

Tubulin Tyrosinolation in Human Polymorphonuclear Leukocytes: Studies in Normal Subjects and in Patients with the Chediak-Higashi Syndrome

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ABSTRACT We have recently reported a specific dose-dependent stimulation of posttranslational incorporation of tyrosine into tubulin α -chains of rabbit peritoneal leukocytes as induced by the synthetic peptide chemoattractant formyl-methionyl-leucyl-phenylalanine (FMLP). The present study reports a similar, specific stimulation of tubulin tyrosinolation in human polymorphonuclear leukocytes (PMN). When compared to normal PMN, both the resting and FMLP-stimulated levels of posttranslational tyrosine incorporation were two- to threefold higher in PMN of three patients with the Chediak-Higashi syndrome (CHS). The concentration of cellular tubulin and the specific activity of tubulin tyrosine ligase were similar in PMN of CHS patients and normal donors and resembled that of other non-neuronal cells. The high levels of tyrosine incorporation in PMN of CHS patients were normalized by the administration of ascorbate, both in *in vitro* and in *in vivo* experiments. *In vitro* addition of ascorbate also inhibited the FMLP-induced stimulation of tyrosine incorporation in both normal and CHS cells. Normalization of higher levels of tyrosine incorporation in PMN of CHS patients and the inhibition of FMLP-induced stimulation of tubulin tyrosinolation in normal and CHS cells as observed with ascorbate could also be affected by other reducing agents such as reduced glutathione, cysteine, or dithiothreitol. These results suggest a possible relationship between cellular redox and tubulin tyrosinolation in PMN.

Tubulin is subject to a reversible posttranslational modification whereby a tyrosine residue is added to the carboxy-terminal glutamate of tubulin α -chain (2, 37). The enzyme that catalyzes this ATP-dependent reaction, tubulin tyrosine ligase (ligase), has been detected in both vertebrate (3, 38) and invertebrate (22) tissues. Another distinctly different enzyme, carboxypeptidase tubulin (CPT), which is believed to be the major cellular enzyme responsible for the removal of the carboxy-terminal tyrosine of tubulin, has also been detected and characterized (1, 23). Although the presence of the tyrosine has not been found to affect the assembly of microtubules *in vitro*, it has been clearly demonstrated that the 6S dimeric tubulin is the substrate for the ligase (21), whereas the detyrosinating enzyme preferentially acts on intact microtubules (23). Moreover, we have observed changes in the state of tyrosinolation of tubulin in cells undergoing cytoskeletal rearrangements such as in differentiating neuronal cells (29) and in HeLa cells during

mitosis (34). Recently, we have studied the levels of tubulin tyrosinolation in rabbit peritoneal leukocytes and have reported (32) a specific, dose-dependent stimulation induced by the synthetic peptide chemoattractant formyl-methionyl-leucyl-phenylalanine (FMLP). The FMLP-induced stimulation of tubulin tyrosinolation in rabbit leukocytes seems to be mediated via receptors and depends on normally functioning pathways of protein and phospholipid methylation (32). The response is also abolished by a variety of reagents which are known to be chemotactic inhibitors. These results strongly suggest a functional link between leukocyte chemotaxis and tubulin tyrosinolation (32).

Microtubules have been implicated in leukocyte chemotaxis (20, 26, 36), and they are believed to be essential for the formation, orientation, and maintenance of pseudopods during the response of the polymorphonuclear leukocytes (PMN) to chemotactic stimuli (26). Our recent findings with rabbit leu-

kocytes (32) encouraged us to examine tubulin tyrosinolation in human peripheral blood PMN. We have also studied and compared the levels of tubulin tyrosinolation in PMN isolated from Chediak-Higashi syndrome (CHS) patients with those of normal PMN. CHS is a rare disorder characterized by giant granules in most granule-containing cells (45, 46). Clinically, the disease can evolve into a lymphomalike phase with death at an early age; patients not developing lymphoma usually develop severe peripheral neuropathy by age 25 (45, 46). A major clinical expression is marked susceptibility to pyogenic infections, which, in the absence of a defect in humoral immunity, has directed attention to phagocytic cells and monocytes (45, 46). The patients are neutropenic (46), and PMN and monocytes isolated from CHS patients have been reported to have defective chemotactic responses (10, 17) and delayed bactericidal activity (39) despite increased oxidative metabolism (40). These abnormalities have often been associated with impaired microtubule function in these cells (6, 35).

The present investigation was undertaken to study the effect of FMLP on human PMN tubulin tyrosinolation and also to compare the reaction in PMN from CHS patients with that of normal PMN. Effects of addition of various reducing agents like ascorbate, reduced glutathione (GSH), cysteine, or dithiothreitol (DTT) were also studied in normal and CHS PMN. During the course of this investigation, a protease (or peptidase) activity was also detected in the particulate fractions of human PMN, which was found to be reduced significantly in CHS patients. While a preliminary report of this work has appeared (33), details of these experiments and possible functional implications of the results are described in the present communication.

MATERIALS AND METHODS

Preparation of PMN

Heparinized human peripheral blood, either from normal donors or CHS patients, was separated into a granulocyte-rich fraction by Hypaque-Ficoll and dextran sedimentation techniques (8). This generally resulted in a cell fraction containing over 95% PMN with >95% viability as determined by the exclusion of trypan blue dye. Most of the PMN from CHS patients were obtained from two brothers with CHS, who have been extensively studied and described (46). In this paper, they will be referred to as CHS₁ and CHS₂. We have also had occasion to study a third, unrelated, CHS patient, whom we shall refer to as CHS₃, who has also been reported previously (45).

Posttranslational Incorporation of [¹⁴C]Tyrosine in Human PMN

Freshly fractionated normal or CHS PMN, suspended in Hanks's balanced salt solution or in Gey's medium, were collected by centrifugation at 1,000 *g* for 5 min. The supernatant was aspirated, and the packed cells were gently resuspended at a cell density of 10⁷/ml in an isoosmotic medium as previously described (29) with bovine serum albumin (BSA) added to a final concentration of 0.1%. All other experimental conditions were identical to those described for rabbit leukocytes (32). Before the addition of [¹⁴C]tyrosine, the cells were routinely incubated with antibiotics (32) for 30 min at 37°C, which effectively inhibited protein synthesis to >99%, without inhibiting chemotaxis.

To study the effect of FMLP on posttranslational incorporation of tyrosine into PMN tubulin in intact cells, we have generally used a cell density of 10⁷/ml with FMLP concentration at 10⁻⁷ M. Although chemotaxis was studied at a lower cell density of 2.5 × 10⁶ cells/ml, the higher concentration of cells was necessary to obtain enough material for reliable determination of [¹⁴C]tyrosine incorporation under our experimental conditions. Unless indicated otherwise, the various agents to be tested were present during the 30-min preincubation with antibiotics.

In some experiments where the effect of oral administration of a large dosage of ascorbate (8 g) on tyrosine incorporation in CHS cells was studied, peripheral blood from the CHS patients was obtained before and 2 h after administration of the ascorbate. The blood levels of ascorbate were also determined at both times.

Other Analytical Procedures

Most of the experimental procedures used in the current study with human PMN were used in our previous work with rabbit leukocytes and have been described in detail (32).

For the ligase assay, freshly separated PMN (~6 × 10⁷ cells) were collected by centrifugation at 1,000 *g* for 5 min and resuspended in 0.5 ml of an isotonic buffer containing 0.34 M sucrose, 25 mM HEPES, 0.5 mM MgSO₄, and 1 mM (DTT), adjusted to pH 7.2. The cells were disrupted by mild sonication (29) and ligase specific activity was determined in the 100,000-*g* supernatant and pellet fractions of the cell-sonicates as previously described (32). The carboxypeptidase (CPT) activity was assayed according to the experimental conditions previously reported from our laboratory (23). The 100,000-*g* supernatant and pellet fractions were obtained as described for the ligase assay, except for using a tubulin reassembly buffer (i.e., 100 mM K⁺ 2-[*N*-morpholino] ethane sulfonic acid, 1 mM EGTA, and 0.5 mM MgSO₄, pH 6.8), to resuspend the cells before they were sonicated and centrifuged.

The procedures for the determination of the specific radioactivity of the intracellular tyrosine pool in normal and CHS PMN were identical to those described for rabbit leukocytes (32). Briefly, the various samples of PMN were incubated with [¹⁴C]-labeled tyrosine in the presence of antibiotics and rapidly filtered and washed with ice-cold phosphate-buffered saline before extraction with 7% TCA. The TCA extracts were lyophilized and reconstituted in desired volume of distilled water. Equal aliquots of each sample were analyzed for tyrosine (by amino acid analyses in a Durrum amino acid analyzer) and radioactivity. Where indicated, the PMN were preincubated with 10⁻⁷ M FMLP for 60 min.

Native tubulin concentration was determined by colchicine binding assay using Whatman DE-23 ion-exchange cellulose columns. The details of the assay have been reported (23). For quantitation of the amount of radioactivity in tubulin bands of normal and CHS cells, the fluorogram was scanned using a quick-scan R and D densitometer (Helena Laboratories, Beaumont, TX). The final recordings were photocopied, and the peak areas (corresponding to tubulin) were cut out and weighed. Polyacrylamide slab gel electrophoresis was performed in an SDS urea system as described by Eipper (11). Cell preparation for the gel electrophoresis and processing of the gels for detection of radiolabeled protein bands by fluorography (24) were similar to those described for rabbit leukocytes (32). Chemotaxis was routinely measured as previously described (14). Protein was determined by the procedure of Lowry et al. (25) standardized with BSA.

[¹⁴C]Tyrosinolated tubulin was prepared by the enzymatic tyrosinolation of tubulin by ligase as recently described (23). [³H]Valine labeled tubulin was prepared from 1-d-old rat brains after intracranial injections of [³H]valine via protein synthesis. The details of the method have been described elsewhere (30).

RESULTS

Ligase and Colchicine Binding Activity

The specific activity of ligase in the 100,000-*g* supernatant of human PMN was around 0.01–0.02 nmol/min per mg of protein, which was similar to that observed in rabbit peritoneal leukocytes (32). About 80% of the total activity was recovered in the soluble fraction of PMN, and the rest (20%) was consistently found to be associated with the particulate fraction. Tubulin concentration as determined by colchicine binding activity in the high-speed supernatant fraction was only around 1% of the total cytoplasmic protein, a result which has so far prevented us from further purification of PMN tubulin to allow us to determine its state of tyrosinolation. However, as in rabbit leukocytes, about half of the total colchicine binding activity of human PMN was associated with its particulate fractions. Both the distribution and concentration of cellular ligase and tubulin were found to be the same in PMN from normals and CHS patients and were similar to that observed in other non-neuronal cells (22, 38). Protein content of normal and CHS PMN were also found to be comparable (data not shown).

Stimulation of Tyrosine Incorporation by FMLP

Fig. 1 shows the basal level of posttranslational incorporation of tyrosine in normal resting PMN and its stimulation induced by the peptide chemoattractant FMLP. The incorporation of [¹⁴C]tyrosine in unstimulated PMN reached a plateau in 90

min, a result which resembled our earlier observation in neuronal cells (29). Fig. 1 also shows that the tyrosine incorporation was stimulated at the end of 30 min by ~50% at an FMLP concentration of 10^{-8} M, and by 100% at 10^{-7} M FMLP. Moreover, unlike in resting PMN, the tyrosine incorporation in stimulated cells did not reach a complete plateau even at 150 min. Fig. 2 shows the time course of stimulation of tyrosine incorporation as induced by FMLP. Unlike in rabbit peritoneal leukocytes where we have observed an initial lag of ~6 min (32), the chemoattractant caused a rapid stimulation of tyrosine incorporation in human PMN. As early as 2 min, the earliest time point studied, tyrosine incorporation was stimulated by 85% above control.

Basal and FMLP-stimulated Levels of Posttranslational Tyrosine Incorporation and Protein Synthesis in Normal and CHS Cells

Fig. 3 compares the resting and FMLP-stimulated levels of tyrosine incorporation in PMN from normal and CHS patients. Basal levels of TCA-precipitable tyrosine incorporation in PMN from CHS_1 and CHS_2 were about threefold greater than that of normal cells. The tyrosine incorporation in PMN of the third CHS patient (i.e., in CHS_3) was about twofold higher

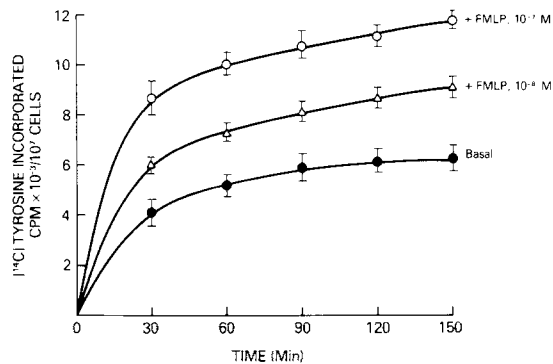


FIGURE 1 Stimulation of posttranslational tyrosine incorporation by FMLP. PMN were preincubated for 30 min at 37°C with antibiotics and FMLP concentrations as indicated. [14 C]tyrosine ($5 \mu\text{Ci} \equiv 0.01 \mu\text{mol}$) was added at 0 min and TCA-insoluble radioactivity was measured at indicated times. The results are means \pm SEM of three separate experiments.

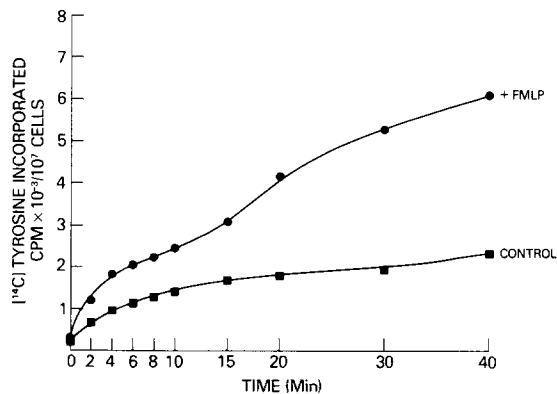


FIGURE 2 Time-course of stimulation of tyrosine incorporation induced by FMLP. PMN at 10^7 cells/ml were preincubated with antibiotics and 10^{-7} M FMLP and $5 \mu\text{Ci}$ of [14 C]tyrosine ($\equiv 0.01 \mu\text{mol}$) were added at 0 time. TCA-insoluble radioactivity was measured at indicated times. The results are the means of duplicate determinations.

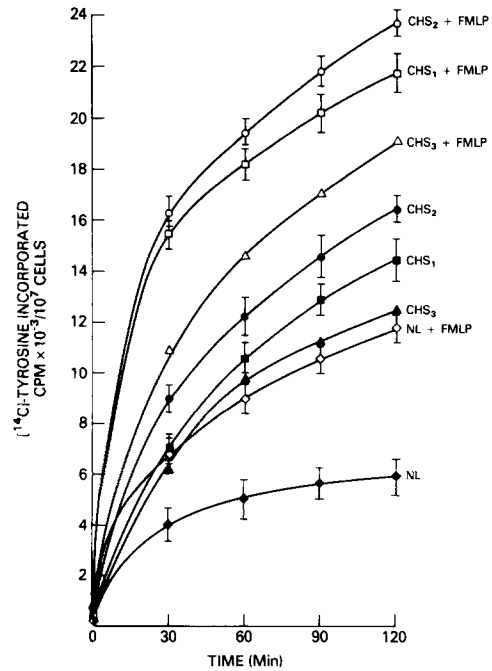


FIGURE 3 Comparison of resting and FMLP-stimulated levels of tyrosine incorporation in PMN from normal (NL) and three Chediak-Higashi syndrome patients (CHS_1 , CHS_2 , and CHS_3). Experimental conditions were the same as those described in Fig. 1. Cells were preincubated for 30 min with antibiotics and 10^{-7} M FMLP before addition of [14 C]tyrosine. Values are expressed as means \pm SEM for three experiments each with CHS_1 and CHS_2 , and for six experiments with different normal individuals. The values for CHS_3 are means of duplicate determinations obtained from a single experiment

TABLE I
Specific Radioactivity of Intracellular Tyrosine Pool in Normal and CHS PMN

Cell sample	Specific radioactivity
	Tyrosine cpm/pmol
Normal - FMLP	151
Normal + FMLP	154
CHS_1 - FMLP	158
CHS_1 + FMLP	155
CHS_2 - FMLP	150
CHS_2 + FMLP	156

PMN, at 10^7 /ml, were preincubated with antibiotics for 30 min at 37°C and then further incubated in the presence of [14 C]tyrosine ($5 \mu\text{Ci}/\text{ml} \equiv 0.01 \mu\text{mol}$) for 60 min in the presence or absence of 10^{-7} M FMLP. Specific radioactivity of the intracellular tyrosine was determined as described in Materials and Methods.

than the normal resting levels. The higher resting levels of tyrosine incorporation in PMN of CHS patients could be further stimulated with FMLP, which was quite similar to that observed in normal PMN. Control studies, designed to assess uptake rates in CHS and normal PMN using radiolabeled sucrose, did not show any significant differences between normal and CHS PMN, a result true for both peptide-stimulated and resting cells (data not shown). The rate of protein synthesis in the presence or absence of FMLP was similar in both normal and CHS PMN as studied by [14 C]leucine incorporation. In addition, in parallel studies, leucine incorporation was inhibited over 98% when the cells were preincubated with anti-

biotics (data not shown) indicating that the incorporation of [^{14}C]tyrosine as measured under our experimental conditions was indeed a posttranslational event involving tyrosination of cellular tubulin (38).

Specific Radioactivity of the Intracellular Tyrosine Pool

Table I shows that the specific radioactivity of the intracellular tyrosine was similar in normal and patient cells, and it did not change in the presence of FMLP. These results demonstrated that neither the observed stimulation of tyrosine incorporation induced by FMLP nor the higher levels of tyrosine incorporation in PMN of CHS patients were attributable to a parallel increase in the specific radioactivity of the intracellular pool of tyrosine.

Specific Tyrosination of Tubulin by Intact Human PMN

The specificity of the reaction in human PMN is demonstrated in Fig. 4. When an equal amount ($\sim 5 \times 10^6$ cells) of [^{14}C]tyrosine-labeled PMN from a normal individual and from CHS₁ patient were electrophoresed on a polyacrylamide slab

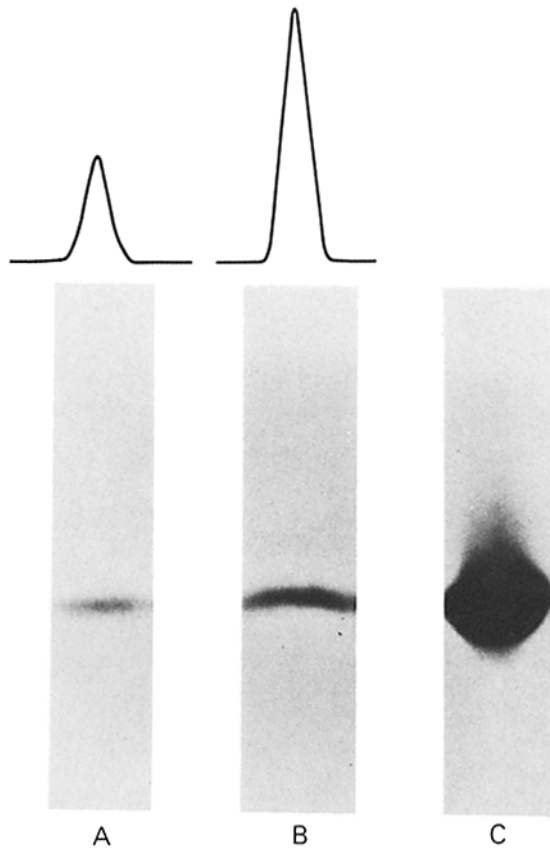


FIGURE 4 Quantitative demonstration of the specific incorporation of [^{14}C]tyrosine into tubulin α -chains of normal and CHS PMN. Equal amounts of [^{14}C]labeled PMN as obtained in the experiment described in Fig. 3 were subjected to gel electrophoresis, and the slab gel was processed for fluorography. Details of the procedures are described in the text. Lane A: normal; Lane B: CHS₁; Lane C: an overexposed sample of authentic assembly-purified [^{14}C]tyrosinated tubulin preparation. The upper panel (in A and B) represents the densitometric scans of the corresponding radioactive bands in the lower panel.

gel and the gel was subsequently dried and processed for fluorography (24), radioactivity was detected only in a single protein band corresponding to authentic [^{14}C]tyrosinated tubulin. Fig. 4 also demonstrates a significantly higher amount of radioactivity in the tubulin band of CHS₁ cells (lane B) as compared to that of normal PMN (lane A). Lane C represents the electrophoretic mobility of a purified sample of [^{14}C]tyrosinated tubulin preparation. Due to the large amount of PMN protein ($\sim 750 \mu\text{g}$) that was subjected to gel electrophoresis to demonstrate the radioactivity in tubulin bands (Fig. 4), the corresponding Coomassie Blue staining patterns of the electrophoresed samples were heavily overstained and did not resolve into discrete bands and, therefore, are not included in the figure. When the radioactive bands corresponding to tubulin (in lanes A and B) were scanned from the original fluorogram and quantitated as described in Materials and Methods, the amount of radioactivity in CHS₁ cells (lane B) was 2.7 times greater than that of lane A (normal PMN), which demonstrates an excellent correlation with the higher levels of [^{14}C]tyrosine incorporated in PMN of CHS₁ as shown in Fig. 3.

Effect of Ascorbate on Posttranslational Tyrosine Incorporation in PMN of CHS Patients

Although reports on the efficacy of ascorbate administration in correcting some of the functional abnormalities of PMN related to CHS have been controversial (5, 15), there is sufficient evidence in the literature to suggest that ascorbate promotes microtubule assembly, both in vivo and in vitro (7), and that it corrects defective adherence, chemotaxis, and degranulation of PMN from CHS patients (5, 18). Since the reaction which we are studying specifically involves cellular tubulin/microtubules, it was of interest to examine the effect of ascorbate on the posttranslational tyrosine incorporation in PMN of CHS patients. Fig. 5 shows typical results of the effect of

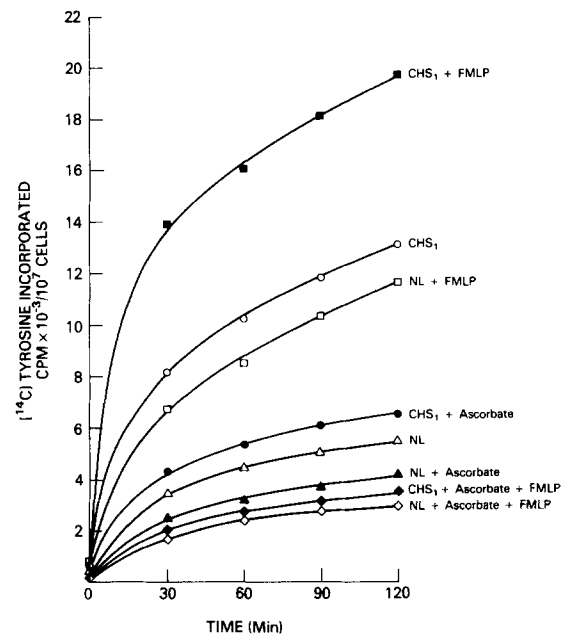


FIGURE 5 Effect of addition of ascorbate in vitro on the resting and FMLP-stimulated levels of tyrosine incorporation in normal and CHS₁ PMN. Where indicated, cells were preincubated for 30 min with 10^{-4} M ascorbate and 10^{-7} M FMLP was added at zero min. Other experimental conditions were the same as in Fig. 1. The results are the means of duplicate determinations.

addition of ascorbate in vitro on tyrosine incorporation in PMN from a normal individual and from CHS₁. Preincubation with 10⁻⁴ M ascorbate had a profound effect both on the rate and the final extent of tyrosine incorporation in PMN of CHS₁ (curve 2), and it essentially resembled the incorporation observed in normal cells (curve 3). Addition of ascorbate also had a small effect on the basal level of incorporation in normal PMN (curve 4), which has been consistently reproducible in PMN obtained from a number of different individuals. Most strikingly, preincubation with 10⁻⁴ M ascorbate completely inhibited the FMLP-induced stimulation of tyrosine incorporation, in both normal PMN and in PMN obtained from CHS₁. Tyrosine incorporation was even less than that in the presence of ascorbate alone. In one experiment, we also studied the effect of in vitro addition of ascorbate in PMN of CHS₂ and obtained similar results (data not shown).

Encouraged by the results reported in Fig. 5, we have also studied the effect of oral administration of 8 g of ascorbate to the CHS patients on the rate and extent of posttranslational tyrosine incorporation in their PMN. Absorption of ascorbic acid from the gastrointestinal tract was documented in each patient. Serum ascorbate levels increased from 70 μM/liter to 205 μM/liter in CHS₁ and from 55 μM/liter to 115 μM/liter in CHS₂ while they were on ascorbate. Fig. 6A demonstrates the levels of TCA-insoluble [¹⁴C]tyrosine incorporation in PMN of CHS₁ and CHS₂ before and 2 h after administration of ascorbate. It clearly shows a significant effect of ascorbate, which was very similar to that observed in Fig. 5, where ascorbate was added in vitro, to the incubation medium. Fig. 6B shows tyrosine incorporation into PMN of the same CHS patients a week after they were withdrawn from ascorbate and demonstrates the typical high levels of incorporation as reported in Fig. 3.

In view of the results presented in Fig. 6, we have also performed a control experiment in which posttranslational incorporation of tyrosine was studied in both normal PMN and in PMN from CHS₁. For this experiment, PMN were preincubated for 1 h in the presence of 10⁻⁴ M ascorbate and

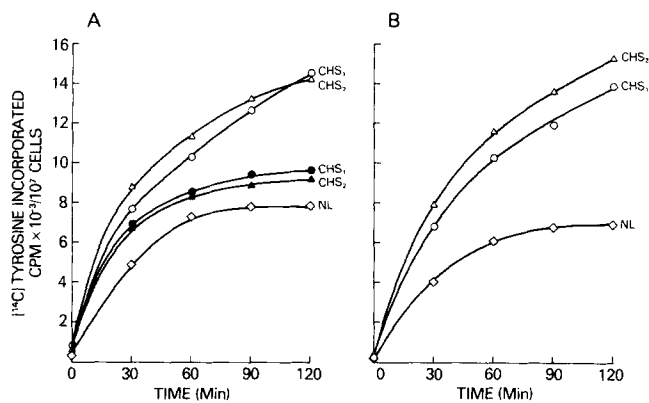


FIGURE 6 Effect of administration of ascorbate in vivo on the resting levels of posttranslational tyrosine incorporation into PMN of CHS patients. PMN at 10⁷/ml were preincubated with antibiotics, and [¹⁴C]tyrosine incorporation was measured at indicated times. Panel A: ○—○ and △—△ represent preascorbate levels of tyrosine incorporation in CHS₁ and CHS₂; ●—● and ▲—▲ represent the corresponding 2-h postascorbate values. ◇—◇ shows resting levels of incorporation in PMN of a normal donor. Panel B shows the corresponding resting levels of tyrosine incorporation in CHS₁ and CHS₂ 1 wk after withdrawal of ascorbate therapy and compares them to the normal levels. The results are the means of duplicate determinations.

then centrifuged and resuspended in either fresh incubation medium without ascorbate or in medium containing 10⁻⁴ M ascorbate. The effect of ascorbate (as shown in Fig. 5) persisted for at least another 90 min after removal of ascorbate from the incubation medium. Resting levels of tyrosine incorporation in the absence of ascorbate resembled the incorporation in the presence of ascorbate, a result true in both normal and CHS₁ PMN (data not shown).

Ascorbate is a powerful reducing agent. To assess whether the ascorbate effect on tubulin tyrosinolation was specific, the effect of other reducing agents on both resting and FMLP-stimulated PMN was studied. Fig. 7 shows that preincubation of normal or CHS PMN with other reducing agents, such as GSH, cysteine, or DTT, produced effects on tyrosine incorporation similar to that of ascorbate (Fig. 5), a result true for both resting and FMLP-stimulated PMN.

Tubulin-specific Particulate Protease/Peptidase Activity of PMN

Since ligase specific activity was not altered in CHS patients, we were interested in measuring the CPT activity (1, 23) which, in brain tissue, has been shown to be the major detyrosinating enzyme responsible for the removal of tyrosine from the carboxy-terminus of α-tubulin (1, 23). It was reasoned that, if indeed there were a microtubule-related functional abnormality in PMN of CHS patients (36), CPT might be altered in such cells. Using [¹⁴C]tyrosinolated tubulin (23) as the substrate, the release of [¹⁴C] carboxy-terminal tyrosine of tubulin α-chains in the presence of high-speed supernatant and pellet fractions of PMN obtained from normal and CHS donors was monitored (Table II). The major detyrosinating activity was associated with the PMN-particulate fractions, and it was reduced significantly in CHS cells. To determine whether the observed release of radioactivity from [¹⁴C]tyrosinolated tubulin was due to the presence of CPT (1, 23) in these subcellular fractions or if it were due to a more nonspecific proteolytic activity, which could also be present in these preparations, three experiments were performed to characterize further the nature of this enzymatic activity: (a) [³H]valine labeled tubulin randomly labeled with [³H]valine via protein synthesis, was used as the

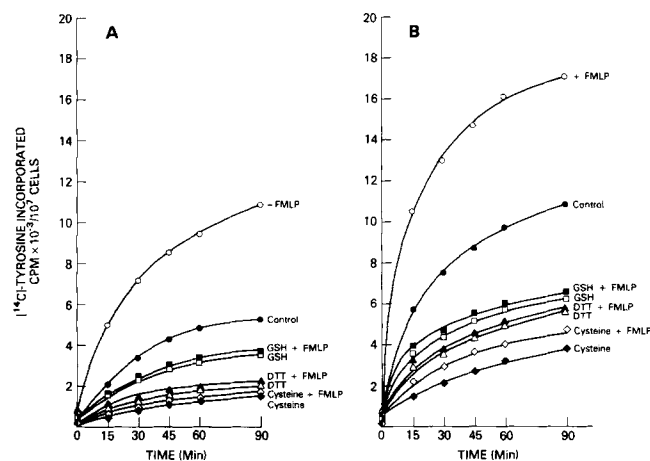


FIGURE 7 Effects of various reducing agents on the resting and FMLP-stimulated levels of tyrosine incorporation in normal and CHS₁ PMN. Where indicated, PMN were preincubated for 30 min with 10⁻³ M GSH, cysteine, or DTT, and 10⁻⁷ M FMLP was added at zero min. Other experimental conditions were the same as in Fig. 1. Panel A: normal PMN; Panel B: CHS₁ PMN. The results are the means of duplicate determinations.

TABLE II
Tubulin-specific Peptidase Activity in PMN from Normals and CHS Patients

Sample	Normals*	CHS*	P‡
	<i>pmol/mg/min</i>		
Supernatant	0.32 ± 0.04	0.26 ± 0.04	>0.05
Pellet	4.30 ± 0.40	0.94 ± 0.62	<0.02

* Data are mean ± SEM of studies in three different normals and three separate preparations of PMN from CHS₁ and CHS₂ and three studies on one preparation of CHS₃.

‡ Significance of difference between CHS and normal; Student's *t* test.

TABLE III
Characterization of the Proteolytic Activity Associated with a PMN-Particulate Fraction

Enzyme sample	¹⁴ C]Tyrosine released			³ H]Valine released	
	Control	+150 mM NaCl	+PMSF	Control	+PMSF
	<i>cpm/mg/min</i>				
Purified CPT	20,000	<50	19,900	0	—
PMN-100,000- <i>g</i> pellet	450	445	<50	780	<80

Freshly separated PMN were suspended in tubulin reassembly buffer and sonicated to disrupt the cells (9). The cell sonicates were centrifuged at 100,000 *g* and the resultant pellet fractions were used for the enzyme assay.

substrate, (b) 150 mM NaCl, which inhibits CPT (28) but not neutral proteases (4, 12) was included in the homogenizing medium, and (c) the PMN pellet fractions were preincubated for 30 min with 1 mM PMSF, which inhibits neutral proteases (4, 12) but not CPT. Using the above criteria, it was concluded that the observed release of tyrosine, as reported in Table II, was not due primarily to the presence of the specific detyrosinating CPT (23), as the PMN-preparations released radioactivity in parallel from [³H]valine labeled tubulin as well. Also, as summarized in Table III, the activity was not significantly inhibited by either the addition of 150 mM NaCl or a 30-min preincubation at 37°C with 1 mM PMSF, which caused ~90% inhibition of the enzymatic activity without inhibiting the purified CPT.

To assess the possibility of whether the increased tubulin tyrosination in PMN from CHS patients was related to the decreased proteolysis of tyrosinated tubulin (Table II), studies were done with normal PMN treated with PMSF. Preincubation with 2 mM PMSF for 30 min at 37°C, which is sufficient to inhibit total PMN proteolytic activity to >60% (41), did not alter tyrosine incorporation in either resting or FMLP-stimulated cells (data not shown).

DISCUSSION

As recently reported for peritoneal rabbit leukocytes (32), the synthetic peptide chemoattractant, FMLP, caused a specific, dose-dependent increase in the rate and the final extent of posttranslational incorporation of tyrosine in peripheral human blood PMN (Fig. 1).

The basal level of tyrosine incorporation in normal PMN reached a plateau in 60–90 min (Fig. 1), which resembled our earlier observations in neuronal cells (29). As revealed by fluorography, the tyrosine, which was incorporated in the absence of protein synthesis, was exclusively incorporated into

tubulin α -chains (Fig. 4), a result true for both normal and CHS cells. The observed stimulation in the presence of FMLP was not related to an increased transport of tyrosine into PMN nor was it due to a higher specific radioactivity of the intracellular tyrosine pool (Table I). Although experimental evidence suggests that the incorporated tyrosine is essentially all in the tubulin α -chains (Fig. 4), we are not sure whether we are measuring turnover of preexisting tyrosine or an incremental fixation. We have so far been unable to chase any fixed radioactivity from either resting or stimulated PMN even with a 1,000-fold excess of [¹²C]tyrosine where we have used a pulse of 10 nM [³H]tyrosine (sp act, 86 Ci/mmol). This is also true in HeLa cells (31) and in rabbit leukocytes (32). This suggests that the observed fixation was an incremental incorporation of tyrosine rather than a turnover or an exchange of the preexisting carboxy-terminal tyrosine of tubulin α -chains. Since tyrosination in cells is always studied in the absence of protein synthesis, it is difficult to correlate posttranslational tyrosination of tubulin in cells (as studied under our experimental conditions) to the recent discovery that some of the tubulin α -chain genes encode a carboxy-terminal tyrosine (43).

Two other important considerations related to the interpretation of the results presented in this paper are the observations that about half of the tubulin from a variety of cells or tissue is not a substrate for enzymatic tyrosination (30, 31), and, as we have observed in brain (30) and also in several cultured cell lines (29, 31), at least part of the tubulin tyrosinated in intact cells is a different species from that which can be tyrosinated in vitro (29, 31) and does not seem to be a substrate for ligase in vitro. Except in HeLa cells where tubulin comprises ~5% of the cellular protein (9), it generally constitutes only 1–2% of the cellular protein in other non-neuronal cells. Due to the small amount of cellular tubulin in PMN (1% of total protein), it has not yet been possible to isolate tubulin in sufficient purity to determine the tyrosine content or the nature of the tyrosinated tubulin in these cells. Another issue related to tubulin tyrosination in cells stems from the fact that, in a variety of cell lines that we have studied so far (29, 31) and also in human PMN and rabbit leukocytes (32), colchicine, podophyllotoxin, or nocodazole, which are all microtubule-disrupting agents and prevent cytoskeletal reorganization in PMN (26, 36), did not inhibit posttranslational tyrosine fixation (J. Nath, unpublished results). This indicates that neither tubulin tread-milling (27) nor intact microtubules (41) are required for the reaction to proceed in intact cells. The results also indicate that the microtubule-related cytoskeletal changes accompanying chemotaxis in PMN (26, 36) are not necessary for the FMLP-induced stimulation of tyrosine fixation to occur.

The abnormally high level of posttranslational tyrosine fixation in PMN of all three CHS patients studied (Fig. 3) is of particular interest. The major defects in PMN function of CHS have been related to its impaired chemotaxis and delayed lysosomal degranulation after phagocytosis (10, 17, 39). In the present context, the most important morphological features of the PMN are its cytoskeletal components, i.e., primarily the microtubules and microfilaments, and the plasma membrane. Although evidence for a microtubule defect in PMN of CHS patients has been controversial and could not be readily demonstrated by morphologic criteria in the patients presented in this paper (6, 13, 16, 35, 45), the results presented in this communication provide evidence for a biochemical alteration in the tubulin of these cells. However, as discussed below, the higher levels of tubulin tyrosination in PMN of CHS patients

may not be a tubulin defect per se but may represent a defect in cellular modulation of the reaction, which could be related to the increased oxidative metabolism characteristic of these cells (40) and subsequent altered redox state. Protein synthesis was found to be normal in CHS cells, and uptake studies performed with radiolabeled sucrose in the presence or absence of FMLP did not show any significant differences between normal and CHS cells (data not shown). The specific radioactivity values of the intracellular pool of tyrosine (Table I) also demonstrate that the higher levels of posttranslational tyrosine incorporation in PMN of CHS₁ and CHS₂ (in the presence or absence of FMLP) is not attributable to a parallel increase in the specific radioactivity of free tyrosine in these cells.

From the specific radioactivity of the tyrosine pool (Table I) and the amount of tubulin per milligram soluble protein (i.e., ~1% or ~10 μg ≡ 0.09 nmol/mg), we can calculate the moles of tyrosine fixed per mole of tubulin. In normal PMN, the basal level of tyrosine incorporation (Figs. 1 and 3) corresponds to 0.25–0.30 mole of tyrosine per mole of tubulin. In CHS cells, the corresponding value ranges from 0.60–0.85 mol/mole of tubulin. The latter amount appears rather high to be entirely attributable to incremental fixation, especially because the fixation has not reached a plateau at this point (Fig. 3).

The proteolytic enzyme, which was not the detyrosinolyzing CPT (Table III), was primarily associated with the PMN particulate fraction (Table II) and was greatly reduced in CHS cells; the enzyme has not yet been characterized sufficiently to have a functional role assigned for it. Human PMN contain a variety of neutral proteases (19) many of which are reported to be associated with particulate fractions (19). In addition, Vasali et al. have reported a reduction in elastase activity, an azurophil granule associated enzyme, in PMN of CHS patients (44). However, the activity reported here is not an elastase type neutral protease (4, 44) as, unlike elastase, it did not dissociate from the particulate fraction of PMN and remained firmly associated with it even after preincubation with 200 mM NaCl which solubilizes elastase (4, 12). Its marked reduction in PMN of all three CHS patients (Table II) warrants further study of the enzyme to better understand its possible role in PMN function. The increased tubulin tyrosinolation in CHS cells is unlikely to be related to the decreased proteolysis since tubulin tyrosinolation was not increased in normal PMN treated with the proteolytic inhibitor PMSF. However, a possible role of the proteolytic enzyme (Table II) cannot be completely ruled out.

Initially, the effect of ascorbate on both resting and FMLP-stimulated posttranslational tyrosine incorporation in PMN of CHS₁ and CHS₂ (Figs. 5 and 6) was studied in view of the reported evidence (7, 18) showing that many of the functional abnormalities in CHS neutrophils, at least in some patients, were correctable by ascorbate. Moreover, ascorbate has also been shown to promote microtubule assembly and to stimulate PMN chemotaxis (7). The latter effect may not necessarily be related to the stimulatory effect of ascorbate on the generation of cyclic guanosine monophosphate in these cells (35, 39) as the stimulation of microtubule assembly and of chemotaxis, were reported to occur when nucleotide levels of PMN were only marginally affected (5). Since ascorbate is a powerful reducing agent, its therapeutic effect could possibly be related to correction of the exaggerated oxidative metabolism in PMN of CHS patients (40). Evidence in support of this possibility was provided by the studies with unrelated reducing agents such as GSH, cysteine, or DTT, which were also found to produce similar effects (as that of ascorbate), on tubulin tyro-

sinolation in resting and FMLP-stimulated normal and CHS PMN (Fig. 7). Thus, the effects of ascorbate on tubulin tyrosinolation were not specific, and the results in Fig. 7 suggest a correlation between cellular redox and tubulin tyrosinolation in PMN.

The biochemical basis and the specificity of our measured levels of posttranslational tyrosine fixation into tubulin α-chains (Fig. 4) and its rapid stimulation in the presence of a chemoattractant (Fig. 2) suggest a functional link between tubulin tyrosinolation and PMN functions, such as chemotaxis, degranulation, or superoxide generation, which are stimulated by chemoattractants. In view of our results with ascorbate and other reducing agents, it is tempting to consider that tyrosinolation of tubulin may be coupled to the early redox changes involved in human PMN activation.

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