# ROLE OF THE TUBULIN-MICROTUBULE SYSTEM IN LYMPHOCYTE ACTIVATION

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ABSTRACT

The role of the tubulin-microtubule system was examined in human peripheral blood leukocytes after activation with phytohemagglutinin (PHA). Soluble tubulin and microtubules were measured with a [$^3$H]colchicine-binding assay. It was found that the tubulin content of PHA-activated lymphocytes was consistently increased relative to total protein content after 36 h of culture. There was no increase in the proportion of total tubulin which was present as microtubules at 36 h. Nevertheless, as a result of increased tubulin synthesis, there was a two- to three-fold increase in total microtubular mass. Colchicine, which disrupts microtubules, was used to assess the role of microtubule assembly in the sequence of events which follow lymphocyte activation, namely lymphokine release, protein synthesis, RNA synthesis, and DNA synthesis. Colchicine consistently inhibited DNA synthesis but did not inhibit release of the lymphokine, osteoclast activating factor (OAF). Protein and RNA syntheses were inhibited much less than DNA synthesis. The fact that some effects of PHA on lymphocytes appear to require intact microtubules and at least one does not suggest that the microtubule-dependent step in PHA-stimulated lymphocyte activation occurs at a stage after propagation of the signal from the membrane to the cell interior.

Microtubule integrity has recently been shown to be necessary for lectin-induced mitogenesis in lymphocytes (5, 17). In addition, microtubules are required for normal secretory function in a variety of cells which release products into the extracellular fluid (9). Mitogen-activated lymphocytes secrete a number of biologically active substances which are presumably important in immune defense. As yet, however, a detailed study of the lymphocyte microtubule system and its possible role in lymphokine release has not been reported.

In the present work we sought to define the relationship between the tubulin-microtubule system and lymphocyte activation by lectins. [$^3$H]-Colchicine was used to measure the total tubulin content of cells (14, 15) and to determine the partition of tubulin between the soluble and assembled forms (11). Unlabeled colchicine was added in culture to disassemble lymphocyte microtubules and determine whether microtubule integrity was necessary for lymphokine secretion and for the increased synthesis of protein, RNA, and DNA which occur after phytohemagglutinin (PHA) activation. We found that PHA reproducibly increased total tubulin concentration relative to other proteins, with a parallel increase in cell microtubule content. We confirmed that lectin-stimulated DNA synthesis was dramatically in-

hibited by colchicine (17), but in the same system the release of a lymphokine (osteoclast activating factor [OAF]) was unaffected.

## MATERIALS AND METHODS

### *Cell Culture*

Peripheral blood leukocytes were cultured by methods described previously, with slight modifications (16). Human peripheral blood leukocytes were obtained from normal donors in the plateletpheresis program of the Connecticut Red Cross (Farmington, Conn.). Erythrocytes were separated from the leukocyte-rich plasma by dextran sedimentation (30 ml of leukocyte- and erythrocyte-rich plasma were added to 4 ml of 6% dextran). The polymorphonuclear leukocytes and residual erythrocytes were removed from the mononuclear cells by Ficoll-Hypaque density sedimentation. The remaining mononuclear cells were resuspended in RPMI 1640 medium containing 20% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.) and incubated at a concn of $1.5 \times 10^6$ cells/ml in plastic petri dishes at 37°C in an atmosphere of 5% $CO_2$ in air for 2 h. The nonadherent cells were then removed by gentle rinsing of the petri dishes with fresh media, and cultured at a concn of $1.5 \times 10^6$ cells per milliliter in RPMI 1640 with 20% fetal calf serum. These cells were assessed as more than 99% lymphocytes in smears stained with Wright's stain. In some experiments, the cells were cultured after dextran sedimentation alone without separation of the lymphocytes from the monocytes and polymorphonuclear leukocytes. The cells were activated with 1% Phytohemagglutinin-M (PHA-M) (Grand Island Biological Co., Grand Island, N.Y.). The cells were usually cultured in 20–30 ml of medium ($1.5 \times 10^6$ cells per milliliter) in 50-ml plastic centrifuge tubes on a rocker. The synthesis of protein, RNA, and DNA was assessed by measurement of the cellular incorporation of $^3$H-amino acids, [$^3$H]uridine, and [$^3$H]thymidine (New England Nuclear, Boston, Mass.), respectively. At the end of the culture period the cells were allowed to sediment for 30 min, and most of the supernatant medium was removed by gentle aspiration without disturbing the sedimented cells. 1 $\mu$Ci of [$^3$H]thymidine, 5 $\mu$Ci of [$^3$H]amino acids or 1 $\mu$Ci of [$^3$H]uridine in 0.1 ml of fresh medium were then added to separate cultures. After a 4-h incubation, the incorporation of $^3$H into the trichloroacetic acid (TCA)-insoluble cell fraction at 4°C was measured. The cell pellet was washed in the culture vial with successive 4-ml vol of phosphate-buffered saline (once), 5% TCA (twice), and methanol (once) and centrifuged at 2,000 rpm for 10 min. The isolated material was dissolved overnight at room temperature in 1 ml of NCS (Nuclear Chicago Solubilizer, Amersham; G. D. Searle & Co., Chicago, Ill.), 4 ml of a toluene base-containing solution (spectrofluor, Amersham; Searle) were added to each sample, and the radioactivity was measured in a liquid scintillation counter.

### *Assay for Osteoclast Activating Factor*

The bioassay for bone resorption to assess OAF in the supernatant media of activated leukocytes has been described in detail elsewhere (12, 16). Timed pregnant rats were injected subcutaneously with 0.4 $\mu$Ci of $^{45}$Ca on the 18th day of gestation. The following day the mothers were sacrificed and the radius and ulna of the fetal rat long bones were separated from the adjacent cartilaginous ends and surrounding soft tissue. These fetal rat long bones were then incubated for 24 h with BGJ medium (Grand Island Biological Co) to allow for exchange of loosely complexed $^{45}$Ca with stable calcium in the culture medium. During the following 48 h the bones were cultured with the cell culture supernates or corresponding control media. At the end of the culture period $^{45}$Ca was measured in the media and in the residual bones. Bone resorption was calculated as the percent of total radioactivity released from four individual bones over a 48-h period compared with four corresponding controls, and the results were expressed as ratios of the test to control bones. Statistical differences were analyzed by Student's $t$ test for nonpaired samples.

Before assay for bone resorption, samples of media were dialyzed exhaustively against fresh BGJ medium to decrease the colchicine concentration in the sample. This was necessary because colchicine is an inhibitor of bone resorption at concentrations greater than $10^{-8}$ M (13). All of the samples that were assayed were subjected to the same dialysis procedure.

### *Tubulin Assay*

Lymphocytes were sonicated for 15 s at 4°C with a microtip sonifier at maximal output (setting 5, Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). The sonicates were stored at −80°C until use. All samples from a given experiment were assayed together by [$^3$H]colchicine (New England Nuclear, Boston, Mass.) binding as previously described (14, 15), except that the assay volume was reduced to 30 $\mu$l to increase assay sensitivity. Under these conditions, less than 50 ng of tubulin can be measured reproducibly.

For measurements of total tubulin, cells were sonicated in TS buffer (0.25 M sucrose, 0.5 mM GTP in 10 mM phosphate pH 6.95). For measurements designed to determine the partition between soluble and microtubule tubulin, cells were sonicated in MTS buffer (50% glycerol, 5% dimethyl sulfoxide, 0.5 mM GTP, 0.5 mM $MgCl_2$, 0.5 mM ethylene glycol-bis($\beta$-aminoethyl ether)$N,N,N',N'$-tetraacetate (EGTA) in 10 mM phosphate pH 6.95), and centrifuged at 50,000 $g_{av}$ for 15 min in an air-driven ultracentrifuge (Airfuge, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) (11, 14, 15). The supernate was diluted 1:2 with TS buffer and stored at −80° until use. The pellet was resonicated in ice-cold TS, incubated for 30 min at 4°C, and then centrifuged as described. The second supernate was stored at −80°C until assay. Protein was determined by the method of Lowry et al. (8).

[$^3$H]Colchicine was purchased from New England Nuclear Corp. Colchicine was obtained from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were reagent grade.

## RESULTS

The first experiment (Table I) shows that, after 72 h in culture, the tubulin concentration of unseparated mononuclear cells decreased to less than half of the original value whereas the tubulin concentration of cells cultured in the presence of PHA did not decrease significantly and was over twice that of the unstimulated cells. To determine whether the decrease in tubulin concentration during culture of unstimulated mononuclear cells represented a decrease in the average tubulin content per cell or was a result of selective loss of cells with high tubulin concentration, adherent and nonadherent mononuclear cells were separated before culture. There was a substantial decrease in the tubulin concentration of the cells after the adherent cells (monocytes) had been removed (Table II). When the nonadherent mononuclear cells (lymphocytes) were cultured for 36 h, there was no detectable decrease in tubulin content, suggesting that the decrease observed during culture of unseparated cells was due to selective loss of monocytes or loss of monocyte tubulin. In these experiments PHA-activation increased the tubulin content of purified lymphocytes from two- to fourfold. Tubulin was increased more than the average lymphocyte protein as indicated by the 60–70% increase in tubulin relative to total cellular protein. Lymphocyte tubulin content did not rise significantly until between 24 and 36 h after stimulation with PHA. In another experiment, PHA had no apparent effect on the partition between the soluble and microtubule forms of tubulin after 36 h in culture (Table III). However, the absolute number of microtubules per cell was increased 2.5-fold, with the increase in total cellular tubulin content.

In order to investigate the possible role of the tubulin-microtubule system in PHA-stimulated lymphocyte secretion, we studied the effects of colchicine ($10^{-9}$–$10^{-5}$ M) on release of a normal lymphokine and on total protein synthesis. The lymphokine assayed was OAF. There was no significant inhibitory effect of colchicine on either OAF release or protein synthesis after 24 h of culture (Table IV). In fact, at higher concentrations, there appeared to be an increase in OAF

TABLE I

*Induction of Tubulin Synthesis by Human Peripheral Blood Mononuclear Cells after Stimulation by PHA*

| Presence of mitogen | Time in culture | Tubulin concn |
|---|---|---|
| | *h* | *% Total protein* |
| – | 0 | 0.55 ± 0.01 |
| – | 72 | 0.23 ± 0.01 |
| PHA | 72 | 0.47 ± 0.03* |

Mononuclear cells were isolated as described in Materials and Methods. After the indicated period in culture, the cells were harvested by centrifugation, sonicated in TS buffer and frozen at −80°C until assay. Values represent the mean ± SE of triplicate cultures.

* Significantly different ($P < 0.01$) from unstimulated lymphocytes.

TABLE II

*Induction of Tubulin Synthesis by Human Peripheral Blood Lymphocytes after Stimulation by PHA*

| Cells | Time in culture | Mitogen | Tubulin content | Tubulin concn |
|---|---|---|---|---|
| | *h* | | *μg/10⁷ cells* | *% Total protein* |
| Exp 1 | | | | |
| Unseparated leukocytes | 0 | – | 6.67 ± 0.035 | 0.66 ± 0.06 |
| Lymphocytes + monocytes | 0 | – | 5.38 ± 0.17 | 0.57 ± 0.02 |
| Lymphocytes | 0 | – | 1.47 ± 0.15* | 0.23 ± 0.03* |
| Lymphocytes | 36 | PHA | 4.06 ± 0.07‡ | 0.37 ± 0.02‡ |
| Lymphocytes | 36 | – | 1.24 ± 0.07 | 0.23 ± 0.01 |
| Exp 2 | | | | |
| Lymphocytes + monocytes | 0 | – | 4.45 ± 0.10 | 0.44 ± 0.02 |
| Lymphocytes | 0 | – | 1.14 ± 0.10* | 0.17 ± 0.02* |
| Lymphocytes | 36 | PHA | 2.37 ± 0.06‡ | 0.27 ± 0.01‡ |
| Lymphocytes | 36 | – | 1.11 ± 0.09 | 0.16 ± 0.01 |

Leukocyte fractions were isolated as described in Materials and Methods. Cells were harvested as described in the legend of Table I. Values represent the mean ± SE of triplicate cultures.

* Significantly different from unseparated leukocytes or lymphocytes plus monocytes ($P < 0.01$).

‡ Significantly different from unstimulated lymphocytes ($P < 0.01$).

TABLE III

*Effect of PHA on Lymphocyte Tubule-Microtubule Partition*

| Treatment | Tubulin content | Tubulin concn | Tubulin in microtubules | Assembled tubulin |
|---|---|---|---|---|
| | *μg/10⁷ cells* | *% Total protein* | *%* | *μg/10⁷ cells* |
| PHA | 6.42, 7.09 | 0.97, 0.76 | 38.8, 34.9 | 2.49, 2.47 |
| Control | 2.73, 3.00 | 0.43, 0.47 | 35.8, 31.0 | 0.98, 0.93 |

Lymphocytes were isolated as described. After 36 h in culture, cells were harvested by centrifugation, sonicated in MTS buffer and fractionated for determination of the tubulin-microtubule partition as described in Materials and Methods. Fractions were stored at −80°C until assay. Cultures were performed in duplicate and individual values are given.

release. In a parallel experiment, colchicine at $10^{-6}$ M almost completely abolished [$^3$H]thymidine incorporation into DNA in PHA-activated lymphocytes after 40 h of culture (Table V). At this time, there was also substantial inhibition by colchicine of both [$^3$H]uridine incorporation into RNA and $^3$H-amino acid incorporation into protein. Colchicine had little or no effect on any of these parameters in the absence of PHA. The effects of colchicine on protein and DNA syntheses was not significant at 72 h although the effect on RNA synthesis persisted.

TABLE IV

*Effect of Colchicine on OAF Production and $^3$H-amino acid Incorporation by PHA-Activated Human Peripheral Blood Leukocytes*

| Colchicine concn | Mitogen | $^{45}$Ca release* | $^3$H-amino acid‡ |
|---|---|---|---|
| | | *Treated/control ratios* | *cpm* |
| – | PHA | 1.65 ± 0.17 | 15,753 ± 3,060 |
| $10^{-9}$ M | PHA | 1.63 ± 0.20 | 16,265 ± 2,700 |
| $10^{-8}$ M | PHA | 1.29 ± 0.17 | 24,663 ± 1,050 |
| $10^{-7}$ M | PHA | 1.90 ± 0.13 | 11,280 ± 2,200 |
| $10^{-6}$ M | PHA | 2.37 ± 0.49 | 17,220 ± 1,150 |
| $10^{-5}$ M | PHA | 2.76 ± 0.11 | 12,439 ± 1,800 |
| – | Unstimulated | 1.13 ± 0.11 | 8,662 ± 1,500 |

Leukocytes were cultured at a concn of 1.5 × $10^6$/ml for 24 h.

* Values are expressed as means ± SEM for four to eight bone cultures. Leukocytes were cultured in BGJ medium supplemented with 10% heat-inactivated fetal calf serum.

‡ Values are expressed as means ± SEM for triplicate leukocyte cultures.

## DISCUSSION

Stimulation of lymphocytes by lectins results in a complex series of biochemical changes including increased synthesis of protein, RNA, and DNA, lymphokine release, and blastic transformation and cell division (3, 7, 10). The precise sequence of events between binding of the lectin to its cell surface receptor and the consequent intracellular changes is not yet known. However, recent evidence suggests that microtubule integrity is required for lectins to stimulate DNA synthesis and mitogenesis in lymphocytes (5, 17) although there is some disagreement about this point (2). Serum stimulation of mitogenesis in neuroblastoma cells also appears to require microtubules (1) since culture of these cells with colchicine ($10^{-6}$ M) inhibits [$^3$H]thymidine incorporation into DNA and prevents cells from leaving $G_0$ as shown by flow microfluorometric analysis.

We have investigated the effects of PHA on tubulin and microtubule content in cultured lymphocytes. PHA caused an increase in total cell tubulin content as measured by [$^3$H]colchicine binding. Although stimulated lymphocytes are actively synthesizing many proteins, the increase in tubulin was always greater than the increase in total cell protein. Presumably, the increase in tubulin concentration is a result of induction of tubulin synthesis, although we cannot rule out decreased degradation of tubulin by PHA-activated cells on the basis of the present experiments.

TABLE V

*Effect of Colchicine on the Activation of Lymphocytes*

| PHA 1% | Colchicine $10^{-6}$ M | $^3$H-Amino acid incorporation | [$^3$H]Uridine incorporation | [$^3$H]Thymidine incorporation |
|---|---|---|---|---|
| | | *cpm* | *cpm* | *cpm* |
| *24 hours* | | | | |
| + | + | 3,937 ± 137 | 14,265 ± 5,417 | 243 ± 69 |
| + | – | 3,563 ± 1,309 | 25,620 ± 2,375 | 440 ± 15 |
| – | + | 2,243 ± 339 | 4,828 ± 739 | 233 ± 24 |
| – | – | 2,855 ± 282 | 6,506 ± 490 | 340 ± 17 |
| *40 hours* | | | | |
| + | + | 8,289 ± 366 | 100,153 ± 11,140 | 1,089 ± 24 |
| + | – | 22,888 ± 856 | 255,091 ± 7,439 | 57,982 ± 1,233 |
| – | + | 2,745 ± 302 | 6,043 ± 347 | 211 ± 16 |
| – | – | 2,773 ± 327 | 8,639 ± 473 | 265 ± 9 |
| *72 hours* | | | | |
| + | + | 5,838 ± 935 | 54,061 ± 9,038 | 27,367 ± 263 |
| + | – | 6,025 ± 2,019 | 150,851 ± 4,752 | 33,637 ± 8,210 |
| – | + | 1,615 ± 210 | 2,314 ± 262 | 237 ± 33 |
| – | – | 2,067 ± 144 | 4,239 ± 269 | 294 ± 33 |

Leukocytes were cultured for periods of 24, 40, or 72 h in RPMI-1640 medium with 20% heat-inactivated fetal calf serum. Values for $^3$H-incorporation into cells were expressed as means ± SE for triplicate leukocyte cultures.

Although no change in the partition between the soluble and microtubule forms of tubulin was detected in PHA-stimulated cells, the absolute amount of microtubule mass per cell was increased more than 2.5-fold after 36 h of exposure to PHA, parallel with the increase in cellular tubulin levels (Table III). It is possible that a transient change in the equilibrium between the soluble and microtubule pools could occur during incubation with PHA and not be detected by our experiments. In any case, the substantial increase in microtubule mass occurring as a result of PHA-activation may be important in mediating some of the subsequent changes induced by PHA, including the increase in DNA synthesis. The observation by Gunther et al. (5) that colchicine inhibits stimulation of DNA synthesis, which we have confirmed, is consistent with this possibility. Thus, the disassembly of microtubules caused by colchicine may prevent mitogenesis. Conversely, an increase in microtubules may be necessary for its full expression.

In order to test whether microtubules were necessary for other lectin-stimulated events in lymphocytes, we measured the effects of colchicine on the release of a lymphokine, OAF, and the increased protein and RNA synthesis which occur early after PHA-activation (6). There was no inhibitory effect of colchicine ($10^{-9}$–$10^{-5}$ M) on OAF production. There was no inhibition of protein synthesis, although RNA synthesis was significantly inhibited (Table V). Nevertheless, RNA synthesis was still greater than that of the corresponding unstimulated control, both at 24 and at 40 h.

DNA synthesis was almost completely inhibited after 40 h of PHA-activation (Table V). However, by 72 h after exposure to PHA, DNA synthesis was no longer significantly inhibited by colchicine. This is in agreement with the results of Wang et al (17), and suggests that the effect of colchicine to disrupt microtubules was incomplete and that the residual microtubule pool was sufficient to allow DNA synthesis to occur, albeit after a delay. Alternatively, there may be a different pathway for lectin-induced DNA synthesis which does not involve microtubules and which is important at a later time in the activation sequence.

If the same population of cells which releases OAF also synthesizes DNA and undergoes mitogenesis in response to PHA, then we may conclude that the signal for OAF production (being independent of microtubules) differs from the mitogenic signal, or that the microtubule-dependent step in mitogenesis occurs at a stage after propagation of the signal from the membrane to the cell interior. This latter possibility is consistent, so far as we know, with all of the available data, including those of Wang et al. (17). These workers showed that the inhibitory effects of colchicine on DNA synthesis and blast transformation are not due to a direct effect on the mitotic spindle, since these effects were apparent before the earliest changes associated with cell division. Greene et al. (4) found that while colchicine inhibited lymphocyte DNA synthesis and $\alpha$-amino-isobutyric acid uptake in response to PHA, it had no effect on lectin binding or calcium uptake and actually increased the cyclic AMP response. Since the role of calcium and cyclic nucleotides in lymphokine release and mitogenesis is unclear, it is difficult at the present time to relate these observations to our own findings. Although these observations together with those of Wang et al. (17) and the present study indicate that microtubules play an important role in lectin-induced lymphocyte activation, the essential intracellular signals are unclear, and exactly how microtubules participate in the process is not yet known. Ongoing experiments in our laboratory are aimed at localizing the step or steps after lymphocyte activation where microtubules are required.

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*Note Added in Proof:* While this manuscript was in review, K. Resch, D. Bouillon, D. Gemsa, and R. Averdunk [1977. *Nature (Lond.).* **265**:349-351] reported that colchicine failed to inhibit the release of a different lymphokine (lymphotoxin) from concanavalin A activated lymphocytes at concentrations which significantly inhibited [$^3$H]thymidine incorporation into DNA.

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