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Research Article

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Regulation of Macrophage Function by Interferon- γ

Somatic Cell Genetic Approaches in Murine Macrophage Cell Lines to Mechanisms of Growth Inhibition, the Oxidative Burst, and Expression of the Chronic Granulomatous Disease Gene

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Abstract

The importance of oxidative cytotoxic mechanisms of phagocytic cells in immune protection against microbial pathogens is uniquely revealed by chronic granulomatous disease (CGD), a genetic deficiency disease of man. This cytotoxic response in mononuclear phagocytes is principally regulated by IFN- γ . A somatic cell genetic approach was taken to select oxidative variants from a cloned murine macrophage cell line, J774.16, which formally permitted us to dissociate three regulatory effects of IFN- γ on these cells: the antiproliferative effect, the antiviral effect, and production of superoxide anion. Half of the variants defective in O_2^- production after phorbol myristate acetate stimulation were also resistant to the antiproliferative effects of IFN- γ . This result suggests that IFN- γ -induced growth inhibition and production of cytotoxic oxygen intermediates are mediated via a common pathway. The somatic cell genetic approach has allowed us to develop in vitro macrophage models for several forms of CGD. One variant characterized in detail, D9, was unable to produce superoxide after stimulation by phorbol esters. At the molecular level, Northern blot analysis revealed that the mRNA encoding the large subunit of the putative CGD gene product, cytochrome b_{558} , was absent in this variant. Another class of variants constitutively unable to produce O_2^- or the cytochrome b_{558} mRNA could be induced to do so by IFN- γ . These somatic mutants may be useful models in clarifying the role of the CGD gene product and its regulation in the production of cytotoxic oxygen intermediates. (*J. Clin. Invest.* 1990. 85:563-569.) superoxide anion • antiproliferative activity • vesicular stomatitis virus • chronic granulomatous disease • cytotoxic oxygen intermediates • antiviral activity

Introduction

Mononuclear phagocytes play an important role in the immune response against infectious agents, indirectly by processing and presenting antigens to T cells, and directly by phagocytosing and killing microbial pathogens by both oxidative and nonoxidative mechanisms (1, 2). IFN- γ is the principal lymphokine involved in regulating macrophage function, activating primary macrophages to become competent to produce oxidative cytotoxic intermediates (3), augmenting expression of major histocompatibility class I and II antigens (4, 5) and Fc

receptors (6), inducing an antiviral state (7), and exerting potent antiproliferative effects on transformed macrophage cell lines (8).

The importance of oxidative cytotoxic mechanisms in protection against microbial infection is underscored by the consequences of chronic granulomatous disease (CGD),¹ a genetic deficiency disease of man in which the oxidative burst of PMN and macrophages after endocytosis of bacteria and parasites is defective (9, 10). CGD is a genetically heterogeneous group of disorders with both X-linked inheritance (66%) and autosomal recessive inheritance (33%) (11-13). Both X-linked and autosomal recessive forms of CGD can be further classified according to whether or not a membrane-associated cytochrome b_{558} is present in the phagocytic cells (14). Both the 91- and 22-kD components of the cytochrome b_{558} have recently been cloned and sequenced (15, 16). Although important information has been derived from the study of primary cells from patients with CGD, there are limitations in the number of such cells available for biochemical and molecular genetic analyses. Because of the potential advantages of continuous cell lines and the power of somatic cell genetic approaches to develop mutant cell models for human genetic diseases, we have for some years studied cloned murine macrophage cell lines. One such clone, J774.16, can be stimulated to oxidize glucose through the hexose monophosphate shunt to produce superoxide and hydrogen peroxide and to kill intracellular parasites, comparable to the IFN- γ -induced activation of primary macrophages (17-19). This cell line was instrumental in establishing that one of the earliest biochemical changes in macrophages exposed to such diverse stimuli as aggregated IgG and phorbol myristate acetate (PMA) was the rapid phosphorylation of a common set of proteins through the action of protein kinase C (PKC) (20).

In this study, we have used a somatic cell genetic approach to isolate variants in oxidative metabolism from the J774.16 cell line, with two aims: (a) To analyze whether the respiratory burst, the antiproliferative and antiviral effects regulated by IFN- γ in macrophages, are mediated by the same or different intracellular pathways; and (b) To develop an in vitro macrophage model useful for studying the regulation of the CGD gene, particularly by gene transfer experiments, to analyze cellular and regulatory requirements for its function in the production of oxidative intermediates.

Methods

Reagents. PMA was obtained from Consolidated Midland Corporation, Brewster, NY. Aliquots of PMA (10 mg/ml in DMSO) were kept at -90°C and diluted 10-fold with DMSO immediately before use.

1. **Abbreviations used in this paper:** CGD, chronic granulomatous disease; NBT, nitroblue tetrazolium; PKC, protein kinase C; VSV, vesicular stomatitis virus.

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Nitroblue tetrazolium (NBT), NP-40, and Tris were purchased from Sigma Chemical Co., St. Louis, MO.

IFNs. Recombinant *Escherichia coli*-derived murine IFN- γ was kindly provided by Genentech Inc. (South San Francisco, CA) and had a specific activity of 1.24×10^7 U/mg. Murine IFN- α was obtained from Lee Biomolecular Research Laboratories, Inc., San Diego, CA (catalogue 22061) and had a specific activity of 2.7×10^6 U/mg. IFN activity was assayed by the inhibition of cytopathic effects of encephalomyocarditis virus on L cells in microtiter wells. IFN activity was routinely titrated once a month to monitor any loss of activity.

Cells. Murine macrophage-like cloned cell lines J774.16 and variant clones were cultured and cloned as single cells as described previously (17). The mouse hepatoma cell line, E₃, was grown in DME supplemented with 10% heat-inactivated FCS (Gibco Laboratories, Grand Island, NY), 5% NCTC, and 1% nonessential amino acids and trypsinized twice a week. Primary rat embryo fibroblasts were cultured in DME supplemented with 10% FCS. Bac1.2.5 is an origin-defective SV40-transformed murine macrophage cell line kindly provided by Dr. Betty Diamond of Albert Einstein College of Medicine.

Selection procedure for variants in oxidative metabolism. The procedure employed is a selection against macrophage clones capable of reducing NBT to the polymer, formazan, which is toxic to the cells as previously described (17). Six independent pools of J774.16 cells were allowed to grow in 100-cm² petri dishes (Falcon Labware, Oxnard, CA) for 3–4 d until confluent, such that there were $\sim 1\text{--}2 \times 10^7$ cells/pool per dish. These cells were incubated with PMA and NBT (6 μ g/ml and 20 mg/ml, respectively) in Krebs Ringer phosphate glucose solution containing 25% FCS, for 1 h at 37°C. The cells were carefully washed with warm Krebs Ringer phosphate glucose solution containing 25% FCS to remove as much NBT and PMA as possible, and viable cells were allowed to grow up for 1 wk. After three successive selections in NBT and PMA over a 3-wk period, cells of each pool were cloned in soft agarose for the isolation of independent colonies. These clones were then tested for their ability to reduce NBT after appropriate stimulation with PMA. Clones J774.16 and mutant D9 have been cloned in agarose at a seeding density of 2,000 cells/60-cm² dish at least four times over the past 2.5 yr, and they have continued to maintain their phenotype. Using the same selection procedure, oxidative variants (2–5, 5–12) were derived from an independent subclone of J774.16 that was previously tagged for other purposes with a defective retrovirus containing a neo^r marker. Reduction of NBT and ferricytochrome *c* by macrophage clones was performed as previously described (17).

Growth curves. Cells in log phase were seeded (7.5×10^3 cells/0.15 ml) in triplicate in 96-well plates (Linbro, Flow Laboratories, Hamden, CT) in the absence or presence of the various IFNs and PMA. At the time intervals indicated, 0.025 ml of [³H]thymidine at 0.04 mCi/ml was added per well (final concentration of 1 μ Ci/well). After a 45-min incubation, the cells were lysed with 0.05 ml of 0.45% NP-40 in PBS (5–10 min), while rotating on an orbital gyrator. Nuclei were harvested onto glass filter paper, dried, and counted with Liquidscint (National Diagnostics, Inc., Somerville, NJ) for 1 min in a liquid scintillation counter. Results for each experimental point are derived from the mean of triplicate wells. (Individual wells varied generally less than 1 SD from the mean.) Preliminary experiments indicated that the inhibition of thymidine incorporation by IFN- α and IFN- γ paralleled the actual growth inhibition ascertained by counting viable cells excluding trypan blue with a hemocytometer.

Viruses. The Indiana (HRC) serotype of vesicular stomatitis virus (VSV) was prepared in chicken embryo fibroblasts, clarified by centrifugation (1,000 g, 10 min), and had a titer of 3.0×10^8 pfu/ml. The stock was divided into aliquots, frozen at -70°C , and diluted into macrophage culture medium before use. Infection of cell lines with viruses and quantitation of viral yields were performed as previously reported (21).

Results

Isolation of variants defective in the respiratory burst. The initial question considered was whether the oxidative burst and

the antiproliferative effects of IFN- γ were regulated via a common pathway. The strategy used to address this question was to derive variants defective in the oxidative burst and test them for their sensitivity to growth inhibition mediated by IFN- γ . The existence of variants defective in both the respiratory burst and growth inhibition mediated by IFN- γ is evidence that these effects may be regulated via a common pathway. In order to select variants from the J774.16 clone defective in oxidative metabolism, we used a suicide-selection strategy similar to that described previously (17), based on the observation that reduced formazan, the product of the reaction between the O₂⁻ anion and the dye, NBT, is toxic to cells. This toxicity permits the selection of variants incapable of reducing NBT, presumably due to the failure to produce superoxide after appropriate stimulation. Six independent pools (A–F) of J774.16 cells ($1\text{--}2 \times 10^7$ cells/pool) were screened by this procedure for oxidative variants. After three successive selections in NBT and PMA over a 3-wk period, cells of each pool were cloned as single cells in soft agarose for the isolation of independent colonies. These clones were then retested for their ability to reduce NBT and for their sensitivity to the antiproliferative effects of IFN- γ . All clones tested ($n = 5$) from at least two independent pools of cells that still maintained their ability to reduce NBT were sensitive to the antiproliferative effects of IFN- γ . In contrast, four NBT-negative clones from independent groups, and $\sim 50\%$ of all the NBT-negative clones tested, were resistant to the antiproliferative effects of IFN- γ after 64 h (Fig. 1). In fact, some of the growth-resistant clones appeared to be slightly stimulated by IFN- γ . The finding that some variants were both defective in the production of superoxide and resistant to the antiprolifer-

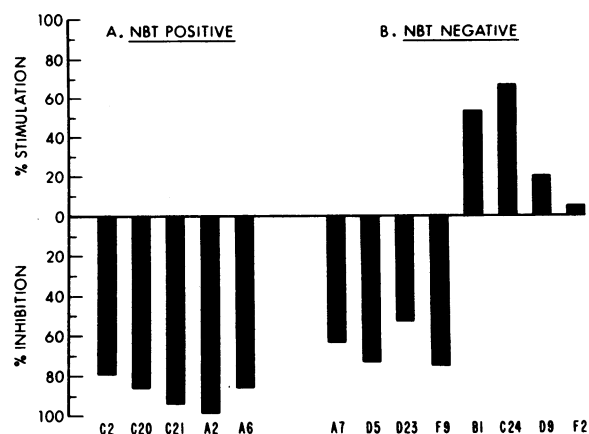


Figure 1. Effect of IFN- γ on the proliferation of macrophage clones. Cells were seeded in triplicate in the presence or absence of IFN- γ (650 IU/ml). After 64 h of incubation, the cells were pulsed for 45 min with tritiated thymidine and harvested. Results are shown as the percent stimulation or percent inhibition of growth by IFN- γ as compared with medium alone. Each clone is represented by an alphanumeric code at the bottom of each bar in which each letter A–F represents an independently selected pool of cells with the numeral referring to a particular clone in that group. The number of counts per minute incorporated of [³H]TdR in the absence of IFN- γ was 5.6, 2.3, 4.6, 4.6, 4.0, 8.2, 11.8, 10.0, 17.1, 4.0, 4.3, 4.5, 3.1, $\times 10^4$ for clones C2, C20, C21, A2, A6, A7, D5, D23, F9, B1, C24, D9, and F2, respectively. (A) Clones that maintained their ability to reduce NBT even after the selective procedure. (B) Clones that were oxidative variants as defined by their inability to reduce NBT.

Table I. Reduction of Viral Yields in Parental and Oxidative Variant Cell Lines

Clone	VSV yields			Reduction of viral yields	
	–	IFN- γ	IFN- α	IFN- γ	IFN- α
		pfu/ml			
J774.16	2.8×10^7	1.5×10^3	1.2×10^3	$\sim 10^4$	$\sim 10^4$
C21	1.1×10^6	7.8×10^2	2.2×10^2	$\sim 10^3$	$\sim 10^4$
D23	3.8×10^6	1.4×10^3	$<10^2$	$\sim 10^3$	$>10^4$
B1	3.9×10^6	8.2×10^3	$<10^2$	$\sim 10^2$	$>10^4$
C24	5.0×10^6	2.5×10^3	1.3×10^3	$\sim 10^3$	$>10^3$
D9	5.3×10^7	2.5×10^3	5.0×10^3	$\sim 10^4$	$\sim 10^4$
2-5	1.1×10^7	7.6×10^4	4.8×10^2	$\sim 10^2$	$\sim 10^4$
5-12	1.6×10^7	1.3×10^5	1.7×10^3	$\sim 10^2$	$\sim 10^4$

Cells (2.5×10^5 IU/ml) were incubated for 21 h in medium alone (–), IFN- γ (500 IU/ml), or IFN- α (100 IU/ml). They were then infected with VSV and cultured for an additional 24 h. The supernatants were collected and assayed for viral yields on Vero cell monolayers.

ative effects of IFN- γ suggests that both of these effects are regulated by a common intracellular pathway. The existence of variants growth-inhibited by IFN- γ but unable to produce O_2^- indicates that mechanisms other than O_2^- toxicity must be responsible for the antiproliferative effects of IFN- γ . These

latter variants also indicate that this common pathway must diverge before final effector functions, i.e., the production of cytotoxic intermediates and growth inhibition, respectively. Further, since the oxidative/antiproliferative variants remain fully protected against VSV infection by both IFN- γ and IFN- α , functional receptors for both IFNs must exist on these cells (Table I). Thus, the receptor-triggered pathway mediating the antiviral response must diverge earlier than the common oxidative/antiproliferative branch. Finally, since the only known molecular function of PMA is to bind and activate PKC (22), and our previous work showed that transcriptional activation of genes in a human macrophage cell line by IFN- γ is blocked by PKC inhibitors (23), the present results suggest that a PKC-mediated pathway is involved in the antiproliferative effects of IFN- γ in these cells.

Molecular analysis of a novel oxidative variant. One of the NBT-negative clones resistant to the antiproliferative effects of IFN- γ , D9, was characterized in greater detail. Fig. 2 illustrates its inability to reduce NBT after stimulation with PMA, as compared with the parental line. This was confirmed by the more precise measurement of O_2^- using SOD-inhibitable cytochrome *c* reduction, as reported previously (17). Clone J774.16 produced ~ 1 nmol/min per 10^6 cells of O_2^- , while clone D9 produced < 0.03 nmol/min, the limit of detection (Fig. 3). When the growth characteristics of parental J774.16 and D9 cells were compared in the presence of PMA and IFN- γ (Fig. 4), we observed that, whereas the parental line was sensitive to the antiproliferative effects of IFN- γ and PMA, the D9 variant grew readily in both. Thus, if one assumes that

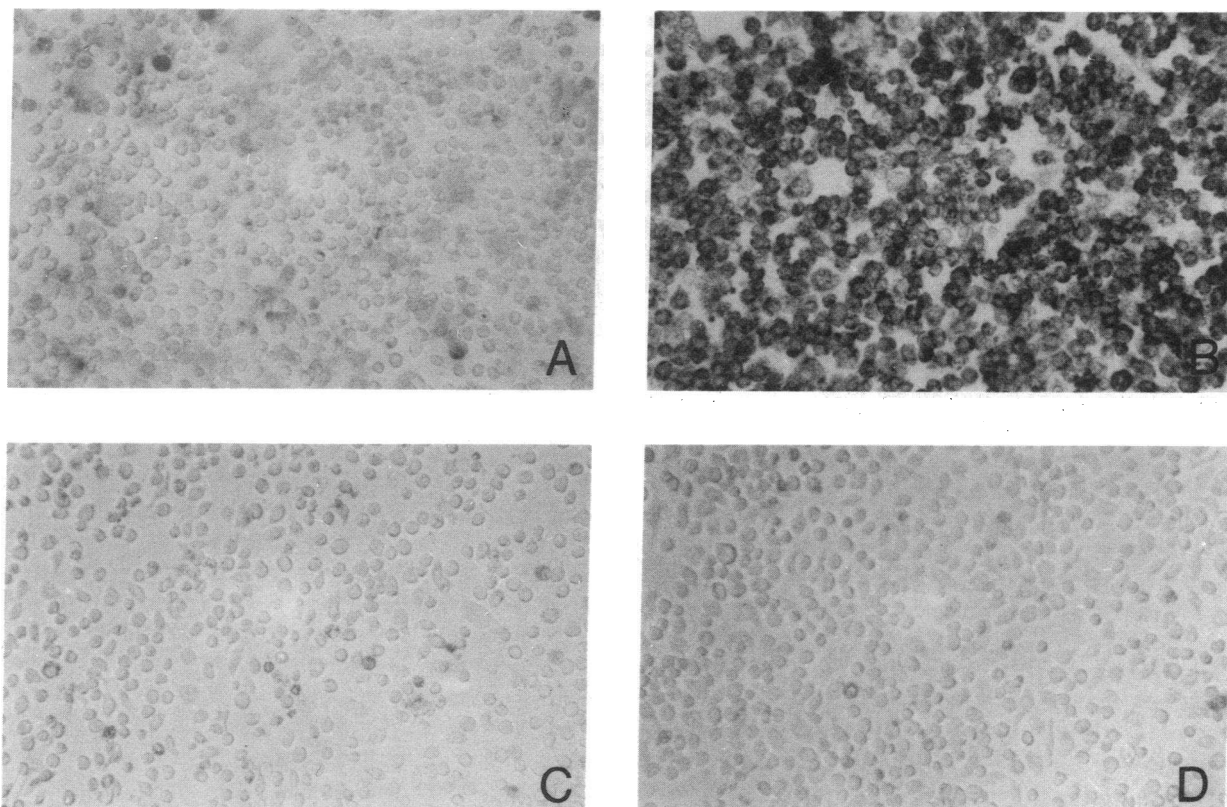


Figure 2. NBT reduction in J774.16 and D9 cells. J774.16 cells (A, B) and D9 cells (C, D) were plated in 96-well tissue culture plates (5×10^4 cells/well) before NBT solution containing either carrier alone, DMSO (A, C), or PMA (B, D) was added. After a 45-min incubation at 37°C , the cells were washed with PBS and then photographed.

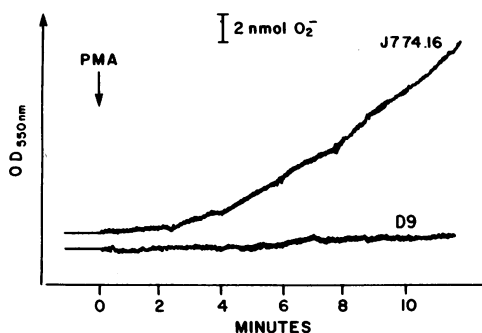


Figure 3. O_2^- production in parental and D9 variant. Superoxide was determined by spectrophotometric assay of cytochrome *c* reduction. Incubation with SOD (30 μ g/ml) totally abrogated reduction of cytochrome *c* (data not shown).

these cells have a single mutation, simultaneous failure of this variant to be growth inhibited by PMA or IFN- γ supports the view that the PMA-induced and IFN- γ antiproliferative effects are mediated by a pathway involving PKC. Until the precise nature of the mutation is elucidated, we cannot formally exclude the possibility that IFN- γ acts at a step distal to PKC in that pathway.

Orkin and colleagues recently reported that the CGD 91-kD subunit gene transcript is induced by both PMA and IFN- γ in the THP-1 human monocytic leukemia cell line (24). Since the D9 variant was unresponsive to both PMA and IFN- γ , the possibility was explored that its inability to produce superoxide was similarly due to a defective 91-kD subunit gene or its regulation. Consequently, RNA preparations from the parental and D9 variant were analyzed by Northern blots (21) for production of the murine homologue of the 91-kD subunit transcript (Fig. 5). The 91-kD subunit gene is constitutively transcribed in the parental J774.16 clone. In contrast, no transcript from this locus in the D9 variant could be observed, even after long exposure times. Southern analysis using a murine probe encoding 1.4 kb of the 5' end of the gene for the 91-kD subunit revealed no detectable changes in its DNA organization in the D9 variant (data not shown).

Using the same selection procedure, additional variants were derived that were unable to produce O_2^- in response to PMA. Two of these variants could be induced by treatment with IFN- γ to reduce NBT and cytochrome *c* after appropriate stimulation (clones 2-5 and 5-12) (Table II). Both clones were found to be growth-inhibited by IFN- γ (Table II) and protected against VSV lysis (Table I). Neither clone constitutively expressed mRNA for the 91-kD subunit, but transcripts were detected after treatment with IFN- γ . This is in contrast to clone D9, which could not be induced to produce O_2^- or the transcript by IFN- γ (Fig. 6). These mutants thus bear an analogy to cells of patients with the X-linked form of CGD that is correctable by treatment with IFN- γ (25, 26).

Discussion

The development of macrophage somatic cell variants described in this paper enabled us to address the question of whether the pleiotropic effects of IFN- γ on macrophages are mediated by a single or multiple pathways. The experimental results formally dissociate three effects of IFN- γ on effector functions of the macrophage: the production of O_2^- , the antiproliferative effect, and the antiviral activity. The mutants have provided evidence for the first time that the regulation of two of these parameters by IFN- γ , the respiratory burst and inhibition of cell growth, occur via a common pathway. Specifically, 50% of the variants defective in their oxidative metabolism were resistant to the antiproliferative effects of IFN- γ . This association is unlikely to be explained by inhibition of cell growth by toxic oxygen metabolites per se, since at least half of the variants unable to produce reactive oxygen intermediates remained fully growth-inhibited by IFN- γ . The addition of superoxide dismutase (30 μ g/ml), in excess of the amount required to abolish all detectable O_2^- , failed to affect the inhibition of growth by IFN- γ in the parental clone (data not shown).

The selection procedure for the isolation of oxidative variants is sufficiently cumbersome as to preclude a fluctuation analysis to determine the true mutation frequency of the oxidative variants. In our previous studies using the same selection procedure, we estimated that the frequency of obtaining

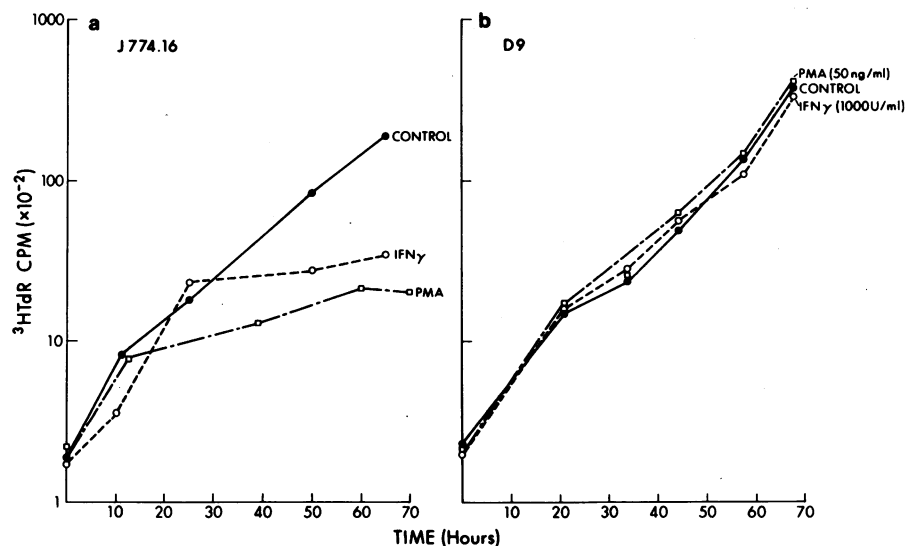


Figure 4. Effect of IFN- γ and PMA on the growth of J774.16 and D9 cells. Cells were seeded at a density of 7.5×10^3 cells/well in $\bullet$, medium alone; $\circ$, IFN- γ (1,000 IU/ml); or $\square$, PMA (50 ng/ml). At the various times indicated the cells were pulsed with [3 H]TdR and harvested. Each point represents the average of triplicate wells.

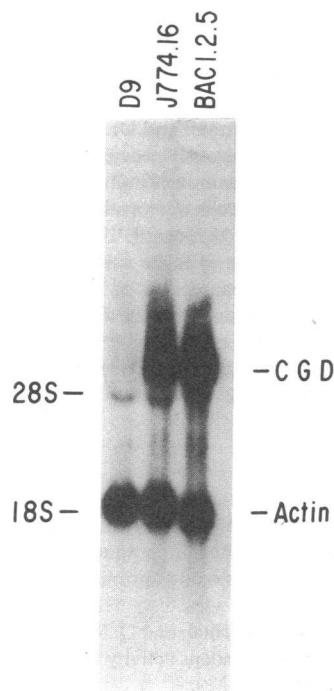


Figure 5. Expression of CGD mRNA in murine macrophage-like cell lines. Total cell RNA (25 μ g) isolated from BAC1.2.5-, J774.16-, and D9-cloned macrophage cell lines was probed with a 1.4-kb murine gene probe homologous to the CGD gene and a murine actin probe. BAC1.2.5 is an origin-defective SV40-transformed murine macrophage cell line.

oxidative variants was on the order of $0.03\text{--}0.3 \times 10^{-5}$ (17). Independent experiments indicate that the frequency of obtaining growth-resistant variants to IFN- γ is on the order of $2.0\text{--}20.0 \times 10^{-5}$ (27). Although the possibility cannot be formally excluded, it is thus extremely improbable that four clones derived from independent cultures of cells were selected in which two random mutational events occurred simultaneously. Since at the present time the exclusive known molecular function of PMA is to bind to and activate PKC (22), our results suggest that a PKC-regulated pathway is associated with the antiproliferative effects of IFN- γ . The precise mechanism by which IFN- γ affects the PKC-mediated pathway remains unclear, but other studies, for example in primary mouse macrophages, reported a three- to fivefold increase of PKC activity

Table II. Effects of IFN- γ on the Oxidative Burst and Growth of Parental and Variant Clones

Clone	A. O ₂ ⁻ production		B. Growth inhibition
	-IFN- γ	+IFN- γ	
	<i>nm/min per 10⁶ cells</i>		
J774.16	0.98	1.15	85
D9	0.01	0.03	0
2-5	0.07	0.65	91
5-12	0.16	0.93	95

(A) Cells were treated with IFN- γ (200 IU/ml) for 48 h before quantitation of cytochrome *c* reduction. (B) Cells were plated in the presence and absence of IFN- γ (200 IU/ml) for 67 h, after which their ability to incorporate [³H]TdR was measured. Results are expressed as the percentage inhibition of growth when comparing cells growing in IFN- γ with growing in medium alone. The number of counts per minute incorporated for cell clones growing in medium alone were in the range of $2\text{--}5 \times 10^4$.

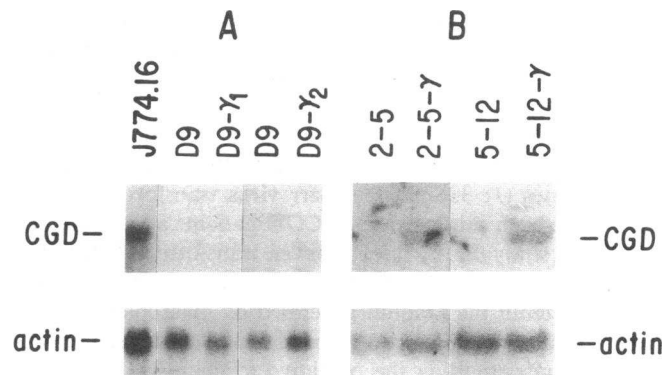


Figure 6. Induction of CGD mRNA by IFN- γ (500 IU/ml) in oxidative variants. D9- γ_1 = D9 cells treated with IFN- γ for 9 h. D9- γ_2 = D9 cells treated with IFN- γ for 3 d. 2-5- γ and 5-12- γ were treated with IFN- γ for 2 d.

after IFN- γ treatment (28). We have recently presented evidence in the human macrophage cell line, U937, that transcriptional activation of many, but not all, IFN- γ -inducible genes occurs by a PKC-dependent pathway (23).

Although multiple mechanisms for the microbicidal activities of mononuclear phagocytes have been proposed, the importance of reactive oxygen intermediates is underscored by CGD, which is characterized by repeated infection and an inability of phagocytic cells to kill intracellular pathogens (10). Both X-linked and autosomally recessive forms of CGD have been described (reviewed in references 13 and 14). The majority of patients with the X-linked form are characterized spectroscopically by the absence of a unique cytochrome *b*₅₅₈ (11). Most patients with the autosomal recessive form have phagocytes that do contain the membrane-associated cytochrome *b*₅₅₈ (29, 30). Recently, the gene deleted in cells from a patient with X-linked CGD has been identified and sequenced (15). The gene encodes a 91-kD glycosylated protein which is transcriptionally regulated by IFN- γ (24). The gene product in the cell associates with a 22-kD constitutively expressed protein forming a complex associated with the cytochrome *b*₅₅₈ spectrum (16). The precise role of the cytochrome *b*₅₅₈ in the production of O₂⁻ by phagocytes has not been conclusively established, but a considerable body of evidence indicates that the cytochrome *b*₅₅₈ is the last component of a short electron transport chain for the transfer of reducing equivalents from NADPH to molecular oxygen by a membrane-bound oxidase (reviewed in reference 31). Confounding this issue is the finding that the 91-kD X-chromosome-derived component of the cytochrome *b*₅₅₈ complex exhibits no significant similarities to other known cytochromes at the amino acid sequence level (15), although the 22-kD component with which it associates does contain a 31-amino acid stretch of 39% identity with polypeptide I of mitochondrial cytochrome *c* oxidase (16). To add to this complexity, deficiencies in the phosphorylation of specific proteins after stimulation of PMNs have been demonstrated in both the X-linked and autosomally transmitted forms of CGD, which may be critically involved in the production of cytotoxic oxygen metabolites (32).

Although much has been learned from the study of primary cells from patients with CGD, direct analysis is intrinsically difficult due to the paucity of patients with this rare genetic disease, limitations in obtaining sufficient quantities of

cells from these generally ill, young patients, and the limited survival in culture of primary cells. Furthermore, if one wants to pursue the mechanisms of the regulation of the macrophage respiratory burst by IFN- γ , primary cells from blood present the additional problem that they already appear primed in vivo for production of oxygen intermediates by IFN- γ or other lymphokines (1, 33). Epstein-Barr virus transformed B cell lines have been produced from CGD patients as somatic cell models to study this disease; however, transformed B cell lines produce very little superoxide after PMA stimulation (less than one tenth the amount generated by macrophages) (34). These results suggest that cell-specific regulation may be crucial for understanding the oxidative mechanisms of macrophages.

The alternative available to us was the study of cloned macrophage-like cell lines, and variants derived from them that have somatic defects resembling different forms of CGD. We initially identified a murine macrophage clone J774.16, constitutively competent to generate reactive oxygen intermediates, and selected genetic variants defective in their ability to produce superoxide anion upon appropriate stimulation. In previous studies, we characterized a mutant clone, C3C, that failed to produce detectable O_2^- upon PMA stimulation (17). Careful spectral analysis of this variant indicated that it produced a cytochrome b_{558} , but one that failed to bind carbon monoxide (18). Thus, the first variant probably contained a point mutation in the cytochrome b_{558} and may be analogous to the cytochrome b_{558} -positive forms of CGD. In the present work a new variant, D9, was found to exhibit characteristics of mononuclear phagocytes from patients with the cytochrome b_{558} -negative form of CGD, which we believe may represent a useful in vitro model for analysis of the function and regulation of the CGD gene. D9 cells are unable to produce O_2^- after PMA stimulation, and at the molecular level, Northern blot analysis revealed that the mRNA encoded in the 91-kD subunit gene locus was absent in this variant. This is similar to findings that monocytes of three of four extensively characterized CGD patients lacked the transcript of the 91-kD subunit, although no alterations in the structural gene were detectable (15). Thus, most X-linked CGD cells examined have been deficient in the mRNA for the cytochrome, at least before treatment with IFN- γ , whereas virtually all non-X-linked CGD cells express the cytochrome. Although the analogy suggests that the defect in the D9 cells will parallel those found in X-linked CGD cells, and likely be associated with expression of the murine cytochrome, further experiments are clearly required to localize the molecular defect in the D9 mutant cells.

Finally, we describe a third class of mutants here that are constitutively unable to produce O_2^- , but can be induced to do so by exposure to IFN- γ , analogous to a form of CGD that is responsive to treatment with IFN- γ (25, 26). These findings, together with those indicating that IFN- γ can activate a PKC-mediated pathway involved in the oxidative burst and induce O_2^- production in phagocytes of some patients with CGD, suggest that the genetic defects in CGD patients may either be structural or regulatory. With the availability of the cloned genes for the two chains associated with the CGD gene product, the mouse macrophage mutants analogous to different forms of human CGD could prove useful for understanding possible tissue-specific requirements for functional reconstitution of macrophage oxidative functions in gene-transfer experiments.

Acknowledgments

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