IMMUNOCHEMICAL DETERMINATION OF TUBULIN

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1. Introduction

Tubulin, the subunit protein of microtubules is found in all eukaryotic cells. Microtubules are functionally important for a wide variety of cellular activities such as mitosis, cell shaping, secretion and motility. They are also abundant in the nervous tissue where neurite outgrowth as well as axoplasmic transport are thought to be dependent on microtubules integrity [1]. Detection and quantitation of tubulin is, thus, of importance in trying to understand differentiation processes. It has recently been shown that during postnatal development of the rat brain, there is a decline in the rate of tubulin synthesis as compared to that of the total protein (2–5). The decrease in the relative amounts of this protein occurs in the soluble fraction and is accompanied by a comparable decline in the percentage of its mRNA (3–5). The relative amounts of rat brain [2,5] or chick brain [6] tubulin have been determined by colchicine binding as well as by electrophoretic resolution on polyacrylamide gels followed by densitometric scanning of the stained gels. An alternative method is the detection and quantitation of tubulin by specific anti-tubulin antibodies, which allows a more sensitive assay as tubulin determinations can be carried out also on membrane bound protein not necessarily in its functional form. Specific tubulin antibodies have been used mainly for visualization of the mitotic spindle, as well as of the microtubular network in the cell [7,8] by immunofluorescence techniques. Immunochemical methods have also been used for the quantitative determination of tubulin in the flagella of *Naegleria gruberi* [9].

In this report we have prepared specific antibodies against a highly purified preparation of tubulin from calf brain. These antibodies were characterized by several immunological assays and were used for the quantitative determination of tubulin during postnatal brain development.

2. Materials and methods

DEAE-Cellulose (DE-52) was obtained from Whatman, Freund's complete adjuvant was from Difco and $Na^{125}I$ for protein iodination (100 mCi/ml) was purchased from the Radiochemical Centre, Amersham.

2.1. *Preparation of calf brain tubulin*

Calf brain tubulin was prepared following the procedure of Eipper [10] with slight modifcations. 300 g of freshly obtained calf brain (omitting the hind brain) were homogenized using an Ultra-Turrax (Ika Werk, Breisgau, Germany) in 600 ml of 0.24 M sucrose/2.5 mM $MgCl_2$/50 mM $NaPP_i$, pH 7.0. The homogenate was centrifuged and fractionated by ammonium sulfate as described [10]. The 50% ammonium sulfate pellet was resuspended in 30 ml of 50 mM $NaPP_i$, pH 7.0/2.5 mM $MgCl_2$, dialysed for 2 h against 70-fold excess of the same buffer and was

loaded onto DEAE-cellulose column (1.5 × 20 cm). The tubulin peak was collected either by elution with 0.26 M NaCl/50 mM $NaPP_i$, pH 7.0/ 2.5 mM $MgCl_2$ or by a linear salt gradient, 100 ml of 0.1 M and 100 ml of 0.6 M NaCl in the same buffer.

2.2. *Preparation of high-speed supernatant fractions from brains*

105 000 × *g* supernatant fractions were prepared in 0.25 M sucrose/10 mM sodium phosphate buffer, pH 7.4/10 mM $MgCl_2$/0.5 mM GTP as described [5].

2.3. *Sodium dodecyl sulfate–polyacrylamide gel electrophoresis*

Samples were mixed with concentrated sample buffer to yield a final concentration of 10% glycerol/ 5% 2-mercaptoethanol/3% sodium dodecyl sulfate/ 0.0625 M Tris–HCl, pH 6.8/0.001% bromophenol blue, and heated at 100°C for 5 min. Samples were subjected to electrophoresis at 3 V/cm at 20°C for 14–16 h on 0.75 mm thick polyacrylamide gel slabs [11], containing a 10–20% polyacrylamide gradient, and 0.1% sodium dodecyl sulfate [12]. The gels were stained with Coomassie Brilliant Blue.

2.4. *Immunochemical methods*

2.4.1. Immunization

Tubulin solutions containing 2 mg (in 1 ml saline) were emulsified with equal volumes of Freund's complete adjuvant and injected intradermally at multiple sites into rabbits. This procedure was repeated at weekly intervals for 1 month, and the animals were bled and their sera separated. Booster injections were given occasionally.

2.4.2. Passive hemagglutination

Passive hemagglutination of tubulin coated formalinized sheep erythrocytes was carried out as previously described [13].

2.4.3. Radioiodination of tubulin

Tubulin was labelled with ^{125}I by the lactoperoxidase method, according to Marchalonis [14]. The iodination mixture contained 10 μl of tubulin solution (2 mg/ml). 5 μl of lactoperoxidase (0.6 mg/ml), 10 μl of $Na^{125}I$ (1 mCi) and 5 μl of hydrogen peroxide (1 mM final concentration) in 100 mM sodium phosphate buffer, pH 7.2, containing 5 mM $MgCl_2$. After 30 min at 37°C the reaction was stopped by the addition of 200 μl of cold phosphate buffered saline (PBS) and followed by gel filtration on Sephadex G-25 column (0.8 × 30 cm) equilibrated with PBS containing 0.1% gelatine (PBS–gelatine). The efficiency of labelling was determined from the relative radioactivities in the first (protein–bound ^{125}I) and the second (free ^{125}I) peaks. 25–40 μCi of ^{125}I were bound per μg of tubulin in different preparations.

2.4.4. Radioimmunoassay

Binding of [^{125}I]tubulin to antibodies was performed as follows: 50 μl of [^{125}I]tubulin with about 16 000 cpm (diluted in PBS–gelatine) were added to 50 μl of antiserum diluted with normal rabbit serum (NRS) in PBS. The final concentration of serum (NRS and immune serum) was kept constant at a dilution of 1:20 in PBS. The mixture was incubated at 37°C for 30 min and goat anti-rabbit IgG was added, in an amount sufficient for the precipitation of all the rabbit IgG (usually 200 μl). The mixture was further incubated 30 min at 37°C and overnight at 4°C. The precipitates were centrifuged, washed twice with cold PBS and the radioactivity was determined by a Packard gamma scintillation spectrometer.

Competition experiments with unlabelled tubulin were performed in a constant anti-tubulin antiserum concentration (usually 1:150). The tested sample, or standard tubulin solutions (50 μl) were mixed with the radioactive tubulin prior to the addition of the antisera, and the assay continued as described above. Non specific binding which was less than 10% (measured by using NRS instead of tubulin antibodies) was subtracted from the experimental values.

3. Results

3.1. *Purification of calf brain tubulin*

Large scale purification of tubulin from calf brain was achieved by fractionation on DEAE-cellulose column. Elution of the tubulin which was adsorbed to the column at low salt concentrations was performed by applying a salt gradient as depicted in fig.1. The eluted protein appeared as two distinct peaks both of which revealed only a single band of tubulin on SDS–polyacrylamide gel electropherograms. Spectrophotometric analysis of the two peaks, namely the

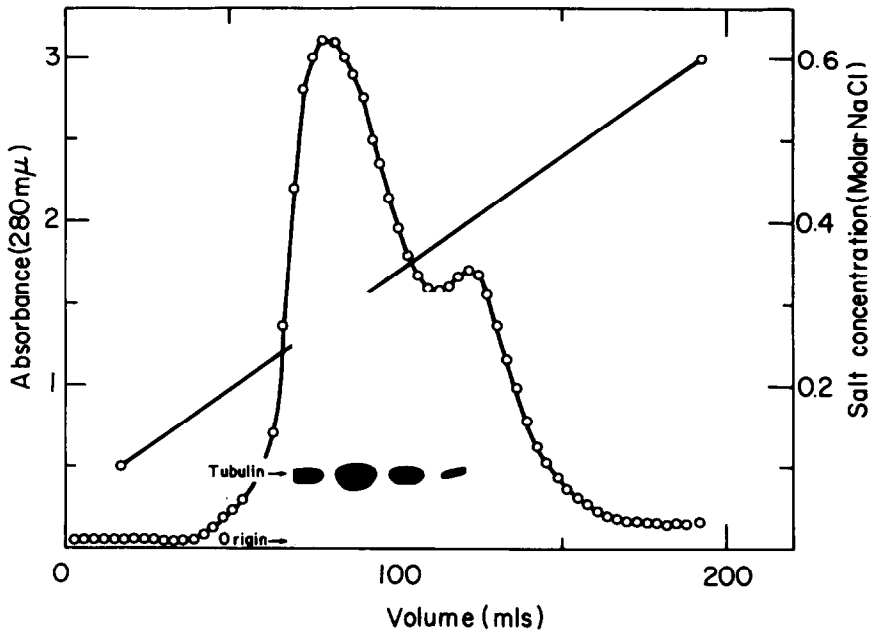

Fig.1. Purification of tubulin: Elution of tubulin from DEAE-cellulose using a linear salt gradient. 100 ml of 0.1 N NaCl – 100 ml of 0.6 N NaCl, in 50 mM $NaPP_i$, pH 7.0/2.5 mM $MgCl_2$. The insert shows the electrophoretic analysis of the eluted protein fractions on SDS–polyacrylamide gel.

absorbance ratio at 260 nm versus 280 nm suggested that they differed in their nucleic acid content, the late eluting peak containing larger amounts. Stepwise elution with 0.26 M NaCl gave tubulin preparation of similar purity, but somewhat lower yield. About 200 mg of pure tubulin were obtained with the gradient elution method from one brain about 300 g.

3.2. *Preparation and immunological characterization of tubulin antibodies*

In search for an improved method for the preparation of anti-tubulin antibodies the following procedures of immunization of rabbits were tried, all in Freund's complete adjuvant: repeated immunizations each with 2 mg tubulin per animal; injection of tubulin covalently coupled with bovine immunoglobulin and electrostatically complexed with methylated bovine serum albumin. These conjugates were prepared by a procedure similar to that described earlier for eliciting anti-tRNA antibodies [15]. We have also tried to enhance tubulin immunogenicity by a mild dinitrophenylation of the protein using fluorodinitrobenzene.

The antibody titers were estimated using the passive microhemagglutination technique with tubulin coated formalinized sheep red blood cells. It was found that either conjugation of tubulin to a carrier protein or chemical modification by dinitrophenylation did not lead to a significant enhancement in the antibody titers. Therefore, for most of the studies reported here we have used antisera of rabbits injected repeatedly with tubulin emulsified in Freund's complete adjuvant. Significant titers were obtained only after 3–4 injections. Optimal conditions for the hemagglutination technique were tested by varying both tubulin concentrations and temperature of coating. We found that 100 μg of tubulin per 1 ml of packed formalinized cells and incubation at 25°C during the coating procedure gave the most sensitive results (table 1). Using these conditions three immunized rabbits had hemagglutination titers between 1/128–1/256, whereas the preimmune sera of these animals did not show significant agglutination (table 1).

It should be noted that the highest titers of anti-

Table 1
Passive microhemagglutination assay of tubulin antibodies

Tubulin concentration used for coating erythrocytes (μg/ml)	Temperature of incubation (°C)	Log_2 hemagglutination titer					
		Rabbits					
		c	c	c	1	2	3
20	4	–	–	–	–	2	2
	25	2	2	2	4	4	4
100	4	2	–	–	7	6	6
	25	2	2	–	8	7.5	7.5
500	4	2.5	–	–	6	6	6
	25	2	–	2	6	6	6
750	4	2	–	–	5	5	5
	25	2	–	–	7	6	7

c = non injected control rabbits

The packed erythrocytes were diluted 3 times in the final incubation mixture and incubations were for 1 h.

bodies to tubulin were obtained shortly following the immunization schedule. Successive bleeding without further booster injection gave lower titers. It is, therefore, advisable to use sera from early bleedings or to boost the animals with further injections in order to maintain a reasonable titer. However, it should be mentioned, that the overall immune response to tubulin is quite low. This may be due to the fact that tubulin is highly conserved throughout evolution [16]. Moreover, the tubulin source which we used is of a mammalian origin and therefore is closely related to that of the immunized rabbits.

3.3. *Radioimmunoassay*

A quantitative and sensitive radioimmunoassay for anti tubulin antibody was developed using [^{125}I] tubulin as the antigen. A representative binding curve is shown in fig.2. With the preparation of labelled tubulin used, significant binding was obtained up to immune serum dilution of about 1:1500. The sensitivity of the assay can be increased further by using antigen preparation with higher specific activity.

For the competition studies increasing amounts (1.5 ng to 100 μg) of unlabelled tubulin were incubated with the radioactive tubulin, prior to the addition of the antisera. The inhibition curve thus obtained is shown in fig.3. The data show that the radioimmunoassay method allows quantitative determination of tubulin from 0.01–20 μg (fig.3). This standard curve was used to estimate the concentration of tubulin in brain extracts and was repeated in each experiment along with the tested samples. The sensitivity of the radioimmunoassay could be increased by a factor of about 5 employing preincubation of the competing, non-labelled tubulin with the antisera prior to the addition of the radiolabelled protein.

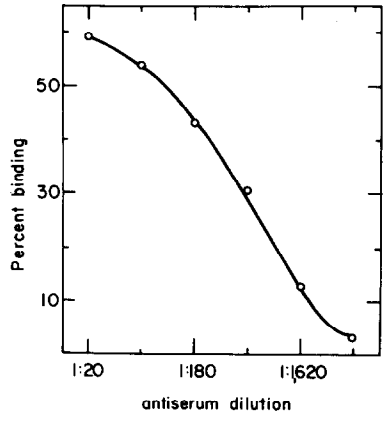

Fig.2. Binding of [^{125}I]tubulin to anti-tubulin antibodies: The binding is calculated as a percentage of the total [^{125}I]tubulin added to the assay mixture, the nonspecific adsorption to NRS is subtracted.

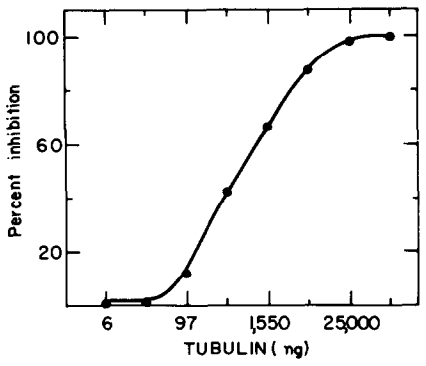

Fig.3. Inhibition of [^{125}I]tubulin binding to antitubulin antibodies by non labelled tubulin: The inhibition is calculated as a percentage of [^{125}I]tubulin bound to the antibodies in the absence of cold tubulin.

3.4. *Determination of tubulin concentration in developing postnatal brain extracts*

The amount of tubulin in postnatal developing rat brain as a function of age was determined (table 2). Various dilutions of brain extracts were tested for their capacity to compete with [^{125}I]tubulin for the binding to the antibodies. Four to five different dilutions of each sample were tested and the concentration of tubulin was computed from the inhibition values which correspond to the linear portion of the standard curve. We found a gradual age dependent decrease in the tubulin percentage in the soluble fraction of the brain with a major decline around the fifteenth day of age. These results are in agreement with results previously obtained [2,5] using [^{3}H] colchicine binding assay and densitometric evaluations of Coomassie Brilliant Blue stained polyacrylamide gels. The colchicine binding assay requires a functionally potent tubulin and decays with time [2,6], whereas the gel electrophoresis analysis depends on the absence of contaminating proteins with similar mobility to that of tubulin. The advantage of the immunological approach resides in the capacity to determine any antigenically active material and does not necessarily require biological activity and/or molecular integrity.

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Table 2
Radioimmune quantitation of tubulin during postnatal rat brain development

Age (days)	Total soluble protein concentration (mg/ml)	Tubulin concentration (mg/ml)	Tubulin (%)
0	4	1.28	32
10	4	1.20	30
15	4	1.05	26
30	4	0.96	24

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