Enzymatic Basis of Macrophage Activation

KINETIC ANALYSIS OF SUPEROXIDE PRODUCTION IN LYSATES OF RESIDENT AND ACTIVATED MOUSE PERITONEAL MACROPHAGES AND GRANULOCYTES*

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To compare the kinetics of the $O_2^-$-generating enzyme in nonactivated and activated macrophages and granulocytes from the mouse peritoneal cavity, we sought conditions in which the activity of this enzyme in cell lysates was comparable to that in intact cells. Pretreatment of macrophages with 10 mM diethyldithiocarbamate inhibited endogenous superoxide dismutase by 70% and enhanced $O_2^-$ secretion up to 15-fold, so that it was comparable to $H_2O_2$ secretion. $O_2^-$ secretion was terminated by detergent lysis and reconstituted by addition of NAD(P)H to the lysates. Optimal detection of $O_2^-$ production in lysates depended on prior stimulation of the respiratory burst, lysis with 0.05% deoxycholate rather than any of 4 other detergents or sonication, acetylation of the cytochrome c used as an indicator, and addition of NADPH rather than NADH.

Kinetic analysis using NADPH-reconstituted deoxycholate lysates, together with spectra of reduced and oxidized cytochrome c, aimed to define, at the enzymatic level, the basis for the marked difference in the ROI secretory capacity of mouse resident peritoneal macrophages compared to activated macrophages and granulocytes. For this purpose we sought assay conditions with lysed cells in which ROI production was as great as with intact cells. The fulfillment of this criterion required changes in reported procedures for estimating macrophage superoxide production. In contrast to the situation with granulocytes, the activity of superoxide dismutase and cytochrome c reductase in mouse peritoneal macrophages, together with the lability of their oxidase after cell disruption, presented major obstacles. We found it necessary to inhibit intracellular superoxide dismutase with DDC (19), to disrupt cells with deoxycholate rather than other detergents or sonication, and to monitor superoxide by the reduction of acetylated rather than native ferricytochrome c (20, 21). Kinetic analysis using these techniques suggested that the increased superoxide-generating capacity of activated compared to resident macrophages can be attributed primarily to an increase in the affinity of their oxidase for NADPH, so that it resembles the affinity of the oxidase in granulocytes. Neither the specific activity of the oxidase nor the specific content of cytochrome b$_{559}$ appeared to play as great a role.

Macrophages and granulocytes share the capacity to kill microbes and tumor cells by generating ROI, such as superoxide and hydrogen peroxide (1–4). The superoxide-generating enzyme of the granulocyte is believed to be a plasma membrane-associated flavoprotein (4, 5) that oxidizes NADPH (1) in concert with a cytochrome b$_{559}$ (6) and an ubiquinone (7). In chronic granulomatous disease (8), decreased ability of granulocytes to produce ROI and thereby to resist infection can result from impairment of NADPH generation (9), absence (10) or abnormality (5, 11) of the cytochrome b$_{559}$, or altered affinity of the oxidase for NADPH (12, 13). Much less is known about the enzymatic basis for production of ROI in macrophages (14–17). The question holds special interest because macrophages are subject to immunologic activation, which can elevate their capacity to secrete ROI from a level like that of chronic granulomatous disease granulocytes to a level comparable to normal granulocytes (3). The position of macrophages along this spectrum is regulated in part by interferon-γ (18). The goal of this study was to define, at the enzymatic level, the basis for the marked difference in the ROI secretory capacity of mouse resident peritoneal macrophages compared to activated macrophages and granulocytes.

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‡The abbreviations used are: ROI, reactive oxygen intermediates; DDC, diethyldithiocarbamate; MEM, Eagle’s minimum essential medium, α variant; HS, horse serum heated at 56°C for 50 min; EDTA, ethylenediaminetetraacetate; 5% HS-MEM, Eagle’s minimum essential medium, α variant, with 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 5% HS; KRPG, Krebs-Ringer phosphate buffer containing 5.5 mM glucose; PMA, phorbol myristate acetate; PBSG, Ca$^{++}$,Mg$^{++}$-free phosphate-buffered saline containing 5.5 mM glucose; S.D., standard deviation.

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MATERIALS AND METHODS

Polyoxyethylene-12-tridecyl ether (Renex 30) was purchased from ICI Americas, Inc. (Wilmington, DE). The following were from Sigma: polyoxyethylene-10-tridecyl ether, Triton X-100, deoxycholate, Nonidet P-40, sodium m-periodate (NaIO₄), DDC, EDTA (tetrasodium salt), sodium hydrosulfite (dithionite), NADPH (tetrasodium salt, type XI), NADH (disodium salt, grade III), ferricytochrome c (type VI), scopoletin (6-methoxy-7-hydroxy coumarin), horseradish peroxidase (type II), superoxide dismutase, and PMA. Sodium caseinate

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was from Eastman, acetic anhydride from Fisher, and proteose peptone from Difco. Diff-Quik staining solutions were from Hanico (Bibbston, NY). MEM (α variant) was from Flow Laboratories (Rockville, MD). Penicillin, streptomycin, and trypan blue were from Gibco Laboratories (Grand Island, NY). HS was from Sterile Systems, Inc. (Logan, UT).

Macrophages from the Perl-Nelson-Collins strain mice (The Rockefeller University, New York, NY) or ICR mice (Camm Research, Wayne, NJ) of either sex were used at > 8 weeks of age. Resident macrophages were washed from the peritoneal cavity with MEM containing 5% HS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (5% HS-MEM). Where indicated, mice were injected intraperitoneally 3 days before harvest with 1 ml of 5 mM NaIO, or 1% (w/v) proteose peptone in 0.9% NaCl or were injected 5 days before harvest with 1 ml of 6% (w/v) sodium caseinate. Granulocyte-rich populations were collected 6 h after injection of 1 ml of 12% sodium caseinate (22).

After centrifugation at 2,000 × g for 5 min, the cell pellet was treated with cold 0.2% NaCl for 30 s to lyse erythrocytes. After tonicity was restored to 300 mOsm, the cells were passed through nylon mesh (200/μm) (Tetko, Inc., Elmwood, NY), centrifuged, and resuspended in 5% HS-MEM. J774G8 murine histiocytoma cells were the gift of J. Unkeless, Rockefeller University. In the case of macrophage-rich populations used as adherent cells, 0.6 × 10^6-2 × 10^6 cells/10 μl were incubated on 13-mm diameter glass coverslips (Clay Adams, Inc., New York, NY) cleaned as described (18). After 2 h at 37 °C in 5% CO₂/95% air, nonadherent cells were removed by agitation of the coverslips in warm medium. The coverslips were incubated overnight in 10 ml of PBSG and transferred to 16-mm wells in 24-well cluster trays (Costar Data Packaging, Cambridge, MA). For macrophages in suspension, 4 × 10^5-5 × 10^6 peritoneal cells/7 μl of 5% HS-MEM were incubated in 100-mm diameter plastic dishes (Nunc, Roskilde, Denmark) for 1-2 h at 37 °C (tissue culture dishes from some other manufacturers were unsatisfactory for recovering the adherent cells). Nonadherent cells were removed during 3 washes with warm PBSG (137 mM NaCl, 2.6 mM KCl, 8.1 mM NaHPO₄, 1.5 mM KH₂PO₄, 5.5 mM glucose, pH 7.4). Where indicated, the monolayers were then treated with 10 mM freshly dissolved DDC in PBSG for 60 min at 37 °C with the addition of 100 ng/ml of PMA, stopped during maximum velocity by the addition of 0.05% (w/v) deoxycholate, and restored 45 s later by the addition of various concentrations of NAD(P)H. All rates reported with NADPH as substrate were completely abolished by 270 units/ml of superoxide dismutase. Activity was expressed as nanomoles of cytochrome c reduced/min/10^6 cells.

Oxidized-Reduced Difference Spectra—Spectra from 2.5 × 10^5-8.0 × 10^5 cells in 1 ml of KRPG were stored in the computer of the Perkin-Elmer 557 spectrophotometer. After addition of a few grains of dithionite to the same cuvette, the spectra were determined and subtracted automatically from those stored.

**RESULTS**

**Characteristics of the Cell Populations**—The adherent peritoneal cells from untreated mice or mice injected with NaIO₄ (28), sodium caseinate, or proteose peptone were recovered in suspension for enzymatic studies. As shown in Table I, morphologically identifiable macrophages comprised 88-94% of the cells and neutrophils ≤0.2%. In contrast, elicited peritoneal granulocyte populations contained 94% granulocytes and ~5% macrophages. Periodate- or caseinate-elicited macrophages released an average of 7.4 times more H₂O₂/90 min/mg of protein than resident or proteose-peptone-elicited cells. After overnight incubation, during which their H₂O₂ secretory capacity declined by about half, the activated macrophages released 16-22% as much H₂O₂ as freshly harvested granulocytes.

Intraperitoneal injection of inflammatory agents might elicit the immigration of different subsets of macrophages than those already present. Therefore, to compare activated and nonactivated macrophages derived from one population, we exposed resident peritoneal macrophages to NaIO₄ in vitro. As shown in Fig. 1, left, 3-day exposure to 0.5 mM NaIO₄, the highest concentration which did not reduce the amount of cell protein adherent to the coverslips, resulted in H₂O₂-releasing capacity 23 times greater than that of control cells. Granulocytes were virtually undetectable in these in vitro-activated populations.

The marked difference between resident and activated cells was preserved over PMA concentrations ranging from 10-100 ng/ml (Fig. 1, right). Negligible ROI secretory activity was observed in cells exposed to vehicle without PMA, as illustrated in Fig. 1, right. The concentration of PMA giving a half-maximal response was 4 ng/ml. Subsequent studies used the optimal concentration, 100 ng/ml.

**Use of DDC to Enhance Detection of Superoxide Secreted by Intact Macrophages**—If H₂O₂ arises from the dismutation of O₂⁻ and if both ROI can be detected outside the cell with equal efficiency, then twice as much O₂⁻ should be detected as H₂O₂ (25). In fact, in the experiment illustrated in Fig. 2, 17 times more H₂O₂ was detected than O₂⁻ secreted by the same cells in response to PMA. We speculated that endogenous superoxide dismutase might be catalyzing the dismutation of O₂⁻ to H₂O₂ before O₂⁻ could react with ferricytochrome c. If so, then inhibition of Cu-Zn-dependent superoxide dismutase with the copper chelator DDC (19) might favor the detection of O₂⁻ at the expense of H₂O₂. This was observed. Exposure of

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TABLE I
Characteristics of the cell populations

| Characteristic                  | Resident (n = 9) | Peptone (n = 4) | NaIO₃ (n = 25) | Casein (n = 4) | Granulocytes (n = 9) |
|--------------------------------|-----------------|----------------|----------------|----------------|---------------------|
| Per cent cells                 | 87.9 ± 4.9      | 93.3 ± 1.4     | 93.3 ± 2.9     | 91.9 ± 4.0     | 93.0 ± 25.0         |
| Macrophages                    |                 |                |                |                |                     |
| Eosinophils                    | 0.5 ± 1.3       | 0.5 ± 0.6      | 2.3 ± 1.1      | 0.6 ± 0.9      | 0.8 ± 0.6           |
| Neutrophils                    | 0.2 ± 6.3       | 0.0 ± 0.0      | 0.1 ± 0.2      | 0.1 ± 0.1      | 93.1 ± 3.2          |
| Lymphocytes                    | 10.6 ± 4.9      | 6.1 ± 1.0      | 4.3 ± 2.7      | 7.6 ± 4.1      | 1.4 ± 1.4           |
| Mast cells                     | 0.5 ± 1.0       | 0.1 ± 0.3      | 0.0 ± 0.0      | 0.0 ± 0.0      | 0.0 ± 0.0           |
| Per cent viability             | 93.6 ± 4.9      | 93.2 ± 5.1     | 93.7 ± 5.7     | 95.7 ± 6.2     | 97.4 ± 2.9          |
| Per cent recovery              | 1.6 ± 5.8       | 21.3 ± 9.2     | 23.8 ± 5.7     | 21.2 ± 2.4     | 101.0 ± 10.8        |
| H₂O₂ release                   | 30 ± 14 (6)     | 56 ± 5 (12)    | 274 ± 64 (12)  | 360 ± 97 (6)   |                     |

* Means ± S.D. of differential counts on stained cytocentrifuge preparations of adherent peritoneal cells elicited, plated, washed, and resuspended as described under "Materials and Methods," for the number of experiments indicated.

† Per cent of resuspended cells excluding 0.2% trypan blue.

‡ Nanomoles/99 min/mg of protein (means ± S.D. for the number of experiments indicated) determined after overnight incubation in order to eliminate virtually all granulocytes. Activity was generally twice as great with freshly harvested macrophages. Granulocytes did not survive overnight incubation; when tested immediately after adherence, they released 1672 ± 72 nmol/90 min/mg of protein.

Fig. 1. In vitro activation of resident mouse peritoneal macrophages with NaIO₃. Left, effect of periodate concentration on H₂O₂-releasing capacity (Ο) of macrophages stimulated with 100 ng/ml of PMA and on the amount of adherent cell protein (Δ), after a 3-day incubation of 0.6 × 10⁶ resident peritoneal cells/coverlip in serum-free MEM. Right, stimulation of H₂O₂ release by different concentrations of NaIO₃ (Ο, Δ) or equivalent concentrations of dimethyl sulfoxide vehicle (●, ▲) applied to resident peritoneal macrophages after 3 days of incubation in MEM with 0 (Δ, ▲) or 0.5 mM NaIO₃ (Ο, ●). Data in both panels are means of triplicates.

Activated macrophages to 1-20 mM DDC for 1 h inhibited endogenous superoxide dismutase activity by approximately 70% (Fig. 2, left). These concentrations were nontoxic, as judged by adherent cell protein (data not shown). In response to PMA, macrophages pretreated with 10 mM DDC released 15 times more O₂ (Fig. 2, center) and 3 times less H₂O₂ (Fig. 2, right) than cells not exposed to the chelator. In fact, after DDC treatment, macrophages released almost exactly as much O₂ as the H₂O₂ they released without exposure to DDC.

Superoxide Generation by Cell Lysates—For a precise comparison of O₂ release by intact and lysed cells, we used a modification of the method of Bellavite et al. (15), in which O₂ production is monitored continuously in the same cuvette before and after lysis of the cells. We first used native ferricytochrome c. Intact macrophages at rest reduced ferricytochrome c at a negligible rate (Fig. 3, left). After addition of PMA, 0.64 nmol of ferricytochrome c was reduced/min/10⁶ macrophages. The addition of 0.6% (w/v) deoxycholate promptly abolished this response. The further addition of 1 mM NADPH resulted in renewed reduction of ferricytochrome c. However, the rate was greater than that with intact cells. Moreover, addition of superoxide dismutase had little effect (Fig. 3, left), suggesting that most of the reduction by lysates may have been due to cytochrome reductase rather than to O₂. In contrast, when acetylated ferricytochrome c was used (20, 21), the rate of cytochrome reduction upon addition of 1 mM NADPH to the lysate was 100% of the value with intact cells and was decreased to 0 by the addition of 270 units/ml of superoxide dismutase (Fig. 3, right).

The ability of exogenous NADPH to restore O₂ production declined rapidly as a function of time after exposure of the cells to deoxycholate (tₙ = 90 s) (Fig. 4). Addition of bovine serum albumin, EDTA, glycerol (29), or N*-a-p-tosyl-L-lysine chloromethyl ketone HCl (29) did not prolong the interval after which NADPH could be added with full effect (data not shown). However, following the addition of NADPH, the rate of O₂ production was constant for ~2 min.

Effect of Detergents and pH—The ability to restore O₂ production by lysed cells to 100% of the value for intact cells was critically dependent on both the choice of detergent and its concentration. In contrast to a report with human granulocytes (30), polyoxyethylene-12-tridecyl ether (Remex 30) was ineffective, as was polyoxyethylene-10-tridecyl ether, Triton X-100, and Nonidet P-40 (Table II). With deoxycholate, 0.025% did not lyse cells, whereas 0.1% lysed cells but did not permit reconstitution of their capacity to release O₂. At 0.05%, deoxycholate both lysed cells completely and permitted full reconstitution (Fig. 5). With sonication of cells, ≤11% as much O₂ was produced after addition of NADPH as was seen with deoxycholate lysis (data not shown).

Full reconstitution of O₂ production after detergent lysis was also critically dependent on the pH of the buffer. As shown in Fig. 6, release of O₂ from intact DDC-treated cells was almost nil at pH ≤ 7.0 and rose steeply to a maximum at pH ≥ 8. O₂ production by lysed cells was optimal at pH ~7.4, and at this pH, the values seen with intact and lysed cells were virtually the same. If the cells were not pretreated with DDC, then the O₂ detected from lysed cells exceeded that from intact cells at all pH <8 (Fig. 6), possibly reflecting the dilution of endogenous superoxide dismutase upon cell lysis.

Based on the above results, kinetic analyses were carried out at pH 7.85, using DDC-pretreated cells lysed with deoxy-
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**FIG. 2.** Effect of DDC on superoxide dismutase (SOD) activity, \(O_2\) release, and \(H_2O_2\) release of macrophages activated in vivo with NaIO\(_4\). Macrophages were incubated overnight, washed, and exposed to the indicated concentrations of DDC as described under "Materials and Methods." Left, superoxide dismutase activity in 0.2% Triton X-100 lysates. Center, \(O_2\) release with 100 ng/ml of PMA (○), with dimethyl sulfoxide vehicle control alone (●), and the difference of these (net release) (△). Right, \(H_2O_2\) release. Symbols same as in center. All data are means of triplicates.

**FIG. 3.** Spectrophotometric record of \(O_2\) generation by activated macrophages monitored by the reduction of (left) native or (right) acetylated ferricytochrome c. 2.5 × 10⁶ macrophages activated in vivo with NaIO\(_4\) were suspended in 1 ml of KRPG containing 2 mM NaN\(_3\) in a dual wavelength spectrophotometer at 37 °C and exposed sequentially to 100 ng/ml of PMA, 0.05% (w/v) deoxycholate (DOC), 1 mM NADPH, and 270 units/ml of superoxide dismutase (SOD), followed 45 s later by the addition of various amounts of NADPH or NADH.

**Kinetic Studies of NAD(P)H Oxidase—**Reduction of acetylated ferricytochrome c by lysates of PMA-stimulated macrophages or granulocytes was 6–14 times faster following addition of NADPH than after the addition of equivalent concentrations of NADH (Table III). The preference of the \(O_2\)-generating enzyme for NADPH was greater than reflected by these figures, because 100% of the cytochrome reduction seen after addition of NADPH, but not after addition of NADH, was abolished by superoxide dismutase (data not shown). \(O_2\) production by lysates of resident macrophages (△), NaIO\(_4\)-activated macrophages (○), or granulocytes (□) was recorded in duplicate samples after adding 1 mM NADPH at the indicated times following the addition of 0.05% deoxycholate. Peak activity was seen when NADPH was added 30–60 s after the detergent and was 100% of the rate before detergent. The data are expressed relative to the rate observed at 30 s. All cells were DDC-treated before assay.

**FIG. 4.** Lability of the oxidase after lysis of macrophages or granulocytes with deoxycholate. \(O_2\) production by lysates of resident macrophages (△), NaIO\(_4\)-activated macrophages (○), or granulocytes (□) was recorded in duplicate samples after adding 1 mM NADPH at the indicated times following the addition of 0.05% deoxycholate. Peak activity was seen when NADPH was added 30–60 s after the detergent and was 100% of the rate before detergent. The data are expressed relative to the rate observed at 30 s. All cells were DDC-treated before assay.
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Table II

Effect of detergents on superoxide production by macrophage lysates

The lysates were adherence-purified macrophages from NaI04-treated mice triggered with 100 ng/ml of PMA.

| Detergent       | Concentration* | O2 release (nmol/min/10^6 cells) |
|-----------------|----------------|---------------------------------|
|                 |                | -NADPH +NADPH                   |
|                 |                |                                 |
| Deoxycholate    | 0.0125         | 96.2                            |
|                 | 0.025          | 115.2                           |
|                 | 0.05           | 0.0                             |
| 12-Tridecyl ether (Renex 30) | 0.00625 | 51.9                            |
|                 | 0.0125         | 30.8                            |
| 10-Tridecyl ether | 0.00625 | 37.7                            |
|                 | 0.0125         | 0.0                             |
| Triton X-100    | 0.00625        | 47.5                            |
|                 | 0.0125         | 24.7                            |
|                 | 0.025          | 0.0                             |
| Nonidet P-40    | 0.00156        | 31.3                            |
|                 | 0.00313        | 0.0                             |

* w/v for deoxycholate; v/v for other detergents.

Fig. 6. Effect of pH on O2 generation by intact and deoxycholate-lysed macrophages. NaI04-activated adherence-purified macrophages were (left) pretreated or (right) not pretreated with DDC as described under "Materials and Methods." They were resuspended in 0.9% NaCl, 5.5 mM glucose, and diluted into KRPG of the indicated pH for assay of O2 production before (O) and after (A) addition of deoxycholate and NADPH as in Fig. 3. Data are means of duplicates.

Fig. 8. Effect of concentration of deoxycholate on O2 production by macrophage lysates. Adherence-purified, DDC-treated, NaI04-activated macrophages were assayed as in Fig. 3, except that the concentration of deoxycholate was varied as shown (per cent w/v). Columns indicate O2 production before addition of detergent. Only 0.05% deoxycholate resulted in abolition of O2 production and lysis of all the cells as judged by microscopic examination. Values are expressed as per cent of the rate before addition of detergent (1.1 nmol/min/10^6 cells).

DISCUSSION

Two aspects of the respiratory burst of macrophages lend particular interest to a study of its enzymatic basis. First, this pathway can account for an important portion of the antitumor, antimicrobial, and inflammatory potential of the macrophage (2, 3). Second, the respiratory burst of the macrophage is highly sensitive to regulation. Thus, interferon-γ, a T lymphocyte product, enhances ROI secretory capacity in both human (18) and murine macrophages, while a tumor cell product has the opposite effect (23). That the enzymatic basis of ROI secretion in macrophages has been much less studied than in granulocytes can be attributed in large part...
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TABLE III

Requirement of the oxidase for PMA and NADPH

After exposure to PMA or dimethyl sulfoxide vehicle alone, cells were lysed with 0.05% deoxycholate. When O₂ release had ceased, the indicated concentration of nucleotide was added and the renewed rate of O₂ production recorded as nanomoles/min/10⁶ cells. The rate of O₂ release by intact cells in each experiment was the same as the highest value obtained after the addition of NADPH to the lysates.

| Concentration of nucleotide | Macrophones* | Macrophones* | Granulocytes* |
|-----------------------------|--------------|--------------|---------------|
|                             | +PMA NADPH   | -PMA NADPH   | +PMA NADPH    | +PMA NADPH    |
|                             | (NADPH)      | (NADPH)      | (NADPH)       | (NADPH)       |
| 1.0                         | 0.54         | 0.06         | 0.57 ± 0.19   | 0.17 ± 0.05   |
| 0.5                         | 0.51         | 0.03         | 0.55 ± 0.01   | 0.04 ± 0.03   |
| 0.25                        | 0.51         | 0.06         | 0.52 ± 0.01   | 0.10 ± 0.10   |
| 0.125                       | ND           | ND           | 0.47 ± 0.06   | 0.11 ± 0.07   |

* Adherence-purified macrophages elicited with NaIO₄. Each datum from 1 assay.
* 100 ng/ml of PMA in 0.033% dimethyl sulfoxide.
* 0.033% dimethyl sulfoxide.
* As in Footnote a, but data are means ± S.D. from 2 experiments, each in duplicate.
* Adherence-purified granulocyte elicited with caseinate. Data are means ± S.D. from 1 experiment done in triplicate.
* ND, not done.

FIG. 7. Lineweaver-Burk plots of NADPH oxidase activity in macrophages and granulocytes. Each point represents the rate obtained after addition of NADPH as in Fig. 3 except that the concentration of NADPH was varied. The cell types grouped in each panel were assayed in the same experiments. Left, granulocytes (△), NaIO₄-activated macrophages (○), and resident macrophages (●). Means of two experiments. Right, casein-activated macrophages (△), NaIO₄-activated macrophages (○), protease-peptone-elicited macrophages (△), and resident macrophages (●). One experiment.

TABLE IV

Summary of kinetic parameters of the NADPH oxidase from macrophages and granulocytes

Means ± S.D. for the intercepts of Lineweaver-Burk plots like those in Fig. 7, for the number of experiments indicated.

| Cell type          | No. of experiments | \( K_m \) (mM) | \( V_{max} \) (nmol \( O_2 \)/min/10⁶ cells) |
|--------------------|---------------------|----------------|------------------------------------------|
| Nonactivated macrophages |                    |                |                                          |
| J774/18 line       | 1                   | 1.43           | 1.00                                     |
| Resident           | 5                   | 0.41 ± 0.26    | 0.71 ± 0.21                             |
| Protease-peptone   | 1                   | 0.20           | 1.00                                     |
| Activated macrophages |                   |                |                                          |
| Casein             | 2                   | 0.053 ± 0.02   | 0.88 ± 0.17                             |
| NaIO₄              | 6                   | 0.047 ± 0.01   | 0.76 ± 0.25                             |
| Granulocytes       | 4                   | 0.043 ± 0.00   | 2.27 ± 0.08                             |

Means ± S.D. for the intercepts of Lineweaver-Burk plots like those in Fig. 7. to difficulty in obtaining activated macrophages free of granulocytes, low values for release of \( O_2 \) compared to \( H_2O_2 \), reduction of indicator cytochrome c by factors other than \( O_2 \), and marked lability of the oxidase after cell disruption. We circumvented each of these problems and reached the conclusion that the enhanced capacity of activated mouse peritoneal macrophages to secrete ROI may be due to an increased affinity of their oxidase for NADPH. We found no evidence that the specific activity of the oxidase was higher in activated than in resident macrophages.

It was first necessary to resolve the problem that \( O_2 \) production by intact cells stimulated with PMA appeared to be much lower than previously reported (30) and than expected from simultaneous measurements of \( H_2O_2 \). This discrepancy has been noted before with both macrophages (23, 34) and
Fig. 8. Difference spectra (reduced – oxidized) of macrophages and granulocytes. Adherence-purified cells were suspended in 1 ml of KRPG. The scan performed before adding dithionite was automatically subtracted from that obtained after adding dithionite, and the difference is displayed. The scale for absorbance units is bracketed between vertical arrows and for wavelength between horizontal arrows. Left, peritoneal granulocytes (5 x 10⁶ cells). The upper trace was recorded immediately after adding dithionite and the lower trace 4 min later. Center, macrophages activated by injection of casein (6.5 x 10⁶ cells, upper trace) or NaIO₄ (8 x 10⁶ cells, lower trace). Right, protease-peptone-elicited macrophages (4.6 x 10⁶ cells, upper trace) and 3774G8 histiocytoma cells (2.5 x 10⁶ cells, lower trace).

granulocytes (35). The present results suggest two explanations. First, the relative recoveries of O₂ and H₂O₂ from the same cell populations varied independently from each other in response to the concentration of DDC toward which the cells had been exposed, over the same range in which endogenous superoxide dismutase was partially inhibited by the chelator (19) (the residual superoxide dismutase activity may have been mitochondrial). That is, the more superoxide dismutase was inhibited, the more O₂ and the less H₂O₂ was detected. This suggests that endogenous dismutase dismutation reacted with O₂ more efficiently (rate constant, 2 x 10⁹ M⁻¹ s⁻¹ (36)) than did exogenous ferricytochrome c (rate constant, 5 x 10⁶ M⁻¹ s⁻¹ (37)). However, we could not detect any superoxide dismutase in the extracellular medium (data not shown). The simplest explanation is that O₂ was produced intracellularly. In the absence of DDC, most of the O₂ reacted with cytosolic superoxide dismutase to form H₂O₂, much of which diffused from the cell. Possible intracellular sites of formation of O₂ include the inner surface of the plasma membrane or vesicles derived from it. A stoichiometric analysis with granulocytes (35) and a cytochemical study with macrophages (38) support the idea that H₂O₂ may arise intracellularly. However, the loci of ROI generation in macrophages and the mechanism of the DDC effect are not defined by our experiments, and additional approaches will be required to test these hypotheses.

A second factor dramatically affecting apparent rates of secretion of O₂ was the pH of the assay buffer. For example, with increasing alkalinity over a range as narrow as from pH 7.25 to 7.50, detectable O₂ increased 4-fold. Indeed, increasing alkalinity would be expected to retard the reaction, 2O₂ + 2H⁺ → H₂O₂ + O₂ (25). However, it seems unlikely that pH would be markedly affected at the intracellular site where most dismutation seems to be occurring. The mechanism of the pH effect is unknown. In any case, the use of slightly more alkaline media in other studies, especially those in which volatile buffers were employed, together with possible variations in cellular superoxide dismutase content, may help explain reported differences in macrophage O₂ secretion rates.

After optimizing conditions for the secretion of O₂ by intact cells, we next endeavored to optimize the production of O₂ by lyzed cells. The comparison was aided by the method of Bellavite et al. (15), in which the same cell population is monitored in a cuvette before and after lysis. We compared sonication and 5 detergents, each under a wide range of conditions such as buffer composition (data not shown) and detergent concentration. The only condition permitting completely lysed cells to produce 100% as much O₂ as they did before lysis was the use of 0.05% deoxycholate. Even so, if the addition of NADPH was delayed by more than 90 s after cell lysis, the capacity to generate O₂ had already declined by 50%. Lability of the oxidase has also been noted after subcellular fractionation of human granulocytes (29, 39). In the case of mouse peritoneal leukocytes, the marked degree of this lability makes both subcellular localization and purification of the oxidase extremely difficult. However, the system was suitable for kinetic studies on unfractionated lysates.

Finally, macrophage lysates contained a superoxide dismutase-resistant principle which rapidly reduced ferricytochrome c in the presence of NADPH, presumably NADPH-dependent cytochrome reductase. Our studies would not have been possible without the use of acetylated cytochrome c (20, 21) to minimize this reaction while preserving the susceptibility of cytochrome c to reduction by O₂. Reduction of acetylated cytochrome c by lysates of PMA-triggered macrophages in the presence of NADPH could not be attributed to radical production by cell-associated DDC, because the Km for NADPH was the same using cells not exposed to DDC (data not shown). Moreover, negligible O₂ production was seen if NADPH was omitted or if PMA was added after rather than before lysis.

There are few previous reports dealing with the kinetics of the macrophage respiratory burst in cell-free preparations. The classic studies of Romeo et al. (14) and more recent work from the same laboratory (15, 16) did not involve direct comparisons among macrophages in different states of activation. In separate studies with resident or casein-elicited macrophages from the peritonea and lungs of guinea pigs and rabbits, these workers found Km values for NADPH ranging from 0.03 to 0.72 mM (14–16). The influence of immunologic activation on the oxidative metabolism of guinea pig or rabbit macrophages has not been explored as thoroughly as in mouse or man. Only Sasada et al. (17), working with mouse peritoneal cells, directly compared the kinetics of the oxidase in cell-free preparations from resident and activated macrophages. The
latter, taken from mice injected with bacterial lipopolysaccharide, displayed a 45% increase in $V_{\text{max}}$ and a 1.9-fold decrease in $K_m$ for NADPH (from 0.094 to 0.049 mm). However, in that study, the cells were disrupted by sonication. The activity recovered was not compared to the initial activity but can be calculated to have been $<4.7\%$ and probably $<0.47\%$ of that of the intact cells. Nonetheless, after overnight culture, the results using that technique (17) were similar to those reported here.

As yet we have no explanation for the markedly enhanced affinity of the oxidase of activated macrophages for NADPH compared to that of resident macrophages. Differences in the degree of triggering of the oxidase by PMA seem unlikely, since PMA receptor number and affinity do not differ between activated and resident macrophages (40), and increasing the PMA concentration did not increase the response of nonactivated cells. An inhibitor of the oxidase (4, 41) may be diminished in activated macrophages. A new oxidase with a lower $K_m$ may be induced by activation, or the existing oxidase may undergo allosteric changes. Activated macrophages may assemble a multicomponent oxidase more efficiently, such as by incorporating more cytochrome $b_559$ or ubiquinone, which might lower the apparent affinity of the electron transport chain as a whole for NADPH. We did not observe a correlation between cytochrome $b_559$ content and capacity to secrete ROI. However, the contribution of mitochondrial cytochromes to the spectra may have obscured critical variations. Such differences should be sought in membrane-rich fractions free of mitochondria.

The change we observed in the $K_m$ of the oxidase for NADPH as resident macrophages (0.41 mm) became activated (0.05 mm) seems likely to be physiologically relevant, when compared with the reported intracellular NADPH concentration in resident and activated macrophages (0.14 and 0.30 mm, respectively, before PMA stimulation, and 50% lower after PMA stimulation (17)). Thus the data provide support for the hypothesis that differences in ROI secretory rates among macrophage populations primarily reflect differences in production of ROI rather than in the proportion of ROI released into the medium, accumulated, or catabolized.

It will now be of interest to compare the changes induced by activation to those induced by deactivation, such as occurs upon exposure of macrophages to factors derived from tumors and some nonmalignant cells (25).

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