

ANALYSIS OF THE NONFUNCTIONAL RESPIRATORY BURST IN MURINE KUPFFER CELLS

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The concept of macrophage activation evolved chiefly from studies on cells from blood, peritoneum, and pulmonary alveoli (1–3). Central to the phenomenon of activation is the ability of lymphokines to enhance the capacity of macrophages to secrete reactive oxygen intermediates and kill intracellular microbial pathogens (4). However, few studies of activation have considered the far larger pool of macrophages in the liver. Recently, surprising evidence emerged that mouse Kupffer cells (KCs)¹ differed from most other macrophages in showing virtually no capacity for a respiratory burst; i.e., almost no increase in consumption of oxygen or production of superoxide or hydrogen peroxide upon challenge with microbes or soluble secretagogues (5, 6). The defect was consequential, since KCs also lacked oxygen-dependent antimicrobial activity (6). Moreover, although KCs responded to rIFN- γ , a well-defined macrophage-activating factor (7), with appropriate changes in surface antigen expression and protein secretion, rIFN- γ failed to induce in KCs either an enhanced respiratory burst capacity or antiprotozoal activity (5). Even the intense immunologic stimulation of an evolving granulomatous response to hepatic infection failed to activate KCs. Instead, respiratory burst and antimicrobial activity in the infected liver were associated with a separate, newly arrived population of inflammatory monocytes (6, 8).

Deficient respiratory burst activity may be a general property of sinus-lining murine macrophages (9). However, it is a mystery how the oxidatively active monocyte (10) can emigrate at random (11) to hepatic sinusoids (12), there to lose both baseline and lymphokine-induced respiratory burst capacity, while monocytes emigrating to other tissues retain these properties. The present study explored the mechanism of this apparent site-determined differentiation by analyzing the biochemical basis for the deficient respiratory burst of KCs.

Materials and Methods

Mice. Female mice (8–12 wk old) were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA (CD, strain) and the Trudeau Institute, Saranac Lake, NY (ICR strain).

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¹ *Abbreviations used in this paper:* CR3, complement receptor type 3; DOC, deoxycholate; HMPS, hexose monophosphate shunt; HPO, horseradish peroxidase; KRPG, Krebs-Ringer phosphate buffer with 5.5 mM glucose; KC, Kupffer cell; MDF, macrophage deactivation factor; PC, peritoneal macrophage; SOD, superoxide dismutase.

Reagents. Murine rIFN- γ (kindly provided by Genentech, Inc., South San Francisco, CA), was purified from *Escherichia coli* and had a specific activity of 5.2×10^7 antiviral U/mg protein. Hybridoma supernates for M1/70 (Mac-1), M18/2, 2.4G2, and FD441.8 (TIB 213) were prepared as described (13). Iodinated F(ab')₂ fragments of goat anti-rat IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) with specific activity of $\sim 10^7$ cpm/ μ g were from S. Wright (The Rockefeller University, New York, NY). [1,2-³H]-2-Deoxy-D-glucose (sp act 30 Ci/mmol), 1-[¹⁴C]D-glucose (sp act 7.6 mCi/nmol), and 5,6,8,9,11,12,14,15-[³H]arachidonic acid ([³H]20:4, sp act 70 Ci/nmol) were purchased from New England Nuclear (Boston, MA). Sodium azide, potassium cyanide, hydrogen peroxide, and acetic anhydride were from Fisher Scientific Co. (Fair Lawn, NJ). The following were from Sigma Chemical Co. (St. Louis, MO): sodium periodate, Tween 20, deoxycholate (DOC), 2-deoxy-D-glucose, EDTA (tetrasodium salt), sodium hydrosulfite (dithionite), NADPH (tetrasodium salt, type X), cytochrome *c* (type VI), scopoletin, horseradish peroxidase (HPO, type II), superoxide dismutase (SOD), DMSO, and PMA.

Cell Preparation. Resident mouse peritoneal macrophages (PCs) were washed from the peritoneal cavity with RPMI 1640 (KC Biological, Inc., Lenexa, KS). After centrifugation at 170 *g* for 10 min at 4°C, the cell pellets were suspended in complete medium: RPMI 1640 containing 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT) and 2 mg/100 ml of gentamycin sulfate (Schering Corp., Kenilworth, NJ), and plated in 35-mm petri dishes (5×10^6 /dish) or 13-mm glass coverslips (5×10^5 /slip) for 2 h at 37°C in 5% CO₂/95% air followed by removal of nonadherent cells by suction. Where indicated, mice were injected intraperitoneally 3 d before harvest with 1 ml of 5 mM sodium periodate.

KCs were isolated according to the method of Lepay et al. (5) with some modifications. In brief, mice were anesthetized with 2 mg pentobarbital intraperitoneally and the peritoneal and thoracic cavities exposed. Perfusate was injected through a 20 G catheter inserted through the right atrium into the inferior vena cava. The portal vein was cut to allow efflux of perfusate after ligating the inferior vena cava caudad to the liver. Perfusion was begun with a prewarmed (37°C) Ca²⁺-, Mg²⁺-free PBS containing 1 mM EDTA and 5.5 mM glucose, pH 7.3, for 2 min at a rate of 7 ml/min, followed by 40 ml of RPMI 1640 containing 100 U/ml of collagenase (Class III, CooperBiomedical Inc., Malvern, PA) at the same temperature and flow rate. The gall bladder was removed. The liver was excised, rinsed in cold RPMI 1640, and teased gently to obtain a cell suspension. Preparations were used only if >70% of the hepatocytes were viable, as determined by trypan blue exclusion. After 4–5 low-speed centrifugations (30 *g*, 4 min, 4°C) to remove parenchymal cells, the supernatant was collected and centrifuged at 170 *g* for 10 min. When erythrocytes were visible, the cell pellet was treated with 0.2% NaCl for 30 s. The cell pellet was washed twice in cold RPMI 1640. Adherent KC monolayers were prepared by plating the cells on 35-mm petri dishes or 13-mm glass coverslips for 30 min in Krebs-Ringer phosphate buffer with 5.5 mM glucose (KRPB) plus 10% HI-FCS. The temperature was kept at 15–18°C during adherence to minimize phagocytosis of liver debris by KCs.

Particulates. Zymosan was purchased from ICN K & K Laboratories, Inc. (Plainview, NY). Unopsonized stock solutions in PBS were prepared according to the method of Bonney et al. (14), and stored at –20°C. For each experiment, an aliquot was thawed and sonicated for 1 min (Branson Astrason, model 6; Branson Cleaning Equipment Co., Shelton, CT). SRBCs were coated with rabbit IgG (EIgG), human C3b (EC3b), or C3bi (EC3bi) as described (15). Monolayers of KCs on 13-mm glass coverslips were washed, overlaid with 3×10^5 erythrocytes, and incubated for 45 min at 37°C in the presence or absence of antibody as indicated. Coverslips were dipped in PBS to remove unattached SRBCs and examined under a phase-contrast microscope.

Glucose Transport. Experiments were performed at room temperature. KCs or PCs adherent to glass coverslips were placed in 16-mm wells in 24-well plastic trays (Corning Glass Works, Corning, NY) containing 0.5 ml Krebs-Ringer phosphate buffer with 2 mM 1,2-[³H]-2-deoxy-D-glucose (1.25 μ Ci). After 1 or 2 min the coverslips were dipped serially in four 250-ml beakers with 0.9% NaCl and drained. The cells were dissolved in 0.3 ml

of 0.2 N NaOH for liquid scintillation counting and protein assay. [1,2-³H]-2-Deoxy-D-glucose uptake was calculated from the differences of radioactivity between 1 and 2 min and expressed as nmol/mg protein per min, which was linear for >2 min in all experiments. When the effect of PMA or zymosan was tested, they were added 3 min before the addition of labeled deoxyglucose.

Glucose Oxidation. The oxidation of ¹⁴C-1-glucose to ¹⁴CO₂ by macrophage monolayers was studied as described previously (16) with minor modifications using cell monolayers in 35-mm petri dishes containing 1 ml of KRP at pH 7.4 with 2 mM glucose and 1 μCi of 1-[¹⁴C]-D-glucose during a 20-min incubation at 37°C. When the effect of PMA or zymosan was tested, they were added before 1-[¹⁴C]-glucose. A background count from cell free control dishes was subtracted and was always <0.01% of the total counts recovered in the NaOH-soaked wick. A correction was also made for trapping efficiency (~70%) as compared with the identical experiment using a screw-capped flask instead of a petri dish. Results were expressed as nanomoles ¹⁴CO₂ released/milligram cell protein per hour.

NADPH Oxidase. O₂⁻ release from KC or PC lysates was measured as described (17) with the modification that NADPH was added before detergent. In brief, 3–5 × 10⁶ cells were suspended in 0.8 ml KRPG with 30 μM acetylated cytochrome C, 2 mM NaN₃, and 1 mM NADPH in a 1-cm light path cuvette with an air-driven stirrer in the thermostatted (37°C) chamber of a Perkin-Elmer/Hitachi model 557 dual wavelength spectrophotometer continuously recording ΔA (550–540 nm) (E = 19.1 mM⁻¹). The reaction was started by the addition of PMA to a final concentration of 100 ng/ml, followed after 1 min by addition of 0.0625% (wt/vol) DOC and 0.0625% (vol/vol) Tween 20. Reduction of acetylated cytochrome C was completely abolished by 300 U/ml SOD.

Cytochrome b₅₅₉ Content. To minimize interference from hemoglobin and mitochondrial cytochromes, a modification of the conventional method was made, which will be detailed elsewhere (Ding, A., manuscript in preparation). In brief, 2–3 × 10⁷ KCs or 5–8 × 10⁷ PCs were suspended in 0.8 ml of KRPG containing 1 mM KCN and 1 mM NaN₃. A few grains of sodium dithionite were added to the suspension, followed by bubbling with air for 2–5 min. Spectra of the suspensions from 400–600 nm were stored in the spectrophotometer. Cytochrome b was reduced again with a second addition of sodium dithionite. The resulting spectra from 400–600 nm were subtracted from the stored spectra for oxygenated cells. Cytochrome b₅₅₉ was estimated by means of its absorbance coefficient of 21.7 mM⁻¹cm⁻¹ at 559 nm (18).

[³H] 20:4 Release. Freshly prepared KC or PC suspensions in complete medium were added to 35-mm petri dishes (5 × 10⁶ in 1 ml/dish). After 2 h at 37°C in 5% CO₂/95% air, the monolayers were washed three times with RPMI 1640. Freshly prepared complete medium containing 0.5 μCi of [³H]20:4 was added (1.2 ml/dish) and the cells were incubated overnight. The monolayers were then washed three times with warm RPMI, overlaid with 1.2 ml RPMI plus 200 μg zymosan, and incubated for 2 h more. 5-μl aliquots were removed at intervals to determine released [³H]20:4. Total labeling was determined as the sum of 0.05% Triton X-100 solubilized counts from the monolayer plus total counts in the medium. The results were expressed as percent of the total label released.

Separation of [³H]20:4-labeled Metabolites by HPLC. Oxygenated products of 20:4 were extracted from culture media according to the method of Unger et al. (19). 1 ml of ethanol and 10 μl of 88% (wt/wt) formic acid were added to 1.2 ml medium containing [³H] 20:4 metabolites. The resultant solution was extracted twice with 1 ml of chloroform containing 0.005% (wt/vol) butylated hydroxytoluene. The lower phases were pooled and evaporated to dryness under N₂. The residue was dissolved in the appropriate starting buffer for reverse-phase HPLC. [³H] 20:4 metabolites were separated and identified using two HPLC systems (20). In the first, a column (4.6 × 250 mm) of Ultrasphere C-18 (Altex Scientific, Inc., Berkeley, CA) was eluted at a flow rate of 1 ml/min with 60 ml of methanol/water/acetic acid (65:35:0.1, vol/vol/vol, pH 5.4), followed by 40 ml of methanol/acetic acid (100:0.01, vol/vol). In the second system, a Fatty Acid Analysis column (3.9 × 300 mm; Waters Associates, Millipore Corp., Milford, MA) was eluted at 2 ml/min with 100 ml of acetonitrile/water/benzene/acetic acid (76.7:23:0.2:0.1, vol/vol/vol/vol). Identification of recovered radiolabeled 20:4 metabolites was accomplished by comparison

of retention times with radiolabeled standards, including 6-keto-prostaglandin $F_{1\alpha}$, thromboxane B_2 , prostaglandin $_{2\alpha}$, prostaglandin E_2 , leukotriene C, and arachidonic acid (20:4) (all from New England Nuclear, Boston, MA), and 12-hydroxyheptadecanoic acid and 12-hydroxyeicosatetraenoic acid prepared from human platelets (a kind gift of N. Pawlowski, University of Pennsylvania, Philadelphia, PA).

RIA of Surface Antigens. The surface antigens of KCs were measured in an RIA using rat hybridoma supernate and ^{125}I -F(ab') $_2$ of goat anti-rat IgG. The following steps were at 4°C. Triplicate coverslips with 1-d-old cultures of KCs or resident PCs were washed with PBS and placed on an inverted lid of a 24-well plate. 30 μl of hybridoma supernatant containing the primary antibody (~10 $\mu\text{g}/\text{ml}$) was added. 50 min later, the monolayers were thoroughly washed and incubated with 1 $\mu\text{g}/\text{ml}$ ^{125}I -F(ab') $_2$ anti-IgG for an additional 50 min. After washing, individual coverslips were transferred to a new 24-well plate and incubated at room temperature for 1 h with 0.3 ml of 0.1 N NaOH, of which 100 μl was used for protein assay and the rest for gamma counting. Background binding was measured in coverslips without cells and was subtracted from all the measurements.

H $_2$ O $_2$ Release. H $_2$ O $_2$ release was measured fluorometrically by the horseradish peroxidase-catalyzed oxidation of fluorescent scopoletin to a nonfluorescent product, as described in detail (21) with the following modifications for use of 24-well rather than 96-well plates. Triplicate coverslips with adherent cells were rinsed in four beakers of 0.9% NaCl, drained, and transferred to 16-mm wells containing 0.3 ml of KRPG with 15–30 μM scopoletin, 1 mM NaN $_3$, 1 purpurogallin U/ml of HPO, and 100 ng/ml of PMA per well. After 60 min of incubation at 37°C, the fluorescence of the supernate was recorded in a fluorometer (model MPF 44A; Perkin-Elmer Corp., Pomona, CA) with excitation 350 nm and emission 460 nm. The protein of cells adherent at the start of the assay was measured by the method of Lowry et al. (22) in every well. Specific release was calculated after subtracting H $_2$ O $_2$ and protein values for cell-free samples and expressed as nanomoles H $_2$ O $_2$ /milligram cell protein/60 or 120 min.

rIFN- γ Injection. rIFN- γ was diluted in normal saline containing 0.1% FCS in a final concentration of 5×10^5 U/ml, and injected into peritoneal cavity of CD $_1$ mice (1 ml/mouse) 18 h before harvesting KCs or PCs. The control mice were injected with 1 ml/mouse of saline containing 0.1% FCS.

Results

Cell Characteristics. Earlier experiments with this procedure for collecting KCs showed that if mice were given intravenous India ink, >90% of the adherent cells obtained were labeled with carbon particles, and thus belonged to the vascular sinus-lining population (5). Although carbon-labeled cells stained in situ with the F4/80 mAb, a marker of mononuclear phagocytes, they were negative for another macrophage marker, Mac-1 (5). Thus, our initial experiments had two goals: to resolve this apparent discrepancy and confirm that our adherent population was nearly pure in macrophages; and to minimize the ingestion of debris during isolation. The latter was essential to decrease the possibility that the respiratory burst might be exhausted by phagocytic triggering during cell preparation (23, 24).

We first compared KCs isolated by means of adherence at 37°C or 15–18°C. Ingestion of debris (probably fragments of hepatocytes) was sometimes extensive at 37°C, but was undetectable at the lower temperature (Fig. 1A). Fig. 1, B–D, documents that after overnight incubation, virtually all the adherent cells displayed receptors for Fc and C3bi but not C3b. The presence of complement receptor type 3 (CR3) was confirmed by the ability of monoclonal anti-CR3 antibody to inhibit rosetting of KCs with EC3bi (Fig. 1E). An RIA provided quantitative evidence that there was comparable expression of Fc and C3bi

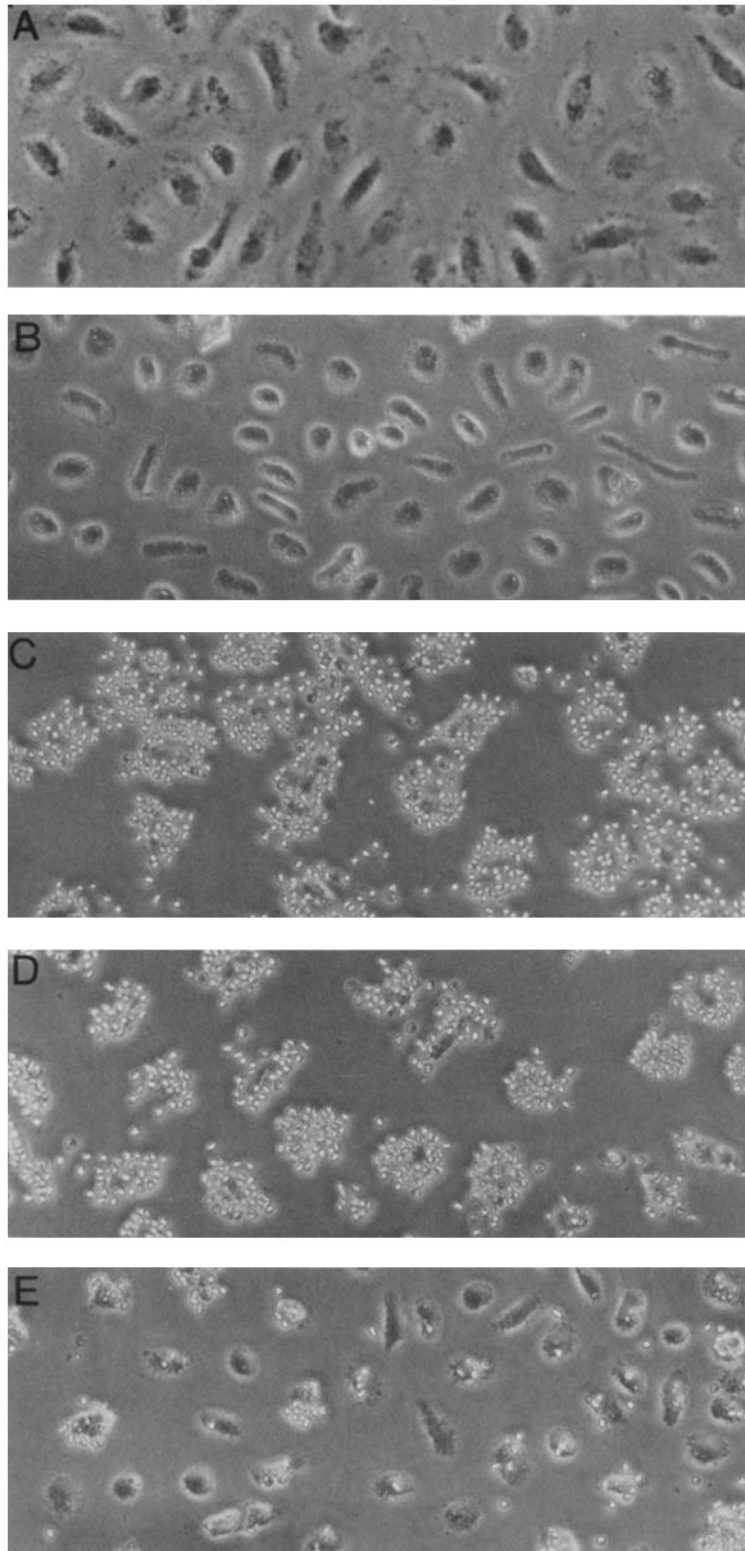


FIGURE 1. Lack of particulate matter in KCs after adherence at low temperature and uniform rosetting with ligand-coated SRBCs. After 1 d in culture, KC monolayers (A) were rosetted (B-E) with ligand-coated SRBCs in the absence (B-D) or presence (E) of 1 µg/ml of M1/70 mAb against CR3. The ligands are (B) human C3b; (C) rabbit IgG; and (D and E) human C3bi.

TABLE I
Surface Antigen Expression

First Ab	Specificity	Antigen expression	
		KCs	Resident PCs
—	—	53 ± 7	17 ± 2
M1/70	α Chain of CR3	869 ± 40	1,098 ± 44
M18/2	β Chain of LFA-1*	1,114 ± 123	1,463 ± 128
FD441.8	α Chain of LFA-1	1,114 ± 106	175 ± 6
2.4G2	FcR II [‡]	576 ± 47	489 ± 42

Monolayers of KCs or PCs were used for RIA after 1 d in culture. Results are expressed as cpm/mg cell protein (means ± SE for triplicates).

* Also recognizes β chain of CR3.

[‡] For IgG1 and IgG2b monomers and immune complexes.

TABLE II
Transport of 2-Deoxy-Glucose

Addition	KCs	Resident PCs	IO ₄ ⁻ -elicited PCs
—	2.4	3.9	3.9
Zymosan*	2.7	4.3	ND
PMA [‡]	2.6	4.0	4.5

Results are representative of three separate experiments and are expressed as nmol 2-deoxy-glucose uptake/mg cell protein per min. Values are means for duplicates (SE <3%).

* 200 μg/ml.

[‡] 100 ng/ml.

receptors on KCs and PCs (Table I). Thus, while CR3 may be blocked or otherwise unexpressed on KCs in situ, CR3 is readily detected on the same cells after overnight incubation in vitro. Lymphocyte function-associated antigen 1, which shares a common β chain with CR3, was sevenfold more abundant per milligram cell protein on KCs than on resident PCs. In this regard, KCs resembled activated rather than resident PCs (25).

Glucose Transport. Lack of increased O₂ consumption during triggering of KCs (5, 26) proved that their failure to release detectable H₂O₂ was due to lack of production of H₂O₂, rather than to its enhanced degradation. Therefore, we looked for a possible defect in each of several biochemically defined steps in H₂O₂ production, beginning with transport of glucose, on which macrophage H₂O₂ production is dependent (27, 28). As shown in Table II, KCs transported 2-deoxy-D-glucose 62% as fast as either resident or activated mouse PCs. Results were similar if the macrophages were at rest or were challenged with zymosan particles or PMA during the assay. KCs are threefold larger than resident PCs (based on the ratios of milligrams protein/cell; unpublished observation). Thus, if the results are expressed on a per cell basis, KCs took up glucose ~80% faster than PCs. Absence of the respiratory burst could not be attributed to a gross reduction in glucose transport.

Hexose Monophosphate Shunt (HMPS). Accordingly, we next examined NADPH-generating capacity, as reflected by the oxidation of the first carbon of

TABLE III
Oxidation of ¹⁴C-1-Glucose

Addition	KCs	Resident PCs	IO ₄ ⁻ -elicited PCs
—	1.4 ± 1.0 (4)	2.8 ± 0.7 (3)	3.1 ± 0.2 (4)
Zymosan*	3.1 ± 0.3 (4)	40.1 ± 0.9 (4)	62.9 ± 2.4 (2)
PMA [‡]	0.9 ± 0.4 (3)	7.7 ± 1.0 (3)	20.3 ± 2.0 (3)

Results are expressed as nmol ¹⁴CO₂ produced/mg cell protein per h and are means ± SE from the number of experiments in parentheses.

* 200 μg/ml.

‡ 100 ng/ml.

TABLE IV
Effect of Detergent Concentration

Concentration of detergent*	Relative rate ΔA550-540/min	Duration of linear rate(s)
0	0.12	—
0.025	0.19	>180
0.040	0.47	>180
0.050	0.64	>180
0.060	0.80	100
0.075	1.0	50
0.100	0.55	25

NADPH oxidase activity in resident PCs was measured spectrophotometrically as described in Materials and Methods, varying the concentration of detergents.

* DOC plus Tween 20; i.e., 0.05% detergents means 0.05% (wt/vol) of DOC plus 0.05% (vol/vol) Tween 20.

glucose to CO₂ via the HMPS (Table III). Baseline HMPS activity in KCs was somewhat lower than in resident or activated PCs on a per mg cell protein basis, although not on a per cell basis. Ingestion of zymosan provoked only a 2.2-fold increase in HMPS in KCs, compared with a 14–20-fold increase in PCs. With PMA, there was a 2.8–6.5-fold increase in HMPS in the PCs, but none in KCs. These results indicated that there was no marked deficiency in the baseline activity of the HMPS in KCs. The lack of a substantial increase in HMPS during exposure to endocytic/secretagogic stimuli might be due to the absence of a respiratory burst, since NADPH is oxidized during the catabolism of H₂O₂ via the glutathione redox cycle (29).

NADPH Oxidase. Thus, we proceeded to test directly the enzymatic activity of the NADPH oxidase that produces superoxide. As detailed in previous reports (17, 30), it is difficult to permeabilize mouse macrophages to exogenous NADPH without destroying the oxidase. Quantitative recovery of oxidase activity in lysed cells depends on the choice of detergent, its concentration, and the duration of detergent treatment before addition of NADPH. The first variable was recently optimized (17). The second was explored in greater detail in Table IV. The third variable was eliminated by adding NADPH to the assay mix before, rather than after, the detergent. These modifications permitted the demonstration of the expected (30) rate of SOD-inhibitable, PMA-induced reduction of acetylated cytochrome c by detergent-lysed resident PCs, but none by KCs (Fig. 2), even at

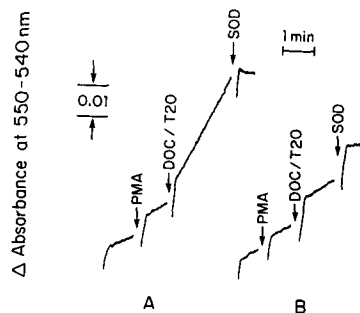


FIGURE 2. NADPH oxidase activity in cell lysates. 4×10^6 resident PCs (A) or KCs (B) were suspended in 0.8 ml of KRPG containing 1 mM NADPH, 2 mM NaN_3 , and $30 \mu\text{M}$ acetylated cytochrome C in a dual wavelength spectrophotometer at 37°C . 100 ng/ml PMA, 0.0625% each of DOC and Tween 20, and 300 U/ml of SOD were added as indicated, and the difference in absorption between 550 and 540 nm was recorded.

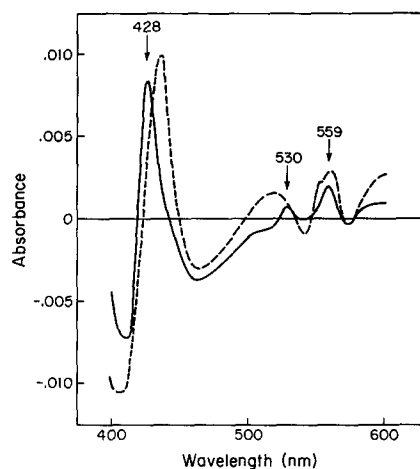


FIGURE 3. Dithionite reduced-minus-oxidized difference spectra of freshly isolated KCs. The dashed line represents the (reduced the first time)-minus-(initial, oxidized) spectrum. The solid line represents the (reduced the second time)-minus-(reoxidized after first reduction) spectrum. Interfering peaks from hemoglobin and mitochondrial cytochromes are eliminated. See text.

1 mM NADPH (well above the intracellular concentration of NADPH in mouse PC [17]). Thus, the oxidase had either been lost during conversion of monocytes to KCs, or rendered refractory to being triggered to an NADPH-reducible state (31).

Cytochrome b_{559} . To try to distinguish between these possibilities, we estimated the cellular content of a component of the oxidase complex that can be detected spectrally, independent of its enzymatic activity, namely, cytochrome b_{559} (cytochrome b_{-245}) (32). Although reduced-oxidized difference spectra of neutrophils give a single cytochrome $b_{559} \alpha$ band at 559 nm, the corresponding region in the macrophage spectrum is complicated both by a shoulder at 550 nm from mitochondrial cytochromes (30) and a coinciding peak at 555–559 nm from hemoglobin, probably from erythrocytes ingested by KCs in vivo. As little as 0.4% contamination with erythrocytes would suffice to double the height of the peak at 559 nm (33). Indeed, in a conventional reduced-oxidized difference spectrum of KCs, massive β (510–515 nm) and γ bands (431–434) characteristic of hemoglobin were recorded (Fig. 3, *dashed line*). To minimize these interferences, we took advantage of the fact that oxidation and reduction of cytochrome b_{559} in the presence of KCN and NaN_3 are reversible, while irreversible changes are induced in mitochondrial cytochromes and hemoglobin under the same conditions. Fig. 3 (*solid line*) displays a (reduced the second time)-minus-(reoxidized after first reduction) difference spectrum of KCs. The interfering peaks

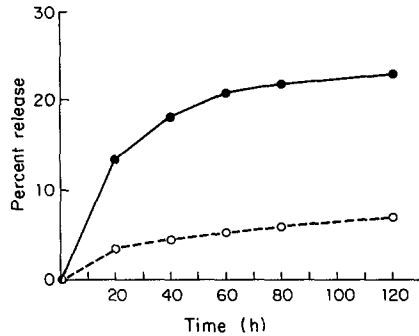


FIGURE 4. Time course of 20:4 release from zymosan-treated KCs (—○—) or PCs (—●—). Macrophages were labeled with [3 H] 20:4 overnight and challenged with 200 μ g/1.2 ml of zymosan. At the time points indicated, duplicate aliquots of the medium were removed and counted. Total labeling was determined by 0.05% Triton X-100-solubilized counts plus the counts from the medium. Values are means from two separate experiments.

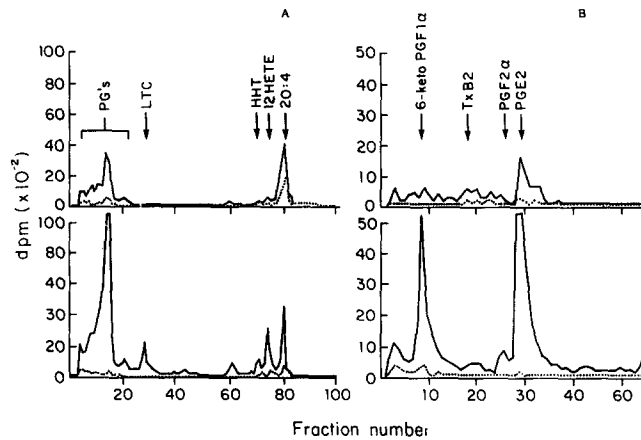


FIGURE 5. HPLC chromatograms of 20:4 metabolites from KCs (upper panels) or resident PCs (lower panels). Cells were labeled overnight with [3 H] 20:4, washed, and incubated with (—) or without (---) zymosan. Conditioned medium was extracted and analyzed on HPLC system I (A) or system II (B). Elution times of known tritiated standards are marked with arrows in the top panel.

were eliminated, and the α (559 nm), β (530 nm), and γ (428 nm) bands of cytochrome b_{559} emerged clearly. This method permitted the use of the extinction coefficient (18) to estimate that the content of cytochrome b_{559} in KCs (4.5 ± 0.4 pmol/ 10^6 cells, mean \pm SE for four experiments) closely resembled that in mouse PCs (3.4 ± 0.2 pmol/ 10^6 in the same four experiments). These results are in agreement with those reported for mouse (30) and human neutrophils (32). Thus, at least one component of the oxidase was unequivocally present in KCs.

Release of Eicosanoids. The foregoing findings focused attention on the possibility that KCs were refractory to triggering. The triggering response in macrophages that most closely parallels secretion of reduced forms of O_2 is secretion of oxygenated forms of arachidonate. That is, the same triggering agents lead to metabolism of molecular O_2 over a similar time course by these two independent (28) pathways. Therefore, KCs and resident PCs were labeled with [3 H]20:4 and challenged with zymosan. KCs released $\sim 7\%$ of their 20:4, compared with $>20\%$ from PCs (Fig. 4). Two HPLC systems were used to identify the major oxygenated metabolites of the cyclooxygenase and lipoxygenase pathways (Fig. 5). Whereas PCs oxygenated 90% of the released 20:4, KCs oxygenated only $\sim 60\%$. Moreover, in contrast to PCs, KCs produced almost no leukotriene C or prostacyclin. These results indicated that zymosan could trigger a secretory response in KCs involving the reduction of O_2 by arachidonate, though not the reduction of O_2 by NADPH. Thus, any defect in triggering must

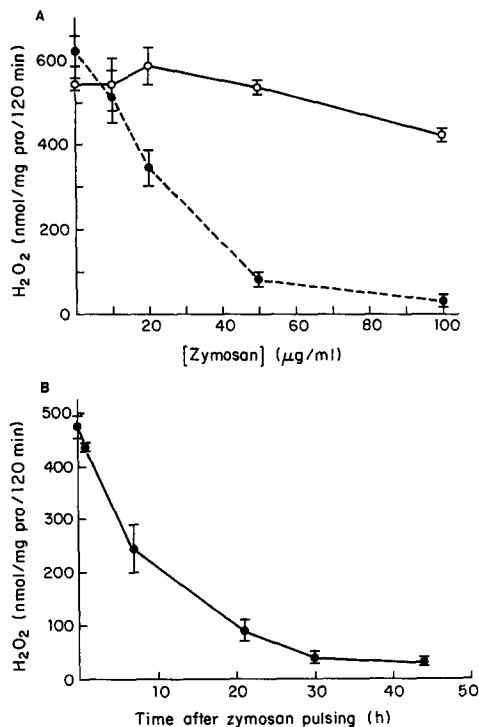


FIGURE 6. Delayed effect of prior exposure to zymosan on subsequent PMA-triggered H_2O_2 release from IO_4^- -elicited macrophages. (A) Periodate-elicited PCs in a 96-well plate were incubated with the indicated concentrations of zymosan for 1 h. PMA-triggered H_2O_2 release was assayed immediately (—○—) or 18 h later (—●—). (B) 50 $\mu g/ml$ of zymosan was added to macrophage monolayers at different time points for 1 h followed by its removal. All H_2O_2 -release assays were performed together 44 h after plating the cells. Time indicates the interval between zymosan pulsing and the assay. The results are representative of three experiments, and are means \pm SE for triplicates.

be relatively specific for the respiratory burst that produces superoxide. Of further interest, the pattern of 20:4 metabolism in KCs was strikingly similar to that reported for PCs recovered from the mouse peritoneal cavity 11–14 d after injection of formalin-fixed propionobacteria or heat-killed mycobacteria (34). Such PCs are probably in a postphagocytic state.

Induction of a Refractory State of the NADPH Oxidase in the PC. The above results suggested that physiologic exposure of KCs to particulates *in vivo* might have induced a selective state of refractoriness to triggering of the respiratory burst. To test this hypothesis, we used PCs and unopsonized zymosan particles. Unopsonized zymosan was selected because it interacts with several macrophage receptors. These include the mannosyl-fucosyl receptor (35) and CR3, via complement synthesized by the macrophages during the assay and fixed by the particles (36). Immediately after a 1-h exposure of activated macrophages to zymosan, the ability of the cells to release H_2O_2 in response to PMA was unimpaired (Fig. 6A). 18 h later, however, PMA-induced H_2O_2 release was almost completely suppressed by prior contact with zymosan, in a dose-dependent manner. H_2O_2 -releasing capacity was suppressed 50%, 18 h after a 1-h exposure to 20 $\mu g/ml$ of zymosan (Fig. 6A). A 1-h exposure to 50 $\mu g/ml$ zymosan induced 50% suppression of H_2O_2 -releasing capacity within 7 h (Fig. 6B). These conditions resulted in only ~29% of the cells containing zymosan particles (~1–3 particles per phagocytic cell).

To test whether a history of endocytic encounters could also influence the responsiveness of macrophages to activation by rIFN- γ , resident PCs were pulsed

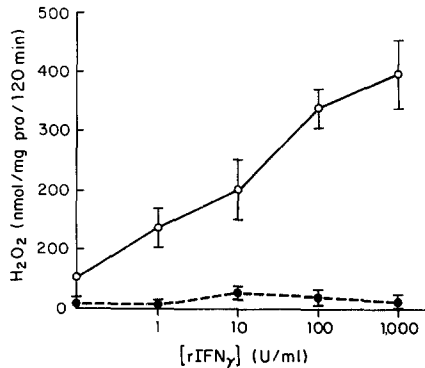


FIGURE 7. Effect of prior exposure of resident PCs to zymosan on their ability to be activated by rIFN- γ . Resident PCs in a 96-well plate were incubated with (—●—) or without (—○—) 50 μ g/ml of zymosan for 1 h. After washing, cells were incubated with different concentrations of rIFN- γ for 2 d before assaying H₂O₂ release in response to PMA. The results are representative of five experiments, and are means \pm SE for triplicates.

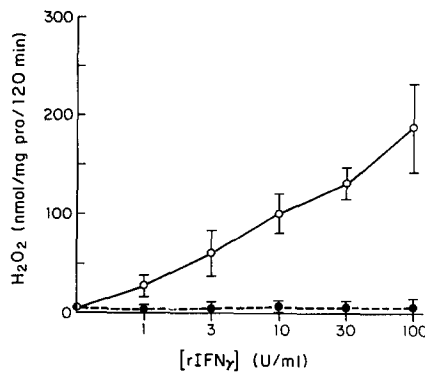


FIGURE 8. Responsiveness of KCs to rIFN- γ after prolonged culture in vitro. Monolayers of KC on 13-mm coverslips were cultured in 24-well plates for 1 (—●—) or 4 (—○—) d, then incubated with the indicated concentrations of rIFN- γ for an additional 3 d before H₂O₂ release was assayed in response to PMA. The results are representative of five experiments, and are means \pm SE for triplicates.

for 1 h with 50 μ g/ml of zymosan, washed, treated for 2 d with 1–1,000 U/ml rIFN- γ , washed again, and triggered with PMA. As shown in Fig. 7, zymosan-pulsed PCs were completely refractory to enhancement of respiratory burst capacity by rIFN- γ at all doses tested, and thus came to resemble KCs.

The experiments illustrated in Figs. 6 and 7 were also performed using hepatic debris in place of zymosan. The debris consisted of the supernate of the low-speed spin of the liver digest from which KCs were isolated. This supernate contained collagenase, perfusate, and broken hepatocytes. Results were the same as with zymosan: the respiratory burst of PCs was neither triggered, nor rendered refractory to triggering when tested immediately after a 1-h exposure to hepatic debris at 37°C. 24–48 h later, however, H₂O₂-releasing capacity was markedly suppressed, and was resistant to reinduction by rIFN- γ (data not shown). These findings suggest that the failure to detect a respiratory burst in KCs immediately after isolation was not a consequence of ingestion of hepatic debris during isolation.

Recovery of Respiratory Burst Capacity in KCs. If the oxidase were present in KCs, but rendered refractory to triggering by means of past phagocytic events, then time in culture in the absence of continued phagocytosis might permit demonstrable oxidase activity to be restored. No PMA-triggered respiratory burst was detected after culture of KC for 4 or 5 d, even with rIFN- γ for the last 3 d (five experiments; Fig. 8). However, culture of KCs for 7–19 d, the last

3 d in rIFN- γ , consistently resulted in substantial PMA-triggered H₂O₂ secretory capacity (nine experiments; Fig. 8).

Response of KCs to rIFN- γ In Vivo. To ascertain whether KCs' refractory state to activation by rIFN- γ antedated their isolation, we injected rIFN- γ intraperitoneally, using a protocol shown to activate intrahepatic inflammatory macrophages (37) as well as alveolar macrophages (38) and PCs (7). When KCs were isolated 1 d later, their PMA-induced H₂O₂ release remained at the limit of detection of the assay (23 ± 2 nmol H₂O₂/mg cell protein per 60 min). In contrast, PCs were fully activated in the same experiments (386 ± 30 nmol/mg cell protein per 60 min, compared with 35 ± 12 for PCs from mice injected with diluent alone). Thus, before they were isolated, KCs were already refractory to induction of respiratory burst capacity by rIFN- γ .

Discussion

Suppression of the respiratory burst may permit macrophages that line vascular sinuses to clear the circulation of senescent cells, immune complexes, and microbial products without inflicting oxidant damage on bystander cells. How could this specialized phenotype arise? We considered three possibilities: (a) by recruitment of sinus-lining cells from a distinct macrophage lineage lacking the NADPH oxidase; (b) by macrophage deactivation in response to cytokines such as macrophage-deactivating factor (MDF; reference 17) or transforming growth factor- β (TGF- β ; Tsunawaki, S., M. Sporn, A. Ding, and C. Nathan, manuscript submitted for publication) that might be released by platelets (39), phagocytizing macrophages (40), or other cells of the blood, vessel wall, or underlying organ; or (c) as a direct consequence of exposure to the ligands in whose clearance sinus-lining macrophages are continually engaged.

With regard to the first possibility, there is no evidence for a distinct macrophage lineage lacking respiratory burst activity. Judging from the cytochemical reaction with nitroblue tetrazolium, the capacity to mount a respiratory burst is a general property of blood monocytes (41), from which KCs arise (12). The presence of normal levels of cytochrome b₅₅₉ is strong evidence that KCs do, in fact, contain at least two components of the NADPH oxidase complex. The only known function of cytochrome b₅₅₉ is as a component of the oxidase; its presence is limited to cell types that normally have respiratory burst capacity; its rare spectral absence from such cells is associated with the absence of respiratory burst capacity and consequent chronic granulomatous disease (32). In the X-linked form of chronic granulomatous disease, loss or abnormality of DNA for the p90 component of the oxidase leads also to loss of the p22 component and loss of the spectral signal for the cytochrome, even though the affected DNA does not encode a protoporphyrin binding site (42). This suggests that the spectral signal of the cytochrome can only be expressed in a phagocyte that contains both of the currently established components of the oxidase-cytochrome complex (p90 and p22). Finally, the recovery of oxidase activity over 7 d in culture, in a nondividing cell population, establishes that KCs are oxidase-positive cells.

For the above studies, it was essential to develop a method for detection of cytochrome b₅₅₉ that would be free of interference from mitochondrial cyto-

chromes and ingested hemoglobin. The double-reduction technique devised here solved this problem. This new method (Ding, A., manuscript in preparation) should prove useful in the estimation of cytochrome b_{559} content in other cell preparations that contain mitochondria or hemoglobin.

The possibility that KCs have been deactivated in situ by either MDF or TGF- β is made unlikely by the distinctive oxidase kinetics in each of these situations. MDF selectively reduces the affinity of the oxidase for NADPH. Addition of mM concentrations of NADPH to permeabilized, MDF-treated macrophages restores the full rate of superoxide production seen with activated macrophages (17). On the other hand, the oxidase activity of permeabilized, TGF- β -treated macrophages is indistinguishable from that of control activated macrophages (Tsunawaki, S., M. Sporn, and C. Nathan, manuscript in preparation). Both patterns stand in striking contrast to the results with KCs, in which no oxidase activity at all could be detected under the same conditions.

PCs displayed a profound defect in the respiratory burst and became refractory to rIFN- γ -mediated enhancement of the burst, as a delayed consequence ($t_{1/2}$, 7 h) of prior ingestion of small numbers of zymosan particles. Suppression ensued under conditions in which the particles themselves triggered no detectable respiratory burst (data not shown). Similarly, lymphokine-induced tumoricidal activity was blocked by prior exposure of PCs to surfaces coated with immune complexes or to erythrocytes treated with glutaraldehyde or coated with IgG or IgM plus complement (43). Likewise, brief exposure of PCs to subnanogram/ml concentrations of bacterial LPS prevented rIFN- γ -induced enhancement of PC H_2O_2 -releasing capacity (44). Each of these observations with PCs may be relevant to the defect in KCs. Erythrophagocytosis is a well-known function of KCs, which left its mark in the present experiments in the form of intense spectral signals from hemoglobin in the absence of morphologically detectable intracellular erythrocytes. KCs are a major site for the clearance of both immune complexes (45) and LPS (46).

Thus, the defect in the respiratory burst of KCs probably arises as a delayed consequence of exposure to senescent erythrocytes, LPS, immune complexes, and/or other ligands destined for endocytosis. In both their eicosanoid metabolism and LFA-1 expression, KCs closely resembled PCs several days after exposure to mycobacteria or propionobacteria, in striking contrast to the pattern in resident PCs (25, 34). These results are consistent with the idea that KCs are in a postendocytic state. A similar mechanism may explain the suppressed respiratory burst of the resident sinus-lining macrophages of murine marrow (9). However, at least one serosal macrophage population, that in the pulmonary alveoli, can show morphologic evidence of extensive endocytosis without loss of respiratory burst capacity or its responsiveness to enhancement by rIFN- γ (47). In addition, ingestion of at least one type of particle, latex beads, does not lead to gradual suppression of the respiratory burst of resident PCs (data not shown). It is not clear what determines these differences.

By what mechanism may continual, low-level endocytosis of certain substances induce selective, gradual, profound, long-lasting, reversible inhibition of the respiratory burst? The mechanism appears to be distinct from the apparent exhaustion of respiratory burst capacity described earlier (23, 24). In the latter

situation, suppression of a second burst was evident immediately upon conclusion of the first burst, and in proportion to the magnitude of the first response. However, it is possible that subliminal triggering of the burst over a long period of time may lead gradually to the same functional state. In any case, this distinctive form of macrophage deactivation remains biochemically unexpected and unexplained.

In susceptible macrophage populations, deactivation after ingestion of small particle loads takes many hours to be expressed, and thus is unlikely to interfere with the antimicrobial action of immigrant monocytes, whose endocytic encounters with microbes should occur early upon entry into an inflammatory site. On the other hand, postendocytic suppression of the respiratory burst persists for days, without impairment in the cell's capacity for further phagocytosis. As such, this phenomenon may play a physiologic role in conditioning macrophages for prolonged service as scavengers of noninfectious particles, not only in certain vascular sinuses, but perhaps also in remodeling tissues and healing wounds.

Summary

Murine Kupffer cells (KCs), which constitute one of the largest populations of tissue macrophages, differ from most other cells of the myelomonocytic lineage in lacking the capacity for a respiratory burst. A collagenase perfusion technique followed by adherence to plastic at low temperature yielded pure cultures of KCs uniformly expressing receptors for Fc and C3bi, and containing virtually no morphologically detectable intracytoplasmic debris. Such KCs took up and oxidized glucose via the hexose monophosphate shunt about the same as peritoneal macrophages (PCs). Respiratory burst stimuli failed to enhance the hexose monophosphate shunt in KCs, probably because no H_2O_2 was produced. Detergent-permeabilized KCs generated no O_2^- in the presence of 1 mM NADPH, in striking contrast to all PC populations studied. Yet, KCs contained at least one component of the O_2^- -producing oxidase, cytochrome b_{559} , in the same quantities as PCs and neutrophils. Cytochrome b_{559} was demonstrated by a novel double-reduction spectral technique that eliminated interference from hemoglobin and mitochondrial cytochromes. Consistent with the presence of the oxidase, KCs acquired normal respiratory burst capacity after prolonged incubation in vitro. The defect in triggering the respiratory burst in KCs was selective for the reduction of O_2 by NADPH, in that reduction of O_2 by endogenous arachidonate was readily demonstrable in response to zymosan. The percent of arachidonate released, the percent oxygenated, and the suppression of prostacyclin and leukotriene C production, as well as the pattern of LFA-1 expression, all resembled the pattern reported with PCs several days after exposure to bacteria. Indeed, exposure of PCs to low numbers of zymosan particles led gradually to complete suppression of respiratory burst capacity and refractoriness to its enhancement by rIFN- γ , as evident in KCs both before and after their explantation. Thus, the modulation of oxidative metabolism that characterizes KCs probably arises from frequent endocytic encounters. This phenomenon may permit macrophages to act as scavengers without oxidative damage to bystander cells.

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