TUBULIN ANTIBODY INHIBITS IN VITRO POLYMERIZATION INDEPENDENTLY OF MICROTUBULE-ASSOCIATED PROTEINS

Janet L. MORGAN, Carter R. HOLLADAY and Brian S. SPOONER

Division of Biology, Kansas State University, Manhattan, KS 66506, USA and *Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, England*

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1. Introduction

Regulation of the assembly and disassembly of microtubules is central to a variety of normal cellular activities, and may be involved in neoplastic transformation [1]. In vitro assembly, drug-binding properties, and amino acid composition and sequencing studies have yielded much information on the tubulin subunit molecule of microtubules [2]. In vivo studies of microtubules using disruptive agents like colchicine, and electron microscopic and immunofluorescent localization of cytoplasmic microtubules have led to a better understanding of the cellular functions of microtubules. Antibodies raised against tubulin have also been used as alternatives to colchicine-binding for tubulin quantitation [3–8] and for assessment of differences and similarities between tubulins from different sources [9,10]. Immunological differences between brain tubulins from lamb, mouse and chick has been demonstrated [11], and evidence provided that chick brain tubulin possesses a unique antigenic determinant in addition to ones common to all these species [11].

The present study examines the effect of tubulin antibodies on in vitro polymerization of microtubules and demonstrates dose-dependent antibody inhibition of assembly. Since inhibition could result from antibody binding to microtubule-associated proteins (MAPs), required for assembly, or from binding to tubulin at MAPs binding sites, we have used radioimmunoassay to compare antibody binding to tubulin in the presence and absence of MAPs. The results imply independence of antibody binding sites and MAPs binding sites on the tubulin molecule.

Address correspondence and reprint requests to: Dr B. S. Spooner, Division of Biology, Kansas State University, Manhattan, KS 66506, USA

2. Experimental procedures

2.1. *Materials*

$Na^{125}I$ was obtained from Amersham/Searle, Arlington, IL., guanosine 5′-triphosphate Type 11-S from Sigma Chemical Co., St Louis, MO. All other chemicals used were reagent grade.

2.2. *Protein isolation and purification*

Brains from day 14 chick embryos were homogenized in 0.1 M Pipes (piperazine-*N*,*N*′-bis [α-ethane sulfonic acid]) pH 6.8, then centrifuged at 40 000 × *g* for 45 min at 4°C. The supernatant was brought to 1 mM GTP, 0.5 mM EGTA (ethylene-glycol-bis-[β-aminoethyl ether] *N*,*N*′-tetraacetic acid), 1 mM $MgCl_2$ and 50% glycerol (v/v) and warmed to 37°C for 30 min. Microtubules were collected by centrifugation at 40 000 × *g*, 30 min at 37°C. The microtubule pellet was dissolved in 0.1 M Pipes, pH 6.8, 1 mM GTP, 1 mM $MgCl_2$, 0.5 mM EGTA and 50% glycerol at 4°C. This solution was warmed to 37°C for 30 min, and microtubules were collected as before. Tubulin was stored as a pellet at −70°C. Prior to use the tubulin pellets were resuspended in 0.1 M Pipes, pH 6.8, and centrifuged at 40 000 × *g* for 30 min at 4°C to remove any insoluble material. Tubulin was used at this purity (PD tubulin) or further purified by chromatography on phosphocellulose [12] (PC tubulin). Follow-

ing PC tubulin isolation and thorough washing of the column (200 ml 0.1 M Pipes/3 ml phosphocellulose), microtubule-associated proteins (MAPs) were eluted from the phosphocellulose with 1 M NaCl in 0.1 M Pipes, concentrated by Amicon filtration (PM-10 filter) and dialyzed overnight against 0.1 M Pipes.

2.3. *Antibody generation and application*

The PC tubulin was treated with glutaraldehyde and injected subcutaneously into the rabbit as in [1,4,5]. Pooled antiserum from a single rabbit, 147, was used for radioimmunoassay (RIA) or processed for immunoglobulin G (IgG) isolation. The globulin fraction of the immune serum was prepared by Na_2SO_4 precipitation [13], or by ammonium sulfate precipitation [14] and the IgG fraction was obtained by chromatography on DEAE-cellulose [15].

Tubulin RIAs were performed as in [5,11]. Tubulin was iodinated by a modification [11] of the method in [16].

2.4. *Polymerization assay*

Polymerization was quantitated by the centrifugation method in [17]. The PD tubulin was used in these experiments. The tubulin was incubated (2.5 mg/ml) for 30 min at 37°C in 0.1 M Pipes, pH 6.8, containing 1 mM $MgCl_2$, 1 mM GTP, and 0.5 mM EDTA, in the presence or absence of varying concentrations of immune or non-immune IgG and the presence or absence of 10^{-5} M colchicine. The tubulin content was constant in all samples and the total reaction volume was maintained at 0.5 ml. Following incubation, the tubes were centrifuged at 45 000 × *g* for 30 min at 37°C, and the protein content of pellets and supernates was determined by the Lowry method [18]. Sodium dodecyl sulfate (SDS) polyacrylamide gels were prepared by the Shapiro method [19].

3. Results

3.1. *Anti-tubulin interactions*

The antiserum against chick brain tubulin did not precipitate antigen in direct quantitative precipitation tests. Similarly, the IgG fraction of the rabbit antiserum failed to produce precipitating antigen–antibody complexes in direct precipitation tests. However, this same antiserum will specifically bind ~90% [^{125}I]tubulin preparations in double antibody coprecipitation tests, using goat anti-rabbit IgG antiserum to precipitate tubulin–antitubulin complexes [11]. Anti-tubulin activity has also been demonstrated in indirect immunofluorescence studies. Thus, the antiserum contains antibodies against tubulin, but they appear to be non-precipitating ones.

3.2. *Antibody effects on tubulin polymerization*

The observation that the chick brain tubulin–antitubulin complexes are non-precipitating provides an opportunity to assess antibody effects on in vitro polymerization, since antigen–antibody complexes do not interfere with tubulin polymer measurements in a centrifugation assay. PD tubulin was incubated for 30 min at 37°C under polymerization conditions, in the presence of anti-tubulin IgG or non-immune IgG. A parallel series simultaneously contained colchicine (10^{-5} M). Microtubules were collected by centrifugation, and protein determinations were made on pellets and supernates. The effects of immune and non-immune IgG are expressed as the percent of control polymerization and the results of a typical experiment are shown in fig.1. All data are corrected for non-colchicine-sensitive pellet protein, which averaged 10% total in this experiment. There is a progressive decrease in % polymer formed as the concentration of anti-tubulin IgG is increased. The antibody is seen to inhibit polymerization by 60% at the highest IgG concentration used, while non-immune IgG has no parallel effect. Preparations of IgG from different bleedings of this rabbit give different results. In one preparation we observed > 90% inhibition of polymerization with high concentrations of immune IgG. The microtubule pellets formed at low and intermediate IgG concentrations were opalescent as would be expected of pellets containing microtubules rather than opaque as an antigen–antibody precipitate would be. In addition, electron microscopy of negatively-stained specimens from each sample in the experiment shown in fig.1, reveals typical microtubules at all IgG concentrations except the highest concentration of immune IgG. Although a quantitative study was not attempted, the number of microtubules seemed to decrease with increasing IgG and, at the same time, the amount of extraneous material associated with the microtubules seemed to increase. Microtubules were absent from all colchicine-treated preparations.

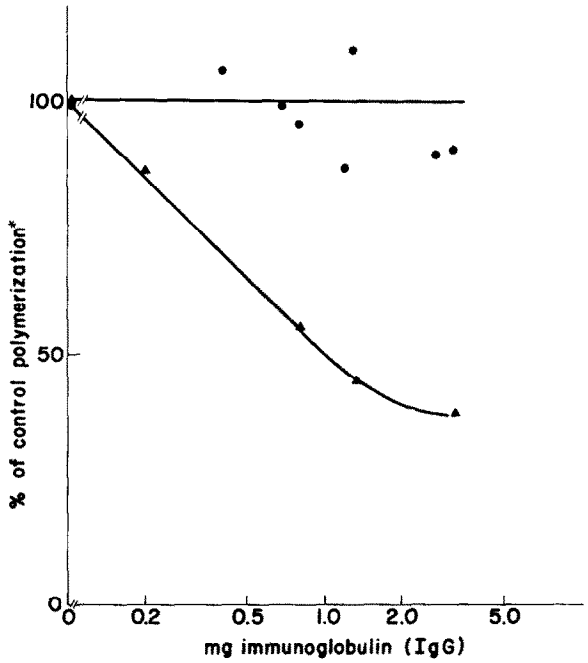

Fig.1. Anti-tubulin IgG inhibits tubulin polymerization in a concentration-dependent manner. Samples of PD tubulin were incubated with increasing amounts of non-immune (•) or immune IgG (▲) under polymerization conditions for 30 min at 37°C. Microtubules were collected by centrifugation at 37°C for 30 min. A parallel series of samples contained 10^{-5} M colchicine. Protein determinations were made on all pellets, and corrections for non-colchicine-sensitive pellet protein included. The data are expressed as % total colchicine-sensitive protein pelleted in the absence of any added IgG (control polymerization) as a function of mg IgG added. Each sample contained 1.25 mg PD tubulin in total vol. 0.5 ml. *Control polymerization in such experiments averages 50% total PD tubulin.

Thus, antibodies to tubulin inhibit in vitro polymerization in a concentration-dependent manner and, at intermediate concentrations, the aggregate that is produced is in the form of microtubules.

3.3. *Effects of MAPs on antibody–tubulin binding*

The possibility that antibody inhibition of tubulin polymerization resulted from antibody binding to MAPs, which can regulate tubulin assembly [20–23], was tested by radioimmunoassay. The binding of [^{125}I]tubulin to antiserum was totally unaffected by purified MAPs at 3500 ng protein/assay. At the same protein concentration, purified tubulin inhibited [^{125}I]tubulin binding by 90% (fig.2). To examine the possibility that antibody binding to tubulin was altered by MAPs (i.e., that they have the same or overlapping binding sites) we compared the abilities of unlabeled highly purified, MAPs-free, PC tubulin and unlabeled MAPs-containing PD tubulin preparations to inhibit [^{125}I]tubulin binding to antiserum by quantitative RIA. The results are shown in fig.2. When expressed per unit unlabeled protein, the inhibition curve for PD tubulin is less efficient than that for PC tubulin. There is an inhibition difference between the curves of 10–12% at all points from 20–3000 ng protein. However, the PC tubulin preparation is 99% tubulin and the PD tubulin preparation is 88% tubulin, determined by SDS–polyacrylamide gel electrophoresis and densitometry of stained gels. Thus, when expressed per unit tubulin, the inhibition curves for MAPs-free PC tubulin and MAPs-containing PD tubulin superimpose. Finally, if MAPs are added back to PC tubulin preparations, the inhibition curve generated as a function of tubulin content superimposes that of PC tubulin alone.

Thus, the antibody binds to tubulin, but not to MAPs, and the presence or absence of MAPs does not alter the efficiency or extent of binding.

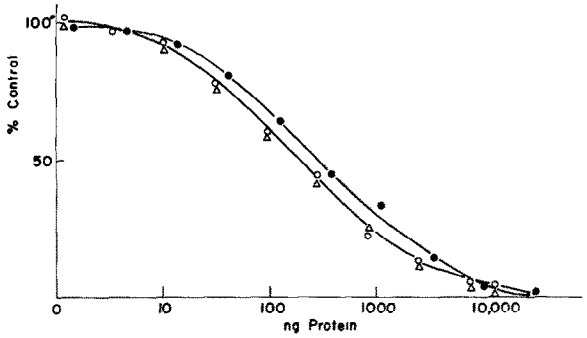

Fig.2. Effects of microtubule-associated proteins (MAPs) on tubulin–anti-tubulin interaction. Inhibition curves, generated using unlabeled chick brain PC tubulin (△) and chick brain PD tubulin (•), are consistent with the amount of tubulin present in the unlabeled tubulin samples as judged by SDS–polyacrylamide gel electrophoresis. The PC tubulin is ~99% homogeneous while the PD tubulin is about 88% tubulin. A third inhibition was generated by PC tubulin to which purified MAPs had been added back. The sample contained 88% PC tubulin and 12% purified MAPs. When only the PC tubulin present in the experiment is plotted (○), the inhibition curve generated superimposes on that generated by PC tubulin alone suggesting that the MAPs proteins do not interfere with the tubulin–anti-tubulin interaction.

4. Discussion

Tubulin–anti-tubulin complexes formed with antiserum or IgG described here are, operationally, not precipitating ones. This is clear from negative results in quantitative direct precipitin tests, while complexes are readily detected in double-antibody coprecipitation and RIA analyses. Whether the failure of complexes to precipitate is a function of small numbers of antigenic determinant sites on the tubulin molecule is unknown. It is clear that chick brain tubulin possesses at least two classes of antigenic determinants [11]. Whatever the reason, the non-precipitating nature of these complexes provides a unique ability to measure antibody effects on in vitro polymerization, since antigen–antibody complexes do not centrifuge out to interfere with tubulin polymer measurements in a centrifugation assay. The results show that anti-tubulin IgG inhibits polymerization in a concentration-dependent manner. The variation in final extent of inhibition observed with IgGs from different bleedings of the same rabbit is best explained by variations in antibody titer. The data may mean that the antibody binding site(s) on the tubulin molecule are at or near the site(s) of tubulin–tubulin interactions. However, it is also possible that the sites are spacially separate and that the presence of antibody blocks sufficiently close contact between tubulin molecules or will not allow a necessary conformational change to take place in the tubulin molecule so that it can polymerize. It is clear that the polymers that are formed at intermediate IgG concentrations are microtubules, since microtubules are observed by electron microscopy, and simultaneous presence of colchicine inhibits their formation.

The existence of MAPs that function in in vitro polymerization [20–23] and probably in vivo as well [24], allow the suggestion that the antibody effect is due to action on MAPs or at MAPs binding sites. However, the antibody does not bind MAPs nor do MAPs alter the antibody–tubulin interaction when sensitively analyzed by RIA. Thus, MAPs-free and MAPs-containing tubulin preparations bind antibody in direct accordance with their tubulin content. We are confident of the reliability of these RIA determinations, since all data points are done in triplicate, RIA results are highly reproducible [5], and the standard error for all data points in such experiments is $< 1\%$ [11]. Therefore, the RIA is specific for tubulin, sensitive and reproducible, and independent of MAPs. Thus, MAPs-binding sites and antibody-binding sites, both of which can affect polymerization are functionally independent. It will be of further interest to examine the drug-binding properties of the tubulin molecule, relative to its antibody-binding properties.

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