) was solubilized in a vehicle composed of 70% polyethylene glycol MW 400, 10% Tween 80, and 20% N,N dimethylacetamide (Sigma, St. Louis, MO).

Treatment was delivered at the indicated doses by continuous i.v. infusion over a period of 14 days by means of a miniosmotic pump (Alzet model 2002, Alza, Paio Alto, CA) implanted in the abdominal cavity and connected to a lumbar vein. Surgery was performed under anesthesia with sodium pentobarbital 40 mg/kg in 0.5 ml PBS injected i.p. EAU was evaluated by histopathologic examination of the eyes and the severity graded from 0.5 to 4.0 as described previously (25). The eyes were collected on day 17 when the treatment started on the day of immunization (day 0), and on day 28 when the treatment started on day 7. The rats were weighed at seven day intervals and the percent variation was calculated in reference to the treatment initiation weight and is reported as the average ± SD of groups of seven rats. The animals were cared for according to the guidelines of the Association for Research in Vision and Ophthalmology.

Lymphocyte proliferation assay
Rats were immunized and the treatment with RAPA 1.0 mg/kg/d or vehicle alone was started on the same day. The draining lymph nodes (DLN) of the immunization site were removed on day 6, 9, 11, 13, and 15, and prepared to single cell suspension. Cells were cultured in flat bottom microtiter plates at 2 x 10^5 cells/well in 200 μl of RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 5 x 10^{-5} M L-mercaptoethanol, and 50 μg/ml gentamycin, and containing 10% FBS (Hyclone, Logan, UT). Cultures were done in quadruplicate in the presence of S-Ag 5 μg/ml, or Con A 2 μg/ml (Boehringer Mannheim, Indianapolis, IN), or medium alone. [3H] thymidine 0.5 μCi/well was added for the last 16 h of the 64 h culture period and the incorporated radioactivity measured by liquid scintillation counting. Results for each time point are for groups of four to eight rats and are given as the average ± SEM of the stimulation indices representing (cpm of Ag-stimulated cultures) / (cpm of non-stimulated cultures).

Measurement of antibody production
Serum antibody levels against S-Ag were measured at the indicated time points by solid phase ELISA following Tuyen et al (28). Serum samples were measured at a 1:100 dilution. The secondary antibodies were peroxidase conjugated sheep anti-rat IgG and IgM (Kirkegaard and Perry, Gaithersburg, MD). Statistical differences were evaluated using unpaired Student's t-test.

RESULTS
Inhibition of EAU induction by RAPA, and immune function correlates
Treatment was initiated at two different time points. The capacity of RAPA to inhibit EAU induction was first tested by implanting the osmotic pumps on the day of immunization. Treatment was also started seven days after immunization when the immune response was already activated to test conditions more pertinent to actual disease. Treatment cannot be delayed until EAU has started because of its brief evolution course. The results obtained with treatment on the immunization day showed a complete inhibition of the disease at a dose of 1.0 mg/kg/day (table 1). When treatment at the same dose was delayed for 7 days, the therapeutic response was also complete (table 1). Graded dose reduction showed that EAU could still be effectively inhibited at a dose of 0.1 mg/kg/d, while a majority of rats had disease at lower doses. The increase in the number of cells contained in the lymph nodes draining the site of immunization was significantly reduced by RAPA treatment (figure 1). The sensitization of lymphocytes to S-Ag during treatment was evaluated by measuring the in vitro proliferative response. The results are depicted in figure 2. There was a shift of
TABLE I

EFFECT OF RAPAMYCIN ON EXPERIMENTAL AUTOIMMUNE UVEORETINITIS

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>Day 0 to 14</th>
<th>Day 7 to 21</th>
</tr>
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<tbody>
<tr>
<td>VEHICLE</td>
<td>8/8 1.78 ±0.35</td>
<td>14/15 3.77 ±0.15</td>
</tr>
<tr>
<td>RAPA 1.00 mg/kg/d</td>
<td>0/8 -----</td>
<td>0/15 -----</td>
</tr>
<tr>
<td>RAPA 0.50 mg/kg/d</td>
<td>2/14 2.50 ±0.50</td>
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<tr>
<td>RAPA 0.10 mg/kg/d</td>
<td>2/14 3.50 ±0.00</td>
<td></td>
</tr>
<tr>
<td>RAPA 0.05 mg/kg/d</td>
<td>5/7 3.69 ±0.21</td>
<td></td>
</tr>
<tr>
<td>RAPA 0.025 mg/kg/d</td>
<td>6/8 2.72 ±0.15</td>
<td></td>
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</table>

*Male Lewis rats were immunized (day 0) in one hind footpad with S-Ag 20 μg/rat emulsified 1:1 v/v in 20 μl Hunter’s adjuvant. Results indicate number of rats positive for EAU on histopathological examination. The severity is given by the average histopathologic score (±SE) of positive eyes only.

† Treatment with Rapamycin or vehicle only was given for 14 days by continuous intravascular infusion in the lumbar vein with an osmotic pump (Alzet model 2002) implanted i.p. either at the time of immunization or 7 days later where indicated.

§ The eyes were removed 17 days after immunization.

‖ The eyes were removed 28 days after immunization.

several days in the proliferation curve as well as a lowering of the peak of the response. Measurement of the antibody production when treatment started on day 0 showed that it was delayed by three days but progressively increased to reach levels close to those of the controls at the end of the treatment period (figure 3-A). When treatment was started on day 7 with various doses of RAPA, there was a dose dependent decrease of the antibody levels for the duration of the three-week observation period (figure 3-B).

General toxicity of RAPA

A limited loss of weight in the rats treated with RAPA was observed in all experiments. Compared to the vehicle control group, rats treated with RAPA 1.0 mg/kg/d from day 0 had a 9% average decrease in weight. The variation in the weight of rats after day 7 treatment with various doses of RAPA is shown in figure 4. During the first week after pump placement, a similar weight loss was noticeable at all therapeutic RAPA doses. However, during the second week of treatment

Figure 1.

Viable cell counts in the DLN of rats, as the average ±SD from groups of 4 to 8 animals immunized with S-Ag 20 μg in Hunter's adjuvant, and treated from day 0 with RAPA 1.0 mg/kg/d or vehicle alone by continuous i.v. infusion with a miniosmotic pump.
Chapter 11

Current Eye Research

Figure 2.
In vitro proliferative response of DLN cells (2 x 10^5 cells/well) to S-Ag 5 μg/ml. Lewis rats were treated from the day of immunization with RAPA 1.0 mg/kg/d or vehicle alone by continuous i.v. infusion with a miniosmotic pump. Results are given as the average S.I. ±SEM of groups of 4 to 8 rats.

DISCUSSION
The increased understanding of the mechanism of uveitis brought about by the study of EAU in animals has allowed for improvement in our therapeutic approach. In particular, the demonstration of the critical role played by T cells has led to the use of CsA with great success (29,30). FK 506 is also currently being clinically evaluated in Japan for the treatment of severe uveitis (31). RAPA is related to CsA and FK 506 by its non-cytotoxic mode of action, as opposed to agents such as cyclophosphamide or chlorambucil. CsA and FK 506 prevent the transcription of T cell IL-2 and IL-4 genes (32-34). RAPA appears to differ from other immunosuppressive agents through its capacity to inhibit lymphocytes by preventing the signal transduction of growth factors (9-12). This effect was reflected by the observed reduction in the number of cells present in the lymph nodes of immunized animals.

EFFECT OF RAPAMYCIN TREATMENT ON ANTIBODY PRODUCTION

Figure 3.
Anti S-Ag antibody levels in the serum of rats treated in A from day 0 and in B from day 7 after immunization with RAPA 1.0 mg/kg/d (■), 0.1 mg/kg/d (▲) or vehicle alone (○). Results are the average ±SEM of groups of 7 rats. The difference between RAPA and vehicle treated Ab levels was statistically significant with p<0.005 at all time points except days 14 and 17 in A.
RAPAMYCIN IN EAU

Current Eye Research

<table>
<thead>
<tr>
<th>% WEIGHT CHANGE</th>
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<tr>
<td>DAY 14</td>
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<tr>
<td>DAY 21</td>
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<td>DAY 28</td>
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Figure 4: Variation in the weight of rats measured at weekly intervals and given as percent change LSTD relative to the day of treatment initiation (day 7 after immunization). Groups of 7 rats were treated with vehicle alone (■), or RAP A at 1.0 (□), 0.5 (■), 0.1 (●), 0.05 (▲), or 0.025 (▲) mg/kg/d by continuous i.v. infusion with a miniosmotic pump.

The decrease in the capacity of the remaining cells to respond to stimulation by S-Ag in culture further indicated that the expansion of the S-Ag specific cell population had been prevented.

Higher levels of antibody production are usually obtained when immunizing with Hunter's adjuvant compared to Freund's adjuvant (27). Although using Hunter's adjuvant, we observed a sizable reduction in the anti-S-Ag antibody levels during RAPA treatment. In previous reports of EAU induced with S-Ag in Freund's adjuvant, CsA treatment did not affect the antibody levels (35), while FK 506 caused a profound depression of these levels (36). In general the persistence of an appreciable antibody production is advantageous in the immunosuppressive treatment of T cell mediated diseases such as uveitis, because it helps to prevent intercurrent complicating infections. On that account cytomegalovirus retinitis, a common opportunistic viral disease in the immunosuppressed, was reported in patients treated with FK 506 (37).

One of the most serious problems in the treatment of autoimmune disease is the need for the immunosuppression to be sustained for prolonged periods of time. The likelihood of developing complications from the toxic side effects of the drugs is increased accordingly. For both CsA and FK 506, the main concern lies with the kidney toxicity (38, 39). In contrast, reports showing a low renal toxicity of RAPA in animals (40-42) suggest that it would be an advantageous addition to our therapeutic arsenal. The results reported here certainly indicate that it could be effective in the treatment of uveitis. The minimal effective dose of 0.1 mg/kg/d for EAU inhibition is 100 to 400 times lower than the intramuscular dose of CsA reported previously for day 7 treatment (35, 43). Even when taking into account the probably increased effectiveness of the i.v. delivery route of RAPA compared to the i.m. administration of CsA, our results indicate a very high degree of therapeutic efficacy of RAPA. The therapeutic effect of RAPA also appears to be prolonged because the rats remained free of disease on day 28, approximately one week after the treatment course was stopped. In regards to the general toxicity, the RAPA treated rats lost weight only during the first week of treatment. It is possible that the weight loss is due to the combined toxicity of the anesthetic used for surgery together with RAPA, since during the second week of treatment there was a net weight gain. Rapamycin thus appears to be a promising alternative for the control of autoimmune diseases.

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REFERENCES


