Cryopexy Enhances Experimental Autoimmune Uveoretinitis (EAU) in Rats

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Treatment of rat eyes with cryopexy enhanced the development of experimental autoimmune uveitis (EAU) in these eyes. The enhancement of EAU by cryopexy was particularly pronounced when the disease was induced by active immunization in rats of a low responder strain (Wistar Furth), or by adoptive transfer with lymphocytes sensitized against S-antigen. The disease enhancement was expressed by earlier onset of clinical symptoms and by more severe inflammatory changes. Histological examination of cryopexy-treated eyes showed focal necrosis and inflammation, confined to the affected sites. Immunohistochemical analysis of the inflammatory infiltration revealed it consists mainly of macrophages and T-lymphocytes of the helper and suppressor subsets. In addition, increased expression of class II antigens was observed in affected areas, on both inflammatory and resident ocular cells. Using electron microscopy and Evans blue angiography we could show breakdown of the blood-retinal barrier at the treated sites. Histological examination of eyes with EAU following cryopexy showed localization of the early inflammation at the injured site. The data are interpreted to suggest that the enhanced EAU in cryopexy-treated eyes is mainly due to the breakdown of the blood–retinal barrier, the accumulation of lymphoid cells and the increased expression of class II antigens, which facilitates antigen presentation. Invest Ophthalmol Vis Sci 30:2165–2173, 1989

Cryotherapy routinely is used in the treatment of a variety of ocular conditions, such as in the treatment of retinal detachment and in the control of inflammation in pars planitis. The use of cryosurgery in the treatment of retinal detachment was first advocated by Bietti in 1933. It was Lincoff, however, who popularized the technique through his early studies on the effects of cryosurgery in rabbits and through his later use of cryopexy in the treatment of retinal detachments in humans. In most cases, the cryosurgical treatment led to various degrees of scarring without much inflammation. It was only when there was excessive freezing that postoperative uveitis occurred. These findings were confirmed by Chignell and his colleagues. Cyropexy was first advocated for the control of inflammation in pars planitis by Aaberg in 1973 and a more recent study has confirmed its beneficial effects in this entity, in particular in cases with neovascularization. Cryotherapy was also reported to be a useful treatment for other neovascular conditions.

Damage is produced by cryosurgery in an attempt to reverse a pathological condition within the eye. Penetrating trauma to the globe, on the other hand, can initiate a pathogenic autoimmune response, which is considered to be the pathogenic mechanism of sympathetic ophthalmia. The present study was aimed at examining the effects of cryopexy on the rat eye. The parameters tested included: (1) monitoring the effect of cryopexy on the development of experimental autoimmune uveitis (EAU), an animal disease which is considered a model for certain uveitic conditions in man; (2) evaluation of the changes in the treated eye, using histological and immunohistochemical procedures; and (3) testing a possible breakdown of the blood–retinal barrier.

Materials and Methods

Animals

Male Lewis or Wistar Furth (WF) rats, 8 to 12 weeks old, were supplied by Harlan-Sprague Dawley (Indianapolis, IN). All procedures involving animals were performed in adherence to the ARVO Resolution on the Use of Animals in Research.

Immunization of Rats

Bovine S-antigen (S-Ag) was prepared according to Dorey et al. Rats were immunized with S-Ag emul-
sified (1:1) in complete Freund's adjuvant containing 2 mg/ml Mycobacterium tuberculosis (H37 RA) (Difco, Detroit, MI), and injected into one hind footpad in a volume of 0.1 ml. A suspension of Bordetella pertussis bacteria (Michigan Department of Public Health, Lansing, MI, lot 91B) was concomitantly injected into the peritoneum, 10^10 organisms per rat.

Evaluation of Disease

The rats immunized with S-Ag were observed daily for clinical ocular changes, and disease occurrence and severity were verified by histological examination as described elsewhere.11

Adoptive Transfer of EAU

Draining lymph node cells from rats immunized with 30 ^g of bovine S-Ag were collected 11 days postimmunization and were used to adoptively transfer EAU after a 3-day incubation with S-Ag. The procedure was similar to that used in our previous studies.12 Briefly, the lymphoid cells were incubated with the antigen (at 3 ^g/ml) in aliquots of 2 ml at 2 X 10^6 cells/ml in 12-well cluster plates (Costar, Cambridge, MA). After incubation for 3 days, the cells were collected, washed and injected i.p. into naive recipients. The characteristic features of development, kinetics and morphology of EAU in recipients of cells sensitized to S-Ag have been described elsewhere.12 The grading of EAU severity in recipient rats was carried out in the same manner as that for actively immunized animals.

Cryopexy

The cryoprobe used in this study was a Beaver Cryo X Tractor (Beaver Waltham, MA), after removal of its freezing unit. The plastic-covered metal rod of the probe was immersed in liquid nitrogen for 1–2 min and applied immediately to one eye of anesthetized rats at two separate spots, supernasally and supertemporally, as posteriorly to the ora serata as possible. Each spot was treated with the probe for 45 sec.

Immunohistochemistry

Frozen sections of rat eyes were prepared and stained with various antibodies by the avidin-biotin peroxidase complex as described in detail elsewhere.13,14 The monoclonal antibodies used in this study, including OX-6, OX-42, W3/25 and OX-8, were obtained from Accurate Chemical and Scientific Corp. (Westbury, NY). Polyclonal antibody against glial fibrillary acidic protein (GFAP) was purchased from Dako Corp. (Santa Barbara, CA). Secondary antibody for the murine monoclonal antibodies was biotin-labelled goat anti-mouse IgG, depleted of antibody cross-reactivity with rat IgG (American Qualex Inc., La Mirada, CA). Secondary antibody for the GFAP antibodies was biotin labelled goat anti-rabbit IgG (Vector Lab, Burlingame, CA).

Transmission Electron Microscopy

A small slit opening was made at the limbus, and the whole eye was immersed in 4% glutaraldehyde. After 5 min of fixation, the lens was removed and the eye immersed again in glutaraldehyde for further fixation. Small pieces of tissue, about 0.5 X 1 mm, were excised from several locations. The pieces were post-fixed in 1% osmium tetroxide solution, dehydrated in graded alcohols and propylene oxide and embedded in epoxy resin. The blocks were oriented to show the radial plane of the retina in the sections. Thick sections were stained with toluidine blue and thin sections with uranyl acetate–lead citrate. Thin sections were then examined with a transmission electron microscope (JEOL 100C).

Evans Blue Technique

One milliliter of Evans blue 1% diluted in balanced salt solution was injected in the inferior vena cava of rats 1 hr and 24 hr after cryopexy. The dye was allowed to circulate for 30 min and the eyes were then enucleated and fixed in 10% formalin.

Results

Enhancement of EAU by Cryopexy

The possible effect of cryopexy on EAU induction was examined by comparing the disease development in cryo-treated eyes and the control untreated eyes of the same rats. EAU was induced in these rats by either active immunization with S-Ag, or by adoptive transfer of S-Ag sensitized lymph node cells.

Table 1 summarizes the data of experiments in which the effect of cryopexy was examined in rats developing EAU following active immunization with S-Ag. The two tested rat strains differ in their susceptibility to S-Ag-induced EAU, with Lewis rats being the “high responders” and WF rats, the “low responders.”15 The difference between the two strains is indicated in this Table by the time of disease onset of the untreated control groups, which was approximately 3 days shorter in Lewis than in WF rats. The disease onset time in the untreated eyes of the experimental rats was similar to that of the corresponding untreated control rats, while remarkably shorter onset time was observed in the eyes treated with cryopexy. The cryopexy effect was more pronounced in the low responder WF rats (shortening of the onset...
Table 1. Effect of cryopexy on EAU development in rats actively immunized with S-Ag*

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Untreated rats</th>
<th>Treated rats</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Lewis</td>
<td>9.7 ± 0.3†</td>
<td>8.0 ± 0.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>WF</td>
<td>13.0 ± 2.1</td>
<td>10.3 ± 1.8</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

* All rats were immunized with 50 μg of bovine S-Ag.
† One eye of each rat was treated with cryopexy, 2 days before immunization.
‡ Day of disease onset, mean of seven rats in each group ± SD.
§ Differences between cryopexy-treated and untreated eyes of the same rats.

Histopathology of Ocular Damage Produced by Cryopexy

The histopathological changes in rat eyes treated with cryopexy were examined at various time intervals following the treatment (Fig. 1). Clear changes were seen as soon as 1 hr after treatment and included marked disorganization of the retinal cellular arrangement of the frozen site, as well as foci of exudation (Fig. 1a). The changes reached their peak at 3 days after cryopexy, with the affected retina becoming necrotic and gliotic and heavily infiltrated with inflammatory cells, mainly phagocytes (Fig. 1b). At 11 days after cryopexy, the inflammatory reaction subsided almost completely, but the damaged retina became atrophied (Fig. 1c). It is noteworthy that the changes at all tested time intervals were confined to the affected areas, with no detectable pathological changes in the tissues surrounding the cryopexy-treated focus.

Immunohistochemical Examination of Cryopexy-Treated Eyes

The changes induced by cryopexy in these rat eyes were further analyzed by immunohistochemical examination (Table 3). The inflammatory infiltrate at the treatment site was found to consist mainly of macrophages (not shown) and T-helper and T-suppressor lymphocytes (Fig. 2a, b, respectively). In addition, the affected tissue showed an increase in expression of class II antigen (Fig. 3b, c).

It was of particular interest that there was increased expression of the GFAP antigen on Müller and glial cells (Fig. 4) of treated eyes. Intense staining with antibodies to GFAP was not limited to the damaged site and was observed throughout. In contrast, no increase in GFAP staining was noted in the untreated control eyes.

Breakdown of the Blood–Retinal Barrier after Cryopexy

Cryopexy was found to cause a breakdown of the blood–retinal barrier, as shown by two methods. By transmission electron microscopy, a widening of the tight junctions between retinal vascular endothelial cells was seen within 1 hr following cryopexy (Fig. 5b). In contrast, no change in the normally tight junctions was noted in the retina immediately adjacent to the cryopexy lesion (Fig. 5a). A breakdown of the blood–retinal barrier was also demonstrated using the Evans blue angiographic technique. A substantial leakage of the Evans blue dye was seen outside the retinal vessels adjacent to the treated area, while no leakage was observed surrounding the untreated areas (Fig. 6).

Histopathological Analysis of EAU in Cryopexy-Treated Eyes

The effects of cryopexy on EAU development were further analyzed by examining the histological changes in eyes of rats in which the disease was enhanced by cryopexy. Figure 7 shows the changes in an eye of such a rat. EAU was induced in these rats by adoptive transfer of sensitized lymphocytes and the eye shown here was enucleated shortly after disease onset. A finding of interest is that the changes at the cryopexy-treated site (Fig. 7a) are profoundly more severe than those at the contralateral, untreated area (Fig. 7b). The section in Figure 7a consists of the cryopexy-treated area and the tissue adjacent to it.

Table 2. Cryopexy enhances EAU induced by adoptively transferred sensitized lymphocytes*

<table>
<thead>
<tr>
<th>Eyes</th>
<th>Onset day†</th>
<th>Severity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>8.1 ± 0.3</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Cryopexy-treated</td>
<td>4.5 ± 0.3</td>
<td>2.3 ± 0.4</td>
</tr>
</tbody>
</table>

* Recipient rats (n = 6) were treated with cryopexy in one eye 24 hr before being injected with 30–120 × 10⁶ sensitized lymphoid cells, as described in Materials and Methods.
† Mean values ± SE.
‡ Differences between untreated and cryopexy-treated eyes of the same rat.
Fig. 1. Histopathological changes at different time intervals following cryopexy. (a) One hour after treatment. Disorganization of the cellular arrangement of the retina as well as foci of exudation. (b) Three days after cryopexy. A pronounced inflammatory response throughout the affected retina, with a few infiltrating cells in the vitreous. The affected area of the retina also shows necrosis and gliosis. Bruch's membrane remains intact, but the choroid exhibits some fibrosis. (c) Eleven days post-treatment. No inflammation is seen, but the affected retina is completely atrophied, forming a scar with the corresponding choroid. Please note the focal nature of the changes, with normal appearance of the tissues surrounding the well-demarcated damaged area (H&E, X160).

The histopathological changes in the adjacent tissue are similar to those typically seen in eyes with severe EAU\(^8,9,12\) and mainly include retinal detachment, with heavy infiltration in the subretinal space and throughout the retinal layers. Unusual changes are seen in the cryopexy-affected area. The characteristic morphology of the retina is completely destroyed and the degenerated tissue is heavily infiltrated. The choroid also is intensely infiltrated and is thickened to a degree not seen in eyes in which EAU is induced by conventional procedures.

Discussion

The data reported here demonstrate that cryopexy enhances the development of EAU in rats. The en-
Table 3. Immunohistochemical characterization of the changes in antigenic markers after cryopexy

<table>
<thead>
<tr>
<th>Days post-treatment</th>
<th>OX-6</th>
<th>OX-42</th>
<th>W3/25</th>
<th>OX-8</th>
<th>GFAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>±</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>±</td>
</tr>
<tr>
<td>3 hr</td>
<td>+</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>±</td>
</tr>
<tr>
<td>1 day</td>
<td>+++</td>
<td>+++++</td>
<td>0</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>3 days</td>
<td>+++</td>
<td>++++</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>5 days</td>
<td>+++</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>8 days</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>++</td>
</tr>
<tr>
<td>12 days</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>++</td>
</tr>
</tbody>
</table>

* The markers' specificity: OX-6, rat class II antigens; OX-42, macrophages; W3/25, helper-inducer T lymphocytes; OX-8, suppressor/cytotoxic T lymphocytes; GFAP, a protein specific for astrocytes and related cells.

Enhancement was particularly clear in rats in which the disease was induced by adoptive transfer of sensitized lymphocytes (Table 2), or in WF rats which are "low responders" to EAU induced by active immunization with S-Ag15 (Table 1). The effect of cryopexy was less pronounced in Lewis rats actively immunized with S-Ag (Table 1). The low effect of cryopexy in actively immunized Lewis rats is assumed to be due to the high responsiveness of these rats to EAU induced by S-Ag.15 The enhancing effect of cryopexy on EAU development was also demonstrated by the finding that the early histopathological changes in eyes developing EAU following cryopexy localized mainly at the affected site (Fig. 7). It is of note that local injury has been found to enhance other organ-specific diseases, experimental allergic encephalomyelitis and adrenalitis.16

The mechanisms whereby cryopexy enhances EAU development are not completely understood, but the data collected in this study provide certain clues. A pivotal change in the cryopexy-treated eye is the breakdown of the blood-retinal barrier, which was demonstrated by electron microscopy and by the Evans blue technique. Our findings in the rat are in line with those of Jaccoma et al17 in the rabbit. The blood-retinal barrier provides a certain degree of sequestration to the retina and its breakdown is assumed to have a dual effect: (1) it allows free exposure of the retinal-specific antigens to the immune system; and (2) it facilitates the infiltration of inflammatory cells into the retina. The infiltrating cells were identified in the present study using the immunohistochemical procedure. A major proportion of these cells were histiocytes and T-lymphocytes, mainly of the helper/inducer subset. These two lymphoid cell populations initiate the majority of immune responses and are the two pivotal components of the

Fig. 2. Immunohistochemical analysis of the infiltrating lymphocytes in an affected area in the posterior segment of a rat eye, 5 days after cryopexy. (a) A section stained with antibody W3/25, specific for the helper/inducer T-lymphocytes. (b) A section stained with antibody OX-8, specific for the suppressor/cytotoxic subset. Lymphocytes typically stained with the antibodies are indicated by arrows. Please note the higher number of lymphocytes of the helper/inducer subset (avidin-biotinperoxidase, X100).
Fig. 3. Expression of MHC class II antigen in the posterior segment of rat eyes treated with cryopexy. (a) An untreated control eye. (b) An eye 3 days after cryopexy, with increased expression of class II antigen on both resident and infiltrating cells. (c) An eye 5 days after cryopexy, showing intense staining with the class II antibody, mainly by infiltrating cells (avidin-biotin-peroxidase, ×160).

Fig. 4. Increased expression of GFAP following cryopexy. An eye 5 days after treatment, showing intense staining of Müller cells. Insert, a control untreated eye (avidin-biotin-peroxidase, ×400).

tion of the sensitized or nonsensitized lymphocytes, thus indicating that the increased localization of lymphocytes in the cryopexy-treated eyes is mostly non-specific (unpublished data).

The immunohistochemical analysis of cryopexy-treated eyes also showed a striking increase in the expression of GFAP by Müller cells throughout the affected eyes. This finding is corroborated by the observations of other investigators in which increased expression of GFAP by Müller cells is found in eyes damaged by a variety of different insults. The role of GFAP in the EAU process is not known, although increased GFAP has been noted in eyes in which EAU was induced by immunization with S-Ag.

A clearer connection to the development of EAU can be made from another immunohistochemical finding, namely, the increased expression of class II MHC antigens in the cryopexy-treated eyes. Class II MHC antigens play a pivotal role in the process in which protein antigenic determinants are recognized by T-lymphocytes; the recognition requires an association between these determinants and the class II antigens, which are expressed on antigen-presenting cells (APC). Since the recognition of autologous antigens by T-lymphocytes is an obligatory step in the initiation of autoimmune diseases, it is assumed that class II antigens should be expressed on APC at the affected sites. This assumption has been corroborated by the finding of increased levels of class II antigens in various organs (eg, brain, thyroid), when affected by pathogenic autoimmune processes. Similarly, increased class II expression was observed on various cells in eyes developing EAU and at least some of these cells could serve as APC in the autoimmune process against the ocular antigens. It is proposed,

type IV immune-mediated inflammation, the basic pathogenic process of EAU.

In another study further evidence for the accumulation of T-lymphocytes in cryopexy-treated eyes was obtained, using the system in which isotope-labelled lymphocytes are injected to naive recipients, to determine their localization in the eyes and in other organs. Preliminary data of that study indicate that isotope-labelled lymphocytes accumulate two to three times more in the cryopexy-treated eyes than in the control eyes. The injected lymphocytes were either spleen cells from normal rats that had been activated in culture with concanavalin A, or cell lines specific toward retinal antigens (S-Ag) or the interphotoreceptor retinoid-binding protein. No significant differences were found between the localization...
therefore, that the increased expression of class II antigen in eyes treated with cryopexy facilitates autoantigen presentation and thus enhances the autoimmune processes of EAU in these eyes.

Both lymphoid and nonlymphoid cells were found to express class II antigens in affected organs and some authors have proposed that even the nonlymphoid cells could serve as functional APC.\textsuperscript{25-27} This notion has recently been challenged, however, and in at least two systems, nonlymphoid cells expressing class II antigens were found to induce unresponsiveness.\textsuperscript{29,30}

The mechanism by which cryopexy induces the expression of class II antigens is unclear, but it is conceivable that lymphokines such as interferon-\(\gamma\) play an important role in this process. Interferon-\(\gamma\) induces class II antigen expression on both lymphoid\textsuperscript{31} and nonlymphoid cells\textsuperscript{32,33} and it is possible

Fig. 5. Electron micrograph showing widening of vascular endothelial tight junctions after cryopexy. (a) Endothelial cells with normal tight junction (arrows) in the retina adjacent to the cryopexy-induced lesion. (b) Retinal endothelial cells, 1 hr after cryopexy. Widening of the tight junction (arrows) between endothelial cells in the treated area. L, lumen of the venule; B, basement membrane. Stained with uranyl acetate and lead citrate.

Fig. 6. Breakdown of the blood-retinal barrier shown by Evans blue albumin leakage. One hour after cryopexy. Dye leakage (arrows) is seen in the affected region, at the upper part of the photograph. In contrast, no leakage is found in the adjacent nonaffected area, at the lower part of the photograph (unstained flat preparation of the retina, \(\times85\)).
that this lymphokine is released by the T-lymphocytes that infiltrate cryopexy-treated eyes. In addition, however, class II antigen expression was observed in tissues affected by noninflammatory injuries such as those occurring in eyes with retinitis pigmentosa, or in blood vessels with atherosclerosis. The mechanism of class II induction in such tissues remains obscure.

In summary, we propose that EAU development is enhanced in the cryopexy-treated eyes by a combination of mechanisms, in particular the breakdown of the blood–retinal barrier, the accumulation of lymphoid cells and the increased expression of class II antigens. It is noteworthy that cryopexy enhanced the development of EAU only in rats in which immunity toward retinal antigens was induced by adoptive transfer of lymphocytes or by active immunization; no disease developed in rats treated by cryopexy alone (data not shown). This finding indicates that cryopexy alone is incapable of inducing the immunopathogenic process that mediates EAU. If these findings are applied to human ocular disease, our data may indicate that cryopexy should not initiate immunopathogenic processes in individuals with no prior sensitization to retinal-specific antigens. On the other hand, cryopexy could have adverse effects in individuals who develop immunity to retinal antigens through other events. Data to support this assumption have been recently provided by another study in this laboratory (S. Hirose et al, in preparation) in which the effect of cryopexy was tested in monkey eyes. No sequelae to cryopexy were detected in the eyes of control monkeys, while inflammation was markedly enhanced in eyes of monkeys treated with cryopexy following immunization against retinal antigens.

Key words: cryopexy, experimental autoimmune uveitis (EAU), blood–retinal barrier, S-antigen, cell markers
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References