Involvement of superoxide generated by polymorphonuclear leukocytes in endotoxin-induced uveitis

Abstract Background: Although superoxide is thought to be involved in the development of endotoxin-induced uveitis (EIU), the role of superoxide generation by polymorphonuclear leukocytes (PMNs) has not been fully elucidated. The purpose of this study was to investigate the role of peripheral blood PMNs in the development of EIU. Methods: EIU was induced in Lewis rats by injection of lipopolysaccharide (LPS) in one hind footpad. Superoxide generation was assayed by measuring the reduction of ferricytochrome c (cyt c). EIU severity was assessed by histological examination, and the relationship between the injected dose of LPS in vivo and the intensity of superoxide generation by peripheral PMNs or intraocular PMNs was studied. Twenty-four hours after the injection of LPS (2, 20, or 200 µg/rat), peripheral blood PMNs were collected and stimulated with phorbol 12-myristate 13-acetate (PMA). The time course of superoxide generation by PMNs after LPS injection (3, 6, 12, 24, 48, and 72 h) was also investigated. To test the possible inhibition of superoxide generation by protein kinase C (PKC) inhibitors, H-7 and staurosporine were added for the incubation. In addition to the measurement of cyt c reduction, western blotting was used to detect PKC activity. The direct effect of LPS on PMNs was tested by priming naive PMNs with LPS in vitro. Results: The intensity of superoxide generation by PMNs and the severity of EIU were dependent on the dose of injected LPS. No apparent superoxide generation was detected from intraocular PMNs. The time course of superoxide generation was similar to that of EIU severity. H-7 or staurosporine inhibited superoxide generation dose dependently and suppressed phosphorylation of PKC. Priming with LPS in vitro prompted minimal superoxide generation by naive PMNs. Conclusion: Superoxide generation by peripheral blood PMNs but not by intraocular PMNs from rats with EIU was demonstrated, and it is suggested that superoxide generation by PKC cascade might be involved in the pathogenesis of EIU.

Introduction

Endotoxin-induced uveitis (EIU) serves as a model for human acute anterior uveitis [22] and is widely used for evaluating the effectiveness of drugs before clinical trials [9, 14]. The main feature of EIU is the infiltration of polymorphonuclear leukocytes (PMNs) and proteinaceous exudates in the aqueous humor and vitreous [2, 10]. To induce EIU in mice or rats, lipopolysaccharide (LPS) from bacteria is injected into a hind footpad [22], which leads to abundant production of proinflammatory cytokines such as tumor necrosis factor α [11, 12, 15] and interleukin 6 [3, 14, 30].
related to the kinetics of cellular infiltration and exudates in the aqueous humor [4, 21]. By contrast, targeted deletion of a cytokine or a cytokine receptor had no apparent effects on the severity of EIU [24], suggesting that complexities of the cytokine network can compensate for dysfunction of one cytokine or its receptor. Although there have been reports about the effectiveness of cytokine administration on the inhibition of EIU [7, 8, 15, 20, 28], it might be premature to conclude that cytokines induced by LPS injection cause EIU.

PMNs are major effector cells in many acute inflammatory diseases [17]; tissue damage results from the nitric oxide [13, 17] or superoxide produced by the PMNs as well as from the microbial activity of, for example, the leukotrienes [10] produced by these cells. Involvement of these oxidative products in the development of ocular diseases was demonstrated for EIU [13, 17] and experimental autoimmune uveoretinitis (EAU) [6, 29]. In EIU, the inhibition of nitric oxide synthesis by the administration of a nitric oxide synthetase inhibitor such as N\(^{G}\)-monomethyl-L-arginine (L-NAME) effectively suppressed the severity of disease [1, 5, 18]. Although the synthesis of superoxide in the retina from rats with EAU was demonstrated by immunohistochemistry [6] and in situ hybridization [29], no data are available for EIU.

Obviously, infiltrated PMNs in the eye were derived from peripheral blood cells, although PMNs that enter the eye may be a unique subset and may not be representative of peripheral blood PMNs. Previous reports focused primarily on the cells in the eye and did not describe the production of nitric oxide or superoxide by peripheral blood PMNs [1, 5, 13, 18, 19]. This information prompted us to investigate the ability of peripheral blood PMNs to produce superoxide, because these cells will eventually be the effector cells in the eye.

**Materials and methods**

**Animals**

Inbred male adult 6- to 8-week-old Lewis rats (Seac Yoshitomi, Fukuoka, Japan) were used in this study. The animals were cared for and handled according to institutional guidelines and the ARVO “Resolution on Use of Animals in Research”.

**Induction of EIU**

LPS from *Escherichia coli* (Difco, Detroit, Mich.) was dissolved in phosphate-buffered saline at 2 mg/ml. Rats were injected in one footpad with LPS (2–200 µg/rat).

**Isolation of PMNs**

After the rats had been anesthetized with diethylether, peripheral blood was collected from three to eight animals and pooled into tubes containing citrate and diluted twice with equal volumes of RPMI 1640 medium (Nikken, Osaka, Japan). The blood was layered on polymorphoprep (Nycomed Pharma, Oslo, Norway) and centrifuged at 500 g for 30 min. The fraction rich in PMNs was collected and washed three times with Krebs-Ringer-phosphate solution (KRP; pH 7.4). The PMNs were then resuspended in KRP containing 1 mM CaCl\(_2\) and 10 mM glucose. The purity (>95%) and number of PMNs were checked and PMN concentration was adjusted to approximately 2x10\(^5\) cells/ml. In case of intraocular PMNs, aqueous humor was collected and combined from 18 eyes (9 rats) using a 26-G needle and pooled. All the harvested cells after repeated washing with buffer were used as above. Experiments were repeated at least three times for every particular condition, including different doses and different times.

**Assay of superoxide generation**

Superoxide generation was assayed by measuring the reduction of ferricytochrome c (cyt c; Sigma Chemical, St. Louis, Mo., USA) at 37°C using a dual-beam spectrophotometer (Shimadzu UV-3000, Shimadzu, Kyoto, Japan) with continuous stirring [25, 31–33]. Phorbol 12-myristate 13-acetate (PMA; Sigma Chemical) was used to stimulate neutrophils. In some forms of FMLP; Sigma Chemical) was also used as a stimulant. The standard assay mixture consisted of 4x10\(^5\) cell/ml, 1 mM CaCl\(_2\), 20 µM cyt c, 10 mM glucose, and a stimulus in a final volume of 2 ml KRP. After preincubation for 3 min, the reaction was started by adding the stimulus and the absorbance change at 550–540 nm (A550–540) was monitored. To examine the effect of LPS on naive PMNs in vitro, naive PMNs were preincubated with LPS [23] for 1 h before PMA stimulation under the above conditions.

**Histopathology**

After the peripheral blood had been collected, the eyes were enucleated, fixed in formalin, and embedded in paraffin. Anteroposterior sections from the optic nerve level were stained with hema-toxylin-eosin. Infiltrated cells in the anterior chamber and the vitreous cavity were counted and the number was used to assess the severity of EIU.

**Dose response**

Twenty-four hours after the various doses of LPS (2, 20, and 200 µg/rat) were injected, the peripheral PMNs were collected and the eyes were enucleated. Infiltrated cells were counted histopathologically as above. Superoxide generation by PMNs that were stimulated with PMA was measured. The amount of superoxide generated by PMNs from normal rats was also measured.

**Time course**

The time course of superoxide generation after the injection of LPS (200 µg/rat) was also examined. The peripheral blood PMNs were collected at various times (3, 6, 12, 24, 48, and 72 h) after LPS injection and stimulated with PMA (2.5 nM), and the superoxide generated was measured. The amount of superoxide generated by PMNs from naive rats was plotted at time 0. Infiltrated cells were counted as above and the number was used to assess the severity of EIU.