

# Effects of Phorbol Myristate Acetate on the Metabolism and Ultrastructure of Neutrophils in Chronic Granulomatous Disease

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**ABSTRACT** Previous investigations have demonstrated that phorbol myristate acetate (PMA), the active principle of croton oil, stimulates alterations in normal polymorphonuclear leukocytes (PMN) that resemble closely the changes that develop in the cells after phagocytosis of bacteria. The present study has compared the effects of PMA and heat-killed bacteria on the oxygen uptake, glucose oxidation, nitroblue tetrazolium (NBT) reduction, and ultrastructure of normal neutrophils and PMN from six patients with chronic granulomatous disease (CGD). PMA stimulated oxygen consumption, hexose monophosphate shunt activity, and NBT reduction in normal cells but failed to produce similar effects in CGD neutrophils. However, PMA did induce formation of cytoplasmic vacuoles in the CGD cells similar to those observed in normal neutrophils. The results indicate that PMA is a useful nonparticulate agent for distinguishing between normal and CGD neutrophils and for studying basic mechanisms of phagocytosis in normal and abnormal PMN.

## INTRODUCTION

Patients with chronic granulomatous disease (CGD)<sup>1</sup> are unusually susceptible to severe recurrent infection with bacterial pathogens that do not ordinarily cause

Dr. Holmes is an Established Investigator of the American Heart Association.

*Received for publication 6 December 1973 and in revised form 18 March 1974.*

<sup>1</sup>Abbreviations used in this paper: CGD, chronic granulomatous disease; DMSO, dimethyl-sulfoxide; HBSS, Hanks' balanced salt solution; HKB, heat-killed bacteria; HMPS, hexose monophosphate shunt; NBT, nitroblue tetrazolium; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes.

illness in the normal population (1-3). A defect in the bactericidal activity of polymorphonuclear leukocytes (PMN) has been demonstrated in CGD, and it appears closely linked to the pathogenesis of frequent infections (4, 5). The abnormal PMN of CGD patients can take up organisms, but fail to develop a burst of metabolic activity associated with the generation of bactericidal levels of hydrogen peroxide in phagocytic vacuoles (6). At the present time, the basis for the failure of CGD neutrophils to respond metabolically to intracellular particulates remains unresolved (7, 8).

Recent investigations have shown that phorbol myristate acetate (PMA), the active principle of croton oil, can stimulate a series of reactions in normal PMN that closely resembles the phagocytic response (9-11). The present study has examined the influence of this potent agent on normal neutrophils and PMN from patients with CGD of childhood. Results indicate that PMA fails to stimulate a significant change in oxygen consumption, glucose oxidation, or reduction of nitroblue tetrazolium (NBT) in the abnormal cells.

## METHODS

Blood for this study was obtained from normal donors and from six patients with CGD. Individuals providing control samples of leukocytes have been tested frequently and were not taking drugs at the time of study. All of the patients, five boys and one girl, were described in previous reports (4-6). They were in good health and off antibiotics when blood was drawn for the present investigation. The diagnosis had been established in each patient by a clinical history of repeated infections, inability of their polymorphonuclear leukocytes to reduce NBT, and failure of the cells to kill bacterial strains destroyed readily by normal neutrophils. The methods used in this investigation were described in detail in earlier publications (5-7, 9, 11-17). Blood obtained by venipuncture was mixed immediately with heparin to a concentration of 40 U anticoagulant/ml. The

sample was placed in a vertical position and allowed to sediment spontaneously for 90 min. Leukocyte-rich plasma was transferred in 5-ml aliquots to plastic tubes and sedimented at 200 *g* for 12 min. The supernatant platelet-rich plasma was discarded and the pellets of leukocytes resuspended in Ca<sup>++</sup> and Mg<sup>++</sup>-free Hanks' balanced salt solution (HBSS) containing 8 U heparin/ml. The cells were washed twice and resuspended in 5 ml of heparinized HBSS. Neutrophils were counted by hemocytometer differential and the sample of leukocytes was diluted with buffer to yield a suspension containing  $1 \times 10^7$  PMN/ml.

Oponins for studies of bacterial phagocytosis were provided by serum separated from clotted blood of normal donors, type AB, pooled and frozen in aliquots at  $-70^\circ\text{C}$ . An aliquot less than 2 wk old was thawed for each experiment, and combined with the suspensions of neutrophils in a final concentration of 10%. *Staphylococcus aureus* 502A, periodically tested for strain identification and contamination, was used to stimulate the phagocytic and metabolic response of PMN. The procedures used to store, regrow, incubate, sediment, wash, resuspend, ultrasonically separate, and dilute the bacteria to obtain suspensions of  $2 \times 10^9$  organisms/ml in heparinized HBSS were described earlier (11, 12). Suspensions of *S. aureus* were heat-killed by incubation in a  $70^\circ\text{C}$  water bath for 30 min and further diluted so that when added to leukocyte samples they would yield bacteria-to-neutrophil ratios of 1:1, 10:1, 50:1, and 100:1. A 1-ml volume of leukocytes, combined with 0.2 ml of pooled serum, and a stirring bar was placed on the oxygen monitor for 4 min before addition of the appropriate number of bacteria in 1.8 ml of HBSS. The total volume of 3 ml contained  $4 \times 10^6$  PMN/ml.

PMA (12-*O*-tetradecanoyl-phorbol-13-acetate, mol wt 616, from Consolidated Midland Corp., Chemical Div., Katonah, N. Y.) was dissolved in dimethyl-sulfoxide (DMSO) at a concentration of 1–2 mg/ml and kept frozen at  $-70^\circ\text{C}$  between experiments. Before use, the stock PMA was thawed and diluted in 0.1 M acetic acid-sodium acetate buffer, pH 6.5, to 10 times the desired final concentrations. At the time of testing, 1.0-ml samples of leukocytes, combined with 0.2 ml of pooled serum and a stirring rod, were allowed to incubate for 4 min on the oxygen monitor before the addition of 1.5 ml of HBSS and 0.3 ml of PMA solution. Final concentrations of PMA were 0.1, 1, and 10  $\mu\text{g/ml}$  of leukocyte suspension ( $4 \times 10^6$  PMN/ml). The cell suspensions combined with PMA were also exposed to the carrier, DMSO, at final concentrations of 0.01, 0.1, and 1% when the stock solution containing 1 mg/ml of PMA was employed, and half those amounts when the stock solution containing 2 mg/ml was used.

Oxygen consumption was measured on a Yellow Springs electrode monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) coupled to a Beckman recorder (Beckman Instruments Inc., Fullerton, Calif.) with moving graph paper advanced at 0.5 in/min. Experimental samples were maintained at  $37^\circ\text{C}$  by a circulating water bath and mixed continuously by a magnetic stirrer. The rate of oxygen consumption in  $\mu\text{l O}_2/\text{h}/4 \times 10^6$  PMN was calculated by measuring the slope of the recorded tracing over a 10-min period. Resting rates were determined for leukocyte samples combined with HBSS, HBSS with serum, HBSS with serum plus DMSO, and DMSO alone. Heat-killed bacteria (HKB) alone, HBS alone, or serum and solutions of PMA and DMSO alone had no effect on the base line of the tracing. Potassium cyanide, 0.001–0.004 M, was added to the neutrophil suspensions before HKB or PMA in some experiments before analysis of oxygen uptake.

Steady states of basal and stimulated oxygen consumption by leukocyte suspensions were reached rapidly and remained steady over the 10-min period of observation. At the end of the analysis, the samples of leukocytes combined with *S. aureus*, with PMA, DMSO, or with HBSS alone were fixed in glutaraldehyde and osmic acid and embedded in Epon 812, (Shell Chemical Co., New York) for study in the electron microscope according to the methods described in several publications (5, 9, 13–15). Thin sections were stained with uranyl acetate and lead citrate before examination in a Philips 200 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.).

The influence of PMA and HKB on the oxidative metabolism of neutrophils was examined with isotopically labeled glucose (16).  $^{14}\text{CO}_2$  produced by oxidation of  $[1-^{14}\text{C}]$ glucose was trapped on filter paper, saturated with 20% sodium hydroxide, and suspended from the stopper of a 15-ml flask containing  $4 \times 10^6$  PMN in 1 ml of HBSS. In control studies  $[6-^{14}\text{C}]$ glucose was substituted for  $[1-^{14}\text{C}]$ glucose. Sensitivity of glucose oxidation stimulated by PMA and HKB to cyanide was determined in some experiments by adding 0.001–0.004 M potassium cyanide to the incubation medium. Analysis was carried out as described previously (16).

The ability of neutrophils to reduce NBT to the blue formazan derivative after exposure to PMA, HKB, DMSO, or HBSS was determined by the method reported earlier (17).

## RESULTS

Oxygen consumption in neutrophil samples from six patients with CGD was analyzed on eight separate occasions. Results of these studies are recorded in Table I and compared with the oxygen uptake by normal PMN. Unstimulated (resting) neutrophils from patients with CGD consumed oxygen at a mean rate of  $0.8 \pm 0.2 \mu\text{l}/\text{h}/4 \times 10^6$  PMN ( $\pm 2$  SEM). This rate of oxygen uptake was about half the resting rate of oxygen consumption by normal neutrophils ( $1.5 \pm 0.2 \mu\text{l}/\text{h}/4 \times 10^6$  PMN) and the difference was significant ( $P < 0.001$ ). The value remained constant over the 10-min period of measurement in each experiment.

The addition of HKB to neutrophils from CGD patients stimulated a slight increase in oxygen consumption by the abnormal cells. At a ratio of one HKB to each neutrophil, the oxygen uptake increased to a rate of  $1.5 \pm 0.3 \mu\text{l}/\text{h}/4 \times 10^6$  PMN. A ratio of 10 HKB per PMN caused an insignificant rise in oxygen utilization, and the higher ratios of 50:1 and 100:1 produced no more of an increase in consumption than had been stimulated by 1 HKB: 1 PMN. Normal neutrophils stimulated by increasing ratios of HKB to PMN under the same conditions as CGD cells responded with incremental increases in oxygen uptake (Table I). At the rate of 100 HKB: 1 PMN, the oxygen uptake by normal neutrophils was 12 times the rate of resting cells. Thus, the difference in the rate of oxygen consumption by normal and CGD cells was apparent in unstimulated samples and in neutrophils exposed to a single bacterium per

TABLE I  
*Oxygen Consumption by Normal and CGD PMN Stimulated with PMA and HKB*

Test conditions	Degree of stimulation	Oxygen consumption	
		Normals	CGD
$\mu\text{l O}_2 \text{ consumed/h/4} \times 10^6 \text{ PMN}$			
Unstimulated PMN	None	1.5±0.2* (30)‡	0.8±0.2 (16)
PMN + HKB	1 HKB: 1 PMN	3.5±0.4 (17)	1.5±0.3 (7)
	10 HKB: 1 PMN	8.4±2.1 (7)	1.8±0.6 (4)
	50 HKB: 1 PMN	14.6±1.5 (24)	1.7±0.3 (9)
	100 HKB: 1 PMN	18.1±2.1 (19)	1.6±0.3 (12)
PMN + PMA	0.1 $\mu\text{g}$ PMA/ml	3.0±0.4 (15)	1.1±0.5 (4)
	1 $\mu\text{g}$ PMA/ml	15.0±1.7 (23)	1.2±0.1 (9)
	10 $\mu\text{g}$ PMA/ml	20.9±2.8 (10)	1.4±0.3 (4)
PMN + PMA + HKB	1 $\mu\text{g}$ PMA/ml + 50 HKB: 1 PMN	21.9±2.0 (21)	1.5±0.5 (5)
Buffer, PMA, HKB, and PMA + HKB in the absence of PMN	HBSS	0.3±0.1 (22)	0.2±0.1 (10)
	HBSS + PMA	0.5±0.1 (20)	0.6±0.3 (9)
	HBSS + HKB	0.1±0.1 (14)	0.2±0.2 (5)
	HBSS + PMA + HKB	0.5±0.1 (10)	0.5±0.1 (8)

\* Two standard errors of the mean.

‡ Number of runs.

Increasing concentrations of PMA and HKB caused significant increases in the rate of oxygen utilization by normal PMN, but produced only slight changes in the amount of oxygen consumed by CGD neutrophils. Addition of both agents to the same sample stimulated increased oxygen uptake by normal PMN, but failed to significantly alter oxygen consumption of CGD cells. The reagents without PMN had little influence on oxygen consumption.

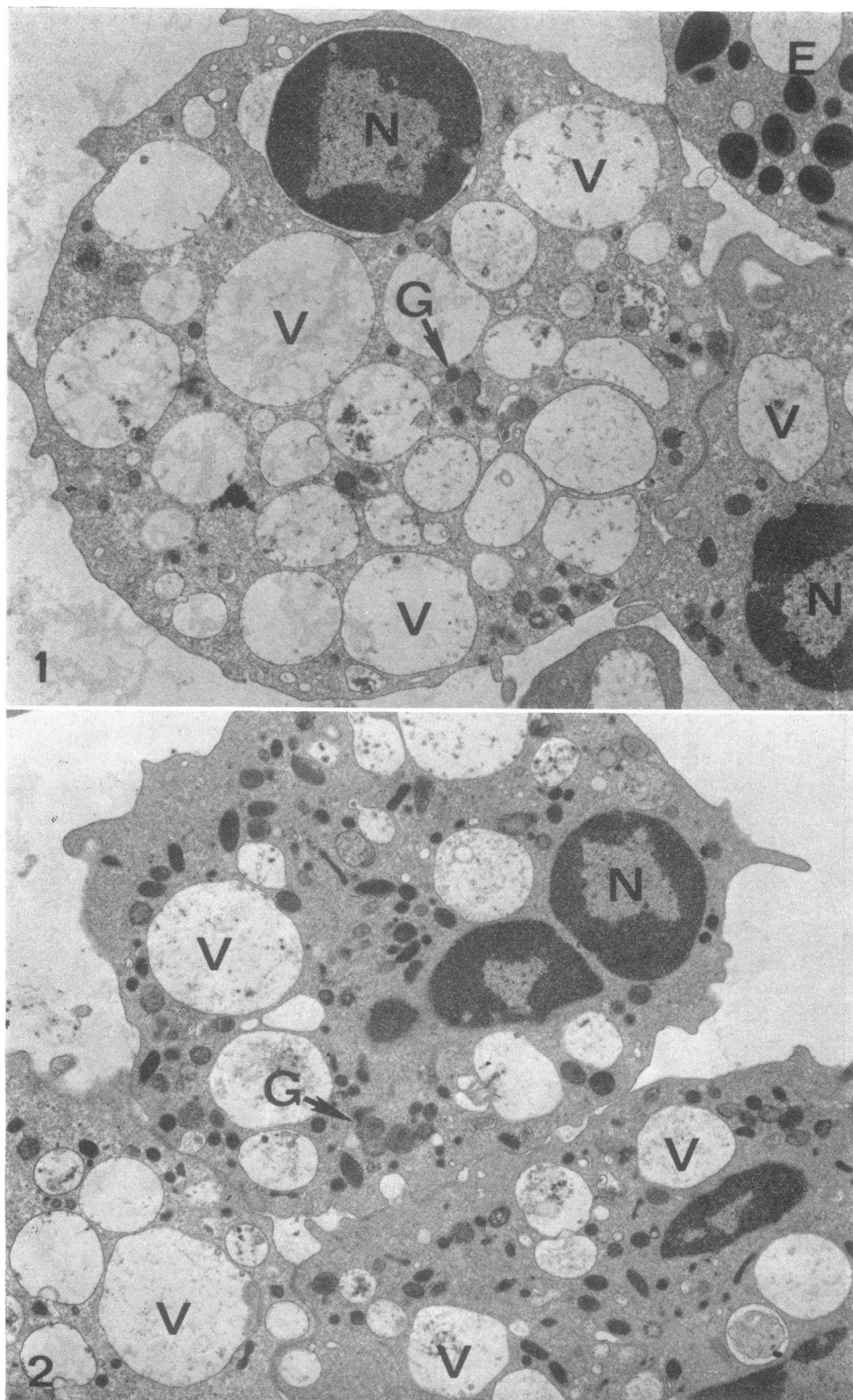
PMN, but was more clearly delineated by the response of normal and abnormal cells to increasing numbers of HKB.

The response of normal and abnormal PMN to stimulation by PMA was nearly identical to the reaction caused by HKB. When added to normal neutrophils, PMA caused an immediate surge in oxygen consumption (Table I). Each increase in the amount of PMA added to the system produced a proportionately greater rise in oxygen uptake by the cells. The highest concentration of PMA used in this study, 10  $\mu\text{g}/\text{ml}$ , yielded a mean rate of  $20.9 \pm 2.8 \mu\text{l/h/4} \times 10^6 \text{ PMN}$ , a value 14 times the rate of oxygen consumption by resting normal neutrophils. PMN from patients with CGD did not respond significantly to stimulation by PMA. Higher rates of oxygen consumption were recorded with each increase in the amount of PMA added to the system. However, the differences were not significant, and even the highest concentration of PMA, 10  $\mu\text{g}/\text{ml}$ , caused only a slight increase, equivalent to the resting rate of oxygen consumption by normal PMN. Potassium cyanide, 0.001–0.004 M, had no effect on oxygen consumption stimulated in PMN by HKB or PMA. DMSO alone at a concentration of 1% did not influence the

resting rate of oxygen uptake by normal or CGD neutrophils.

Although CGD neutrophils failed to develop a burst of oxygen consumption after stimulation by PMA, they did undergo morphological changes which resembled alterations observed in PMA-treated normal PMN (9, 11). PMA-stimulated normal and CGD cells, fixed for study in the electron microscope immediately after removal from the oxygen monitor, revealed alterations not apparent in unstimulated neutrophils prepared for study under identical conditions (Figs. 1 and 2). Neutrophils exposed to PMA were frequently clumped together in large aggregates. The cytoplasm of normal and CGD neutrophils contained large numbers of vacuoles not evident in untreated control cells. All three concentrations of PMA produced similar effects. The number of cells affected and the number and size of cytoplasmic vacuoles varied with the amount of PMA added to the system (9). Normal PMN appeared to develop more and larger cytoplasmic vacuoles than CGD neutrophils exposed to the same concentration of the agent. 1% DMSO alone had no apparent effect on the morphology of normal or CGD neutrophils.

The PMA-induced formation of vacuoles in normal PMN was associated with reduction of NBT and acti-



FIGURES 1 and 2 Neutrophils from a normal control (Fig. 1) and a patient with CGD (Fig. 2) fixed on the oxygen monitor 10 min after addition of PMA at a final concentration of 1  $\mu\text{g/ml}$  of leukocyte suspension ( $4 \times 10^6$  PMN/ml). A portion of an eosinophil (E) is

TABLE II  
*Oxidation of [1-<sup>14</sup>C] Glucose by Normal and CGD PMN Stimulated with PMA and HKB*

Test conditions	Degree of stimulation	Rate of oxidation	
		Normals	CGD
<i>cpm/20 min/4 × 10<sup>6</sup> PMN</i>			
Unstimulated PMN	None	49±7* (27)‡	23±5 (6)
PMN + HKB	1 HKB: 1 PMN	82±6 (4)	14 (2)
	50 HKB: 1 PMN	601±77 (25)	24±6 (6)
PMN + PMA	0.1 µg PMA/ml	207 (2)	—
	1 µg PMA/ml	2,106±225 (28)	60±15 (6)
Buffer, PMA, HKB, in absence of PMN	HBSS	6±4 (6)	9 (4)
	HBSS + PMA	8±3 (6)	4 (4)
	HBSS + HKB	5±3 (6)	6 (4)

\* Two standard errors of the mean.

† Number of runs.

The resting rate of glucose oxidation by CGD neutrophils was approximately  $\frac{1}{2}$  that of normal PMN in this study. CGD neutrophils responded slightly to stimulation by the highest concentration of PMA used (1 µg/ml), but did not react significantly to the lower concentration of PMA or to either of the two ratios of HKB employed. Normal PMN developed a slight increase in glucose oxidation when exposed to HKB at a ratio of one organism to one neutrophil, and dramatically increased glucose oxidation on incubation with the higher ratio of HKB to PMN and with both concentrations of PMA. In the absence of PMN the reagents had little influence on glucose oxidation.

vation of the hexose monophosphate shunt (HMPS) (10, 11). Since PMA caused vacuoles to develop in CGD cells without increasing their rate of oxygen consumption, it seemed worthwhile to determine if HKB and PMA influenced NBT reduction and glucose metabolism in the abnormal cells. Results of these studies are recorded in Tables II and III. Normal neutrophils exposed to HKB or PMA rapidly produced <sup>14</sup>C-labeled carbon dioxide from [1-<sup>14</sup>C]glucose, and the oxidation of [1-<sup>14</sup>C]glucose was sensitive to 0.004 M potassium cyanide. The oxidation of [6-<sup>14</sup>C]glucose was stimulated slightly in normal cells by PMA or HKB. Glucose oxidation was not stimulated significantly in CGD neutrophils by either HKB or PMA. DMSO alone at a concentration of 1% did not influence the oxidation of glucose by normal or abnormal cells. A similar dichotomy was observed in the capacity of the normal and CGD neutrophils to reduce NBT dye after stimulation by HKB and PMA. Both agents caused a significant number of normal PMN to reduce NBT but failed to stimulate dye conversion by CGD neutrophils. DMSO alone did not cause NBT reduction by normal or abnormal cells.

## DISCUSSION

PMA is the 12-0-tetradecanate and 13-acetate of the 20-carbon alcohol, phorbol (18, 19). The fatty acid ester of phorbol is best known for its capacity to enhance

TABLE III  
*Reduction of NBT dye by Normal and CGD PMN Stimulated with PMA and HKB*

Test conditions	Degree of stimulation	Percentage NBT-positive PMN	
		Normals	CGD
<i>per 100 cells counted</i>			
Unstimulated PMN	None	10±5* (4)‡	0 (4)
PMN+HKB	50 HKB: 1 PMN	50±5 (4)	0 (4)
PMN+PMA	0.1 µg PMA/ml	90±10 (4)	0 (4)

\* Two standard errors of the mean.

† Number of runs.

A concentration of PMA (0.1 µg/ml) which stimulated 90% of normal PMN to convert the dye failed to cause CGD neutrophils to reduce NBT. HKB also stimulated NBT reduction by normal PMN without affecting neutrophils from patients with CGD.

present with two neutrophils in the thin section of PMA-treated normal neutrophils. Lobes of the nucleus (N), the surface membranes, and some cytoplasmic granules (G) remain intact in the altered cells. Large and small vacuoles (V) fill the cytoplasm of the normal neutrophils after exposure to PMA and are also present in some eosinophils. PMN from the patient with CGD (Fig. 2) also contain numerous cytoplasmic vacuoles (V). Fig. 1 × 11,500; Fig. 2 × 9,700.

the tumor-promoting activity of carcinogens (20–22). Efforts to define the influence of PMA on carcinogenesis have revealed a wide variety of actions in several cell systems. PMA is a surface-active agent that binds to cell membranes, but can be removed by methanol treatment (23). It causes refractile changes in the membranes (24), stimulates the activities of  $\text{Na}^+$  and  $\text{K}^+$ -dependent surface ATPase and 5' nucleotidase (25), and initiates proliferation of contact-inhibited 3T3 fibroblasts (26). The response of cultured cells may reflect intracellular effects of PMA, for the agent stimulates RNA (27) and DNA (22, 28) synthesis and mitosis (29). In some cell systems PMA causes a rapid fall in the intracellular level of cyclic 3',5'-AMP (30) and in others stimulates increased levels of cyclic 3',5' GMP (31). High concentrations of PMA have been noted to damage cell membranes, labilize lysosomes, and disrupt mitochondria (32), while small amounts can stimulate platelet aggregation and secretion without causing the cells to lose cytoplasmic enzymes that would suggest injury or increased permeability (33, 34). Several other effects of PMA on cells and subcellular organelles have been observed (35–37), and the multiplicity of actions has made it difficult to determine the specific mechanism by which it influences carcinogenesis.

The present investigation has extended the range of knowledge about this versatile agent to the evaluation of abnormal PMN from patients with CGD. Earlier studies demonstrated that PMA caused the development of vacuoles in the cytoplasm of normal neutrophils in association with a burst of oxygen consumption, a marked increase in HMPS activity, and the reduction of NBT dye (9–11). In this study, similar concentrations of PMA added to CGD neutrophils produced the cytoplasmic vacuoles but failed to stimulate the metabolic response of the cells significantly. The highest concentration of PMA employed increased the rate of oxygen uptake by CGD neutrophils to the resting rate of oxygen consumption by normal cells. Normal PMN exposed to the same amount of PMA consumed oxygen at a rate 14 times that of resting cells. A concentration of PMA which stimulated a marked increase in the cyanide-insensitive conversion of  $[1-^{14}\text{C}]$ glucose to  $^{14}\text{C}$ -labeled carbon dioxide by normal PMN had little influence on glucose oxidation in CGD neutrophils. Over 90% of normal PMN reduced NBT after exposure to PMA, but the same amount of agent did not stimulate dye conversion by CGD cells.

Many chemical substances besides PMA have been found to stimulate a response resembling phagocytosis in normal PMN. Surface-active agents, such as digitonin, deoxycholate, and saponin, cause increased oxygen consumption and glucose oxidation by PMN, while others, including Triton X-100, Tween-80, cetylpyri-

dinium chloride, lauryl sulfate, and vitamin A alcohol, have little or no effect on metabolism (38). Electron microscope studies have revealed marked changes in the surface membranes of PMN treated with digitonin, but none of the surface active agents stimulating neutrophil metabolism has been reported to initiate cytoplasmic vacuole formation. Macromolecular species that attach to cell surfaces such as endotoxin (39), concanavalin A (40), and immune complexes (41) also increase PMN oxygen uptake and glucose metabolism without causing the affected cells to form vacuoles. Epinephrine, norepinephrine, adrenochrome, DOPA, ascorbic acid (42) and phenazine methosulfate (43, 44) activate PMN glucose metabolism and elevate the rate of oxygen consumption, but do not produce large vacuoles in neutrophils. Ascorbic acid is one of the few chemical substances evaluated for its influence on the metabolism of CGD neutrophils, and the abnormal PMN responded to the same degree as normal cells when exposed to this agent (45, 46). Thus PMA appears to be the first chemical stimulant of neutrophil oxygen consumption and glucose oxidation to cause simultaneous development of intracellular vacuoles and to possess the capacity to discriminate between normal and CGD cells.

The effects of PMA and HKB on normal and CGD neutrophils in this and in previous investigations (9–11) were similar but not identical. PMA produced a more rapid onset of increased oxygen consumption than HKB and did not require calcium ions or serum opsonins for its influence on PMN. The chemical agent also caused selective labilization and discharge of products confined to specific granules, whereas the uptake of HKB or other particulates into phagocytic vacuoles stimulated the release of substances from azurophilic lysosomes as well as specific granules. Yet similarities in the response of normal and abnormal CGD neutrophil to HKB and PMA appear as compelling as the differences. Even the reduced size of vacuoles in PMA-treated CGD cells resembled the smaller average size of phagocytic vacuoles in abnormal PMN after ingestion of organisms (5). Also, the capacity of PMA to stimulate vacuole formation without increasing glucose oxidation or oxygen consumption in CGD neutrophils is essentially identical to the effect of the particulates on the abnormal cells. Thus, PMA is interesting not only because it is the first chemical agent to distinguish between normal and CGD cells, but also because it provides a new tool for deciphering basic aspects of the phagocytic process in normal and abnormal PMN.

In conclusion, the findings of this investigation have shown that a chemical agent, PMA, is as efficient as HKB in detecting the metabolic abnormality in PMN from patients with CGD. The capacity of PMA to discriminate between normal and CGD neutrophils sug-

gests that the drug may stimulate membranes through mechanisms activated by particulates, but at present the precise mode of drug action in this cell system remains uncertain. Additional investigations currently in progress may clarify the mechanism of PMA action and extend the usefulness of the agent to other problems of neutrophil dysfunction. Preliminary studies, for example, on obligate carrier relatives of patients with CGD have revealed that PMA can detect partial metabolic deficiencies as well as any other available test of PMN function.

## ACKNOWLEDGMENTS

This work was supported by U. S. Public Health Service grants HL-11880, AM-15010, AM-15317, HL-06314, CA-11996, CA-08832, AI-10033, National Science Foundation GB-33923X, American Heart Association, American Cancer Society PRA-57, Minnesota Medical Foundation, and Twin Cities Diabetes Association.

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