Effects of Cyclosporine on T-Cell Subsets in Experimental Autoimmune Uveitis

Robert D. Nussenblatt* and Irwin Scherf

The effective inhibition of S-antigen (S-Ag) induced experimental autoimmune uveitis (EAU) by Cyclosporine (CsA) suggests strongly the important role of T-cells in the modulation of this disease. The authors evaluated the changes in T-cell subsets induced by this agent in S-Ag immunized Lewis rats. Using the fluorescence-activated cell sorter and monoclonal antibody preparations directed against rat T-cell subsets, a comparison was made between lymphocyte populations obtained from CsA or olive oil treated S-Ag immunized rats taken 5, 10, 12, and 14 days after antigenic challenge. The T-cell subpopulations of lymphocyte preparations from the spleen and peripheral blood of CsA-treated and control animals appeared to parallel each other, with both groups showing an increase in the suppressor/cytotoxic fraction beginning on day 12 and approaching the percentage of inducer cells by day 14. Lymphocyte preparations from lymph nodes draining the site of S-Ag immunization from CsA-treated animals demonstrated a different T-cell subset profile than did controls. Beginning on day 10, the control group was noted to have an increased inducer cell fraction as compared with the CsA group. This increase in the inducer fraction paralleled an increase in the in vitro proliferative responses to the S-Ag. These data suggest that CsA appears to prevent the development of inducer cells in the lymph nodes draining the S-Ag immunization site, the T-cell subgroup the authors have seen capable of inducing EAU. Invest Ophthalmol Vis Sci 26:10-14, 1985

Experimental autoimmune uveoretinitis is induced with the retinal S-antigen, tentatively identified as rhodopsin kinase. This immune mediated model of ocular inflammatory disease can be transferred by T-cells, but not by hyperimmune serum, suggesting the important role of the T-cell in the expression of this disorder. To further support this hypothesis, we have additionally reported the effective abrogation of S-antigen-induced experimental autoimmune uveitis with Cyclosporine (CsA), a drug with essentially specific anti-T-cell characteristics. These studies firmly reemphasized the mandatory role of the T-cell in this model for human ocular inflammatory disease. We have noted changes in the proliferative response to the immunizing antigen in animals treated with CsA. These findings suggested that CsA was interfering with the recruitment of immunoreactive cells. The fluorescence activated cell sorter can evaluate the potential shifts of T-cell populations by quantitatively evaluating the number of T-cell subsets present as defined by their staining characteristics with monoclonal antibody preparations specific to the various T-cell subfractions. We report here our findings in a longitudinal study in experimental autoimmune uveitis, in which T-cell subsets were followed in animals treated with Cyclosporine utilizing the fluorescence activated cell sorter and were correlated with proliferative responses and disease.

Materials and Methods

Animals

A total of 48 female Lewis rats, weighing approximately 200 g each, were used for this study. At least three animals were included in each test group. All animals were immunized with a total of 30 μg of S-antigen, prepared as outlined elsewhere, mixed in an equal volume of complete Freund's adjuvant (Difco; Detroit, MI) augmented with mycobacterium HR37, so that the final concentration of mycobacteria was raised to a total of 5 mg per ml. CsA therapy was given as outlined previously. A total of 10 mg per kg of CsA was given daily until killing. Control animals received olive oil daily.

Cellular Preparations and Monoclonal Antibodies

At various points after immunization, the animals were killed and cellular preparations were derived from peripheral blood, lymph node, and spleen. The

From the Clinical Ophthalmic Immunology Section, National Eye Institute, National Institutes of Health, Bethesda, Maryland* and Merck, Sharp & Dohme Research Laboratories, Rahway, New Jersey.†

Submitted for publication: February 29, 1984.

Reprint requests: Robert B. Nussenblatt, MD, National Eye Institute, National Institutes of Health, Building 10, Room 10N202, Bethesda, MD 20205.
peripheral blood was separated utilizing a centrifugation gradient as reported elsewhere. The cells then were washed twice and prepared for either staining with monoclonal antibodies or in vitro culture. The draining lymph nodes from the site of S-antigen immunization were teased gently and washed three times. Spleens also were taken and teased gently. This preparation was macrophage depleted by a 45-minute incubation in a petri dish with 5% fetal calf serum, and the nonadherent fraction then was washed twice in preparation for monoclonal antibody staining.

Monoclonal Antibody Preparations to Rat T-Cell Subsets

The MAS 031C (Accurate Chemical and Scientific Corp., Westbury, NY) was utilized at the concentration of 1:100. This monoclonal antibody specifically adheres to the inducer fraction of the T-cell population. The MAS 041C, which delineates the putative suppressor/cytotoxic cell fraction was utilized at a concentration of 1:500. The cells were prepared in a manner as has been reported elsewhere. At least 10⁸ cells were used for staining with each monoclonal preparation. A mouse-derived monoclonal antibody of the same class, with no known specificity to rat T-cells was utilized as a control. At least 10⁶ cells from each sample were examined with the fluorescence-activated cell sorter, with nonviable lymphocytes excluded from evaluation. A computer subtraction program determined the frequency of specifically labelled T-cells as a percent of the T-cell population, with the methodology described in detail elsewhere.

Cell Cultures

At various points after S-antigen immunization as indicated in the results section, cell cultures were prepared from the same cell preparations as were evaluated with the fluorescence activated cell sorter. The spleen cultures were prepared before macrophage depletion had occurred. Cell culture preparations contained 1.5 × 10⁶ cells per ml. Cell cultures were prepared in microtiter wells containing 0.2 cc of RPMI 1640 medium with 10% pooled heat inactivated AB+ human serum, and were incubated four days at 37°C, 5% CO₂ with tritiated thymidine added 14 hours before termination of culture. A concentration of S-antigen of 1 μg per ml was added to the appropriate wells at the time of culture. The proliferative responses to the S-antigen are presented as stimulation indices that were derived as reported elsewhere.

The determination of statistical significance was evaluated utilizing the Student's t-test.

The animal investigations described in this article conform to the ARVO Resolution on the Use of Animals in Research.

Results

Cell suspensions prepared from the blood, lymph node, and spleen of the same animals were prepared with the various anti-T-cell monoclonal antibody preparations and T-cell subfractions were determined by the fluorescence-activated cell sorter on days 5, 10, 12, and 14 after S-antigen immunization. The animals receiving oil injections alone and killed on day 14 all had clinical evidence of uveitis. This was in contradistinction to the group receiving CsA, which were all without ocular inflammatory disease on day 14. The results of the T-cell subset fractions from the various cellular preparations can be seen in Figure 1. At various points after S-antigen immunization as indicated in the results section, cell cultures were prepared from the same cell preparations as were evaluated with the fluorescence activated cell sorter. The spleen cultures were prepared before macrophage depletion had occurred. Cell culture preparations contained 1.5 × 10⁶ cells per ml. Cell cultures were prepared in microtiter wells containing 0.2 cc of RPMI 1640 medium with 10% pooled heat inactivated AB+ human serum, and were incubated four days at 37°C, 5% CO₂ with tritiated thymidine added 14 hours before termination of culture. A concentration of S-antigen of 1 μg per ml was added to the appropriate wells at the time of culture. The proliferative responses to the S-antigen are presented as stimulation indices that were derived as reported elsewhere.

The determination of statistical significance was evaluated utilizing the Student's t-test.

The animal investigations described in this article conform to the ARVO Resolution on the Use of Animals in Research.

Results

Cell suspensions prepared from the blood, lymph node, and spleen of the same animals were prepared with the various anti-T-cell monoclonal antibody preparations and T-cell subfractions were determined by the fluorescence-activated cell sorter on days 5, 10, 12, and 14 after S-antigen immunization. The animals receiving oil injections alone and killed on day 14 all had clinical evidence of uveitis. This was in contradistinction to the group receiving CsA, which were all without ocular inflammatory disease on day 14. The results of the T-cell subset fractions from the various cellular preparations can be seen in Figure 1. At various points after S-antigen immunization as indicated in the results section, cell cultures were prepared from the same cell preparations as were evaluated with the fluorescence activated cell sorter. The spleen cultures were prepared before macrophage depletion had occurred. Cell culture preparations contained 1.5 × 10⁶ cells per ml. Cell cultures were prepared in microtiter wells containing 0.2 cc of RPMI 1640 medium with 10% pooled heat inactivated AB+ human serum, and were incubated four days at 37°C, 5% CO₂ with tritiated thymidine added 14 hours before termination of culture. A concentration of S-antigen of 1 μg per ml was added to the appropriate wells at the time of culture. The proliferative responses to the S-antigen are presented as stimulation indices that were derived as reported elsewhere.

The determination of statistical significance was evaluated utilizing the Student's t-test.
the CsA treated group was noted on day 14 as compared with the controlled oil-treated rats. This difference also was not statistically significant.

The proliferative responses to the S-Ag from the same cell preparations that were evaluated in the fluorescence activated cell sorter can be seen in Figure 2. All control wells (ie, receiving no antigen or mitogen stimulation) for both groups had proliferative values that fall between 400–800 CPM. Figure 2, top, shows the proliferative responses to the S-Ag from the peripheral blood obtained in culture. A proliferative response statistically significantly above that of the control values was noted to be present on days 13 and 14 from the peripheral blood preparations obtained from animals receiving oil injections. This pattern of response corroborates that noted in a previous communication. These findings could not be correlated with differences in T-cell subsets as defined by the fluorescence-activated cell sorter. The lymph node responses to the S-Ag were quite marked (Fig. 2, middle). The proliferative responses from the lymph node cell preparations obtained from animals receiving only oil injections peaks on day 11. This peak occurred somewhat earlier than that seen in the blood. The lymph node in vitro proliferative response to the S-Ag in the CsA-treated animals is dampened as compared with the oil-treated group with a small-but-significant peak seen on day 12. The larger proliferative response to the S-Ag observed in the oil-treated S-Ag immunized animals appears to correlate with the increase in the inducer cell fraction that becomes present at this time, as was noted in Figure 1, middle. Splenic cultures did not demonstrate proliferative responses in the presence of the S-Ag above that of control wells (Fig. 2, bottom).

Discussion

The use of the fluorescence-activated cell sorter (FACS) enables the observer to quantify graphically the percent of various cellular components in a given preparation. The monoclonal antibodies used in this study have been shown to recognize different T-cell populations with different functional characteristics. The MAS 010C antibody has been found to bind to all mature rat T-cells. However, the MAS 031C+ subgroup of cells has been identified to aid in antibody responses and mediate graft-vs-host reactions, and the MAS 041C, MAS 031C− subgroup appear to
suppress antibody formation in F₁ hosts. The use of these markers coupled with the FACS provided us with an opportunity to follow kinetically the potential cellular changes that might occur in various cellular compartments as CsA is administered to S-Ag immunized rats. The results demonstrate a profound decrease in the number of T-cells bearing the MAS031C marker in the lymph node draining the site of S-Ag immunization in CsA versus control animals. Additionally, these changes were concomitant with a marked decrease in the proliferative response to the immunizing antigen. However, no statistically significant difference could be seen in the CsA-treated and control rats when the MAS041C subset was compared. It is interesting to note that this subset percentage increases in both groups towards the end of 2 weeks, when disease expression occurs. These cells could be thought to correlate broadly with the OKT8+ fraction of human T-cells. Indeed, we have noted an increase in the OKT8 fraction at the acute phase of posterior uveitis in humans. Our preliminary evidence further suggested that CsA-treated uveitis patients had a further increase in their OKT8 fraction. This also has been noted by Routhier and colleagues in their treatment of patients of primary biliary cirrhosis. However, an increase in the suppressor/cytotoxic fraction in the blood was not prognostic. Further, the human studies compare an activated population (uveitis) with a putatively “inactive” group, controls. In this study, the comparison is between two S-Ag immunized groups, the consequence of which is profound immune activation. It would seem that the clinical and laboratory situations are not totally comparable. It should be added further that the effectiveness of FACS analysis is dependent upon the relative specificity of the monoclonal antibody used to define subpopulations of cells. Newer monoclonal antibodies are being sought to further subdivide the suppressor/cytotoxic subset. It may be that a small reactive clone important to the immune kinetics of this model could not be defined with these antisera. By contrast, the data from the lymph node studies seem to indicate that an increased inducer T-cell population and the acquisition of blastogenic responsiveness in the blood in the oil-treated rats corresponded with the onset of disease.

The results of CsA therapy and its effects on T-cell subsets should not be extended automatically to other species. The effects of CsA administration may vary depending on the species studied. Green and colleagues have reported the induction of a tolerant state
in rabbits receiving renal allografts when given CsA. However, tolerance was not obtainable in a similar allograft system in dogs. We have reported that CsA therapy of rats after S-Ag immunization prevented the expression of the disease at least 40 days after CsA therapy had ceased, suggesting that active suppression, extending beyond the agents pharmacologic life, had been induced. Additionally, a non-adherent splenic T-cell fraction transferred from CsA-treated rats to naive recipients will prevent S-Ag induced EAU, further supporting the notion that active suppression has occurred (manuscript in preparation). This putative suppressor cell cannot as yet be defined with antisera generated against rat T-cell subsets now available. It is possible that further dissection of these established subsets with newer monoclonal preparations to further subdivide these populations will better delineate the reactive clone(s), probably small in number, responsible for modulation of the model of ocular inflammatory disease.

**Key words:** experimental autoimmune uveitis, cyclosporine, T-cell subsets, monoclonal antibodies, inducer T-cell subset, suppressor/cytotoxic T-cell subset

**References**