Sulfatide activation of the oxygen radical generating system of leucocytes

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1. INTRODUCTION

Phagocytosing cells, such as polymorphonuclear leucocytes (PMN), monocytes and macrophages, are known to exhibit a respiratory burst that results in formation of oxygen intermediates, such as O_2^-, H_2O_2 and $^{\cdot}OH$ [1–6]. Generation of these oxygen intermediates is catalysed by a membrane-bound NADPH oxidase [3,7–9]. Similar metabolic changes can be induced by treating intact phagocytes with surfactants of sterol type [10,11], fatty acids [12] or phorbol myristate [13]; i.e., agents that bind to cell membranes of phagocytes by hydrophobic attraction, resulting in activation of membrane-bound NADPH oxidase to form oxygen radicals. These active oxygen intermediates are important in the bactericidal mechanism because of their oxidizing activity or action in cleaving carbon–carbon double bonds of lipids [14,15]. They also seem to be important in understanding the pathogenesis of some inflammatory diseases that are invariably associated with damage of the cell membrane. We found that sulfatide, a major component of the myelin membrane, can activate leucocytes to release the oxygen radical that may damage the membrane. Certain aspects of the allergic demyelinating process may be interpreted in the light of this effect of sulfatide in membrane damage.

Abbreviations: CSE, sulfatide; seminolipid, (1-*O*-alkyl-α-*O*-acyl-3-*O*-β-D-(3′-sulfo)galactopyranosyl-*sn*-glycerol; HRP, horseradish peroxidase; PMN, polymorphonuclear leukocytes; KRP, Krebs–Ringer phosphate buffer.

2. MATERIALS AND METHODS

2.1. *Preparation of sulfatide and related compounds*

Sulfatide (galactocerebroside-3′-sulfate, cerebroside sulfuric ester, CSE) was prepared from bovine brain as described previously [16]. Galactocerebroside-6′-sulfate was synthesized from bovine brain galactocerebroside by the method of Taketomi and Yamakawa [17]. Cholesterol sulfate was synthesized as the sodium salt by the carbodimide method described previously [18]. Seminolipid of boar testis was kindly provided by Dr. I. Ishizuka, Department of Biochemistry, Teikyo University, Tokyo. Galactose-6-sulfate was purchased from Seikagaku Kogyo, Tokyo.

2.2. *Other materials*

Horseradish peroxidase (HRP) was purchased from Boehringer Co. (Mannheim) as a purified, lyophilized dry powder. Catalase (purified from bovine liver) was obtained from Boehringer Co. as a crystalline suspension in water, saturated with thymol. Superoxide dismutase was obtained from Sigma Chemical Co. (St. Louis, MO) as a purified, lyophilized dry powder. Ferricytochrome *c* was purchased from Boehringer Co. as a purified, lyophilized dry powder (from horse heart), and 63% of its lysine residues were acetylated as reported previously [19]. *Escherichia coli* were heat-killed, washed and used as reported previously [20]. Other chemicals were of analytical grade.

2.3. *Cell preparation*

Guinea pig PMN were prepared as reported

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previously [1,12]. Sixteen hours after peritoneal injection of sterilized 2% sodium caseinate in saline, peritoneal exudates were collected in a plastic tube and centrifuged at 120 × *g* for 5 min at 4°C. The cell pellet was treated for 30 s with 0.2% NaCl solution to lyse red cells, and then promptly mixed with an equal volume of 1.6% NaCl solution to restore the isotonicity. The cell suspension was recentrifuged at 120 × *g* for 5 min at 4°C, and the resulting pellet of cells, more than 98% of which were PMN, was suspended in Ca^{2+}-free KRP (pH 7.4) and kept in an ice-bath until use.

2.4. *Assay of H_2O_2 generation*

The rate of H_2O_2 release from cells was measured by recording the rate of formation of an HRP–H_2O_2 complex, as reported previously [20]. A plastic cuvette of 10 mm light path containing 1.6 ml of cell suspension in the medium described below was attached to a windmill cell mixer and maintained at 37°C by means of a jacketted holder connected to a constant temperature water bath. The assay medium contained 6 μM HRP, 3 × 10^6 cells/ml, and 5 mM glucose in Ca^{2+}-free KRP. First H_2O_2 release in the resting state was recorded and then formation of the HRP–H_2O_2 complex was followed at 417–403 nm in a double-beam spectrophotometer (Hitach model 556) after addition of various concentrations of CSE or related compounds as concentrated solutions in distilled water (10–15 mg/ml).

2.5. *Assay of O_2^--generation*

The rate of O_2^- release from PMN was measured in the cuvette described above. The assay medium contained 15 μM 63%-acetylated cytochrome *c*, 5 μg/ml catalase, 3 × 10^6 PMN/ml, and 5 mM glucose in 1.6 ml of KRP. The reduction of acetylated cytochrome *c* was measured at 37°C at 550–540 nm after addition of various concentrations of sulfatide or related compound in a double beam spectrophotometer as described previously [19].

3. RESULTS

When CSE was added to the incubation medium, ferricytochrome *c* was precipitated, probably due to ionic interaction of CSE with cytochrome *c*. However, CSE did not give a precipitate with acetylated ferricytochrome *c*, and so this modified

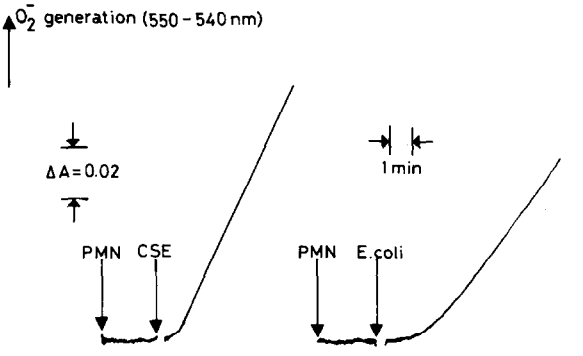


Fig.1. Trace (left) of O_2^- formation followed as reduction of acetylated ferricytochrome *c* on addition of CSE to PMN. The reaction mixture contained 15 μM 63%-acetylated ferricytochrome *c*, 5 μg/ml catalase, 3 × 10^6 cells/ml, and 5 mM glucose in 1.6 ml of Ca^{2+}-free KRP buffer (pH 7.4). Reduction of cytochrome *c* was measured at 37°C at 550–540 nm. After recording the trace in the resting state, CSE was added at 0.1 mg/ml to the cell suspension. Trace (right) of O_2^- formation by phagocytosing PMN on the addition of heat-killed *E. coli* (about 100 bacteria/cell).

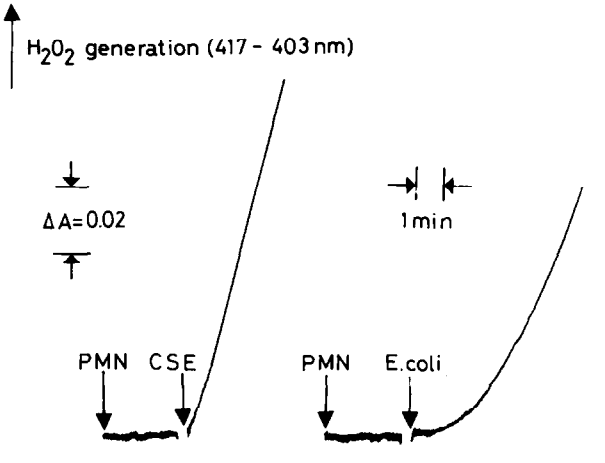
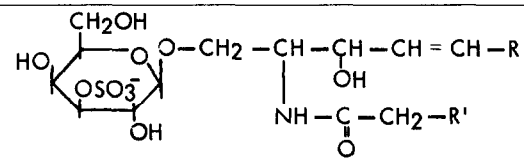
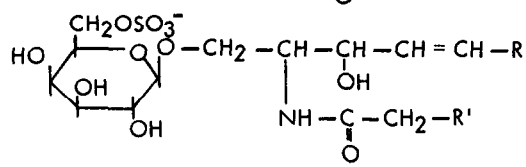
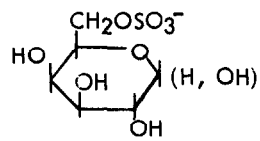
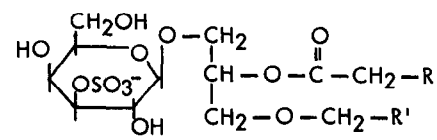
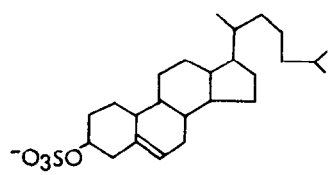


Fig.2. Trace (left) of H_2O_2 formation by PMN by followed as formation of the HRP–H_2O_2 complex on addition of CSE (0.2 mg/ml). The reaction mixture contained 6 μM HRP, 3 × 10^6 cells/ml, and 5 mM glucose in 1.6 ml of Ca^{2+}-free KRP buffer. Trace (right) of H_2O_2 formation by phagocytosing PMN. The reaction medium was as described above. *Escherichia coli* were added to cells in the same ratio of bacteria to cell as for fig.1. Formation of the HRP–H_2O_2 complex was measured at 37°C at 417–403 nm.

cytochrome *c* was employed for assay of O_2^- formation by PMN treated with CSE. The spectrophotometric trace in fig.1 shows the formation of O_2^- by PMN upon addition of CSE solution at a final concentration of 0.1 mg/ml. Marked stimulation of O_2^- formation was observed in the cells soon after the addition of CSE. In contrast, there was a short lag time before O_2^- began to be released when the cells were exposed to bacteria (heat-killed *E. coli*; about 100 bacteria/cell). Figure 2 shows the formation of H_2O_2 from PMN on addition of CSE at 0.2 mg/ml. No precipitate was formed after formation of the HRP–H_2O_2 complex until the concentration of added CSE was increased to 1.0 mg/ml. H_2O_2 formation was more rapid in CSE-treated cells than in phagocytizing cells, and only the latter showed a lag time of about 20 s after started addition of *E. coli* before H_2O_2 generation. Figure 3 shows a plot of the rate H_2O_2 formation by PMN against the CSE concentration added to the cell suspension. The rate of H_2O_2 formation increased with increase in CSE

Table 1

H_2O_2 generation by PMN upon addition of CSE and its related compounds

CSE and related compounds	Structure	Rate of H_2O_2 generation (nmol H_2O_2 . min^{-1} . 10^7 $cells^{-1}$)
CSE (galactocerebroside-3'-sulfate)		7.52
Galactocerebroside-6'-sulfate		0.56
Galactose-6-sulfate		neg
Seminolipid		neg
Cholesterol sulfate		14.1

The rate of H_2O_2 formation by PMN was measured as for fig.2. CSE and related compounds were added at 0.1 mg/ml to suspension of PMN.

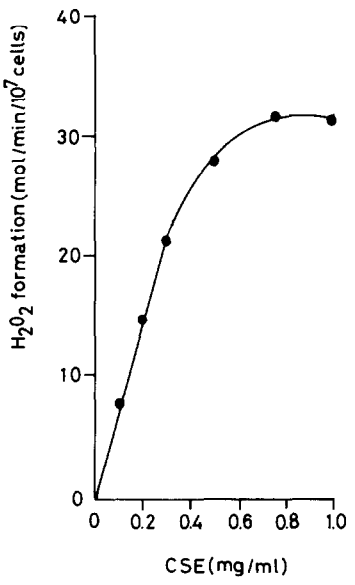


Fig.3. Plot of the rate of H_2O_2 generation by PMN against the CSE concentration added to the cell suspension. The reaction mixture was the same condition as for fig.2, except that it contained various concentrations of CSE.

concentration to a plateau at 0.68 mg/ml (1.4 mg/10^7 cells).

Next, the stimulatory effects of CSE and related compounds on the O_2^- and H_2O_2 generating system of PMN were tested. Table 1 summarizes results on H_2O_2 formation by PMN upon addition of CSE and related sulfated compounds at a concentration of 0.125 mg/ml (0.55 mg/10^7 cells). Galactocerebroside-6′-sulfate has a simulatory effect on PMN, causing formation of 0.56 nmol $H_2O_2 \cdot min^{-1} \cdot 10^7$ cells^{-1}, although this is far less than the formation of 7.52 nmol $H_2O_2 \cdot min^{-1} \cdot 10^7$ cells^{-1} induced by CSE. This indicates the necessity of some particular sulfated carbohydrate structure for the stimulation. Galactose-6-sulfate had no ability to induce H_2O_2 generation, indicating that the hydrophobic moiety of CSE may be necessary for stimulation of PMN, because of its attraction to the PMN cell membrane. However, another sulfated glycolipid, seminolipid, which has a hydrophobic chain of the glyceride type, had no effect on PMN when added at a similar concentration to that of galactocerebroside-6′-sulfate. Cholesterol sulfate induced marked stimulation of H_2O_2 formation.

4. DISCUSSION

It is widely accepted that the hydroxyl radical (·OH) splits carbon–carbon double bonds of lipids by a chain scission reaction because of its higher redox potential than those of other oxygen intermediates such as O_2^- and H_2O_2 [14,15]. ·OH is thought to be formed by a Haber–Weiss reaction of O_2^- with H_2O_2 [5,21]. Superoxide (O_2^-) is dismuted to form H_2O_2 at high velocity ($k = 10^5$ $M^{-1} \cdot s^{-1}$) even in the absence of superoxide dismutase. O_2^- reacts with many substances acting as either an oxidizing or reducing agent [14]. H_2O_2 is a reactive oxidizing agent and is converted to an even stronger oxidizing agent (HOCl) in the myeloperoxidase–H_2O_2–Cl system [22,23]. When phagocytosing cells, including PMN, monocytes [2] and macrophages [3,6], come in contact with infectious bacteria, their oxidative metabolism is promptly activated with generation of these toxic oxygen intermediates. This is an important host-defense mechanism. It is known that some hydrophobic agents induce marked enhancement of oxidative metabolism leading to formation of greater amounts of the oxygen intermediates than those formed on stimulation by phagocytosis [12,19,24].

As shown in the present experiments, brain sulfatide and cholesterol sulfate greatly induce oxidative metabolism of leucocytes, resulting in generation of the oxygen radicals that may attack the membrane. It is particularly interesting that galactose-6-sulfate, an analogue of the sugar moiety of sulfatide, was not stimulatory and also that seminolipid, a sulfatide with the glyceride but not the ceramide structure of CSE was not stimulatory. These findings suggest that the interaction between sulfatide and the leucocyte cell membrane necessary for induction of H_2O_2 formation requires some particular forms of not only hydrophobic but also anionic hydrophilic structures.

Tissues of the nervous system, and particularly the myelin membrane, are rich in sulfatide. In various types of demyelinating diseases, such as multiple sclerosis, post-vaccinal encephalomyelitis, and experimental autoallergic central or peripheral demyelination, a number of phagocytes, including polymorphonuclear leucocytes, monocytes and macrophages, appear in demyelinating lesions [25,26]. These phagocytes appearing in the lesions may be in an active state, in all probability like

those reported in cases of many inflammatory diseases. These activated cells possibly release O_2^- and other membrane-toxic oxygen intermediates, which in turn may release sulfatide molecules from the membranes in a state that causes a second activation of the phagocytes leading to a further release of oxygen intermediates. Thus, a myelin-destructive chain reaction may be evoked by the interaction between sulfatide and phagocytes. Further investigations with myelin itself or modified or artificial sulfatide-containing membranes are needed to clarify this problem.

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