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Presence of cytochrome *b*-558 in guinea-pig alveolar macrophages – subcellular localization and relationship with NADPH oxidase

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The assignment of cytochrome *b*-558 as a component of the O_2^- (H_2O_2) -generating enzyme in guinea-pig alveolar macrophages was investigated. Guinea pig alveolar macrophages contained 76 pmol cytochrome *b*-558/mg protein, a value very similar to that of neutrophils. The rate of myristic acid-stimulated O_2^- generation by alveolar macrophages, calculated per cytochrome *b*-558, was only one-fourth that of neutrophils. An analysis of Percoll density gradient centrifugation profiles showed that the H_2O_2-generating activity of myristic acid-activated alveolar macrophages was concentrated in a single peak which was consistently associated with 5′-nucleotidase activity, a plasma membrane marker enzyme. A little H_2O_2-generating activity was seen with unactivated alveolar macrophages. Furthermore, the cytochrome *b*-558 of both myristic acid-activated and unactivated alveolar macrophages was also predominantly associated with 5′-nucleotidase activity and was found in trace amounts in a peak containing lysozyme activity, a marker of lysosome granules. Only about 6% of the cytochrome *b*-558 in plasma membranes from myristic acid-activated guinea-pig alveolar macrophages was anaerobically reduced by 0.5 mM NADPH, while under the same conditions about 30% of the heme protein of myristic acid-activated neutrophils was reduced. These results suggest two conclusions: firstly, that in both activated and unactivated alveolar macrophages, cytochrome *b*-558 is located in the plasma membrane, and the translocation of cytochrome *b*-558 does not occur during the activation of NADPH oxidase; and secondly, that a smaller part of cytochrome *b*-558 is associated with the activated NADPH oxidase of guinea pig alveolar macrophages compared with neutrophils.

Introduction

Alveolar macrophages constitute the primary host defense mechanisms against infections of microorganisms invading the lung. It has been proposed that microbiocidal activity of alveolar macrophages is different from that of other phagocytes such as neutrophils and peritoneal macrophages [1]. Phagocytosing neutrophils generate and release active oxygen species such as O_2^- and H_2O_2, which are potent bacteriocidal agents [2]. This active oxygen generation is driven by the reduction of O_2 catalyzed by an NADPH oxidase located in the plasma membrane of neutrophils [3]. Some resident and BCG-activated alveolar macrophages generate and release these active

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonylfluoride; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; EGTA, ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; LDH, lactate dehydrogenase.

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oxygen species upon addition of bacteria or soluble stimulants [4–6]. However, it has been shown that rabbit and guinea pig alveolar macrophages do not contain myeloperoxidase as an H_2O_2-dependent bacteriocidal enzyme [7], indicating that the bacteriocidal activity of alveolar macrophages, in contrast to neutrophils, is not dependent on the myeloperoxidase-H_2O_2. Andrew et al. [8] reported that superoxide dismutase-inhibitable NADPH-dependent nitroblue tetrazolium reductase was localized in the endoplasmic reticulum and mitochondria of rabbit alveolar macrophages. If this activity is contributed by the NADPH oxidase of alveolar macrophages, the active oxygen-generating system in alveolar macrophages is markedly different from that of neutrophils.

Flavin and a *b*-type cytochrome (*b*-558) have been proposed to be components of the neutrophil NADPH oxidase [2,9,10]. Some investigators [11–13] revealed that peritoneal macrophages also contained this cytochrome *b*-558. On the other hand, it has been proposed that the major portion of cytochrome *b*-558 in resting neutrophils is located in specific granules, and that during the activation, the cytochrome *b*-558 is translocated into the plasma membrane from these granules [14,15]. If this translocation of cytochrome *b*-558 into the plasma membrane is a trigger for the activation of NADPH oxidase, the location of cytochrome *b*-558 of alveolar macrophages must be the same as reported in neutrophils. However, it is well-known that the types and contents of cytoplasmic organelles of alveolar macrophages differ markedly from those of neutrophils. Furthermore, we and another group have reported that no translocation of cytochrome *b*-558 in neutrophils occurs during a short activation [16,17].

Previously, we showed that guinea pig alveolar macrophages generated and released a considerable amount of H_2O_2 upon addition of bacteria or fatty acid (myristic acid) [4]. We report here the location of cytochrome *b*-558 and the assignment of this heme protein as the NADPH oxidase in guinea pig alveolar macrophages.

Materials and Methods

Materials. Cytochrome *c* (Type VI, from horse heart), superoxide dismutase, phenylmethylsulfonylfluoride (PMSF) and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) were purchased from Sigma Co., St. Louis. NADPH and catalase were obtained from Boehringer Co., Mannheim. Percoll was obtained from Pharmacia Fine Chem. Co., London. Ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and myristic acid were purchased from Wako Pure Chem. Co., Tokyo, Japan. Myristic acid was dissolved in dimethylsulfoxide to 20 mM. Hexamethylene-diamide phosphate, an anti-denaturing agent of protein [18], was obtained from Tokyo Kasei Co., Japan. Cytochrome *c* peroxidase was purified and crystallized from baker's yeast according to Yonetani [19]. All other reagents were of analytical grade.

Cell preparation. Alveolar macrophages were prepared from male guine pigs weighing 400–700 g, by tracheobronchial lavages according to Myrvik et al. [20]. Guinea pig peritoneal neutrophils were collected from the peritoneal cavity as described by Sbarra and Karnovsky [21], except for the use of 6% casein solution instead of 12% casein solution. Finally, cells were suspended in 17 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffered saline (pH 7.4) containing 5 mM KCl, 5 mM glucose and 1.2 mM $MgCl_2$.

Activation and fractionation of cells. The fractionation of cells was performed according to a previous report [22], with a slight modification. Cell suspensions ($2 \cdot 10^6$ cells per ml Hepes-buffered saline) were incubated for 5 min at 37 °C and then the cells were activated with 150 μM myristic acid (for alveolar macrophages) or 60 μM myristic acid (for neutrophils) for 30 s in the presence of 1 mM dimethylthiourea as an antioxidant [23]. After incubation with myristic acid, each cell suspension was loaded on a chilled layer of silicone oil (1.02 g/cm^3) and 0.34 M sucrose and centrifuged immediately at $1000 \times g$ for 3 min. Sedimented cells were suspended in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 0.1 mM TLCK and 0.2 mM hexamethylenediamide phosphate. Subsequent procedures were performed at 0–4 °C. Resting cells were treated using the same procedure but without myristic acid. Both resting and activated cells were homogenized in an ice bath with a Teflon pestle homogenizer at 1000 r.p.m. (for a total of 40 strokes), and then a 1/100

volume of 0.1 M PMSF was slowly added to the homogenates. To remove cell debris, the cell homogenates were centrifuged at $1000 \times g$ for 10 min. Resulting supernatants were mixed with EGTA (1 mM final) and loaded on 0–50% Percoll density gradient in disruption buffer containing 1 mM EGTA. The density gradient centrifugation was performed using a Beckman SW-27 rotor at 12000 r.p.m. for 60 min. After centrifugation each sample was fractionated (1.2 ml per tube). The density was determined using dextran marker beads (Pharmacia).

The assay of O_2^-- and H_2O_2-generating activities. The rate of O_2^- generation by intact cells was measured by following the SOD-inhibitable reduction of cytochrome *c* at 37 °C as described in Ref. 24 with a windmill mixer [25]. The reaction mixture contained $2 \cdot 10^6$ cells/ml of Hepes-buffered saline containing 50 μM ferricytochrome *c* and 5 μg/ml catalase. The rate of H_2O_2 generation by subcellular fractions was assayed according to the cytochrome *c* peroxidase methods [26] at 25 °C. The basal assay medium contained 5 μM cytochrome *c* peroxidase, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM NADPH and an aliquot of the test samples in 65 mM sodium potassium phosphate buffer/0.17 M sucrose (pH 7.0).

Enzymatic assay. 5′-Nucleotidase activity was measured as a plasma membrane marker [27]. The phosphorus released was measured by the method of Youngburg and Youngburg [28]. NADPH cytochrome *c* reductase and cytochrome *c* oxidase were measured as microsomal and mitochondrial markers, respectively, as described by Omura and Takesue [29] and by Orii and Okunuki [30]. Lysozyme was assayed as a granule (lysosome) marker [31].

Spectral study. Difference spectra under various conditions were measured at room temperature in a Hitachi 557 spectrophotometer with a data processor. After the absolute spectra were measured and stored, a few grains of dithionite were added to the sample and the reduced-minus-oxidized spectra were recorded using the subtraction mode. The content of cytochrome *b*-558 was calculated by using an absorption coefficient of 21.6 $mM^{-1} \cdot cm^{-1}$ at 558 nm [32]. For anaerobic spectra, as suspension of plasma membranes in 0.17 M sucrose/65 mM potassium sodium phosphate buffer (pH 7.2)/1 mM $MgCl_2$/0.1 mM EGTA was placed in an anaerobic cuvette and flushed with O_2-free argon gas. An oxidized spectrum was measured and stored in a data processor, then 0.5 mM NADPH was added to the suspension. After 30 min, the reduced-minus-oxidized spectra were recorded using the subtraction mode.

Protein. Protein was determined by the method of Lowry et al. [33] with bovine serum albumin as standard. Interference by Tris or other components of the text mixtures was avoided by using samples precipitated with 5% trichloroacetic acid.

Results

O_2^- generation by guinea pig alveolar macrophages

The O_2^- generation and release by guinea pig alveolar macrophages was measured following addition of myristic acid. Fig. 1 shows the rate of O_2^- generation by guinea pig alveolar macrophages under various concentrations of myristic acid. The maximum rate of O_2^- generation was observed with about 150 μM myristic acid, 59.1 ± 16.7 nmol O_2^-/min per 10^7 cells (mean ± S.D., $n = 6$). Guinea pig peritoneal neutrophils generated and released a maximum 96 ± 14 nmol O_2^- per min per 10^7 cells ($n = 5$) with about 60 μM of myristic acid.

Spectral study at cellular level

Fig. 2 shows the reduced-minus-oxidized spectra of guinea pig alveolar macrophages compared with guinea pig peritoneal neutrophils. In the case of alveolar macrophages, there were broad absorption maxima (α and β bands) in the visible region, and two peaks at 426 and 442 nm in the Soret region. However, we could not detect an absorption peak at 474 nm, characteristic of myeloperoxidase, in agreement with previous reports on rabbit alveolar macrophages [7]. On the other hand, the differential spectrum of neutrophils revealed typical absorption maxima of myeloperoxidase (474 nm) and cytochrome *b*-558 (558, 529 and 426 nm). From these spectral data it remained unclear whether or not guinea pig alveolar macrophages contained cytochrome *b*-558; therefore we analyzed the derivative curves of reduced-minus-oxidized spectra. Fig. 3a shows the 4th derivative spectra of guinea pig alveolar macrophages and peritoneal neutrophils. Clearly, the

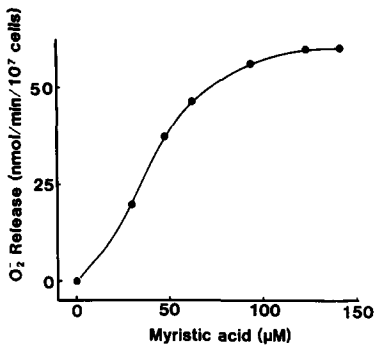


Fig. 1. Generation of O_2^- by guinea pig alveolar macrophages stimulated with myristic acid. The rate of O_2^- release was measured after stimulation with different concentration of fatty acid as described under Materials and Methods.

alveolar macrophages have a sharp peak at 558 nm, identical to that of neutrophils, as well as another peak at 550 nm. We compared the neutrophils' cytochrome *b*-558 content with the absorption at 558–563 nm in the 4th derivative curve of reduced-minus-oxidized spectra. Fig. 3b shows that the neutrophil cytochrome *b*-558 content calculated by these two methods clearly correlated, indicating that the 4th derivative spectra are useful for the calculation of cytochrome *b*-558 content. The contents of cytochrome *b*-558 in guinea pig alveolar macrophages and neutrophils, calculated by the relative absorption of 558–563 nm, is summarized in Table I. The specific contents of cytochrome *b*-558 in guinea pig alveolar macrophages (per mg protein) were similar to that of peritoneal neutrophils. It is noted that the cytochrome *b*-558 content per cell of alveolar macrophages was about 2.5-fold higher than that of peritoneal neutrophils because the protein content of guinea pig alveolar macrophages is about 2.5-fold higher than that of neutrophils.

Subcellular localization of NADPH oxidase and cytochrome b-558 guinea pig alveolar macrophages

To clarify the subcellular localization of cytochrome *b*-558 in alveolar macrophages, postnuclear supernatants isolated from both myristic acid-activated and unactivated alveolar macrophages were fractionated by Percoll density gradient centrifugation. The results are shown in Fig. 4. The NADPH-dependent H_2O_2 generating activity from MA-activated alveolar macrophages was found mainly in a single peak at about 1.04 g/cm^3. All fractions of unactivated alveolar macrophages showed much lower H_2O_2-generating activity. Distribution patterns of 5′-nucleotidase as a plasma membrane marker from both activated and unactivated alveolar macrophages were similar to those of the NADPH oxidase activity of myristic acid-activated alveolar macrophages and to those of guinea pig neutrophils [22]. Furthermore, the cytochrome *b*-558 of both myristic acid-activated and unactivated alveolar macrophages was also associated with 5′-nucleotidase activity, but was not found in the granule-containing fractions, located using lysozyme as a marker. NADPH-cytochrome *c* reductase activity was also found in the light fraction in both types of alveolar macrophages. The peak of NADPH cytochrome *c* reductase was slightly lighter than the peaks containing 5′-nucleotidase, cytochrome *b*-558 and the H_2O_2-generating activity of activated alveolar macrophages; these peaks were separated by only

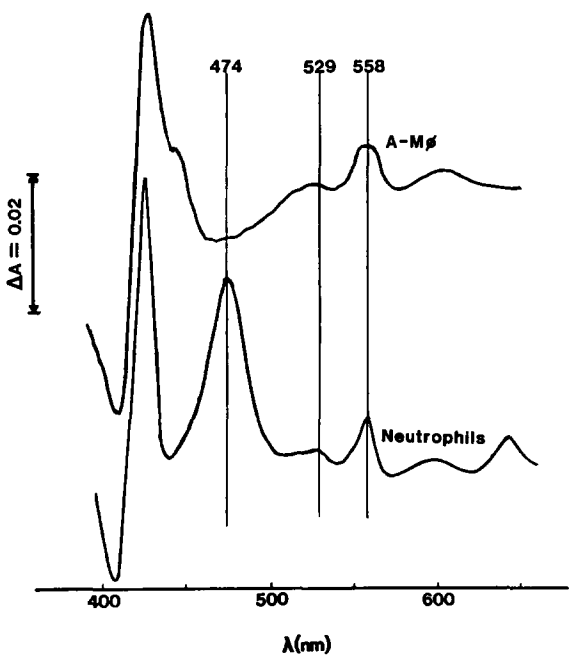
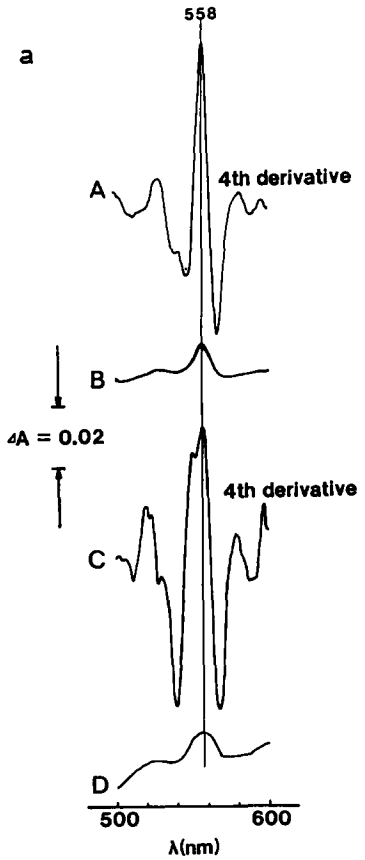


Fig. 2. Reduced-minus-oxidized spectra of whole cell homogenates of guinea pig alveolar macrophages and neutrophils. Alveolar macrophages and neutrophils contained 1.8 and 2.3 mg protein/ml, respectively.



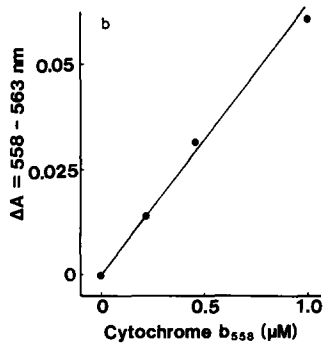


Fig. 3. (a) Fourth derivative reduced-minus-oxidized spectra of alveolar macrophages and neutrophils. Reduced-minus-oxidized spectra of whole cell homogenates of alveolar macrophages (D) and neutrophils (B) was measured as described in Fig. 2. Traces A and C, respectively, are the 4th derivatives of traces B and D. Alveolar macrophages and neutrophils contained 2.8 and 2.6 mg protein/ml, respectively. (b) Correlation of cytochrome *b*-558 content and absorption height of 558–563 nm in 4th derivative spectra in guinea pig neutrophils. Cytochrome *b*-558 content was calculated as described in Materials and Methods.

two fractions from the peak of NADPH cytochrome *c* reductase. Lysozyme was found mainly in the denser fractions and only to a small extent in the main fractions of 5′-nucleotidase. Cytochrome *c* oxidase, a mitochondrial marker enzyme, showed bimodal distribution of myristic acid-activated alveolar macrophages; the less dense peak of cytochrome *c* oxidase was found in a slightly heavier fraction than that of the plasma membrane marker and the other peak was close to the bottom fraction. On the other hand, the cytochrome *c* oxidase of unactivated alveolar macrophages was located mainly in slightly lighter fractions than lysozyme. Presumably, one possible reason for the difference of cytochrome *c* oxidase distribution between myristic acid-activated and unactivated alveolar macrophages might be due to the binding of myristic acid to mitochondria of activated cells. Because fatty acids such as myristate are well-known detergents, so that a portion of bound myristate on the plasma membrane may be removed into soluble fraction by its detergent effect during the homogenization or the fractionation [22]. Therefore, the released myristate may bind to the mitochondrial membranes by its high affinity to mitochondria [34]. Lactate dehydrogenase (LDH) activities of both alveolar macrophages were found in the top fractions.

NADPH oxidase and cytochrome b-558 in plasma membranes of alveolar macrophages

Fractions with the highest 5′-nucleotidase activity were pooled and collected by centrifugation at $105\,000 \times g$ for 60 min as plasma membranes. Table II shows marker enzyme activities and cyto-

TABLE I

CYTOCHROME *b*-558 CONTENTS IN GUINEA PIG ALVEOLAR MACROPHAGES AND PERITONEAL NEUTROPHILS

	Cytochrome *b*-558 (pmol/mg protein)
Alveolar macrophages	76.3 ± 11.6 [a]
Neutrophils	70.0 ± 11.2

[a] Mean ± S.D. from four experiments.

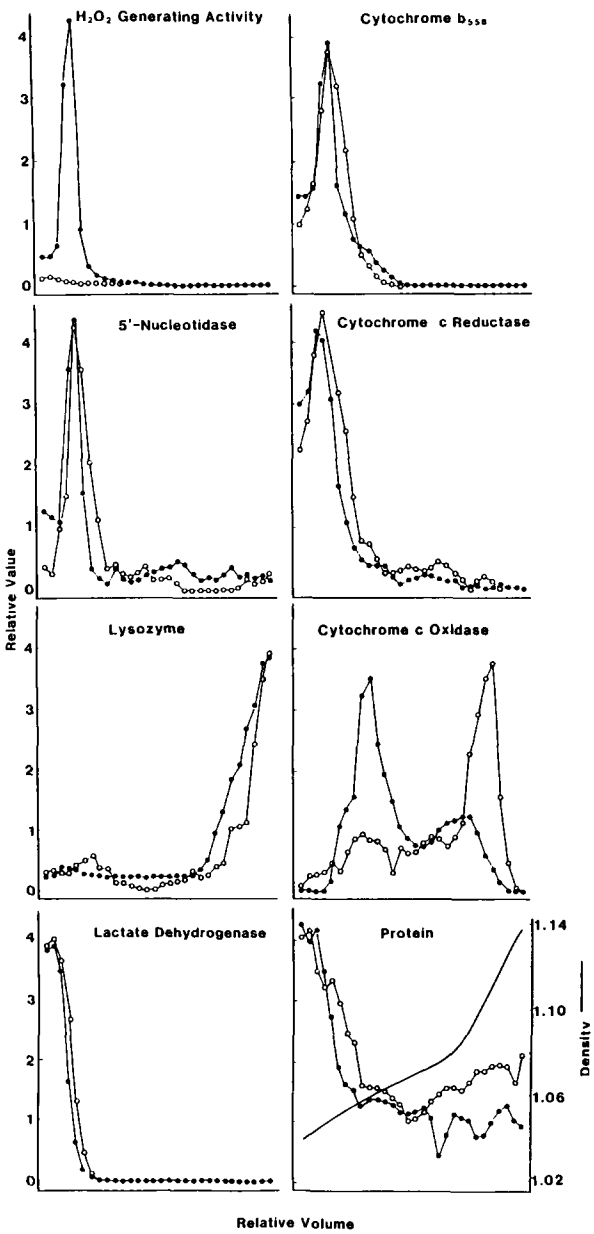


Fig. 4. Distribution of enzyme activities in guinea pig alveolar macrophages. Graphs are the normalized distribution patterns of alveolar macrophages as a function of the volume collected. Radial distance increases from left to right. The ordinate is the concentration in the fraction relative to the concentration corresponding to a uniform distribution throughout the gradient. Closed and open circles show the distribution of enzymes from myristic acid-activated and unactivated alveolar macrophages, respectively.

chrome *b* content in the plasma membrane fraction from myristic acid-activated and unactivated alveolar macrophages. The Percoll density gradient centrifugation provided significant increases in 5′-nucleotidase, H_2O_2-generating activity and cytochrome *b* in the plasma membrane fraction from myristic acid-activated cells with amplification factors of 10.2, 10.9 and 9.9, respectively. Similar increments in 5′-nucleotidase activity and cytochrome *b*-558 content, but not H_2O_2-generating activity, were observed in the plasma membranes from unactivated alveolar macrophages. On the other hand, the specific activity of cytochrome *c* reductase in the plasma membrane fraction of myristic acid-activated and unactivated cells was 4.1- and 3.8-fold higher than those of whole cell homogenates, respectively. Cytochrome *c* oxidase activities were dramatically decreased in the plasma membrane form both myristic acid-activated and unactivated alveolar macrophages.

Fig. 5 shows the reduction of cytochrome *b*-558 by the addition of 0.5 mM NADPH in anaerobic condition. Only about 6% of the cytochrome *b*-558 in plasma membranes from myristic acid-activated alveolar macrophages was reduced by the addition of NADPH, compared with 29% of cytochrome *b*-558 in plasma membranes from myristic acid-activated neutrophils. It might be noted that there is a factor 5 difference between the percentage of NADPH-reducible cytochrome *b*-558 in alveolar macrophages and in neutrophils.

The K_m value of NADPH oxidase from neutrophils has been reported to be 20–50 μM [2,26]. We determined the apparent K_m value of NADPH oxidase in plasma membranes of guinea pig alveolar macrophages by a method employing cytochrome *c* peroxidase [26]. Fig. 6 shows the rate of H_2O_2 generation by plasma membranes from both activated and unactivated alveolar macrophages, as a function of the concentration of added NADPH. The rate of H_2O_2 generation by both plasma membranes is proportional to NADPH concentration until a maximum is reached at 0.1 mM. The apparent K_m value for NADPH of myristic acid-activated alveolar macrophages was 35.0 ± 5.3 μM (mean $\pm$ S.D., $n = 4$), and the V_{max} was 0.42 to 0.93 μmol per min per mg protein. The apparent K_m value for NADPH of unactivated cells was 50.5 ± 7.8 μM ($n = 4$), and the

TABLE II

SPECIFIC CONTENTS OF MARKERS IN PLASMA MEMBRANES FROM GUINEA PIG ALVEOLAR MACROPHAGES

Numbers are means ± S.D. from three experiments.

	H_2O_2-generating activity (μmol/min per mg protein)	5′-Nucleotidase activity (μmol/min per mg protein)	Cytochrome *b* (pmol per mg protein)	NADPH cytochrome *c* red (nmol/min per mg protein)	Cytochrome *c* oxidase (nmol/min per mg protein)
Myristic acid-activated					
Cell homogenates	0.11 ±0.03	0.81 ± 0.11	0.079 ± 0.014	33 ± 8	31.2 ± 8.4
Plasma membranes	1.2 ±0.34	8.3 ± 1.8	0.78 ± 0.12	134 ± 28	2.8 ± 0.7
Resting cells					
Cell homogenates	0.005 ± 0.002	0.77 ± 0.06	0.076 ± 0.011	38 ± 6	28.0 ± 3.2
Plasma membranes	0.021 ± 0.01	7.9 ± 0.32	0.77 ± 0.15	145 ± 15	1.4 ± 0.6

V_{max} was 0.012 to 0.023 μmol per min per mg protein.

Discussion

Guinea pig alveolar macrophages generated and released lower amount of O_2^- than peritoneal neutrophils upon addition of myristic acid, as reported Kaneda et al. [4]. In the present study, analysis of the 4th derivative of the difference spectra suggests that guinea pig alveolar macrophages also contained 76 pmol cytochrome *b*-558 per mg protein, as estimated by the absorption height at 558–563 nm in the 4th derivative curve of the reduced-minus-oxidized spectra (Table I). Since the cytochrome *b*-558 content per cell of alveolar macrophages was about 2.5-fold higher, the O_2^- generating activities for this heme protein in guinea pig alveolar macrophages and peritoneal neutrophils stimulated by myristic acid were calculated to be about 300 nmol/min per nmol cytochrome *b*-558 and 1200 nmol/min per nmol cytochrome *b*-558, respectively, suggesting that the O_2^--generating activity was independent of the

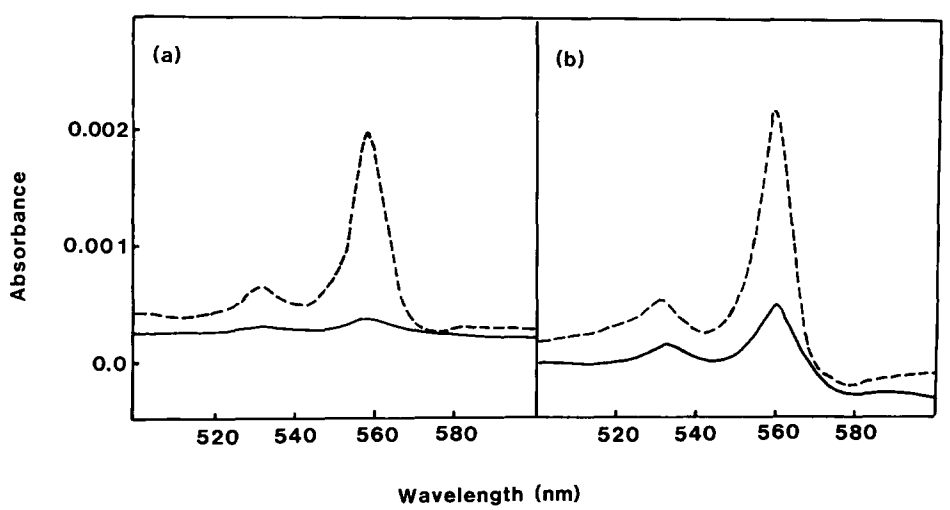


Fig. 5. The reduction of cytochrome *b*-558 in guinea pig alveolar macrophages and neutrophils. (a) Plasma membranes from alveolar macrophages. (b) Plasma membranes from neutrophils. Solid line shows difference spectra after addition of 0.5 mM NADPH. Dotted line is after addition of a few grains of dithionite.

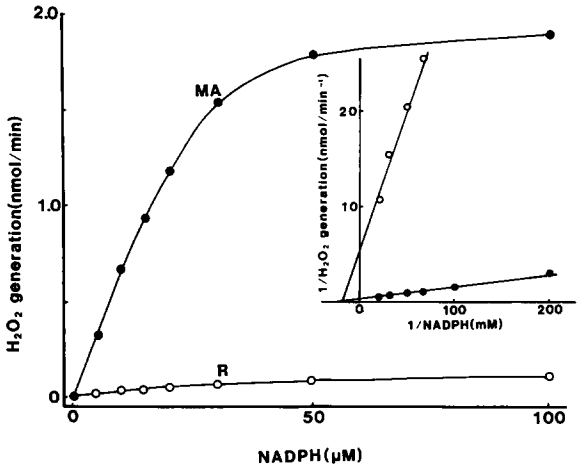


Fig. 6. The rate of H_2O_2 production by plasma membranes of both activated and unactivated alveolar macrophages plotted against the NADPH concentration added. The reaction mixture was as in Materials and Methods by using plasma membranes of myristic acid-activated (MA) and unactivated (R) alveolar macrophages contained 2.0 μg protein. Inset shows the Lineweaver-Burk plot of H_2O_2-producing activity in both plasma membranes.

content of cytochrome *b*-558. Tsunawaki and Nathan reported that the content of cytochrome *b*-558 in the peritoneal macrophages did not retain the capacity of the cells to generate oxygen radicals [12]; for example, protease peptone-elicited macrophages, which released little O_2^-, appeared to contain about 108 pmol of cytochrome *b*-558 per mg protein. The low activity of O_2^- generation in guinea pig alveolar macrophages might be due to an alteration of the activation process or to loss of some other factor of the NADPH oxidase. In the present study, only about 6% of the cytochrome *b*-558 in plasma membranes of myristic acid-activated alveolar macrophages was reduced by addition of NADPH in anaerobic condition, while 29% of cytochrome *b*-558 of myristic acid-activated neutrophils was reduced. The percentage of NADPH-reducible cytochrome *b*-558 in guinea pig alveolar macrophages was about one-fifth of that in neutrophils, indicating that a considerable but minor portion of the cytochrome *b*-558 might contribute to the activated NADPH oxidase in guinea pig alveolar macrophages. Recently, from the studies of ESR signals of flavin in myristic acid-activated and unactivated porcine neutrophil membranes, Kakinuma et al. [35] suggested that stimulation of neutrophils brings the flavin free radical and some metal center(s) into closer relationship in the membrane. Therefore, one possible explanation is that the lower activity of O_2^- generation per cytochrome *b*-558 by guinea pig alveolar macrophages, compared to neutrophils, is due to the association of a small part of this heme protein with the flavin in the membrane.

The NADPH-dependent O_2^--generating activity in activated neutrophils is located in the plasma membrane and is dormant in resting cells [3,17]. In the present study, the H_2O_2-generating activity of myristic acid-activated guinea pig alveolar macrophages was found in the same peak of 5′-nucleotidase as a plasma membrane marker, while the H_2O_2-generating activity of unactivated cells was very low over all fractions. The cytochrome *b*-558 from both activated and unactivated alveolar macrophages was located in the same peak of the plasma membrane and was scarcely detected in the bottom fractions (lysosomal granules) or the peaks of cytochrome *c* oxidase activity. The peak of cytochrome *b*-558 and H_2O_2-generating activity in myristic acid-activated alveolar macrophages was slightly dissociated from that of the microsomal marker NADPH cytochrome *c* reductase. The increment rates in the specific content of cytochrome *b*-558 and H_2O_2-generating activity from the whole cell homogenates to plasma membranes were very close to that of 5′-nucleotidase activity, except for the case of H_2O_2-generating activity of unactivated alveolar macrophages. Therefore, the cytochrome *b*-558 and NADPH oxidase of guinea pig alveolar macrophages appear to be co-located in the plasma membrane, and this heme protein was not located in the granules of either activated or unactivated cells. Several groups have reported that the distribution of cytochrome *b*-558 in neutrophils was bimodal [13–15,36], i.e., in plasma membranes and specific granules. Furthermore, some investigators have proposed that cytochrome *b*-558 translocates into plasma membranes from granules during the activation of NADPH oxidase [13–15]. From the present results, the cytochrome *b*-558 of guinea pig alveolar macrophages appears to be located in the plasma membrane even in the resting state, unlike neutrophils, so that the trans-

location of this heme protein does not occur during the activation of guinea pig alveolar macrophages.

Several investigators have reported various K_m values of NADPH oxidase in peritoneal macrophage, alveolar macrophages and monocytes [12,37–39] by measuring the O_2^- generation using a method employing cytochrome *c*. In the present study, we investigated the kinetics of the NADPH oxidase in guinea pig alveolar macrophages using a method employing cytochrome *c* peroxidase [26] because cytochrome *c* peroxidase forms stable complexes with H_2O_2. It is well-known that macrophages contain relatively higher microsomal and mitochondrial enzyme activities than neutrophils which might disturb the O_2^--generating assay by the reduction of cytochrome *c*. On the other hand, the assay of H_2O_2 generation by the cytochrome *c* peroxidase method is not disturbed by these enzyme activities. The apparent K_m value for NADPH of NADPH oxidase in myristic acid-activated alveolar macrophages was about 35 μM, which was very close to that of neutrophil NADPH oxidase (Fig. 6). Furthermore, V_{max} value of the NADPH oxidase in plasma membranes of myristic acid-activated alveolar macrophages was 30–40-times higher than that of unactivated alveolar macrophages. These results indicate that the affinity of the NADPH oxidase of guinea pig Mø is very close to that of neutrophils. Therefore, low activity of O_2^- generation by guinea pig alveolar macrophages per cytochrome *b*-558 might not be due to the low affinity to the NADPH oxidase for NADPH.

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