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Production of superoxide anions by a CNS macrophage, the microglia

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Microglia have been implicated in both physiological and pathological processes of the brain. Their possible roles have been compared to those of macrophages and granulocytes. Here we demonstrate the specific ability of microglia to secrete the superoxide radical ion in response to a complement activated agent, opsonized zymosan, and to phorbol myristate acetate. As in other organs, this endogenously produced reactive oxygen intermediate could have both beneficial and deleterious effects.

Microglia; Superoxide anion; Macrophage; Phorbol myristate acetate; Oxygen burst

1. INTRODUCTION

The role of microglia in inflammatory and non-inflammatory pathological processes in the central nervous system (CNS) is not clear. Del Rio Hortego [1] and others [2-4] have suggested that microglia accumulate at the site of neuronal damage where they serve as CNS-specific macrophages. Since cultured microglia exhibit phagocytosis [3-5], it is possible that one of their actions in the CNS is to ingest cellular debris at the injured site. Although debated [6,7], evidence to support this role of microglia stems from the fact that these cells have many of the same anatomical, biochemical and specific surface markers as circulating monocytes [2-4,8-10]. Because of the similarity of microglia to monocytes and to tissue macrophages it is also possible that microglia may serve to kill invading organisms. It is well known that monocytes, macrophages and neutrophils secrete reactive oxygen intermediates, e.g. the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and oxidized halides, e.g. HOCl [11-13]. These highly reactive compounds plus the hydroxyl free radical (OH^-) (produced by interaction of superoxide and hydrogen peroxide in the presence of metal ions) have been shown to be very effective bactericidal agents [10]. Microglial secretion of reactive oxygen intermediates could thus serve as a protection against infective organisms in the CNS. We examined the ability of microglia to secrete oxygen intermediates by exposing cultured microglia cells to activating agents and assaying for superoxide anion production.

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2. MATERIALS AND METHODS

Primary glial cell cultures were prepared from 2-day-old rat cerebral hemispheres essentially as described [5,14,15]. The cortices were removed and placed in Leibovitz's (L-15) medium. Using a binocular microscope, the meninges and any blood vessels were carefully removed thus limiting contamination of the culture with blood monocytes. The cortices were then placed in fresh

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L-15, mechanically dissociated and plated into 75 cm^2 Falcon flasks. Cells were grown at 37°C in a 10% carbon dioxide, 90% air environment using a modified Dulbecco's medium (DMEM) containing 10% fetal calf serum. These conditions were not favorable to neuronal growth [14]. After 10–14 days, the flasks were placed on a rotary shaker (200 rpm) and shaken for 15–20 h at 37°C. The superantant containing loose cells was collected, centrifuged and the cells resuspended in DMEM. This method produced a cell fraction composed from 70 to 90% microglia. Microglia were identified by standard histochemical and fluorescent antibody techniques [16–18] and were shown to be positive for non-specific esterase, a monocyte marker [18], and negative to peroxidase (a second monocyte marker [19]) in the resting (non-activated) state and negative to the following specific glial antibodies; GFAP, A_2B_5 and galactocerebroside (GalC). The results from the histochemical and fluorescent antibody techniques support the idea that microglia, like other resident macrophages, are derived from blood borne monocytes but may assume different characteristics (e.g. peroxidase negative at rest) once in the tissue [19].

The microglia-enriched fraction was counted and seeded at variable densities into 96-well culture dishes. The adhered cells remaining in the flask were removed by scraping, centrifuged and also resuspended in DMEM. This remaining fraction, composed primarily of type I (GFAP positive) and type II, (GFAP and A_2B_5 positive) astrocytes, was also counted and seeded at the same densities as the microglia fraction in 96-well culture dishes.

After adhering overnight, the cells in each well were assayed for superoxide using a microcytochrome *c* reduction assay developed by Pick and Mizel [20]. Specificity for superoxide anion is provided by comparing the amount of cytochrome *c* reduction in the presence and absence of superoxide dismutase (SOD), the enzyme which degrades superoxide. An automated plate reader (Titer Tek) was used to read the absorbance at 550 nm in each of the culture wells. Data are expressed as nmol superoxide/mg protein. Protein content of the wells was determined by digesting the cells overnight at 37°C with 0.1 N NaOH. The Lowry assay [21] was used to determine the protein content with bovine serum albumin as the standard.

3. RESULTS AND DISCUSSION

The production of superoxide by the microglia-enriched fraction under resting (i.e. non-stimulated) and stimulated conditions is shown in fig.1. The stimulus for secretion in this case was 0.5 mg/ml of opsonized zymosan, prepared using rat serum in the standard way [22]. Opsonized zymosan was chosen as an activating agent because it has been shown to stimulate superoxide production in macrophages and neutrophils [22,23]. As shown, superoxide concentration increased at a rate of 3.7 nmol/mg protein per min until a maximum secretion was reached at about 2–3 h. Superoxide secretion in the resting state increased only slightly over the same time period. Superoxide secretion of the astrocyte fraction is also shown in fig.1. In contrast to the microglia, these cells did not significantly respond to opsonized zymosan by secreting superoxide. In addition to being specific to this cell type, superoxide production by the microglia-enriched fraction varied with cell density (fig.2) and concentration of stimulant (fig.3). Finally, the microglia were responsive to stimulation by phorbol myristate acetate (fig.4) but not to stimulation by concanavalin A (from 10 to 100 μg/ml) or by formylmethionylleucylphenylalanine (f-Met-Leu-Phe) (from 10^{-8} to 10^{-5} M).

These results suggest that microglial production of superoxide has many of the same characteristics as neutrophil or monocyte superoxide production. In other words, superoxide secretion can be stimulated by both membrane-associated complement factors (e.g. opsonized zymosan) and by phorbol esters. These activating agents have been extensively examined for both neutrophils and monocytes [12,13,22–25]. In addition, the rate of superoxide secretion is similar to that seen in neutrophils and monocytes where values ranging from 1 tot 4 nmol/mg protein per min are found [23]. However, not all agents which activate neutrophils or monocytes, i.e. concanavalin A or f-Met-Leu-Phe, induced superoxide production in microglia. This suggests that, at least at this stage of development, microglia have unique characteristics.

The presence of a superoxide producing cell in the CNS may have significant functional consequences. For example, the amount of superoxide released by the microglia into the relatively con-

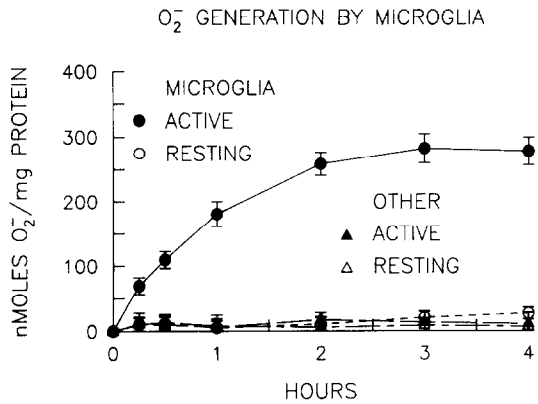

Fig.1. Production of superoxide by cultured glia. Data points represent the average concentration of superoxide/mg protein (± SE) produced over 4 h by a microglial-enriched cell fraction at rest (i.e. unstimulated) (○) and after stimulation by 0.5 mg/ml opsonized zymosan (●) and by an astrocyte-enriched fraction after stimulation by 0.5 mg/ml opsonized zymosan (▲) and at rest (△). Number of wells assayed (*n*) equals at least 8.

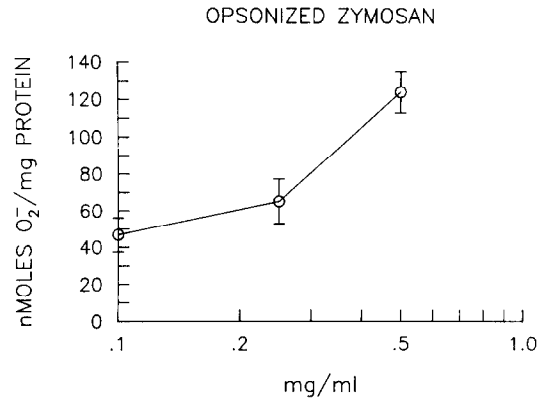

Fig.3. Dose response relationship for the effect of opsonized zymosan on superoxide production. Data points represent average values (± SE) for superoxide production/mg protein obtained after 60 min of exposure to varying concentrations of opsonized zymosan. Number of wells assayed (*n*) equals at least 6.

fined extracellular spaces of the CNS is equivalent to those concentrations of superoxide secreted by neutrophils and monocytes [23,26]. Microglia, thus, are likely to serve a similar anti-infective function but are specific to the CNS. However, superoxide secretion can also have deleterious effects as shown in peripheral tissues such as the lung [27,28]. Halliwell and Gutteridge [29,30] have recently discussed the possible mechanisms of reactive oxygen intermediate damage in the CNS. Lipid peroxidation of membrane is one potentially important damaging action of oxygen intermediates, especially in those areas of the brain which are rich in transition metals such as iron and copper [31-34]. Consistent with this hypothesis, synaptic changes are seen in in vitro neural systems during exposure to oxygen intermediates [35,36]. Free

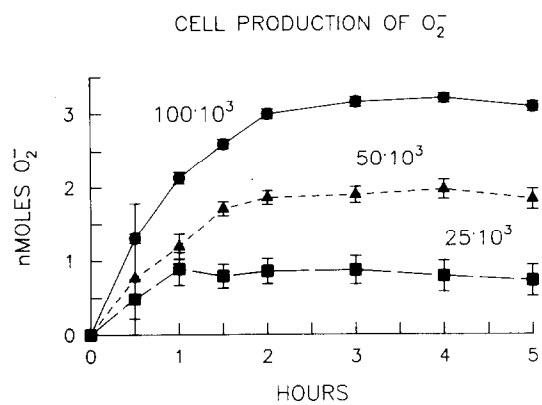

Fig.2. Effect of cell density on superoxide production. Values represent the average concentration of superoxide (± SE) produced with time at three different cell densities: (●) 100×10^3 cells/well; (▲) 50×10^3 cells/well and (■) 25×10^3 cells/well. Number of wells assayed (*n*) equals 8.

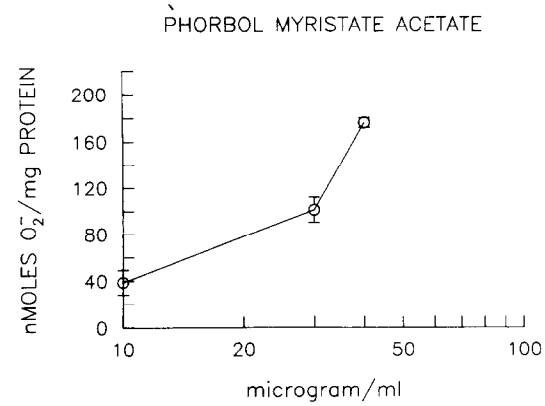

Fig.4. Dose response relationship for the effect of phorbol myristate acetate on superoxide production. Data points represent the average values (± SE) for superoxide production/mg protein obtained after 60 min of exposure to varying concentrations of phorbol myristate acetate. Number of wells assayed (*n*) equals at least 6.

radicals have also been implicated in pathological alterations of the cerebral circulation as in acute severe hypertension [37,38] and in CNS ischemia [39]. If microglia have the same ability to secrete superoxide in whole brain as in tissue culture, it may be reasonable to suggest that part of the free radicals are produced by the microglia.

Finally, one of the responses to injury in the CNS is overgrowth of the damaged area with glia, a process called gliosis [2]. Halks-Miller et al. [40] have recently shown that α-tocopherol, a lipid soluble free radical scavenger, diminishes gliosis and reduces neuron damage and loss in mixed neuronal/glia cultures. They proposed that the trigger for gliosis may involve a free radical mechanism, the source of which was not defined. One possible source, as indicated by this study, is the activated microglia.

Overall, superoxide secretion by CNS microglia is similar to that seen in neutrophils and monocytes and could produce both anti-infective and deleterious effects. The level of antioxidant protection may be the determining factor in balancing the potential desirable and undesirable consequences of microglia activation.

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