Isolation of Platelet Microtubule Protein by an Immunosorptive Method*

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A rapid and highly specific method for the isolation of human platelet tubulin by immunosorption was developed. Platelet tubulin isolated by successive cycles of polymerization was used as antigen. Densitometric quantification of the antigen subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed 96% tubulin of molecular weight 55,000 and 4% high molecular weight proteins (m, = 240,000 to 250,000) which co-purified with platelet microtubule protein.

Platelet tubulin bound 0.57 pmol of colchicine/100 mg of protein. Monospecific antibody of human platelet tubulin was prepared in rabbits. After absorption with tubulin co-purifying high molecular weight proteins, and serum proteins, the rabbit anti-tubulin serum gave a single precipitin line on double immunodiffusion against platelet tubulin and the high speed supernatant of a platelet sonicate (platelet extract). The antiserum precipitated the colchicine-binding activity of platelet extracts. The γ-globulin fraction of the absorbed antiserum was linked to an agarose matrix. Platelet extracts applied to such immunosorptive columns showed the disappearance of a single protein which was eluted with 0.5 g/liter of Triton X-100 and identified as platelet tubulin. Its colchicine-binding activity was retained in full.

Electron microscopic examination revealed that the ability of platelet tubulin to polymerize and form tubules was not impaired in the presence of 0.5 g/liter of Triton X-100. This simple isolation procedure of platelet tubulin has great advantages in terms of purity and yield and can readily be adapted for use with other cell systems.

During the past few years studies of microtubules have rapidly increased in number and considerable progress has been made in elucidating their cellular function, their structure, and to a limited degree, their mode of assembly from component protein subunits. These developments were in no small measure the result of improved methods of purification which permit the isolation of relatively large amounts of microtubule protein from tissues rich in tubulin (1, 2). In platelets these structures are believed to play an important role in maintaining the discoid shape (3). In addition microtubules may participate in some manner in platelet aggregation, one of the primary functions of thrombocytes. To date, the electron microscope has been the main research tool in their study in platelets. Attempts at investigating microtubules from a biochemical standpoint have been largely frustrated by the difficulty of isolating sizable quantities of tubulin from platelets, which contain only small quantities of this protein. We have found conventional methods of extraction and isolation based upon ammonium sulfate fractionation and separation on DEAE-cellulose (4) unsatisfactory because of low yield and the length of the procedure. We have therefore tried to develop a new method of isolation which is rapid, highly specific, and which may be adapted for use with other cell types having low concentrations of tubulin.

METHODS AND MATERIALS

Isolation of Platelet Tubulin

Platelet tubulin was prepared by a modification of the method of Shalanski et al. (2). Platelets were isolated at 22° from blood anticoagulated with 15% (v/v) acid/citrate/dextrose (U.S.P. Formula "A") obtained from normal healthy volunteers by standard methods used in our laboratory (5). Generally, 4 to 5 units of blood were used for one preparation. Red and white cell contamination was reduced by brief centrifugation for 870 x g max, min. Platelets were washed twice with 0.1 M Pipes' buffer containing 4 mM EGTA and 2 mM MgSO4 and were resuspended in Pipes buffer containing 2 mM GTP. This platelet suspension was exposed to 80 KHz ultrasound for 15 s at an energy output of the probe of 150 watts followed by 30 s homogenization at 4° with a motor-driven, Teflon-coated pestle. Disrupted platelets were kept at 4° for 30 min and then centrifuged for 3.3 x 10^6 x g max, min. The supernatant (from here on referred to as platelet extract) glycerol was added to a final concentration of 4M. The glycerol-containing platelet extract was then incubated at 37° for 30 min to allow polymerization, followed by centrifugation for 1.65 x 10^6 x g max, min at 25°. The resultant pellet (H,P) was resuspended in Pipes buffer containing 2 mM GTP and was exposed to 4° for 30 min to depolymerize.

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The abbreviations used are: Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(2-aminoethyl ether) N,N'-tetraacetic acid; SDS, sodium dodecyl sulfate.
The absorbance at 260 nm was negligible. Platelet extract, chilled to 4°C, was tested by Ouchterlony double immunodiffusion performed at room temperature for 20 h in a moisture chamber. The y-globulin fraction of serum was first absorbed once at 4°C with red cells coated with human serum proteins, then once with red cells coated with high molecular weight ribonuclease. The resultant pellet was subjected to electron microscopy examinations. The specimens were processed in 1% glutaraldehyde in phosphate buffer, pH 7.4, for 1 h. Post-fixation was accomplished in 2% osmium tetroxide in phosphate buffer, pH 7.4, for 1 h. Specimens were dehydrated through a graded series of alcohols and embedded in Epon 812. Thin sections were stained with 5% uranyl acetate and 1% lead citrate, and examined on a Phillips 201-C transmission electron microscope.

Identification of Isolated Platelet Tubulin

Polyacrylamide Gel Electrophoresis—Continuous SDS-polyacrylamide gel electrophoresis was performed on 7.5% gels using 0.1 M phosphate buffer (pH 7.0) containing 0.1% SDS electrode solution with a current of 8 to 10 mA/gel. Samples for electrophoresis were solubilized in 1% SDS containing 2% 2-mercaptoethanol at a concentration of 1 to 2 mg of protein/ml. Bromphenol blue was used as tracking dye. Fixation and staining with Coomassie blue were performed as described by Fairbanks et al. (6). The molecular weight of platelet tubulin was determined from its relative rate of migration in this gel system. β-Galactosidase, bovine serum albumin, pepsin, and ribonuclease were the standard proteins used to calibrate the gel system. Platelet tubulin reduced with 2-mercaptoethanol and alkylated with iodoacetate according to Renaud et al. (7) was electrophoresed in a discontinuous SDS system essentially according to Laemmli (8), but using an acrylamide concentration of 7.5%. Gel densitometry was performed with an ISCO model UA-5 absorbance monitor or scanning the Coomassie blue-stained gels through a 0.1-mm slit at 580 nm.

Colchicine Binding Assay—Aliquots of platelet extract, C,S and C,H, 5 mg, 5 mg, and 200 μg of protein, respectively, were incubated for 60 min at 37°C with [3H]colchicine (approximately 106 cpm) at a final concentration of 4 × 10⁻⁶ M. The tubulin-colchicine complex was separated by gel filtration on columns (1 × 20 cm) of Sephades G-100 (9) equilibrated with Pipes buffer. The effluent was collected in 1.5-ml fractions which were assayed for protein and radioactivity. Protein was determined by the method of Lowry et al. (10). The standard curve was prepared with bovine serum albumin. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. The samples solubilized by addition of Bio-Solv BBS-3 were collected in toluene containing 0.4% 2,5-diphenylxazole and 0.05% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)-1 benzene.

Antibody Preparation

Human platelet tubulin purified by the temperature-dependent polymerization-depolymerization method was used as antigen to sensitize rabbits. Approximately 400 μg of platelet tubulin in Pipes buffer mixed with an equal volume of complete Freund adjuvant was injected into several sites intramuscularly into rabbits, followed by three booster injections of 400 μg each at weekly intervals. Two weeks after the last injection the rabbits were bled via cardiac puncture. The serum was first absorbed once at 4°C with red cells coated with human serum proteins, then once with red cells coated with high molecular weight tubulin co-purifying proteins which were recovered from acrylamide gels as described by Feit (11). Specificity of the antibody was tested by Ouchterlony double immunodiffusion performed at room temperature for 20 h in a moisture chamber. The γ-globulin fraction of the serum was purified by precipitation with ammonium sulfate (12). It was then dialyzed against 0.07 M phosphate buffer (pH 7.0) containing 0.1 M NaCl and stored at -20°C.

Immunosorption

Immune γ-globulin fraction of rabbit serum, 10 to 15 mg in 25 ml of 0.1 M phosphate buffer (pH 7.0), was added to a vial containing 1 g of Affi-gel 10 (Bio-Gel A-15 m agarose gel beads of exclusion limit 15 to 200 mesh, to which had been attached a 10-A long aliphatic spacer arm terminating in carboxy N-hydroxysuccinimide active ester. After coupling was completed the material was poured into a column which was washed with 0.1 M phosphate buffer (pH 7.0) until the absorbance at 260 nm was negligible. Platelet extract, chilled to 4°C, containing 5 to 10 mg of protein was applied to the immunosorbent column measuring 12 × 1.5 cm. The antigen-antibody reaction was allowed to proceed for 1 h. Platelet tubulin was eluted with 0.5 g/liter of Triton X-100 in 0.1 M phosphate buffer, pH 7.0. Aliquots were collected and protein was monitored by the method of Lowry et al. (10).

Electron Microscopy

C,S was incubated at 37°C for 30 min in the presence or absence of 0.5 g/liter of Triton X-100 followed by centrifugation for 1 65 × 10⁴ × gₘₚₐₜ min at 25°C. The resultant pellet was subjected to electron microscopy.
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Fig. 1 (top left). Electrophoretograms of platelet extracts C$_2$S and C$_1$S. Samples containing 100 µg of protein were run on SDS-polyacrylamide gels (7.5%) as described under "Methods and Materials."

Fig. 2 (center). Densitometric tracings of platelet extract (A) and C$_2$S (B) subjected to SDS-polyacrylamide gel electrophoresis on 7.5% gels as described under "Methods and Materials." The Coomassie blue-stained gels were scanned at 580 nm and the areas under the peaks quantified by planimetry.

Fig. 3 (right). Molecular weight determination of platelet tubulin on 7.5% polyacrylamide gels containing SDS. The gels were calibrated with the proteins indicated in the figure. The molecular weight of the platelet tubulin subunit was estimated to be 55,000.

Fig. 4 (bottom left). Electrophoretograms of thrombothenin and C$_2$S. Thrombothenin was isolated according to the method of Bettex-Galland et al. (18). Samples containing approximately 100 µg of protein were prepared for SDS-polyacrylamide gel electrophoresis by addition of the following (to the stated final concentrations): 1% SDS, 5 to 10% sucrose, 10 mM Tris/HCl (pH 8.0), 1 mM EDTA, and 40 mM dithiothreitol. Bromphenol blue was used as tracking dye. Gels were prepared according to the method of Fairbanks et al. (6). Conditions of electrophoresis and the method of staining of gels were as described under "Methods and Materials." Arrow A indicates platelet actin; Arrow B, platelet tubulin.
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of sulfhydryl-reducing agents. Electrophoresis of reduced and alkylated platelet tubulin in a discontinuous buffer system (8) revealed two closely spaced bands, indicating that platelet tubulin, similar to microtubule protein derived from other tissues, is composed of two dissimilar subunits.

Because of the relative abundance of contractile protein in platelets and the possible concomitant extraction of actin with platelet microtubule protein, the electrophoretic migration of platelet tubulin was compared to that of actin (Fig. 4). The two proteins clearly had different rates of relative migration on 5.6% gels containing SDS.

Preparation of Platelet Tubulin Antibody—The rabbit antiserum absorbed with human serum proteins and with the two high molecular weight electrophoretic co-migrant proteins of platelet tubulin was specific for the latter as revealed both by immunodiffusion and immunosorption methods. Double immunodiffusion of the absorbed antiserum against platelet extracts C₅S and C₆S, showed only one precipitin line, identical for all these fractions. No precipitin line was observed against human serum and thrombosthenin (Fig. 5). Coupling of the γ-globulin fraction of rabbit anti-tubulin serum to agarose beads via a spacer arm ending in carboxy N-hydroxysuccinimide further documented the specificity of the antibody. One milliliter of platelet extract containing 7 mg of protein was applied to the immunosorbent column. The void volume was collected and concentrated by filtration through collodion bags (S & S collodion bag No. 100; Schleicher & Schuell) with retention size of molecular weight 25,000. Aliquots of the concentrated proteins (100 to 200 μg) were subjected to SDS-acrylamide gel electrophoresis. Comparison of the densitometric tracings of platelet extract before and after immunosorption with anti-tubulin rabbit γ-globulin fraction revealed the absence of a single protein band corresponding to a polypeptide of molecular weight 55,000 from platelet extract which had passed through the immunosorbent column (Fig. 6). The specificity of this absorption was manifested by the failure of this protein to be removed when platelet extract was passed through a column of agarose coupled with nonimmune rabbit γ-globulin.

The specificity of the antiserum was also demonstrated by the ability of the antibody to precipitate colchicine-binding protein from platelet extract. The latter was incubated with 0.5 volume of anti-tubulin serum at 4° overnight, resulting in a visible precipitation of proteins. The supernatant of this mixture obtained by centrifugation for 3.4 × 10⁴ × gₘₚₙₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚₚ¢rophy of 0.15 M NaCl for 24 h, fixed with 0.5% tannic acid (w/v) for 10 min, and photographed.
Lowry et al. (10). Absorbance at 750 nm was plotted.

Triton X-100 was found to be a satisfactory alternate eluant of the absorbed tubulin. One single protein peak was eluted from the immunosorbent column (Fig. 7). When 7 mg of platelet extract was applied, 0.2 mg of protein was eluted. SDS-polyacrylamide gel electrophoresis revealed a single Coomassie blue-stainable band which corresponded in relative rate of migration to tubulin isolated by the temperature-dependent polymerization-depolymerization method. No high molecular weight contaminants eluted with tubulin by this method (Fig. 6). The biological integrity of tubulin was well preserved as demonstrated by complete retention of its colchicine-binding activity in the presence of 0.5 g/liter of Triton X-100 (Table I). Under saturating conditions platelet tubulin eluted from the immunosorptive column bound 0.64 pmol of colchicine/100 mg of protein. The effect of Triton X-100 on the ability of tubulin to polymerize, one of its most important biological attributes, was examined by measuring the increase in absorbance at 500 nm. In the presence of 0.5 g/liter of Triton X-100 this ability was not affected, but at higher concentrations polymerization of tubulin was decreased (Fig. 8). Electron microscopy verified the tubular nature of the polymerized tubulin both in the presence and absence of 0.5 g/liter of Triton X-100 (Fig. 9, A and B).

**Discussion**

Our studies have shown that the temperature-dependent assembly-disassembly method can be applied to the isolation of microtubule protein from platelets in which this protein is not abundant. The poor yield obtained with the method of Weisenberg et al. (14) has also been noted by Castle and Crawford (19) who extracted tubulin from porcine platelets. As platelets contain considerable amounts of actin, the isolation procedure used must not yield high contamination with this protein. This apparently is the case when vinblastine is utilized to precipitate tubulin from platelet extract. The temperature-dependent polymerization-depolymerization method, although quite specific for tubulin, does give rise to some contamination with other proteins, particularly a pair of high molecular weight proteins. While the presence of GTP and EGTA are both necessary for the extraction of tubulin from platelets, the yield of this protein can be greatly increased (2- to 3-fold) with the inclusion of 4 M glycerol in the extraction medium.

The fact that the molar binding ratio of the alkaloid colchicine per tubulin dimer of molecular weight 110,000 did not approach unity as reported by Weisenberg et al. (14) for 6 S sedimentable protein suggests that our preparation of tubulin contained a considerable portion of high molecular weight tubulin polymers (30 S to 36 S), which according to Kirschner et al. (20) do not ligate colchicine very readily. The purity of the extracted tubulin was demonstrated by electrophoretic analysis of SDS-solubilized material, which showed an average 96% of the protein of C, S as tubulin. High molecular weight tubulin polymers dissociate completely under these conditions (20). The subunit molecular weight, as determined by SDS-polyacrylamide gel electrophoresis, conforms to tubulin isolated from other sources, as does the heterodimer structure indicated by the electrophoretic behavior of reduced and alkylated microtubule protein (11, 21-23).

High molecular weight proteins co-purifying with tubulin by successive cycles of temperature-dependent assembly-disassembly have been described in preparations from brain, clams, and sea urchins and also from porcine platelets (15, 19, 24). The exact function and nature of these proteins is not clear although axonemeal material with similar electrophoretic migration characteristics has been identified as dynein (24). Recently it has been suggested that high molecular weight proteins stimulate microtubule assembly by facilitating the formation of ring structures which are intermediates in tubulin polymerization (25).

The relative abundance of these proteins appears to vary considerably from 17% in C, S preparations of porcine brain to 4% in human platelets. The reason for this is not clear, but among the possibilities considered the most plausible one...
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Fig. 9. Electron micrographs of platelet microtubules. Platelet tubulin isolated by two successive cycles of polymerization-depolymerization was incubated at 37°C for 30 min (A) without Triton X-100; (B) with 0.5 g/liter of Triton X-100 followed by centrifugation for 1.65 x 10^5 g, min at 25°C. The resultant pellet was examined by electron microscopy as described under "Methods and Materials." Magnification was (A) x 21,000; (B) x 41,000.

seem to us a species-related difference or the effect of disruption of platelets by sonication. There are also differences in biochemical behavior depending upon the source from which tubulin co-purifying proteins derive. Thus, such proteins from chick brain were good substrates for an endogenous cyclic AMP-dependent protein kinase (26), but not those extracted from human platelets (27).

The immunosorptive method for isolation of microtubule protein was found to be a highly specific and very efficient technique for extracting tubulin from thrombocytes, which have only low concentrations of this protein. Production of antibody to tubulin has been reported previously (28, 29) and at least one publication provides good documentation for the specificity of the antibody which was used to demonstrate mitotic spindles by immunofluorescence (30). The specificity of antibody produced to structural proteins is generally thought to be less than that to soluble cellular proteins. In this respect the antibody produced to human platelet tubulin was very specific, as it did not react with actin, a closely related structural protein which is abundant in platelets. Cross-reactivity of the antibody to platelet tubulin with microtubule protein from other sources has not been determined yet, but on the basis of a reported universal antigeneic determinant in tubulin (28) we expect that our antibody will cross-react. It may be possible to utilize antibody to tubulin derived from a source abundant in this protein for the isolation of microtubule protein by immunosorptive techniques from tissues or cells which cannot yield specific antigen in sufficient quantity for the preparation of antibody.

Of the methods available for dissociation of antigens from their specific antibodies, Triton X-100 and reduction in pH were both found effective. Dissociation of tubulin from its column-bound antibody by 0.5 g/liter of Triton X-100 yielded a protein which retained its full biological capacity with respect to colchicine binding and ability to polymerize, functions which were greatly diminished when tubulin was eluted by reduction of H^+ concentration.

Whether for in vitro polymerization of tubulin certain non-tubulin factors are needed or not is currently a topic of much discussion. The platelet tubulin isolated by the described immunosorptive method is free from high molecular weight proteins. This technique of isolating tubulin, therefore, presents a good experimental tool to determine the factors involved in in vitro polymerization of tubulin, an area which is under investigation in our laboratory.

The immunosorptive method of isolation of microtubule protein described by us represents a rapid and simple technique for obtaining pure tubulin. Its usefulness extends beyond the isolation of tubulin to a rapid method for identification of microtubule protein in cell extracts. The potency and specificity of the antibody will also allow its application to histochemical studies in intact cells.

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REFERENCES
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