Effect of Deferoxamine on Retinal Lipid Peroxidation in Experimental Uveitis

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Purpose. To examine the effect of deferoxamine, an effective iron chelator, on experimental autoimmune uveitis. Because deferoxamine has been shown to reduce iron-catalyzed hydroxyl radical generation, the in vivo effect was sought in the experimental autoimmune uveitis-mediated retinal lipid peroxidation, which is presumably induced by the inflammatory cell-derived oxygen radicals including hydroxyl radicals.

Methods. The experimental uveitis was induced in Lewis rats by retinal S-antigen. Deferoxamine infusion by osmotic pumps was started 2 days before the onset of the disease and was continued for 7 days. The extent of retinal lipid peroxidation was measured by the production of conjugated dienes, ketodienes, and thiobarbituric acid reactive substances. The inflammation associated free radical activity was measured by the luminol-amplified chemiluminescence.

Results. Levels of conjugated dienes, ketodienes, and thiobarbituric acid reactive substances were significantly decreased in the deferoxamine-treated animals. With Student’s t test, the P values are < 0.025 for conjugated dienes between deferoxamine- and sham-treated animals; < 0.025 for ketodienes between deferoxamine- and sham-treated animals; and < 0.01 for thiobarbituric acid reactive substances between deferoxamine- and sham-treated animals. With in vitro addition of 10 mM deferoxamine, the free radical generation of inflamed retina was suppressed by nearly 40%.

Conclusions. The administration of deferoxamine resulted in reduction of retinal lipid peroxidation. Because photoreceptors contain a high proportion of polyunsaturated fatty acids, deferoxamine, in turn, will act to ameliorate the experimental autoimmune uveitis-mediated retinal degeneration.


Tissue damage that accompanies inflammation is believed to result from oxygen metabolites, such as superoxide and hydrogen peroxide, that are released by activated neutrophils and macrophages. On release, these reactive oxygen species can be converted to hydroxyl radicals, which, in turn, can cause tissue damage through a number of mechanisms, including peroxidation of membrane lipids and proteins, and generation of other free radicals. In vitro studies indicate that the hydroxyl radicals are formed through an iron catalyzed reaction, and various animal models have been used to demonstrate that the inflammation can be minimized by eliminating available iron through the use of chelating agents such as deferoxamine.

Retinal S-antigen-induced uveitis in rats is one of the models in which deferoxamine has been shown to be effective. This experimental uveitis is a well-characterized entity that has been used to study the pathogenesis and treatment of uveitis in humans. Using histologic and morphometric analyses of choroidal inflammation, it was demonstrated that deferoxamine clearly reduces the degree of choroidal inflammation and retinal destruction. We have subsequently attempted to better understand the effect of deferoxamine in the experimental autoimmune uveitis (EAU) related retinal lipid peroxidation by using biochemical methods to assay for conjugated dienes (CD), ketodienes (KD), and thiobarbituric acid reactive substances (TBARS). For the assessment of in vivo lipid peroxidation, the use of multiple parameters is often encouraged, because any one parameter may not be the true representation of this complex process. We
have chosen three most commonly used parameters for this assessment. CD measure the structural feature associated with the fatty acid hydroperoxides, major products in lipid peroxidation. Both KD and TBARS measure the secondary products derived from hydroperoxides. KD reflect the fraction of hydroperoxide rearranged to compound containing KD structure. The TBARS represent secondary degradation products from hydroperoxide. We have also used luminol amplified chemiluminescence (LAC) to measure the level of reactive oxygen metabolites and excited lipid species generated by inflammatory cells.

**MATERIALS AND METHODS**

Female Lewis rats (VAF, Charles River Laboratory, Wilmington, MA) weighing approximately 175 g, were injected in the footpads with 50 μg of retinal S-antigen in Freund’s complete adjuvant. The animals used in the LAC experiments also received a tail vein injection of 1 μg of pertussis toxin (List Biological Laboratory, Campbell, CA) on the day of S-antigen immunization. A total of 56 animals were used for the study.

For lipid peroxidation and light microscopic studies, 38 immunized and 6 naive animals were used. The immunized animals were further divided into two subgroups (19 each), namely, deferoxamine-treated and sham-operated groups. For the measurement of CD and KD (both parameters can be read from the same sample), eight deferoxamine-treated and eight sham-operated animals were used. Retina and choroid of both eyes from the same animal were combined to give one number each of CD and KD. Therefore, n = 8 for data pertaining to all four groups, CD/deferoxamine, CD/sham, KD/deferoxamine, and KD/sham. Separately, three naive animals (also 2 eyes/determination) were used to obtain n = 5 for both CD/control and KD/control data. For the determination of TBARS, another set of eight deferoxamine- and eight sham-treated animals were used. Two sets of retina/choroid from the same animal were again combined to give one number. In TBARS determinations, three deferoxamine-treated and three sham-operated animals were used.

For the LAC study, 12 additional immunized animals were used. A total of six sets of retina and choroid from three animals were combined to obtain LAC without deferoxamine, and another six retina/choroid for LAC with deferoxamine. The experiment was performed twice.

Ten days after S-antigen immunization, 38 rats underwent subcutaneous implantation of osmotic pumps (#2ML1, Alza Corp., Palo Alto, CA). A group of 19 rats received pumps containing 2 ml of deferoxamine mesylate (250 mg/ml; Desferal, Ciba, Summit, NJ), and the other 19 animals received 2 ml of saline. Although the rate of release of enclosed fluid by the osmotic pumps was specified by the manufacturer for that particular lot, the rate was also independently checked by monitoring release of phenol red (absorption maximum at 431 nm) before use. The rate of release was found to be relatively constant (10 to 11 μl/hr) throughout the 7-day period, and at the end of this period, the pump had been emptied of 90% of its contents. With 50 μg of S-antigen prepared according to the Dorey’s rapid purification method, Lewis rats normally display onset of disease at 12 to 13 days and maximum disease development at 16 to 17 days after immunization. Therefore, in the intervention experiment with deferoxamine, the deferoxamine pumps were implanted on day 10, at least 2 days before onset of disease and the infusion was continued until day 17, thus covering the period of maximum disease development. The evidence of onset as well as continued development of disease was monitored clinically by slit-lamp observation beginning on day 8 after immunization. All animals were killed 17 days after immunization. All animal procedures conformed to the Association for Research in Vision and Ophthalmology Resolution on Use of Animals in Research.

The details of the measurement of CD, KD, and TBARS have been described elsewhere. Briefly, for measuring CD and KD, the retinas and choroids from two globes of the same animal were combined, homogenized, and extracted with 2 ml of chloroform/methanol (2:1) containing 0.5 mg of butylated hydroxytoluene per 100 ml of solvent. The pooled extracts were then washed with 0.4 ml of water and centrifuged. Solvents were removed and the residue dissolved in 1 ml of ethanol for measuring the optical density. The absorption maxima were recorded using a Shimadzu spectrophotometer model UV-160 (Shimadzu Corp., Kyoto, Japan). For CD, the absorption maximum at 235 nm and molar extinction coefficient of 25,200 were used, and for KD the same numbers used were 280 nm and 20,000, respectively. The same samples were used to measure absorptions for both CD and KD. For the measurement of TBARS, the retina and choroid from two eyes were combined and homogenized in 1.2% potassium chloride to make up to 10% w/v tissue homogenate. To this mixture, 3 ml of 1% phosphoric acid and 1 ml of 0.6% thiobarbituric acid were added. The mixture was then heated in a 95°C bath for 45 minutes. After cooling, the reaction mixture was extracted with 4 ml of n-butanol. Using a Shimadzu UV-160 spectrophotometer, the absorption of butanol phase was measured at 535 nm and a molar extinction coefficient of 156,000 was used for the calculation. For verifying the molar extinction coefficient used, TBARS standard was prepared by the hydrolysis
of 1,1,3,3-tetraethoxypropane (97% pure, Sigma Chemical Co., St Louis, MO).

The details of the procedure for measuring LAC of retina and choroid have been described elsewhere. Twelve sets of retinas and choroids were briefly minced, combined and then divided into two equal samples in two counting vials; one to be measured with and one without the addition of deferoxamine. Exactly 10 ml of luminol (5-amino-2,3-dihydro-1,4-phthala-zinedione, Sigma) (0.2 µg/ml) dissolved in 0.01 M phosphate buffered saline (pH 7.4) was added to the tissue sample immediately before counting with a Packard 1500 Liquid Scintillation Analyzer (Packard Instruments, Downers Grove, IL) with operation mode in single photon counting. After the establishment of the same level of count in two vials, deferoxamine was added to one vial to give final concentration of 10 mM. Both vials were counted for 60 minutes. The experiment was carried out twice with different groups of immunized animals. The LAC of naive animals with and without addition of deferoxamine was also measured using 12 retinas and choroids.

RESULTS

The effect of deferoxamine on EAU-mediated retinal lipid peroxidation is shown in Figure 1. All three parameters, including CD, KD, and TBARS were markedly decreased after administration of deferoxamine. In case of CD, this suppression amounts to 71% of the EAU-mediated increase (represented by the difference between sham and control level); this suppression for KD was 72%; and for TBARS, 90%. Using Student's t tests, the P values were found to be < 0.025 between deferoxamine CD and sham CD; < 0.025 between deferoxamine KD and sham KD; and < 0.01 between deferoxamine TBARS and sham TBARS.

To examine the effect of deferoxamine on the free radical activity of inflamed tissues, 10 mM of deferoxamine (final concentration) was added in vitro to the

FIGURE 1. Effect of deferoxamine on conjugated dienes (A), ketodienes (B), and thiobarbi-turic acid reactive substance (C) production in experimental uveitis. The quantities produced are in nmoles per eye; all bars represent mean ± 2 SD. Two eyes from each animal were combined for one determination. For each parameter, eight determinations for the deferox-amine-treated, eight determinations for the sham-operated, and three determinations for the unimmunized control animals were performed.
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FIGURE 2. Effect of deferoxamine on luminol amplified chemiluminescence of inflamed retina and choroid. Chemiluminescence expressed as cpm/six retinas and choroids is shown as (■) without deferoxamine, (○) with 10 mM deferoxamine added, and (★) nonimmunized normal retina/choroid preparation. Note that deferoxamine not only decreases the total counts, but also completely quenches the surge of counts seen upon exposure of the vials to light. The experiments were performed twice and a representative curve is shown.

retina/choroid preparations from EAU-animals (Fig. 2). The in vitro experiment was carried out with a parallel counting procedure. There is normally a brief surge of counts after the addition step caused by the momentary exposure of the vial to light when deferoxamine is added. The vials with or without the addition of deferoxamine were subjected to the same period of light exposure. However, deferoxamine completely quenched this surge and maintained a marked suppression of counts, which remained consistent for the period of measurement. This decrease is on the magnitude of 105,000 counts, approximately one third of the count before the addition of deferoxamine. The change caused by the addition of deferoxamine to nonsensitized control animals was negligible. The LAC remains one of the most sensitive tools for measuring radicals and excited species generated by inflamed tissues. However, as noted previously by us and by others, the beginning counts between runs of the same inflamed tissues are highly variable. However, once basic counts were established, the level of suppression by a given agent was consistent. For this reason, the mean ± SD type statistical analysis is not feasible, and the extent of suppression is customarily shown as measurement for the entire period; and this can be confirmed by repeating the experiment, as was done in the current case.

Figures 3 and 4 are representative histologic sections from the EAU animals treated with normal saline and with deferoxamine, respectively. All sham-operated controls developed disease and show marked mononuclear infiltration in the retina and choroid, with destruction of the outer retinal layers (Fig. 3). The deferoxamine-treated animals show only mild cellular infiltration, with remarkable preservation of the photoreceptors and outer retina (Fig. 4). Only a small number of animals was used for the histology, because the details of the histopathologic and morphometric analyses of the deferoxamine administration have been reported. In the current study, the major focus was on the EAU-associated lipid peroxidation and chemiluminescence; six animals were included merely to provide support for the lipid peroxidation data and the inflammatory process noted in treated and sham-operated groups.

DISCUSSION

Despite recent advances in immunology, the treatment of uveitis still relies largely on nonspecific approaches, such as corticosteroids or immunosuppressive agents. Ideally, treatment would interfere specifically with the function of that subset of immunocompetent cells responsible for the immune response in ocular tissue. If this is not possible, an attempt should be made to specifically block the final

FIGURE 3. Histology of retina and choroid of experimental uveitis. Control animal without treatment shows marked inflammatory cell infiltration and destruction of retina and choroid (hematoxylin-eosin, X 400).
common pathway of the immune response, namely, the production of tissue destroying substances.

In previous studies we showed that phagocyte-derived reactive oxygen metabolites can induce peroxidation and the destruction of retinal tissues in experimental uveitis. In fact, this process can be demonstrated before actual tissue damage is seen morphologically, which implies a causative role for free radicals in inflammation-induced tissue damage. That many of these processes are iron-dependent, and that the iron chelator, such as deferoxamine clearly decreases the gross manifestation of the immune response, led us to explore the effects of this compound as the biochemical modulator of tissue damage. As would be predicted, deferoxamine markedly decreased the production of free radicals, as shown by LAC. There was also a subsequent decrease in lipid peroxidation, as shown by the indices of lipid peroxidation, CD, KD, and TBARS. All of this is consistent with a marked reduction of available free radicals, with resulting preservation of retinal membrane lipid structure.

It is believed that deferoxamine acts primarily by inhibiting the iron-catalyzed generation of hydroxyl radicals and subsequent lipid peroxidation. The LAC activity in experimental animals, however, appears to stem from a combination of all photoemissive species, including hydroxyl radicals, superoxide, hydrogen peroxide, singlet oxygen, and excited carbonyl radicals. This probably explains why the decrease in chemiluminescence was only partial, inasmuch as deferoxamine affects primarily the hydroxyl radical portion of oxygen metabolites.

As was evident from the histology and chemiluminescence results, deferoxamine administration appears to reduce phagocyte recruitment in the inflammatory sites. Although deferoxamine does not modulate this process directly, the lipid peroxidation products are chemotactic to neutrophils. Lipid hydroperoxides are produced in significant quantities during the development of EAU by phagocyte-mediated reactive oxygen species. Deferoxamine administration was found to reduce the level of lipid peroxidation and the formation of lipid peroxides. This leads to a reduction in available chemotactic factors at inflammatory sites, and thus to fewer neutrophil and mononuclear cell infiltration.

Deferoxamine has also been shown to decrease the level of photic injury in rats, another process believed to be mediated by oxygen free radicals and lipid peroxidation.

Reports of the toxicity of deferoxamine administered in vivo are scarce. A recent experiment showed a reversible electroretinographic b-wave reduction after a single 100-mg or 150-mg subcutaneous injection of deferoxamine. In this case, the damage demonstrated appears to be a combined effect of deferoxamine and readily absorbed high energy light. Therefore, the toxicity of deferoxamine reported has no direct relationship to the current study, because the effect was induced with a large single dose and without subsequent exposure to high energy light, the damaging process does not occur.

Therefore, it is conceivable that the administration of deferoxamine in proper dose will result in amelioration of EAU-mediated retinal damage by reducing the lipid peroxidation. The oxidative destruction of membrane lipids in photoreceptors could be one single important factor in EAU-associated retinal degeneration considering that photoreceptors contain a high proportion of docosahexaenoic acid, which is extremely susceptible to peroxidation.

**Key Words**

deferoxamine, experimental uveitis, lipid peroxidation, free radicals, S-antigen

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