

MACROPHAGE DEACTIVATION

Altered Kinetic Properties of Superoxide-producing Enzyme after Exposure to Tumor Cell-conditioned Medium

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The capacity of macrophages to kill microbes and tumor cells is reflected by and in many instances dependent on the quantity of reactive oxygen intermediates (ROI)¹ the macrophages can secrete (1, 2). Cytokines closely regulate these functions. IFN- γ mediates immunologically induced activation (3). Suppression is exerted by a protease-sensitive factor in medium conditioned by a wide variety of malignant and some nonmalignant cells (4). In a selective and nontoxic manner, this macrophage deactivation factor (MDF) inhibits the capacity of macrophages to undergo a respiratory burst and to kill intracellular protozoal pathogens (5).

The molecular mechanisms of macrophage activation and deactivation are unknown, although it can be anticipated that an understanding of either will illumine the other. Activation is associated with a marked increase in the affinity of the superoxide-producing enzyme (the NADPH oxidase) for its substrate, NADPH (6-9), with little change in its maximal velocity (6-9) or the apparent cellular content of its cytochrome b component (7, 10). Our present work focused on the metabolism of ROI by deactivated macrophages. Deactivation had no effect on the ability of macrophages to catabolize H₂O₂, transport glucose, or maintain intracellular concentrations of NADP(H) or cytochrome b₅₅₉. In contrast, the affinity of the oxidase for NADPH decreased an order of magnitude in response to MDF. This increase in K_m appeared to account quantitatively for the deactivated macrophage's deficiency in secretion of ROI.

Materials and Methods

Tumor Cell-conditioned Medium (TCM). TCM containing MDF was prepared from P815 mouse mastocytoma cells (4) in α -MEM (Flow Laboratories, Rockville, MD; or KC Biological, Lenexa, KS) containing 10% heat-inactivated horse serum (HS) (Sterile Systems, Logan, UT), 100 U/ml of penicillin, and 100 μ g/ml of streptomycin (10% HS-MEM). Cells were centrifuged at 200 *g* for 5 min, resuspended at 1.5×10^6 cells/ml, and

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¹ *Abbreviations used in this paper:* DOC, deoxycholate; HS, horse serum; KRP(G), Krebs-Ringer phosphate buffer (with 5.5 mM glucose); MDF, macrophage-deactivating factor; PBSG, Ca²⁺ and Mg²⁺-free PBS with 5.5 mM glucose; PDBu, phorbol dibutyrate; ROI, reactive oxygen intermediates (primarily O₂⁻, H₂O₂, and its myeloperoxidase products, and OH \cdot); TCM, tumor cell-conditioned medium.

cultured in 10% HS-MEM for 2 d. Only viable cells (>90%) were used. TCM was collected from the P815 culture by centrifugation at 500 *g* for 15 min, dialyzed in Spectra/Por 6 tubing (1 kD cutoff) (Spectrum Medical Industries, Inc., Los Angeles, CA) against MEM at 4°C, filtered (0.45 μ m Millex-HA; Millipore, Bedford, MA), stored at 4°C, and used within 2 wk.

Macrophages. Activated macrophages were washed from the peritoneal cavity of female ICR mice (>8 wk old) (Camm Research, Wayne, NJ) with 5% HS-MEM 3–5 d after injection of 1 ml of autoclaved 6% (wt/vol) sodium caseinate (practical grade; Eastman Kodak Co., Rochester, NY) in 0.9% NaCl. After centrifugation at 200 *g* for 5 min, cold 0.2% NaCl was added to the cell pellet for 30 s to lyse erythrocytes. After tonicity was restored to 300 mosM, the cells were passed through nylon mesh (200/in²) (Tetko, Inc., Elmsford, NY), centrifuged, and resuspended in 5% HS-MEM. $0.78\text{--}1.5 \times 10^6$ cells per 75 μ l on 13-mm-diam glass coverslips (Clay Adams, Inc., New York) or $35\text{--}45 \times 10^6$ cells in 100-mm-diam plastic dishes (Nunc, Roskilde, Denmark) (7) were incubated for 1.5–3 h at 37°C in 5% CO₂ in 95% air. Nonadherent cells were removed by agitating the coverslips or plastic dishes in warmed MEM. The medium was replaced with 1 ml (for 16-mm wells in 24-well cluster trays [Costar Data Packaging, Cambridge, MA]) or 15–20 ml (for 100-mm plastic dishes) of 10% HS-MEM or various dilutions of TCM, and the cells were cultured for 2 d. Detached cells were removed either by the agitation of coverslips in four beakers of 150 ml 0.9% NaCl, or by the washing of plastic dishes three times with warm PBSG (PBS with glucose; 137 mM NaCl, 2.6 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 5.5 mM glucose, pH 7.4). For studies with macrophages in suspension, unless otherwise indicated, the monolayer cells in plastic dishes were then treated with 10 mM diethyldithiocarbamate for 60 min at 37°C to inhibit superoxide dismutase, exposed to 5 mM EDTA for the final 15 min, dislodged by pipetting, washed with PBSG at 4°C, and suspended in cold Krebs-Ringer phosphate buffer with glucose (KRP). KRP was formulated as described (7).

H₂O₂ Release and Consumption. The oxidation of scopoletin by H₂O₂, catalyzed by horseradish peroxidase, was monitored fluorometrically as described (7). To measure the H₂O₂ releasing capacity of macrophages, 0.9% NaCl-washed monolayers were stimulated with 167 nM PMA or phorbol dibutyrate (PDBu) in 16-mm wells containing 1.5 ml of KRP in the presence of 0.44 purpurogallin units per milliliter of peroxidase. After 90 min at 37°C, the fluorescence in 1 ml of the supernatant was recorded in a Perkin-Elmer MPF4A fluorometer (excitation 350 nm, emission 460 nm). The remaining 0.5 ml of reaction mixture together with the monolayer was used for determination of the protein content by the method of Lowry et al. (11) using BSA as a standard. H₂O₂ release by cells is expressed as nanomoles of product per 90 min per milligram of cell protein. For H₂O₂ consumption, monolayers were incubated with 25 nmol reagent H₂O₂ (Fisher Chemical Co., Fairlawn, NJ) in KRP without phorbol esters at 37°C, and the amount of H₂O₂ remaining in the supernatant was determined by its ability to oxidize scopoletin.

Glucose Transport. 2-Deoxy-D-glucose uptake by macrophages adherent to coverslips was measured as described (12, 13). (1,2)-[³H]-2-deoxy-D-glucose (30–60 Ci/mmol in 90% ethanol) (New England Nuclear, Boston, MA) was evaporated and redissolved to 50 mM (0.54 mCi/mmol) with cold 2-deoxy-D-glucose. Macrophages adherent to glass coverslips were preincubated in glucose-free KRP for 30 min at 37°C to deplete endogenous stores. Coverslips were then transferred to 0.5 ml prewarmed KRP containing 0.25–4 mM 2-deoxy-D-glucose with or without 167 nM PMA in the presence or absence of 2 mM NaF. After precisely 6 min, the coverslips were washed, drained, and the macrophages were dissolved in 0.3 ml 0.5 N NaOH for liquid scintillation counting (Rackbeta 1218; LKB, Paramus, NJ) and protein measurement. For each condition, nonspecific uptake in the presence of 100 μ M cytochalasin B was subtracted. Nonspecific uptake was maximal (10.5%) at the highest concentration of 2-deoxy-D-glucose used (4 mM), and was proportionately less at lower 2-deoxy-D-glucose concentrations. Activity is expressed as nanomoles of 2-deoxy-D-glucose transported per 6 min per milligram protein.

NADP(H) Content. Intracellular concentrations of NADP(H) were determined by the enzymatic cycling methods of Burch (14) with minor modifications. Macrophages on coverslips were incubated for 90 min at 37°C in KRP or the same buffer lacking glucose

(KRP) with or without 167 nM PMA. Cells were dissolved in 0.3 ml 0.04 N NaOH containing 0.5 mM cysteine (Fisher Chemical Co.) and 0.05% Triton X-100 for 15 min at 4°C. For NADPH, 50 μ l aliquots of cell lysate were heated at 60°C for 10 min. For NADP, 50 μ l aliquots were made 30 mM in ascorbic acid, mixed with 50 μ l 0.02 N H₂SO₄/0.1 N Na₂SO₄ and heated for 30 min at 60°C. 5 μ l of sample for NADPH determination or 10 μ l of sample for NADP determination were added to 100 μ l cycling reagent (100 μ g/ml glutamic dehydrogenase, 50 μ g/ml glucose-6-phosphate dehydrogenase [both from Boehringer Mannheim, Indianapolis, IN], 5 mM α -ketoglutarate [Calbiochem Behring, San Diego, CA], 1 mM glucose-6-phosphate, 0.1 mM ADP, 0.025 M ammonium acetate, and 0.2 mg/ml BSA in 0.1 M Tris-HCl buffer, pH 8.0) at 4°C, and cycled for 60 min at 37°C. The reactions were stopped by heating the tubes at 100°C for 3 min. To measure the cycled product (6-phosphogluconate), 6-phosphogluconate dehydrogenase reagent (1 μ g/ml 6-phosphogluconate dehydrogenase [Boehringer Mannheim], 0.03 mM NADP, 0.03 M ammonium acetate, 0.1 mM EDTA, and 0.2 mg/ml BSA in 0.02 M Tris-HCl buffer, pH 8.0) was added to the tubes for a 30 min incubation at 37°C. NADP reduced by the final 6-phosphogluconate product was determined fluorometrically (excitation 340 nm, emission 450 nm). The same procedure was followed for NADP(H) standards. 0.05% Triton X-100 did not interfere with the enzymatic reactions used in cycling. The amplification factors for both NADPH and NADP were $\sim 1.67 \times 10^4$ -fold, so that 10^5 cells per assay were sufficient. Cellular concentrations of NADP(H) were expressed in millimolar terms after measuring the volume of cell water.

Cell Water Volume. This was measured according to Rottenberg (15), using 14–33 $\times 10^6$ adherence-purified macrophages that had not been exposed to diethyldithiocarbamate and that were >90% viable by trypan blue dye exclusion. The cells were suspended in 200 μ l of KRPG containing 10 μ Ci/ml [³H]water (25 mCi/g) and 0.4 μ Ci/ml D-[¹⁴C(U)]-sorbitol (346 mCi/mmol) (both from New England Nuclear) in 250- μ l Eppendorf tubes. After 3 min at 4°C, the tubes were spun for 1 min, the volume of supernatant was determined by its weight, and the ³H and ¹⁴C in 10 μ l supernatant and in the total cell precipitate (dissolved with 0.1% Triton X-100) were measured by liquid scintillation counting. Cell water volumes (μ l/mg protein) of macrophages cultured for 2 d with the concentrations (vol/vol) of TCM indicated in parentheses were: 3.50 \pm 0.89 (0%), 4.07 \pm 1.51 (12.5%), 3.62 \pm 0.88 (25%), 3.53 \pm 0.64 (50%), 3.52 \pm 0.62 (75%), and 3.50 \pm 0.38 (100%) (mean \pm SD for three experiments, each in duplicate).

NADPH Oxidase. Ferricytochrome c was acetylated with acetic anhydride (16) (200 mol/mol of cytochrome) and NADPH oxidase measured by a modification of the reported method (7). Macrophages, treated with diethyldithiocarbamate to inhibit endogenous superoxide dismutase, were suspended at 1.4–2.9 $\times 10^6$ cells/ml of KRPG with 30 μ M acetylated cytochrome c and 2 mM NaN₃ in a cuvette fitted with an air-driven stirrer (17) in the 37°C thermostat-controlled chamber of a Perkin-Elmer (Hitachi) 557 dual wavelength spectrophotometer for recording of ΔA (550–540 nm) ($\epsilon = 19.1/\text{mM}$). Unless otherwise indicated, the reaction was started by the addition of 167 nM PMA, stopped 1.5 min later by addition of final 0.0625% (wt/vol) deoxycholate and 0.0625% (vol/vol) Tween 20, and restored 1 min later with NADPH. Initial velocity was recorded. Reduction of cytochrome c was inhibited completely by addition of 270 U/ml superoxide dismutase. Activity is expressed as nanomoles of cytochrome c reduced per minute per milligram protein.

Oxidized-reduced Difference Spectra. Macrophages were purified by adherence as described above, but diethyldithiocarbamate treatment was omitted. Spectra from 4.0–9.6 $\times 10^7$ cells suspended in a 1-ml mixture of equal volumes of 0.25 M sucrose and KRPG were stored in the computer of the spectrophotometer as described (7). After the addition of a few grains of dithionite to the same cuvettes, the spectra were redetermined and automatically subtracted from those stored. The cytochrome b₅₅₉ content was calculated based on the reported extinction coefficient ($\epsilon = 21/\text{mM}$) (18) using the peak at 559 nm. Content is expressed as picomoles of cytochrome b₅₅₉ per milligram of cell protein.

Reagents. Where not otherwise indicated, reagents were from Sigma Chemical Co., St. Louis, MO.

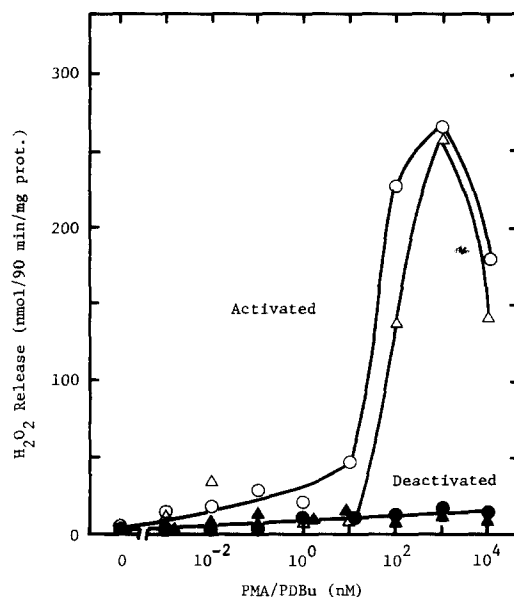


FIGURE 1. Suppression of H_2O_2 release from macrophages previously exposed to TCM. Increased concentrations of phorbol diesters were unable to overcome suppression. Casein-activated macrophages on coverslips were incubated without TCM (open symbols) or with 100% TCM (solid symbols) for 2 d, washed, and stimulated with the indicated concentrations of PMA (○, ●) or PDBu (△, ▲). H_2O_2 release was measured after 90 min at 37°C in KRPG. Data are means of triplicates.

Results

Inability of Increased Phorbol Ester Concentration to Overcome Suppression of Macrophage H_2O_2 -releasing Capacity by TCM. In earlier work (4, 5), suppression of macrophage H_2O_2 -releasing capacity after incubation in TCM was demonstrable whether the respiratory burst was triggered with PMA, yeast cell walls, toxoplasma trophozoites, or leishmania promastigotes. For the present studies, we focused on phorbol diesters rather than particles, to bypass possible alterations in the signal transduction capacity of cell surface receptors for phagocytic particles, and to avoid interference with spectrophotometry. We first confirmed that TCM induced profound suppression of H_2O_2 -releasing capacity from caseinate-activated macrophages challenged either with PMA or PDBu. The concentrations of TCM producing 50% inhibition of H_2O_2 release triggered by 167 nM PMA or PDBu were $21 \pm 11\%$ ($n = 8$) and $13 \pm 3\%$ ($n = 2$), respectively (percent vol/vol; means \pm SD for n experiments). Suppression could not be overcome by increasing the concentration of phorbol diesters up to 300-fold (for PMA) or 137-fold (for PDBu) above that which gave 50% of the maximal response in the controls (Fig. 1). Thus, deactivation was not manifest simply as a rightward shift in the dose-response curve for triggering. We therefore proceeded to compare ROI metabolism in activated and deactivated macrophages.

H_2O_2 -consuming Activity. To study the basis for defective H_2O_2 secretion by TCM-treated macrophages, it was necessary to distinguish between increased catabolism or decreased anabolism of H_2O_2 . Previous work revealed no changes in catalase, glutathione, glutathione peroxidase, or glutathione reductase that

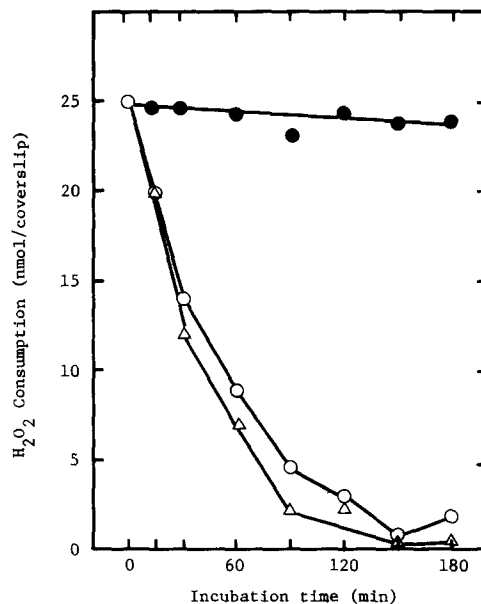


FIGURE 2. H_2O_2 consumption by macrophages. Macrophages cultured for 2 d (10^6 peritoneal cells per coverslip) with 0 (○) or 100% TCM (△) were incubated with 25 nmol reagent H_2O_2 in 1.5 ml KRPG at 37°C . At indicated times, supernatant was removed to determine remaining H_2O_2 as described in Materials and Methods. (●) indicates the cell-free control. Mean adherent cell protein values of 0% TCM- and 100% TCM-treated macrophages were 51.9 and 62.9 $\mu\text{g}/\text{coverslip}$, respectively. Data are means of triplicates.

could explain the decreased H_2O_2 release from TCM-treated macrophages (4). Consistent with this, when reagent H_2O_2 (25 nmol) was added to macrophage cultures (10^6 peritoneal cells/coverslip) in an amount typically produced in response to PMA, the H_2O_2 was degraded at the same rate ($t_{1/2} = 35\text{--}40$ min) whether or not the cells had been exposed to TCM (Fig. 2). Because the factors that govern H_2O_2 uptake are unknown, these experiments were repeated after lysing the macrophages with 0.05% Triton X-100. Under these conditions, H_2O_2 was degraded faster ($t_{1/2} = 12\text{--}18$ min) than by intact cells, but incubation in TCM still did not lead to an enhanced capacity to degrade H_2O_2 (not shown). Accordingly, we turned next to possible causes for decreased production of ROI.

Glucose Uptake. Chemically diverse inhibitors of the respiratory burst in mouse peritoneal macrophages share a common mechanism of action: interference with glucose uptake, on which the respiratory burst depends (12). To explore whether this might also be the locus of action of TCM, 2-deoxy-D-glucose uptake was determined in activated and deactivated macrophages as a function of 2-deoxy-D-glucose concentration. From double reciprocal plots, both the K_m (1 mM) and V_{\max} (~ 100 nmol per 6 min per milligram protein) were determined and found to be invariant with exposure to TCM, PMA, or NaF (an inhibitor of glycolysis) (Table I).

Intracellular Concentration of NADP(H). Although their avidity and capacity for glucose uptake were similar, it was still possible that deactivated macrophages might maintain lower concentrations of NADPH than activated cells, or a lower ratio of NADPH/NADP, to account for the apparently decreased function of

TABLE I
2-Deoxy-D-glucose Uptake by TCM-treated Macrophages

TCM	NaF	K_m^*		V_{max} (nanomoles per 6 min per milligram of protein)*	
		With PMA [‡]	Without PMA [§]	With PMA	Without PMA
%	mM	mM			
0	0	1.0 ± 0	1.0 ± 0	101.0 ± 14.2	97.2 ± 19.6
	2	1.0 ± 0	1.0 ± 0	101.0 ± 14.2	97.2 ± 19.6
100	0	1.0 ± 0	1.0 ± 0	93.5 ± 9.2	105.5 ± 7.8
	2	1.0 ± 0	1.0 ± 0	105.5 ± 7.8	105.5 ± 7.8

Macrophages on coverslips treated with 0 or 100% TCM were preincubated for 30 min in KRP to deplete glucose. Monolayers were then transferred to KRP containing 0.25–4 mM 2-deoxy-D-glucose (0.54 mCi/mmol) and 0 or 167 nM PMA in the presence or absence of 2 mM NaF for 6 min. Nonspecific uptake in 100 μ M cytochalasin B was subtracted from each value (see Materials and Methods). K_m and V_{max} values were obtained by Lineweaver-Burk analysis. Data are means \pm SD from two experiments, each in triplicate.

* For 2-deoxy-D-glucose.

[‡] 167 nM PMA in 0.033% DMSO.

[§] 0.033% DMSO.

their NADPH oxidase. To investigate this, we measured intracellular NADP(H) content in macrophages after incubation in 0–100% TCM, both at rest and after stimulation with PMA, in the presence or absence of 5.5 mM glucose. These values were converted to millimolar terms by relating them to measurements of cell water volume, as described in Materials and Methods (Fig. 3). After a 90-min incubation of activated macrophages (0% TCM) at 37°C in the presence of glucose and absence of PMA, NADPH was 0.59 ± 0.12 mM and NADP 0.11 ± 0.19 mM (mean \pm SD, $n = 5$ experiments). The ratio of NADPH/NADP (5.4) changed little after stimulation with PMA (4.9) despite release of 381 ± 89 nmol H_2O_2 per 90 min per milligram protein, suggesting adequate generation of NADPH through the hexose monophosphate shunt during the respiratory burst. In contrast, in the absence of exogenous glucose, NADPH decreased and NADP increased in resting cells (ratio, 1.54), and these changes were accentuated by exposure to PMA (ratio, 0.78). Deactivation of macrophages with TCM had little or no effect on these results (Fig. 3).

Kinetic Properties of the O_2^- -generating System—The foregoing results made it highly likely that suppression of ROI secretion by TCM was due to an effect on the function of the oxidase itself. Measurement of the kinetic parameters of the oxidase in macrophages is hindered by the low yield of oxidase activity with reported methods of subcellular fractionation. We recently described a modification (7) of an assay (19) using deoxycholate (DOC) lysates in which provision of NADPH could restore the full initial rate of O_2^- production exhibited by intact macrophages. However, oxidase activity still decayed rapidly after addition of NADPH (7). Therefore, we tried to improve the method for the present work. In contrast to results with neutrophils, addition of 5–20% glycerol (20) or ethylene glycol (21) was not effective. However, by combining DOC (an anionic detergent) with a nonionic detergent of the Tween series (21), the stability of the oxidase was improved (Fig. 4). After lysis of macrophages with optimal concentrations of DOC and Tween-20, 51–65% of the oxidase activity recover-

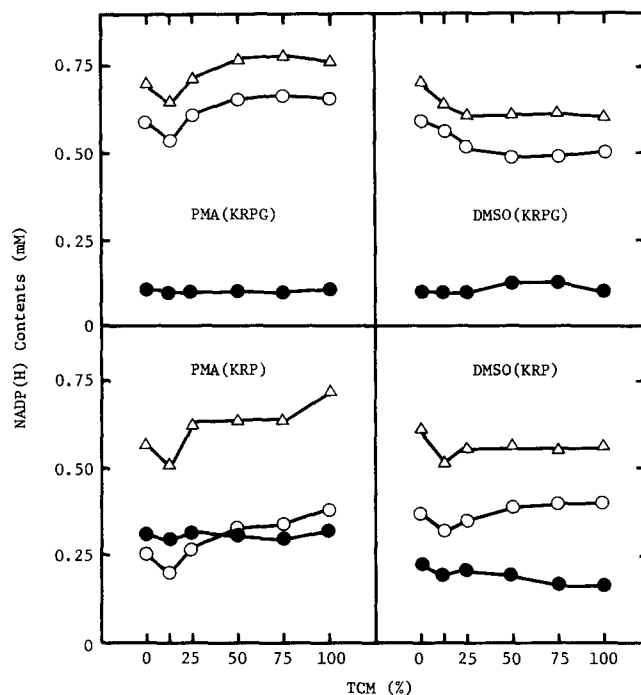


FIGURE 3. Intracellular NADP(H) concentration. Macrophages on coverslips treated with the indicated concentrations of TCM were incubated in 1.5 ml KRPG with 167 nM PMA or 0.033% DMSO for 90 min at 37°C. Cells were washed, lysed and used for enzymatic cycling as described under Materials and Methods to measure NADP(H) per milligram cell protein. Intracellular concentrations (mM) were determined using the values for volume of cell water given in Materials and Methods. NADPH (○), NADP (●), and total NADP(H) (Δ), the sum of measured NADPH and NADP. Data are means of five experiments done in triplicate.

able by adding NADPH at 1 min was still observed when NADPH was withheld until 6 min, while with DOC alone, only 7% of activity remained. Once NADPH was added, the oxidase functioned 7 min later at 86% of the rate displayed at 1 min (not shown). The optimal detergent concentrations were identical for activated and deactivated macrophages (0.0625% [wt/vol] DOC and 0.0625% [vol/vol] Tween-20). The preference of the oxidase for NADPH over NADH (22) was preserved under these conditions (K_m sixfold lower and V_{max} twofold higher with NADPH than with NADH, data not shown). These conditions were used for the following experiments on the effect of TCM.

The reciprocal of the rate of O_2^- production by lysates of PMA-stimulated macrophages treated with 0–100% TCM was a linear function of the reciprocal of NADPH concentration (Fig. 5). With increasing concentrations of TCM, the K_m of the oxidase for NADPH rose in proportion to the inhibition of H_2O_2 releasing capacity of the intact cells (maximal increase in K_m , 14.1-fold), with a relatively much smaller decrease in V_{max} (maximal decrease, fourfold). In three such experiments, exposure to 100% TCM increased the K_m of the oxidase for NADPH by a mean of 11.2-fold, and decreased the V_{max} by a mean of 1.67-fold (Table II).

Using the mean kinetic parameters from Table II and the measured values for total intracellular concentration of NADPH in the presence of PMA and glucose

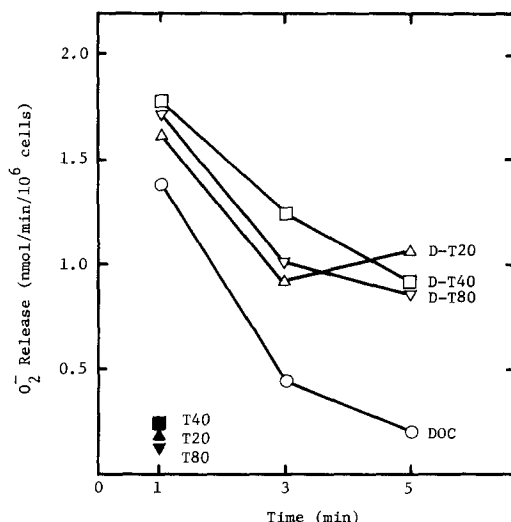


FIGURE 4. Lability of the oxidase after cell lysis with detergents. Adherence-purified macrophages cultured without TCM were brought into suspension (2.1×10^6 cells/ml) and stimulated with 167 nM PMA for 90 s. Detergent was then added (time 0), followed 1, 3, or 5 min later by 1 mM NADPH. Thereupon, O_2^- production was recorded. 0.0625% DOC was used alone (\circ) or together with 0.0625% Tween 20 (Δ), Tween 40 (\square) or Tween 80 (∇). Solid symbols indicate the same Tweens used without DOC. Data are means of duplicates.

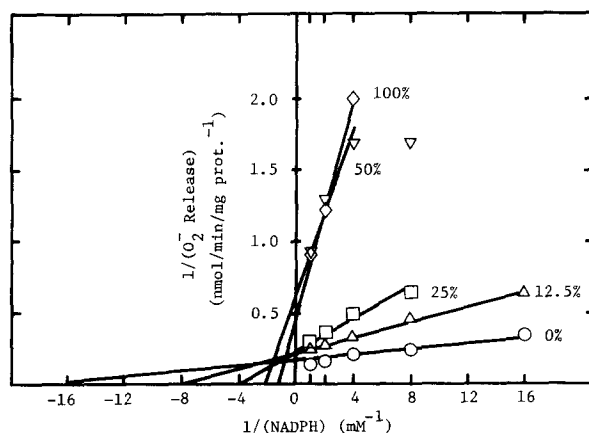


FIGURE 5. Lineweaver-Burk plots of NADPH oxidase activity in macrophages cultured with TCM. Adherence-purified macrophages were recovered in suspension (viabilities were 86–92%) and stimulated with 167 nM PMA for 90 s. Each point represents the rate of O_2^- release obtained after addition of the indicated concentrations of NADPH. NADPH was added 1 min after 0.0625% DOC and 0.0625% Tween 20. Macrophages had been treated for 2 d with 0% (\circ), 12.5% (Δ), 25% (\square), 50% (∇), or 100% (\diamond) TCM. One of three similar experiments is illustrated.

(0.62 ± 0.05 mM, mean for all TCM concentrations tested in five experiments [Fig. 3]), production of H_2O_2 by intact cells could be predicted by the Michaelis-Menten equation. For this calculation, it was noted that O_2^- production continues steadily for 90 min (not shown), and it was assumed that each mole of H_2O_2 derives from two moles of O_2^- (23). The calculated release of H_2O_2 for caseinate-activated cells (301 ± 93 nmol per 90 min per milligram protein) compared

TABLE II
Modification of Kinetic Parameters of NADPH Oxidase by TCM

TCM	Number of experiments	K_m	V_{max}	H ₂ O ₂ release (percentage of control)*	
				Calculated [‡]	Measured [§]
%		mM NADPH	nmol O ₂ ⁻ /min/mg		
0	3	0.06 ± 0.00	7.38 ± 2.28	100	100
12.5	1	0.13	4.55	73 [‡]	82 [‡]
25	3	0.14 ± 0.10	4.58 ± 1.50	56 ± 4	62 ± 19
50	3	0.30 ± 0.14	2.78 ± 1.22	31 ± 22	35 ± 34
100	3	0.67 ± 0.23	4.42 ± 2.17	35 ± 18	20 ± 18

* Means ± SD for number of experiments shown.

[‡] H₂O₂ release over 90 min was calculated using an NADPH concentration of 0.62 mM (see text) and the K_m and V_{max} data shown, and was expressed as a percent of the value for 0% (control) TCM (301 ± 93 nmol per 90 min per milligram protein).

[§] Determined with PMA-stimulated macrophages on coverslips in the same experiments, and expressed as a percent of the value for 0% TCM (267 ± 48 nmol per 90 min per milligram protein).

[‡] As in [‡], except that the control value was 255 nmol per 90 min per milligram protein.

[§] As in [§], except that the control value was 213 nmol per 90 min per milligram protein.

TABLE III
Cytochrome b₅₅₉ Content

TCM	Cytochrome b ₅₅₉ *
%	pmol/mg protein
0	109.6 ± 26.4
100	94.6 ± 41.7

Adherence-purified macrophages (prepared without exposure to diethylthiocarbamate) were suspended in 1 ml of 1:1 0.25 M sucrose and KRPG. Their initial spectrum was automatically subtracted from that recorded after reduction by dithionite.

* Estimated from the extinction coefficient ($\epsilon = 21/\text{mM}$). Means ± SD of three experiments.

favorably with the observed release (267 ± 48). By the same calculations, the suppression of H₂O₂ secretion by each concentration of TCM could largely be accounted for by the change in kinetic parameters of the oxidase (Table II).

Cytochrome b₅₅₉ Content. Phagocyte production of O₂⁻ from O₂ and NADPH is believed to depend on an electron transport chain containing a unique b-type cytochrome b₅₅₉. Deficiency of this cytochrome is the commonest cause of inadequate ROI production by phagocytes in chronic granulomatous disease (24). To determine whether the effect of TCM on the oxidase was exerted through a decrease in cytochrome b₅₅₉, difference spectra between oxygenated and dithionite-reduced cells were recorded. Activated and TCM-treated macrophages both exhibited a striking peak at 559 nm with a slight shoulder at 550 nm, as reported for other mouse macrophage populations (7, 10), and were estimated to contain equivalent amounts of cytochrome b₅₅₉ (Table III).

Discussion

We conclude that cytokines both enhance and suppress ROI-dependent macrophage effector functions by acting on the same control point: the affinity of

the superoxide-producing enzyme for its reducing cosubstrate, NADPH. The results reported here ascribe the deactivation of macrophages by MDF to an order of magnitude decrease in affinity of the oxidase. This mirrors the increase in affinity accompanying activation by IFN- γ or other stimuli (6–9).

The action of MDF on the kinetic properties of the oxidase was selective, in that there was no change at any of several alternative control points at which the secretion of ROI could have been regulated. The unaffected steps were H₂O₂ catabolism, glucose transport, intracellular concentration of NADPH and NADP, and cytochrome b₅₅₉ content. Although the capacity of the oxidase in substrate excess (V_{\max}) was suppressed by MDF, the degree of suppression was small ($40 \pm 20\%$). In contrast, the increase in K_m of the oxidase for NADPH brought about by MDF averaged $1,117 \pm 383\%$. The altered kinetic properties of the oxidase could account quantitatively for the deficiency of deactivated macrophages in secretion of ROI (4), and by extension, in the ROI-dependent killing of toxoplasma and leishmania (5).

Knowledge of the intracellular concentration of NADPH was important in permitting the calculation that the MDF-induced alteration in the kinetic properties of the oxidase could account for the change in H₂O₂-releasing capacity of the cells. The intracellular concentration of NADPH (0.59 mM) measured here for caseinate-activated macrophages at rest in 5.5 mM glucose is double that reported (6) for macrophages activated with bacterial LPS under comparable incubation conditions (0.30 mM). It is not clear if this reflects the difference in cell populations, duration of culture in vitro, or techniques for the measurement of the nucleotide and cell water. We did not observe the marked decline in intracellular NADPH reported upon PMA stimulation of macrophages in glucose-containing medium (6). However, simultaneous measurements of NADPH and NADP (apparently the first to be reported in macrophages) demonstrated a drop in the NADPH/NADP ratio upon incubation in glucose-free medium, even without PMA stimulation. The fall in ratio was exaggerated by PMA. This emphasizes the critical dependence of the redox state of the mouse peritoneal macrophage upon an external source of glucose (12, 25).

Although there have been no previous reports of the kinetics of the oxidase during macrophage deactivation, concordant findings have emerged from four studies of oxidase kinetics during activation. These studies used mouse peritoneal macrophages (6–8) or human blood monocyte-derived macrophages (9). In the latter work, an increase in the K_m of the oxidase for NADPH with little change in the V_{\max} was observed when monocytes differentiated into macrophages. The rise in K_m was reversed when the macrophages were activated by IFN- γ (9). Thus, the affinity of the oxidase for its substrate appears to be a point of control for the respiratory burst of mononuclear phagocytes from both extravascular and intravascular sites, in at least two species, and in response to at least two cytokines (MDF and IFN- γ) and four other kinds of activating agents (sodium periodate, sodium caseinate, *C. parvum*, and bacterial LPS), as well as for some of the apparently spontaneous changes believed to be part of the differentiation program of the cell lineage.

Kitagawa and Johnston (26) recently reported that a spontaneous decline of superoxide-releasing capacity during 3 d of culture of activated mouse peritoneal macrophages could be reversed by replacing Na⁺ with K⁺ in the assay buffer at

the end of the culture period. This suggests that all components of the oxidase were present in nonlimiting amounts in activated and spontaneously deactivated macrophages, but were differentially used in the more physiologic buffer. TCM suppressed macrophage H_2O_2 -releasing capacity equally well whether the assay was conducted in KRPG ($92 \pm 4\%$ suppression) or in the high- K^+ buffer described by Kitagawa and Johnston ($90 \pm 6\%$ suppression) (means \pm SEM for three paired experiments, data not shown). This observation suggests that spontaneous deactivation and the more rapid deactivation induced by MDF differ fundamentally in mechanism. Nonetheless, the results in these two systems complement each other in supporting the hypothesis that macrophage deactivation, however achieved, is unlikely to result from changes in the cellular content of the oxidase.

Attention must now focus instead on the mechanisms responsible for changes in the affinity of the oxidase for NADPH. Current understanding of the chain that transports electrons from NADPH to O_2 in the plasma membrane (22) implies that there are at least three points for affecting the apparent affinity of the transport chain as a whole for NADPH: electron flow from NADPH (-320 mV) to flavoprotein (-280 mV) (27, 28), from flavoprotein to cytochrome b_{559} (-245 mV) (24), or from cytochrome b_{559} to O_2 . Judging from reduced-minus-oxidized difference spectra, neither the cellular content (7, 10) nor potentiometric properties (10) of cytochrome b_{559} differ among macrophage populations in a manner that could account for their differences in oxidase kinetics. On the other hand, Kakinuma et al. (28) have recently studied the electron spin resonance properties of the dominant plasma membrane flavoprotein in neutrophils under anaerobic conditions. With dithionite as a reductant, no differences were found in midpoint redox potential for the flavin from resting and stimulated neutrophils (-280 mV at pH 7.0). In striking contrast, the flavin from resting but not stimulated neutrophils was completely refractory to reduction by NADPH. This suggests that stimuli which trigger the respiratory burst in neutrophils remove a gate which, in the resting cell, selectively bars NADPH from the flavoprotein. Differences in the degree to which this gate is removed at the time of triggering could explain the changes in apparent affinity of the oxidase that accompany macrophage activation and deactivation.

How the respiratory burst is triggered is unknown. Recent evidence (12) discounts the idea that arachidonate or one of its oxygenation products mediates triggering in macrophages. In neutrophils, protein kinase C may be a major component of the triggering mechanism (29). It will be of interest to examine the effect of macrophage deactivation on the function of protein kinase C.

Summary

Incubation of activated mouse peritoneal macrophages with tumor cell-conditioned medium (TCM) results in their deactivation, as measured by ability to release reactive oxygen intermediates and kill protozoal pathogens. The mechanism of suppression by macrophage deactivation factor (MDF) was studied. Inhibition of H_2O_2 release could not be overcome by increasing the concentration of phorbol diesters used to trigger the respiratory burst. Deactivated macrophages consumed H_2O_2 at the same rate as activated cells ($t_{1/2}$, 35–40 min for 25 nmol H_2O_2 per 10^6 peritoneal cells). They transported glucose with the same kinetics (K_m , 1 mM; V_{max} , ~ 100 nmol per 6 min per milligram cell protein), and

maintained similar intracellular concentrations of NADPH and NADP (~0.62 mM and ~0.11 mM, respectively), as measured by enzymatic cycling methods and determinations of the volume of cell water (3.6 μ l/mg cell protein). To study the kinetics of the PMA-triggered NADPH oxidase in cell lysates, mixed detergents were used (deoxycholate and Tween 20). These stabilized the oxidase for ~3.3-fold longer than deoxycholate alone, which was used in previous studies. Incubation of activated macrophages in MDF resulted in a marked increase in the K_m of the oxidase for NADPH, from 0.06 mM to 0.67 mM. The V_{max} fell ~1.7-fold. These kinetic changes, together with the measured intracellular concentration of NADPH, account quantitatively for the suppression of H₂O₂ release by deactivated macrophages, and are nearly the mirror image of the kinetic changes observed during macrophage activation.

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