

Studies in Normal and Chronic Granulomatous Disease Neutrophils Indicate a Correlation of Tubulin Tyrosinolation with the Cellular Redox State

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ABSTRACT A specific stimulation of tubulin tyrosinolation in human polymorphonuclear leukocytes (PMN) is induced by the synthetic peptide chemoattractant, *N*-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe), and this stimulation of tyrosinolation in PMN is completely inhibited in the presence of various reducing agents. Further studies to characterize the mechanism of stimulation of tyrosinolation in PMN have revealed that conditions that inhibited the respiratory burst in stimulated PMN, e.g., an anaerobic atmosphere, or addition of antioxidants such as cysteamine, azide, or 2,3-dihydroxybenzoic acid, also inhibited the peptide-induced stimulation of tyrosinolation in these cells. Moreover, the sulfhydryl reagent, *N*-ethylmaleimide, depressed tyrosinolation in resting PMN and completely inhibited the fMet-Leu-Phe-induced stimulation. In contrast, addition of diamide, which preferentially oxidizes cellular glutathione, significantly stimulated tyrosinolation both in resting and fMet-Leu-Phe-stimulated PMN. Furthermore, resting levels of tyrosinolation in seven patients with chronic granulomatous disease (CGD), whose oxidative metabolism is severely depressed, were 35–45% lower ($P < 0.01$). Most strikingly, PMN from CGD patients failed to respond to fMet-Leu-Phe or the Ca^{2+} -ionophore A23187, which also induced stimulation of tyrosinolation in normal resting PMN. Methylene blue normalized the depressed tyrosinolation in resting CGD PMN, although it did not increase tyrosinolation in stimulated PMN. These results are consistent with the idea that the characteristic activation of the oxi-

dative metabolism and the associated changes in the redox state in stimulated PMN are coupled to the induction of stimulation of tubulin tyrosinolation in these cells.

INTRODUCTION

In recent years, it has become increasingly clear that neutrophil function is influenced not only by the immune system and the availability of cytoplasmic and granule-associated enzymes, but also by the dynamic properties of the cell membrane (1–4), cytoplasmic microtubules (1, 5–7), and microfilaments (8), which are the major constituents of the cytoskeleton. The processes of chemotaxis, phagocytosis, oxidant generation, and lysosomal degranulation are of central importance to neutrophil function (1). Their initiation depends on the existence on the plasma membrane of receptors that recognize and bind surface ligands (9), setting in motion a variety of cellular events. It includes the activation of membrane-associated enzyme systems (10, 11), ion fluxes (12, 13), enhanced oxidative metabolism (14), and the specific assembly and reorganization of microtubules (6) and microfilaments (8). The motile and bactericidal functions are subsequently expressed.

Tubulin, the protein dimer that is the major component of the microtubules, is subject to a unique reversible modification, whereby in an ATP-dependent reaction, a tyrosine residue is specifically added through a peptide linkage to the α -carboxyl of the α -chain carboxy-terminal glutamate (15, 16). The enzyme that catalyzes this reaction, tubulin tyrosine ligase (ligase), has been detected both in vertebrate (15, 17) and invertebrate (18) cells and tissue. Although the biological

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importance of this reaction has not yet been established, changes in the state of tyrosinolation of tubulin have been observed in cells undergoing cytoskeletal reorganization (19, 20). We have recently reported a specific, dose-dependent stimulation of tubulin tyrosinolation in rabbit peritoneal neutrophils (21) and also in human peripheral blood neutrophils (22), as induced by the synthetic peptide chemoattractant, *N*-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe).¹ In addition, resting peripheral blood polymorphonuclear leukocytes (PMN) from patients with the Chediak-Higashi syndrome (CHS) were found to have two- to threefold higher levels of tubulin tyrosinolation when compared with normal PMN (22), and the higher tyrosinolation in CHS cells could be corrected by the addition of ascorbate or other reducing agents such as reduced glutathione (GSH), cysteine, or dithiothreitol (DTT) to the reaction medium (22). More significantly, the fMet-Leu-Phe-induced stimulation of tubulin tyrosinolation was completely inhibited in the presence of ascorbate and other reducing agents, a result that was true in both normal PMN and in PMN from CHS patients (22). The abnormally high levels of tubulin tyrosinolation in PMN of CHS patients (22), whose oxidative metabolism is reportedly exaggerated (14, 23), together with the observed inhibitory effects of ascorbate and various reducing agents (22), suggested a possible relationship between cellular oxidative metabolism and tubulin tyrosinolation in PMN.

Since cytoskeletal changes involved in PMN chemotaxis are not necessary for stimulation of tubulin tyrosinolation (21, 22) and in view of the results obtained with various reducing agents (22), it was possible that the fMet-Leu-Phe-induced stimulation of tubulin tyrosinolation (21, 22) was somehow coupled to the increased oxidative metabolism and associated cellular redox changes of these cells (14). The latter event includes an enhanced oxygen consumption via the hexosemonophosphate shunt (HMPS) and the production of reduced oxygen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (14, 23). The primary enzymatic system involved in this process is a membrane-bound pyridine nucleotide (NADPH)-dependent oxidase (10, 11, 24, 25). Upon perturbation of the PMN cell membrane, the oxidase converts from an inactive to an active form that transfers electrons from NADPH to molecular oxygen, reducing it to the free radical O_2^- (10, 11, 26,

27). The activation of NADPH-oxidase is coupled to the activation of the HMPS (14, 28) and to the effective operation of the glutathione redox system (7, 14, 29).

Therefore, to further characterize and understand the modulation of tubulin tyrosinolation in PMN, we have studied tyrosinolation in resting and stimulated PMN after manipulation of their oxidative metabolism and redox state and/or thiol-disulfide status. We have also studied tyrosinolation in PMN from seven patients (four male and three female) with chronic granulomatous disease (CGD) whose oxidative metabolism is severely depressed because of an impaired function of the membrane-associated NADPH-dependent oxidase (24, 30, 31). In addition, we have studied the effect of the Ca^{2+} -ionophore A23187, which stimulates PMN oxidative metabolism (32), on tubulin tyrosinolation in normal resting PMN and in PMN from patients with CGD. The details of these experiments and possible functional implications of the results are described.

METHODS

Preparation of PMN. Heparinized, human peripheral blood, either from normal donors or patients with CGD, was separated into a granulocyte-rich fraction by Hypaque-Ficoll and dextran sedimentation techniques (33). For all the experiments to be described, freshly prepared cell fractions containing >95% PMN with >95% viability were used. Cell viability was determined by the exclusion of trypan blue dye. PMN from four male and three female patients with CGD have been used in this study. Diagnosis of their disease was already confirmed by established *in vitro* metabolic and functional criteria (34–36).

Posttranslational incorporation of [^{14}C]tyrosine in human PMN. Since tubulin tyrosinolation is a posttranslational reaction (15, 16), it can be studied in intact functional cells by measuring incorporation of radiolabeled tyrosine into a trichloroacetic acid (TCA)-insoluble fraction, in the absence of protein synthesis (19, 21, 22). Therefore, before the addition of [^{14}C]tyrosine to the reaction medium, cells were routinely incubated for 30 min at 37°C with a mixture of cycloheximide, puromycin, and chloramphenicol (21, 22), which inhibited protein synthesis to >99% without inhibiting chemotaxis (21, 22). As previously reported, the radiolabeled tyrosine was specifically incorporated into tubulin under these experimental conditions (21, 22). Moreover, most of the fixed radioactivity could be released upon digestion with carboxypeptidase A, indicating a carboxy-terminal location of the incorporated tyrosine (37).

For studying tyrosinolation in PMN, freshly fractionated normal or CGD cells were collected by centrifugation at 1,000 *g* for 5 min and resuspended at a cell density of 10^7 /ml in an isoosmotic medium, as previously described (21), with bovine serum albumin added to a final concentration of 0.1%. All other experimental conditions were identical to those described for studying tubulin tyrosinolation in rabbit leukocytes (21). Where indicated, 10^{-7} M fMet-Leu-Phe was added at zero time along with [^{14}C]tyrosine (5 μ Ci/ml). Unless indicated otherwise, the various agents, the effects of which were to be studied on resting and fMet-Leu-Phe-stimulated tyrosinolation, were also present during the 30-min incubation with antibiotics. To study the effect of the Ca^{2+} -ionophore A23187 on tubulin tyrosinolation, the procedures

¹ **Abbreviations used in this paper:** BESA, 2-bromoethane sulfonic acid; CGD, chronic granulomatous disease; CHS, Chediak-Higashi syndrome; DHB, dihydroxybenzoic acid; DTT, dithiothreitol; fMet-Leu-Phe, *N*-formyl-methionyl-leucyl-phenylalanine; HMPS, hexosemonophosphate shunt; NEM, *N*-ethylmaleimide; O_2^- , superoxide anion.

were identical to those described for fMet-Leu-Phe. Where indicated, 10^{-6} M A23187 was added at 0 min.

Tubulin tyrosinolation under anaerobic conditions. To study tubulin tyrosinolation under anaerobic conditions, PMN samples (10^7 cells/ml) were continuously flushed with a gentle stream of nitrogen (N_2) throughout the entire course of the incubation period. This was achieved by using incubation tubes with tightly fitting flexible rubber stoppers through which fine needles could be inserted. These tubes were connected by rubber tubings to a cylinder containing N_2 . A series of tubes could be set up in this fashion by interconnecting them. At the end of the incubation period with antibiotics, various additions were made by injection through the rubber stoppers by using appropriate Hamilton syringes (Hamilton Co., Reno, NV). The PMN samples were then incubated with [^{14}C]tyrosine (5 μ Ci/ml) for 60 min in the presence or absence of 10^{-7} M fMet-Leu-Phe under a steady stream of N_2 . The reaction was carried out in a shaking water bath at 37°C, as usually used for studying tubulin tyrosinolation in intact cells (19). Along with the anaerobic samples, duplicate samples of PMN were incubated in parallel to monitor and compare tyrosine incorporation in the presence or absence of fMet-Leu-Phe under usual aerobic conditions (22).

At the end of the incubation period, the PMN samples were chilled on ice, and they were rapidly filtered through Whatman GF/B filters (Whatman Laboratory Products, Inc., Whatman Paper Div., Clifton, NJ; 2.4-cm diam) that were placed on a vacuum filtration manifold (Hoefer Scientific Instruments, San Francisco, CA). The filters were then washed rapidly with three 10-ml portions of ice-cold 10% TCA, followed by two 5-ml portions of ethanol. The filters were carefully removed from the filtration manifold and briefly dried under a heat lamp. The TCA-insoluble radioactivity of the dried filters were counted in a Beckman scintillation spectrometer (Beckman Instruments, Inc., Palo Alto, CA) using 10 ml of the scintillant 3a20 (Research Products International Corp., Elk Grove, IL).

Determination of the specific radioactivity of the intracellular pool of tyrosine. The procedures for the determination of the specific radioactivity of the intracellular tyrosine pool in normal and CGD PMN were identical to those previously described for rabbit leukocytes (21) and human PMN (22). Briefly, the different samples of PMN were incubated with [^{14}C]tyrosine in the presence of antibiotics and then were rapidly filtered and washed with ice-cold phosphate-buffered saline. The tyrosine was then extracted with 7% TCA, and the TCA extracts were lyophilized and reconstituted in a desired volume of distilled water. Equal amounts of each sample were analyzed in a Durrum amino acid analyzer (Dionex Corp., Sunnyvale, CA) for their tyrosine content and corresponding radioactivity. Where indicated, 10^{-7} M fMet-Leu-Phe was added to the PMN samples at 0 min. Incubation was carried out for 90 min at 37°C in a shaking water bath.

Chemicals. Cycloheximide, chloramphenicol, puromycin, 3,5-dihydroxybenzoic acid (DHB), cysteamine, and diamide were purchased from Sigma Chemical Co. (St. Louis, MO). The synthetic peptide attractant, fMet-Leu-Phe, was from Peninsula Laboratories, Inc. (San Carlos, CA). N-ethylmaleimide (NEM) and 2,3-DHB were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI), and L-[U- ^{14}C]tyrosine was obtained from Amersham Corp. (Arlington Heights, IL). The Ca^{2+} -ionophore A23187 was purchased from Calbiochem-Boehringer Corp. (San Diego, CA). Methylene blue was obtained from Fisher Scientific Co. (Fairlawn, NJ).

RESULTS

Effects of various antioxidants and anaerobic atmosphere on posttranslational incorporation of tyrosine in resting and stimulated PMN. In view of our earlier studies that demonstrated the marked inhibitory effects of various reducing agents on fMet-Leu-Phe-induced stimulation of tyrosinolation (22), we have further tested the effects of antioxidants such as cysteamine (38, 39), azide (40), and 2,3- and 3,5-DHB (41, 42) on fMet-Leu-Phe-stimulated tyrosinolation in PMN. Similar to the reported results with other reducing agents (22), all of the above antioxidants at concentrations of 10^{-4} – 10^{-3} M caused significant inhibition of the peptide-induced stimulation of tyrosinolation in PMN (data not shown).

Anaerobic conditions reportedly inhibit the characteristic "respiratory burst" in stimulated PMN (24). In view of the results obtained with various reducing agents (22) and antioxidants, we studied tyrosinolation under anaerobiosis in an atmosphere of N_2 . As shown in Table I, the fMet-Leu-Phe-induced stimulation of tyrosine incorporation, as observed under our routine experimental conditions (i.e., in air), was completely inhibited under anaerobic conditions. These results strongly suggest a correlation between tubulin tyrosinolation and oxidative-reductive reactions in PMN.

Effects of sulfhydryl reagents on tyrosinolation in PMN. Thiol-disulfide status of PMN has been reported to play an important functional role in these cells (43–45), and modification of tubulin sulfhydryl groups also affects microtubule-related cell motility in other cells (46). Therefore, we have studied the effect of NEM, which irreversibly alkylates available (intracellular) sulfhydryl groups and inhibits O_2^- production

TABLE I
Effect of Anaerobic Atmosphere on the Posttranslational Incorporation of Tyrosine in PMN

PMN sample	[^{14}C]tyrosine-fixed cpm/ 10^7 cells
In air	
Resting	3,680±105
+ 10^{-7} M fMet-Leu-Phe	8,480±190
In nitrogen	
Resting	2,700±85
+ 10^{-7} M fMet-Leu-Phe	2,500±75

PMN at 10^7 /ml were incubated for 60 min with [^{14}C]tyrosine (5 μ Ci/ml = 0.01 μ mol) in the presence of antibiotics. As indicated, 10^{-7} M fMet-Leu-Phe was added at zero min. TCA-insoluble radioactivity was determined as described in Methods. The data are means of duplicate determinations.

in PMN (47), and 2-bromoethane sulfonic acid (BESA), which selectively blocks extracellularly exposed sulfhydryl groups by forming stable thioether bonds (48), on tubulin tyrosinolation in PMN. Since both reagents react in an irreversible manner, for the experiments shown in Fig. 1 PMN samples were incubated for 10 min in the presence or absence of 10^{-3} M NEM or BESA, and the cells were centrifuged at 1,000 g and resuspended in fresh incubation medium before studying tubulin tyrosinolation. As shown in Fig. 1, prior incubation with 10^{-3} M NEM severely depressed resting levels of tyrosine incorporation, and the cells failed to respond to fMet-Leu-Phe. The NEM-treated PMN were still viable as determined by the exclusion of trypan blue dye, although morphologic examination revealed a rounded appearance of the cells with markedly smooth cell surfaces (not shown).

In contrast to the effects of NEM, BESA, a lipid-

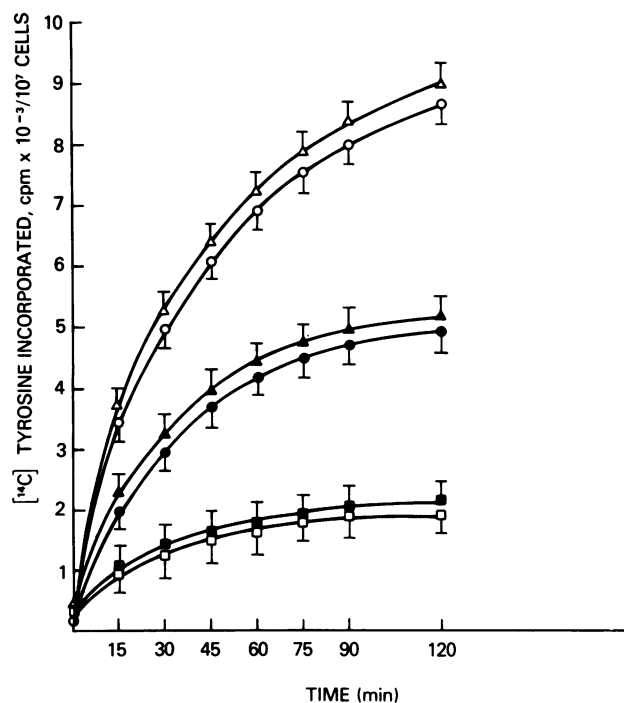


FIGURE 1 Effects of NEM and BESA on posttranslational incorporation of tyrosine in resting and fmet-leu-phe-stimulated PMN. Where indicated, cells at 10^7 /ml were incubated with 10^{-3} M NEM or BESA for 10 min at 37°C and centrifuged and resuspended in fresh incubation medium. PMN samples were then incubated with antibiotics for 30 min at 37°C . [^{14}C]tyrosine ($5 \mu\text{Ci} = 0.01 \mu\text{mol}$) and/or fMet-Leu-Phe were added at 0 min and TCA-insoluble radioactivity was measured at indicated times. The data are means \pm SEM for three different experiments. Δ , +BESA+fMet-Leu-Phe; \circ , +fMet-Leu-Phe; \blacktriangle , +BESA; \bullet , control; \blacksquare , +NEM+fMet-Leu-Phe; \square , +NEM.

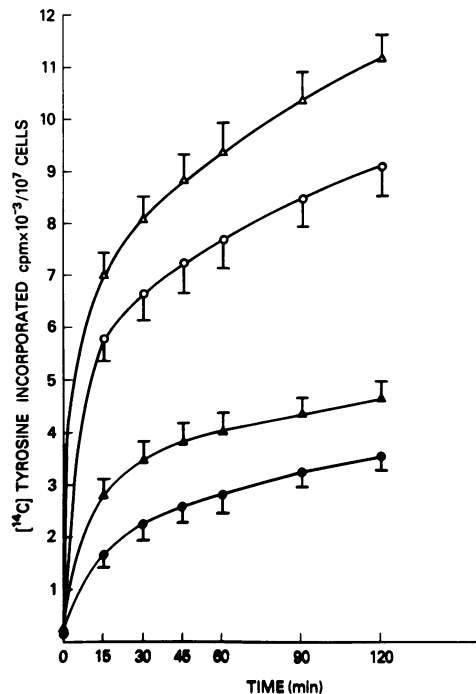


FIGURE 2 Effect of diamide on resting and fMet-Leu-Phe-stimulated incorporation of tyrosine in PMN. Cells were incubated with antibiotics for 30 min at 37°C in the presence or absence of 10^{-4} M diamide. [^{14}C]tyrosine ($5 \mu\text{Ci} = 0.01 \mu\text{mol}$) and/or fMet-Leu-Phe were added at 0 min. TCA-insoluble radioactivity was determined at indicated times. Data are means \pm SEM of three separate experiments. Δ , +diamide+fMet-Leu-Phe; \circ , +fMet-Leu-Phe; \blacktriangle , +diamide; \bullet , control.

insoluble, highly polar compound that does not penetrate the cell membrane, and, therefore, primarily reacts with sulfhydryl groups on the cell surface (48), did not inhibit tyrosine incorporation in either resting or fmet-leu-phe-stimulated PMN (Fig. 1).

Effect of diamide on posttranslational incorporation of tyrosine in PMN. Diamide is a chemical oxidant of GSH that preferentially oxidizes cellular GSH (49) to glutathione disulfide (GSSG). Since GSH peroxidase-catalyzed reduction of H_2O_2 plays an important functional role in PMN redox changes (43–45), we studied the effect of diamide on tubulin tyrosinolation in resting and fMet-Leu-Phe-stimulated PMN. As shown in Fig. 2, 10^{-4} M diamide caused a significant stimulation ($P > 0.02$) of both resting and fMet-Leu-Phe-stimulated incorporation of tyrosine in PMN, providing further evidence in support of a causal relationship between cellular redox and modulation of tubulin tyrosinolation in PMN.

Posttranslational incorporation of tyrosine in PMN of patients with CGD. In view of the results pre-

sented in Table I and Figs. 1 and 2, we studied tubulin tyrosinolation in PMN obtained from patients with CGD. Unlike normal stimulated PMN (14, 50), failure to activate the membrane-bound NADPH-dependent oxidase (30, 31) leads to a primary functional defect in PMN of CGD cells (34, 35, 50). These cells do not respond to particulate and soluble stimuli with a "respiratory burst" (30) and, therefore, also fail to reduce molecular oxygen to the free radical O_2^- that results in the generation of other toxic cellular metabolites, such as H_2O_2 and hydroxyl radical (30, 31). Thus, neutrophils from these patients should be in a relatively reduced state with higher concentrations of NADPH and GSH, the effective oxidation of which is coupled to the activation of the oxidative metabolism via the HMPS pathway (14, 29). As shown in Fig. 3, resting levels of posttranslational incorporation of tyrosine was 35–45% lower ($P < 0.01$) in the PMN of seven patients with CGD (four male and three female). Most strikingly, PMN from all seven patients with CGD failed to respond to 10^{-7} M fMet-Leu-Phe, a concentration that induces a maximal stimulation of tyrosinolation (two- to threefold) in normal PMN (22).

We also studied the effect of addition of methylene blue on tyrosinolation in PMN of CGD patients. Methylene blue, an electron acceptor, has been used to by-

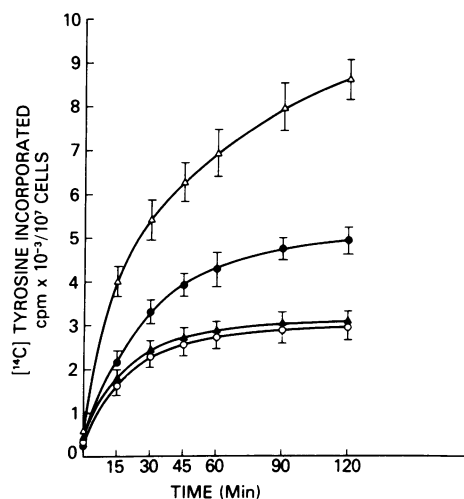


FIGURE 3 Resting and fMet-Leu-Phe-stimulated post-translational incorporation of tyrosine in normal and CGD PMN. Cells at 10^7 /ml were incubated with antibiotics for 30 min at 37°C , and [^{14}C]tyrosine was added at 0 min. Where indicated, 10^{-7} M fMet-Leu-Phe was also added at 0 min. TCA-insoluble radioactivity was determined at indicated times. Data are means \pm SEM of studies in PMN of seven different normal individuals and in seven different patients with CGD. Δ , normal PMN+fMet-Leu-Phe; \bullet , normal PMN; \blacktriangle , CGD PMN+fMet-Leu-Phe; \circ , CGD PMN.

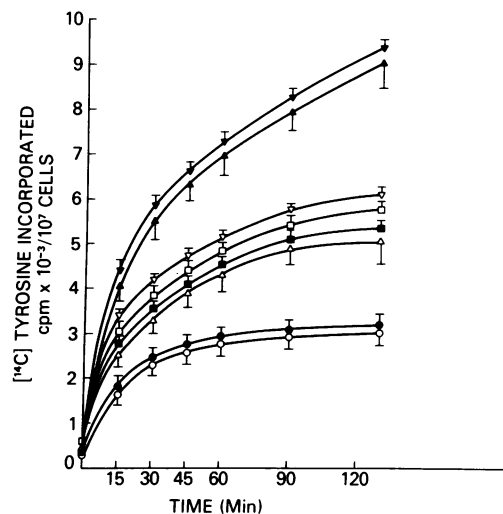


FIGURE 4 Effect of methylene blue on resting and fMet-Leu-Phe-stimulated incorporation of tyrosine in normal and CGD PMN. Cells at 10^7 /ml were incubated with antibiotics for 30 min at 37°C . 10^{-4} M methylene blue was added 15 min before addition of [^{14}C]tyrosine ($5 \mu\text{Ci} = 0.01 \mu\text{mol}$) and/or fMet-Leu-Phe at 0 min. TCA-insoluble radioactivity was measured at indicated times. Data are means \pm SEM of three different experiments with different normal individuals and CGD patients. ∇ , normal PMN+methylene blue+fMet-Leu-Phe; \blacktriangle , normal PMN+fMet-Leu-Phe; ∇ , normal PMN+methylene blue; \square , CGD PMN+methylene blue+fMet-Leu-Phe; \blacksquare , CGD PMN+methylene blue; Δ , normal PMN; \bullet , CGD PMN+fMet-Leu-Phe; \circ , CGD PMN.

pass the oxidative block due to the impaired function of the NADPH-dependent oxidase in these cells (51). Thus, methylene blue will correct the depressed HMPS pathway in PMN of CGD patients (51), although it will not induce an enhanced production of oxygen radicals such as O_2^- and H_2O_2 . Fig. 4 shows the effect of addition of 10^{-4} M methylene blue on resting and fMet-Leu-Phe-stimulated PMN from three patients with CGD. It was particularly noteworthy that methylene blue could normalize the depressed resting levels of tyrosinolation in PMN of CGD patients, but failed to induce the stimulation in the presence of fmet-leu-phe. In studies with PMN from three CGD patients, diamide did not have any significant effect on either resting or fMet-Leu-Phe-stimulated tyrosine incorporation (data not shown).

Specific radioactivity of the intracellular tyrosine pool in normal and CGD patients. In view of the results obtained with PMN from CGD patients (Fig. 3), we determined the specific radioactivity of the intracellular pool of tyrosine in normal and CGD cells. Table II shows that the specific radioactivity was very similar in normal and CGD patient cells, and it did not change in the presence of fMet-Leu-Phe, a result

TABLE II
Specific Radioactivity of Intracellular Tyrosine Pool
in Normal and CGD PMN

Cell sample	Specific radioactivity
	<i>cpm/pmol tyrosine</i>
Normal	
– fMet-Leu-Phe	213
+ fMet-Leu-Phe	210
CGD	
– fMet-Leu-Phe	220
+ fMet-Leu-Phe	230

PMN at 10^7 /ml were incubated with antibiotics for 30 min before the addition of [14 C]tyrosine ($5 \mu\text{Ci}/\text{ml} = 0.01 \mu\text{mol}$) and further incubated for 90 min in the presence or absence of 10^{-7} M fMet-Leu-Phe. Specific radioactivity of the intracellular tyrosine pool was determined as described in Methods.

true in both normal PMN and PMN of CGD patients. These results clearly demonstrated that the lower resting levels and the absence of fMet-Leu-Phe-induced stimulation of tyrosinolation in PMN of CGD patients, were not attributable to a parallel decrease in the specific radioactivity of the intracellular pool of tyrosine. Moreover, the ligase specific activity and the protein and tubulin concentrations of normal and CGD PMN were found to be highly comparable (data not shown).

Effect of the Ca^{2+} -ionophore A23187 on post-translational incorporation of tyrosine in normal and CGD PMN. Since the Ca^{2+} -ionophore A23187 stimulates PMN oxidative metabolism (32) without stimulating chemotaxis, we studied the effect of this agent on tubulin tyrosinolation in PMN. As shown in Fig. 5, addition of 10^{-6} M A23187 caused a marked stimulation of tyrosine incorporation in normal resting PMN, which was abrogated in the presence of a reducing agent like dithiothreitol. The A23187-induced stimulation of tyrosine incorporation was similarly inhibited in the presence of other reducing agents such as cysteine, GSH, or ascorbate (data not shown). These results were identical to our reported observations in fMet-Leu-Phe-stimulated PMN (22). Fig. 5 also shows the characteristic depressed levels of tyrosine incorporation in the resting PMN of CGD patients, which, in striking contrast to normal PMN, failed to respond to the Ca^{2+} -ionophore. Moreover, these results provide further support to the idea of a causal relationship between activation of oxidative metabolism and stimulation of tubulin tyrosinolation in PMN and, as indicated in our earlier studies with various antitubulin drugs (22), further demonstrate that the stimulation of tyrosinolation in PMN can be dissociated from the microtubule-related cytoskeletal changes involved in chemotaxis.

DISCUSSION

Our recent studies on tubulin tyrosinolation in normal PMN and also in PMN of CHS patients (22) suggested a link between PMN oxidative metabolism and tubulin tyrosinolation in these cells. The present data showing inhibition of the fMet-Leu-Phe-induced stimulation of tyrosinolation in the presence of various antioxidants and scavengers of reactive oxygen metabolites provide further evidence in support of such a correlation.

A number of reports have provided evidence in support of a functional role of the glutathione redox system in PMN activation. Oliver and her associates (43–45) have suggested that GSH homeostasis is critical during physiological events such as phagocytosis, which simultaneously induce the assembly of microtubules and the production of agents like H_2O_2 that can oxidize GSH. Moreover, oxidation of GSH markedly stimulated the HMPS pathway in PMN, which in turn resulted in a rapid regeneration of GSH (43–45). In a recent report, Wedner et al. (52) presented data to indicate that normal physiological GSH levels

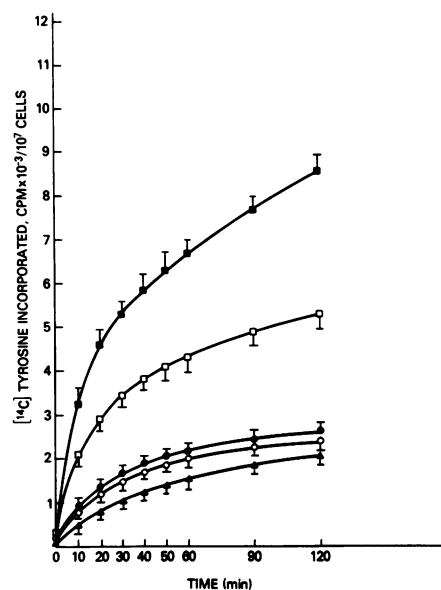


FIGURE 5 Effect of the Ca^{2+} -ionophore A23187 on post-translational incorporation of tyrosine in normal and CGD PMN. Cells at 10^7 /ml were incubated with antibiotics for 30 min at 37°C , and [14 C]tyrosine ($5 \mu\text{Ci} = 0.01 \mu\text{mol}$) and/or 10^{-6} M A23187 were added at 0 min. Where indicated, 10^{-3} M DTT was added during the initial incubation period with antibiotics. TCA-insoluble radioactivity was determined at indicated times. Data are means \pm SEM of three different experiments using PMN of different normal individuals and different CGD patients. ■, normal PMN + A23187; □, normal PMN; ●, CGD PMN + A23187; ○, CGD PMN; ▲, normal PMN + DTT + A23187.

were necessary for the transduction of the activation signal from the exterior to the interior of the PMN. It is also well-documented in the literature that changes in cellular GSH levels affect the thiol-disulfide status of the cell, and such alterations have significant effects on microtubule-related functions in PMN (43–45) and in other cells (46). The results presented in Figs. 1 and 2 indicate that changes in available sulfhydryl groups, or in cellular GSH levels, significantly affected tyrosine incorporation in both resting and fMet-Leu-Phe-stimulated PMN, indicating a possible association of the PMN thiol-disulfide status in modulation of tubulin tyrosinolation in these cells.

Studies involving seven patients with CGD (four male and three female), provided further evidence to suggest that activation of PMN oxidative metabolism was coupled to the induction of stimulation of tyrosinolation in PMN (22). As shown in Fig. 3, the resting levels of tyrosine incorporation in PMN of CGD patients were 35–45% lower ($P < 0.01$) when compared with normal levels. In striking contrast with normal PMN, the PMN of CGD patients failed to respond to fMet-Leu-Phe (Fig. 3), and this was not attributable to a parallel decrease in the specific radioactivity of the intracellular pool of tyrosine in CGD PMN (Table II).

Assuming that ~1% of the total PMN protein is tubulin (22), and based on the data presented in Fig. 3 and Table II, we can calculate the amount of tyrosine fixed per mole of tubulin in normal and CGD PMN. In normal resting PMN, the values amount to 0.15–0.17 mol of tyrosine/mol of tubulin, which increased to 0.30–0.34 mol of tyrosine/mol of tubulin when stimulated with fMet-Leu-Phe. The corresponding values are only ~0.10 mol of tyrosine/mol of tubulin in resting and stimulated PMN of CGD patients. As we have previously reported, none of the fixed radioactivity, either in rabbit peritoneal neutrophils (21) or in human peripheral PMN (22), could be chased in the presence of a large excess of [^{12}C]tyrosine. This is particularly intriguing since all of the incorporated [^{14}C]tyrosine appears to be in the tubulin α -chains (21, 22). As we have demonstrated in related studies in other cells and tissues (37, 53), the inability to chase the posttranslationally fixed radioactivity could possibly be related to the “nonsubstrate” nature of the *in vivo* tyrosinolated tubulin (53). Due to the small amount of tubulin in PMN, it has not yet been possible to purify and characterize the nature of the tyrosinolated tubulin species in these cells.

The results of the anaerobic experiment (Table I) are particularly relevant in view of the results obtained with other antioxidants and scavengers of oxygen metabolites and the failure of CGD PMN to respond to fMet-Leu-Phe (Fig. 3) and A23187 (Fig. 5). Since both

normal PMN under anaerobic conditions and the PMN of CGD patients fail to elicit the characteristic “respiratory burst” when stimulated (24, 30), the present data, together with the data showing that the electron acceptor methylene blue, which bypasses the oxidative block in those cells (51), corrects the defect of CGD PMN tyrosinolation under basal conditions (Fig. 4), provide strong evidence in support of a close association between the activation of PMN oxidative metabolism (and associated changes in cellular redox state) and the induction of stimulation of tubulin tyrosinolation in PMN. Further studies on the mechanism whereby PMN tubulin tyrosinolation is modulated via its oxidative-reductive reactions seem clearly warranted.

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