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Activation of mouse macrophages causes no change in expression and function of phorbol diesters' receptors, but is accompanied by alterations in the activity and kinetic parameters of NADPH oxidase

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Summary. Mouse peritoneal macrophages activated *in vivo* by the injection of *Corynebacterium parvum* release larger amounts of superoxide anion (O_2^-) than macrophages from control mice when stimulated with phorbol myristate acetate (PMA). The biochemical bases for this enhanced response of activated macrophages have been investigated by studying the expression and function of receptors for the stimulant, and the activity of the enzyme NADPH oxidase which is responsible for the production of O_2^- in leucocytes. Studies of binding of phorbol dibutyrate, an agent closely related to PMA, showed that the affinity constants (Kds) and the number of binding sites were the same in resident and activated peritoneal macrophages. The activity of the NADPH oxidase was, however, different in the two macrophage populations which differ in their capacity to release O_2^-. NADPH oxidase activity was studied in macrophage monolayers after lysis with deoxycholate. The main features of this activity were as follows:

(i) stimulation of macrophages with PMA or zymosan caused an increase in NADPH-dependent O_2^- production;

(ii) NADPH oxidase activity in the lysates followed the same dose-response curve for different concentrations of PMA as O_2^- release by intact macrophages;

(iii) O_2^- release by intact macrophages could be fully accounted for by NADPH-dependent O_2^- production by macrophage lysates;

(iv) activity was strictly substrate-specific, in that NADH could not substitute for NADPH;

(v) after stimulation with PMA or zymosan, NADPH oxidase activity was higher in lysates of *C. parvum*-activated macrophages than in lysates of resident macrophages;

(vi) NADPH oxidase activities of activated and resident macrophages differed markedly in their kinetic parameters. The NADPH oxidase of macrophages activated by *C. parvum* or trehalose dimycolate of mycobacterial origin displayed a five to seven times lower Km compared to the enzyme in resident macrophages.

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INTRODUCTION

The microbicidal and cytotoxic properties of neutrophils and macrophages depend, to a large extent, on the generation of reactive oxygen intermediates. The molecular bases of oxygen metabolism have been investigated extensively in neutrophils. Reduction of oxygen to superoxide anion (O_2^-) is due to activation of an NADPH oxidase which has been proposed to reflect the activity of an electron transport chain whose components have been only partially defined (Rossi,

Patriarca & Romeo, 1980; Babior, 1984). NADPH oxidase activity has also been demonstrated in macrophages. Subcellular particles isolated from phagocytosing guinea-pig and rabbit peritoneal and alveolar macrophages oxidize NADPH at a faster rate than subcellular particles from resting macrophages (Romeo *et al.*, 1973). NADPH-dependent O_2^- production has been described in subcellular particles isolated from resident and inflammatory guinea-pig peritoneal macrophages (Bellavite *et al.*, 1981; Berton *et al.*, 1982).

Macrophages vary considerably in their response to appropriate stimuli. Peritoneal macrophages obtained from mice after injection of intracellular pathogens (Nathan & Root, 1977; Johnston, Godzik & Cohn, 1978) and bacterial products (Cummings, Pabst & Johnston, 1980; Lepoivre *et al.*, 1982) release larger amounts of O_2^- and/or H_2O_2 after treatment with phorbol myristate acetate (PMA) or zymosan, than resident macrophages from control mice. The biochemical bases for these differences between different macrophage populations are still poorly understood. Macrophage activation is accompanied by profound changes in the expression of surface antigens and receptors (Ezekowitz & Gordon, 1982). There seems, however, to be no positive correlation between expression of receptors for stimulants of the respiratory burst, and the actual macrophage response to surface stimulation (Ezekowitz, Bampton & Gordon, 1983; Weinberg & Misukonis, 1983). Little attention has been paid to the activities of enzyme(s) responsible for the respiratory burst in relation to the phenomenon of macrophage activation. Recently, NADPH oxidase of peritoneal macrophages elicited with LPS or inflammatory stimuli has been shown to display a lower Km for NADPH than NADPH oxidase of resident cells (Sasada, Pabst & Johnston, 1983; Tsunawaki & Nathan, 1984).

In the present paper, we report studies on NADPH-oxidase activities for macrophage populations which differ in their ability to release O_2^- after stimulation. The results obtained indicate that, while binding of the stimulant is not changed in activated macrophages, modification of the activity and kinetic properties of NADPH-oxidase does accompany macrophage activation.

MATERIALS AND METHODS

Media and reagents

Dulbecco's modification of Eagle's minimum essential medium (DMEM) was obtained from Flow Laboratories (Irvine, Scotland). Horse serum (HS) from the same source was heat inactivated at 56° for 30 min before use. Streptomycin (50 μg/ml) and penicillin (50 μg/ml) were added to the media. Phosphate-buffered saline (PBS) was used routinely without calcium and magnesium. Krebs ringer phosphate buffer (KRP) contained 123 mM NaCl, 1·23 mM $MgCl_2$, 4·90 mM KCl and 16·7 mM Na-phosphate buffer, pH 7·4. Stock solutions were kept at 4° with 5 mM glucose and 0·5 mM $CaCl_2$ added before use (KRPGCa). Phorbol myristate acetate (PMA), phorbol dibutyrate (PDBU), zymosan A, NADH, NADPH and cytochrome *c* were purchased from Sigma (Taufkirchen, West Germany). Superoxide dismutase purified from human red cells was a kind gift of Dr J. V. Bannister, of the Department of Inorganic Chemistry, University of Oxford, Oxford.

Peritoneal cells

Swiss mice of both sexes bred at the Institute of General Pathology of the University of Verona were used, aged 2–3 months. Peritoneal macrophages were obtained by washing the peritoneal cavity with 5 ml PBS. Animals were either untreated or had been i.p. injected 7–20 days previously with 1 mg *Cornybacterium parvum* (Wellcome Research Lab., Beckenham, England), or with 50 μg trehalose dimycolate from *Mycobacterium*, a kind gift of Dr J. F. Petit, Institut de Biochemie, Université de Paris-Sud, Paris, Peritoneal cells were freed of contaminating erythrocytes with hypotonic saline. Macrophage monolayers were obtained by plating peritoneal cells in DMEM + 10% HS in tissue culture plastic 24-well trays Space Saver (Flow Laboratories).

Superoxide anion (O_2^-) release

This was assayed by the spectrophotometric measurement of cytochrome *c* reduction (Babior, Kipnes & Curnutte, 1973). Adherent macrophages were assayed as described (Berton & Gordon, 1983a) in a reaction mixture prepared in KRPGCa and containing 2 mM NaN_3 and 80 μM cytochrome *c*, plus or minus 40 μg/ml superoxide dismutase.

NADPH oxidase activity

Previous work (Bellavite *et al.*, 1981; Berton *et al.*, 1982) has shown that deoxycholate lysis of macrophages in suspension provides a suitable way to study activities of the enzymatic system(s) responsible for respiratory burst activity in response to PMA stimula-

tion. In the present study, the same procedure was applied to macrophage monolayers. Macrophages cultivated overnight, as described above, were washed twice with PBS and overlayed with 0·45 ml KRPGCa + 2 mM NaN_3, i.e. the same reaction mixture used to study O_2^- release from intact cells. After 5 min incubation at 37°, 0·05 ml KRP alone, when the oxidase of resting cells was assayed, or KRP containing the appropriate stimulant (PMA or zymosan), was added. After 30 min (or different times as specified) at 37°, the KRP solution was aspirated and replaced by 0·3–0·5 ml of a reaction mixture containing 50 mM HEPES, pH 7·0, 1 mM diethylenetriaminepentaacetic acid (DTPA, Sigma), a metal chelator, 2 mM NaN_3, 0·05% recrystallized deoxycholate, 80–160 μM cytochrome *c*, and 0·15 mM NADPH. All assays were performed in duplicate with or without 50 μg/ml superoxide dismutase. Controls included assays in which NADPH was omitted, and cell-free blanks. After 15 min (or longer as specified) at room temperature, the reaction was stopped by addition of an equal volume of 50 mM HEPES pH 7·0/1 mM DTPA/2 mM NaN_3 containing 2 mM p-chloromercuribenzoate (PCMB, Sigma), an inhibitor of O_2^- production by macrophages and of NADPH oxidase of macrophage lysates (Berton *et al.*, 1982). Appropriate controls showed that, when PCMB was included from the beginning, the cytochrome *c* reduction was the same as for cell-free blanks. The difference of cytochrome *c* reduction in the absence and in the presence of superoxide dismutase was used to calculate the amount specifically reduced by O_2^-. The reduced cytochrome *c* was measured by difference in absorbance at 550−468, using an extinction coefficient of 24·5 mM^{-1} cm^{-1} (Bellavite *et al.*, 1983). Activity of NADPH oxidase was assayed at different substrate concentrations and graphed according to the traditional Lineweaver-Burk double reciprocal plot. Linear regression was used to fit the best straight line to the data, and Michaelis constant (Km) and maximal velocity (V_{max}) were calculated from the interception of the line with the abscissa and the ordinate, respectively.

Binding of [^{3}H]PDBU

PDBU [20-^{3}H(N)] was purchased from New England Nuclear (Dreieich, W. Germany) at a specific activity of 13·7 Ci/mmol. Adherent macrophages in a 24-well Space Saver (Flow) were incubated with different concentrations of [^{3}H]PDBU in 0·25 ml KRPGCa plus 2 mM NaN_3, with or without 10 μg/ml PMA. After incubation at 4° or 37° for different periods (as specified), the cells were washed rapidly three times with cold PBS and lysed in 250 μl of 1 M NaOH. The cell-associated radioactivity was determined in Picofluor 30 (Packard, Downers Grove, IL) with a 240 CL/D Packard scintillation counter. The radioactivity of control wells without cells treated as above, was measured in parallel and subtracted from cell-associated radioactivity. Affinity constant (Kd) and maximal binding of [^{3}H]PDBU to macrophages were calculated from data of specific binding (binding in the absence minus binding in the presence of 10 μg/ml PMA) by means of Scatchard analysis.

Miscellaneous

Cell proteins were assayed in cell lysates by the Lowry method, and PMA and zymosan were prepared and stored as described (Berton & Gordon, 1983a).

RESULTS

Binding of [^{3}H]PDBU to, and associated O_2^- release by, resident and *C. parvum*-activated peritoneal macrophages

Mouse peritoneal macrophages (PM) have been shown to express specific saturable receptors for [^{3}H]PDBU (Berton & Gordon, 1983a). PMA effectively competes with [^{3}H]PDBU for binding to mouse macrophages and induces selective down-regulation of [^{3}H]PDBU receptors. Figure 1 shows the binding of [^{3}H]PDBU to resident PM (a) and *C. parvum* PM (b) at 4°. Binding was saturable and PMA effectively competed with [^{3}H]PDBU. As reported previously (Berton & Gordon, 1983a), binding at 4° was maximal after 10 min and remained constant up to 2 hours (data not shown).

Activation of macrophages by i.p. injection of *C. parvum* was not accompanied by any change in affinity and number of receptors for PDBU. From Scatchard analysis of [^{3}H]PDBU binding, we calculated an affinity constant (Kd) of 32·4 ± 12 nM ($n = 3$) and a maximal binding of 2·2 ± 0·6 pmoles/mg cell protein for resident PM. For *C. parvum* PM, the Kd was 28·0 ± 9 nM ($n = 3$) and maximal binding 2·5 ± 1·2 pmoles/mg cell proteins.

Figure 2 shows a comparison of time course of specific binding at 37° of saturing concentrations of [^{3}H]PDBU to resident PM and *C. parvum* PM, and O_2^- release by the same macrophage populations. Specific binding was maximal after 5 min and remained fairly

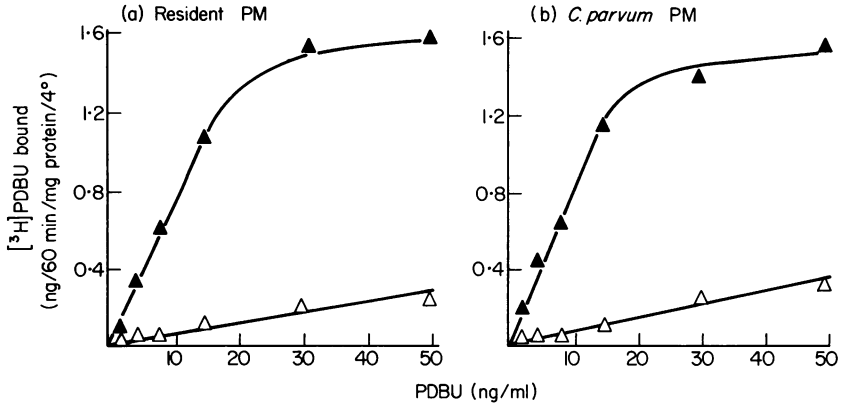

Figure 1. Ligand concentration dependence of [^{3}H]PDBU binding to (a) resident PM and (b) *C. parvum* PM. Binding of [^{3}H]PDBU to macrophages cultivated for 24 hr was measured at 4°, as described in the text. Mean binding in the absence (▲——▲) and in the presence (△——△) of 10 μg/ml PMA of duplicate assays is reported. One of three similar experiments.

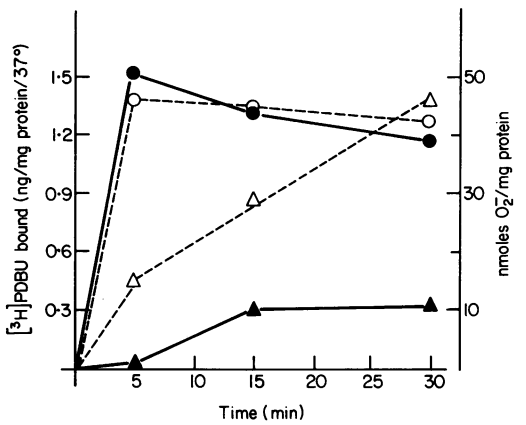

Figure 2. Time-course of [^{3}H]PDBU specific binding to resident PM and *C. parvum* PM, and of O_2^- release. Specific binding to resident PM (●——●) and *C. parvum* PM (○——○) was measured at 37° with saturating concentrations of [^{3}H] PDBU (30 ng/ml), as described in the text. O_2^- release by resident PM (▲——▲) and *C. parvum* PM (△——△) was measured in parallel using 30 ng/ml PDBU. The means of duplicate assays which varied $< 10\%$ are reported. One of two similar experiments.

constant up to 30 min. The graphs for binding to resident PM and *C. parvum* PM were superimposable. *C. parvum* PM released three to five times more O_2^- than resident PM at all the times tested.

O_2^- release by different macrophage populations in response to PMA

Peritoneal macrophages from mice injected with *C. parvum* released more O_2^- than macrophages of control mice also in response to PMA. After 24 hr of cultivation as adherent monolayers, both resident PM and *C. parvum* released negligible amounts of O_2^- spontaneously. The O_2^- (nmoles/mg cell protein) released in the extracellular medium after a 30 min treatment with 100 ng PMA/ml was: resident PM, $24{\cdot}4 \pm 6{\cdot}2$ (SD) ($n = 10$), *C. parvum* PM, $94{\cdot}2 \pm 34{\cdot}5$ (SD) ($n = 13$). Maximal stimulation was obtained at concentrations of PMA between 20 and 100 ng/ml. *C. parvum* PM released three to six times more O_2^- than resident PM at all concentrations (5–200 ng/ml) of PMA tested. The difference in the activity of the two populations was already evident 10 min after the addition of the stimulant and lasted up to 60 min. Time and stimulant concentration-dependence of the macrophage response to PMA is shown in Fig. 3.

NADPH-dependent O_2^- production by lysates of macrophage monolayers

In order to assay activity of the enzyme responsible for O_2^- production in macrophages cultivated as adherent cells, we used a modification of the method applied previously to macrophages in suspension (Bellavite *et al.*, 1981; Berton *et al.*, 1982).

The assay of NADPH oxidase activity in deoxycholate lysates of macrophage monolayers was found to be reproducible and suitable to study the enzymatic basis of O_2^- production in cultivated macrophages. Appropriate controls were examined as follows.

(i) The addition of 0·05% deoxycholate to PMA-stimulated macrophages stopped the O_2^- release by

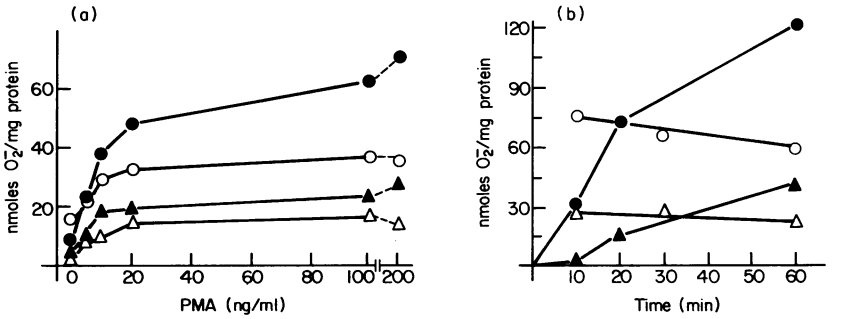

Figure 3. (a) O_2^- release by intact macrophages and NADPH-oxidase activity of macrophage lysates after stimulation with different concentrations of PMA. O_2^- release by resident PM (▲——▲) and *C. parvum* PM (●——●) is in nmoles O_2^-/30 min/mg proteins. NADPH oxidase activity of resident PM (△——△) and *C. parvum* PM (○——○) is in nmoles O_2^-/15 min/mg proteins. NADPH concentration was 0·15 mM. Means of duplicate assays which varied < 10%. (b) O_2^- release by intact macrophages and activation of NADPH oxidase after stimulation with 100 ng/ml PMA for various times. (▲——▲), (●——●): O_2^- release by intact resident PM and *C. parvum* PM, respectively. (△——△), (○——○): NADPH oxidase activity of resident PM and *C. parvum* PM, respectively. Activity is in nmoles O_2^-/mg proteins/15 min. Means of duplicate assays which varied < 10%. O_2^- release by macrophages in the absence of PMA was < 2 nmoles O_2^-/mg proteins at all the times tested. NADPH-oxidase activity of unstimulated cells was: resident PM, 7·0 nmoles O_2^-/15 min/mg proteins; *C. parvum* PM, 15·3 nmoles O_2^-/15 min/mg proteins.

intact cells, and no cytochrome *c* reduction was measurable if NADPH was not included in the reaction mixture used to assay the activity of macrophage lysates;

(ii) With 0·15 mM NADPH, the production of O_2^- was linear up to 60 min;

(iii) Concentrations of deoxycholate of 0·05% were shown to be optimal.

(iv) The O_2^- producing activity of macrophage lysates was assayed also by using 0·15 mM NADH. Total cytochrome *c* reduction was higher when NADH was used instead of NADPH. Superoxide dismutase did not, however, inhibit cytochrome *c* reduction, either in unstimulated or in PMA-treated macrophages. When NADPH was used as substrate, superoxide dismutase inhibited cytochrome *c* reduction by 30–50%.

Table 1 shows NADPH-dependent O_2^- production by macrophage monolayers lysed with 0·05% deoxycholate after stimulation with a single concentration of PMA or zymosan. Activities of unstimulated cells were variable but low, and did not differ significantly. After stimulation with PMA or zymosan, NADPH-dependent O_2^- production by macrophage lysates

Table 1. O_2^- release by intact macrophages and NADPH-dependent O_2^- production by macrophage lysates

	Resident macrophages	*C. parvum*-activated macrophages	
(a) NADPH-oxidase (nmoles O_2^-/15 min/mg protein)			
Unstimulated	11·0±3·1 (4)	19·1±11·2 (6)	NS
PMA (100 ng/ml)	23·6±9·2 (7)	57·4±30·3 (9)	$P<0·01$
Zymosan (100 μg/ml)	17·4±7·0 (3)	37·3±9·3 (3)	$P<0·05$
(b) O_2^- release by intact cells (nmoles O_2^-/30 min/mg protein)			
Unstimulated	2·7±1·1	5·9±4·9	
PMA	24·1±5·7	89·9±32·3	
Zymosan	32·2±7·6	72·8±15·4	

Macrophages cultivated for 24 hr were assayed as described in the text. The means ± SD for the number of experiments reported are shown in parentheses. Mean values of O_2^- release by intact cells refer to the same experiments where NADPH-oxidase activity was assayed. NADPH-oxidase was assayed in the presence of 0·15 mM NADPH.

increased 1·6–2·3, and 1·9–3·0 times in resident PM and *C. parvum* PM, respectively. NADPH oxidase activity in the lysates of *C. parvum* PM after prior stimulation of the cell with PMA or zymosan was significantly (2·1–2·4 times) higher than activity of resident PM lysates.

The data given in Table 1 indicate that the O_2^- released by intact macrophages can be accounted for by the NADPH oxidase activity of macrophage lysates. By extrapolating to 30 min, i.e. the time of exposure of macrophages to PMA before lysis and assay, the activity of the NADPH oxidase, an amount of O_2^- even higher than that measured in intact cells in the same group of experiments, is obtained; although, with a different type of assay, we had the same results with guinea-pig peritoneal macrophages (Berton *et al.*, 1982). Other properties of the NADPH oxidase activity of macrophage lysates are shown in Fig. 3. NADPH-dependent O_2^- production was proportional to PMA concentration up to 10–20 ng PMA/ml, with saturation of response reached between 20 and 100 ng PMA/ml (Fig. 3a). O_2^- release by intact cells in response to PMA followed the same concentration-dependence.

As shown in Fig. 3b, activity of NADPH oxidase was already maximal after 10 min of exposure to maximal stimulatory concentrations of PMA. Since O_2^- release by intact macrophages is linear up to about 60 min, it could be that, once activated by PMA, the oxidase functions for relatively long times. In our conditions of assay, activity of NADPH oxidase was, in effect, linear up to 60 min. Any extrapolation to the *in vivo* situation is, however, impossible. O_2^- release by intact macrophages could, in fact, reflect the recruitment by PMA of new oxidase molecules whose activity takes the place of that due to oxidase molecules which underwent inactivation.

Measurement of NADPH oxidase activity at physiological substrate concentrations

The concentrations of NADPH in LPS-elicited and resident mouse peritoneal macrophages after PMA stimulation have been reported to be 0·15 and 0·09 mM, respectively (Sasada *et al.*, 1983). We therefore compared O_2^- release by intact cells with NADPH oxidase activity, also assayed at lower NADPH concentrations more representative of physiological conditions. After PMA stimulation, NADPH oxidase activity in the presence of 0·1 mM NADPH as nmoles ($\bar{x} \pm$ SD) O_2^-/30 min/mg proteins was: resident PM, 24·4 ± 7·2 ($n = 2$), *C. parvum* PM, 73·0 ± 3·2 ($n = 2$). In the same experiments, the O_2^- released by intact cells, also expressed in nmoles ($\bar{x} \pm$ SD)O_2^-/30 min/mg proteins, was: resident PM, 31·0 ± 4·9; *C. parvum* PM, 73·5 ± 8·5.

Kinetic properties of NADPH oxidase in resident PM, *C. parvum* PM and trehalose dimycholate-activated macrophages

Demonstration of measurable levels of NADPH oxidase at physiological substrate concentrations allowed us to examine kinetic properties of the enzyme in more detail.

Figure 4 shows NADPH oxidase activities of PMA-stimulated resident PM and *C. parvum* PM assayed in the presence of different concentrations of NADPH

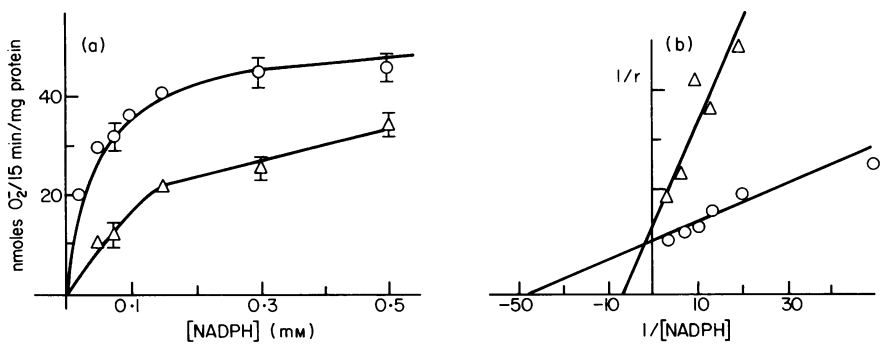

Figure 4. (a) O_2^- release by lysates of macrophages stimulated for 30 min with 100 ng/ml PMA in the presence of different concentrations of NADPH. Results show means ± SD of three to four independent experiments for resident PM (△——△) and *C. parvum* PM (○——○). (b) Linewaever-Burk plots of NADPH-oxidase activity of resident PM (△——△) and *C. parvum* PM (○——○). Results show means of three independent experiments.

Table 2. Kinetic properties of the NADPH oxidase of different macrophage populations which differ in their capacity to release O_2^- after PMA stimulation

	Resident PM	*C. parvum* PM	Trehalose dimycolate-activated PM
Km for NADPH (mM)	0·151 ± 0·062 (4)	0·035 ± 0·014 (4)	0·018 ± 0·002 (2)
V_{max} (nmoles O_2^-/15 min/mg protein)	38·0 ± 14·6	54·5 ± 14·8	40·5 ± 8·5
Superoxide release by intact cells (nmoles O_2^-/30 min/mg protein	34·9 ± 6·8	70·7 ± 4·9	85·3 ± 14·2

Macrophages were assayed after 24 hr cultivation. NADPH oxidase activity was assayed in macrophages stimulated for 30 min with 100 ng/ml PMA. The means ± SD for the number of experiments reported in parentheses are shown. Mean values of O_2^- release by intact cells refer to the same experiments where NADPH oxidase activity was assayed. In the same experiments, activity of NADPH oxidase in macrophage lysates of unstimulated cells in the presence of 0·15 mM NADPH was: resident PM, 12 ± 1·0; *C. parvum* PM, 18·5 ± 6·2; trehalose dimycolate-activated PM, 18·2 ± 0·4 nmoles O_2^-/15 min/mg protein. O_2^- release by intact cells in the absence of PMA was 2·8 ± 4·8, 5·5 ± 5·0, and 2·0 nmoles O_2^-/30 min/mg protein in resident PM, *C. parvum* PM and trehalose dimycolate-activated PM, respectively.

(a). Analysis of their kinetic properties, according to Lineweaver-Burk double reciprocal plots (b), shows that the NADPH oxidase of *C. parvum*-activated macrophages displayed a higher V_{max} and a lower Km for NADPH than that of resident PM.

Table 2 reports kinetic properties of NADPH oxidase from three different mouse peritoneal macrophage populations. A reasonable correlation was shown between capability to release O_2^- and the Km of NADPH-oxidase. As with *C. parvum*-activated macrophages, macrophages from animals injected i.p. with trehalose dimycholate of mycobacterial origin released larger amounts of O_2^-, and their NADPH oxidase displayed a Km for NADPH markedly lower than that of resident PM. Trehalose dimycholate has been shown to be effective in activating macrophage capability to release H_2O_2 and to express cytotoxic activity (Lepoivre *et al.*, 1982).

DISCUSSION

Macrophages vary considerably in their capacity to release reactive oxygen intermediates. Peritoneal macrophages activated by *in vivo* injection of intracellular pathogens (Nathan & Root, 1977; Johnston *et al.*, 1978) or bacterial derived products (Cummings *et al.*, 1980; Lepoivre *et al.*, 1982) release larger amounts of O_2^- and H_2O_2 when challenged with particulate or surface-active agents than resident peritoneal macrophages. Ability to release reactive oxygen intermediates correlates well with microbicidal (Murray & Cohn, 1980) and cytotoxic (Nathan, 1980) properties of macrophages. Although in leucocytes, the amount of O_2^- and/or H_2O_2 released extracellularly can be affected by different pathways of degradation (Rossi *et al.*, 1979), enhanced release of reactive oxygen intermediates by activated macrophages probably reflects enhanced production. Increased respiratory burst activity in activated mouse peritoneal and rabbit alveolar macrophages has, in fact, also been shown by measuring total oxygen consumption and HMP shunt activity (Drath & Karnovsky, 1975; Rossi, Zabucchi & Romeo 1975; Berton & Gordon, 1983b). The biochemical basis for differences in respiratory burst activity between different macrophage populations remains obscure. Structural and functional modifications which could be responsible for this phenomenon may be at the level of surface receptor expression and function, of transduction mechanisms which transfer

signals from stimulant-receptor complexes to appropriate targets, or of the enzymatic system which reduces the oxygen molecule.

Activation of mouse peritoneal macrophages by injection of BCG or *C. parvum* is accompanied by profound changes in expression of surface receptors and in expression of self antigens (Ezekowitz & Gordon, 1982). However, no positive correlation between expression of receptors for stimuli which trigger the respiratory burst and O_2^- release have been demonstrated. For example, although BCG-activated macrophages express the same or a lower number of Fc receptors for IgG2a and IgG2b than thioglycollate-elicited macrophages, they release larger amounts of O_2^- upon interaction with immobilized immune complexes (Ezekowitz *et al.*, 1983). Also, as previously reported for BCG-activated macrophages (Weinberg & Misukonis, 1983), we have shown that number and affinity of receptors for phorbol diesters calculated from binding studies at 4° do not differ in *C. parvum*-activated and resident macrophages. Furthermore, a comparison of time-course of PDBU binding and O_2^- release at 37° shows that, although *C. parvum*-activated macrophages bind the same amount of the ligand, they release three to four times more O_2^- than resident macrophages.

Results obtained in different laboratories have provided strong evidence that a calcium-activated phospholipid-dependent protein kinase (protein-kinase C) may serve as a receptor for phorbol diesters which directly activate the enzyme (Nishizuka, 1984). Our results, and those obtained by other laboratories, exclude the possibility that enhanced response to PMA of activated macrophages may be due to enhanced expression or binding activity of protein kinase C. It is not known, however, if protein kinase activity is higher in activated macrophages, irrespective of its binding capacity for phorbol diesters.

Stimulation of macrophage respiratory burst has been shown to be accompanied by an increase in the activity of NADPH oxidase assayed as oxidation of NADPH (Romeo *et al.*, 1973) or production of O_2^- (Bellavite *et al.*, 1981; Berton *et al.*, 1982). Studies on NADPH oxidase activity in macrophage populations which differ in their ability to release O_2^- have only recently been reported (Sasada *et al.*, 1983; Tsunawaki & Nathan, 1984). In the present paper, we have extended these studies to analysis of NADPH-dependent O_2^- production in lysates of the different macrophage monolayers. Results obtained showed that stimulation of macrophage monolayers with appropriate particulate or surface-active agents caused an increase in NADPH-dependent O_2^- production. Several features of this activity indicated that NADPH oxidase was the enzyme responsible for the production of O_2^- by the macrophage populations studied.

(i) Activation of NADPH oxidase by PMA followed the same dose-response curve as stimulation of O_2^- release by intact cells;

(ii) O_2^- release by intact macrophages was fully accounted for by NADPH oxidase activity over a range of substrate concentrations, including those reported as physiological (Sasada *et al.*, 1983);

(iii) Production of O_2^- by macrophage lysates was strictly substrate-specific, in so far as NADH could not substitute for NADPH;

(iv) There was a good correlation between the capacity of different macrophage populations to release O_2^- in response to PMA, and the NADPH oxidase activity. Macrophages activated *in vivo* by *C. parvum* release higher amounts of O_2^- in response to appropriate stimuli than resident macrophages, and their stimulated NADPH oxidase activity is increased in parallel.

The most striking feature of NADPH oxidase activity of macrophages activated with *C. parvum* or trehalose dimycholate of mycobacterial origin is that Km for NADPH is five to seven times lower than that of NADPH oxidase of resident macrophages. Altered kinetic properties of NADPH oxidase of activated macrophages can reflect alterations of NADPH oxidase components or of plasma membrane environment and, hence, oxidase-substrate and/or oxidase-components interactions. It is not known if these alterations reflect new protein synthesis. Macrophage activation *in vitro* usually requires relatively long exposure (hours or days) to appropriate bacterial or cellular molecules. However, enhancement of O_2^- release in response to PMA has been demonstrated within 20 min of exposure to proteases (Johnston, Chadwick & Cohn, 1981).

In recent years, a great deal has been learnt about the role played by reactive oxygen intermediates in the killing of micro-organisms and tumour cells by macrophages. Thus far, the molecular basis for the enhanced ability of activated macrophages to release reactive oxygen intermediates has remained largely unexplored. The studies reported in this paper extend and strengthen the observation that macrophage activation is accompanied by modifications of kinetic properties of NADPH oxidase. Further studies are needed to establish the reasons for these modifications

and their significance in relation to the process of macrophage activation.

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