QUANTITATIVE DETERMINATION OF TUBULIN BY RADIOIMMUNOASSAY

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1. Introduction

Tubulin is the protein subunit of microtubuli. During interphase these ubiquitous structural components of eukaryotic cells make up the cytoplasmic microtubular complex (CMTC) [1]. Microtubuli are also essential elements of the mitotic spindle and of cilia and flagella. In addition to their role in cytoskeleton formation, cell division and cell motility, they are thought to be involved in the transmembrane control of receptors [2]. As such they would form part of the surface modulating assembly (SMA) [3]. According to some [4,5], oncogenic transformation would disturb this assembly both structurally and functionally. Others [6] have presented evidence that no essential structural modification is involved here. We have recently [7] applied immunoperoxidase techniques to the study of the CMTC and found no indication for structural impairment (De Mey, Joniau and De Brabander, unpublished observations). A primary requirement for the solution of this controversy will be the quantitative determination of tubulin.

Tubulin has been quantitated in cell extracts by densitometry of the monomer (mol. wt 55 000) band on SDS–polyacrylamide gels. The main technique, however, has been the colchicine binding assay. Tubulin dimer (mol. wt 110 000) binds 1 equivalent of this antimitotic drug. This binding, although strong ($K_{ass} \simeq 10^6$ liters.mol^{-1}), is slow, requires incubation at 37°C, competes with concurrent thermal denaturation of the binding site, and occurs only with the dimer. Assay procedures using [^{3}H] colchicine binding [8] yield linear binding curves in the range of 0.1–1.5 mg tubulin/ml.

A test based on the antigenic rather than on the drug-binding properties of tubulin would not suffer from these many drawbacks. Two reports [9,10] mention the use of anti-tubulin antibody, one of which [9] describing a sandwich immunoprecipitation assay.

We have previously described the use of an antiserum specific for tubulin in cytochemical studies [7]. Here we report on its application in a radioimmunoassay for tubulin useful in the concentration range of 0.5–20 μg tubulin/ml. We will discuss the advantages of this technique over the classical colchicine binding assay in terms of sensitivity and non-susceptibility to the degree of polymerization and thermal denaturation of tubulin allowing its application to cell culture homogenates.

Abbreviations: CMTC, cytoplasmic microtubular complex; SMA surface modulating assembly, PBS, phosphate-buffered saline, pH 7.0; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate

≠ In the polymerization buffer of Shelanski et al., MES was replaced by phosphate. We will refer to polymerization buffer as to 0.1 M phosphate pH 6.4 containing 0.5 mM $MgSO_4$, 1 mM EGTA and 1 mM GTP. Phosphate buffer is polymerization buffer minus GTP

2. Materials and methods

2.1. *Tubulin*

Tubulin was prepared from brain homogenates of rats (Wistar), pigs or rabbits, according to Shelanski et al. [11] and invariably involved two polymerization–depolymerization cycles. Preparations containing 1 to 5 mg protein/ml were mostly stored in polymerization buffer≠ in liquid nitrogen. Occasionally

a preparation was made 3 M in glycerol and stored at −70°C. Yields averaged 40 mg of twice-polymerized tubulin/100 g wet brain tissue. Purity as measured by densitometry on SDS–polyacrylamide gels was better than 95%.

2.2. *Antibodies*

Rabbits were injected intradermally either with twice-polymerized rat brain tubulin in the presence of complete Freund's adjuvant (rabbit No. 105), or with the mol. wt 55 000 band of a series of SDS–polyacrylamide gels to which twice-polymerized rat brain tubulin had been applied (rabbit No. 116). From individual serum pools immunoglobulin fractions were prepared by sodium sulfate precipitation [12]. These fractions (e.g. Ig 105-A and -B) were used as such in immunoprecipitin experiments. They showed single precipitin lines in double diffusion against rat brain tubulin, and extensive crossreaction with pig brain tubulin.

Purified anti-tubulin antibodies (AB 105-A or -B and AB 116-A), used in radioimmunoassay, were prepared from the immunoglobulin fractions by affinity chromatography on twice-polymerized rat brain tubulin fixed to CNBr-activated Sepharose [13]. Purified anti-ovalbumin antibodies (AB 22-A or -B), used as a blank in radioimmunoassay, were isolated as described [14].

2.3. *Precipitin curves*

In conical 1 ml pyrex tubes (Sorvall) 100 μl of the salt-precipitated immunoglobulin fraction of either a rabbit anti-rat brain tubulin serum or a pool of normal rabbit sera (Ig NRS) were mixed with 100 μl of a series of dilutions of tubulin in phosphate-buffered saline, pH 7.0 (PBS), containing from 0.01–2 mg tubulin/ml. After mixing, the tubes were stored at 4°C overnight. Precipitates were centrifuged, washed, dried overnight in a desiccator, dissolved in 0.5 ml 0.1 N NaOH, and analyzed for protein content by the Lowry method. Precipitin curves are plotted as protein concentration of redissolved precipitate against the logarithm of the concentration of added tubulin.

2.4. *Colchicine binding assay*

[^{3}H]Colchicine binding was carried out as described by Sherline et al. [8]. [*Ring C-methoxy*-^{3}H]Colchicine, a product of Amersham (England), had a specific radioactivity of 18 mCi/mg. The charcoal used for adsorption of unbound colchicine was Norit-A supra (Serva, Heidelberg). Before use, it was dextran-treated: a 1% charcoal suspension was prepared in water containing 0.05% dextran (Serva) and 0.02% sodium azide, and stored at 4°C.

After incubation and precipitation of the free colchicine with charcoal, the radioactivity of the supernatant solution was measured by mixing 100 μl with 10 ml scintillation mixture (Corusolve, ICN) and counted in a liquid scintillation counter (Packard, type 2425).

2.5. *Radioiodination of tubulin*

Labeling was done according to Hunter and Greenwood [15] and largely as described [16]. As a rule 100 μg of twice-polymerized rat brain tubulin were mixed with 3–5 mCi of [^{125}I]iodide (Amersham) and iodinated by addition of chloramine-T. The resultant mixture, after addition of bovine serum albumin (BSA, RIA-grade, Sigma) to a final 1%, was applied to a Sephadex G-25 column. Protein-bound ^{125}I was eluted with 0.1% BSA-containing PBS and stored frozen.

2.6. *Radioimmunoassay*

According to Catt and Treager [17], polystyrene hemolysis tubes (10 × 70 mm, Falcon Plastics No. 2038) were coated with 1 ml of a solution of purified rabbit antibody against either rat brain tubulin or chicken ovalbumin both at 50 μg/ml in 0.01 M bicarbonate buffer, pH 9.8. After standing overnight at 4°C the contents were syphoned off and the tubes filled to the top with a 0.5% solution of BSA in PBS. After standing for 1 h at 4°C the tubes were emptied, washed once with PBS, and used for the binding test that same day. A standard dilution series of tubulin was made up in PBS containing 0.5% BSA and 0.2% Tween-80, with tubulin concentrations ranging from 0.1–100 μg/ml. To each standard was added ^{125}I-labeled rat brain tubulin to a final concentration of 0.1 μg/ml. Fractions (300 μl) of each standard solution were applied to both types of coated tubes. These were rotated at an angle of 30° from horizontal at 30 rev/min for 2 h at 4°C. After emptying they were washed extensively with PBS containing 0.2% Tween-80. Finally, they were counted dry in a crystal

scintillation spectrometer (Packard, type 5230). In the absence of unlabeled tubulin (i.e. the 100% binding reference) adsorbed radioactivity regularly ranged from 30–50 X 10^3 cpm for tubes coated with anti-tubulin, as compared to 1.5–2 X 10^3 cpm for anti-ovalbumin coated ones. After accounting for this non-specific binding, the percentage binding was computed and plotted against the logarithm of unlabeled tubulin concentration. Each value plotted was the result of two determinations (two tubes). Tubulin from different animal sources was used to set up the standard dilution series but only ^{125}I-labeled rat brain tubulin was used as the indicator.

2.7. *Thermal denaturation experiments*

Four stock solutions containing resp. 1000, 400, 40 and 4 μg rat brain tubulin/ml were prepared in PBS containing 0.5% BSA. Samples of these solutions were either used as such or after incubation at 56°C for 4 h or at 37°C for overnight (20 h). After cooling in ice-bath they were further diluted to the regular standard dilution series (0.1–100 μg/ml) with the same medium but with addition of Tween-80 to a final 0.2%. Then they were analyzed for tube-binding as described.

Similarly, a stock solution of rat brain tubulin containing 1.5 mg/ml was incubated for 4 h at 56°C, or overnight at 37°C. After cooling to 0°C a dilution series from 0.1–1.5 mg/ml was set up in the buffered medium described by Sherline et al. [8] and 100 μl fractions analyzed for [^{3}H]colchicine binding. The same was done with an untreated sample of the same preparation. Thirdly, in the case of immuno-precipitation analysis 100 μl fractions of serial dilutions of rat brain tubulin, containing from 0.01–2 mg tubulin/ml, were either used as such or after incubation for 4 h at 56°C, or overnight at 37°C. After cooling in ice-bath, 100 μl of a suitable dilution of the immunoglobulin fraction of the antiserum was added to each tube and the immunoprecipitation analysis carried out.

3. Results

Figure 1 shows a curve (circles) obtained as the average of two radioimmunoassay experiments using twice-polymerized rat brain tubulin and tubes coated with AB 105-A. The useful portion of the S-shaped

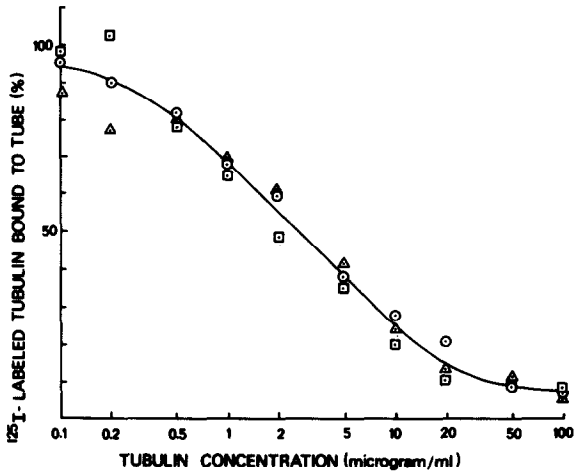

Fig.1. Radioimmunoassay of rat brain tubulin. Effect of thermal denaturation of the antigen. Polystyrene hemolysis tubes were coated with affinity-purified rabbit antibodies either against rat brain tubulin (AB 105-A) or against ovalbumin (AB 22-B). After correction for non immuno-specific adsorption, tube-adsorbed radioactivity, expressed as % of maximum, was plotted (⊙) against the logarithm of unlabeled tubulin concentration. Similar results were obtained when the same tubulin preparation was first incubated for 20 h at 37°C (⊡) or 4 h at 56°C (▲).

inhibition curve extends from about 0.5–20 μg tubulin/ml with optimal accuracy (inflection point) around 3 μg/ml. As our assay needs 300 μl of tubulin solution, we can optimally determine 1 μg tubulin. In fig.2a colchicine binding curve (circles) is shown for the same preparation of rat brain tubulin. The plot is rectilinear only for tubulin concentrations below 1.5 mg/ml. As this binding test requires 100 μl solution, the useful range extends from 10–150 μg tubulin.

Figure 1 also contains the radioimmunoassay curves obtained for rat brain tubulin that has been subjected to various schemes of thermal denaturation. These manipulations do not seem to appreciably affect the antigenic properties and thus the competitive behaviour in the tube-binding assay. This can be deduced in a more direct way from immunoprecipitation analysis (fig.3). Differently denatured samples of the same tubulin preparation display a nearly identical immuno-precipitation behaviour after due correction for spontaneous precipitation of the antigen, induced by thermal treatment. We also measured the effect of thermal denaturation of tubulin on colchicine binding

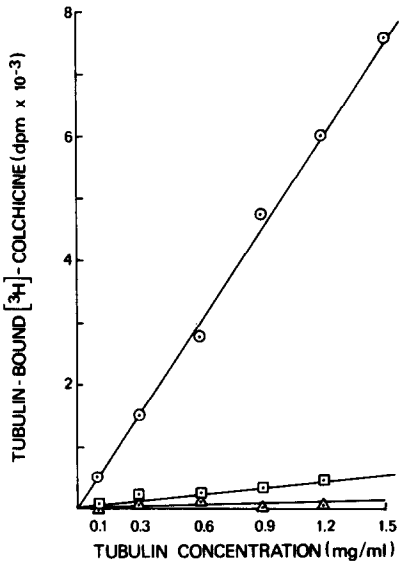

Fig.2. [^{3}H]Colchicine binding to rat brain tubulin following the method of Sherline et al. [8]. Effect of thermal denaturation of tubulin. Tubulin-bound [^{3}H]colchicine (dpm), measured in duplicate, was plotted (⊙) as a function of protein concentration (shown by densitometry of an electropherogram to contain at least 95% tubulin). Different curves were obtained with samples of the same tubulin preparation after incubation for 20 h at 37°C (⊡) and 4 h at 56°C (△).

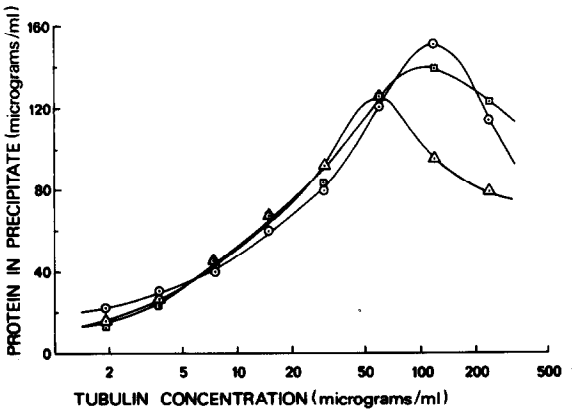

Fig.3. Immunoprecipitation of rat brain tubulin with anti-rat brain tubulin immunoglobulins (Ig 105-B). Effect of thermal denaturation of the antigen. The protein concentration of the redissolved precipitate, as measured by Lowry's method, was plotted against the logarithm of the concentration of tubulin added (⊙). Similar precipitin curves were obtained with samples of the same preparation of rat brain tubulin that had been incubated for 20 h at 37°C (⊡) or 4 h at 56°C (△). Here, some spontaneous precipitation of tubulin, occurring in the absence of antibody and induced by thermal treatment, was accounted for.

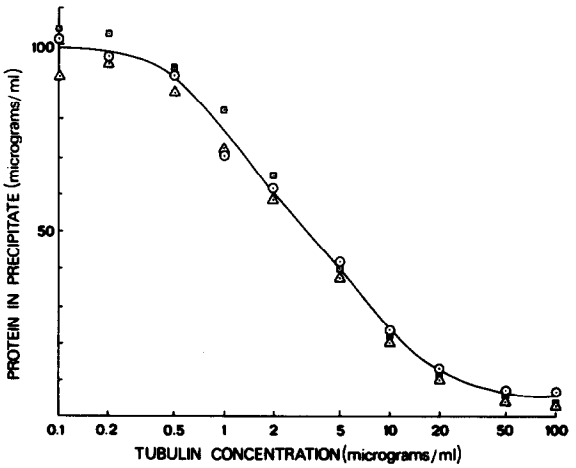

Fig.4. Radioimmunoassay of tubulins from different animal sources. Polystyrene tubes were coated with antibodies against rat brain tubulin (AB 105-B) or against ovalbumin (AB 22-B). To dilution series of the tubulin under investigation, ^{125}I-labeled rat brain tubulin was invariably added as the indicator. The curve was drawn to fit the experimental values for rat brain tubulin (⊙). Also shown are the results for rabbit (△) and pig (⊡) brain tubulins. Each point is the mean of two determinations.

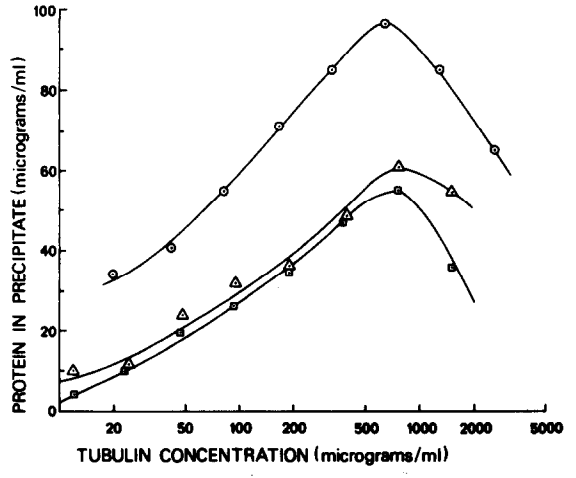

Fig.5. Immunoprecipitation of brain tubulins of different species with rabbit anti-rat brain tubulin immunoglobulins (Ig 105-B). The three curves were obtained with, respectively, rat (⊙), pig (⊡) and rabbit (△) tubulins.

(fig.2). When the same tubulin preparation as that used for radioimmunoassay was subjected to denaturation at 37°C or 56°C, colchicine binding capacity was reduced to 7% and 0.2%, respectively, of the original. Next, for brain tubulins of different origin the capacity to compete antigenically with ^{125}I-labeled rat brain tubulin for tube-bound anti-rat brain tubulin antibodies was measured. Figure 4 shows that both pig and rabbit brain tubulins are nearly equivalent antigenically to the immunogen derived from rat brain. A more accurate determination of the relative antigenic value of these three kinds of tubulin was done by immunoprecipitation analysis (fig.5). Both pig and rabbit brain tubulin precipitate a substantial part (53% and 59%, respectively) of the rabbit anti-rat brain tubulin antibodies.

4. Discussion

From a comparison of figs 1 and 2 it can be deduced that our radioimmunoassay is on the average 50–100 times more sensitive than the colchicine binding method. This may be a critical factor when analyzing tubulin contents of cells grown in vitro. Using the drug binding assay, we were unable to accurately determine the tubulin content of homogenates obtained from $2-5 \times 10^6$ cells grown as monolayers (about the content of a 20 cm^2 surface culture flask, Falcon Plastics No. 3013), whereas with the radioimmunoassay this amount largely sufficed for an accurate determination.

As evident from fig.1 thermal denaturation of tubulin in conditions as described here has little or no effect on its radioimmunoassay in contrast with its pronounced effect on colchicine binding (fig.2). Immunoprecipitation analysis (fig.3) offers a further proof of this observation. It should be mentioned however that, although more straightforward, immunoprecipitation may in this case turn out to be a complex phenomenon. Although no accurate determinations were done, we found by turbidity measurements and electron microscopy that the differently denatured tubulin samples contained different amounts of aggregates, that may require different antibody concentrations at equivalence.

The lack of effect of thermal denaturation of tubulin on its radioimmunoassay may be of interest when the tubulin content of cells or tissues has to be measured. The extraction procedures applied inevitably expose the labile tubulin molecule (half-life at 0°C is 11 h) [18] to thermal denaturation. Even with the difficulties of mechanical extraction (disruption of membranes, desorption from organelles, depolymerization) resolved, the thermal denaturation remains to be accounted for if the measurement would depend on a colchicine binding assay. Besides, the possibility exists that cytoplasmic tubulin may in part exist in a precursor form or in a non-functional 'denatured' or irreversibly aggregated form, both probably lacking colchicine binding activity but extensively cross-reacting antigenically. A radioimmunoassay procedure such as the one described here would allow the quantitation of these colchicine-insensitive and/or non-depolymerizable tubulins. In addition, the possible presence of colchicine binding proteins different from tubulin may seriously disturb the correct measurement of the latter. On the other hand, there is at present no evidence of any cytoplasmic protein antigenically cross-reactive with tubulin.

When comparing the antigenic activities of brain tubulins of different animals, the most intriguing observation is the pronounced reaction of rabbit tubulin, on which we have reported previously [7]. It raises the question of the possible involvement of tubulin in autoimmune diseases. The fact that tubulins from different sources extensively cross-react as evidenced by others [19] extends the possible application of our radioimmunoassay far beyond the limits of the homologous (rat) system. We have begun to systematically determine tubulin contents of normal and transformed cells in in vitro culture. Although an absolute measurement would require a reference curve to be constructed with tubulin extracted from the culture under investigation, it is possible to accurately measure relative tubulin concentrations using rat brain tubulin as a standard.

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