Inhibition of Autoimmune Uveitis by Anti-CD4 Antibody

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In this study, rats with S-antigen-induced uveitis were treated with W3/25, a monoclonal antibody that recognizes the CD4 molecule expressed by helper/inducer cells. Treatment was started on day 5 after administration of S-antigen. Groups of animals were killed 18 or 31 days after S-antigen injection. The enucleated globes were studied histologically, and in vitro T-cell response and anti-S antibody levels were determined. Results showed that the antibody treatment prevented development of experimental autoimmune uveitis (EAU) in all animals. Furthermore, there was no evidence of disease development for 14 days after cessation of therapy. These results suggest that CD4+ cells are important in the initiation of EAU, and that monoclonal antibodies directed to this subset may provide effective treatment of autoimmune uveal inflammation. Invest Ophthalmol Vis Sci 31:1264-1270, 1990

It has been reported that helper/inducer T-cells mediate S-antigen-induced uveoretinitis.¹ Helper/inducer T-cells express the CD4 molecule on their surfaces. The CD4 determinant is involved in the recognition of antigens in conjunction with major histocompatibility complex (MHC) class II molecules.²,³ In vitro treatment with anti-CD4 antibodies has been shown to block antigen-induced proliferation of lymphocytes, and in vivo treatment with these antibodies to reduce or abolish the development of experimental autoimmune encephalomyelitis (EAE).⁴ Several monoclonal antibodies that recognize T-cell-specific surface determinants have been used as immunosuppressive agents⁵,⁶ to reverse organ transplant rejection⁷-⁹ and autoimmune diseases¹⁰-¹² in experimental animal models. In EAE, lymphocytes bearing the CD4 marker are implicated in the pathogenesis of the disease, since anti-CD4 monoclonal antibody treatment has been successful in reducing the incidence of EAE in mice and in rats.⁴,¹⁰-¹³

In this study, we were able to block the progression and subsequent expression of EAU in rats by treatment with a mouse monoclonal antibody directed to the CD4 molecule. Moreover, the rats remained disease-free for 14 days after cessation of therapy.

Materials and Methods

Thirty Lewis rats weighing approximately 175 g each were sensitized with 50 μg of retinal S-antigen in complete Freund’s adjuvant containing heat-killed tubercle bacilli, 2 mg/ml (Difco, Detroit, MI). The S-antigen was isolated from bovine retina according to the method described by Dorey et al.¹⁴ Each rat received 0.1 ml of the suspension containing S-antigen and complete Freund’s adjuvant (1:1) in one of the hindfoot pads. The rats were divided into five groups of six each and injected as follows: groups 1 and 2 (controls) were injected with culture medium (RPMI-1640) or 1000 μg mouse IgG, respectively. Group 3 received anti-CD4 (W3/25, isotype IgG) in a dose of 1000 μg per rat. These agents were injected intraperitoneally on days 3, 5, 8, 11, 14, and 17 after S-antigen injection. The animals were inspected daily for perilimbal conjunctival hyperemia and anterior chamber inflammatory reaction. On day 18 after S-antigen injection, the animals were anesthetized and blood samples were collected to determine the titer of anti-S-antibody, as described by Mochizuki et al.¹ The eyes were enucleated and processed for histologic examination. The spleens were processed individually for lymphocyte transformation assay in the presence of S-antigen.¹⁵ Identification of CD4⁺ and CD8⁺ cells was performed on frozen tissue from spleens and eyes by immunohistochemical staining, as described below.
The remaining rats (groups 4 and 5) were injected with anti-CD4 or with RPMI, respectively, on the same days as the other groups, but were killed 13 days later, on day 31 after S-antigen injection.

All procedures were performed in accordance with the ARVO Resolution on the Use of Animals in Research.

Monoclonal Antibodies

The anti-CD4 monoclonal antibody (W3/25) producing cell line was originally provided by Dr. Allan Williams (Oxford University, Oxford, England). Isotype switch variants of this hybridoma were produced by one of us (LS), and the IgG1 was used in this study. Anti-CD8 (OX8) was obtained from Accurate Chemical (Westbury, NY). The antibody-producing hybridomas were grown in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and antibiotics. Upon reaching confluency the cell cultures were resuspended in serum-free medium and incubated for 48 hr, at which time supernatants containing the monoclonal antibodies were collected. Protein concentration of the supernatants was measured using the standard Biorad assay. The supernatants were concentrated using ultrafiltration with 10 filters (Amicon, Danvers, MA); dialyzed against phosphate buffered saline (PBS), pH 7.4, using membranes of molecular weight 12–14 kD cut-off (Spectrum, Los Angeles, CA); sterilized by passage through 0.22-μm filters (VWR Scientific, Cerritos, CA); aliquoted; and stored at −70°C prior to use.

Determination of Antibody Specificity

Specificity of the monoclonal antibodies was determined using a technique similar to that described by McMaster and Williams. Briefly, 5 × 10⁵ spleen mononuclear cells were incubated with 50 μl antibody. The cells were washed twice with 1.5 ml PBS containing 0.5% bovine serum albumin and resuspended in 50 μl fluorescein-conjugated rabbit F(ab')2 anti-mouse IgG antibody, 25 μg/ml. After two additional washes, the cells were analyzed by flow cytometry (FACS IV; Becton Dickinson, Mountain View, CA). This analysis, as has been reported, revealed the presence of 41% of lymphocytes were CD4⁺ and 63% of monocytes were CD4⁺ in the spleen.

Detection of Antibodies to S-Antigen

Serum samples were titrated for antibody to S-antigen by an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well polyvinylchloride plates (Dynatech Laboratories, Alexandria, VA) were coated with S-antigen (50 ng/well in carbonate buffer). Plates were incubated at 37°C for 1 hr, and then washed five times with PBS containing 0.05% Tween-20. After incubation with PBS-Tween-20 at room temperature for 30 min, diluted serum samples (100 μl) were added. After incubation for 1 hr at 37°C, plates were washed five times with PBS-Tween; 100 μl peroxidase-conjugated anti-rat IgG (Dako, Santa Barbara, CA) in a dilution of 1:6000 was added to each well, after which the plates were incubated at room temperature for 1 hr. One hundred microliters of the substrate 4-phenylenediamine (Sigma, St. Louis, MO) in citrate buffer containing 3% H₂O₂ was added to the wells after washing. The reaction product was measured by absorbance at 490 nm. Antibody levels were expressed as the absorbance values at 1:400 dilution of each serum sample.

Lymphocyte Proliferation Assay

Rat mononuclear cells were isolated from single-cell spleen preparations by Ficoll-Paque gradient (Pharmacia, Piscataway, NJ). Proliferation assays were performed in flat-bottomed 96-well culture plates (Costar, Cambridge, MA) using RPMI-1640 supplemented with 10% FCS and 5 × 10⁻⁵ M 2-mercaptoethanol. One hundred microliters of mononuclear cell suspension (1 × 10⁶ cells/ml) and then 100 μl of appropriately diluted S-antigen or mitogen concanavalin A (ConA) reagent were dispensed into each well. Mitogen-stimulated cultures were incubated for 3 days, and antigen-stimulated cultures were incubated for 6 days at 37°C. At the end of the culture periods, the cells were pulsed for 6 hr with 1.0 μCi ³H-thymidine, the cultures harvested, and incorporated counts per minute (CPM) were assessed. Results were expressed as stimulation index (SI):

\[
SI = \frac{³H\text{ (cpm), cultures with S-antigen (or mitogen)}}{³H\text{ (cpm), control culture}}
\]

Indirect Immunoperoxidase Technique

Eight-micron sections of frozen spleens and eyes were prepared. The sections were fixed for 10 min in 99.5% acetone (reagent-grade; VWR), air-dried, and rinsed with PBS, pH 7.3. Primary antibodies specific for CD4 (undiluted) and for CD8 (diluted 1:100) were applied to tissue sections for 30 min. The slides then were washed with PBS and incubated with peroxidase-conjugated goat anti-mouse IgG, 1:50 (Tago, Burlingame, CA) at room temperature for 30 min. Aminoethylcarbazol (AEC) substrate in 1% hydrogen peroxide was added to the slides to develop the color. Slides were mounted in glycerogel and coverslipped.
Results

Clinically, on day 13 and 14 after S-antigen injections, the control animals in groups 1 and 2 (treated with RPMI and mouse IgG) developed conjunctival hyperemia. Cellular exudate in the anterior chamber and haziness of the vitreous cavity occurred within the next 48 hr. No animals in group 3 (treated with W3/25) showed evidence of conjunctival hyperemia or cells in the anterior chamber or in the vitreous. Histopathologically, uveitis and retinitis were noted in all of the animals in groups 1 and 2. These animals had marked inflammatory cell infiltrate in the iris, ciliary body (Fig. 1A), choroid, and retina (Fig. 1B).
The infiltrating cells were polymorphonuclear leukocytes, mononuclear cells, and a few plasma cells. Perivasculitis as well as vasculitis of the retinal vessels also were present. Destruction of the photoreceptor cell layer and loss of outer retinal layers with serous detachment of the retina were observed in several eyes. In group 3 (anti-CD4 treatment), no evidence of intraocular inflammation was observed. Uveal tracts and retinas were normal in all animals of this group (Fig. 2A,B). The results are summarized in Table 1. Histologic sections of the kidneys obtained from all animals, regardless of treatment group, showed no evidence of glomerulonephritis.

Anti-S-antibody levels and lymphocyte transformation SI s were determined on two randomly selected animals from groups 1 and 3. As summarized

Fig. 2. Animal treated with anti-CD4 (group 3). Ciliary body (A) appears normal (hematoxylin and eosin, × 325), as does retina and choroid (B) (hematoxylin and eosin, × 275).

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the signs of uveitis were apparent at day 14 after S-antigen injection in all six animals of group 5, but none of the six in group 4 showed inflammation (Table 1).

Hematoxylin and eosin-stained sections of the enucleated globes showed intraocular lesions of eyes. In contrast to group 5 animals, in which no lesions were observed in contrast to group 5 animals, in which no lesions were observed in group 4 (anti-CD4 treated), very few CD4+ cells were found. CD8+ cells were present in spleen sections of all groups.

Indirect immunohistochemical studies were done to localize CD4+ and CD8+ cells after treatment. Spleens of groups 1 and 2 animals revealed large numbers of CD4+ cells. In contrast, in animals of group 3 (anti-CD4 treated), very few CD4+ cells were found. CD8+ cells were present in spleen sections of all groups.

Immunohistochemical staining of frozen sections of the choroids of control animals showed the presence of CD4+ and CD8+ cells. Choroids of treated animals (group 3) exhibited no intraocular inflammation and no positive staining.

In group 4 (anti-CD4-treated animals) no disease was observed in contrast to group 5 animals, in which the signs of uveitis were apparent at day 14 after S-antigen injection. Hematoxylin and eosin-stained sections of the enucleated globes showed intraocular inflammation in all six animals of group 5, but none of the six in group 4 showed inflammation (Table 1).

**Discussion**

EAU is an autoimmune disease mediated by helper T-cells. The current studies clearly establish the effectiveness of W3/25 monoclonal antibody in the prevention of S-antigen-induced experimental uveitis. The complete inhibition of the disease by anti-CD4 monoclonal antibodies confirms the importance of these cells in the induction of this type of uveitis.1

Similar to our results in EAU, the mouse anti-rat CD4 antibody W3/25 was previously shown to have beneficial effects in the amelioration of EAE.4 It has been demonstrated that the demyelinating process can be prevented and that the ongoing demyelination can be reversed by this therapy.6,8,10,12,19 Such an effect was found to be due to selective binding or depletion of CD4 cells by these monoclonal antibodies. Similar beneficial effects have been noted in the treatment of other diseases as well.11,20 It has been reported also that immunotherapy with monoclonal antibodies to the lymphocyte subset may not only halt the progression of the autoimmune disease but may lead to long-term reversal of the disease after therapy has ended.20

The role played by the immunoglobulin isotype in the therapy of autoimmune disease has been established clearly by Waldor et al.21 Heavy-chain class switch variants of the IgM mouse anti-rat CD4 antibody W3/25 were isolated in order to assess the importance of antibody isotype in the anti-CD4 treatment of EAE. Although all isotypes showed some beneficial effects in the amelioration of the EAE, some isotypes were superior to others in the treatment of EAE. Therefore, the selection of the appropriate isotype of the anti-CD4 antibody may be critical for a good response.

Localization by an immunoperoxidase technique of CD4+ cells in sections of spleens from animals treated with anti-CD4 revealed selective loss of this marker or of the cells bearing this molecule. It is possible that, in the current experiment, inhibition of uveitis development was caused by the depletion of the CD4 cell subset or by binding of the monoclonal antibody to this subset. However, recent reports have established that cell elimination is not necessary for successful immunotherapy. Loss of antigens may rep-

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>Dosage per rat*</th>
<th>Day of enucleation</th>
<th>No. of rats with EAU/total†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RPMI 1640</td>
<td>0.5 ml</td>
<td>18</td>
<td>6/6</td>
</tr>
<tr>
<td>2</td>
<td>IgG</td>
<td>1000 μg</td>
<td>18</td>
<td>6/6</td>
</tr>
<tr>
<td>3</td>
<td>Anti-CD4 (W3/25)</td>
<td>1000 μg</td>
<td>18</td>
<td>0/6</td>
</tr>
<tr>
<td>4</td>
<td>Anti-CD4 (W3/25)</td>
<td>1000 μg</td>
<td>31</td>
<td>0/6</td>
</tr>
<tr>
<td>5</td>
<td>RPMI 1640</td>
<td>0.5 ml</td>
<td>31</td>
<td>6/6</td>
</tr>
</tbody>
</table>

* Injections were given in a volume of 0.5 ml intraperitoneally, beginning 5 days after S-antigen injection and repeated on days 8, 11, 14, and 17.† Histopathologic examination of hematoxylin and eosin-stained preparations of eyes.

in Table 2, both the SIs and the antibody levels in the treated groups were markedly diminished as compared with those in the control group.

**Table 2. Effect of anti-CD4 treatment on antigen-specific and polyclonal immune responses**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Anti-S antibody, OD</th>
<th>S-antigen</th>
<th>Con A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RPMI</td>
<td></td>
<td>8.7 ± 0.14</td>
<td>34.7 ± 0.04</td>
</tr>
<tr>
<td>3</td>
<td>Anti-CD4 (W3/25)*</td>
<td>0.27 ± 0.10, P &lt; 0.001†</td>
<td>1.5 ± 0.20, P &lt; 0.001†</td>
<td>26.9 ± 0.18, P &lt; 0.1</td>
</tr>
</tbody>
</table>

* W3/25 was injected at a dose of 1000 μg/injection.† Student t-test (two-tailed).‡ S-antigen was used in a concentration of 27 μg/ml and Con A in a concentration of 5 μg/ml. Plates were incubated for 3 days in the Con A assay and for 6 days in the S-antigen assay. Mean stimulation index CPM stimulated cells and CPM cells in medium.
resent capping and shedding of the marker or blocking of epitopes by monoclonal antibodies.\(^2\) It has been observed that the anti-CD4 treatment, by depleting CD4 cells, inhibits protective immunity as well as autoimmunity.\(^23,24\) Hence, suppression of normal immune function persists for a prolonged period, even after treatment is stopped. Long-standing inhibition of uveoretinitis after cessation of therapy suggests that antibody treatment protects the uvea from further destruction. Although antibodies to CD4 induce tolerance to protein antigens,\(^25\) such tolerance occurs only when the initial exposure to antigen occurs after antibody treatment.\(^26\) It is possible that sustained reduction of CD4\(^+\) T-cells create an environment that allows dominant suppressive mechanisms to emerge.\(^26\)

The specific reduction in responsiveness to the S-antigen indicates a specific blocking of antigen responses, and not an overall loss of reactive cells, since the concanavalin A (ConA) SI was not significantly different in the treated than in the control group (Table 2). On the other hand, the ConA-responsive cells belong also to the CD8 subset, which accounts for the less marked significant change in ConA responsiveness.

In addition to inhibition by monoclonal antibodies against helper T-cells, suppression of EAU in rats by anti-IA antibody treatment has been demonstrated.\(^27\) Detailed studies were done by Rao et al.,\(^18\) who reported that complete inhibition of uveitis was noted in approximately 92\% of animals treated with anti-IA antibodies. Successful immunotherapy was found to depend on selection of the appropriate antibody with regard to affinity, isotype, and dosage. In the current study, we found that treatment with anti-helper antibodies led to inhibition of the disease in 100\% animals, which may be due to the fact that the CD4 antibody isotype is an appropriate one for immunotherapy. Moreover, treatment with the monoclonal antibodies did not cause any toxic side effects, and none of the treated animals developed anaphylaxis or glomerulonephritis.

In conclusion, treatment with the monoclonal antibody W3/25, which is directed against the CD4 antigen on the surface of the helper T-cell, specifically suppressed the immune response in this experimental autoimmune uveitis.

**Key words:** autoimmune uveitis, anti-CD4 antibody, helper/inducer cell, rat, retinal S-antigen

**References**


