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Malondialdehyde and antioxidant enzyme levels in the aqueous humor of rabbits in endotoxin-induced uveitis

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PURPOSE. *To investigate the role of oxidative stress in endotoxin-induced uveitis.*
METHODS. *Lipopolysaccharide was injected intravitreally into the right eyes of rabbits. Sterile saline was injected intravitreally into the left eyes as a control. Inflammation was assessed according to clinical score, aqueous humor cell count, and protein levels. Malondialdehyde, superoxide dismutase, glutathione peroxidase, catalase, and nitrite levels were measured in the aqueous humor.*
RESULTS. *The clinical grade ($p<0.01$), inflammatory cell count ($p<0.001$), and protein content ($p<0.001$) were significantly higher in the aqueous humor of eyes with uveitis than in that of controls. Malondialdehyde ($p<0.01$) and nitrite ($p<0.001$) levels in the aqueous humor of eyes with uveitis were significantly higher than in the control group. Superoxide dismutase ($p<0.001$), glutathione peroxidase ($p<0.001$), and catalase ($p<0.001$) levels were significantly lower in the aqueous humor of eyes with uveitis than in that of the controls.*
CONCLUSIONS. *Oxygen free radicals may be implicated as a mediator of inflammation in endotoxin-induced uveitis. The increase in free radicals in the aqueous humor may play a role in the pathogenesis of endotoxin-induced uveitis. (Eur J Ophthalmol 2003; 13: 779-83)*

KEY WORDS. *Endotoxin-induced uveitis, Free radical, Antioxidants, Aqueous humor*

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INTRODUCTION

Uveitis is the most commonly seen intraocular inflammatory disease in humans. Immunologically or biochemically active factors in uveitis are important in tissue damage development. The factors involved in tissue damage due to inflammation in uveitis have not been fully identified.

Endotoxin-induced uveitis (EIU), a model of intraocular inflammation elicited by lipopolysaccharide (LPS), is a useful experimental model for the understanding of acute anterior uveitis in humans, and is used in studies examining the pathophysiology of this disease (1, 2). LPS produces uveitis when it is injected into the vitreous of the rabbit eye, as characterized by dilation of the iridal blood vessels, the breakdown of blood-ocular barriers, increased vascular permeability, plasma protein leakage, and inflammatory cell infiltration into the aqueous humor (AH) (3). Many proinflammatory mediators have been implicated in the pathogenesis of EIU, including cytokines, prostaglandins, leukotrienes, oxygen free radicals, and other toxic substances. Although the ocular effects of LPS are well documented, the molecular mechanisms of EIU are not fully understood.

Although the exact pathogenic mechanisms underlying uveitis are unknown, free radicals appear to be involved in this inflammatory disorder. Numerous oxy-

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gen metabolites have been detected in ocular tissues and media in various studies of experimental autoimmune uveitis (4). Despite numerous studies, the roles of free radicals in uveitis are unclear. The characterization of the influx of free radicals into the anterior chamber of eyes with uveitis has provided important insights into the mechanisms of this disease. There have been a few reports on the relationship between uveitis and free radicals. To our knowledge, this is the first report on the measurement of antioxidant enzyme levels in the AH of rabbits in EIU.

The purpose of this study was to experimentally produce EIU in rabbits and then measure the levels of nitrite, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and malondialdehyde (MDA) in the AH. We hoped to elucidate the oxidative damage occurring in EIU.

METHODS

Nine female New Zealand white rabbits (2 to 3 kg) were anesthetized with intramuscular injections of xylazine (12 mg/kg) and ketamine (20 mg/kg). The right eye of each rabbit was injected intravitreally (2 mm posterior to the limbus) with endotoxin (2 µg in 10 µl; LPS from *Salmonella typhimurium*, L 6511, Sigma Chemical Co., St. Louis, MO). For controls, 10 µl sterile saline was injected intravitreally into the left eye. Experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

The degree of the anterior uveitis intensity was clinically assessed by two investigators 20 hours after the LPS injection. All eyes were examined with a slit-lamp and scored clinically for vascular, pupillary, and exudative signs. The intensity of the signs of intraocular inflammation was graded using a clinical scoring system described previously (5). At 24 hours post LPS injection, the rabbits were killed with intracardiac pentobarbital. Approximately 0.2 to 0.3 ml of AH samples were obtained from each eye with a 26-gauge needle and a 1-ml tuberculin syringe by means of anterior chamber paracentesis under a surgical microscope. An AH sample of 10 µl was taken for a cell count before centrifugation. After drying, the AH was dyed by Wright's method. Inflammatory cells per microliter were counted under the microscope. The AH was then centrifuged at 15,000 rpm for 3 minutes.

Because there was a limited amount of AH, it was diluted with phosphate-buffered saline for measurement. The GPx activity of the AH was determined using cumene hydroperoxide as substrate. The method is based on the NADPH-coupled reaction by which oxidized glutathione produced by the activity of GPx is converted to reduced glutathione by exogenous glutathione reductase and NADPH. The rate of NADPH oxidation was measured at 340 nm (6). The results were expressed as U/mg protein.

The SOD activity of the AH was determined with commercially available kits (Randox Lab. Ltd., Ireland, Cat. No. SD125). SOD estimation was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activity of the AH was measured by degree of inhibition of a reaction that catalyses superoxide radical generation by xanthine and xanthine oxidase. The results were expressed in international units per milligram of aqueous protein (U/mg protein).

The lipid peroxidation levels of the AH were determined by the thiobarbituric acid (TBA) method (7). MDA, an end product of fatty acid peroxidation, reacts with TBA to form a colored complex that has maximum absorbance at 532 nm. MDA values were calculated from the absorbance coefficient of the MDA-TBA complex at 532 nm, 1.56×10^5 $cm^{-1}mol^{-1}$. The values were expressed as nanomoles MDA formed per milliliter of aqueous. CAT activity was determined by the technique described by Aebi (8), taking the catalytic activity of the catalase that can be measured by following the decomposition of H_2O_2. The values were expressed as U/mg protein.

The protein content of the AH was determined by the Lowry method (9). Nitrite levels were determined by a colorimetric assay based on the Griess reaction (10).

Data were analyzed by Wilcoxon and paired *t*-test, with $p<0.05$ considered statistically significant.

RESULTS

Twenty hours following injection, fibrinous exudation and heavy flares were seen in the anterior cham-

ber of all eyes injected with LPS (median clinical score 7.50 (range 5-11)). In eyes injected with saline, conjunctival hyperemia was seen (median clinical score 1.00 (range 0-2)). The clinical score of the endotoxin group was significantly higher than that of the control group (w=-2.68, p<0.01). Our study showed that the AH cell count and protein and nitrite levels had increased significantly at 24 hours in eyes with uveitis, to 3478±556 cell/µl, 26.0±2.74 mg/ml, and 12.11±2.03 mM/ml, respectively. In eyes without uveitis, the AH cell count and protein and nitrite levels were determined to be 9.44±5.17 cell/µl, 1.61±0.38 mg/ml, and 3.11±1.69 mM/ml (t=18,719, p<0.001, t=25,964, p<0.001, t=11,513, p<0.001), respectively. The clinical score, AH cell count, and protein and nitrite levels are shown in Table I.

It was determined that MDA levels were significantly higher in the AH of eyes with uveitis than in eyes injected with saline (t=5,407, p<0.01). SOD, GPx, and CAT levels were significantly lower in the AH of eyes with uveitis than in that of the controls (t=-7,125, p<0.001, t=-5,752, p<0.001, t=-31,215, p<0.001, respectively). The AH levels of antioxidant enzymes and MDA are shown in Table II.

DISCUSSION

Anterior uveitis can be induced in rabbits by single-dose intravitreal LPS injection. Eighteen to 24 hours following LPS injection, the AH inflammatory reaction reaches its maximum, disappearing within 5 to 7 days. Our study showed that the clinical score and AH protein level increased significantly at 20 to 24 hours in eyes with uveitis compared to those of the controls.

EIU is characterized by a massive cell increase in the AH, consisting predominantly of PMN and a few monocytes/macrophages (11). In this study, we demonstrated that injection of LPS leads to cell increase in the AH. It was determined that 88% of the cells into the AH were composed of PMN.

Free radicals arising from activated monocytes/macrophages and PMN in uveitis initiate a variety of cytotoxic reactions and can exacerbate the inflammatory response, cause the inflammation to become chronic, and lead to tissue damage (12). Free radicals are atoms or molecules in outer orbit or with one or more unpaired electrons (13). The unpaired electrons make these molecules extremely toxic, damage

TABLE I - RESULTS OF CLINICAL SCORE AND AQUEOUS HUMOR CELL COUNT, PROTEIN, AND NITRITE LEVELS IN ENDOTOXIN-INDUCED UVEITIS

		Aqueous humor		
Group	Clinical score	Cell/µl	Protein, mg/ml	Nitrite, mM/ml
LPS	7.50[a] (5-11)	3478±556[b]	26.0±2.74[c]	12.11±2.03[d]
Control	1.00 (0-2)	9.44±5.17	1.61±0.38	3.11±1.69

Values are median (range) or mean±SD. [a]w=-2.68, p<0.01 (Wilcoxon test); [b]t=18,719, p<0.001 (Paired t-test); [c]t=25,964, p<0.001 (Paired t-test); [d]t=11,513, p<0.001 (Paired t-test); LPS = Lipopolysaccharide

TABLE II - AQUEOUS HUMOR LEVELS OF ANTIOXIDANT ENZYMES AND MALONDIALDEHYDE (MDA) IN THE CONTROL AND LIPOPOLYSACCHARIDE (LPS) GROUPS

	LPS group	Control group	Paired t-test	
			t	p
MDA, nmol/ml	13.18±2.78	7.91±0.95	5,407	<0.01
Superoxide dismutase, U/mg protein	1.30±0.91	4.08±1.33	-7,125	<0.001
Glutathione peroxidase, U/mg protein	0.24±0.11	0.59±0.18	-5,752	<0.001
Catalase, U/mg protein	1.88±0.19	5.89±0.31	-31,215	<0.001

their structures by attaching to molecules such as those of protein and nucleic acid, and, by impairing the cell membrane's permeability and integrity, leading to cell death. An organism is protected from the harmful effects of free radicals by antioxidant enzymes such as SOD, CAT, and GPx (14). When the balance between free radicals formation and the mechanisms for cleansing these radicals is upset, free radical-related tissue damage is initiated. Various studies have demonstrated the role of inflammatory free radicals during uveal inflammation.

The superoxide radical is a highly toxic free radical that plays a variety of roles in the uveitis. It has been experimentally demonstrated that the superoxide radical increases vascular permeability and exacerbates inflammation. In this study, we found significantly lower SOD levels in the AH of eyes with uveitis than in the control eyes. SOD activities indicate the production of superoxide radicals during EIU. Superoxide radicals accumulate and activate neutrophils at an inflammatory site (15). The low SOD levels in the AH may be an indication that insufficient detoxification of the superoxide radicals in the environment has contributed to the development of tissue damage. PMN that have infiltrated the iris, the ciliary body, and the AH may contribute to the high levels of superoxide radicals in the AH.

Superoxide anions and hydrogen peroxide are among the most important primary species generated by phagocytes (2). Superoxide radicals produced in excessive amounts during oxidative stress are converted to H_2O_2 by SOD. If the H_2O_2 produced exceeds the capacity of peroxide cleaning enzymes, the hydroxyl radical is formed, which is even more toxic. Hydroxyl radicals have been implicated as the ultimate cytotoxic agent because of their high reactivity (16). When hydroxyl radicals derived from superoxide rise above a certain level in the cell, they affect the unsaturated fatty acids present in the cell membranes, causing lipid peroxidation. In this study, MDA levels were two times higher in the AH of eyes with uveitis than in that of the controls. The most important source of MDA, which we determined to be present in high amounts in the AH during EIU, are PMN that have infiltrated the anterior segment.

Glutathione-dependent enzymes play an important role in the detoxification of substances capable of forming reactive metabolites. They protect membrane lipids from the oxidation of peroxides by breaking up GPx, H_2O_2, and lipid peroxides (17). In a number of experimental cataract studies, a decrease in lens GPx levels prior to the onset of opacification has been demonstrated. As glutathione levels decrease, so does the resistance of the lens to oxidative stress (18). In our study, GPx levels were found to be lower in the AH 24 hours after intravitreal injection of LPS than in the control eyes.

NO, a multifunction free radical with a short half-life, is rapidly transformed into more stable compounds such as nitrite (19). In the present study, high nitrite levels in the AH were determined. NO has been shown to be a potent oxidizing agent, capable of initiating the peroxidation of lipids in membranes. Increased NO production is found in the enhancement of inflammation through its effects on vasodilatation, neutrophil adhesion, and alteration of vascular permeability, leading to tissue damage in many endotoxin-induced acute inflammations (20). The high nitrite levels determined may be due to inflammatory cells that have infiltrated the AH. Inflammatory cells, mainly PMN, have been shown to generate NO and superoxide simultaneously in experimental autoimmune uveitis (16). The simultaneous generation of NO and superoxide may be an important mediator of inflammation. NO reacts with superoxide anions to produce peroxynitrite and the subsequent hydroxyl radical.

Free radicals may play an important role as mediators of inflammation in the pathogenesis of EIU. In this study, MDA levels were shown to increase and antioxidant enzyme levels to decrease in the anterior chamber of eyes injected with LPS. The low levels of antioxidant enzymes such as SOD, GPx, and CAT are insufficient to prevent the formation of free radicals, leaving the eye exposed to oxidative damage. The increase in free radicals and the decrease in antioxidant enzymes in the anterior chamber may be important factors in the development of uveitis-related complications.

These findings suggest that free radicals play a role in EIU-related inflammatory reactions. The influx of inflammatory cells such as PMN and monocytes/ macrophages in the AH may contribute to the production of various free radicals during EIU. In inflammation, the free radicals released by the cells into their environment play a role in the pathogenesis of EIU.

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