RADIOIMMUNOASSAY FOR TUBULIN DETECTION

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1. Introduction

A fundamental step towards the understanding of differentiation mechanisms in neural systems relates to tubulin metabolism and microtubule organization during the outgrowth of neurites. Several studies have been devoted to this problem involving chiefly the use of labelled colchicine to explore eventual changes in tubulin levels accompanying neurite outgrowth [1,2]. These techniques are however lacking sensitivity especially when dealing with small variations in tubulin concentrations. In addition colchicine binds specifically to 6 S tubulin at a site which is probably blocked when this protein is assembled into microtubules [3] or present in an aggregated material [4]. These considerations have prompted us to develop a radioimmunoassay making use of an iodinated antigen and a high affinity antiserum. The present report will discuss the sensitivity and validity of the assay.

2. Materials and methods

2.1. *Preparation of immunogen*

Tubulin was obtained from mouse brain (A/J strain) by repeated cycles of polymerization as was previously described by Weingarten et al. [5]. Furthermore, 6 S tubulin dimers were purified by chromatography through a phosphocellulose column according to Wiengarten et al. [6]. The column was equilibrated and washed in MES–EDTA buffer (25 mM MES, 0.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1 mM EDTA, pH 6.4). Tubulin was eluted in MES–EDTA buffer. This purified material was distributed in aliquots for immunization, iodination or standard calibration and stored in liquid nitrogen in 100 mM MES, pH 6.4, 2 mM EGTA, 0.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1 mM EDTA and 1 mM GTP buffer.

Abbreviations: MES, morpholinoethane sulfonic acid; EDTA, diethylaminoethylcellulose; EGTA, ethyleneglycolbis-(β-aminoethylether)N,N'-tetraacetic acid; PBS, phosphate buffer saline, phosphate 0.1 M, pH 7.4, NaCl 0.15 M

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2.2. *Immunization procedures*

Rabbits were immunized with three monthly injections of 25 μg purified antigen emulsified in Freund's complete adjuvant. Bleedings were made three weeks after each injection and the sera tested for antibody titers.

2.3. *Iodination*

Iodinated tubulin was prepared at room temperature by using the chloramine T method [7]. To 10 μl (2.5 μg) of tubulin, were added successively: 10 μl of phosphate buffer 0.5 M pH 7.4 containing: NaCl 0.15 M; 2 μl $Na^{125}I$ (New England Nuclear, 200 μCi); 10 μl chloramine T (40 μg in PBS). The reaction was stopped after 1 min by addition of 100 μl sodium metabisulfite (250 μg in PBS). The radioactive mixture was immediately filtered at 4°C through a

Sephadex G-25 column (30 X 1.2 cm) equilibrated and eluted with PBS containing $CaCl_2$ 1 mM, $MgCl_2$ 0.6 mM, bovine serum albumin 0.5%. The iodinated protein solution was stored in separate samples of 1 μCi/ml at -30°C.

The yield of tubulin iodination calculated after gel filtration was about 50% corresponding to a specific radioactivity of 40 Ci/g. ^{125}I tubulin storage during 4 weeks in liquid nitrogen does not affect the binding parameters of the iodinated antigen–antibody reaction. Non-specific binding (> 30%) occurred when high concentrations of MES buffer were used instead of phosphate buffer.

2.4. *Purification of anti-polysaccharide rabbit antibodies*

Specific rabbit anti type II *Pneumococcus* antibodies were purified from the total antiserum by affinity chromatography [8] through a Sepharose column coated with the corresponding polysaccharides [9].

2.5. *Radioimmunoassay*

All the immunological reactions were performed in PBS supplemented with $CaCl_2$ 1 mM, $MgCl_2$ 0.5 mM, bovine serum albumin 0.5% and GTP 1 mM.

For a standard experiment the following procedure was used: to 50 μl iodinated tubulin (17 000 dpm) were added successively: unlabelled tubulin or a test sample, 100 μl; diluted antiserum (100 μl); plus anti-*Pneumococcus* antibodies (50 μl of a preparation containing 100 μg antibodies). These latter antibodies were used as carrier during the complex antigen–antibody precipitation reaction. The mixture was stirred gently and incubated for 18 h at 4°C. Then, 20 μl goat rabbit globulin antiserum (Institut Pasteur, Paris) were added in each tube for an additional incubation period of 5 h at 4°C. All the tubes were centrifuged in the cold at 2200 X g for 20 min, the supernatants discarded and the radioactivity found in the pellets was determined in a gamma counter.

3. Results and discussion

Analysis by acrylamide electrophoresis, under denaturing conditions (fig.1), shows that tubulin, eluted from a phosphocellulose column, is resolved

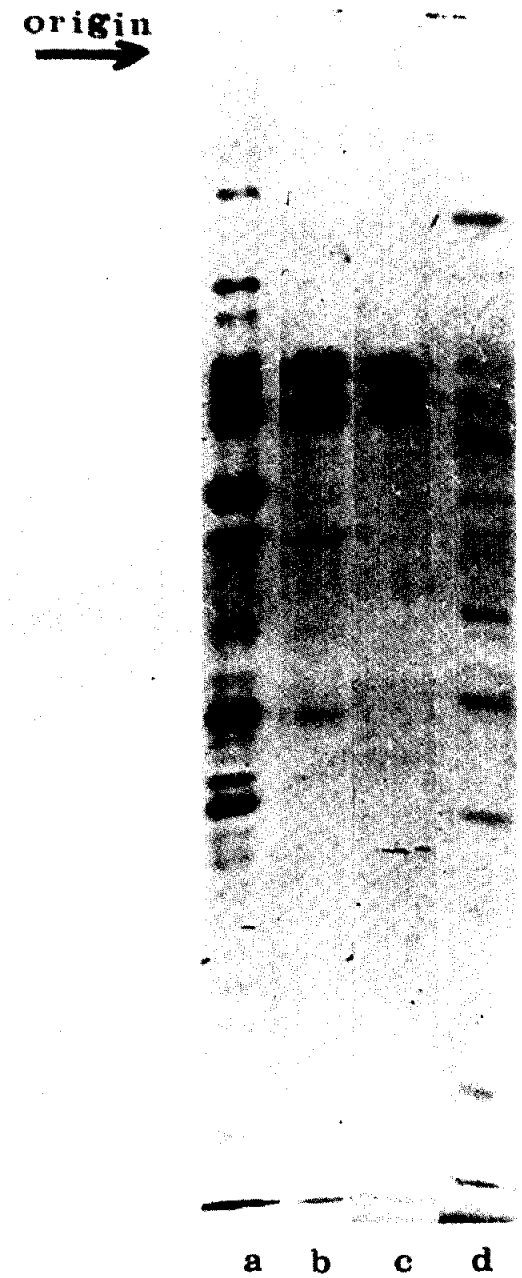

Fig.1. SDS–gel electrophoresis of different tubulin preparations. Slab gel electrophoresis was done in gels of 15% acrylamide and 0.087% bisacrylamide containing 0.1% $NaDodSO_4$ [13]. Gels were stained with Coomassie blue. (a) Mouse brain crude extract. (b) Tubulin obtained after two cycles of polymerization and depolymerization according to procedure [5]. (c) Purified tubulin separated on phosphocellulose column [6]. (d) Associated tubulin proteins retained on phosphocellulose column and eluted with MES–EDTA, 1 M NaCl buffer.

into two components designated as α and β tubulin subunits as previously described by Weingarten et al. [6]. These subunits have been shown to form microtubules in vitro, when appropriate association cofactors are present [6]. α and β subunits of tubulin, prepared in these conditions, were injected for immunization.

Six weeks after initial immunization, rabbit sera began to display an increasing capacity in the binding of [^{125}I]tubulin which was about twice that of unimmunized rabbits sera. The natural anti-tubulin antibody level, present in normal rabbit sera as previously described [10], can explain the low antibody concentration arising after immunization. The optimal sensitivity of the radioimmunoassay is obtained with an antiserum dilution of 1/200. For this dilution, 40% of the [^{125}I]tubulin (500 pg) present in the reaction mixture is bound to antibodies.

A typical dose response curve is shown in fig.2.

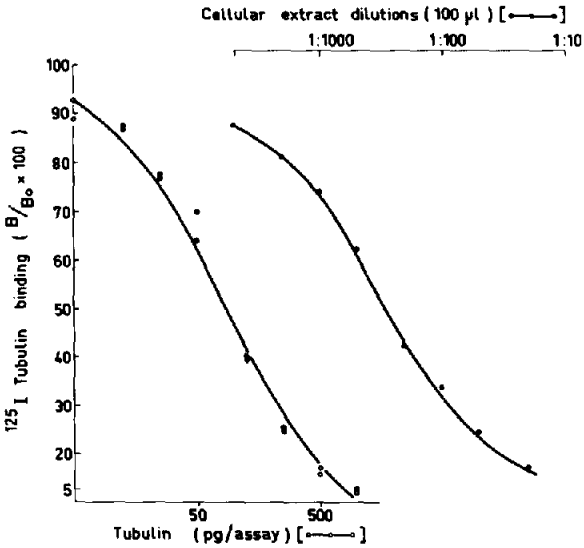

Fig.2. Inhibition of [^{125}I]tubulin fixation to antitubulin antibodies by increasing amounts of unlabelled tubulin (o–o–o) or dilutions of mouse brain cellular extract (•–•–•). The standard curve, obtained with unlabelled tubulin as inhibitor, has been calculated from the experimental values reported on the figure. The curve obtained with dilutions of cellular extracts reflects the mean of duplicate experimental values. Experiments were performed in presence of 1 mM GTP. (Bo) initial binding corresponding to 40% of [^{125}I]tubulin fixed to the antibodies (see Results and discussion). (B) Per cent of residual [^{125}I]tubulin bound in presence of different concentrations of unlabelled tubulin.

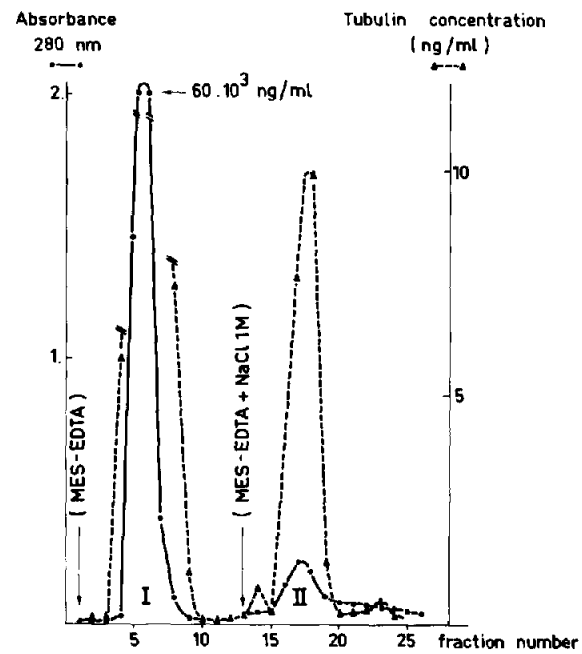

Fig.3. Determination of the tubulin concentration in the protein fractions eluted through the phosphocellulose column. Tubulin obtained after two cycles of polymerization according to procedure [5] was resuspended in MES–EDTA buffer and applied to a phosphocellulose column equilibrated in MES–EDTA. The tubulin (peak I) was eluted with this buffer and the remaining proteins were removed by 1.0 M NaCl in MES–EDTA (peak II). For each fraction, optical density at 280 nm (•–•–•) and immunoreactive tubulin (▲– – –▲) were determined.

The sensitivity of the radioimmunoassay permits measurement of quantities as low as 50 pg tubulin.

The specificity and avaibility of the radioimmunoassay have been tested by determination of the tubulin concentration present in a crude mouse brain extract (fig.2), in which tubulin represents about 10% of the total protein. Different dilutions of this extract were found to inhibit the fixation of [^{125}I]tubulin to the antibodies. The inhibition curve corresponding to these experimental values closely parallels the standard curve obtained when unlabelled purified tubulin was used as inhibitor (fig.2). Other samples corresponding to different steps of tubulin purification after one or more cycles of polymerization–depolymerization according to the procedure described by Weingarten et al. [5] have been tested for their

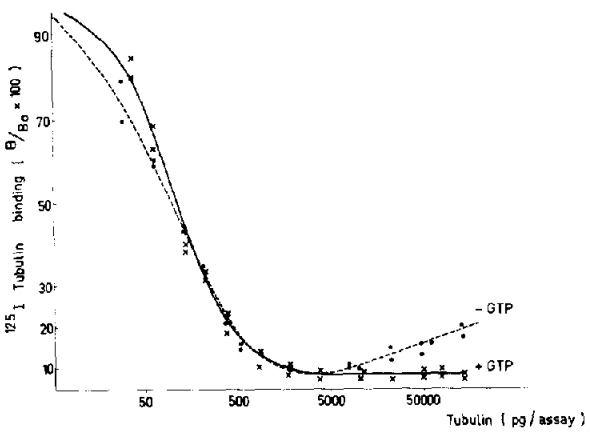

Fig.4. Influence of GTP on the shape of the log dose curve using the tubulin radioimmunoassay. These experiments were performed in PBS buffer described in Materials and methods, supplemented (X–X–X) or not (•– – –•) with 1 mM GTP.

capacity to displace the iodinated tubulin. In these cases too, the inhibition curves obtained with each sample show a close parallel with the standard curve. Another experiment to demonstrate the sensitivity of the radioimmunoassay technique is described as follows: we know that the tubulin can be completely purified by a passage on a phosphocellulose column equilibrated with MES–EDTA buffer [6]; the contaminants remain bound to the column and can be eluted with a buffer (MES–EDTA) containing 1 M NaCl. As is shown in fig.3 the tubulin concentration is calculated to be 10 ng/ml in the peak obtained after addition of 1 M NaCl to elute the remaining proteins. In the major peak fraction of the column corresponding to the tubulin eluted with MES–EDTA without salt addition the concentration was 60×10^3 ng/ml.

An interesting observation is related to the shape of the log dose–response curve of the radioimmunoassay. We found a displacement of the dose–response curve when RIA buffer was depleted of GTP (fig.4). These data could reflect a modification of the immunological properties of tubulin in relation to conformational changes induced by GTP as has already been described [6,11].

Recently another laboratory has independently developed a radioimmunoassay technique for tubulin determination [12], but our method is approx. 5000-fold more sensitive than that used [12].

This difference could be attributed to the variations in the tubulin preparation, the iodination procedures, or the RIA test which is used here in the presence of GTP.

The present radioimmunoassay would seem particularly appropriate to monitor eventual variations in tubulin concentrations during expression of cell differentiation or to analyse the products resulting from the in vitro translation of tubulin-mRNA in cell-free systems. Due to its high sensitivity, the method is particularly convenient to the study of tubulin synthesis in cultures of primary neurons in which small amounts of material are available. Experiments aimed at determining the tubulin synthesis in C1300 neuroblastoma cells during exponential growth or neurite formation are actually in progress.

Acknowledgements

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