

Cell Surface Tubulin in Leukemic Cells: Molecular Structure, Surface Binding, Turnover, Cell Cycle Expression, and Origin

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ABSTRACT We report here new characteristics of cell surface tubulin from a human leukemia cell line. These cells (CEM cells) possess tubulin that is readily iodinated on the surface of living cells, turns over at a rate identical to that of other surface proteins, and is present throughout the cell cycle. When removed with trypsin, it rapidly returns to the surface. Peptide mapping of iodinated surface tubulin indicates that it possesses a similar, but not identical, primary structure to total CEM and rat brain tubulin. Living CEM cells are able to bind specifically a subfraction of CEM tubulin from metabolically labeled high speed supernatants of lysed CEM cells. Surface tubulin is more basic than the total tubulin pool. The binding, which is saturable, is inhibited by unlabeled CEM high speed supernatants but not by excess thrice-cycled rat or bovine brain tubulin. Surface tubulin is also shown to bind to living nontransformed normal rat kidney cells but not to normal, circulating, mononuclear white cells. Activated lymphocytes produce a tubulin that binds to CEM cells. Since CEM tubulin was detected in the media of 6-h cultures of CEM cells, we must conclude that at least some of the surface tubulin comes from the media. We further conclude that these leukemic cells produce an unusual tubulin that may bind specifically to any membrane. The presence of iodinated surface tubulin, however, appears to require both the production of a unique tubulin and the presence of a "receptor-like" surface binding component.

Over the past ten years, numerous authors have suggested that tubulin can exist as a membrane protein. Tubulin has been reported to be associated with surface membranes of nerves (5, 13–15, 39), flagellar surface membranes (1), mammalian thyroid tissue total membrane (4), transformed human lymphoid cells (2), platelet plasma membrane (32), intracellular membranes (17, 31, 35), and even artificial liposomes (7). Several of these studies are based on the co-purification of tubulin with a given membrane preparation and thus contamination from intracellular tubulin pools has been difficult to eliminate. To avoid this potential artifact, surface labeling of living cells has been used as a method for identifying tubulin on the plasma membrane (2, 28). The possibility of artifactual labeling of cytoplasmic tubulin has been shown to be remote (28).

Unresolved aspects of membrane tubulin research concern the chemical nature and function of membrane tubulin. It has been reported to be either insoluble in some nonionic detergents such as Triton X-100 (2, 39) or differentially

soluble in agents such as Nonidet P-40 (13). Other reports demonstrate the presence of enzymatically added tyrosine on tubulin believed to be associated with membrane systems (23). In studies by Stephens (33, 34), a systematic protein analysis of membrane versus nonmembrane tubulin shows that ciliary membrane tubulin is different from axonemal tubulin and that the association of ciliary tubulin to the membrane is highly specific.

We have previously reported that cells from a human lymphoblast cell line established from the peripheral blood of a leukemic patient (CCRF-CEM, henceforth referred to as CEM) can be externally labeled with ^{125}I using three different iodination methods. In these cells, tubulin is one of the most heavily labeled components; whereas normal, intact, mononuclear white cells from human peripheral blood and bone marrow do not possess iodinated tubulin (24, 28, 29). These labeling procedures do not label soluble, intracellular proteins. The labeled tubulin on the living cell surface can be easily removed by mild proteolytic treatment. In addition, we have

shown by the same methods that some circulating leukemia cell types in patients also possess surface tubulin. These cells, along with our CEM line, undergo cytolysis after exposure to very low concentrations of anti-tubulin drugs (18). In the present study, we present new evidence on the origin, structure, turnover, and surface binding properties of surface tubulin in CEM cells.

MATERIALS AND METHODS

Cell Culture: Log phase cultures of lymphoblasts of the CEM cell line (T-cell origin) were propagated in Eagle's minimal essential medium supplemented with 10% fetal calf serum and the antibiotics penicillin and streptomycin (11, 12). The total mononuclear white cell population from normal volunteers was obtained by ficol centrifugation and was activated with phytohemagglutinin and 10% T-cell growth factor for 4 d before labeling with [³⁵S]-methionine.

Iodination: Cells were washed in Hanks' balanced salt solution (HBSS)¹ and recovered by centrifugation at 100 g for 10 min. Cell pellets were resuspended in 1 ml HBSS and iodinated with 200 μCi ¹²⁵I catalyzed by lactoperoxidase (LPO) in the presence of glucose and glucose oxidase for 15 min at room temperature (16). The reaction was stopped by washing the cells three times in large volumes of HBSS containing phenylmethylsulfonyl fluoride. Membrane permeability of the cells was monitored before and after any iodination procedure by Trypan blue dye exclusion.

Electrophoresis: One-dimensional polyacrylamide gel electrophoresis (1-D PAGE) was performed according to the method of Laemmli (21). CEM cells were solubilized and proteins separated by two-dimensional (2-D) PAGE as described elsewhere (27, 28). The gels were stained with 0.1% Coomassie Blue-R250 in 25% isopropanol and 10% acetic acid and destained in a solution of 25% methanol and 10% acetic acid. 2-D gels from iodinated cell samples were dried on filter paper and autoradiographed using Kodak X-Omat XAR film.

Immunoblot and Immunolocalization: Protein electroblotting and immunolocalization were performed according to the Bio-Rad Immunoblot Assay Kit (Bio-Rad Laboratories, Richmond, CA). Briefly, after completion of 2-D PAGE, duplicate slab gels of CEM whole cell homogenate were electrophoretically transferred overnight at 4°C to nitrocellulose paper. One of the nitrocellulose sheets was stained with 0.1% Amido black in 45% methanol, 10% acetic acid to visualize the whole cell staining pattern. The other sheet was washed in a 0.05% Tween-20 solution and then put in a blocking solution of 3% gelatin in Tris-buffered saline. The primary antibody used was rat anti-alpha tubulin (Clone Y1 1/2, Sera Lab) at 15 μg/ml. This antibody is specific to tyrosinated tubulin only. The secondary antibody was rabbit anti-rat IgG horseradish peroxidase conjugate (Accurate Antibodies, Westbury, NY) in a 1:100 dilution. The antibodies were localized by the addition of 60 mg horseradish peroxidase color development reagent in 100 ml Tris-buffered saline with 60 μl of ice cold 30% hydrogen peroxide.

Peptide Mapping: Presumptive CEM cell tubulin was compared to twice-cycled bovine brain tubulin prepared by the method of Shelanski et al. (30). CEM whole cell homogenate and the purified brain tubulin were each run independently by 2-D PAGE. The alpha and beta subunits of tubulin were cut out of the stained gels and run on a second 15% 1-D slab gel. Peptide fragments were produced by proteolytic digestion with *Staphylococcus aureus* V8 protease by the procedure of Cleveland et al. (9). Peptide maps were also used to compare CEM cytoplasmic tubulin with cell surface tubulin. Intact, viable cells were surface labeled by a routine iodination method. A high speed supernatant was prepared from a second group of cells by sonication on ice for 15 s followed by centrifugation at 100,000 g for 1 h. Soluble proteins were iodinated, dialyzed overnight against 0.01 M ammonium acetate, pH 7.2, and lyophilized. Solubilized samples from the two preparations were run on 2-D PAGE and the tubulin subunits were cut out and proteolytically digested as described above. The resulting peptide map was fluorographed using PPO (6) and exposed on Kodak XAR film to detect weak, secondary beta-like emissions from ¹²⁵I which represent 93% of the total emissions of this isotope. Further enhancement was obtained by using rare earth screens (36).

Trypsinization: Twice-washed (HBSS) CEM cells were iodinated by the routine LPO catalyzed method and divided into equal aliquots, to which increasing concentrations of trypsin were added. After a 5-min incubation at 37°C, the cells were cooled to 0°C, and soybean trypsin inhibitor was added at twice the trypsin concentration. The cells were washed twice in large volumes

of HBSS and solubilized for 2-D PAGE. The stained gels were autoradiographed using Kodak XAR film and rare earth screens.

Tubulin Turnover Experiments: CEM cells were washed twice in HBSS and trypsinized (7.5 μg/ml) for 10 min at 37°C. The reaction was stopped with soybean trypsin inhibitor and the washed cells were reincubated in roller bottles with fresh complete media. Aliquots of the culture were withdrawn at 6-h intervals over a 24-h period. The aliquots were iodinated, run on 2-D gels, and autoradiographed. The reverse experiment was performed by iodinating a large number of cells and returning them to culture for 24 h. Aliquots were withdrawn at 6-h intervals, washed free of media and unbound radioactivity, run on 2-D gels, and autoradiographed.

Cell Synchronization by Elutriation: Log phase L-1210 mouse leukemic cells were separated into synchronized subpopulations by centrifugal elutriation by methods which have been recently reported in detail (20). Aliquots of the different cycle phases were stained with propidium iodide and analyzed with flow cytometry for homogeneity of each fraction (19). Each fraction was radioiodinated, as described above, and radioautographed.

Specific Binding of Tubulin: CEM cells were metabolically labeled by incubating 10 ml (0.7 × 10⁸ cells/ml) in low methionine medium with 10% dialyzed fetal calf serum using 200 μCi [³⁵S]methionine for 20 h. Cells were washed twice in HBSS, resuspended in 0.8 ml HBSS containing 1 mM phenylmethylsulfonyl fluoride and 0.1 mM methionine, sonicated, and centrifuged at 100,000 g for 1 h at 4°C. An unlabeled batch of CEM cells was then exposed to the labeled high speed supernatant for 15 min at room temperature. The cells were washed exhaustively (eight times) until counts in the washes were equivalent to the background. Samples of the solubilized cells, as well as of the [³⁵S]methionine-labeled proteins of the soluble supernatant, were run on 2-D gels. The stained gels were fluorographed, dried on filter paper, and exposed on Kodak XAR film at -80°C.

The procedure just described for metabolic labeling and preparation of a labeled high speed supernatant was used in a series of competitive binding studies. CEM cells were preincubated with saturating concentrations of twice-cycled brain tubulin or unlabeled high speed supernatant from CEM whole cell homogenates before the addition of ³⁵S-labeled high speed supernatant. After five washes in HBSS, a sample of the cell pellet was counted using Aquasol (New England Nuclear, Boston, MA) and a Beckman LS 2800 scintillation counter. A more rapid method was also used. This involved spinning the cells once through 500 μl of dibutyl phthalate in a 0.8-ml microfuge tube on a Beckman microfuge (Beckman Instruments, Inc., Palo Alto, CA). Saturation curves for CEM tubulin binding were determined both at room temperature and at 4°C. A mixing experiment was done using CEM cells and normal rat kidney (NRK) cells in which metabolically labeled high speed supernatants from these two cell lines was incubated with viable cells from each of the cell types. Tubulin binding was compared by 2-D PAGE analysis and direct scintillation counts.

2-D PAGE Quantitation: 2-D gel radiofluorographs were quantitated by the method of Mariash et al. (22). The program was essentially the same as described and kindly provided by Dr. Mariash. No effort was made to obtain absolute DPM values and thus the data is expressed as a percentage of the total radioactivity in arbitrary units.

RESULTS

Protein Identification

We found that spots which co-migrate with purified brain tubulin contain label when living cells are radioiodinated by one of several surface iodination methods. As previously shown (28), tubulin is the most prominently labeled surface component (Fig. 1A). This remains the case even when the labeling is done in the cold (Fig. 1A, lower panel). The small amount of apparent actin labeling is lost when the iodination is done in the cold. The two major iodinated polypeptides observed after surface labeling of intact CEM cells were initially identified as tubulin by co-migration with twice-cycled brain tubulin in 2-D PAGE. The presumptive CEM tubulin was further verified as being composed of alpha- and beta-tubulin subunits by immunoblotting (Fig. 1B) and by the similarity in peptide maps from twice-cycled brain tubulin subunits cut from stained 2-D gels (Fig. 2). However, there are some differences in the peptide fragments seen in the higher molecular weight region of the alpha subunit map.

¹ Abbreviations used in this paper: HBSS, Hanks' balanced salt solution; LPO, lactoperoxidase; NRK, normal rat kidney.

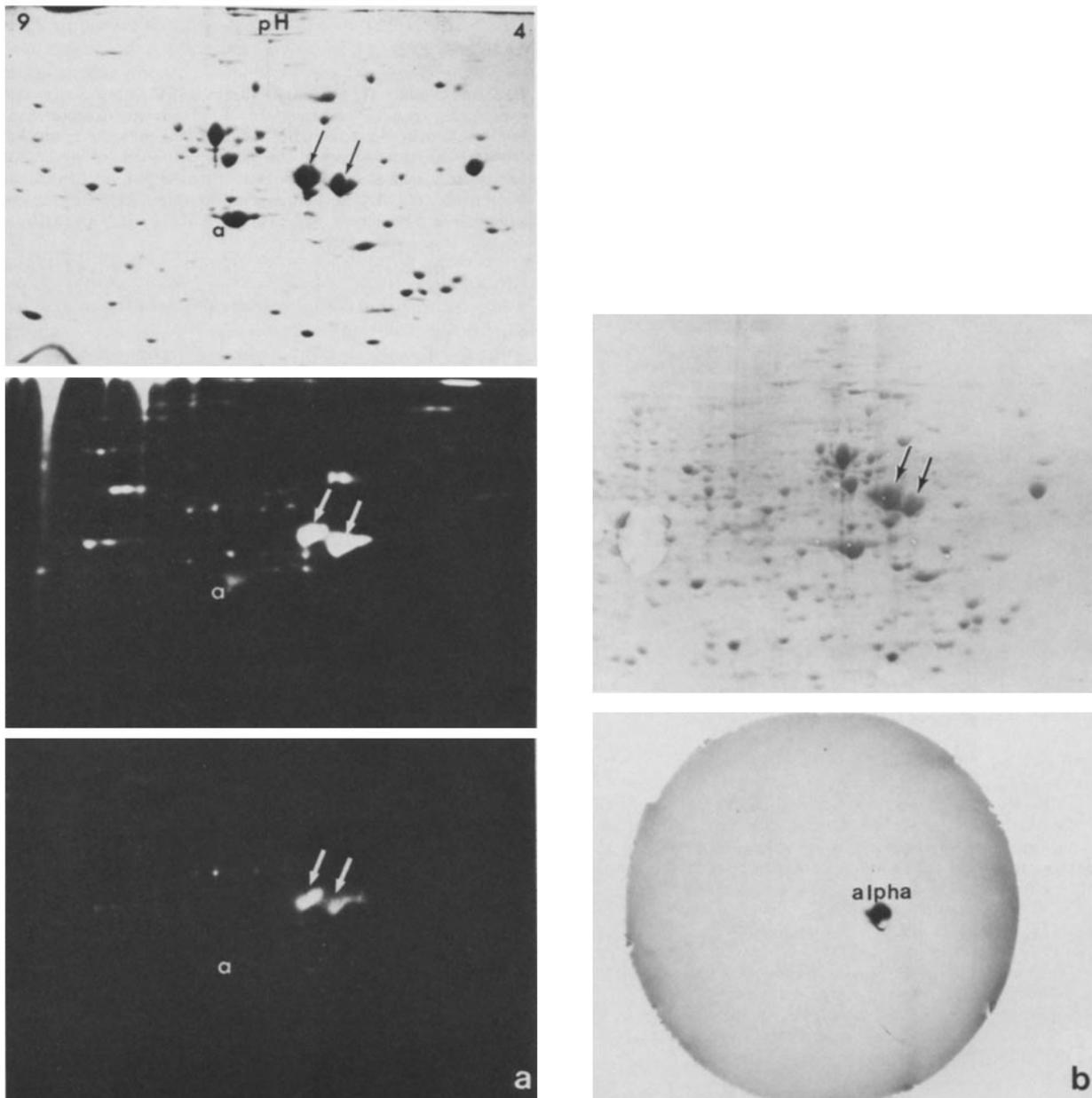


FIGURE 1 (a) Surface radioiodination of CEM cells. Living CEM cells were washed in HBSS and radioiodinated by the LPO method. The cells were then solubilized and run on 2-D gels. The upper panel shows the staining pattern from such a gel. The resulting gel was then dried for autoradiography (*middle panel*). Note that tubulin (arrows) is the most prominently labeled component. The lower panel is an autoradiograph of a replicate experiment in which the iodination was carried out at 4°C to eliminate endocytosis. Note that tubulin is even more prominent relative to the other spots. a, actin. (b) Alpha-tubulin immunoblot. Alpha-tubulin from 2-D gels of CEM cells was further identified by immunoblot. 2-D gels of CEM cells were transferred to nitrocellulose papers. One was stained with Amido black (*top panel*) and the other was incubated with anti-alpha-tubulin (Accurate Antibodies Co.). This was visualized using horseradish peroxidase staining (*lower panel*). The protein labeled by the anti-alpha tubulin antibody in the lower panel corresponded to the spot indicated by the left arrow in the upper panel.

This is probably not surface tubulin but does demonstrate that the spot we find co-migrating with alpha-tubulin does contain tubulin which is not highly contaminated by nontubulin proteins. Interestingly, the Western blot indicates that the antigen represents only part of the alpha spot pointing to the possibility that other isomers of alpha-tubulin may be present in this region.

To further establish the identity of the presumed surface tubulin and to learn something about its structure, peptide maps of the surface tubulin (defined by iodination in living cells) were also made and compared with those from intracel-

lular, soluble CEM tubulin taken from 2-D gels. It would appear, based on the similarity of peptide fragments observed (see Fig. 3), that surface tubulin and intracellular, soluble tubulin are very similar in primary structure and that spot contamination by nontubulin proteins on our 2-D gels is minimal.

Surface Tubulin Turnover Studies

Having demonstrated the apparent presence of a specific tubulin on the surface of these cells, studies were done on the

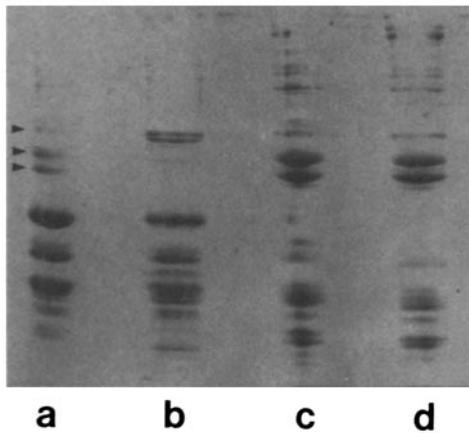


FIGURE 2 Total tubulin peptide maps: Coomassie Blue staining of *S. aureus* protease peptide maps compares alpha and beta subunits of tubulin from twice-cycled bovine brain and from CEM whole cell homogenate. (a) Bovine brain alpha; (b) CEM alpha; (c) bovine brain beta; (d) CEM beta. Note homology of the peptide fragments produced in the alpha subunits (a and b) and in the beta subunits (c and d) from the two sources. Arrows denote clear differences in alpha subunits.

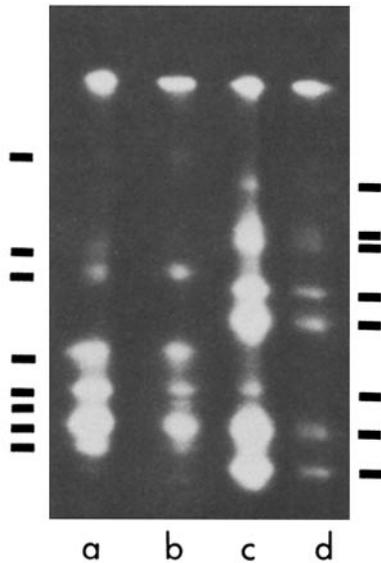


FIGURE 3 Surface tubulin peptide maps: autoradiograph of peptide map from soluble intracellular tubulin versus surface membrane tubulin. The protocol is given in the Materials and Methods section. Labeled peptide fragments produced are from (a) intracellular soluble alpha-tubulin, (b) surface-labeled alpha-tubulin, (c) intracellular soluble beta-tubulin, and (d) surface-labeled beta-tubulin. No prominent differences can be detected in the labeling of the intracellular versus surface tubulin (compare a with b and c with d). Dashes in the margin indicate areas of homology seen clearly in the original autoradiographs.

properties of this membrane tubulin and its possible binding site. To do so, it was necessary to find a way to perturb this site specifically or at least remove the surface tubulin with as little cellular damage as possible. Previous experiments have shown that trypsin could be used to remove surface tubulin from living cells (28). By decreasing the trypsin concentration and the time of trypsinization, we found that the surface tubulin could be removed without appreciably affecting the viability or growth rate of the cells.

In our previous studies using 2-D gels to analyze surface

iodination of live cells, we noted the predominance of moderate and high molecular weight, multicharged species which show a characteristic even spacing of spots which gradually increase in molecular weight toward the acid end of the gels. Whenever such cells are treated with neuraminidase, these multicharged families move toward the basic end of the gel or become one spot at the position of the most basic component (8, 10). This is interpreted to mean that these multicharged families of surface proteins are sialoglycoproteins. Along with tubulin, these glycoproteins are a prominent feature on the surface of CEM cells and are readily removed along with tubulin by our gentle trypsinization procedure (see Fig. 4).

To study surface tubulin turnover, CEM cells were trypsinized under conditions that remove all detectable label from the tubulin spots on 2-D gel radiofluorograms and then reincubated in normal media. Aliquots were removed for iodination every few hours. The data suggest that when surface tubulin is experimentally removed, it reappears to near-normal levels within 12 h (Fig. 4; Table I). Membrane tubulin turnover was also studied with cultures that were not trypsinized. Cells were radioiodinated, washed, re-incubated, and sampled periodically over 24 h for 2-D PAGE. The results (Fig. 5) are more ambiguous in that the iodination process inhibits growth although no reduction in Trypan blue excluding cells was observed. Even in the absence of any detectable cell doubling, there is considerable reduction of labeled tubulin when expressed as cpm/cell. This must be due to degradation or loss to the media. However, when pre-iodinated, it is clear that the cells do not replace the surface tubulin at the same rate as when trypsinized.

Expression during the Cell Cycle

Since drug cytotoxicity has frequently been shown to be cell cycle and proliferation dependent (37), it was of interest to examine the expression of surface tubulin during the cell cycle. Murine leukemic L-1210 cells which possess surface tubulin can be synchronized readily by elutriation. L-1210 cells were separated by centrifugal elutriation and subpopulations containing predominantly G₁, S, and G₂-M cells iodinated with LPO. The results (not shown) demonstrate the presence of label over the tubulin spots throughout the cell cycle in apparently equal amounts.

Origin of CEM Surface Tubulin

Experiments were designed to investigate the origin of surface tubulin in CEM cells. There are only two logical sources for this tubulin. It could originate from within the cell and be actively inserted through (or into) the plasma membrane by some normal biosynthetic process. Alternatively, it could come from the media and be adsorbed to, or specifically bound onto, the surface membrane. A combination of both alternatives is also possible. To distinguish between these two hypotheses, unlabeled CEM cells were added to a solution of metabolically labeled CEM proteins (Fig. 6). Analysis of the high speed supernatant (top panel) shows that this fraction is highly heterogeneous containing, as expected, many hundreds of proteins with many constituents exhibiting the same or similar isoelectric point as tubulin (Fig. 6). The results also clearly show that cold CEM cells act as a live tubulin affinity matrix (Fig. 6). The cells selectively remove only labeled tubulin from the high speed supernatant, which contains the

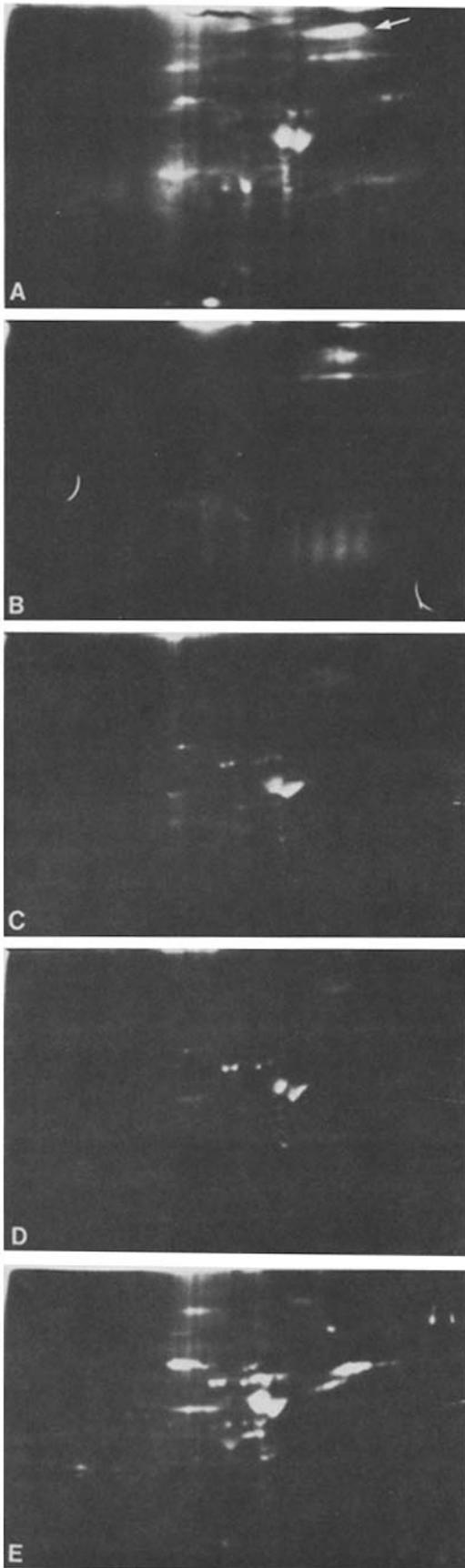


FIGURE 4 Replacement of surface tubulin after trypsin treatment. CEM cells were trypsinized (7.5 $\mu\text{g/ml}$, 37°C, 5 min) and put back into roller bottle culture. Equivalent aliquots were withdrawn at

TABLE I. Tubulin Turnover Rate in Trypsinized CEM Cells

	Experiment 1		Experiment 2	
	Alpha	Beta	Alpha	Beta
	%	%	%	%
Control	100	100	100	100
Time 9	0.77	1.6	0.41	0.49
1 h			15.2	15.3
2 h	28.3	46.1		
3 h			20.5	26.5
6 h	34.8	68.1	24.1	33.2
12 h			83.2	26.5
24 h	211	150		

CEM cells were trypsinized (7.5 $\mu\text{g/ml}$, 5 min), washed in HBSS, and put back into culture with fresh media. Equivalent aliquots were withdrawn at various time intervals and iodinated. Labeled alpha and beta spots were cut from the gels and counted directly in a gamma counter. After subtraction of the gel background counts, the cpm have been expressed as a percentage of the control sample which was not trypsinized. Time 0 represents an aliquot of cells that were trypsinized immediately after iodination, indicating the effectiveness of the trypsin in removing labeled tubulin from the cell surface.

total soluble protein fraction of these cells. This bound, labeled tubulin contains fewer nontubulin spots than any thrice-cycled brain tubulin preparation we have ever analyzed. Beta-tubulin, which has 19 methionine residues, is always more heavily labeled than alpha-tubulin, which has only 11.

Approaches to Finding the Origin of Surface Tubulin

We then looked for the presence of tubulin in CEM culture media using metabolically labeled CEM cells incubated in serum-free media. The results indicate that many proteins, including tubulin, are present in the medium within a few hours after the inoculation of a fresh culture. Whether the tubulin is actively secreted by the cells or is released by damage to the cells in culture has not been determined. An attempt was also made to sequester any tubulin which might be released into the media of a growing cell culture. Excess monoclonal antibody to tubulin (shown to bind to CEM tubulin, Fig. 1B) or lactoperoxidase covalently bound to sepharose beads, which selectively binds soluble tubulin (25, 26), was added to the growing culture. These treatments did not prevent the re-expression of surface tubulin 6 h after the removal by trypsinization. Since the efficiency of sequestration of tubulin in the media was not determined, these negative results were not conclusive. Attempts were also made to inhibit the re-expression of surface tubulin by inhibiting protein synthesis. Cyclohexamide at levels that inhibited >97% of the incorporation of methionine into the total trichloroacetic acid-pelletable fraction was used. The use of cyclohexamide did not affect the percentage of non-dye-excluding cells in these experiments (<3%). Cells were pre-treated in the drug for 30 min, then trypsinized to remove surface tubulin, and placed back into culture in the presence of the drug. At periodic intervals, a portion of the culture was sampled and iodinated for 2-D PAGE and radiofluorography. No inhibition of the re-expression of surface tubulin could be obtained (not shown).

various times over 24 h and iodinated. (A) Control—not trypsinized; (B) at time 0; (C) at 2 h; (D) at 6 h; and (E) at 24 h posttrypsinization treatment. Table I summarizes data from two such experiments.

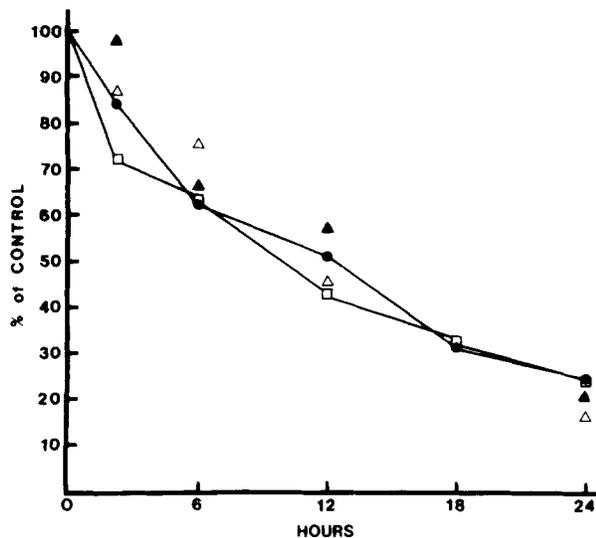


FIGURE 5 Loss of surface tubulin in culture: CEM cells were labeled with ^{125}I and then placed back in roller bottle culture for 24 h. Equivalent aliquots were withdrawn at 6-h intervals, washed free of media and unbound counts, and run on 2-D gels. The labeled alpha and beta subunits, along with two glycoproteins, were cut out of the gels and counted directly in a gamma counter. The CPM from each of the proteins is expressed as a percentage of the initial counts in the control sample measured at time 0. The apparent loss of membrane-labeled tubulin over 24 h seems to parallel the turnover of two other randomly selected membrane glycoproteins. ●, alpha-tubulin; ■, beta-tubulin; △ and ▲, two glycoproteins.

Membrane-Tubulin Interaction

Since CEM tubulin in high speed supernatants bound so specifically to live CEM cells (>95% of the total radioactivity on 2-D gels of CEM cells exposed to these supernatants is tubulin), it was possible to study the binding of CEM tubulin to live cells by simply counting the total radioactivity in a washed cell pellet exposed to a labeled high speed supernatant (Fig. 7). It is apparent that tubulin binds with a low affinity that is not inhibited by preincubation with a great excess of unlabeled thrice-cycled bovine tubulin. Scatchard analysis of this data does not produce a linear plot, suggesting the binding phenomenon is complicated and may represent more than one interaction. Repeating these experiments in the cold produced identical results except that saturation was achieved at 200 $\mu\text{g}/\text{ml}$ total protein rather than 800. Unlabeled high speed supernatants, however, did compete for labeled CEM tubulin binding (Fig. 8). This competition was not complete, with some tubulin apparently binding even in the presence of 10-fold excess of cold CEM tubulin.

The failure to inhibit this binding with unlabeled brain tubulin was surprising and led us to suspect that there might be something unique about CEM surface tubulin. Therefore, samples from these binding experiments were run on 2-D gels at high protein loading levels to try to observe any heterogeneity in the isoelectric focusing or molecular weight characteristics of the bound tubulin. The results of this analysis suggested that the alpha subunit of CEM membrane-binding tubulin is indeed different from the total CEM alpha-tubulin (Fig. 9). It is apparent that the staining and fluorographic patterns of the alpha spot are different, with the bound tubulin occupying the basic side of the total alpha tubulin spot.

We have further characterized the specificity of the CEM

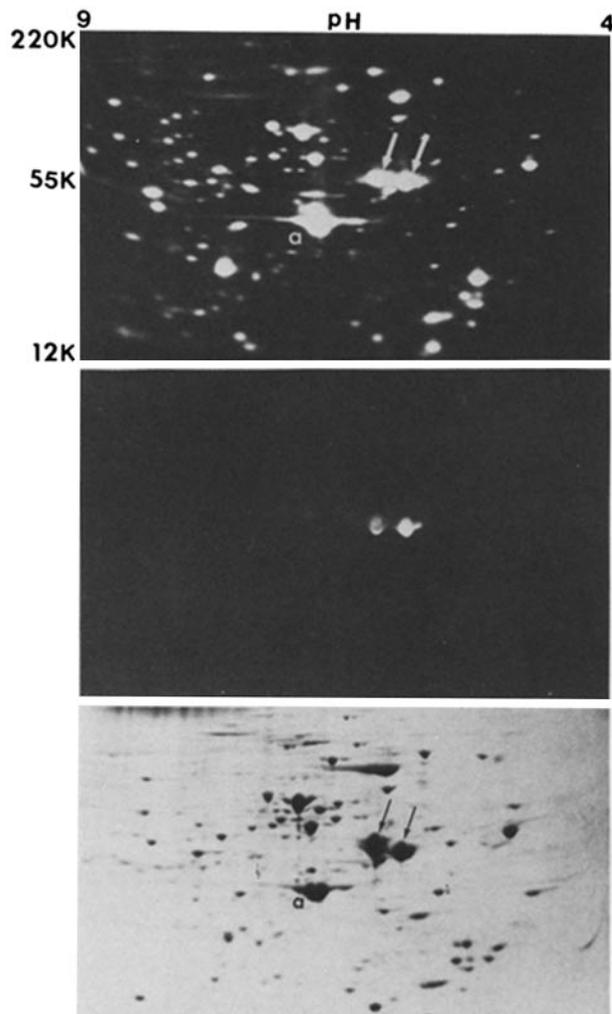


FIGURE 6 Affinity of CEM plasma membrane for CEM tubulin: CEM cells were incubated with ^{35}S methionine for one generation before homogenization. A high speed supernatant of the metabolically labeled proteins was prepared and the upper panel shows the fluorograph of the 2-D gel from the labeled soluble intracellular proteins. Unlabeled living CEM cells were placed in this labeled supernatant for 10 min at room temperature and then washed exhaustively and solubilized for 2-D gel analysis. Tubulin is essentially the only labeled component adherent to the CEM cells, as seen in the fluorograph (middle panel). The staining pattern of the fluorograph is shown in the lower panel. a, actin. Arrows indicate alpha- and beta-tubulin.

tubulin-membrane interaction by incubating CEM cells in metabolically labeled high speed supernatants from NRK cells and doing the reciprocal experiment with labeled CEM high speed supernatants. The results show that the isoelectric variant of CEM tubulin does bind (as do some other proteins) to live NRK cells, but NRK tubulin does not appear to bind to CEM cells with any specificity (Fig. 10). These experiments have been repeated using isolated circulating mononuclear white cells from normal individuals and the results were the same (no CEM tubulin binds to the white cells). The reverse experiment to determine whether circulating white cells possess tubulin that will specifically bind to CEM cell surfaces was not possible as we could not obtain sufficient methionine incorporation into the quiescent white cells. However, we were able to label these cells after mitogenic activation. These

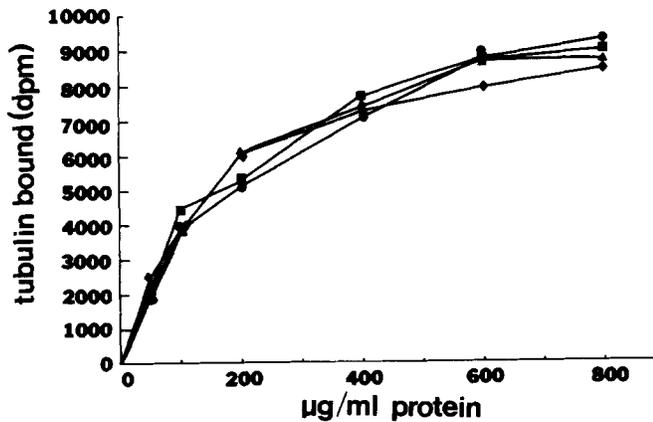


FIGURE 7 Tubulin binding curve. Increasing concentrations of ^{35}S -labeled high speed supernatant were incubated with CEM cells. CEM tubulin denoted only by diamond and square. In lines labeled with a triangle and circle, purified brain tubulin (at 1 mg/ml) was incubated with the cells 5 min before and during the addition of the labeled high speed whole cell supernatant. Each experiment is shown in duplicate. The volumes and the concentrations of the high speed supernatant total protein were kept equal for both treatment groups. Washing was done using the dibutyl phthalate method. Similar results were obtained using HBSS washing. Exogenous bovine brain tubulin therefore is not a competitive inhibitor of CEM tubulin binding.

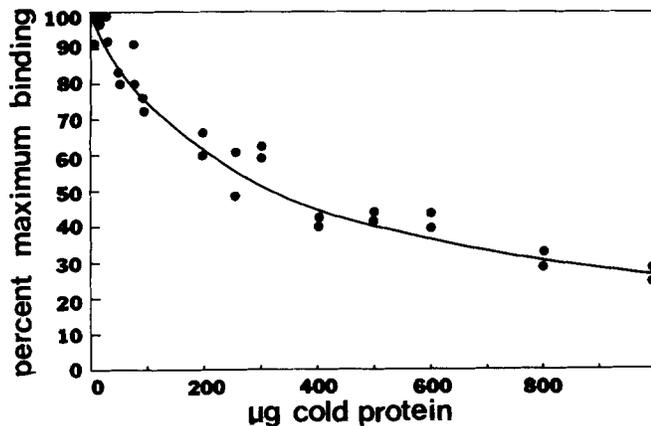


FIGURE 8 CEM tubulin competition. CEM cells were incubated with increasing amounts of whole cell, high speed supernatant 5 min before and during the addition of ^{35}S -labeled whole cell, high speed supernatant at saturation concentration. Points on the graph are the average of two experiments showing the competitive inhibition of binding of labeled CEM tubulin by unlabeled CEM tubulin. Washing was done using the dibutyl phthalate method.

cells did bind some CEM tubulin and CEM cells did bind some tubulin from high speed metabolically labeled supernatants made from the activated white cells. The binding in both cases was much less specific than the binding of CEM tubulin to CEM cells but there was clearly a great enhancement of the tubulin spots over that seen in the high speed supernatant of the white cells (Fig. 11).

DISCUSSION

Evidence is accumulating to support the view that subclasses of many cytoskeletal proteins associated specifically with various membrane systems (1-5, 7, 13-15, 17, 28-35, 38, 39). These may represent isoproteins coded by different genes or,

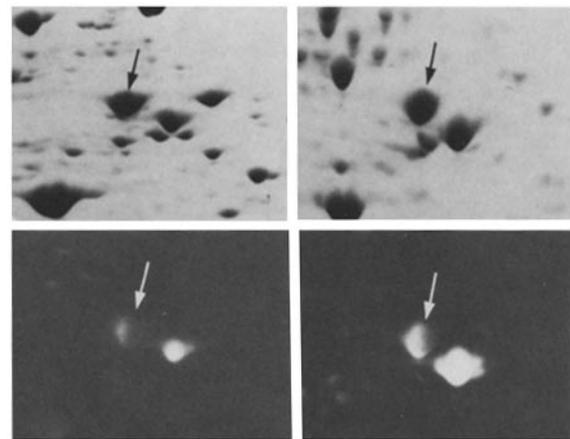


FIGURE 9 Surface tubulin is a more basic isomer of the total tubulin pool. Upper panels show Coomassie Blue staining of tubulin region of two replicate 2-D gels of CEM cells. Autoradiographs (lower panels) show the selective adsorption of alpha- and beta-tubulin from a radiolabeled CEM whole cell high speed supernatant. Arrows indicate alpha-tubulin spot. Note only the basic half of alpha spot covered by radioactivity.

alternatively, by posttranslational modification of normal proteins. In the present report, evidence is provided that in at least one cell type tubulin can exist in or on the outer surface of living cells in culture. We conclude that this tubulin is structurally similar (although clearly an isoelectric variant) to soluble intracellular tubulin and is fully exposed on the outer surface. This is based on the peptide mapping data (Figs. 2 and 3). At the very least, surface tubulin has the same number of exposed tyrosines as the soluble dimer and is released more readily by proteases than other membrane proteins.

In addition, the turnover data support the view that surface tubulin is rapidly replaced by new tubulin every 6 to 12 h (see Figs. 4 and 5 and Table I). This result may not represent classical turnover, since the ultimate fate of the surface tubulin is unknown. At least some of this tubulin originates in the media, probably from lysed cells since CEM tubulin binds so specifically to living cells and CEM tubulin is found in the media. We are unable to determine if any surface tubulin originated from a normal synthetic pathway via insertion of stores of tubulin into the membrane. The experiments in which the cells were iodinated and then returned to culture show that the surface tubulin is not simply internalized and put back into the intracellular tubulin pool. Fluorographs of these whole cell homogenates would detect the internalized tubulin, although these results do not preclude the possibility of internalization followed by immediate degradation.

Lastly, the results of this study also support contention that CEM cells possess a specific CEM tubulin "receptor" on the cell surface. It is important to distinguish this from classical designations of receptors in the pharmacological sense. By "receptor" we mean only a component(s) on the surface that selectively binds or adsorbs soluble CEM tubulin. Competitive inhibition of binding by CEM high speed supernatants, but not brain tubulin, suggests that what causes these leukemia cells to possess surface tubulin is that they produce an unusual tubulin isomer that has a specific membrane receptor. We cannot be sure that this competitive inhibition was caused exclusively, or even partially, by CEM tubulin. The fact that membrane tubulin is the only protein from high speed supernatants that binds to the live cells does not preclude other

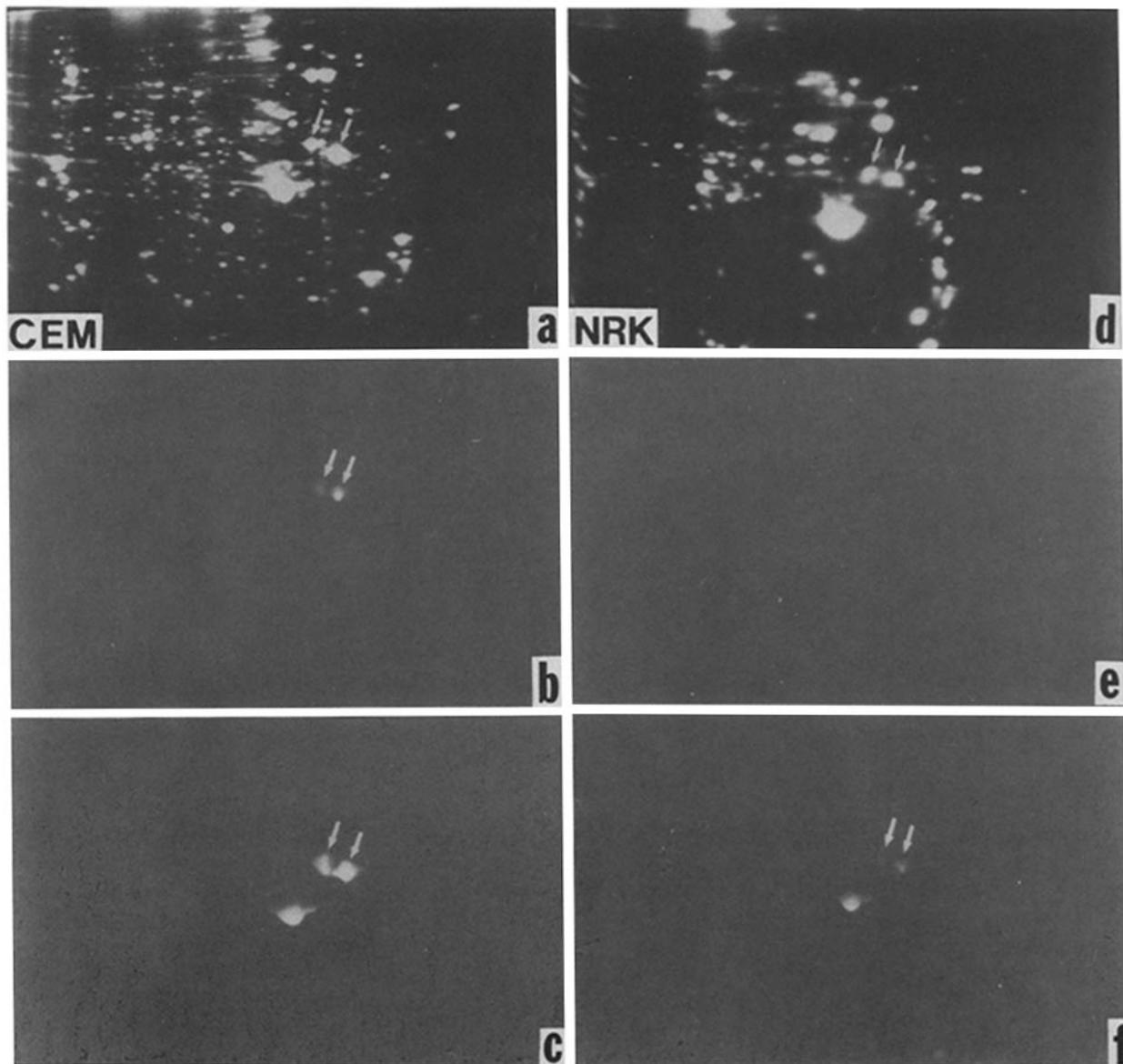


FIGURE 10 CEM and NRK tubulin binding to living CEM and NRK cells. (a) Fluorograph of a [35 S]methionine-labeled CEM lysate high speed supernatant. CEM (b) and NRK (c) cells were incubated in this labeled supernatant for 5 min, washed extensively, and solubilized for 2-D PAGE fluorography. Note that CEM tubulin (arrows) binds specifically only to CEM cells but does bind nonspecifically to NRK cells as does actin. (d) Fluorograph of a [35 S]methionine-labeled NRK cell lysate high speed supernatant. CEM (e) and NRK (f) cells were incubated in the labeled NRK high speed supernatant as described above. Note the absence of NRK tubulin binding to CEM cells. Some binding of both NRK tubulin and actin to NRK cells is apparent after long exposure.

components from inhibiting the binding. Since the CEM tubulin competition was not 100%, it may be that there is also some adsorption of CEM tubulin to the outer surface that does not require a specific CEM tubulin "receptor." Some of this residual, noncompetitively bound material may simply be methionine within the cellular pool. That liposomes alone can specifically bind soluble tubulin *in vitro* (7) emphasizes the vague nature of this definition. It is also of interest that one fraction of CEM tubulin can also bind to the unrelated "normal" NRK cells. Another possibility is that [35 S]methionine in the metabolically labeled high speed supernatant becomes incorporated into the intracellular amino acid pool and is not removed by brief exposure to the unlabeled high speed supernatant. Preliminary experiments indicate that, depending on the care taken in removing cell pellets from the

quick wash tube, 5 to 15% of the counts remaining with the cell pellet are trichloroacetic acid soluble. The adsorption of some tubulin from mitogen-activated white cells to CEM cells and the binding of some CEM tubulin to these white cells (but not unactivated primary lymphocytes) suggests that mitogen activation of the cell cycle induces the appearance of some tubulin receptors on the surface and perhaps the synthesis of some membrane tubulin. This is not surprising in light of the report that lymphocyte activation induces the new appearance of iodinated surface tubulin (2). CEM cells may then represent an extreme example of this expression.

The nature of the surface tubulin-binding component and the reason for its presence remains unknown. Methods to quantitate the amount of surface tubulin and the number of available tubulin binding sites using tubulin antibodies, and

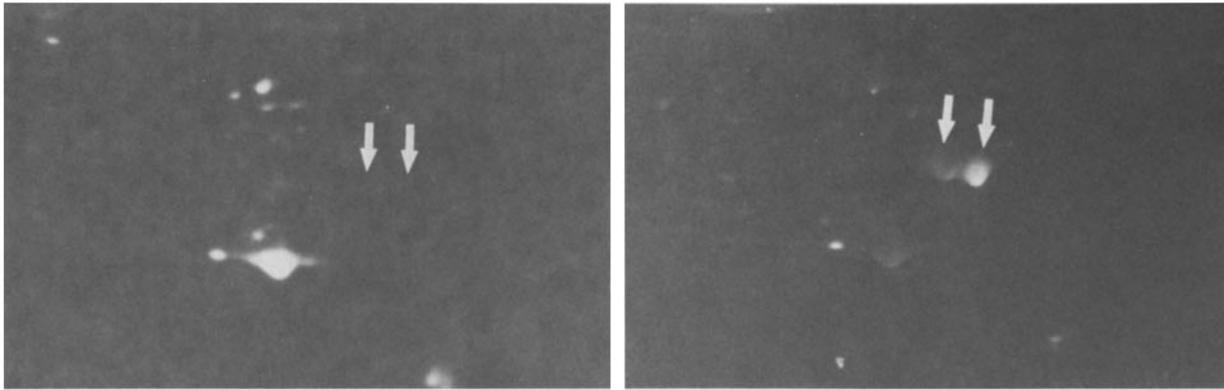


FIGURE 11 Mononuclear white cell tubulin binding to CEM cells. A similar experiment to that shown in Fig. 10 was undertaken with circulating mononuclear white cells. No CEM tubulin or any other protein was bound to these cells (not shown). These cells do not take up methionine. Therefore they were activated with phytohemagglutinin and T-cell growth factor before labeling. (Left) Autoradiograph of the high speed supernatant from the labeled, activated lymphocytes. Note the almost complete absence of tubulin. (Right) Autoradiograph of the lymphocyte proteins bound to living CEM cells. Note that tubulin, among other proteins, is present. Arrows denote alpha- and beta-tubulin (right) and its absence (left).

iodinated tubulin, need to be established. This will be difficult considering that the tubulin that binds to CEM surfaces appears to be a different protein (more basic) than the bulk of CEM intracellular tubulin or purified brain tubulin. In another report (2), immunolocalization of surface tubulin on cultured human leukemic cells was observed, although only qualitatively. We have also attempted to demonstrate CEM affinity binding to colchicine beads as has been reported for avian erythrocytes (38) without success. We could not demonstrate competitive inhibition of cellular binding on addition of exogenous colchicine for either avian erythrocytes or CEM cells. Some of these negative results may be the result of an altered secondary and tertiary structure of membrane tubulin.

The apparent presence of surface and membrane tubulin in primary human leukemia cells *in vivo*, in the membranes of cilia and flagella of invertebrates, and in platelets (1, 32, 33) as well as the membrane systems of nervous tissue (13–15, 31) further suggests the importance and wide-spread nature of the phenomenon. Taken together, the work reported here and that of others cited strongly suggests that membrane tubulin is real and plays some cellular function. The apparent similarity of its primary structure to soluble and invertebrate axonemal tubulin (though some chemical differences have been reported by Stephens [33]) argues that over evolutionary time, membrane tubulin has retained some microtubule-like function. One intriguing possibility is that membrane tubulin represents a normal tubulin gene product which has been secondarily modified to increase its lipid solubility. The selective adsorption or binding to CEM surfaces and liposomes (7), and its co-purification into isolated reconstituted ciliary membranes (34) supports such a hypothesis. However, membrane tubulin is also frequently reported to be insoluble in nonionic detergents (we have confirmed this for CEM surface tubulin). Once in or on the membrane, there may be an ionic interaction of tubulin with other as yet undetermined membrane-associated components.

It is not yet possible to propose a function for surface or membrane tubulin with confidence. The demonstration that surface tubulin can be removed while the cells remain viable and completely re-express the surface tubulin may allow us to study the functions of this unique membrane protein directly.

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