

INCREASED SUPEROXIDE ANION PRODUCTION BY IMMUNOLOGICALLY ACTIVATED AND CHEMICALLY ELICITED MACROPHAGES*

BY RICHARD B. JOHNSTON, JR.,[‡] CATHLEEN A. GODZIK, AND ZANVIL A. COHN

(From The Rockefeller University, New York 10021, Departments of Pediatrics and Microbiology, University of Alabama Medical Center, Birmingham, Alabama 35294, and National Jewish Hospital and Research Center and the Department of Pediatrics, University of Colorado School of Medicine, Denver, Colorado 80206)

Certain microorganisms, including mycobacteria, listeria, and some viruses and protozoa, are pathogenic, at least in part because they can survive inside mononuclear phagocytes. Resistance to these intracellular parasites appears to depend upon "activation" of macrophages by lymphocytes (1). Thus, lymphocytes sensitized by previous experience with a microorganism will, in the presence of that organism, stimulate macrophages to greater physiological activity, and in particular, to enhance killing of the ingested parasite.

The biochemical basis of the enhanced microbicidal activity of activated macrophages has not been defined. In fact, the mechanism by which macrophages kill intracellular parasites is poorly understood. The killing of most extracellular and at least some intracellular organisms by polymorphonuclear neutrophils depends upon this cell's capacity to convert oxygen to microbicidal metabolites. The initial step in this phagocytosis-associated enzymatic process appears to be the formation of superoxide anion (O_2^-)¹, the one-electron reduction product of oxygen. Further interactions lead to the formation of hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and, probably, singlet oxygen (2, 3). Peroxide forms the basis for an efficient microbicidal system (2), and evidence has been presented that $\cdot OH$ is an essential bactericidal species (4). Peripheral blood monocytes generate these same oxygen metabolites (5-9), and alveolar and peritoneal macrophages can generate H_2O_2 and O_2^- (10-13). We have studied the capacity of murine peritoneal macrophages to produce O_2^- after stabilization by *in vitro* cultivation. We report here that macrophages elicited by injection of thioglycollate or endotoxin, or obtained after Bacille Calmette-Guérin (BCG) infection have enhanced capacity to generate O_2^- , compared to resident cells from normal mice.

* Supported by U. S. Public Health Service grants AI 07012, AI 14148, CA 16673, and CA 13148.

[‡] This research was done in part while Dr. Johnston was a Josiah Macy, Jr. Foundation Faculty Scholar, 1976-77.

¹ *Abbreviations used in this paper:* BCG, Bacille Calmette-Guérin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; hkBCG, heat-killed BCG; HMP, hexose monophosphate; LPS, lipopolysaccharide; O_2^- , superoxide anion; PMA, phorbol myristate acetate.

Materials and Methods

Macrophage Harvesting and Cultivation. Peritoneal macrophages were obtained from Swiss mice maintained at The Rockefeller University. Cells were harvested as previously described (14), except that the mice were killed with chloroform, and the cells were removed after intraperitoneal injection of 5 ml of Dulbecco's phosphate-buffered saline (Grand Island Biological Co., Grand Island, N.Y.). Washed cells, $2-6 \times 10^6$ in a total volume of 1.5 ml, were plated on 35-mm diameter polystyrene tissue-culture Petri dishes (Nunc, Copenhagen, Denmark; actual measurement of the culture surface, 32 mm diameter). Dulbecco's modified Eagle's medium (DMEM; Grand Island Biological Co.) supplemented with 20% heat-inactivated (56°C, 30 min) fetal calf serum (FCS; Flow Laboratories, Inc., Rockville, Md.) was used for all experiments with murine cells. After incubation for 140 min at 37°C in 5% CO₂-95% air, adherent macrophages were washed vigorously with medium twice, and then cultured in medium with 20% FCS, penicillin, 100 U/ml, and streptomycin, 100 µg/ml. For long-term cultures, the medium with FCS and antibiotics was changed after 24 h, and thereafter at 2-day intervals.

Elicited Macrophages. Macrophages were elicited by intraperitoneal injection of 1 ml of 2.4% Brewer's thioglycollate medium (Difco Laboratories, Detroit, Mich.), or 0.3 ml (30 µg) of endotoxin from *Salmonella abortus-equi* (LPS; Difco Laboratories). The two different batches of this LPS (referred to as A and B) were obtained from the manufacturer several months apart. Cells were harvested 4 days after injection of either material and processed as described above.

BCG Infection. Mice were infected with BCG by injection of $2-4 \times 10^7$ freshly thawed organisms into the tail vein. The BCG strain used (TMC 1011) was obtained from the Trudeau Institute, Saranac Lake, N. Y., and was stored at -70°C. On the 21st day after injection, some of the mice were given intraperitoneally $2-4 \times 10^7$ BCG killed by heating at 56°C for 60 min (hkBCG). A second group of the infected mice received an intraperitoneal injection of an equal volume (0.4 ml) of saline on the 21st day, or animals were simply placed in a separate cage without injection. Cells from these saline-injected and uninjected subgroups gave equal activity in the O₂ assay, and the results were therefore pooled. In early experiments, a control group of animals was placed in separate cages at the time of the initial BCG injection. Superoxide anion generation by cells from this last group was not greater than that of cells from other normal, uninjected mice. Peritoneal cells were harvested from each of these groups 24 days after the injection of viable BCG and processed as described above.

Human Monocytes. Monocyte-lymphocyte fractions were prepared by centrifugation of heparinized peripheral blood through a Ficoll-Hypaque mixture (4, 7). The percentages of monocytes in washed preparations were estimated from smears stained with Wright's stain, and $1-4 \times 10^6$ monocytes were plated on 35-mm diameter tissue culture dishes in DMEM with 10% heat-inactivated (56°C, 30 min) autologous serum (15). After incubation for 60 min at 37°C in 5% CO₂-95% air, the monolayers were washed twice by vigorous swirling with DMEM and cultured overnight in DMEM with 10% fresh autologous serum or plasma (15), 100 U penicillin/ml, and 100 µg streptomycin/ml. This culture medium was changed the next day and every 2 days thereafter.

Murine Cell Lines. The macrophage-like tumor lines J774.1 and PU5-1.8, and the more primitive macrophage-like tumor line WEHI-3, were obtained from Dr. Peter Ralph, Sloan-Kettering Institute, Rye, N. Y. (16); the macrophage-like tumor line P388D1 was obtained from Dr. Jay Unkeles, The Rockefeller University. Cells from each of these four lines actively phagocytized opsonized zymosan or latex particles or both. L cells, a primary culture of embryo-derived cells (BALB), the 3T3 fibroblast line (Swiss), and the 3T3, clone A31 endothelial cell line (BALB) were obtained from Dr. Daniel Rifkin, The Rockefeller University. Cell lines were cultured in DMEM with 10 or 20% FCS. Each cell line was tested for its capacity to generate O₂ at varying cell densities, from scant to nearly confluent, on 35- and 60-mm diameter dishes, and after growth for 1 or 2 days in the same medium.

Assay for Superoxide Anion Generation. Plated cells were washed quickly with vigorous swirling, once with DMEM and a second time with Hanks' balanced salt solution (HBSS; Grand Island Biological Co.) without phenol red. The second wash was aspirated and replaced with a 1.5-ml reaction mixture containing 80 µM ferricytochrome c (Sigma Chemical Co., St. Louis, Mo.) in HBSS. The standard reaction mixture for stimulated cultures contained, in addition, either opsonized zymosan (4) at a final concentration of 1.3 mg/ml, or phorbol myristate acetate (PMA; Consolidated Midland Corp., Brewster, N. Y.), at a final concentration of 2-4 µg/ml. PMA was

maintained at -70°C in a stock solution of 1 mg/ml in dimethylsulfoxide (DMSO; Sigma Chemical Co.). DMSO alone did not stimulate cytochrome c reduction by cultured cells. Some assay mixtures contained bovine erythrocyte superoxide dismutase (Sigma Chemical Co.), final concentration 30 $\mu\text{g}/\text{ml}$, in addition to one of the stimuli.

Each reaction was run in duplicate. Controls included six cell-free dishes, each incubated with HBSS, cytochrome, and opsonized zymosan, PMA, or additional HBSS. After a 90-min incubation at 37°C in 5% CO_2 -95% air, the reaction mixture was removed, placed into iced microcentrifuge tubes, and promptly cleared by centrifugation at 8000 g in an Eppendorf microcentrifuge. The optical density of the supernates was determined spectrophotometrically using mixtures from plates without cells as blanks (4), and the concentration of cytochrome c reduced was determined using the equation $\Delta E_{550\text{nm}} = 2.1 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$.

During centrifugation of the reaction mixtures, adherent cells in the original culture dishes were washed twice with HBSS, then covered with 1 ml of 0.05% triton. After 20-30 min incubation, cells were removed by scraping with a rubber policeman, and the protein content of the digest was determined by the method of Lowry et al. (17) using egg lysozyme as standard. The protein content of dishes containing unstimulated cells did not differ significantly from that of dishes containing cells stimulated by PMA: 68.3 $\mu\text{g}/\text{dish}$ and 71.7 $\mu\text{g}/\text{dish}$, respectively, means of 110 comparisons ($n = 17$ -27 for each of the five different cell types). Protein concentrations from dishes incubated with opsonized zymosan averaged $\approx 10\%$ higher than those from unstimulated or PMA-stimulated cells in the same experiment.

Results obtained in the O_2^- assay with dishes containing <30-35 μg protein occasionally showed large variability among replicates. Dishes containing <100-120 μg protein showed minimal cell aggregation by phase microscopy, even with the largest cells (thioglycollate-elicited). Therefore, we selected 40-100 μg protein/dish as standard and compared results with cell types only when the protein content of the dish fell within this range.

Comparison of Cell Number and Protein Content. The number of macrophages adherent to the surface of a culture dish and the protein content of the dish were compared for each of the five cell types. Cells within six representative $\times 200$ fields were counted using inverted phase microscopy. The number of cells adherent to the entire dish was calculated from the surface area of the field, which was determined by placing the grid of a leukocyte counting chamber beneath the bottom of the dish. Counts were obtained with unstimulated and PMA-stimulated cells after washing once, before addition of the reaction mixture. After termination of the experiment, the plates were processed for protein measurement as described above, and results with dishes which contained 40-100 μg protein were collated. The estimated protein content for 10^6 cells, based on comparison of mean values, was, for resident 87 μg ; for thioglycollate-elicited, 143 μg ; for LPS-elicited, batch A, 120 μg ; for LPS-elicited, batch B, 130 μg ; for BCG-infected, 128 μg ; and for BCG-infected, hkBCG challenged, 124 μg .

Determination of Cell Types Plated. In two separate experiments, suspensions of each of the five cell types were plated on multiple 13-mm diameter glass cover slips. After the initial 140 min incubation or after 24 h in culture, the coverslips were washed twice with HBSS and stained with Wright's-Giemsa stain. Differential counts were performed on 400 cells of each type from each experiment. Results in the two experiments were almost identical and showed that after 140 min, cultures from BCG-infected mice given hkBCG 3 days before cell harvest contained a mean of 16% granulocytes. None of the other cell preparations contained >3% granulocytes, and after 1 day in culture, granulocyte contamination was <2% in all preparations. As will be described below (Fig. 4), release of O_2^- by cells from mice given BCG and hkBCG was equivalent whether measured after 140 min or 1 day in culture. Thus, it seems unlikely that granulocyte contamination significantly influenced results with any of the cell preparations studied here. Similarly, the percentage of cells not identifiable as macrophages or granulocytes at 140 min and at 24 h did not exceed 5 and 4%, respectively, of adherent cells. Therefore, adherent lymphocytes should have contributed little to protein determinations.

Results

Comparison of O_2^- Release by Resident, Elicited, and Infection-Activated Peritoneal Macrophages. The release of O_2^- , measured as superoxide dismu-

TABLE I
Release of O_2^- by Mouse Peritoneal Macrophages during Phagocytosis of Opsonized Zymosan or upon Contact with PMA

Cell type	Ferricytochrome c reduction*				
	Unstimulated cells	Stimulated cells			
		PMA	Opsonized zymosan	PMA	Opsonized zymosan
	nmol/mg protein‡	nmol/mg protein‡		nmol/10 ⁶ cells§	
Resident	3 ± 10 (13)	49 ± 34 (21)	233 ± 80 (22)	4.3	20.3
Thioglycollate-elicited	6 ± 10 (15)	493 ± 168 (11)	304 ± 78 (8)	70.5	43.5
LPS-elicited (A)	0 (7)	127 ± 47 (11)	421 ± 119 (11)	15.2	50.5
LPS-elicited (B)	0 (5)	570 ± 71 (5)	454 ± 99 (4)	74.1	59.0
BCG	1 ± 3 (9)	518 ± 187 (10)	305 ± 88 (10)	66.3	39.0
BCG-hkBCG	2 ± 9 (14)	492 ± 150 (13)	356 ± 119 (12)	61.0	44.1

* O_2^- was quantitated by its capacity to reduce ferricytochrome c. Superoxide dismutase, 33 μ g/ml, eliminated cytochrome reduction with each cell type; heat denaturation of the enzyme removed \approx 90% of this activity. Incubation was for 90 min, with HBSS (unstimulated cells), PMA, 2–4 μ g/ml, or zymosan, 1.3 mg/ml. Two batches of *S. abortus-equi* LPS (A and B) were used. All cells were assayed after \approx 24 h in culture.

‡ Nanomoles of cytochrome c reduced per mg of protein on the culture dish. Values are expressed as mean \pm SD of averages obtained with duplicate dishes; the number of experiments is given in parentheses. Only results with dishes which contained 40–100 μ g cell protein were collated.

§ Values were derived from the relationship between number and protein content of each of the cell types, which was determined by paired measurement of cell protein and microscopic estimation of cell number, as described in Materials and Methods.

tase-inhibitable reduction of ferricytochrome c, was studied in peritoneal macrophages cultivated in vitro. Cells for study were obtained from normal mice (resident macrophages), from mice given an intraperitoneal injection of thioglycollate broth or LPS 4 days previously (elicited macrophages), or from mice given live BCG intravenously 24 days earlier (infection-activated macrophages). Some of the last group were given an intraperitoneal challenge with hkBCG 21 days after the intravenous dose. The results are shown in Table I. Under resting (unstimulated) conditions, there was minimal O_2^- release from any of the five cell types. When incubated with the surface-active agent PMA or allowed to phagocytize opsonized zymosan particles, however, each cell type consistently released significant quantities of O_2^- . Results with cells elicited chemically or with cells from BCG-infected mice were significantly greater than those achieved with resident cells using either PMA or zymosan as stimulus ($P < 0.005$ for each cell type compared to resident cells, *t* test). Since elicited and BCG cell types were larger than resident macrophages (Materials and Methods), the enhanced O_2^- response of these "activated" macrophages was even more prominent when considered as a function of cell number rather than quantity of protein on the culture dish (Table I).

Effect of Cell Density on O_2^- Release. The effect of cell density, measured as μ g cell protein per culture dish, on the extent of O_2^- release is shown in Fig. 1.

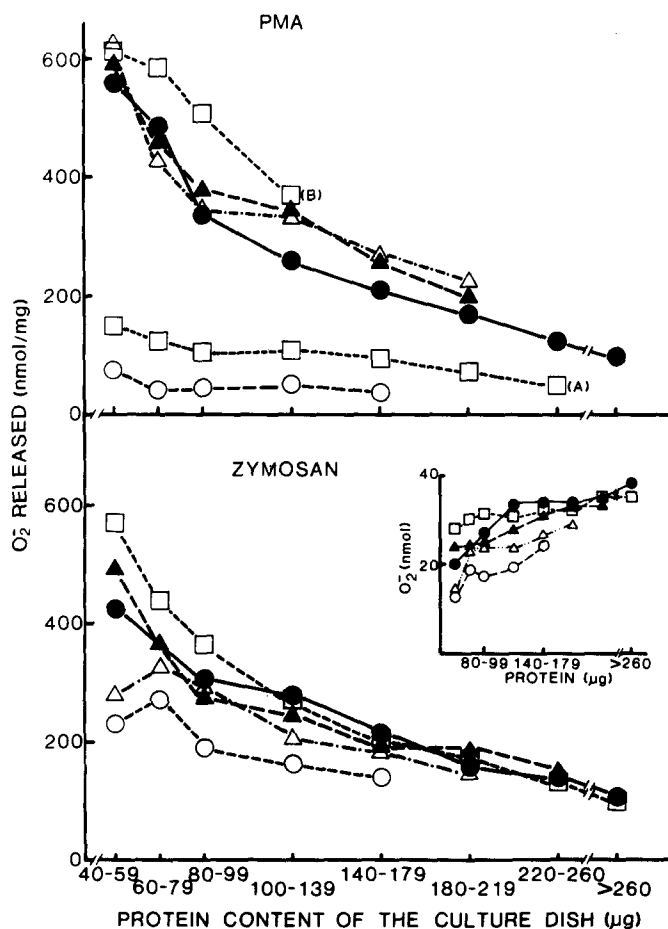


FIG. 1. Extent of O_2 release (nmol ferricytochrome c reduced/mg cell protein) on stimulation by PMA or opsonized zymosan plotted as a function of the quantity of protein on the culture dish. The points represent the means of values achieved in 2-10 experiments, done in duplicate, with dishes containing protein within the ranges noted. In the inset, the absolute quantity of O_2 released upon stimulation with zymosan (nmol) is plotted as a function of cell protein, within the same ranges. Two batches of LPS were used (A and B); results with the two batches differed significantly with PMA stimulation (plotted individually), but not with zymosan stimulation (collated and plotted together). (O), resident; (●), thioglycollate; (□), LPS A and B; (Δ), BCG; (▲), BCG-hkBCG.

As expected, a higher cell density resulted in more O_2 release, as shown for zymosan stimulation in the inset of Fig. 1. (The same effect was noted with PMA.) When expressed as specific activity (nmol/mg protein), however, O_2 release in general varied inversely with cell density. That is, the greater the density, the lower the efficiency of stimulated O_2 release.

The enhanced release of O_2 by elicited and BCG macrophages was most evident when the cells were least crowded on the dishes (<100 μg protein). In fact, zymosan-stimulated O_2 release by activated cells at higher densities fell below that of resident cells at lower densities, emphasizing the importance of comparing results obtained within a prescribed range of cell densities (40-100

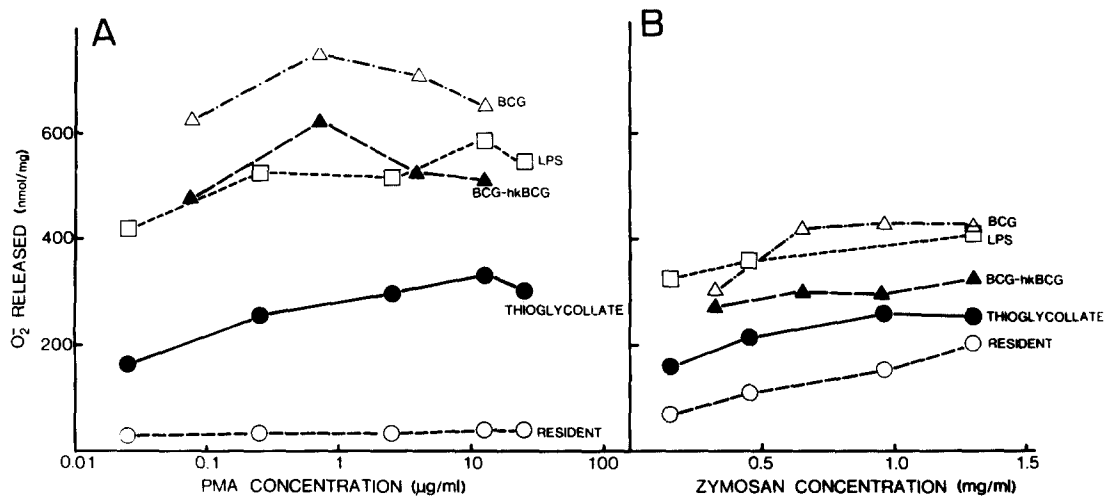


FIG. 2. Extent of O_2^- release plotted as a function of the concentration of PMA (A) or opsonized zymosan (B) used as stimulus. The experiments shown are representative of three to five performed in duplicate with each cell type. Batch B of LPS was used.

$\mu\text{g}/\text{dish}$ for the studies reported here).

Effect of PMA and Opsonized Zymosan Concentrations. The enhanced oxidative response of elicited and BCG macrophages was noted over a wide range of concentrations of PMA and opsonized zymosan (Fig. 2). As shown, higher concentrations of PMA were associated with a slight but consistent decline in O_2^- release by activated cells. Concentrations of PMA of 2–4 $\mu\text{g}/\text{ml}$ and of zymosan of 1.3 mg/ml were used in other experiments reported here.

Response with Time. The kinetics of stimulated O_2^- release by the five cell types is shown in Fig. 3. The greater response by elicited or BCG macrophages than by resident macrophages was noted at the earliest time point studied (20 min) was most pronounced in the first 30–60 min, and persisted through 120 min.

O_2^- Release during Long-Term Cultivation. The greater O_2^- response of elicited or BCG macrophages was present when cells were compared after their initial 140-min adherence to tissue-culture dishes (day 0, Fig. 4). Over the next 3 days in culture, the response of resident cells improved markedly, whereas the response of the activated cell types remained approximately the same. There was a general decline in O_2^- response by days 6–8, then a gradual return toward higher values by days 12–16 (Fig. 4). The decline in the efficiency of O_2^- release, expressed as nmol/mg , shown for thioglycollate-elicited macrophages after day 3 was due to an almost twofold increase in the protein content of these cultures during 2 wk in vitro. It is not clear what contribution fibroblast contamination might have made to this protein increase. With the other cell types, the protein content of the culture dishes remained approximately the same as that of day 0, or it decreased slightly. All cell types continued to release O_2^- vigorously when stimulated by PMA or zymosan for as long as studied; 16 days for resident and BCG-activated cells, 21 days for LPS-elicited cells, and 42 days for thioglycollate-elicited and BCG, hkBCG-stimulated cell types.

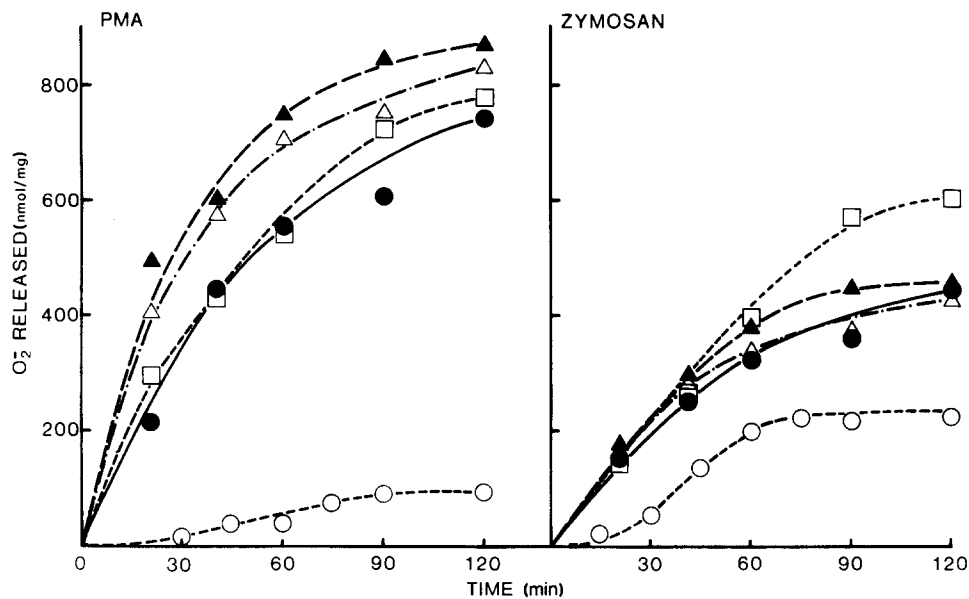


FIG. 3. Extent of O_2^- release plotted as a function of time, with PMA or opsonized zymosan as stimulus. The experiments shown are representative of two to four performed in duplicate with each cell type. Batch B of LPS was used. (O), resident; (●), thioglycollate; (□) LPS; (△), BCG; (▲), BCG-hkBCG.

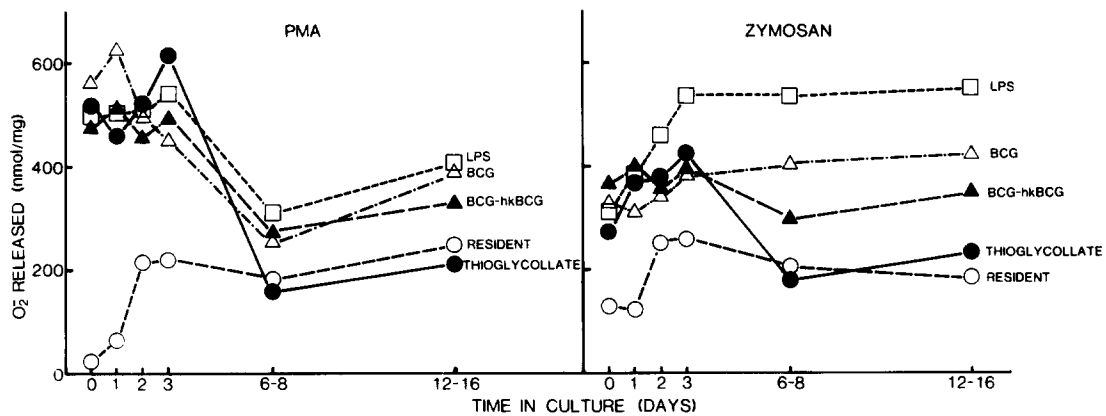


FIG. 4. Release of O_2^- after initial 140-min adherence to tissue culture dishes (day 0) and upon long-term cultivation, in response to stimulation by PMA or opsonized zymosan. The values plotted represent the means of two to five individual experiments, done in duplicate, for which results were available on days 0, 1, 2, 3, 6-8, and 12-16. Batch B of LPS was used.

O_2^- Release by Murine Tumor and Cell Lines and Human Monocytes. The capacity to effectively elaborate O_2^- in culture appears to be confined to "professional" phagocytes. As summarized in Table II, three of four macrophage tumor lines and human peripheral blood monocytes cultivated in vitro released O_2^- upon exposure to PMA or opsonized zymosan. In contrast, fibroblast and endothelial cell lines and a primary culture of embryonic cells did not. We have previously shown that human peripheral blood lymphocytes also lack this

TABLE II
Release of O₂⁻ by Murine Tumor and Cell Lines and Cultured Human Monocytes during Phagocytosis or upon Contact with PMA

Cell type		Ferricytochrome c reduction	
		PMA	Zymosan phagocytosis
<i>nmol/mg protein*</i>			
J774.1	Macrophage-like tumor	77 ± 28 (6)	103 ± 24
PU5-1.8	Macrophage-like tumor	13 ± 15 (5)	86 ± 55
P388D1	Macrophage-like tumor	10 ± 13 (3)	38 ± 21
WEHI-3	Primitive macrophage-like tumor	0 (5)	0
L cells	Fibroblast line	0 (3)	0
3T3	Fibroblast line	0 (3)	0
3T3, clone A31	Endothelial line	0 (3)	0
	Primary embryo culture	0 (3)	0
Human monocytes, day 1‡		24 ± 20 (4)	210 ± 105
Human monocytes, day 3‡		529 ± 304 (4)	326 ± 202

* Mean ± SD. The number of experiments (done in duplicate) is given in parentheses. The reaction mixtures and incubation time used to study mouse peritoneal macrophages were used here.

‡ Only results with dishes which contained 40–100 µg cell protein were collated. The same four culture sets were studied on day 1 and day 3.

capacity (7). None of the cell types released O₂⁻ if unstimulated. The greater O₂⁻ response by human monocytes after 3 days in culture compared to 1 day (Table II) correlates with the increase in spreading, visible ruffled membrane, lysozyme secretion, and 5'-nucleotidase activity noted for human monocytes during this period (15).

Superoxide Dismutase Activity of Supernates of Cultured Cells. We explored the possibility that differences in O₂⁻-mediated cytochrome reduction among cell types might be due to variable release of superoxide dismutase, which removes O₂⁻. Supernates obtained from 2–5 cultures of each cell type after stimulation with PMA or zymosan were tested for superoxide dismutase activity. The standard reaction mixtures were used, but the volumes were reduced to 1 ml and cytochrome-c was omitted. No definite activity was detected in any of the supernates by the assay of McCord and Fridovich (18), using up to 800 µl of sample. Purified superoxide dismutase incubated at 7 µg/ml for 90 min with stimulated cultures of each cell type gave easily detectable activity with 20 µl of sample, and activity was equivalent from culture to culture. It therefore seems unlikely that the decreased cytochrome reduction by resident macrophages can be attributed to greater release of superoxide dismutase by this cell type.

Discussion

Macrophages obtained from animals with enhanced resistance to intracellular parasites exhibit an increase in size, spreading on surfaces, ruffling of the plasma membrane, numbers of mitochondria and lysosomal granules, content of hydrolytic enzymes, stimulated protease secretion, pinocytosis, phagocytosis

(of some particles), antimicrobial and tumoricidal activity, and other manifestations of a state of activation (1, 19-21). Macrophages elicited by injection of materials that induce inflammation have been reported to exhibit most but not all of these properties (13, 21-23).

Resident and activated macrophages have also been compared in regard to certain manifestations of the phagocytosis-associated burst of oxidative metabolism. Normal resident macrophages have been shown to undergo increased oxygen consumption, H_2O_2 generation, hexose monophosphate (HMP) shunt activation, and O_2^- generation during ingestion (10-13) or upon contact with soluble substances capable of plasma membrane perturbation, such as phospholipase c and PMA (11-13). Macrophages from animals previously infected with BCG or listeria, or injected with casein have shown an increase relative to resident cells in activation of the HMP shunt at rest, during phagocytosis, or upon exposure to supernates from stimulated lymphocytes or to phospholipase c (10, 24-27). Murine peritoneal macrophages elicited chemically or obtained from infected animals have shown increased oxygen consumption during phagocytosis (10), and increased H_2O_2 production when stimulated by phagocytosis or PMA (13). In contrast, release of O_2^- during phagocytosis was apparently not increased in peritoneal macrophages from listeria-infected mice as compared to normal mice (12).

It seems clear from the studies reported here, however, that chemically elicited or immunologically activated macrophages have an increased capacity to elaborate O_2^- , compared to resident macrophages from normal mice. The extent of O_2^- release from cells stimulated by phagocytosis or contact with PMA, when expressed on the basis of cell protein, was from 30% to 12-fold higher in elicited or activated cells, depending upon the cell type and stimulus. When expressed on the basis of estimated cell number, O_2^- release was 2- to 17-fold higher in these cells. The greater responses were noted over a wide range of PMA and zymosan concentrations, on incubation times with the stimuli of from 20 to 120 min, and with cells cultured for 140 min to 16 days.

Cell density on the culture dishes appears to be a critical variable when comparing O_2^- release by different cell types and different cultures of the same cell type. Although the release of O_2^- increased with greater numbers of cells on the dish, the rise was not proportional to that in cell number. Thus, as the number of culture cells increased, the efficiency of O_2^- release declined markedly (Fig. 1). The percentage and size of adherent macrophages is greater in peritoneal leukocyte preparations elicited chemically or obtained from infected animals than in preparations from normal mice. Therefore, plating approximately the same number of peritoneal cells would result in relative crowding of elicited or activated macrophages and less efficient O_2^- elaboration, even to a point at which release from these cell types, expressed as nmol/mg, might be less than that from resident cells. This could explain the reported lack of increased O_2^- release from peritoneal macrophages of listeria-infected mice (12), since equivalent numbers of peritoneal leukocytes were plated from normal and infected animals.

The mechanism for the suppressive effect of increased cell density is not known. Possibilities include a relative deficiency of nutritional factors in more

densely populated cultures, or increased release of O_2^- -scavenging substances, or of cytotoxic materials from greater numbers of cultivated cells.

The release of O_2^- from elicited or activated macrophages stimulated after 1, 2, or 3 days in culture did not vary greatly from release obtained with cells stimulated after the initial 2-h adherence. In contrast, by day 3 in culture, O_2^- release by resident macrophages had doubled in response to zymosan phagocytosis and had risen ninefold in response to PMA. This accentuated response parallels the increases in size, spreading, specific activity of hydrolytic enzymes, and numbers of granules, mitochondria, and lipid droplets that develop in mouse resident peritoneal macrophages over 3–4 days in culture (14). A similar increase in O_2^- release (Table II) and in membrane ruffling, lysozyme secretion, and 5'-nucleotidase activity (15) occurs in cultured human monocytes during this period.

The mechanism by which elicited or infection-activated macrophages are modified to respond with greater oxidative metabolic activity remains to be determined. One possibility might be an alteration in the plasma membrane, coincident with that recognized as increased ruffling, such that contact with or binding of the PMA or zymosan particles is increased. In this case, the augmented response would reflect a quantitative increase in the triggering of the enzyme responsible for converting oxygen to its toxic metabolites.

A second possible explanation for the enhanced oxidative response of elicited and activated macrophages would be an alteration in the intracellular concentration or the intrinsic activity of this respiratory burst enzyme. An NADPH oxidase capable of generating O_2^- (28) and oxygen radical-dependent chemiluminescence (29) has been demonstrated in a particulate fraction from neutrophils (30–33) and macrophages (34). This enzyme is stimulated to greater activity by brief exposure of the cells before disruption to opsonized particles or PMA. The differentiation of macrophages that occurs consequent to inflammation or infection could include synthesis of increased amounts of this enzyme or enhancement of its inherent capacity to respond to plasma membrane perturbation.

Because of the rather firm association of the phagocytic respiratory burst with microbicidal activity in neutrophils, involvement of oxygen by-products in phagocytic killing by macrophages has been suspected. In support of such an involvement, monocytes from patients with chronic granulomatous disease, which do not undergo the normal respiratory burst, kill ingested microorganisms poorly (35). In addition, marked reduction of the partial pressure of oxygen in the reaction mixture has been reported to inhibit the killing of some bacteria by macrophages (35). However, others have found no effect of oxygen deprivation on macrophage bactericidal activity (35), so that the precise relationship between oxidative metabolism and the antimicrobial capacity of macrophages remains to be defined. Nevertheless, the enhanced capability of activated macrophages to resist infection (1) and tumors (36, 37) is associated with a remarkable increase in the production of oxygen by-products in response to surface contact or phagocytosis. These oxygen metabolites can disrupt the plasma membrane of microorganisms and mammalian cells (38). Thus, the greater oxidative metabolic response of the activated macrophage could play a significant role in its accelerated capacity to effect cell-mediated immunity.

Summary

We studied the capacity of cultured mouse peritoneal macrophages to generate superoxide anion (O_2^-), the initial product of conversion of oxygen to microbicidal species, during phagocytosis of opsonized zymosan or upon contact with the membrane-active agent phorbol myristate acetate (PMA). Macrophages from mice infected with Bacille Calmette-Guérin (BCG) or injected intraperitoneally with thioglycollate broth or endotoxin, released up to 12 times more O_2^- than did resident peritoneal macrophages, depending upon the cell type and whether the stimulus was zymosan or PMA. There was little if any O_2^- release from resting (unstimulated) macrophages. The density of cells on culture dishes was an important variable since crowding of the dish markedly reduced the efficiency of O_2^- production. The enhanced O_2^- release of chemically elicited and infection-activated macrophages was noted after stimulation with a wide range of concentrations of PMA and zymosan, at all time points studied (up to 120 min), and with cells maintained for 140 min to 16 days in culture. The O_2^- response of resident cells improved twofold to zymosan and ninefold to PMA during the first 3 days in culture. The capacity to release O_2^- appears to be limited to actively phagocytic cell types: murine macrophage-like tumor lines and cultured human monocytes released O_2^- when stimulated by PMA or zymosan, fibroblast and endothelial lines and embryo-derived cells did not. Activity of superoxide dismutase, which removes O_2^- , was not detectable in culture supernates of any cell type, and thus, differences in detectable O_2^- could not be attributed to variations in the release of this enzyme.

We conclude that the phagocytosis-associated respiratory burst is significantly enhanced in mononuclear phagocytes obtained after chemical inflammation or BCG infection. Increased capacity to generate O_2^- and other oxygen radicals during phagocytosis could contribute to the improved microbicidal and tumoricidal activity of activated macrophages.

We thank Ms. Donna Chadwick, Ms. Barbara Mei, and Dr. Warren D. Johnson, Jr. for their assistance in the cultivation of human monocytes; Doctors Peter Ralph, Jay C. Unkeless, and Daniel B. Rifkin for the generous gift of murine tumor and cell lines; Dr. Martha Fedorko for her guidance in the determination of cell types in stained smears; and Dr. Paul J. Edelson for his many helpful suggestions.

Received for publication 10 March 1978.

References

1. Mackaness, G. B. 1970. The mechanism of macrophage activation. *In* Infectious Agents and Host Reactions. S. Mudd, editor. W. B. Saunders Company, Philadelphia. 61.
2. Klebanoff, S. J. 1975. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. *Semin. Hematol.* 12:117.
3. Johnston, R. B., Jr., and J. E. Lehmeyer. 1977. The involvement of oxygen metabolites from phagocytic cells in bactericidal activity and inflammation. *In* Superoxide and Superoxide Dismutases. A. M. Michelson, J. M. McCord, and I. Fridovich, editors. Academic Press Inc. Ltd., London. 291.
4. Johnston, R. B., Jr., B. B. Keele, Jr., H. P. Misra, J. E. Lehmeyer, L. S. Webb, R. L. Baehner, and K. V. Rajagopalan. 1975. The role of superoxide anion generation in phagocytic bactericidal activity. *J. Clin. Invest.* 55:1357.

5. Baehner, R. L., and R. B. Johnston, Jr. 1972. Monocyte function in children with neutropenia and chronic infections. *Blood*. 40:31.
6. Sagone, A. L., Jr., G. W. King, and E. N. Metz. 1976. A comparison of the metabolic response to phagocytosis in human granulocytes and monocytes. *J. Clin. Invest.* 57:1352.
7. Johnston, R. B., Jr., J. E. Lehmeyer, and L. A. Guthrie. 1976. Generation of superoxide anion and chemiluminescence by human monocytes during phagocytosis and on contact with surface-bound immunoglobulin G. *J. Exp. Med.* 143:1551.
8. Nelson, R. D., E. L. Mills, R. L. Simmons, and P. G. Quie. 1976. Chemiluminescence response of phagocytizing human monocytes. *Infect. Immun.* 14:129.
9. Weiss, S. J., G. W. King, and A. F. LoBuglio. 1977. Evidence for hydroxyl radical generation by human monocytes. *J. Clin. Invest.* 60:370.
10. Karnovsky, M. L., J. Lazdins, and S. R. Simmons. 1975. Metabolism of activated mononuclear phagocytes at rest and during phagocytosis. In *Mononuclear Phagocytes in Immunity, Infection and Pathology*. R. vanFurth, editor. Blackwell Scientific Publications Ltd., Oxford, England. 423.
11. Rossi, F., G. Zabucchi, and D. Romeo. 1975. Metabolism of phagocytosing mononuclear phagocytes. In *Mononuclear Phagocytes in Immunity, Infection and Pathology*. R. vanFurth, editor. Blackwell Scientific Publications Ltd., Oxford, England. 441.
12. Drath, D. B., and M. L. Karnovsky. 1975. Superoxide production by phagocytic leukocytes. *J. Exp. Med.* 141:257.
13. Nathan, C. F., and R. K. Root. 1977. Hydrogen peroxide release from mouse peritoneal macrophages: dependence on sequential activation and triggering. *J. Exp. Med.* 146:1648.
14. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes: morphology, cytochemistry, and biochemistry. *J. Exp. Med.* 121:153.
15. Johnson, W. D., Jr., B. Mei, and Z. A. Cohn. 1977. The separation, long-term cultivation, and maturation of the human monocyte. *J. Exp. Med.* 146:1613.
16. Ralph, P., M. A. S. Moore, and K. Nilsson. 1976. Lysozyme synthesis by established human and murine histiocytic lymphoma cell lines. *J. Exp. Med.* 143:1528.
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265.
18. McCord, J. M., and I. Fridovich. 1969. Superoxide dismutase: an enzymic function for erythrocyte hemocuprein (hemocuprein). *J. Biol. Chem.* 244:6049.
19. Leake, E. S., and Q. N. Myrvik. 1968. Changes in morphology and in lysozyme content of free alveolar cells after the intravenous injection of killed BCG in oil. *J. Reticuloendothel. Soc.* 5:33.
20. Blandin, R. V. 1968. Modification of macrophage function. *J. Reticuloendothel. Soc.* 5:179.
21. Karnovsky, M. L., J. Lazdins, D. Drath, and A. Harper. 1975. Biochemical characteristics of activated macrophages. *Ann. N. Y. Acad. Sci.* 256:266.
22. Gordon, S. 1976. Macrophage neutral proteinases and chronic inflammation. *Ann. N. Y. Acad. Sci.* 278:176.
23. Bianco, C., and P. J. Edelson. 1978. Plasma membrane expressions of macrophage differentiation. In *Molecular Basis of Cell-Cell Interaction*. R. A. Lerner, editor. Alan R. Liss, Inc., New York. In press.
24. Evans, D. G., and Q. N. Myrvik. 1968. Studies on glucose oxidation during the interaction of alveolar macrophages and bacteria. *Ann. N. Y. Acad. Sci.* 154:167.
25. Ratzan, K. R., D. M. Musher, G. T. Keusch, and L. Weinstein. 1972. Correlation of increased metabolic activity, resistance to infection, enhanced phagocytosis, and inhibition of bacterial growth by macrophages from *Listeria*- and BCG-infected mice. *Infect. Immun.* 5:499.

26. Stubbs, M., A. V. Kühner, E. A. Glass, R. J. David, and M. Karnovsky. 1973. Metabolic and functional studies on activated mouse macrophages. *J. Exp. Med.* 137:537.
27. Nathan, C. F., M. L. Karnovsky, and J. R. David. 1971. Alterations of macrophage functions by mediators from lymphocytes. *J. Exp. Med.* 133:1356.
28. Babior, B. M., J. T. Curnutte, and B. J. McMurrich. 1976. The particulate superoxide-forming system from human neutrophils. *J. Clin. Invest.* 58:989.
29. McPhail, L. C., L. R. DeChatelet, J. R. Lehmeyer, and R. B. Johnston, Jr. 1977. NADPH-dependent chemiluminescence of a particulate fraction from human neutrophils. *Clin. Res.* 25:343A. (Abstr.).
30. Patriarca, P., R. Cramer, S. Moncalvo, F. Rossi, and D. Romeo. 1971. Enzymatic basis of metabolic stimulation in leucocytes during phagocytosis: the role of activated NADPH oxidase. *Arch. Biochem. Biophys.* 145:255.
31. Hohn, D. C., and R. I. Lehrer. 1975. NADPH oxidase deficiency in x-linked chronic granulomatous disease. *J. Clin. Invest.* 55:707.
32. DeChatelet, L. R., L. C. McPhail, D. Mullikin, and C. E. McCall. 1975. An isotopic assay for NADPH oxidase activity and some characteristics of the enzyme from human polymorphonuclear leukocytes. *J. Clin. Invest.* 55:714.
33. McPhail, L. C., L. R. DeChatelet, and P. S. Shirley. 1976. Further characterization of NADPH oxidase activity of human polymorphonuclear leukocytes. *J. Clin. Invest.* 58:774.
34. Romeo, D., G. Zabucchi, T. Marzi, and F. Rossi. 1973. Kinetic and enzymatic features of metabolic stimulation of alveolar and peritoneal macrophages challenged with bacteria. *Exp. Cell Res.* 78:423.
35. Klebanoff, S. J., and C. B. Hamon. 1975. Antimicrobial systems of mononuclear phagocytes. In *Mononuclear Phagocytes in Immunity, Infection and Pathology*. R. vanFurth, editor. Blackwell Scientific Publications Ltd., Oxford, England. 507.
36. Keller, R. 1974. Mechanisms by which activated normal macrophages destroy syngeneic rat tumor cells in vitro: cytokinetics, non-involvement of T lymphocytes, and effect of metabolic inhibitors. *Immunology.* 27:285.
37. Evans, R. 1975. Macrophage-mediated cytotoxicity: its possible role in rheumatoid arthritis. *Ann. N. Y. Acad. Sci.* 256:275.
38. Slater, T. F. 1972. Free radical mechanisms in tissue injury. Pion Ltd., London.