Hydrogen Peroxide Metabolism in Human Monocytes during Differentiation In Vitro

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ABSTRACT The capacity of human blood monocytes to secrete hydrogen peroxide (H_2O_2) and superoxide (O_2^-) was measured as the cells differentiated during 4 wk of culture. Morphologic transformation of monocytes into macrophages, epithelioid cells, and multinucleated giant cells accompanied a steady increase in the content of protein per cell, from 0.77 mg/10^7 cells on days 0 to 11.77 mg/10^7 cells on days 20 to 29. In contrast, secretion of H_2O_2 by adherent monocytes was 859±73 nmol/60 min per mg protein (mean ±SEM, n = 18) on day 0, rose 40% on day 3, and then fell rapidly, remaining below 6% of the initial values after day 10. The decline in capacity to secrete reactive oxygen intermediates was observed whether H_2O_2 or O_2^- were measured, whether the cells were challenged with phorbol myristate acetate or with opsonized zymosan, and whether the results were expressed per milligram cell protein or per cell.

Superoxide dismutase activity tripled in adherent monocytes from day 0 to day 3, and thereafter remained elevated through at least day 16. In contrast, the activity of myeloperoxidase declined rapidly, catalase and glutathione peroxidase declined more gradually, and glutathione reductase and glutathione remained constant throughout the period of observation. Thus, the decline in capacity to secrete H_2O_2 could not be attributed to increases in cellular levels of these antioxidants.

On the first day of culture, H_2O_2 release was enhanced up to fourfold by inclusion of sodium azide or potassium cyanide in the assay medium. This enhancement appeared to be due to inhibition of monocyte myeloperoxidase, rather than catalase. This conclusion was based on the kinetics and dose-response relationships for the effects of azide and cyanide on H_2O_2 release and on the activities of catalase and myeloperoxidase.

Thus, the differentiation of human monocytes into macrophages in vitro is accompanied by an apparent reduction in the capacity to produce H_2O_2 and O_2^-. In this regard, the human monocyte-derived macrophage comes to resemble the resting tissue macrophage previously characterized in the mouse peritoneal cavity.

INTRODUCTION

More than 50 years ago, investigators observed blood monocytes differentiate into macrophages, epithelioid cells, and giant cells over days or weeks in vitro (1–3). Such observations have been confirmed repeatedly (4, 5) and extended to document increases in cell size, protein content, lysosomal and ectoenzyme activity, surface receptor expression, and phagocytic capacity (4, 6–12). This differentiation sequence in vitro closely parallels that observed when monocytes emigrate from the vasculature into the tissues (13–15).

The freshly isolated human blood monocyte responds to soluble and particulate surface-active stimuli by the secretion of superoxide anion (16–20) and hydrogen peroxide (17) in amounts similar to those released by granulocytes (17, 18). The production of these reactive oxygen intermediates is of considerable interest, in view of mounting evidence for their involvement in some of the antimicrobial (21–24) and antitumor (25–27) activities of mononuclear phagocytes, together with the recent finding that monocyte superoxide release is suppressed in some patients with cancer and elevated during certain infections.[1]

In the mouse, macrophages from uninflamed tissues secrete little O_2^- and H_2O_2 (28–30). When they have

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[1] Nakagawara, A. Submitted for publication.

been activated by exposure to inflammatory agents or lymphocyte mediators, their capacity to secrete these substances increases toward the high levels observed with human blood monocytes (23, 29–31). We hypothesized, therefore, that the ability of the blood monocyte to secrete large amounts of O_2^- and H_2O_2 might (*a*) normally involute during its maturation into a macrophage; and (*b*) that this capacity might be retained or regained in the presence of lymphocyte mediators. The present study tests the first part of this proposed sequence of differentiation.

METHODS

Preparation of mononuclear leukocytes (MNL).[2] Healthy adult volunteers of both sexes were phlebotomized at the Greater New York Blood Center. 450 ml blood was collected into 63 ml of anticoagulant CDPA-1 (hydrous dextrose, 1.61 g; sodium citrate dihydrate, 1.66 g; anhydrous citric acid, 0.19 g; sodium biphosphate, 0.14 g; adenine, 0.017 g). We obtained the buffy coat 2–3 h after venipuncture, and separated MNL by a modification of the method of Böyum (32). Buffy coat was diluted with an equal volume of 0.9% NaCl, and 30 ml of the cell suspension was layered on 15 ml of Ficoll-Hypaque (d = 1.077, Pharmacia Fine Chemicals, Piscataway, N. J.) in 50-ml polypropylene conical tubes (No. 2533, Corning Glass Works, Corning, N. Y.). After centrifugation at 700 *g* for 20 min at 25°C, the upper cell layer was collected and centrifuged at 400 *g* for 10 min at 4°C. The sedimented cells were suspended in 20 ml of RPMI 1640 (Flow Laboratories, Rockville, Md.) and centrifuged at 100 *g* for 10 min at 4°C. The final pellet was resuspended in RPMI 1640 containing 25% human serum (see below), 100 μg/ml streptomycin and 100 U/ml penicillin ("medium") to give 1×10^7 MNL/ml. The average yield of MNL at this stage was 3.5×10^8 cells (mean of 13 experiments), with a differential count of 32% monocytes, 67% lymphocytes, and 1% granulocytes after staining with Diff-Quik (Harleco, American Hospital Supply Corp., Philadelphia, Pa.). Of the mononuclear cells, ~27% (mean of nine experiments) were peroxidase positive by the method of Kaplow (33).

Preparation of serum. 450 ml of human blood of any blood type was obtained from the Greater New York Blood Center immediately after collection without anticoagulant. The blood was held at room temperature for 60 min, and then at 4°C for 75 min, before centrifugation at 140 *g* for 10 min at 4°C. The supernate was centrifuged again at 1,500 *g* for 15 min at 4°C, filtered (0.45 μm, Nalge Co., Rochester, N. Y.), aliquotted, and stored at −80°C until use.

Culture of monocytes. 13-mm Diam glass coverslips (Clay-Adams, New York) were soaked in 70% ethanol for at least 5 d, then dipped in 95% ethanol, flamed, and placed in 100-mm plastic petri dishes. 1×10^6 MNL in 0.1 ml medium were layered carefully over each coverslip. After incubation for 2 h at 37°C in 5% CO_2/95% humidified air, the petri dish was filled with Hanks' balanced salt solution (HBSS, Flow Laboratories, McLean, Va.) at 37°C and gently agitated. The HBSS was removed, and the rinsing process repeated twice more. Individual coverslips were then transferred to 16-mm Diam plastic wells in 24-well trays (Costar Data Packaging, Cambridge, Mass.) in 0.3 ml of medium. The medium was replaced on days 1, 3, 5, and 7, and thereafter every 4 d. For enzyme and glutathione assays, 4×10^7 MNL were plated in 2 ml medium in 35-mm Diam Pyrex glass petri dishes, or 1×10^8 MNL in 6 ml medium were plated in 85-mm Diam Pyrex dishes, and otherwise processed as for coverslips.

Cell counts and protein measurements. Coverslips were washed four times in 0.9% NaCl, drained briefly on absorbent paper, and transferred to a dry Costar well. 0.05–0.20 ml of nuclear staining solution was layered over each coverslip, consisting of 0.1 M citric acid, 0.05% naphthol blue black (Allied Chemical Corp., New York), and 1.0% Triton X-100. After 30 min at room temperature, the suspension of nuclei was mixed in a pipette tip, transferred to a hemocytometer, and counted. Separate coverslips stained with Diff-Quik were used to determine the average number of nuclei per cell after counting 400 cells. The number of nuclei per coverslip was divided by the average number of nuclei per cell to give the number of cells per coverslip. Matched coverslips were rinsed in 0.9% NaCl as above, dried, dissolved in 0.3 ml of 0.5 N NaOH, and assayed for protein by the method of Lowry et al. (34) with bovine serum albumin as a standard. Determinations of cell number and of adherent cell protein were performed in triplicate at each time point.

Measurement of H_2O_2 release. In preliminary experiments, H_2O_2 release from monocytes adherent to 13 × 27-mm glass coverslips was observed continuously by measuring the oxidation of scopoletin (Sigma Chemical Co., St. Louis, Mo.) in the presence of 1 purpurogallin unit (PU) of horseradish peroxidase (HPO), Type II (Sigma) in 3.0 ml of modified Krebs-Ringer phosphate buffer with glucose (KRPG) (137 mM NaCl, 4.9 mM KCl, 0.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.5 mM glucose, 5.7 mM sodium phosphate, pH 7.35) in the presence of 1 mM NaN_3 (Fisher Scientific Co., Fair Lawn, N. J.) in a 37°C thermostated Perkin-Elmer-Hitachi MPF-44A fluorometer, as described (28). After stimulation with phorbol myristate acetate (PMA) (Consolidated Midlands, Brewster, N. Y.) or opsonized zymosan (see below), H_2O_2 release commenced within 3 min, continued at a nearly constant rate for ~40 min, and ceased at ~70–90 min. Therefore, standard assays were performed as follows. Adherent monocytes on 13-mm Diam coverslips were washed thoroughly by swirling the coverslip in four successive beakers of 0.9% NaCl at room temperature, drained on absorbent paper, and placed in 16-mm Costar wells containing 1.5 ml KRPG, from 2 to 35 nmol scopoletin, 0.5 PU HPO, and 150 ng PMA (dissolved in dimethylsulfoxide [DMSO], with final DMSO concentration of 0.033%). As controls, cell-free coverslips were rinsed in the same manner and carried through the assay. The Costar trays were placed in a 37°C water bath for 60 min, the supernatant fluid removed, and its fluorescence determined at room temperature as described (28). To use opsonized zymosan as a stimulus, 40 mg zymosan (Sigma) was suspended in 5 ml 0.9% NaCl with 10% human serum prepared as described above. After 30 min at 37°C, the particles were washed twice by centrifugation in 0.9% NaCl and stored at −80°C until use. 2.0×10^7 zymosan particles were added to the H_2O_2 reaction mixture instead of PMA. The Costar plate was centrifuged at 340 *g* for 2 min at 25°C to initiate contact of the particles with the monocytes. All H_2O_2 release assays were performed with triplicate coverslips. In preliminary experiments, no H_2O_2 release was detected in the absence of PMA or zymosan. These controls were omitted from subsequent experiments.

Measurement of O_2^- release. 13-mm Diam coverslips containing adherent monocytes were rinsed as for the H_2O_2 assay and transferred to Costar wells containing 0.75 ml

[2] *Abbreviations used in this paper*: GPO, glutathione peroxidase; GR, glutathione reductase; MNL, mononuclear leukocytes; HPO, horseradish peroxidase; KRPG, Krebs-Ringer phosphate buffer with glucose; MPO, myeloperoxidase; PMA, phorbol myristate acetate; SOD, superoxide dismutase.

KRPG with 100 μM ferricytochrome *c* (Sigma) with or without 40 μg superoxide dismutase (SOD) (Sigma). After 10 min at 37°C, PMA (75 ng) or opsonized zymosan (2.0×10^7) were added. The particles were sedimented by centrifugation at 340 *g* for 2 min at 25°C. After 60 min at 37°C, the trays were cooled to 4°C and the zymosan removed from the medium by centrifugation at 400 *g* for 5 min. Superoxide release was determined from the optical density at 550 nm of samples without SOD, minus the OD_{550} of matched samples containing SOD, using a molar extinction coefficient of 21.0×10^3 cm^{-1}. All samples were assayed in triplicate.

Myeloperoxidase. The 0.05% Triton X-100 lysate of rinsed, adherent monocytes was assayed for peroxidatic activity using *o*-dianisidine (Sigma) as substrate by the method of Steinman and Cohn (35). Triton did not interfere with the assay.

Catalase. The 1.0% Triton X-100 lysate was centrifuged at 4°C for 15 min in an Eppendorf microfuge (Brinkmann Instruments, Westbury, N. Y.). The supernate was assayed for catalase by the method of Baudhuin et al. (36). Triton did not interfere with the assay. In addition, the generation of oxygen from exogenous H_2O_2 in the presence of cell lysate was measured polarographically with a Clark type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) by the method of Roos et al. (37).

Glutathione peroxidase (GPO) and glutathione reductase (GR). The supernatant of 0.2% Triton X-100 lysates was assayed for GPO by the method of Paglia and Valentine (38) and for GR by the method of Roos et al. (39).

Superoxide dismutase. The supernate of the 0.2% Triton X-100 lysate was assayed according to McCord and Fridovich (40) using sufficient lysate to obtain 50% inhibition of the rate of reduction of cytochrome *c*, which was defined as 1 U of SOD under the stated conditions (40). Under these conditions, there appeared to be no significant contribution by cytochrome oxidase or cytochrome peroxidase, because the observed activity was unaffected by 20 μM KCN (41).

Glutathione. The 0.05% Triton X-100 lysate was deproteinated with 2.5% sulfosalicylic acid, and the supernate analyzed by the method of Tietze (42). Values are reported as nanomoles of the tripeptide (reduced plus oxidized) per milligram cell protein.

Statistics. Data shown from individual experiments are the means of triplicate samples except as noted in the figure legend. Results of pooled experiments are given as means ±SEM where each datum is the mean of triplicates. In Fig. 5, results were shown as means±SD. Significance was determined by Student's *t* test.

RESULTS

Differentiation of monocytes into macrophages in vitro. Our culture conditions supported the long-term survival and maturation of monocytes into macrophages, epithelioid cells, and giant cells, as assessed by morphology and measurement of protein content per coverslip and per cell. As shown in Fig. 1, the adherent protein content per coverslip was nearly constant for the first 3 d of culture, then declined to ~40% by day 7. The nonadherent cells collected during the 1st wk were 60% viable by trypan blue exclusion. Their ability to release H_2O_2 is discussed below. After day 7, the adherent protein content per coverslip increased steadily, coincident with the appearance of binucleate cells (from days 5–6) and of giant cells with 3–16 nuclei (from day 7). Giant cell formation was observed in the

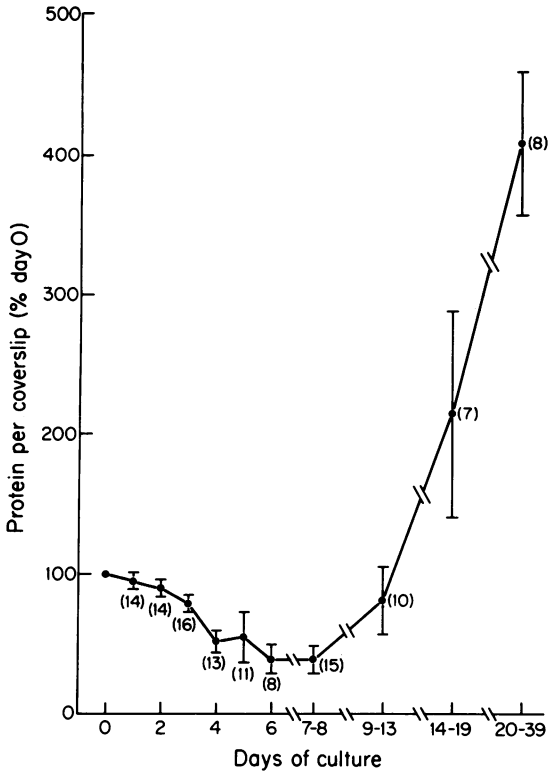

FIGURE 1 Change in adherent cell protein per coverslip during culture of human monocytes. The day 0 value was 18.2±1.1 μg/coverslip (mean±SEM, n = 17). Values are expressed as means±SEM for the number of experiments shown in parentheses. Each experiment had triplicate coverslips.

cultures from ~60% of donors; in these samples, giant cells comprised from 2 to 80% of the cells after the 2nd wk. The protein content per adherent cell increased from 0.77±0.05 mg/10^7 cells (n = 7) on day 0 to values that were approximately twice as great on day 4, three times as great on day 7, and 15 times as great on days 20–29 (Fig. 2).

H_2O_2 release from adherent monocytes: assay conditions. In matched coverslips, values for H_2O_2 release exceeded those for O_2^- release (Table I, line 1). With complete recovery of both substances, the expected ratio would be the reverse, namely $2:1::O_2^-:H_2O_2$ (41). Therefore, we devoted most experiments to measurements of H_2O_2 release, which apparently provided a better estimate of the cells' capacity to reduce molecular O_2. In preliminary experiments, concentrations of PMA over the range 0.01 to 1,000 ng/ml and of opsonized zymosan over the range 10 to 150 particles per cell, were tested. Half-maximal stimulation by PMA required 2.5 ng/ml; maximal stimulation was seen with 10 ng/ml (16.7 nM). Maximal stimulation by opsonized zymosan followed the addition of 40 particles/cell. Under these conditions, >95% of the cells ingested zymosan particles, whether tested on day 0

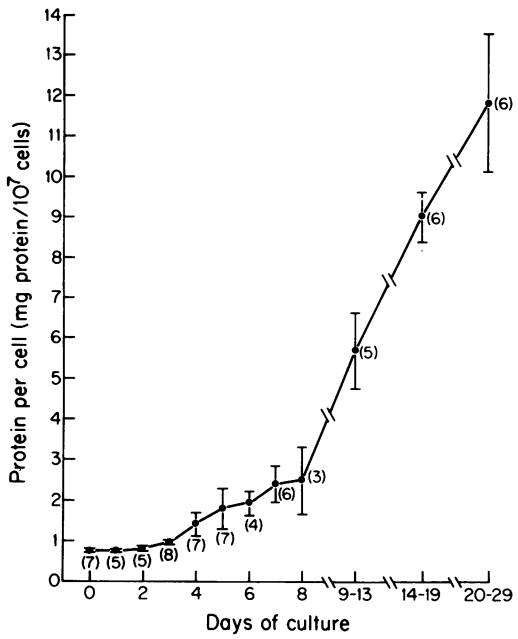

FIGURE 2 Change in protein per cell during maturation of human monocytes in vitro. Cell number per coverslip was determined as in Methods. Values given are means±SEM for the number of experiments shown in parentheses (each in triplicate).

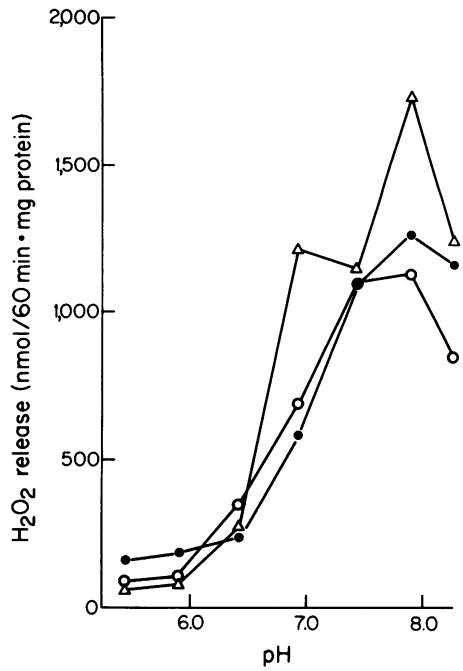

FIGURE 3 Effect of pH on H_2O_2 release by monocytes or monocyte-derived macrophages. The pH of phosphate buffer in KRPG was adjusted to the indicated values while holding phosphate concentration constant. The reaction was triggered by 100 ng/ml PMA. ●, day 0; ○, day 1; △, day 5.

or on day 14 of culture. The dependence of H_2O_2 release upon pH of the assay buffer was studied on day 0, day 1, and day 5, and gave similar profiles at each time (Fig. 3), with optimal values between pH 7.2 and 8.2. The abrupt decrease in H_2O_2 release at acid pH was not due to the inhibition of HPO by azide at low pH, because authentic H_2O_2 oxidized the same amount of scopoletin in the range from pH 5.3 to pH 8.0. Cell density has been reported to affect O_2^- release from mouse macrophages (29). Therefore, we compared H_2O_2 release from adherent monocytes as a function of the number of cells per coverslip. As shown in Fig. 4,

TABLE I

Comparison of Superoxide and Hydrogen Peroxide Release from Monocytes Induced by PMA or Opsonized Zymosan at Different Times in Culture

Days of culture	PMA*		Opsonized zymosan‡	
	O_2^-	H_2O_2	O_2^-	H_2O_2
	nmol/60 min/mg protein			
0	344±36	877±32	199±17	612±33
3	195±48	1,001±121	143±31	364±87
14	4±2	20±10	0±0	31±6

* 100 ng/ml.
‡ 100 particles added per cell.
§ Mean±SEM for triplicates.

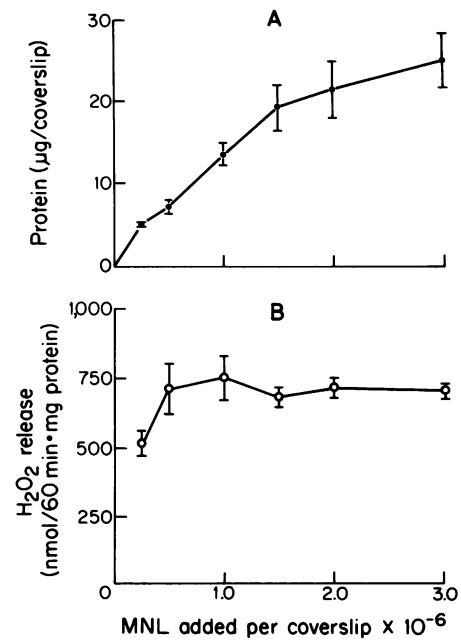

FIGURE 4 Effect of cell density on H_2O_2 release by day 0 monocytes. The various concentrations of mononuclear leukocytes suspended in culture medium were mounted on coverslips, and incubated for 2 h at 37°C in 5% CO_2-air. After vigorous washing, (A) the protein content and (B) PMA-induced H_2O_2 releasing activity were measured. Values are expressed as means±SD from four experiments.

H_2O_2 release per milligram protein was independent of the number of monocytes adhering.

Changes in H_2O_2 release with time in culture. On day 0, adherent monocytes from 18 donors released an average of 859±73 nmol H_2O_2/60 min per mg protein after stimulation by PMA. As shown in Fig. 5, there was a statistically significant decline to 73% of the initial value on day 1, followed by a statistically significant increase to 140% of the initial value on day 3. Thereafter, H_2O_2 release decreased rapidly. After day 10, values were below 6% of the day 0 value. The foregoing values were expressed per milligram cell protein. However, overall results were similar if the values were expressed per cell, even though the cells increased progressively in size during the same period (Fig. 6). The decline in H_2O_2-releasing capacity of adherent cells was not due to a selective detachment of more active cells. When the cells detaching from day 0 to day 5 were collected and assayed in suspension, they released 6.9 nmol H_2O_2/60 min per 10^5 cells, a value almost the same as that of the adherent cells tested on day 5.

Using opsonized zymosan as a stimulus instead of PMA, H_2O_2 release also declined markedly from day 0 to day 14 (Table I). However, values on day 3 were lower than on day 0, rather than being somewhat elevated, as seen with PMA (Table I). The time-dependent decline in H_2O_2 release in response to opsonized zymosan could not be attributed to differences in phagocytosis of the particles. The percentage of phagocytic cells remained constant (~95%), and the mean number of particles per cell actually increased from day 0 (5) to day 14 (35) (means of two experiments).

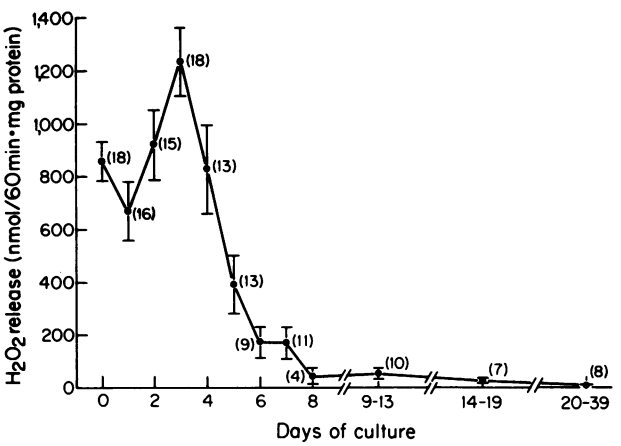

FIGURE 5 Change in H_2O_2 release based on mg cell protein during culture of human monocytes in vitro. Values are expressed as means±SEM for the number of experiments shown in parentheses, each done in triplicate. The reaction was triggered by 100 ng/ml PMA. Except for days 2 and 4, each point is significantly different from the day 0 point. The *P* values are: <0.1 (day 1), <0.01 (days 3 and 5), and <0.001 (all other points).

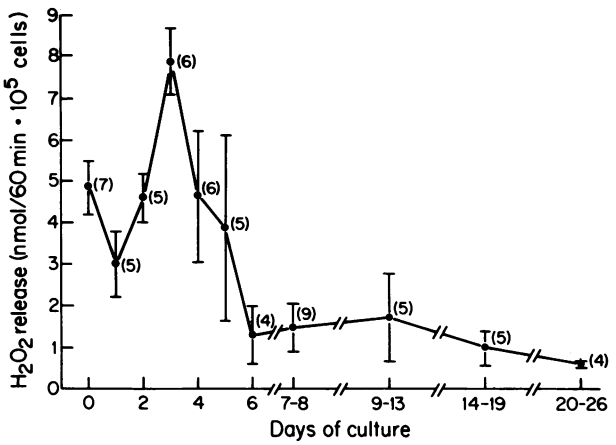

FIGURE 6 Change in H_2O_2 release based on cell number during culture of monocytes. PMA (100 ng/ml) was used as the triggering agent. Means±SEM (number of experiments).

Changes in O_2^- release with time in culture. With either PMA or opsonized zymosan as a stimulus, O_2^- release was about one-third as great as H_2O_2 release on day 0, and declined progressively to barely detectable levels by day 14 (Table I).

Changes in scavengers of O_2^- and H_2O_2 during differentiation. As shown in Table II, the specific cellular activity of SOD increased an average of threefold on day 3 compared with day 0. This was of considerable interest, in view of the increased release of H_2O_2 and the decreased release of O_2^- observed on day 3. Thereafter, SOD levels remained nearly constant through day 16. In contrast, catalase levels declined after day 3, reaching <10% of initial values by the 3rd wk of culture. GPO decreased less markedly, reaching about one-third of the initial value by day 22. GR and glutathione levels were nearly constant throughout the same period. Finally, myeloperoxidase (MPO) activity decreased rapidly after day 0 (Table III). Thus, there was no evidence that an increase in peroxide-catabolizing pathways could account for the decreased release of H_2O_2 observed during differentiation of monocytes in culture.

Importance of inhibition of myeloperoxidase for measurement of H_2O_2 release. As noted, changes in the activities of H_2O_2-catabolizing substances with time in culture did not appear to account for decreases in H_2O_2 release after day 4. However, intracellular H_2O_2-scavengers did exert a profound effect on observed levels of H_2O_2 release during the first 3 d of culture. This was reflected by the fact that H_2O_2 release was 4.3-fold greater on day 0 in the presence of 1 mM NaN_3 than in its absence. The H_2O_2 release-enhancing effect of azide decreased with time in culture in parallel with the decrease in specific activity of myeloperoxidase (Table III), but without relation to levels of

TABLE II

*Changes in O_2^- and H_2O_2 Scavengers during Differentiation of Human Monocytes In Vitro**

Days of culture	H_2O_2 release	SOD	Catalase	GPO	GR	GSH + GSSG‡
	nmol/60 min/ mg protein	*U/mg protein*	*$\times 10^{-1}$ BU/ mg protein§*	*nmol/min/mg protein*	*nmol/min/mg protein*	*nmol/mg protein*
0	750±16	13±3	25.1±0.4	264±8	71±2	41.3±0.4
3	1339±81	41±10	28.7±1.2	243±6	66±2	37.4±1.0
10	194±19	36±12	15.5±1.1	160±10	76±7	45.6±1.2
22	ND‖	46±23**	2.2±0.1	80±7	77±2	32.9±0.5

* Mean±SD for triplicates from a single donor, except three separate experiments for SOD on day 0 and two experiments for SOD on days 3, 10, and 16.
‡ GSH, reduced glutathione; GSSG, oxidized glutathione.
§ BU, Baudhuin Units.
‖ Not done.
** Tested on day 16.

catalase (Table II). To investigate further whether the enhancement of H_2O_2 release by azide was due primarily to inhibition of MPO or to inhibition of catalase, the dose-response curves for all three effects of azide were compared for monocytes on day 0 (Fig. 7). The release of H_2O_2 was augmented twofold, that is, 50% of the maximal increase, by 5 μM azide. Essentially the same dose of azide (4.2 μM) afforded 50% inhibition of MPO activity. Maximal stimulation of H_2O_2 release coincided with 86% inhibition of MPO activity, at 50 μM azide. In contrast, catalase was inhibited 50% by as little as 0.5 μM azide, a dose that enhanced H_2O_2 release only by 24%. 90% inhibition of catalase by 5 μM azide was associated with only a 2.1-fold increase in H_2O_2 release. There was a small decrease in H_2O_2 release with concentrations of azide >1 mM. This was not due to inhibition of the HPO used in the assay for H_2O_2 (data not shown).

The differential effects of inhibition of MPO and of catalase were even more striking using KCN instead of NaN_3, as shown in Fig. 8. Again, inhibition of MPO and augmentation of H_2O_2 release followed precisely the same dose-response curve. In contrast, complete inhibition of catalase by lower doses of KCN afforded no increase in H_2O_2 release. Thus, MPO rather than catalase appears to be responsible for profoundly reducing the amount of H_2O_2 that can be detected in the extracellular medium when monocytes are assayed within the first 3 d of culture.

TABLE III

*Effect of Azide on H_2O_2 Release: Correlation with Spontaneous Decline in MPO Activity**

Days of culture	H_2O_2 Release			Peroxidase activity
	+NaN_3 (1 mM)	−NaN_3	+NaN_3/−NaN_3	
	nmol/60 min/mg protein			*$\times 10^{-2}$ U/ mg protein*
0	599	139	4.31	24.8
1	291	90	3.23	14.6
2	510	439	1.16	8.6
3	670	511	1.31	3.2
4	102	118	0.86	2.2
6	28	41	0.68	1.2

* Values are means of triplicates.

DISCUSSION

Recent evidence implicating reactive oxygen intermediates in some of the antitumor and antimicrobial functions of mononuclear phagocytes (21–27) prompted this study of oxidative metabolism during the differentiation of human blood monocytes in vitro. As monocytes matured into macrophages, their capacity to release O_2^- and H_2O_2 declined precipitously. Over the same time, their specific activity of SOD increased;

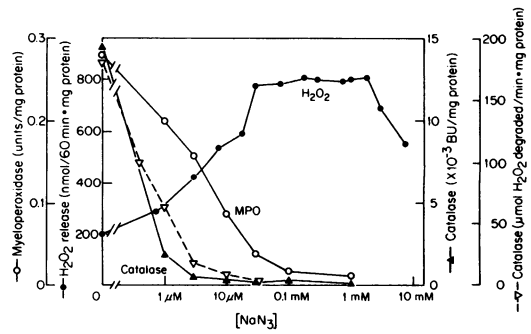

FIGURE 7 Effect of sodium azide on H_2O_2 release, catalase and myeloperoxidase activities of day 0 monocytes. H_2O_2 release was triggered with PMA (100 ng/ml). Catalase was measured both spectrophotometrically and polargraphically (Methods). Data are means from four experiments, in one of which the same cell preparation was used for intact cells and for lysates. BU, Baudhuin units.

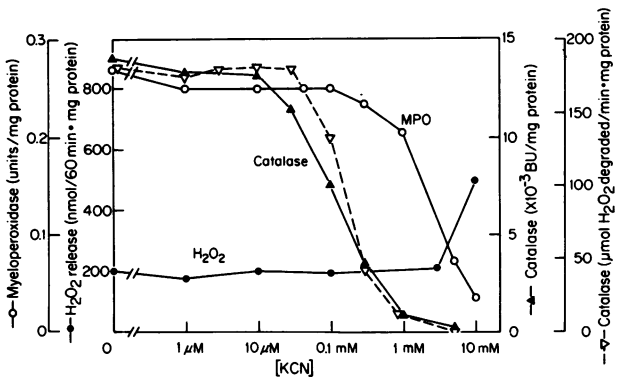

FIGURE 8 Effect of potassium cyanide on H_2O_2 release, catalase and myeloperoxidase activities of day 0 monocytes. See legend to Fig. 8.

MPO, catalase, and GPO decreased; and GR and the content of glutathione per milligram cell protein remained the same. Thus, the decrease with time in H_2O_2 release can probably not be attributed to increased catabolism of H_2O_2. Most likely, diminished capacity to release O_2^- and H_2O_2 reflected diminished capacity to produce them.

The validity of these conclusions rests on the suitability of the assay conditions for adherent monocytes. The culture conditions supported the morphologic differentiation of monocytes into macrophages and frequently into giant cells, with a 15-fold increase in the protein content per cell over a 4-wk period. We compared the release of O_2^- and H_2O_2, in response to both soluble and particulate stimuli at optimal doses, under conditions not limited by pH or cell density, with inhibition of intracellular peroxidase and catalase. The same trends were observed whether the results were expressed per milligram cell protein or per cell.

At each time point tested, the H_2O_2 detected in the extracellular medium markedly exceeded the O_2^- detected from monocytes on matched coverslips. If H_2O_2 arose from dismutation of O_2^- outside the cell, and if both compounds were detected with comparable efficiency, then one would expect to detect twice as much O_2^- as H_2O_2 (41). Our contrary findings may suggest that oxygen is reduced to O_2^- within the monocyte, at a site accessible to SOD, so that H_2O_2 is more likely to be secreted than O_2^-, when H_2O_2-catabolizing enzymes are inhibited. In support of this possibility was the decline in O_2^- release on day 3, at a time when SOD activity increased threefold and H_2O_2 release increased 1.4-fold, compared with day 0. Alternatively, some portion of oxygen reduction in monocytes may proceed by a 2-electron reaction, without an O_2^- intermediate. Finally, neither the assay for O_2^- nor the assay for H_2O_2 approaches 100% efficiency (43). The ratios of the two compounds detected may simply reflect the relative efficiencies of the assays under the conditions used. Reiss and Roos (17) reported that freshly isolated human monocytes stimulated in suspension with opsonized zymosan released ~0.8 nmol of both H_2O_2 and O_2^-/min per 10^6 cells. Our value for H_2O_2 release on day 0 agrees closely with theirs. The difference between the findings for O_2^- release may be attributed in large part to our use of adherent cells (unpublished observations).

The predominant enzymatic scavengers of H_2O_2 in monocytes are probably MPO, catalase, and GPO. We do not know of a specific inhibitor of GPO, a selenoenzyme (44). Both azide and cyanide inhibit the heme enzymes, MPO and catalase. We found that MPO was much more important than catalase in limiting the amount of H_2O_2 detected in the extracellular medium. This conclusion was based on two findings. First, the dose-response curves were identical when comparing (*a*) the inhibition of MPO by azide or cyanide and (*b*) their augmentation of H_2O_2 release. The same studies dissociated the inhibition of catalase from the augmentation of H_2O_2 release. Second, the enhancing effect of azide on H_2O_2 release decreased over 2 d, precisely in parallel with the decline in MPO levels, but without relation to the decline in catalase, which was more gradual. The implications of the differential scavenging effects of MPO and catalase are not yet clear as regards the probable site of H_2O_2 formation in the monocyte. MPO is located in granules (45). However, some MPO is released into the extracellular medium during stimulation of the cells (46). We detected 6% of the MPO in the medium after exposure to PMA. MPO may then adhere to the outer face of the plasma membrane. The location of catalase in human monocytes is not certain. It may be cytosolic (47) or localized in granules (48). In a study of catalase-deficient subjects, Roos et al. concluded that catalase protects monocytes from external oxidative stress, whereas the glutathione redox cycle is more predominently involved in scavenging H_2O_2 during phagocytosis (37).

At least four explanations could be offered for the decline in O_2^- and H_2O_2 releasing capacity after the 4th d of culture of monocytes. First, this could reflect functional heterogeneity of monocytes (49), with selective survival of cells less active in release of oxygen intermediates. This is unlikely to be the sole explanation. We could detect no difference in the H_2O_2-releasing capacity of adherent and nonadherent monocytes over the first 5 d of culture. Approximately 65% of the monocytes adherent on day 4, when H_2O_2 release was comparable to that on day 0, were still adherent on day 7, when it was minimal. Second, there could be increased activity of H_2O_2 scavengers. As already noted, the specific activity of the presumed major scavengers of H_2O_2 in monocyte lysates actually declined or remained constant. However, it is possible that there

were shifts in intracellular location of H_2O_2 scavenging systems that could promote their efficiency, or that there were increases in the activity of scavengers more important than those we tested. Third, there could be an acquired defect in the ability of the oxidase to be triggered by the soluble and particulate stimuli we used (24). Fourth, the number or activity of NAD(P)H oxidase molecules or their cofactors (50) might decrease, or the number or activity of regulatory molecules might increase (51, 52). Distinctions among these possibilities will probably have to await the development of assays for both the activity and the content of the oxidase, its cofactors, and its regulators.

Several other laboratories are also studying changes in monocyte oxidative metabolism with time in culture (20, 29). Some of their preliminary reports appear similar to our results (20), while others may possibly be discrepant (29). Comparison of culture conditions and assay methods will be desirable.

In the light of our findings, it is of considerable interest that many workers have noted decreased antimicrobial activity in human macrophages after 5 or more days in culture, compared with monocytes studied during the first 3 d of incubation. Such observations have been reported for *Toxoplasma gondii* (24), *Leishmania*,[3] *Herpes simplex* (53), and *Cryptococcus neoformans* (54). The multiplication of microbes such as *Mycobacterium leprae* and *Ricksettia mooseri* within human macrophages after several weeks in culture has also been described (55, 56). However, some investigations have reported an increased bactericidal activity during culture of human monocytes (57, 58). Studies are needed in which monocyte antimicrobial activity and oxidative metabolism are studied simultaneously.

The appearance of giant cells in cultures of monocytes is a striking phenomenon that has been repeatedly observed (1–3, 6, 7, 9). We know very little about the metabolism or functions of these multinucleated cells or their responses to regulatory molecules. Also of considerable interest was the substantial portion of viable monocytes that became nonadherent after 4 d of culture. The nonadherent monocyte population is frequently overlooked in studies of monocyte physiology.

The changes in oxidative metabolism observed here stand in sharp contrast to increases in cell size, lysosomal hydrolase and ectoenzyme activity, endocytic capacity, and expression of cell surface receptors that are underway at the same time (4, 6–11). As has often been observed with tissue macrophages of laboratory animals, various features of cell structure and function do not necessarily change in a coordinate fashion during the differentiation of mononuclear phagocytes.

[3] Murray, H. Personal communication.

Based on the data presented here and earlier,[1] we propose the following tentative, partial sequence of human monocyte differentiation. The normal blood monocyte has a capacity to secrete O_2^- and H_2O_2 comparable to that of a granulocyte (17, 18) or an activated mouse tissue macrophage (28, 29). This capacity is decreased in monocytes collected from some patients with cancer, and elevated in some patients with infection, by mechanisms not yet understood.[1] When the monocyte migrates from the vasculature into an inflammatory site, its oxidative capacity may be retained under the influence of lymphocyte mediators or other humoral factors. When the monocyte enters noninflamed tissue, its capacity to produce reactive oxygen intermediates may involute to resemble that of the resident peritoneal macrophage of the mouse (28, 29). During the remainder of the cell's lifespan, the remarkable capacity to secrete large amounts of O_2^- and H_2O_2 may be reinduced by inflammatory mediators (23, 29–31). The above hypothesis contains key elements that have not yet been tested. Experiments are underway to do so.

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