Published October 1, 1981

Stimulation of Tubulin Tyrosinolation in Rabbit Leukocytes Evoked by the Chemoattractant Formyl-methionyl-leucyl-phenylalanine

JAYASREE NATH, MARTIN FLAVIN, and ELLIOTT SCHIFFMANN
Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, and Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205

ABSTRACT Cellular tubulin is subject to a posttranslational modification involving the reversible addition of tyrosine through peptide linkage to the C-terminal glutamate of the α-chain. The synthetic peptide chemoattractant, N-formyl-methionyl-leucyl-phenylalanine, causes a specific, dose-dependent stimulation of tubulin tyrosinolation in rabbit leukocytes. This stimulation is prevented by carbobenzoxy-phenylalanyl-methionine, benzoyl-tyrosine ethylester, and nordihydroguaiaretic acid, which are all inhibitors of chemotaxis presumed to act via membrane-associated events. The combination of 3-deazaadenosine and homocysteine thiolactone, which inhibits phospholipid methylation, and quinacrine, an inhibitor of phospholipase A_2, also abolishes the response to the peptide. Colchicine, however, which causes a marked disassembly of cellular microtubules in these cells and also inhibits chemotaxis, does not have any inhibitory effect on the basal or peptide-stimulated rate of tubulin tyrosinolation. In contrast, taxol, a microtubule-stabilizing agent, has an inhibitory effect on both the basal and peptide-stimulated tyrosine incorporation. Taxol also inhibits chemotaxis in rabbit leukocytes. The results strongly suggest the role of closely linked membrane-cytoskeleton interactions in leukocyte chemotaxis, in which tyrosinolation of tubulin may be functionally involved.

Chemotaxis, the directed migration of cells in response to a chemical gradient, has been demonstrated in a variety of cells, including bacteria (1), the cellular slime molds (7, 18), leukocytes (47, 48), fibroblasts (30), and neuronal (36), embryonal (2), and tumor (37) cells. Chemotaxis in general has been shown to be receptor mediated (4, 14, 21, 49), and the activated receptor in stimulated cells is believed to deliver a signal to the motility elements of the cells (such as microfilaments and microtubules), resulting in their orientation and subsequent directional migration towards the chemoattractant (12, 13, 45). The evidence for a functional role of microtubules in leukocyte chemotaxis has been somewhat controversial. However, work from several laboratories has suggested that microtubules play an important role in leukocyte chemotaxis (16, 22, 28). It was shown that exposure of leukocytes to attractants induces microtubule assembly before locomotion. This assembly was blocked by colchicine, which also prevented their proper orientation toward chemoattractants (9, 22, 29, 32). These results suggested that microtubules were required for the initial orientation and subsequent cell stability during sustained locomotion (22). However, the molecular basis for the essential role of microtubules in these processes is not known.

Tubulin in vertebrate (6, 33, 34) and in some invertebrate (17) cells and tissues is uniquely subject to a reversible posttranslational modification whereby a tyrosine residue is added to the α-carboxyl of the tubulin α-chain carboxy-terminal glutamate (3, 35). The enzyme that catalyzes this reaction, tubulin tyrosine ligase, has been detected in extracts of every avian and mammalian tissue so far examined (31, 34), and in cultured neuronal (24), HeLa (8, 26), and myoblast cells (46). More recently, the enzyme has also been detected in sea urchin eggs (17). Although the presence or absence of the tyrosine has not been found to affect assembly into microtubules in vitro (34), it could well influence assembly in vivo. Because cellular microtubules can form and dissolve rapidly in the presence of a constant pool of tubulin, reversible posttranslational modifications are good candidates to modulate microtubule assembly or function in vivo. We had previously found that changes in

THE JOURNAL OF CELL BIOLOGY · VOLUME 91 OCTOBER 1981 232-239

Published October 1, 1981

tubulin tyrosinolation[1] accompany the cytoskeletal reorganizations that occur during neurite extension by differentiating neuronal cells (24), and during mitosis in HeLa cells (26). It was, therefore, possible that changes in the level of tubulin tyrosinolation might occur during chemotactic stimulation of the leukocyte, which in some manner could be related to the known increased assembly, or function, of microtubules in such cells. In this paper, we present data showing that the synthetic peptide chemoattractant, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), causes a specific stimulation of rabbit leukocyte tubulin tyrosinolation, and that this stimulation is prevented by a variety of known inhibitors of chemotaxis.

MATERIALS AND METHODS

Leukocyte Preparation

Rabbit peritoneal leukocytes were obtained as previously described (43). Washed cells were suspended in Gey's balanced salt solution containing 0.1% bovine serum albumin (BSA) in 0.015 M HEPES buffer at pH 7.4, at a concentration of 8–11 × 10^6 cells/ml. Chemotaxis was routinely measured in modified Boyden chambers as described (44). Cell viability was determined by trypan blue exclusion.

[^{14}C]Tyrosine Incorporation in Rabbit Leukocytes

Freshly harvested rabbit peritoneal leukocytes washed as described above were collected by centrifuging at 1,000 *g* for 5 min. The supernate was discarded, and packed cells were resuspended at a cell density of 10^7 cells/ml, in an isoosmotic, buffered medium, as previously described for neuroblastoma-glioma hybrid (NG) cells (24), containing a complete amino acid mixture less tyrosine. For the leukocyte experiments, we also included 0.1% BSA in the medium. As we have demonstrated before (24), the presence or absence of the amino acid mixture did not have any effect on the rate or extent of [^{14}C]tyrosine fixation in cells under our experimental conditions. In studying tubulin tyrosinolation in cells in vivo, it is of crucial importance to have protein synthesis effectively inhibited. In all of the experiments (except as indicated otherwise), the leukocytes were preincubated for 30 min at 37°C, in a medium containing a mixture of 50 μg/ml each of cycloheximide and puromycin, and 25 μg/ml of chloramphenicol, before ^{14}C-labeled tyrosine was added to the cells. This combination of antibiotics inhibits protein synthesis very effectively to the extent of >98% without inhibiting chemotaxis.

In most experiments, we have used cells at a density of 10^7/ml and FMLP at a concentration of 10^{-8} M. Unless otherwise indicated, various compounds to be tested were also present during the 30-min preincubation with antibiotics. At the end of the preincubation, the cells were quantitatively transferred to tubes containing [^{14}C]tyrosine (or [^{14}C]leucine), 5 μCi ≡ 0.010 μmol/ml, and further incubated for the indicated times. At intervals, 75- to 80-μl aliquots were taken to determine the amount of TCA-insoluble [^{14}C]tyrosine fixed into various cell samples. All other experimental procedures were similar to those described for NG cells (24).

Tubulin Tyrosine Ligase Assay in Rabbit Leukocytes

For ligase assay, freshly harvested and washed leukocytes (30–40 ml of cells at a cell density of 5 × 10^6 cells/ml) were centrifuged at 1,000 *g* for 5 min and resuspended in 1.0 ml of an isotonic buffer containing 0.34 M sucrose, 25 mM HEPES, 0.5 mM $MgSO_4$, 1 mM dithiothreitol (DTT), adjusted to pH 7.2. The cells were disrupted by mild sonication (24) and ligase specific activity was determined in the supernate and pellet fractions obtained after centrifugation of the cell-sonicate at 100,000 *g* for 45 min. The 100,000 *g* pellet fractions were washed twice with 10 ml of the homogenizing buffer and finally resuspended in 500 μl of the same buffer with 0.1% Nonidet P-40. Addition of the detergent was necessary to facilitate dispersion of the leukocyte 100,000 *g* pellet fraction into a homogeneous suspension, which was otherwise highly sticky in nature and difficult to resuspend. The concentration of detergent used does not affect ligase assay under our experimental conditions (25). Where indicated, the cells were preincubated with 5 × 10^{-9} M FMLP. Conditions for ligase assay have been previously described (35).

Determination of the Specific Radioactivity of Intracellular Tyrosine Pool

Freshly harvested leukocyte suspension at 10^7 cells/ml in Gey's medium (with 0.1% BSA) was preincubated with antibiotics as described above. To each of four plastic tubes containing 5 μCi of [^{14}C]tyrosine (≡ 0.01 μmol), 1 ml of cell suspension was added, with or without the addition of 10^{-8} M FMLP, and 3-deazaadenosine (DZA) + homocysteinethiolactone (HCyL) at 10^{-4} M and taxol at 10^{-5} M. The cells were incubated for 75 min at 37°C, with gentle shaking. The cell suspensions were then chilled to 0°C and rapidly filtered through Whatman GF/B filters (2.4 cm diameter) placed on a vacuum filtration manifold (Hoefer Scientific Instruments, San Francisco, Calif.). The filters were then washed rapidly with two 10-ml portions of ice-cold phosphate-buffered saline. The moist filters were placed in glass vials and triturated successively with two 1-ml portions of 7% TCA, kept at 0°C. The TCA extracts were combined and filtered through disposable 0.22-μm Millex (Millipore Corporation, Bedford, Mass.) to remove debris. The filtrates were lyophilized and the residue was used for the determination of tyrosine and its specific radioactivity. [^{14}C]Tyrosine uptake was determined by counting an aliquot of the water-solubilized lyophilizate from the previous steps. For determination of the specific radioactivities of the free tyrosine in different cell samples an aliquot of each sample was analyzed for tyrosine, using a Durrum amino acid analyzer (Dionex Corp., Sunnyvale, Calif.). An identical aliquot was processed through the ion-exchange resin, without ninhydrin development, and the effluent was collected in 1-ml fractions. These were each counted (after mixing with 12 ml of Aquasol), in a Beckman scintillation spectrometer (Beckman Instruments, Inc., Palo Alto, Calif.).

Other Analytical Procedures

Native tubulin was determined by colchicine binding assay with Whatman DE-23 ion exchange cellulose columns. Assay conditions were the same as described before (25). Polyacrylamide slab gel electrophoresis was done in a SDS-urea system (10). To prepare the cell samples for gel electrophoresis, the ^{14}C-labeled leukocytes were chilled to 0°C and centrifuged at 2,000 *g* for 5 min and then resuspended in 100–200 μl of ice-cold isotonic buffer (as described for ligase assay) containing 0.1 mM each of tosyl-L-phenylalanyl chloromethane ketone and tosyl-L-lysyl chloromethane ketone to prevent any proteolysis that could occur during subsequent steps of sample preparation. The cell samples were then prepared for gel electrophoresis as previously described (25). At the end of the electrophoresis run, the slab gel was stained with Coomassie Blue and destained in acetic acid. The gel was then processed for fluorography (19), to identify the labeled protein bands. Protein was determined by the procedure of Lowry et al. (20), using bovine serum albumin as the standard.

Chemicals

The synthetic peptide attractant, FMLP, was obtained from Peninsula Laboratories, Inc. (San Carlos, Calif.) and was composed of L-amino acids. Cycloheximide, chloramphenicol, benzoyl-tyrosine-ethyl ester (BTEE), HCyL, puromycin, and quinacrine were obtained from Sigma Chemical Co. (St. Louis, Mo.). Colchicine and L-tyrosine were purchased from Calbiochem-Behring Corp. (San Diego, Calif.). L-[U-^{14}C]tyrosine and [^{3}H]colchicine were obtained from Amersham-Searle (Chicago, Ill.), and L-[U-^{14}C]leucine from New England Nuclear (Boston, Mass.). Taxol was obtained from Dr. John Douros of the National Cancer Institute. DZA was a gift from Dr. F. Hirata (National Institute of Mental Health). Carbobenzoxy-phenylalanyl-methionine (Z-Phe-Met) was purchased from Vega-Fox Biochemicals Div., Newbery Energy Corp. (Tucson, Ariz.)

RESULTS

We first determined the amounts of both tubulin and ligase activity in rabbit leukocytes. As shown in Table I, the specific activity of the ligase was not demonstrably different in extracts of control and FMLP-treated cells. The low activity of 0.01 nmol/min·mg is comparable to that of other nonneuronal tissues or cells that have been examined (26, 31). Also, as observed for other tissues and cells, the enzyme was primarily recovered (90%) from the soluble fraction of leukocytes. How-

[1] "Tyrosinolation" is the correct biochemical nomenclature for the modification we are studying and refers to the same reaction that has been called "tyrosylation" in earlier publications from our laboratory and "tyrosination" by others (see Nomenclature of α-amino acids, *Biochemistry* [1975] 14:450, Section 1.4.4).

Published October 1, 1981

ever, ~10% of the total enzyme activity was consistently found to be associated with the particulate fraction as well. Table II describes the concentration and distribution of cellular tubulin in control and FMLP-treated leukocytes in terms of their colchicine-binding activity. Again, there were no significant differences in the cellular distribution of tubulin between the control and the peptide-stimulated cells. The low colchicine-binding values of the high-speed supernates correspond to only ~1% of the total cytoplasmic protein as tubulin. This did not allow us to further purify the leukocyte tubulin for reliable measurement of its state of tyrosinolation, or its acceptor capacity (25). Another significant point to note is that about half of the total colchicine-binding activity was in the particulate fraction of these cells (last column); this high amount has been found elsewhere only in brain (11, 25).

Next, we measured the rate of [^{14}C]tyrosine incorporation in vivo into leukocyte tubulin α-chains. Fig. 1 shows that, in the presence of inhibitors of protein synthesis, there was detectable incorporation of [^{14}C]tyrosine into the TCA-insoluble fraction of rabbit leukocytes and that about a threefold stimulation in the rate of fixation was observed in the presence of FMLP.

TABLE I

Tubulin Tyrosine Ligase Activity in Rabbit Peritoneal Leukocytes, after Preincubation in the Presence or Absence of FMLP

Sample assayed	Total protein	Ligase specific activity	Total units of ligase	Cellular distribution
	mg	*nmol/min · mg*	*nmol/min*	*%*
High-speed supernate				
Control	7.2	0.0120	0.086	90.5
+ FMLP	7.0	0.0100	0.070	88.6
High-speed pellet				
Control	12.9	0.0007	0.009	9.5
+ FMLP	13.5	0.0007	0.009	11.4

Rabbit peritoneal leukocytes were preincubated for 30 min at 37°C, in the presence or absence of FMLP. At the end of the incubation period, the cells were centrifuged down, incubation medium was removed, and the packed cells were resuspended in 1 ml of buffer containing 0.34 M sucrose, 25 mM HEPES, 1 mM DTT, and 0.5 $MgSO_4$, pH 7.2. The cell suspensions were sonicated and the cell sonicates centrifuged at 100,000 × g for 30 min. The high-speed supernates were carefully separated and the pellets were washed and resuspended in 500 μl of the same buffer containing 0.1% NP-40. Ligase assays were performed with the high-speed supernates and the pellet suspensions. Other details are given in the text.

TABLE II

Colchicine Binding Activity in Rabbit Peritoneal Leukocytes after Preincubation in the Presence or Absence of FMLP

Sample assayed	Total protein	Colchicine bound	Total amount of colchicine bound	Cellular distribution
	mg	*nmol/mg*	*nmol*	*%*
High-speed supernate				
Control	7.2	0.065	0.47	48
+ FMLP	7.0	0.070	0.49	49
High-speed pellet				
Control	12.9	0.040	0.51	52
+ FMLP	13.5	0.038	0.51	51

Rabbit peritoneal leukocytes were preincubated as described for the ligase assays and the same high-speed supernates and pellet suspensions (as in Table I) were used for colchicine binding assays.

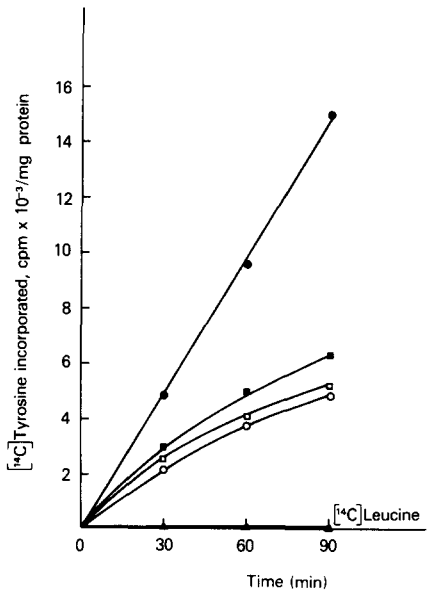

FIGURE 1 FMLP-induced stimulation of tubulin tyrosinolation in vivo and its inhibition by Z-Phe-Met and BTEE. Freshly harvested leukocytes were preincubated for 30 min at 37°C with antibiotics, in the absence or presence of the various compounds, and then further incubated in the presence of [^{14}C]tyrosine (5 μCi ≡ 0.01 μmol/ml). [^{14}C]tyrosine incorporation was measured by following TCA-insoluble radioactivity at indicated times. Results are the means of duplicate determinations. (○) Control cells; (●) with FMLP; (□) with FMLP + 10^{-4} M Z-Phe-Met; (■) with FMLP + 10^{-4} M BTEE. The bottom curve (▲) shows the complete inhibition of [^{14}C]leucine incorporation under the same experimental conditions (with or without FMLP added to the incubation medium).

These results are typical for a large number of experiments with chemotactically competent cells, where we have always observed such two- to threefold stimulation in the presence of the chemoattractant. Occasionally, if a particular batch of cells was not chemotactically responsive toward FMLP (in Boyden chamber assay), it also failed to demonstrate the stimulated [^{14}C]tyrosine incorporation in the presence of the peptide. Thus, there seems to be a 100% correlation between the leukocyte's ability to exhibit chemotaxis and its ability to show stimulated tyrosinolation. Unlike the events in NG cells, [^{14}C]tyrosine incorporation did not reach a plateau in 60 min (24) but continued essentially in a linear fashion during the 90–120 min in which [^{14}C]tyrosine fixation was measured in these cells. Under the same experimental conditions incorporation of the unrelated amino acid leucine was completely inhibited, in both the presence and absence of FMLP (Fig. 1, bottom curve). This indicated that protein synthesis was very effectively inhibited while [^{14}C]tyrosine incorporation was measured. Fig. 1 also shows that the enhanced rate of incorporation of [^{14}C]tyrosine in the presence of the chemoattractant was essentially abolished by the addition of 10^{-4} M Z-Phe-Met, which is known to specifically block the FMLP receptors in these cells (41). BTEE, a chymotryptic substrate and an inhibitor of chemotaxis in these cells (5), also prevented the stimulated response.

Fig. 2 shows the time-course of stimulation of tyrosine incorporation in the presence of FMLP. There was an initial lag of ~6 min before a noticeable stimulation was observed. Also, there seemed to be an early, somewhat rapid phase of incorporation (0–2 min), followed by a period of slower incor-

Published October 1, 1981

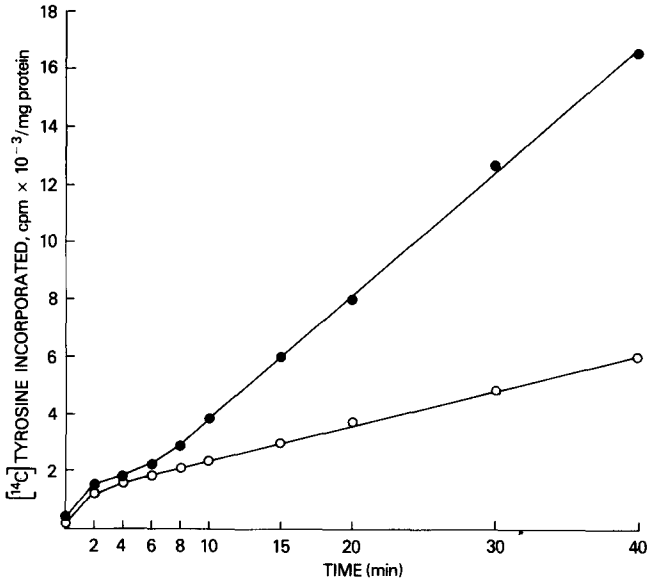

FIGURE 2 Time-course of stimulation of tyrosine incorporation induced by FMLP. Rabbit leukocytes at 10^7 cells/ml were preincubated with antibiotics and 10^{-8} M FMLP and 5 μCi of [^{14}C]tyrosine (≡ 0.01 μmol) were added at 0 time. TCA-insoluble [^{14}C]tyrosine incorporation was measured at indicated times. (○) Control cells; (●) with FMLP.

poration (2–6 min), before the peptide-induced stimulation of tyrosine incorporation in these cells. As observed in Fig. 1, the peptide-stimulated tyrosine incorporation is not a transient phenomenon but is sustained over a considerable length of time, as indicated by the linear rate of incorporation up to 40 min (and much longer as observed in other experiments). Because chemotactic behavior can occur within a very short time, tyrosinolation is more likely to be associated with sustaining locomotion than with its induction.

In a control experiment, we have also compared the rates of protein synthesis in the presence and absence of the chemoattractant. As shown in Fig. 3, FMLP had a significantly inhibitory effect on the rate of both [^{14}C]tyrosine and [^{14}C]leucine incorporation under conditions that allowed protein synthesis to continue. These results suggest that the attractant did not act by increasing the rate of uptake of tyrosine into these cells. To rule out any possible differences in the rate of tyrosine uptake in the presence and absence of FMLP (which would have changed the specific activity of the radioactive tyrosine inside the cells), we have also compared the rates of [^{14}C]-tyrosine uptake in the presence and absence of the chemoattractant. In two separate experiments, there were no significant differences in the rates of tyrosine uptake, in the presence and absence of FMLP (data not shown), indicating that the enhanced rate of tubulin tyrosinolation in the stimulated cells was not attributable to a parallel increase in transport of labeled tyrosine into the cells.

We have also determined the specific radioactivity of the intracellular tyrosine pool in both unstimulated and FMLP-stimulated leukocytes. The specific activity of the radioactive tyrosine in peptide-stimulated cells was similar after 75-min incubation with FMLP (Table III). The specific radioactivity of intracellular tyrosine also was similar in both DZA + HCyL and taxol-treated leukocytes (Table III). In fact, the specific radioactivity of tyrosine was slightly higher in taxol-treated leukocytes. These results clearly confirmed that any changes observed in the rate of tyrosine incorporation (i.e., stimulation or inhibition) were *not* attributable to parallel changes in its intracellular specific radioactivity.

The results in Fig. 4 demonstrate that the specific stimulation of tubulin tyrosinolation in response to FMLP is dose-dependent. For the various concentrations of the chemoattractant added in this particular experiment, the cell density was maintained at 5×10^6 cells/ml. Although chemotaxis is usually assayed with a cell density of 2.2×10^6 cell/ml, we have here used the higher level to obtain enough material for measurement of tyrosine incorporation. The results show that, at concentrations of 1, 2, and 5 nM FMLP, tyrosine incorporation was stimulated at the end of 30 min by ~60, 70, and 80%, respectively, above controls. Because the optimal response for rabbit peritoneal exudate cells has been shown to occur over a range of 1–4 nM (43), it is possible that the levels of tyrosine incorporation in the presence of 1, 2, and 5 nM FMLP are associated with sustained locomotion of the cell. The higher levels (10 and 20 nM) could induce release of lysosomal enzymes as well (43).

Fig. 5 shows the photograph of a fluorogram obtained with [^{14}C]tyrosine-labeled cell samples from the experiment described in Fig. 4. In the original fluorogram of the slab gel, a

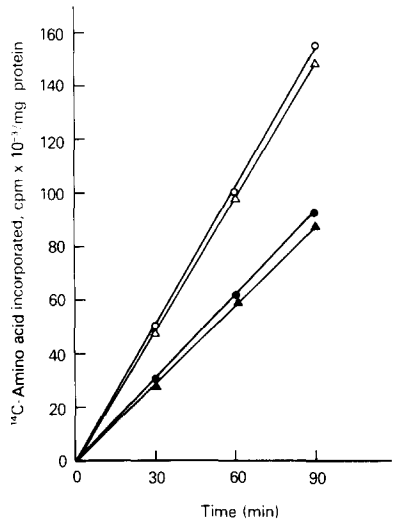

FIGURE 3 Effect of FMLP on protein synthesis in rabbit leukocytes. Leukocytes were incubated in the presence of [^{14}C]tyrosine or [^{14}C]leucine, under conditions that allowed protein synthesis to continue. The rate of amino acid incorporation was measured by following TCA-insoluble radioactivity at indicated times. (○) [^{14}C]-Tyrosine, control cells; (●) [^{14}C]tyrosine with FMLP; (△) [^{14}C]leucine, control cells; (▲) [^{14}C]leucine, with FMLP.

TABLE III

Specific Radioactivity of Intracellular Tyrosine Pool under Different Conditions

Cell sample	Specific radioactivity
	cpm/pmol tyrosine
Control	145
+ FMLP, 10^{-8} M	160
+ DZA + HCyl, 10^{-4} M	120
+ Taxol, 10^{-5} M	180

Leukocytes, at 10^7/ml, were preincubated with antibiotics for 30 min and then further incubated in the presence of [^{14}C]tyrosine (5 μCi/ml ≡ 0.01 μmol) for 75 min at 37°C, in the absence or presence of indicated compounds. Specific radioactivity of the intracellular tyrosine was determined as described in Materials and Methods.

Published October 1, 1981

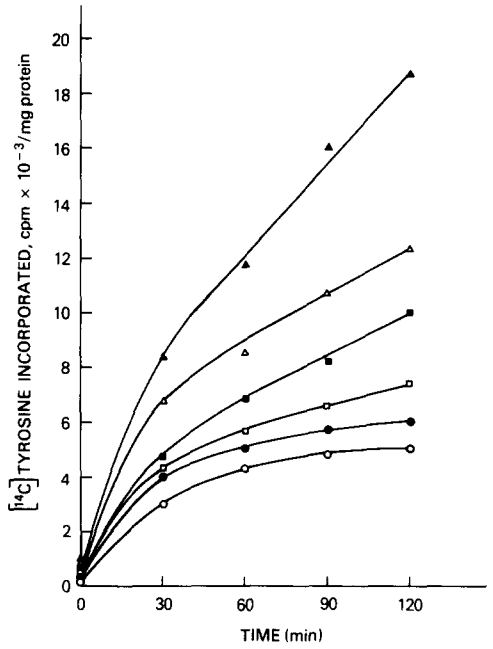

FIGURE 4 Stimulation of tyrosine incorporation as a function of FMLP concentration. The leukocytes were preincubated for 30 min at 37°C with antibiotics alone and further incubated in the presence of [^{14}C]tyrosine (5 μCi ≡ 0.01 μmol) in the absence or presence of increasing concentrations of FMLP, at a constant cell density of 5 × 10^6 cells/ml. TCA-insoluble radioactivity was measured at indicated times. The results are the means of duplicate determinations. (○) Control cells; (●) with 1 × 10^{-9} M FMLP; (□) with 2 × 10^{-9} M FMLP; (■) with 5 × 10^{-9} M FMLP; (△) with 1 × 10^{-8} M FMLP; (▲) with 2 × 10^{-8} M FMLP.

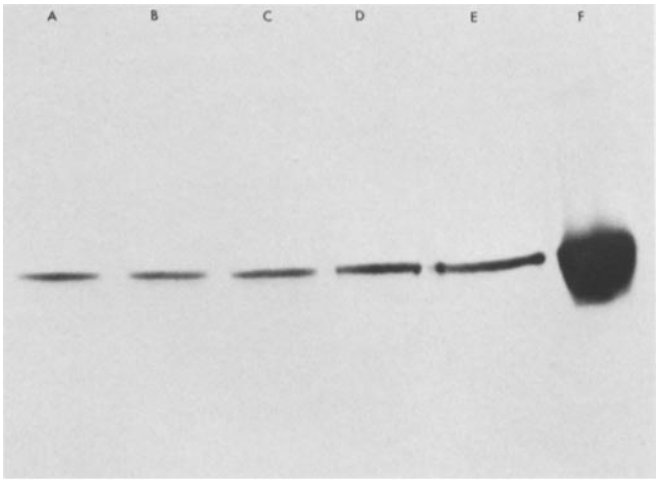

FIGURE 5 Fluorographic demonstration of [^{14}C]tyrosine fixation into tubulin α-chains as a function of the concentration of FMLP. ^{14}C-labeled leukocytes, as obtained in the experiment described in Fig. 4, were subjected to gel electrophoresis, and the slab gel was processed for fluorography. Details of the procedures are described in the text. Lanes *A* through *E* represent ^{14}C-labeled leukocyte samples as obtained in the experiment of Fig. 4, with 1 × 10^{-9} M to 2 × 10^{-8} M FMLP, respectively. Lane *F* shows an overexposed sample of authentic, assembly-purified [^{14}C]tyrosinolated tubulin preparation.

progressive increase in the intensity of the radioactive tubulin bands was quite visible. Because of technical limitations imposed by photographic reproduction, the gradual increase in the intensity of the radioactive tubulin bands was not that apparent at lower concentrations of 1, 2, and 5 nM FMLP (Fig. 5, lanes *A–C*). However, at high concentrations of the peptide (i.e., at 10 and 20 nM), an increase in the amount of radioactivity could still be observed (Fig. 5, lanes *D* and *E*). Fig. 5 also demonstrates the specificity of the reaction in rabbit leukocytes.

Fig. 6 summarizes the results we have obtained by adding specific inhibitors of various reactions involved in chemotaxis to the incubation medium and examining their effects on tyrosinolation of tubulin in peptide-stimulated and unstimulated cells. Methylation of specific protein dicarboxylic acid γ-carboxyls and membrane phospholipids is believed to be involved in leukocyte chemotaxis (15, 27). The combination of DZA and HCyL effectively inhibits the transfer of methyl groups from adenosylmethionine to acceptor molecules such as membrane phospholipids (42). Fig. 6 shows that the combined application of DZA and HCyL completely abolishes the FMLP-stimulated response on tubulin tyrosinolation. In the absence of FMLP, DZA and HCyL together also caused a slight inhibition of the basal level of tyrosinolation in control cells. Quinacrine, a known inhibitor of phospholipase A_2 (38), also blocked the stimulation of tubulin tyrosinolation by FMLP. Nordihydroguaiaretic acid (NDGA), which has been recently reported to inhibit specifically the influx of extracellular calcium induced by FMLP (23), also prevented the peptide-stimulated response upon tyrosinolation.

It has been previously reported that intact microtubules are required for turnover of C-terminal tyrosine of α-tubulin in cultured muscle cells (46). However, in our earlier studies with NG cells, we could not demonstrate any inhibitory effect of colchicine, which depolymerizes microtubules, on the in vivo rate of tyrosine fixation into tubulin α-chains (24). We have reexamined the effect of colchicine on tubulin tyrosinolation

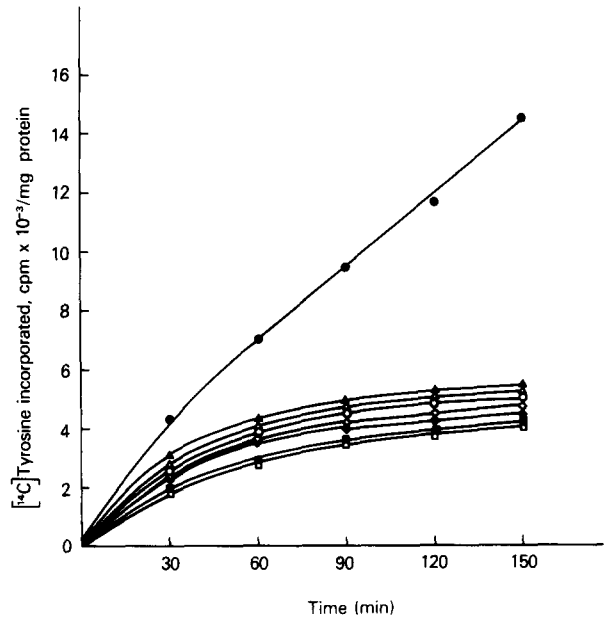

FIGURE 6 Effects of various inhibitors on the FMLP-stimulated tyrosine incorporation in rabbit leukocytes. Leukocytes were incubated in the presence or absence of various inhibitors as described in the text and their effects on the basal and FMLP-stimulated rates of tyrosine fixation were measured. (○) Control cells; (●) with FMLP; (□) with 10^{-4} M DZA + 10^{-3} M HCyL; (■) with FMLP + 10^{-4} M DZA + 10^{-3} M HCyL; (△) with 10^{-4} M quinacrine; (▲) with FMLP + 10^{-4} M quinacrine; (◇) with 10^{-5} M NDGA; (◆) with FMLP + 10^{-5} M NDGA.

Published October 1, 1981

in rabbit leukocytes. When leukocytes are preincubated with 10^{-6}–10^{-5} M colchicine for 30 min, chemotaxis is reportedly inhibited (9, 22, 29, 32), and the inhibition is accompanied by a marked disassembly of centriole-associated microtubules (22). Yet, as can be seen from Fig. 7, preincubation with colchicine did not cause any inhibition of either the basal or the FMLP-stimulated tyrosinolation of tubulin in these cells.

On the contrary, we observed a modest stimulation in the presence of colchicine, which was quite reproducible. In the experiment of Fig. 7, the leukocytes were preincubated with antibiotics for 30 min in the presence or absence of 10^{-5} M colchicine, before the chemoattractant was added to the incubation medium, and the cells were then further incubated for 30 min before [^{14}C]tyrosine was added. The drug did not have any inhibitory effect on either the basal or the FMLP-stimulated rate of tyrosine incorporation. Similar results were obtained when the order of FMLP and colchicine addition was reversed during the preincubation, or when both compounds were present during the entire preincubation period.

Fig. 7 also shows the effect of taxol on basal and FMLP-stimulated rates of tyrosine incorporation in rabbit leukocytes. Taxol, a low molecular weight compound (isolated from the plant *Taxus brevifolia*), has recently been shown to promote microtubule assembly and to stabilize microtubules, both in vitro (39) and in vivo (40). Taxol has also been reported to inhibit cell division and fibroblast migration (40), presumably via its stabilizing effect on cellular microtubules. It was therefore of interest to study the effect of taxol on leukocyte tubulin tyrosinolation and also on chemotaxis. Under routine conditions of assay, 10^{-6}–10^{-5} M taxol effectively inhibited chemotaxis in rabbit peritoneal leukocytes, without destroying their viability (data not shown). As can be seen from Fig. 7, taxol also had a marked inhibitory effect on the basal rate of tyrosine incorporation. The effect of taxol on the FMLP-stimulated rate of tyrosine incorporation was especially intriguing. When the leukocytes were preincubated, with FMLP first, followed by taxol, the peptide stimulation was completely abolished and the rate of tyrosine incorporation was essentially similar to that observed in the presence of taxol alone. However, when the leukocytes were preincubated with taxol only, followed by taxol + FMLP, a stimulated rate of incorporation, comparable to that of cells treated with FMLP alone, was observed (Fig. 7). This differential effect of taxol, depending on the sequence of additions during preincubation, has been observed in three separate experiments with different batches of cells.

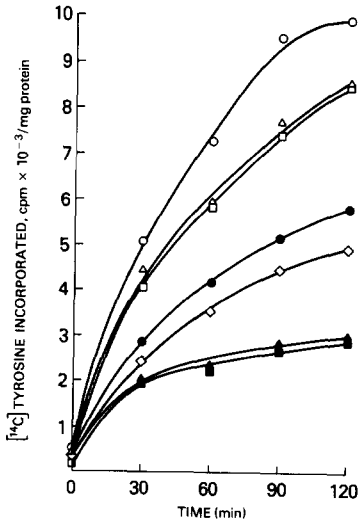

FIGURE 7 Effect of colchicine and taxol on the basal and FMLP-stimulated tyrosine incorporation in rabbit leukocytes. 10^7 cells/ml were preincubated in the presence or absence of the various compounds as described in the text and TCA-insoluble radioactivity was determined at indicated times. Results are the means of duplicate determinations: (◇) control cells; (●) with 10^{-5} M colchicine; (□) with FMLP; (○) with FMLP + 10^{-5} M colchicine; (■) with 10^{-5} M taxol; (▲) with FMLP alone, followed by 10^{-5} M taxol; (△) with 10^{-5} M taxol alone, followed by FMLP.

DISCUSSION

We have demonstrated that exposure of rabbit peritoneal leukocytes to a peptide chemoattractant specifically stimulates a biochemical modification of the cell's tubulin. The stimulation was manifested as an increased rate of tyrosine fixation at the α-chain C-terminus. The increase began ~6 min after addition of the peptide (Fig. 2) and is therefore not one of the earliest biochemical events in the chemotactic response but might be related to a phase of cytoskeletal rearrangement and sustained locomotion. The increased rate was maintained throughout the period we have studied (150 min), whereas in unstimulated cells tyrosine fixation tended to plateau after 90 min (Figs. 4 and 6).

It is important to specify what is, and what is not, known about the biochemical basis of our measured rates of posttranslational tyrosine fixation. We have shown that the stimulation by chemoattractant is not attributable to an increased tyrosine transport into the leukocytes. Also, the specific activity of the free tyrosine was found to be similar in control and in peptide-stimulated cells. The result clearly shows that the stimulation of tyrosine fixation cannot be explained by a higher specific activity of the free tyrosine. The specific radioactivity of intracellular tyrosine was also similar in DZA + HCyL- and taxol-treated cells (Table III), where we have obtained a marked inhibition of TCA-insoluble radioactivity (Figs. 6 and 7). Combined application of DZA + HCyL is known to inhibit chemotaxis by preventing specific membrane-associated protein and phospholipid methylation reactions (42), whereas taxol presumably inhibits chemotaxis via its stabilizing effect on cellular microtubules (40). That neither of the two inhibitors (which are widely different in their effective site of action) had any effect on the specific radioactivity of tyrosine in these cells confirms beyond doubt that the changes observed in the rate of tubulin tyrosinolation (stimulation or inhibition) are not attributable to a parallel change in the specific radioactivity of the intracellular free tyrosine. Furthermore, these results reaffirm a link between chemotaxis and tubulin tyrosinolation.

It is not known 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 control or stimulated cells by adding up to a 50-fold excess of [^{12}C]tyrosine after a 60-min pulse with 10 μM [^{14}C]tyrosine. This was also true in a chase with 1,000-fold excess of [^{12}C]-tyrosine, where we have used a pulse of 10 nM [^{3}H]tyrosine (sp act: 86 Ci/mmol). Despite this result, it is not certain that all the observed fixation is incremental. We can calculate the moles of tyrosine fixed per mole of tubulin from the specific activity of the tyrosine pool (Table III), and the amount of tubulin per milligram soluble protein, which the colchicine-binding data in Table II show to be ~10 μg, or 0.09 nmol. Unstimulated leukocytes fixed a maximum of 4,000 cpm/mg protein (Figs. 4 and 6) corresponding (Table III) to 0.27 mol tyrosine/mol of tubulin. Stimulated leukocytes fixed a maxi-

mum of 18,000 cpm/mg, corresponding to 0.55 mol tyrosine/mol of tubulin. The latter amount seems rather high to be entirely attributable to incremental fixation, especially because the fixation has not reached a plateau at this point (Figs. 4 and 6).

One more consideration bearing on the biochemical interpretation of the results in this paper is the observation that in brain (25) and, as we have also recently shown, in several cultured cell lines, at least part of the tubulin tyrosinolated in vivo is, in some way not yet elucidated, a different species from that which can be tyrosinolated in vitro. Because of the small amount of tubulin in leukocytes (Table II), we have not yet been able to determine whether this is also the case for these cells.

The chemotactic response in leukocytes may occur in three stages: interaction of the attractant with receptor, processing and amplification of the signal generated by the activated receptor, and activation of the cellular motility elements to produce directed movement. The stimulation of tyrosine fixation is mediated via receptors, appears to depend on normally functioning pathways of protein and phospholipid methylation, and is abolished by a variety of inhibitors of chemotaxis, with the interesting exception of colchicine. Colchicine presumably inhibits chemotaxis by causing disassembly of cytoplasmic microtubules (22), whereas, with the exception of taxol, the chemotactic inhibitors tested (Figs. 1 and 6) are all known to be inhibitors of membrane-associated events. These results, therefore, suggest a link between tubulin tyrosinolation and membrane phenomena. That intervention at different sites of the motile response affects the rate of tyrosinolation of tubulin suggests, but does not establish, that chemotaxis and tyrosinolation are linked. It is, of course, also possible that membrane-associated events such as methylation reactions could modulate tyrosinolation that, in turn, could play a role in sustaining chemotaxis.

Colchicine, under conditions causing microtubule disassembly, stimulated both control and peptide-stimulated tyrosine fixation (Fig. 7). Similar stimulation has also been observed with a variety of neuronal and nonneuronal cells in culture (J. Nath and M. Flavin, unpublished results). This does not support the hypothesis (46) that intact microtubules are required for rapid tyrosine fixation in vivo, nor does our finding that taxol, a microtubule-stabilizing agent, inhibited tyrosine fixation in control cells (Fig. 7). The immediate effect of taxol is to stabilize or "freeze" the existing microtubules (40); over a longer time-span than we have employed, it can also lead to increased numbers, and changed orientation, of microtubules (Susan Horwitz, personal communication). Together, the above results suggest that tyrosine fixation is facilitated in proportion to tubulin present as unassembled subunits.

The effect of taxol on peptide-stimulated tyrosine fixation gives a somewhat different picture (Fig. 7), however. When taxol was added after preincubation with the chemoattractant, stabilizing the microtubules after they had had the opportunity to undergo cytoskeletal rearrangements involved in chemotaxis, tyrosine fixation was inhibited; conversely, when taxol was added first, presumably stabilizing the relatively small proportion of assembled microtubules, subsequent addition of the chemoattractant was fully effective in stimulating tyrosine fixation (Fig. 7). These are not the results that would be expected if tyrosinolation were coupled to the distribution of tubulin between assembled and unassembled states in a simple manner. Because the more obvious cytoskeletal changes accompanying chemotaxis do not seem to be necessary for the enhanced tyrosine fixation to occur, the role of this reaction in cell migration presents an intriguing problem.

We would like to thank Guy Hawkins of the National Institute of Dental Research, for his expert assistance in amino acid analyses.

Received for publication 26 January 1981, and in revised form 8 June 1981.

REFERENCES

1. Adler, J. 1975. Chemotaxis in bacteria. *Annu. Rev. Biochem.* 44:341–356.
2. Altman, L. C., and H. Kirchner. 1972. The production of a monocyte chemotactic factor by agammaglobulinemic chicken spleen cells. *J. Immunol.* 109:1149–1151.
3. Arce, C. A., H. S. Barra, J. A. Rodriguez, and R. Caputto. 1975. Tentative identification of the amino acid that binds tyrosine as a single unit into a soluble brain protein. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 50:5–7.
4. Aswanikumar, S., B. A. Corcoran, E. Schiffmann, A. L. Day, R. J. Freer, H. J. Showell, E. L. Becker, and C. B. Pert. 1977. Demonstration of a receptor on rabbit neutrophils for chemotactic peptides. *Biochem. Biophys. Res. Commun.* 74:810–817.
5. Aswanikumar, S., E. Schiffmann, B. A. Corcoran, and S. M. Wahl. 1976. Role of a peptidase in phagocyte chemotaxis. *Proc. Natl. Acad. Sci. U. S. A.* 73:2439–2442.
6. Barra, H. S., C. A. Arce, J. A. Rodriguez, and R. Caputto. 1974. Some common properties of the protein that incorporates tyrosine as a single unit and the microtubule proteins. *Biochem. Biophys. Res. Commun.* 60:1384–1390.
7. Bonner, J. T., D. S. Barkley, E. M. Hall, T. M. Konijn, J. W. Mason, G. O'Keefe, and P. B. Wolfe. 1969. Acrasin, acrasinase and the sensitivity to acrasin in *Dictyostelium discoideum*. *Dev. Biol.* 20:72–87.
8. Bulinski, J. C., J. A. Rodriguez, and G. G. Borisy. 1980. Test of four possible mechanisms for the temporal control of spindle and cytoplasmic microtubule assembly in HeLa cells. *J. Biol. Chem.* 255:1684–1688.
9. Caner, J. E. Z. 1965. Colchicine inhibition of chemotaxis. *Arthritis Rheum.* 8:757–764.
10. Eipper, B. A. 1974. Properties of rat brain tubulin. *J. Biol. Chem.* 249:1407–1416.
11. Feit, H., and S. H. Barondes. 1970. Colchicine-binding activity in particulate fractions of mouse brain. *J. Neurochem.* 17:1355–1364.
12. Gallin, J. I., and A. R. Rosenthal. 1974. The regulatory role of divalent cations in human granulocyte chemotaxis: evidence for an association between calcium exchanges and microtubule assembly. *J. Cell Biol.* 62:594–609.
13. Goldstein, I. M., S. Hofstein, J. I. Gallin, and G. Weissman. 1973. Mechanisms of lysosomal enzyme release from human leukocytes: microtubule assembly and membrane fusion induced by a component of complement. *Proc. Natl. Acad. Sci. U. S. A.* 70:2916–2920.
14. Hazelbauer, G. L., and J. Adler. 1971. Role of the galactose binding protein in chemotaxis of *E. coli* toward galactose. *Nature New Biol.* 230:101–104.
15. Hirata, F., B. A. Corcoran, K. Venkatasubramanian, E. Schiffmann, and J. Axelrod. 1979. Chemoattractants stimulate degradation of methylated phospholipids and release of arachidonic acid in rabbit leukocytes. *Proc. Natl. Acad. Sci. U. S. A.* 76:2640–2643.
16. Hoffstein, S., I. M. Goldstein, and G. Weissman. 1977. Role of microtubule assembly in lysosomal enzyme secretion from human polymorphonuclear leukocytes. A reevaluation. *J. Cell Biol.* 73:242–256.
17. Kobayashi, T., and M. Flavin. 1981. Tubulin tyrosylation in invertebrates. *Comp. Biochem. Physiol. B Comp. Biochem.* 69B:387–392.
18. Konijn, T. M., J. G. C. Meene, J. T. Bonner, and D. S. Barkeley. 1967. The acrasin activity of adenosine-3′,5′-cyclic phosphate. *Proc. Natl. Acad. Sci. U. S. A.* 58:1152–1154.
19. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335–341.
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
21. Malchow, D., and G. Gerisch. 1974. Short-term binding and hydrolysis of cyclic-AMP by aggregating *Dictyostelium* cells. *Proc. Natl. Acad. Sci. U. S. A.* 71:2423–2427.
22. Malech, H. L., R. K. Root, and J. I. Gallin. 1977. Structural analysis of human neutrophil migration. *J. Cell Biol.* 75:666–693.
23. Naccache, P. H., H. J. Showell, E. L. Becker, and R. I. Shaafi. 1979. Pharmalogical differentiation between the chemotactic factor induced intracellular calcium redistribution and transmembrane calcium influx in rabbit neutrophils. *Biochem. Biophys. Res. Commun.* 89:12240–1230.
24. Nath, J., and M. Flavin. 1979. Tubulin tyrosylation *in vivo* and changes accompanying differentiation of cultured neuroblastoma-glioma hybrid cells. *J. Biol. Chem.* 254:11505–11510.
25. Nath, J., and M. Flavin. 1980. An apparent paradox in the occurrence and the *in vivo* turnover, of C-terminal tyrosine in membrane-bound tubulin of brain. *J. Neurochem.* 35: 693–706.
26. Nath, J., J. Whitlock, and M. Flavin. 1978. Tyrosylation of tubulin in synchronized HeLa cells. *J. Cell Biol.* 79(2, Pt. 2):294 *a* (Abstr.).
27. O'Dea, R. F., O. H. Viveros, J. Axelrod, S. Aswanikumar, E. Schiffmann, and B. A. Corcoran. 1978. Rapid stimulation of protein carboxymethylation in leukocytes by a chemotactic peptide. *Nature (Lond.).* 272:462–464.
28. Oliver, J. M. 1978. Cell biology of leukocyte abnormalities—membrane and cytoskeletal function in normal and defective cells. *Am. J. Pathol.* 93:221–259.
29. Phelps, P. 1970. Polymorphonuclear leukocyte motility *in vitro*. IV. Colchicine inhibition of the formation of chemotactic activity after phagocytosis of urate crystals. *Arthritis Rheum.* 13:1–9.
30. Postlethwaite, A. E., J. M. Seyer, and A. H. Kang. 1978. Chemotaxic attraction of human fibroblasts to type I, II, and III collagens and collagen-derived peptides. *Proc. Natl. Acad. Sci. U. S. A.* 75:871–875.
31. Preston, S. F., G. G. Deanin, R. K. Hanson, and M. W. Gordon. 1979. The phylogenetic distribution of tubulin: tyrosine ligase. *J. Mol. Biol.* 13:233–244.
32. Ramsey, W. S., and A. Harris. 1973. Leukocyte locomotion and its inhibition by antimitotic drugs. *Exp. Cell Res.* 82:262–270.
33. Raybin, D., and M. Flavin. 1975. An enzyme tyrosylating α-tubulin and its role in

microtubule assembly. *Biochem. Biophys. Res. Commun.* 65:1088–1095.
34. Raybin, D., and M. Flavin. 1977. Modification of tubulin by tyrosylation in cells and extracts and its effect on assembly in vitro. *J. Cell Biol.* 73:492–504.
35. Raybin, D., and M. Flavin. 1977. Enzyme which specifically adds tyrosine to the α-chain of tubulin. *Biochemistry.* 16:2189–2194.
36. Roisen, F. J., and R. A. Murphy. 1973. Neurite development *in vitro*: II. The role of microfilaments and microtubules in monophosphate and nerve growth factor stimulated maturation. *J. Neurobiol.* 4:397–412.
37. Romualdex, A. G., and P. A. Ward. 1975. A unique complement derived chemotactic factor for tumor cells. *Proc. Natl. Acad. Sci. U. S. A.* 72:4128–4132.
38. Rubin, D. P., and S. G. Laychock. 1975. *In* Calcium in Drug Action. G. B. Weiss, editor. Plenum Publishing, New York. 135–155.
39. Schiff, P. B., J. Fant, and S. B. Horwitz. 1979. Promotion of microtubule assembly *in vitro* by taxol. *Nature (Lond.).* 277:665–667.
40. Schiff, P. B., and S. B. Horwitz. 1980. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. U. S. A.* 77:1561–1565.
41. Schiffmann, E., B. A. Corcoran, and S. Aswanikumar. 1978. Molecular events in response of neutrophils to FMet peptides. *In* Leukocyte Chemotaxis. J. I. Gallin, and P. G. Quie, editors. Raven Press, New York. 97–111.
42. Schiffmann, E., and J. I. Gallin. 1979. Biochemistry of phagocyte chemotaxis. *Curr. Top. Cell. Regul.* 15:203–261.
43. Showell, H. J., R. J. Freer, S. H. Zigmond, E. Schiffmann, S. Aswanikumar, B. Corcoran, and E. L. Becker. 1976. The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretion for neutrophils. *J. Exp. Med.* 143: 1154–1169.
44. Snyderman, R., J. Phillips, and S. E. Mergenhagen. 1970. Polymorphonuclear leukocyte chemotactic activity in rabbit serum and guineapig serum treated with immune complexes. *Infect. Immun.* 1:521–525.
45. Stossel, T. P. 1978. The mechanism of leukocyte locomotion. *In* Leukocyte Chemotaxis. J. I. Gallin, and P. G. Quie, editors. Raven Press, New York. 143–160.
46. Thompson, W. C., G. G. Deanin, and M. W. Gordon. 1979. Intact microtubules are required for rapid turnover of carboxyl-terminal tyrosine of α-tubulin in cell cultures. *Proc. Natl. Acad. Sci. U. S. A.* 76:1318–1322.
47. Ward, P. A., and L. J. Newman. 1969. A neutrophil chemotactic factor from human C'5. *J. Immunol.* 102:93–99.
48. Wilkinson, P. C. 1973. Recognition of protein structure in leukocyte chemotaxis. *Nature (Lond.).* 244:512–513.
49. Williams, L. T., R. Snyderman, M. C. Pike, and R. J. Lefkowitz. 1977. Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. *Proc. Natl. Acad. Sci. U. S. A.* 74:1204–1208.