MACROPHAGE VARIANTS IN OXYGEN METABOLISM*

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The precise molecular mechanisms by which phagocytic cells effect their microbicidal function is unknown. Potential mechanisms that have been identified include low intracellular pH, lysozyme, cationic proteins, lysosomal hydrolases, and oxygen metabolites (1–4). Largely from the study of leukocytes of patients with rare genetic deficiency diseases—which include chronic granulomatous disease (CGD),[1] gutathione peroxidase, and the reductase deficiencies, glucose-6-phosphate dehydrogenase deficiency and myeloperoxidase deficiency—it has emerged that there exists a close correlation between the respiratory burst in normal phagocytic cells and their cytocidal activity, both of which are impaired in CGD (4–7). It has long been established that one of the early sequelae of the phagocytic event in macrophages is a stimulation of the hexose monophosphate (HMP) shunt, and recent studies indicate that the ability of macrophages to kill parasites intracellularly and possibly tumor cells extracellularly is dependent upon the oxidative burst and production of oxygen radicals including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical, and, possibly, singlet oxygen; $^1O_2$ (1–4, 8–10). The nature of the cellular enzyme or molecule(s) responsible for the initial reduction of oxygen, the biochemical nature of the electron transferring molecule, and the intracellular localization of the oxidative bactericidal mechanism and the mechanisms of its regulation in the macrophage remain important problems.

In previous studies (11, 12) we have endeavored to design selective genetic strategies for producing variants in defined functions of cloned macrophage-like cell lines, in the hope that they may yield tractable models for the study of the molecular basis for various macrophage functions. In the present report we describe a cloned, macrophage-like cell line derived from a murine reticulum cell sarcoma that can be stimulated to oxidize glucose via the HMP shunt, and produce O_2^- and H_2O_2, which we believe represents a useful model of activated primary macrophages. A simple strategy was employed to select for variants lacking the ability to reduce nitroblue tetrazolium (NBT), presumably by O_2^-, which has led to development of a series of clones defective in oxidative metabolism. These macrophage-variant clones lack the

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[1] *Abbreviations used in this paper:* CCP, cytochrome *c* peroxidase; BCG, Bacille Calmette-Guérin; CGD, chronic granulomatous disease; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; HMP, hexose monophosphate; KRPG, Krebs-Ringer phosphate glucose; NBT, nitroblue tetrazolium; O_2^-, superoxide anion; $^1O_2$, singlet oxygen; PMA, phorbol myristate acetate; PMN, polymorphonuclear cells; PBS, phosphate buffered saline; RBC, erythrocytes; SOD, superoxide dismutase; TdR, thymidine.

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ability to generate O_2^- or H_2O_2 by the HMP shunt and may prove useful in both defining the susceptibility of different pathogens to oxidative killing mechanisms of the macrophage and, for biochemical studies, to define and characterize the mechanisms of macrophage cytotoxicity.

Materials and Methods

Cell Lines. The cell lines used for these experiments were all derived from the murine reticulum cell sarcoma, J774 (13), and adapted to in vitro culture by L. Frank and M. Scharff (Albert Einstein College of Medicine, Bronx, N.Y.). The cells were cloned in soft agar as previously described (14).

All cell lines and clones were grown in Dulbecco's modified Eagle's medium that was supplemented with penicillin, streptomycin, glutamine, nonessential amino acids, and 20% heat-inactivated horse serum (all from Grand Island Biological Co., Grand Island, N. Y.). The cells were cultured in a humid incubator in 5% CO_2 in balanced air at 37°C in 60-mm Petri dishes (1007; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) at a starting density of 5×10^5/plate. For best results, cells were cultured for at least 1 wk in the same Petri dishes.

Primary Macrophages. For some experiments, in which the oxidative metabolism of the cell lines was compared with that of primary macrophages, resident macrophages were obtained from the peritoneal cavities of BALB/c mice by peritoneal lavage. Macrophages were also induced by inoculation of either 3 ml of Brewer's thioglycolate broth 3 d before harvest, or by sensitizing the animal with 10^7 living Bacille Calmette-Guérin (BCG) intravenously (The Trudeau Institute, Saranac Lake, N. Y.) and harvesting the peritoneal cells at 3 wk.

Reagents. Phorbol myristate acetate (PMA) (Consolidated Midland Corp., Brewster, N. Y.) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml and stored at −90°C in small aliquots. In the amounts used for experiments, DMSO alone at comparable volumes had no detectable effect on the cell lines studied.

Zymosan particles (Sigma Chemical Co., St. Louis, Mo.) were suspended in phosphate-buffered saline (PBS) at 10 mg/ml, boiled for 60 min, washed three times, and suspended at 50 mg/ml. They were then opsonized according to the method of Johnston et al. (15) with one part of zymosan and four parts fresh human serum. The final suspension was 10 mg/ml, and 1 mg/ml was present with the cells for the assays. Aggregated immunoglobulins were prepared by the method of Ishizaka et al. (16). Cytochrome *c* (horse heart, VI), catalase, NBT, and superoxide dismutase, were purchased from Sigma Chemical Co.

Preparation of Cytochrome c Peroxidase (CCP). CCP was prepared with minor modification by the procedure of Nelson et al. (17). Fresh commercial Baker's yeast was crumbled and allowed to dry until it lost ~40% of its original weight. It was allowed to autolyse in the presence of ethyl acetate, and released supernatant material was clarified by centrifugation and filtration. The soluble extract was chromatographed through a DEAE-column equilibrated with 0.1 M sodium acetate buffer, pH 5.0. The adsorbed red CCP band was cut out of the column, repoured into a smaller column, and eluted with 0.5 M sodium acetate buffer, pH 5.0. The concentrated enzyme solution was then subjected to gel filtration on a Sephadex G-75 column. The major enzyme-containing fractions were concentrated on DEAE cellulose, eluted in 10 ml (concentration of ~1 mM CCP), frozen, and stored in liquid nitrogen. The yield of a representative preparation was 260 mg of purified enzyme from 7.75 kg of yeast (0.033%). The heme:protein ratio (purity index: $A_{408\ nm}/A_{280\ nm}$) was 1.2.

NBT Reduction by Cell Lines. A modification of the quantitative NBT test of Baehner and Nathan (18) was used. Cells of each clone were seeded in triplicate at a concentration of 5×10^5 cells/well in flat-bottom Linbro tissue culture plates (76-033-05; Linbro Chemical Co., Hamden, Conn.). After the cells had attached and spread for 4–5 h, the monolayer in each well was washed once with Hanks' solution, and 0.45 ml of a 0.05% solution of NBT dissolved in PBS that contained 25% fetal calf serum (FCS) was added. PMA (6 μg/ml) or opsonized zymosan particles (1 mg/ml) was added to the NBT solution as required.

The Linbro plates were incubated for 1 h in a 37°C incubator in air with continuous gentle rocking (5 rpm) shielded from light. The reaction was stopped by adding 2 ml of 0.5 N HCl to

each well. The cells were then scraped from the plastic with a rubber policeman, and media from duplicate wells were collected and centrifuged at 2,000 rpm in the cold. The supernate was removed, and the precipitate was extracted for 10 min with 2 ml of pyridine in a boiling water bath under an exhaust hood. Tubes were again centrifuged at 2,000 rpm to remove debris. The absorbance of the resulting supernate was measured at 515 nm in a Guilford spectrophotometer against a pyridine blank. Values were expressed as A per 5×10^5 cells at 515 nm and converted to nanomoles of NBT reduced per 10^6 cells.

Qualitative NBT staining was performed either on duplicate cultures of cells attached to the bottom of the wells of Linbro plates or on 0.5-in. diameter coverslips. The wells were washed once with Hanks' after the NBT assay, fixed in 10% formalin-buffered saline for 30 min, and stained for 5 min with 0.1% safranin O in 1% acetic acid. After drying, the cells were examined microscopically under oil immersion. The percentage of positive cells was determined by light microscopy.

Selection Procedure for Variants in Oxidative Metabolism. The procedure employed is basically a selection against those clones capable of reducing NBT to formazan. The NBT-positive clone, J774.16, was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0.3 μg/ml per 10^6 cells) for 30 min, cultured for 2 d, and then plated at 5×10^6 cells/100-mm tissue culture dish that contained 28 round 0.5-in. diameter coverslips. When the cells were nearly confluent, generally 2 d after plating, the selection procedure was applied. The coverslips were washed twice with Hanks' solution, and 10 ml of the NBT-FCS solution, described above, that contained 6 μg/ml of PMA was added. The dish was rocked at 37°C in air that was shielded from the light. After various periods of time, the optimum being 2 h, of incubation, the NBT solution was aspirated, and the cells were washed twice with Hanks' solution. Coverslips were then transferred to 24-well Linbro plates, culture medium was added to the wells, and cells that were killed by the NBT staining were detached from the coverslips with vigorous pipetting. The medium was replaced with 0.3 ml of fresh culture medium that contained 25% conditioned medium from other cultures of J774 cells. The washing procedure was repeated 1 and 4 d later to remove cells that were damaged or killed by formazan. After each washing, 25% conditioned medium, i.e., medium from 3-d cultures of J774 lines, was readded.

The cells were allowed to grow out in each well and transferred to larger dishes that contained coverslips. Qualitative NBT tests were performed on coverslips from each well. From those dishes, where few NBT-positive cells were detected, the cells were cloned in soft agar as described previously. One clone, C3, was selected for detailed study, which was recloned, and five subclones from soft agar were derived (C3A, C3B, C3C, C3D, or C3E).

The clones were grown up and stored in liquid N_2 in 10% DMSO. At 3- to 6-mo intervals, clones were replaced either by fresh samples from frozen stock, or recloned. Some clones were recloned up to four times and retained their phenotype. Cells were seeded at a concentration of 0.5×10^6 cells/well in 24-well Linbro plates. After 4–5 h, the monolayers were washed in Hanks' solution and exposed to 0.5 ml of either NBT-FCS solution that contained either PMA alone (6 μg/ml) or NBT alone (0.05%), or PMA and NBT. The NBT assay was carried out as usual. At various intervals, the cells were pulsed with 1.0 μCi of [^{3}H]thymidine (specific activity 3 Ci/mM) for 30 min at 37°C in an air incubator. Incorporation was stopped with 1 ml of ice-cold Hanks' solution. The cells detached 0.25% trypsin-EDTA and collected on a filter paper, washed with 20% cold TCA and PBS, and counted in a scintillation counter as previously described.

Assay for [1-^{14}C]Glucose, [6-^{14}C]Glucose, and Oxidation. The assay for glucose oxidation has been described previously (19). Radioactive glucose was obtained from New England Nuclear (Boston, Mass.). [1-^{14}C]Glucose had a 0.05 mCi/1.3 mg sp act (NEC 043). [6-^{14}C]Glucose (NEC 095; New England Nuclear) had a 0.05 mCi/mg sp act. All cells were labeled with ~0.25 μmol/ml glucose. In the case of [1-^{14}C]glucose, cold glucose carrier was added at a ratio of 20:1, and for [6-^{14}C]glucose, 18:1. Cells were labeled in PBS that contained 0.5% bovine serum albumin without glucose at a density of 1×10^6 cells/ml. Cells, either stimulated with PMA or opsonized zymosan or incubated in control medium, were labeled for 30 min. CO_2 was released by the addition of 0.5 ml of 1 M H_2SO_4, and the CO_2 released was trapped with 0.2 ml of DL-alpha-phenylamine on filter paper.

Detection of O_2^- by Cytochrome c Reduction. A modification of the method of Babior et al. (20)

was performed. 10^7 cells in culture medium were seeded in 75-cm^2 Corning tissue culture bottles (25110; Corning Glass Works, Science Products Div., Corning, N. Y.) and allowed to attach and spread for 4–5 h in a 5% CO_2-air incubator. The monolayer was then washed twice with Hanks' solution that contained bicarbonate without phenol red. Cytochrome *c* (0.08 mM) (1 mg/ml) (Sigma grade III or grade VI) was added in a vol of 3 ml. Samples without cytochrome *c* or of cytochrome *c* without cells to be used as controls were kept on ice. Stimulating agents, PMA, and opsonized zymosan were added as required. In some experiments, superoxide dismutase (SOD) (30 μg/ml) or catalase (2,000 U/ml) was added. After 45 min, the samples were removed from the bottles, transferred to tubes in ice, and centrifuged to remove detached cells or debris. Absorbance was measured at 550 nm against the cell-free blank, and the absorbance of sample minus control values was converted to nanomoles of cytochrome *c* reduced with $\Delta E_{550} = 2.1 \times 10^4\ M^{-1}$. Virtually identical results were obtained with the method of Johnston et al. (15).

Measurement of H_2O_2 Production by CCP. Macrophages cultured in the same Petri dishes for at least 1 wk (two feedings) were removed, washed with Krebs-Ringer phosphate glucose (KRPG) buffer, resuspended to 1×10^6 cells/ml, in KRPG, and kept on ice. 1 ml of cells was added to two cuvettes, and a base-line absorbance spectrum was taken from 500 to 350 nm in a Cary model 14 double-beam recording spectrophotometer (Varian Associates, Instrument Div. Palo Alto, Calif.). 20 μl of CCP solution (410 nmol/ml) was added to the experimental cuvette, and the cells in both the reference and experimental cuvettes were mixed. After 10 min, a second spectral reading scan was made, which indicated the resting level of H_2O_2 production. A frozen stock solution of PMA was thawed immediately before use and diluted to 1 mg/ml with DMSO. Various amounts, e.g., 6 μl, were added to both reference and experimental cuvettes, the cells were mixed, and spectral scans were made immediately and at 10-min intervals for 50 min. To confirm the activity of the CCP in each experiment after no further shift in spectrum was seen, 70 μl of a 1:100 dilution of a standardized H_2O_2 solution was added to convert all the CCP to the compound I form.

The decrease in absorbance at 408 nm in relation to the isosbestic point at 450 nm was measured, $\Delta A = A_{408} - A_{450}$, which permitted the quantitation of H_2O_2 generated from a standard curve (Fig. 3) (21, 22).

Assay of SOD and Catalase. SOD activity was measured by the method of McCord and Fridovich (23) in which the initial velocity of O_2^--dependent reduction of cytochrome *c* generated by xanthine oxidase and xanthine is measured, and its 50% inhibition is estimated. Catalase activity was measured spectrophotometrically by the method described by Beers and Sizer (24).

Macrophages to be assayed for these activities were cultured in 100-mm tissue culture dishes for 4 h and washed twice with PBS to remove contaminating cells, and the adherent cells were cultured for 20 h. After two further washes in Hanks' solution without phenol red, they were solubilized with 0.3% Triton X-100 plus 2 mM phenylmethylsulfonylfluoride in 0.05 M potassium phosphate buffer (pH 7.8) (1 ml/dish) for 10 min at 0°C. Cell debris remaining on the dishes was removed with a rubber policeman, and the extracts were centrifuged at 1,500 rpm for 10 min. Aliquots of the supernatant extract were assayed.

Results

When independent, unselected clones from a reticulum cell sarcoma that had been adapted to culture, J774, were screened for their ability to reduce NBT, considerable differences were found between individual clones (Table I). Whereas all clones had Fc receptors for IgG and phagocytized opsonized sheep erythrocytes (RBC), latex particles, and zymosan (data not shown), clone J774.16 showed a marked increase in NBT reduction after stimulation with PMA in comparison with J774.2, which showed only a very slight reduction. These differences suggested that it might be possible to select for variants of the parental J774.16 clone that had impaired oxygen metabolism.

Toxicity of Reduced Formazan for the Cell Lines. Yellow NBT dye is ingested by stimulated macrophages or polymorphonuclear leukocytes and reduced to a blue,

TABLE I

Screen of Some Independent Clones of the Macrophage-like Cell Line, J774, for NBT Reduction

Clone	Minus PMA	Plus PMA
	nmol NBT reduced/10^6 cells/60 min	
J774.16	1.03 ± 0.24	13.3 ± 1.06
J774.2	1.03 ± 0.08	2.52 ± 0.38
J774.9	2.44 ± 0.24	8.94 ± 0.54
J774.45d	2.90 ± 0.46	21.08 ± 1.98

Average of three experiments ± SE. PMA concentration: 6 μg/ml. The extinction coefficient at 515 nm = 2.62×10^4 M^{-1} cm^{-1} determined in pyridine.

insoluble dye, formazan, primarily inside phagocytic vacuoles (18, 25). Positive cells under light microscopy appear to be those with large disintegrating nuclei and blue granular cytoplasm (25) (G. Damiani, C. Kiyotaki, W. Soeller, M. Sasada, J. Peisach, and B. R. Bloom. Unpublished observations.). This led us to investigate the possibility that cells capable of reducing NBT were killed either by the reaction or by the resulting reaction product, formazan. When cell lines were stimulated with PMA in the presence of NBT, and [^{3}H]thymidine (TdR) incorporation was used to measure viability, it became clear that the more strongly NBT reactive cells, e.g., J774.16, survived least well, being largely killed within 2 h. From these observations, a strategy for selecting variants in oxidative metabolisms from J774.16 was applied, based on the rationale that the toxicity of reduced formazan for oxidative cells should permit the survival of variants either incapable of interiorizing or reducing NBT. The selection procedure consisted of applying the NBT test to J774.16 cells cultured on small Petri dishes in the presence of PMA, as described in Methods and Materials. The selection procedure was applied in five independent experiments on clone J774.16. In two experiments, the selection failed, and the majority of cells in all wells were NBT positive, similar to the parental clone. In one experiment, in which the selection period was extended to 3 h, no cells survived the selection procedure. In two experiments, using a 2-h selection, NBT-negative wells were obtained. In one of these experiments, for example, whereas the vast majority of cells were killed within 2 h and detached in the multiple washings, after 7–10 d, clusters of cells were observed in 20 of 24 wells. The cells in each well were transferred, grown, and screened for oxidative function by qualitative NBT staining. 12 wells were found to be NBT negative, and 8 were found to be positive. One of the wells that appeared to have the least staining cells, C3, was grown to mass culture and cloned in semi-soft agar. Random clones were picked, grown again in Linbro wells, and again screened for NBT reduction. Five subclones were grown to mass culture and studied in greater detail. Clone C3A was strongly NBT positive, whereas subclones C3B, C3C, C3D, and C3E were negative (Table I). Reselection of the clones with NBT plus PMA indicated that clones 16 and C3A were exquisitively sensitive to PMA-induced NBT toxicity, whereas C3C was the most resistant (Fig. 1). Although the dose of PMA (6 μg) used to stimulate the cells is toxic for primary macrophages, it appeared, in fact, to be somewhat stimulatory for the growth of the macrophage-like cell lines after exposure. The parental clone and subclones, over the course of 3 yr, were recloned

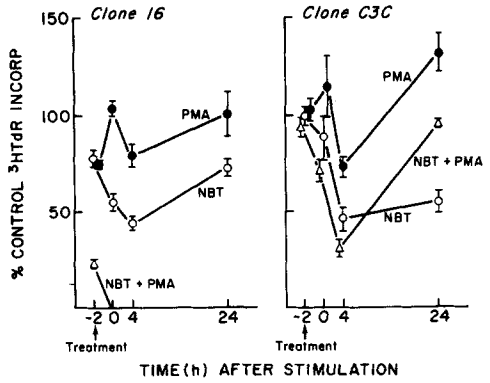

FIG. 1. Toxicity of the NBT selection on parental clone 16 and variant clone C3C. Cells were cultivated for 2 h with PMA (6 μg/ml), NBT (0.05%), or PMA and NBT. At various times, the cells were pulsed for 30 min with [^{3}H]TdR. The results are expressed at the percent incorporation (INCORP) of untreated control cells (0 time: 957 cpm; 24 h: 13,084 cpm).

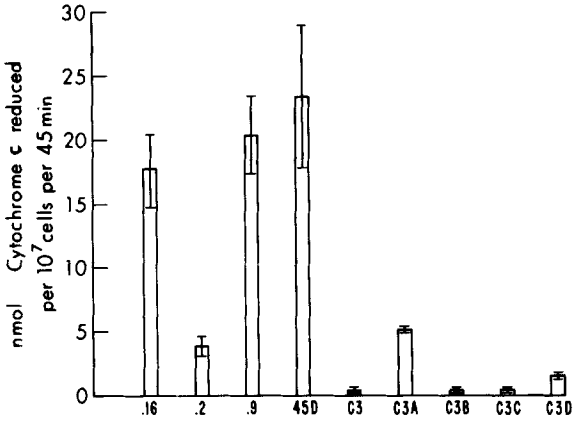

FIG. 2. O_2^- production by parental clone 16 and subclones. O_2^- was determined by spectrophotometric assay of cytochrome *c* reduction. The results represent the mean ± SE of at least three experiments for each clone after stimulation of 10^7 cells by 6 μg/ml PMA for 45 min. Incubation with SOD (30 μg/ml) totally abrogated reduction of cytochrome *c* (data not shown). .16, J774.16; .2, J774.2; .9, J774.9; and 45D, J774.45d.

two to four times, and their oxidative metabolism phenotype remained stable, thus indicating that it is a genetic property of the clone.

Production of O_2^-. Although NBT reduction is believed to be primarily a result of the O_2^-, the conversion from NBT to formazan can be carried out by other cellular-reducing intermediates, and the quantitative precision of the assay is limited. Consequently, SOD-inhibitable reduction of cytochrome *c* after stimulation, a more refined assay for O_2^- (15, 20), was carried out on the parental clone J774.16, the selected variant C3, and its subclones. As shown in Fig. 2, whereas J774.16 shows increased cytochrome *c* reduction after stimulation with PMA, clones C3, C3B, C3C, C3D, and C3E showed minimal, if any, reduction. Parental clones J774.9, 0.45d, and subclone C3A showed values comparable to J774.16. Thus, with a more quantitative assay, it appears that all but one of the subclones selected were markedly deficient in their abilities to generate O_2^- after stimulation as suggested by the NBT assay.

Determination of H_2O_2 Production with CCP. The precise determination of H_2O_2 production by living cells has been difficult, and a wide variety of assays have been employed. Because of its sensitivity, specificity, and relative freedom from quenching (26), the spectrophotometric measurement of the oxidation of CCP was chosen to assay the parental clones and selected macrophage subclones. The basis for this assay is the stoichiometric reaction of 1 mol of H_2O_2 with 1 mol of CCP, which produces a spectral shift from 408 nm to 418 nm, measured in the double-beam spectrophotometer (21, 22) (Fig. 3). With enzymes prepared from yeast by the method of Nelson et al. (17), it has been possible to efficiently measure H_2O_2 production by living cells stimulated with PMA. The type of results obtained with clone 16 and C3C is illustrated in Fig. 4. The data presented in Fig. 5 indicate that appropriate stimulation of J774.16 and C3A results in significant increases in H_2O_2 production, and that the NBT-selected subclones C3B, C3C, and C3D show a marked deficiency in H_2O_2 production. That the defect is a characteristic of the cell and not merely insusceptability to inducing agent (PMA) is illustrated in Fig. 6 in which aggregated immunoglobulins are able to stimulate H_2O_2 production in the parental clone 16 but not in the variant clone C3C. Thus, the selected variants lack the capacity, upon appropriate stimulation, to produce significant amounts of either O_2^- or H_2O_2.

Oxidation of [1-^{14}C]Glucose. The failure of leukocytes from patients with chronic granulomatous disease to exert a bactericidal effect or to produce O_2^- and H_2O_2 has been attributed to a failure to show a respiratory burst upon appropriate stimulation. The inability of the selected variants from J774.16 to produce O_2^- or H_2O_2 could represent a defect in the respiratory burst, or could result from the breakdown of O_2^- and H_2O_2 before assay. This could occur if the variants had increased levels of SOD, which accelerates the dismutation of O_2^- and/or catalase, which catalyzes the conversion of H_2O_2 to water and oxygen.

When levels of these enzyme activities were compared (Table II), the SOD levels of

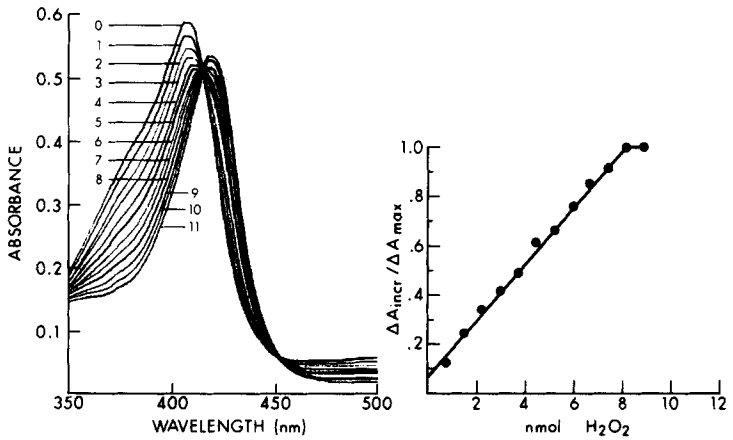

FIG. 3. Titration of H_2O_2 by CCP and standard curve for measurement of H_2O_2. To 5.9 nmol of purified CCP was added a series of 10-μl aliquots of 0.59 nmol of reagent H_2O_2 standardized with 0.1 M potassium permanganate. The decrease in absorbance at 408 nm in relation to the isosbestic point at 450 nm was plotted against the amount of H_2O_2 added to prepare the standard curve. It will be seen that 1.39 mol of H_2O_2 was required to convert CCP quantitatively into the compound I form. A, absorbance; incr, increase; and max, maximum.

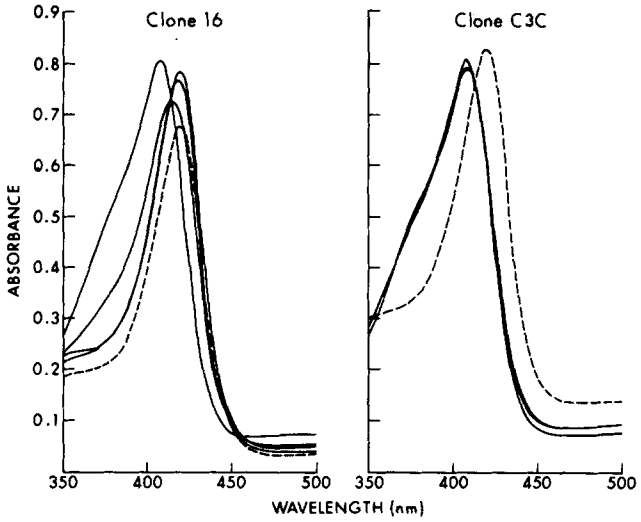

FIG. 4. Experimental data on production of H_2O_2 by parental clone 16 and variant clone C3C. In this experiment, 8.2 nmol CCP was added to 1×10^6 cells for the background reading. Similar aliquots were treated with 6 μg/ml PMA, and readings were taken at 10-min intervals (solid lines). At 40 min, standardized reagent peroxide was added to ascertain that all the CCP was still active and capable of reacting with H_2O_2. (dashed line).

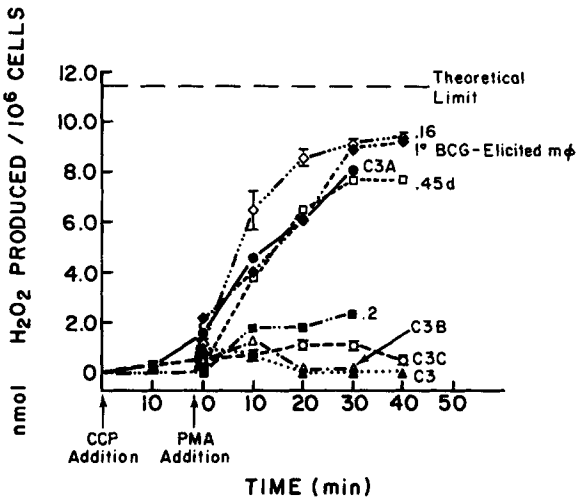

FIG. 5. H_2O_2 production by parental clones, variant subclones, and BCG-primed mouse primed peritoneal exudate cells. Data represent H_2O_2 produced by 10^6 cells after stimulation with 6 μg PMA at 25°C. 1°, primary; mφ, macrophage; .16, J774.16; and .45d, J774.45d.

both cell lines were below those for primary macrophages, although the catalase levels were slightly higher. The similarity of levels in the parent clone and the C3C variant suggested that the variant was unlikely to be degrading O_2^- and H_2O_2 more rapidly than the parent.

Consequently, the oxidative metabolism of the selected macrophage variants through the HMP shunt was assessed by measuring the oxidation of [1-^{14}C]glucose (Table III), and the oxidation through the classical glycolytic pathway was assessed

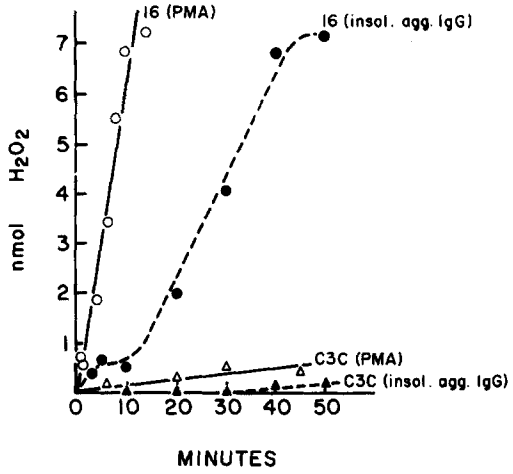

FIG. 6. H_2O_2 production in clone 16 and variant clone C3C after exposure to *bis*-diazobenzidine-aggregated rabbit IgG (90 μg/ml) or PMA (6 μg/ml). insol. agg., insoluble aggregate.

TABLE II

SOD and Catalase Activities in Primary Macrophages and Macrophage-like Clones

	SOD		Catalase	
	U/mg protein	*U/10^6 cells*	*U/mg protein*	*U/10^6 cells*
Primary macrophages				
Resident	9.3	0.70	57.4	4.3
Thioglycolate induced	4.9	0.37	32.0	2.4
BCG induced	6.7	0.50	32.0	2.4
J774 clones				
16	1.9	0.28	70.5	10.5
C3C	2.5	0.37	84.6	12.7

TABLE III

Stimulation of [1-^{14}C]Glucose Oxidation in Macrophage Clones by PMA and Opsonized Zymosan

Clone	$^{14}CO_2$ production		
	Control	Plus PMA	Plus Zymosan
	nmol/10^6 cells/60 min		
J774.16	2.40 ± 0.13	11.86 ± 1.50	6.42 ± 0.73
J774.2	1.15 ± 0.23	2.15 ± 0.33	1.48 ± 0.17
J774.9	1.90 ± 0.09	10.18 ± 2.42	6.64 ± 0.33
C3	1.55 ± 0.15	1.64 ± 0.21	1.33 ± 0.12
C3A	0.62 ± 0.05	11.84 ± 1.31	5.49 ± 0.81
C3B	1.54 ± 0.21	2.10 ± 0.33	2.42 ± 0.29
C3C	1.43 ± 0.24	2.56 ± 0.64	1.86 ± 0.19
C3D	3.32 ± 0.70	4.10 ± 0.65	3.31 ± 0.49

by measurement of oxidation of glucose-6-^{14}C. While J774.16 exhibited a twenty-fold increase in glucose-1-^{14}C following stimulation with PMA, the NBT-negative variants exhibited a minimal level of [1-^{14}C]glucose. Little difference between the cell lines was seen in oxidation of [6-^{14}C]glucose (data not shown). These observations support the interpretation that the NBT selection scheme has selected for variants lacking the ability to undergo a respiratory burst after stimulation with either PMA or zymosan. In addition, the variants similarly failed to undergo a respiratory burst after Fc-mediated phagocytosis of opsonized sheep RBC or opsonized *Candida parapsilosis* (data not shown). At a ratio of cells:fungi of 3:1, ingestion of *C. parapsilosis* was complete by 60 min and equivalent in clones 16, 2, C3A, C3B, and C3D as determined by counting cells on glass coverslips double-stained with Wright's and May-Giemsa stains (27).

Discussion

Although a wide variety of potentially cytocidal mechanisms are available to phagocytic cells, recognition of the paramount importance of oxidative cytocidal mechanisms derives largely from studies on the nature of the bactericidal defect in patients with a number of genetic deficiency diseases, which include CGD, glucose-6-phosphate dehydrogenase deficiency, glutathione peroxidase and reductase deficiencies, and myeloperoxidase deficiency (1–6). In CGD, perhaps the paradigm of these conditions, there is an almost total inability of polymorphonuclear cells (PMN) or monocytes to exert cytocidal activity on a wide variety of microorganisms, which can lead to persisting infections, particularly due to *Staphylococcus aureus*, enterobacteriaciae, candida, aspergillus, and atypical mycobacteria (2, 28). The large literature on the biochemical and functional defects in phagocytic cells of these patients has indicated that chemotactic migration and phagocytosis by PMN (29), vacuole formation, and lysosomal fusion (28, 30) can be essentially normal, while oxidative metabolism via the HMP shunt and bactericidal actvity are impaired.

For the past several years we and others (11–13) have studied continuous macrophage-like cell lines as models for the study of macrophage function. Because of their continuous growth in culture, individual clones of macrophage-like cells possessing or lacking a variety of properties can be obtained, and genetic selective techniques can be employed to derive variants or mutants in these properties. For example, it has been possible to develop macrophage variants in their Fc-mediated phagocytosis function, and to show that cyclic AMP was able to correct the defect in some but not all clones (14). It has also been possible to select for variants with altered enzymes such as adenylate cyclase and protein kinases (31). In addition, Unkeless et al. (32) have derived variants lacking the specific Fc receptor for IgG_{2b}, and we have isolated variants with diminished expression of the IgG_{2a} receptor (J. Schneck, B. Diamond, O. Rosen, and B. R. Bloom. Manuscript in preparation.). Because of the importance of oxidative cytocidal mechanisms in killing by PMN, and the difficulty of obtaining sufficient numbers of primary monocytes or macrophages from patients with the genetic deficiencies in cytocidal activity for detailed biochemical studies, we have attempted to develop continuous cloned macrophage-like cell lines possessing properties in common with primary activated macrophages and with deficiencies in oxygen metabolism.

J774 is a BALB/c reticulum cell sarcoma that had been induced and carried in ascitic form in mice by Ralph (13). It is highly phagocytic, has Fc and C3 receptors,

a full complement of lysosomal hydrolases, and the ability to secrete neutral proteases (11). Whereas the majority of cells (clones) of J774 lacked the ability to produce a significant respiratory burst, as measured by reduction of NBT, it was possible to isolate a clone, 16, from the original tumor, which was highly active upon stimulation with PMA in reducing NBT to formazan. The low level of NBT-reducing capacity of other (e.g., J774.2) independent clones suggested that it might be possible to derive mutants in oxidative metabolism.

The selection procedure employed was that of treating the parental clone, 16, with the NBT reagent itself. Concomitant with PMA stimulation, many previous studies on the localization of the reduced reaction product of the NBT reagent, formazan, indicated that it can be found both associated with the plasma membrane and in intracellular vacuoles of phagocytic cells (18, 19, 23). As demonstrated in Fig. 1, the stimulating agent, PMA, had a very low degree of toxicity, and, indeed, appeared to be somewhat mitogenic for the macrophage cell lines. The NBT reagent alone was toxic, thus reducing TdR incorporation by ~50% during the 2 h of treatment. However, treatment with PMA plus NBT had a marked degree of cytotoxicity within the 2-h period on the parental clone, ([^{3}H]TdR incorporation was decreased by four logs) with essentially no greater effect on the C3C variant subclone than NBT alone. We have performed the selection in only a limited number of experiments, and significant numbers of culture wells containing a majority of NBT-negative cells were found in only two of five selections. It appears that the time of exposure of NBT plus PMA determines the overall viability of the resulting cells; too limited an exposure in two experiments led to the emergence of many clones with normal NBT-reducing capacity, and too long an exposure (3 h) led to the killing of all cells. After selection and allowing the cells to regrow in mass culture, a number of clones were isolated and tested for the ability to reduce NBT. 1 of 12 such clones chosen for detailed study, C3, demonstrated a marked reduction in the ability to reduce NBT after PMA stimulation, and the clone was recloned to ascertain that the oxidative capacity was a true genetic trait. Of five subclones examined in detail, four were found to be markedly defective in reducing NBT. The fifth clone, C3A, was essentially as active as the parental clone in reducing NBT, and represented either a parental contaminant remaining after the selection, or more likely, a revertant to wild-type phenotype. At present, we are not in a position to evaluate the efficiency of the method used here to select the variants in oxidative metabolism. For example, while cells were treated before selection with a mutagenic agent, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, as in previous experiments designed to derive variants in phagocytosis (14), the present methodology was sufficiently cumbersome to preclude a fluctuation analysis to establish whether the treatment with mutagen increased the frequency of the variants. Indeed, for the same reason, it was not possible to determine by fluctuation analysis the true mutation frequency of the oxidative variants. If one assumes that 80% of the cells plated in the dish adhere to the coverslips that were subjected to the NBT plus PMA selection procedure, and that each well that contained NBT-negative cells represents a single clone, then a mutation frequency on the order of $0.3\text{–}3 \times 10^{-6}$ might be surmised. In any case it is clear that treatment with PMA alone or NBT alone in a number of attempts failed to result in selection for NBT-negative cells.

When the parental clone 16 and the five selected subclones were compared for production of oxygen intermediates after appropriate stimulation, it was found that

four of the clones had markedly reduced ability to produce O_2^-, as measured by SOD-inhibitable reduction of ferricytochrome *c*. Production of H_2O_2 by these cells was measured spectrophotometrically with the oxidation of CCP. This assay system was chosen for four reasons: (*a*) it directly measures the stoichiometric reaction of H_2O_2 with the enzyme; (*b*) it has been shown to be as, or more, sensitive than other assays (26); (*c*) it is a more specific reaction of H_2O_2 than other tests; and (*d*) it is less sensitive to quenching effects. The results with this assay for H_2O_2 production revealed that whereas clone 16 and C3A produced H_2O_2 in close to comparable amounts to BCG-activated primary macrophages in our, and other published (33), experiments, four of the subclones were markedly deficient in their ability to generate H_2O_2. The method may offer the additional possibility of ultimately measuring intracellular and intralysosomal levels of H_2O_2, because it may be possible to permit macrophages to interiorize the CCP before activation. In view of the differential sensitivity of different forms of parasites to H_2O_2 and different levels of production by macrophages activated in different fashions recently demonstrated by Nathan et al. (8) it may prove to be important to be able to measure intralysosomal levels of H_2O_2 after activation or infection. A disadvantage of the assay, however, is that it appears impossible, because of light scattering, to make reliable measurements on cells stimulated by opsonized zymosan.

Finally, direct measurement of glucose-C1 and glucose-C6 oxidation after stimulation with PMA or zymosan revealed that the parental clone, clone 16, and clone C3A had high levels of glucose-C1 oxidation, whereas the four NBT-negative clones showed markedly reduced glucose-C1 oxidation (Table II). In contrast, there was little difference between clones in oxidation of glucose-C6, thus indicating that the defect was localized to the HMP shunt. Because of the failure of the selected variants to produce O_2^-, H_2O_2, or demonstrate glucose-C1 oxidation, we believe that they represent appropriate models for study of macrophage oxidative cytocidal mechanisms. Consistent with our interpretation that the variants were defective in the reduction of O_2 by the HMP shunt, are the data that levels of SOD and catalase were similar in the parent clone 16 and the C3C variant, and were in fact lower for SOD than resident, thioglycolate, or BCG-induced primary macrophages.

The quantitative aspects of oxygen metabolism will require further, more-detailed, study. It should be emphasized that the various tests done on the cells in the present work, namely measurement of O_2^- production, H_2O_2 production, and glucose oxidation, were performed under widely different experimental conditions, with different numbers of cells, adherent and nonadherent, and different temperatures. It is very difficult to compare these data meaningfully with those in the literature on primary macrophages for the same reasons.

Perhaps the most obvious discrepancy between the data from the cell lines and from primary activated macrophages (33, 34) is the lower value for O_2^- relative to H_2O_2 produced by the lines. There could be several explanations, including: (*a*) local effects of SOD reducing the amounts of O_2^- secreted or detected outside the cells; (*b*) the known inefficiency of cytochrome *c* to detect O_2^-—20% of O_2^- produced by xanthine oxidase and xanthine in free solution can be detected by cytochrome *c* before dismutation to H_2O_2 (35); or (*c*) the possibility that macrophages may have an enzyme system capable of reducing molecular O_2 to H_2O_2 directly, as has been suggested, but not established, for neutrophils (36).

We believe that continuous macrophage cell lines possessing the properties of clones 16 and C3C should prove to be useful models for the study of macrophage cytocidal mechanisms for several reasons: *(a)* They are homogeneous cloned cells that are free from the inevitable granulocyte contamination present in most primary macrophage populations. *(b)* The cells grow rapidly in culture and can be cultured in sufficient quantity to carry out biochemical studies on the protein(s) or enzyme(s) responsible for initiating the reduction of molecular oxygen. In this regard, it is a great advantage to have a parental cell and a mutant derived from the same cell, and which presumably differ only in one genetic function. *(c)* Studies on the fate of a wide variety of bacteria, protozoa, and viruses in the oxidative metabolism variants may be helpful in defining the spectrum of microorganisms for which oxidative metabolism is essential for intracellular killing, and for defining nonoxidative mechanisms that are active against some species. *(d)* It should be possible, as has been reported previously for PMN derived from patients with CGD, to reconstitute the cells in vitro with defined enzyme systems capable of generating one or another oxygen radical to define the nature of the enzymes in some parasites that permit them to elude the natural cytocidal mechanisms of the host. For example, Klebanoff and Hamer (1) were able to show that neutrophils from patients with CGD were capable of iodinating and killing microorganisms that produced H_2O_2, and Johnston and Baehner (37) and Baehner et al. (38), in elegant experiments, were able to add glucose oxidase absorbed to latex particles to correct the defect in CGD granulocytes. *(e)* Finally, with such continuous cell lines, it may be possible to screen for pharmacologic agents capable of blocking enzymes evolved by microorganisms, such as SOD or catalase, enabling them to resist oxidative killing mechanisms. The hope would be to render them susceptible to the normal oxidative cytocidal mechanisms of the host.

Summary

Whereas phagocytic cells from normal individuals have the capacity to kill ingested bacteria and parasites, those from patients with several uncommon genetic deficiency diseases are known to be defective in bactericidal activity. Studies on neutrophils of these patients have revealed fundamental defects in their ability to reduce molecular oxygen and metabolize it to superoxide anion, hydrogen peroxide, and oxygen radicals.

In the present experiments, we describe a clone of a continuous murine macrophage-like cell line, J774.16, that, upon appropriate stimulation, activates the hexose monophosphate shunt, and produces superoxide anion and hydrogen peroxide. With nitroblue tetrazolium to select against cells capable of being stimulated by phorbol myristate acetate to reduce the dye to the polymer—formazan—which is toxic for cells, we have selected for variants that are defective in oxygen metabolism. Four of these subclones have been characterized and found to be lacking in the ability (*a*) to generate superoxide anion, as measured by cytochrome *c* reduction; (*b*) to produce hydrogen peroxide, as measured by the ability to form complex I with cytochrome *c* peroxidase; and (*c*) to be stimulated to oxidize glucose via the hexose monophosphate shunt. These variants appear to represent a useful model for studying the molecular basis for macrophage cytocidal activity.

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