0022-1767/86/1364-1393$02.00/0
The Journal of Immunology

Vol. 136, No. 4, February 15, 1986
Printed in U.S.A.

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MOLECULAR BASIS OF MACROPHAGE ACTIVATION. EXPRESSION OF THE LOW POTENTIAL CYTOCHROME b AND ITS REDUCTION UPON CELL STIMULATION IN ACTIVATED MACROPHAGES[1]

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The expression of the novel b-type cytochrome, which is part of the superoxide anion (O_2^-)-generating system in phagocytes, has been investigated in populations of mouse peritoneal macrophages heterogeneous in their capability to produce O_2^-. Reduced minus oxidized difference spectra of intact cells showed the appearance of a b-type cytochrome with major peaks in the α region at 558 to 559 nm and in the γ region at 426 to 428 nm. Resident peritoneal macrophages, as well as thioglycollate broth-elicited and Corynebacterium Parvum-activated macrophages and neutrophils expressed about 50 pmol cytochrome b/10^7 cells. In intact macrophages and neutrophils, Na-dithionite reduced >75% of the cytochrome b measurable in disrupted cells. No correlation was found between capability to produce O_2^- by different population of macrophages and their content of cytochrome b. When stimulated in strictly anaerobic conditions with phorbol myristic acetate, macrophages activated in vivo by i.p. injection of Corynebacterium Parvum reduced approximately 40% of their total cytochrome b. In resident peritoneal macrophages that produced five times lower amounts of O_2^-, cytochrome b reduction was instead undetectable. Potentiometric properties of cytochrome b was investigated in macrophage subcellular particles. Both resident and Corynebacterium Parvum-activated macrophages revealed the presence of b cromophores with very low potentials of −255 and −244 mV, respectively, whose content was not different in the two populations. These results show that resident and activated macrophages express the same amount of cytochrome b, but upon stimulation with PMA, activated macrophages recruit a higher number of cytochrome b molecules in parallel with an enhanced production of O_2^-.

Upon interaction with appropriate stimuli, macrophages and granulocytes produce reactive oxygen species such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which exert powerful microbicidal and cytotoxic effects. The molecular basis of this phenomenon generally known as "the respiratory burst" has been extensively investigated in neutrophils. There is now a general agreement that reduction of oxygen to O_2^- depends on the activity of an oxidase that uses NADPH as substrate (1, 2). Extensive evidence has been accumulated in the last few years also indicating that a b-type cytochrome whose peculiar feature is a low mid-point potential ($Em_{7.0}$ = −245 mV) participates in the transfer of reducing equivalents to oxygen (3–6).

The molecular basis of O_2^- generation in macrophages are less known. Evidence has been provided by different investigators that NADPH oxidase is the enzyme responsible for the respiratory burst also in mononuclear phagocytes (7–13). Products derived by lymphocytes (now thought to reflect the action of interferon-γ; 14) or of bacterial origin (15–17) prime macrophages to produce larger amounts of O_2^- and H_2O_2 upon triggering with phagocytosable particles or other appropriate stimuli (reviewed in Reference 18). Only recently, data have started to be accumulated on the properties of NADPH oxidase in relation to the process of macrophage activation. Alterations of the kinetic properties of the enzyme with an increase of the affinity for NADPH seem to accompany macrophage activation (11–13).

Because a b-type cytochrome with spectroscopic properties similar to those described in neutrophils has been also described in mononuclear phagocytes from different species, we have investigated the properties of this cytochrome in relation to the phenomenon of macrophage activation. In this paper, we present data on the expression of cytochrome b and its reduction in intact cells after stimulation with phorbol myristic acetate (PMA) in macrophage populations that differ in their ability to produce O_2^- upon triggering. These data show that macrophage activation is not accompanied by enhanced expression of cytochrome b, but in activated macrophages, more cytochrome b molecules are recruited in the transfer of electrons to oxygen upon stimulation by PMA.

MATERIALS AND METHODS

Cells. Macrophages were obtained from the peritoneal cavity of Swiss mice as described (13). Animals were either untreated or had been injected i.p. 7 to 20 days previously with 1 mg Corynebacterium Parvum (Wellcome, Beckenham, UK), or had been injected 4 days previously with 1 ml of thioglycollate broth (Oxoid, Basingstoke, UK). Neutrophils were obtained from the peritoneal cavity of mice 12 hr after the injection of 1% sodium caseinate (Merck, Frankfurter, FRG); L929 cells were kindly donated by Dr. Siamon Gordon of the Sir William Dunn School of Pathology of the University of Oxford,

Received for publication August 15, 1985.
Accepted for publication November 5, 1985.
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[1] This work was supported by grants from Consiglio Nazionale delle Ricerche of Italy (Progetto finalizzato "Oncologia", gruppo Immunologia, n. 84.00774.44) and Progetto Finalizzato Medicina Preventiva e Riabilitativa Meccanismi di Invecchiamento n. 84.02200.56 and Ministero Pubblica Istruzione (fondi 40%).
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UK. Hepatocytes were obtained from mouse livers with a modification of the procedure described by Howard et al. (19). Liver slices were obtained as described (19) and were placed in 50 ml plastic tubes (Flow Laboratories, Irvine, Scotland) in a shaking water bath at 37°C, and then were incubated for 20 min in a collagenase solution (19). During the incubation, 100% oxygen was blown over the solution. After transfer in a Petri dish, the liver slices were broken into two to three pieces with a rubber policeman, the whole suspension was gently pipetted two to three times, and finally was filtered through a gauze. Hepatocytes were washed five times in phosphate-buffered saline without calcium and magnesium (PBS) containing 1 mM EDTA by centrifugation at 50 × G for 1 to 2 min, and finally was resuspended in PBS. Lymphocytes were obtained from mouse spleens by standard procedures, and after lysis of contaminating erythrocytes, were cultivated for 2 hr at 37°C/5% CO_2 (see below) to left contaminating macrophages adhere. Red cells were obtained from heparinized human blood. Cells viability was checked by trypan blue exclusion.

Polymorphonuclear leukocyte depletion of macrophage population. Macrophages were assayed immediately after harvest or after cultivation on normal or gelatin-coated tissue culture plastic (see below). In the former case, peritoneal washouts were centrifuged, and the cells were resuspended in PBS at 10×10^6/ml. Neutrophils were separated from mononuclear cells by centrifugation on Ficoll-Hypaque. Contaminating erythrocytes were lysed by hypotonic shock, and the cells were finally resuspended in Krebs Ringer phosphate buffer or PBS for metabolic or spectroscopic studies, respectively (see below). Macrophage populations contained between 25 and 45% lymphocytes and negligible amounts of neutrophils.

Macrophage cultivation. Macrophages were cultivated for 4 to 24 hr at 37°C in the presence of 5% CO_2 in Dulbecco's modification of Eagle's minimum essential medium (Flow Laboratories) containing 10% heat-inactivated (56°C for 30 min) horse serum and 50 μg/ml of penicillin and streptomycin. Macrophage cultivation was performed either in tissue culture plastic 30 mm dishes (Flow Laboratories), or in 25 cm^2 gelatin-coated flasks from the same source. In the former case, adherent macrophages were washed twice with PBS and were detached by scraping with a rubber policeman. When macrophages were cultivated in flasks coated with gelatin (20), they were detached after washing of nonadherent cells with 10 mM lidocaine (20) or 0.1% trypsin plus 1.5 mM EDTA.

O_2^- production. The O_2^--dependent cytochrome c reduction was measured as originally described by Babior et al. (21). Macrophages were assayed immediately after harvest or after cultivation and recovery in suspension in a reaction mixture prepared as described (13). The assay was done in plastic tubes incubated in a shaking water bath (37°C) or in 1 ml plastic cuvettes. In the former case, the reaction was stopped after 30 min with ice-cold KRP, and after centrifugation, the supernatants were read at 550 nm. In the latter, the kinetics of O_2^- release was monitored at 37°C in a 576 Perkin-Elmer double-beam spectrophotometer.

Spectroscopy with intact cells. Na-dithionite reduced minus oxidized difference spectra of intact cells were measured with a Beckman DU-8 spectrophotometer. Samples containing 10 to 20×10^6 cells were analyzed in a glass microcuvette in a final vol of 0.3 ml. Cells were suspended in PBS. When hepatocytes or L929 cells were used, cells were diluted 1/1 with Ficoll-Hypaque to delay sedimentation. Controls done with macrophages showed that this did not affect the assay. Absorbance of samples in the oxidized state was first measured and was memorized in the computer program. Reduction of samples was then accomplished by addition of a few grains of Na-dithionite, and the difference spectrum (reduced minus oxidized) was immediately recorded.

Oxidation-reduction potential measurement. This was determined in macrophage subcellular particles with phenosafranin dye as potential indicator according to Light et al. (22). Resident or Corynebacterium Parvum-activated macrophages were washed in PBS, and after lysis of contaminating erythrocytes, were placed in 30 mm Petri dishes, and were cultivated as described above. After 18 hr cultivation, the adherent cells were washed three times with PBS and were overlayed with 50 mM Na-phosphate buffer pH 7.0 containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 2 mM NaN_3. Cells were scraped with a rubber policeman, were sonicated for three 5-sec pulses at 100 W with a Branson sonifier, and then were centrifuged at 400 × G for 10 min to remove nuclei and cellular debris. After centrifugation at 100,000 × G for 30 min, the pellet was resuspended in the above buffer and was analyzed. Oxidation-reduction potential measurements were done with a 576 Perkin-Elmer double-beam spectrophotometer. Details of the procedure used will be reported elsewhere (23). The concentration of proteins in the sample was 0.5 mg/ml. Cytochrome b concentration at potentials below −200 mV was calculated from OD at 410 minus 426 nm by using an ϵ of 151 mM^{-1} cm^{-1}.

Reduction of cytochrome b in intact macrophages. Resident and Corynebacterium Parvum-activated macrophages were washed in PBS, and after lysis of contaminating erythrocytes, were depleted of neutrophils on Ficoll-Hypaque. Cells (8×10^7) in 2 ml of KRP containing 2 mM NaN_3, 5 mM glucose, 0.5 U/ml heparin, and 1 mM EDTA were distributed in 3 ml plastic cuvettes, and these were placed in the sample and reference-holder of the 576 Perkin-Elmer spectrophotometer turbid samples compartment. The apparatus was thermostated at 37°C, and the cell suspensions in the sample were stirred by a micro-stirring bar driven by an external magnetic field placed under the cuvette. The sample cuvette was made anaerobic by flushing oxygen-free nitrogen through a 1.1 x 40 mm needle inserted in a ribbon plug that stoppered the cuvette. Outlet of nitrogen was allowed through another needle of the same dimensions, which also served for the various additions. After addition of glucose oxidase (25 mg/ml) to ensure complete anaerobiosis, the sample was stirred for a couple of minutes, and after interruption of the stirring, the first spectrum in a range between 403 and 433 nm was recorded. Spectra were then automatically recorded every 38 sec by setting the spectrophotometer on repetitive scan. Every 20 to 30 sec, the sample was magnetically stirred for a few seconds, and the reference cuvette was agitated manually. After 15 min from the addition of glucose oxidase, 200 ng/ml PMA were injected in the sample cuvette, and other spectra were recorded. When the reduction of cytochrome b reached a plateau, total cytochrome b was measured by adding few grains of Na-dithionite to the sample cuvette. Cytochrome b was calculated from OD at 410 minus 426 nm as described above. O_2^- release by the macrophage population assayed as described above was measured in the same KRP mixture in aerobic conditions and without the addition of glucose oxidase.

Miscellaneous. To study the effect of phagocytosis on cytochrome b expression, 2×10^7 macrophages in 25 cm^2 gelatin-coated flasks were overlayed with cold KRP plus 5 mM glucose and 0.5 mM $CaCl_2$ containing 2 to 3 mg of zymosan (Sigma, Taufkirchen, FRG). After incubation at 4°C for 30 min to let zymosan settle on the cells, the flasks were transferred to 37°C for 15 to 30 min. After extensive washing with cold PBS, the cells were detached with trypsin-EDTA (see above) at 4°C, and cytochrome b was measured as described above. Control cells were treated in parallel exactly as described but omitting the addition of zymosan. In some experiments, trypsin-EDTA treatment was prolonged up to 30 min at 4°C. Proteins were assayed with the method of Lowry and PMA and zymosan were prepared as described (24).

RESULTS

Reduction of cytochrome b with Na-dithionite in different populations of macrophages. Reduction of intact cells with Na-dithionite has been used to detect a unique b-type cytochrome in human neutrophils (25). As shown in Figure 1, also reduced minus oxidized difference spectra of resident peritoneal macrophages revealed the presence of a major cytochrome b-type chromophore with major peaks in the α region at 558 to 559 nm and in the γ one at 426 to 428 nm. These spectral characteristics did not change significantly upon disruption of the cell by sonication, which enhanced the cytochrome b-like spectrum by less than 25% (see Table I for additional details).

Table I shows that the b-type cytochrome reduced from the outside of the cell in different populations of macrophages and neutrophils was comparable. This was approximately 50 pmol/10^7 cells in peritoneal neutrophils and resident, thioglycollate-elicited and Corynebacterium Parvum-activated macrophages, a value similar to that previously reported for human blood neutrophils and monocytes (26). In all the populations studied, the cytochrome b measured in intact cells accounted for 75 to 90% of that detectable in cell sonicates.

Expression of cytochrome b in different macrophage populations and neutrophils was not correlated with the capability to produce O_2^- upon challenge with appropriate stimuli. As shown in Table II, Corynebacterium Parvum-activated macrophages and neutrophils produced

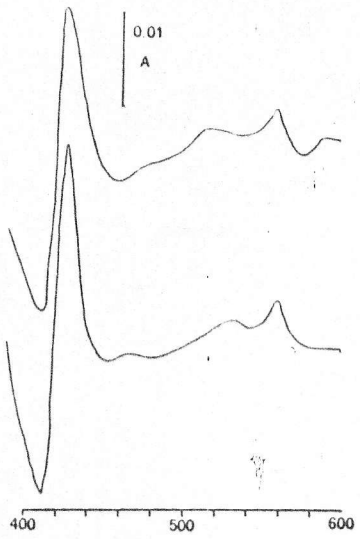

Figure 1. Reduced minus oxidized difference spectra of intact (*upper trace*) and sonicated (*lower trace*) macrophages. Resident peritoneal macrophages were assayed immediately after harvesting after centrifugation on Ficoll-Hypaque gradients to remove possibly contaminating granulocytes. The concentration of cells was 45×10^6/ml in PBS, and 0.3 ml were assayed. Reduction was accomplished by addition of a few grains of Na-dithionite.

TABLE I

Expression of cytochrome b by different populations of macrophages and neutrophils[a]

	pmol Cytochrome b/10^7 Cells	
	Intact cells	Sonicated cells
Resident macrophages	49.5 ± 9.6 (8)	62.0 ± 13.7
Corynebacterium Parvum-activated macrophages	53.8 ± 8.1 (5)	60.8 ± 2.0
Thioglycollate broth-elicited macrophages	54.3 ± 6.7 (2)	60.2 ± 3.4
Neutrophils	52.4 ± 12.0 (4)	68.5 ± 13.1

[a] Macrophages were cultivated as described in the text and were detached by scraping with a rubber policeman after removal of nonadherent cells. Neutrophils were freed of contaminating erythrocytes by hypotonic shock and were purified by centrifugation on Ficoll-Hypaque. Detection of cytochrome b in intact macrophages was not affected by the procedure used to obtain them as a suspension of cells. In resident macrophages purified on Ficoll-Hypaque and assayed immediately after harvesting from the peritoneal cavity, cytochrome b was 53.8 ± 12.0 ($\bar{X}$ ± SD; n = 3) pmol/10^7 cells. In thioglycollate-elicited macrophages cultivated on gelatin and detached by lidocaine, cytochrome b was 54.0 ± 5.2 ($\bar{X}$ ± SD; n = 3) pmol/10^7 cells. The mean results ± SD of the number of experiments shown in parentheses are reported.

four to six times more O_2^- in response to PMA or zymosan than peritoneal resident macrophages, but expressed comparable amounts of cytochrome b.

Although the kinetics of permeation of Na-dithionite in phagocytes is not known and the appearance of a b chromophore upon its addition to intact cells could reflect reduction of intracellular chromophores, we tryed to obtain possible evidence that Na-dithionite reduced a unique cytochrome b on the surface of macrophages. Addition of Na-dithionite to other cell types rich in intracellular chromophores such as erythrocytes, hepatocytes, and L929 cells, reduced only a minor amount (at most 25%) of these chromophores (not shown). Also spleen and peritoneal lymphocytes were negative for the presence of chromophores reducible from the outside of the cell. Conversely, we have been able to detect a cytochrome b-like spectrum in intact human, guinea pig, and mouse neutrophils, and guinea pig and mouse macrophages. Controversial data were obtained, however, when we tried to selectively remove this putative plasma membrane-located cytochrome b from the cell surface by protease digestion or phagocytosis. Trypsin treatment (1 mg/ml at 4°C for 30 to 60 min) of intact macrophages actually increased the cytochrome b expression reducible from the outside by 33 and 48% in two independent experiments, whereas phagocytosis of zymosan reduced cytochrome b expression in intact macrophages by 34 and 40% in two independent experiments, without causing any decrease in the amount measurable in macrophage sonicates (not shown).

Reduction of cytochrome b in intact macrophages upon stimulation of O_2^- production by PMA. Stimulation of neutrophils respiratory burst in anaerobic conditions is accompanied by reduction of cytochrome b (5, 25). As shown in Figure 2, in Corynebacterium Parvum-activated macrophages also, the stimulation with PMA in anaerobic conditions caused the increase of a b-type chromophore with a peak of absorption at 426 to 428 nm, comparable with that revealed by Na-dithionite.

Figure 3 shows the kinetics of cytochrome b reduction in macrophages stimulated with PMA. When the cells were maintained in anaerobic conditions in the absence of PMA, a small increase of b-type chromophores spectra was detected both in resident and Corynebacterium Parvum-activated macrophages. These did not change with time up to 30 min (not shown) and could possibly be due to anaerobic reduction of mitochondrial cytochromes. Addition of maximal stimulatory concentrations of PMA to Corynebacterium Parvum-activated macrophages caused reduction of cytochrome b, which increased with time up to 10 min after the addition of the stimulant at which time a plateau was reached. Reduction of cytochrome b was not detectable when the same number of resident macrophages was used. This was not due to a complete lack of respiratory burst activation in resident macrophages. In fact, when tested in an assay in kinetics in a similar reaction mixture (see *Materials and Methods*) but in aerobic conditions, resident macrophages produced 3.1 ± 1.5 ($\bar{x}$ ± SD; n = 3) nmol O_2^-/min/10^7 cells compared with 16.6 ± 5.7 ($\bar{x}$ ± SD; n = 3) nmol O_2^-/min/10^7 cells produced by Corynebacterium Parvum-activated macrophages. Undetection of cytochrome b reduction after PMA stimulation of resident macrophages was probably due to the sensitivity limits of the assays. In fact, when we used a lower number of Corynebacterium Parvum-activated macrophages, which produced an amount of O_2^- comparable with that of resident macrophages, reduction of cytochrome b upon addition of PMA was also undetectable.

In Corynebacterium Parvum-activated macrophages, PMA reduced less than 40% of the total cytochrome b measurable with Na-dithionite. In three independent experiments, we obtained a value of 21.2 ± 3.4 pmol cytochrome b/10^7 cells ($\bar{x}$ ± SD) compared with 58.4 ± 11.1 ($\bar{x}$ ± SD) pmol cytochrome b/10^7 cells after reduction with Na-dithionite.

Oxidation-reduction potential of the cytochrome b of mouse macrophages. Oxidation-reduction potential of cytochrome b of mouse macrophages was measured in

TABLE II

Expression of cytochrome b by macrophages and neutrophils heterogenous in their capability to produce O_2^-[a]

Experiment	Additions	nmol O_2^-/30 min/10^6 Cells[b]			pmol Cytochrome b/10^7 Cells
		Nil	PMA	Zymosan	
1	Resident macrophages	0.5	2.7	ND[c]	50.3
	Corynebacterium Parvum-activated macrophages	0.9	10.6	ND	48.1
2	Resident macrophages	0.4	5.6	1.1	51.3
	Corynebacterium Parvum-activated macrophages	1.5	21.6	4.8	53.0
	Neutrophils	3.6	29.1	7.7	40.6
3	Resident macrophages	0.3	6.0	ND	69.4
	Neutrophils	1.0	28.1	ND	65.0

[a] Cells were obtained and cytochrome b was measured in intact cells as described in the legend to Table I.
[b] The assay of O_2^- release was done in 10 ml plastic tubes in a shaking water bath (temperature = 37°C) as described in the text.
[c] ND, not detected.

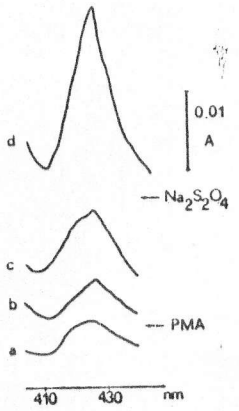

Figure 2. Reduction of cytochrome b in intact macrophages upon stimulation of the respiratory burst with PMA. Corynebacterium Parvum-activated macrophages were assayed immediately after harvesting after centrifugation on Ficoll-Hypaque gradients to remove possibly contaminating granulocytes. The concentration of cells was 4×10^7/ml. Absorption in the range 403 to 433 nm is shown. *Line a*, 15 min after incubation in anaerobic conditions; *line b*, 5 min; *line c*, 15 min after addition of PMA (0.5 μg/ml); and *line d*, after addition of a few grains of Na-dithionite.

subcellular particles of resident and Corynebacterium Parvum-activated cells by using phenosafranin as potential indicator. As shown by others (24) and by us with guinea pig macrophages (23), phenosafranin is a particularly suitable indicator for these measurements. Figure 4 reports the potentiometric tritation of cytochrome b in macrophage subcellular particles. The $Em_{7.0}$ of cytochrome b was −244 and −255 mV in Corynebacterium Parvum-activated and resident macrophages, respectively. When the reduced cytochrome b was quantified at potentials around the midpoint (−250 mV), we calculated that both resident and Corynebacterium Parvum-activated macrophages contained about 150 pmol cytochrome b/mg proteins, thus confirming that populations of macrophages heterogenous in their capability to produce O_2^- contain comparable amounts of the low potential cytochrome b.

DISCUSSION

Macrophages are heterogeneous in their capability to produce O_2^- and H_2O_2 upon stimulation with appropriate stimuli. In the last few years, extensive evidence has accumulated that production of these toxic oxygen molecules is correlated with macrophage capability to kill intracellular pathogens (27) and tumor cells (28). A great deal has been also learned about factors capable of regulating the release of toxic oxygen molecules in vivo and in vitro (see Reference 18 for review).

Only recently the molecular basis of macrophage heterogeneity and activation by environmental stimuli has become the object of intensive investigations. In vivo and in vitro activation of mouse macrophages is accompanied by profound changes in surface expression of receptors that trigger the respiratory burst, such as the mannose-fucose receptors (MFR) (29, 30) and the type I receptors for Fc of immunoglobulin G (31). Enhanced expression of antigens whose function is still unknown has been also reported (32, 33).

It is now clear that the capability of different receptors to trigger the respiratory burst varies considerably. For example, the type I Fc receptor is very efficient (31), whereas receptors for C3 fragments are not (34). Although macrophage activation is accompanied by enhanced expression of type I Fc receptors (31), capability of activated macrophages to produce higher amounts of toxic oxygen molecules clearly reflects events beyond receptor expression. There is in fact no correlation between enhanced capability of activated macrophage to produce O_2^- upon triggering and expression of receptors such as type II Fc receptors (31), MFR (30), and phorbol diesters receptors (13).

Data obtained in three different laboratories (11–13) have recently indicated a correlation between the capability to produce O_2^- and/or H_2O_2 upon triggering and the kinetic properties of NADPH oxidase, the enzymatic system responsible for the generation of O_2^- in leukocytes. We found that NADPH oxidase activity in macrophage lysates increased in parallel with O_2^- production by intact cells and could account for differences between resident and Corynebacterium Parvum-activated peritoneal macrophages (13). The affinity of the oxidase for NADPH also increased, the Michaelis-Menten constant (km) being five to seven times lower in activated macrophages. The same alterations of the oxidase affinity for its substrate has been reported for macrophages activated with lipopoly-

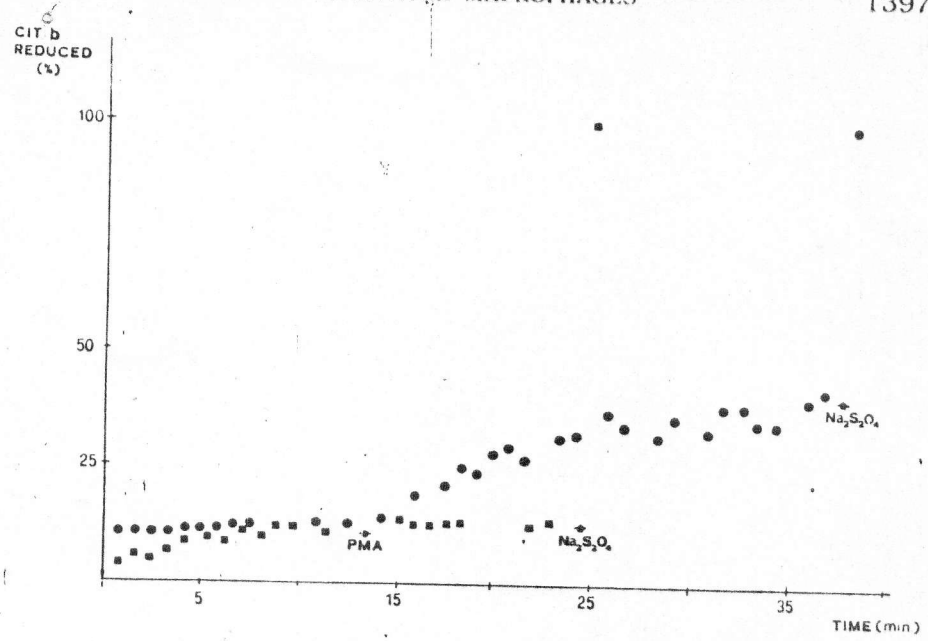

Figure 3. Time course of reduction of cytochrome b in intact macrophages upon stimulation of the respiratory burst with PMA. Percent of cytochrome b reduced taking as 100% that reduced by dithionite. ●, Corynebacterium Parvum-activated macrophages; ■, resident macrophages. The results of one of three similar experiments is reported. Cytochrome b reduced by Na-dithionite in intact macrophages in this group of experiments was 58.4 ± 11.1 pmol/10^7 cells for Corynebacterium Parvum-activated macrophages and 54.0 ± 4.5 pmol/10^7 cells for resident macrophages. Assays conditions were described in the legend to Figure 2.

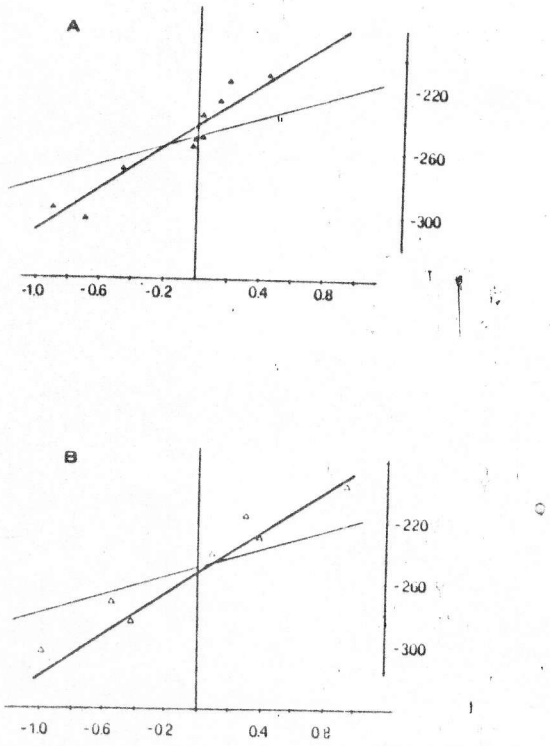

Figure 4. Potentiometric titration of cytochrome b of Corynebacterium Parvum (▲—▲, *Panel A*), and resident macrophages (Δ—Δ, *Panel B*). Titrations were done with macrophage subcellular particles as described in the text. The *thin line* shows the potentiometric titration of phenosaphranin, which was used as an indicator of reduction-oxidation potential. *Abscissa*, log cytochrome b oxidized/cytochrome b reduced. *Ordinate*, E (mV).

saccharide (11) or inflammatory stimuli (12).

There is now a general agreement that in neutrophils, the NADPH oxidase activity also depends on the participation of a peculiar b-like cytochrome with a very low (−245 mV) midpoint potential. The presence of a chromophore with similar spectroscopic characteristics has been also observed in human monocytes (26), and the promelocytic cell line HL60 upon differentiation in vitro (35), mouse macrophage cell lines (36), and peritoneal macrophages. We recently completed experiments (23) showing that the typical cytochrome b of neutrophils is also present in guinea pig peritoneal macrophages in which it represents the major cellular chromophore, copurifies with NADPH oxidase activity after solubilization and gel filtration, and is characterized by a midpoint potential of −247 mV.

The main conclusions that emerge from the data presented in this paper are first that macrophages and neutrophils heterogeneous in their capability to produce O_2^- upon challenge with PMA or zymosan express comparable amounts of cytochrome b as measured by sodium-dithionite reduction of intact cells, and then macrophage activation is apparently not accompanied by enhanced expression of this oxidase component. Second, the enhanced production of O_2^- by Corynebacterium Parvum-activated macrophages is paralleled by a higher reduction of cytochrome b when the cells are stimulated by PMA in anaerobiosis. This suggests that in activated macrophages, a higher number of cytochrome b molecules are involved in the transfer of reducing equivalents from NADPH to oxygen, possibly due to alterations in the activity of the NADPH binding component as revealed by kinetic studies (11–13). Third, the cytochrome b of both resident and Corynebacterium Parvum-activated mouse macrophages is characterized as those of neutrophils (4) and guinea pig macrophages (23) by a low midpoint potential of −255 and −244 mV, respectively. Specific measurement of the low potential cytochrome b showed that its amount was comparable in resident and activated macrophages, thus strengthening the conclusion that macrophage activation is not accompanied by enhanced expression of this oxidase component.

Reduction of intact cells by Na-dithionite was originally used to detect cytochrome b in neutrophils and was used as evidence of its surface localization (25). Subsequently, evidence has been produced that in resting neutrophils, cytochrome b is associated with the specific granules (37). A comparative study in neutrophils and monocytes has revealed that although in neutrophils cytochrome b shows a bimodal distribution in the specific granules and the plasma membrane, monocytes selectively express cytochrome b on the cell surface (38).

The data presented in this paper are in accord with this last observation. We observed that Na-dithionite easily

reduced a cytochrome b in intact macrophages, which accounted for more than 75% of the b-like chromophore measured in disrupted cells. Significantly, in the same assay conditions, Na-dithionite did not reduce intracellular chromophores of other cell types such as erythrocytes, L929 cells, and epatocytes. Preliminary experiments indicated that phagocytosis of zymosan partly affected the reduction of cytochrome b from the outside of the cell, and insensitivity of cytochrome b to trypsin treatment can be conceived in the light of functional data that showed that treatment of macrophages with proteases actually increased their capacity to produce O_2^- upon challenge (39). Additional studies are required, however, to establish a possible surface localization of cytochrome b.

As measured by total chemical reduction with Na-dithionite, cytochrome b content is comparable in populations of macrophages heterogeneous in their capability of producing O_2^-. It is therefore likely that heterogeneity of macrophages in responding to environmental stimuli with O_2^- production is not due to a quantitative alteration of cytochrome b expression. This conclusion is strengthened by the observation that when specifically measured during potentiometric titration, the amount of the low potential cytochrome b in cellfree particles of resident and Corynebacterium Parvum-activated macrophages was the same.

The oxidation-reduction midpoint potential of leukocyte cytochrome b is unusually low for a b-type cytochrome. This property is in favor of the participation of cytochrome b in O_2^- formation as a terminal oxidase, because the O_2/O_2^- couple as a −330 mV potential. Therefore the difference of O_2^- production between macrophages in different states of activation could theoretically be attributable to differences of their cytochrome b. Our findings of similar oxidation-reduction properties of resident and Corynebacterium Parvum-activated macrophages exclude the possibility that the regulation of the respiratory burst activity lies at this level. Other qualitative alterations such as the CO binding properties, however, have been reported in the cytochrome b of plasma membrane-enriched fractions from macrophage cell lines variants, which do not undergo a respiratory burst upon challenge (36).

On the basis of the hypothesis that cytochrome b reduction in intact cells reflects the activation of the O_2^- generating system (5, 25), we have investigated possible differences in the cytochrome b reduction in different macrophage populations. The results presented in this paper demonstrate that in activated macrophages, a higher number of cytochrome b molecules is reduced during stimulation of the respiratory burst.

A precise model of the oxidative-reductive system responsible for the generation of O_2^- in phagocytes, which can help in interpreting these results is still lacking. It is widely believed that the NADPH oxidase is composed of a flavoprotein and cytochrome b_{-245}. We have presented substantial evidence that the molecular ratio of this two components is in large favor of cytochrome both in neutrophils (40) and macrophages (23). Taken together, the observations that activated macrophages do not express a higher number of cytochrome b molecules but are able to reduce them more effectively upon triggering of the respiratory burst could suggest that macrophage activation is accompanied by alterations of the NADPH-dependent flavoprotein that transfers electrons from NADPH to cytochrome b. This suggestion is in accord with data obtained by others (11, 12) and by us (13) that showed that the NADPH oxidase of activated macrophages challenged with PMA displays a higher affinity for its substrate.

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