Quantitation and Characterization of Antibody Binding to Tubulin*

Livingston Van De Water, III and J. B. Olmsted
From the Department of Biology, University of Rochester, Rochester, New York 14627

A goat antiserum, raised to native hog brain tubulin, was characterized by conventional immunological techniques and by employing a method for precipitation of tubulin-anti-tubulin complexes with heat-inactivated Staphylococcus aureus. Antiserum dilution experiments indicated that antibodies to native tubulin were raised, and maximal binding was observed to microtubules fixed with 1 mM glutaraldehyde. Competition experiments, using iodinated fixed microtubules as tracer, demonstrated that equivalent binding occurred with microtubules at protein concentrations 100- to 1000-fold lower than those for monomeric tubulin.

A rabbit antiserum raised to sodium dodecyl sulfate-treated axonemal tubulin was also characterized. The serum bound maximally (90%) to either sodium dodecyl sulfate-treated or native iodinated hog brain tubulin, and competition for antibody binding has been observed with tubulin from diverse sources (Tetrahymena pyriformis ciliary axonemes, Lytechinus pictus flagellar axonemes, and mouse neuroblastoma extracts). Using these two antisera, radioimmunoassays are being developed for quantitation of polymeric and total tubulin in cellular systems.

Microtubules are found in all eukaryotic cells and have been implicated in multiple functions, including cellular shape changes, intracellular transport, secretion, and mitosis (1-3). Although the mechanisms involved in microtubule assembly in vitro have been studied extensively, the factors regulating assembly in vitro are largely unknown. Both in vitro (4) and in vivo (5) studies indicate that there is a dynamic equilibrium between the assembled and disassembled forms of tubulin, and the regulation of this equilibrium is of probable functional importance. Recently, several different experimental approaches have been used to attempt to estimate the proportion of the cellular tubulin in each pool. Using microtubule stabilization media and colchicine binding assays, a method has been devised for quantitation of stabilized and unstabilized forms of tubulin (6). Assembled tubulin has also been measured using ultrastructural stereological techniques and the total tubulin dimers per cell assayed by colchicine binding (7). However, the limitations of colchicine binding include the liability of the colchicine-binding complex and ambiguity in quantitating binding to polymeric or insoluble forms of tubulin. Consequently, several laboratories (8-13) have developed radioimmunoassays for tubulin as an alternative to the colchicine binding method. In addition, tubulin antibodies have been employed in a number of studies to identify the distribution of microtubules in cells (see Ref. 14 for examples).

The present paper reports the use of an immunoassay which exploits the capacity of protein A-bearing strains of Staphylococcus aureus to bind IgG molecules efficiently (15). This immunoassay has been used to characterize: 1) a goat antiserum, raised to native hog brain tubulin, which preferentially binds polymeric forms of tubulin; and 2) a rabbit antiserum, raised to Tetrahymena pyriformis ciliary tubulin, which binds either native or SDS-treated tubulin.2

EXPERIMENTAL PROCEDURES

Preparation of Tubulin and Microtubule Samples—Porcine microtubule protein was obtained by two cycles of polymerization-depolymerization (16) and tubulin purified free from microtubule-associated proteins by chromatography on DEAE-Sephadex A-50 (17). As shown in Fig. 1, SDS polyacrylamide gel electrophoresis (18, 19) of these preparations revealed a single band corresponding to tubulin.

Preparation of SDS-treated tubulin was carried out using a method of Hunter and Greenwood (21) and freed of excess iodine by chromatography on Sephadex G-25. Iodinated protein usually had a specific activity of 10,000 cpm/ug, and both labeled and unlabeled preparations were stored frozen at -80°C. Protein concentrations were determined by the method of Lowry et al. (22) using bovine serum albumin (bovine serum) as a protein standard.

Production of Antiserum—Purified tubulin (Fig. 1) in 5 mM Pipes (pH 6.9) was concentrated to 2 mg/ml and then sonicated with two volumes of complete Freund’s adjuvant to obtain a uniform emulsion. Immunization was carried out by injecting a male Nubian-Sannan goat with a total of 4 mg of protein at 20 intradermal sites on each flank (70 ug/site) and 1 ml (0.6 mg) intramuscularly in each thigh. After 25 days, serum was collected and stored frozen at -20°C. The goat was boosted at multiple sites with 2.5 mg of tubulin in incomplete Freund’s adjuvant 61 days after the initial injection. All experiments reported were carried out using goat serum from the first bleeding. Preimmune serum was obtained prior to the initial injection.

Antiserum to SDS-treated tubulin was raised in a rabbit using T. pyriformis axonemal tubulin in SDS sample buffer (18) as an immunogen. The antiserum was the generous gift of S. Guttman and M. A. Gorovsky, Department of Biology, University of Rochester.

Preparation of Bacterial Adsorbent—Protein A adsorbent was prepared from S. aureus (Cowman strain) by the method of Kessler (15). The amount of each preparation of bacterial adsorbent necessary

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1 The abbreviations used are: IgG, immunoglobulin G; SDS, sodium dodecyl sulfate; Pipes, 1,4-piperazinediethanesulfonic acid.

2 A preliminary report of these data was presented at the American Society of Cell Biology Meetings, San Diego, California, November, 1977 (1977) J. Cell Biol. 75, 980a
to bind the immune complexes quantitatively was routinely determined.

Antibody Dilution Assays—Serial dilutions of goat or rabbit serum were incubated with iodinated purified tubulin and the immune complexes collected with Protein A adsorbent. Typically, 1 to 5 ng of iodinated tubulin in 40 μl of saline/borate buffer (0.15 M NaCl, 0.05 M borate, 0.02% sodium azide containing 5 mg/ml of albumin at pH 7.4) was mixed with 40 μl of serum diluted between 1/4 and 1/1024 in the same buffer. Following incubation for 15 min at 4°C, a volume (40 μl) of 10% v/v Protein A adsorbent was added to each reaction tube and incubation continued for 10 min at 4°C. The suspension was then centrifuged at 3600 \times g for 10 min, and the pellet was resuspended in saline/EDTA/Tris buffer (0.15 M NaCl, 5 mM EDTA, 0.05 M Tris, 0.02% sodium azide containing 5 mg/ml of albumin at pH 7.4) containing 0.05% Nonidet P-40. The suspension was washed through 0.2 μm Cellogate filters (Millipore Corp.) and the filters were then dried and counted. Changes in volumes or temperatures are noted in the figure legends. In all assays, the total amount of radioactive protein in the samples was established by trichloroacetic acid precipitation of a standard aliquot.

Competition Assays—Competition assays were carried out by varying the amounts of competitor protein to be incubated with dilutions of goat or rabbit serum under conditions of antigen excess. Typically, 10 ng to 1 μg of iodinated tubulin in 40 μl of saline/borate buffer was mixed with 40 μl of diluted goat or rabbit serum and incubated for 90 min at 4°C. Iodinated tubulin (2 ng) in 20 μl of saline/borate buffer was added and incubation continued for 15 min at 4°C. Protein A adsorbent was then incubated with the antigen and antibody mixture for 10 min at 4°C, and samples were processed as described previously.

RESULTS AND DISCUSSION

To characterize the reactivity of the goat antiserum with tubulin, a number of standard immunological techniques were initially employed. Using rocket immunoelectrophoresis (23) or Ouchterlony assays (24), distinct immunoprecipitin lines were visible with partially purified preparations of tubulin. In the Ouchterlony assay, inclusion of iodinated purified tubulin with partially purified tubulin yielded superimposable patterns of radioactivity with precipitin lines, demonstrating that antibodies to tubulin were present (data not shown). Although faint precipitin lines were seen with pure tubulin, the observation that the tubulin anti-tubulin complexes were weakly precipitating necessitated the development of a more sensitive method for quantitating the binding reaction. A number of double precipitation methods were explored, and binding of immune complexes by S. aureus (Cowan I strain) (15) proved to be the most satisfactory. Conditions were established (outlined under "Experimental Procedures") in which the bacteria bound labeled tubulin goat antibody complexes quantitatively, and low backgrounds of binding were obtained with preimmune serum and buffer controls.

Using the Protein A adsorbent method, the goat antiserum was initially characterized by incubating fixed amounts of labeled purified tubulin (e.g. Fig. 1) with increasing dilutions of immune or preimmune serum. These antibody dilution curves (Fig. 2) exhibited the characteristic sigmoidal shape with plateaus at points of antibody excess, and indicated that antibodies to native tubulin were produced. A low level of binding was also observed in the control serum, suggesting that antibodies to native tubulin were present in the goat before immunization; this is consistent with other recent observations (25). However, at the dilution of immune serum used for competition assays (1/100 to 1/250), little binding to preimmune serum was observed. Dilution curves obtained using sera from several bleeds indicated that only about a 2-fold change in titer occurred, even after boosting.

At saturating amounts of serum, variations in the extent of binding were observed. Typically, 30 to 40% of the trichloroacetic acid-precipitable counts were bound by the goat serum, suggesting that only a fraction of the labeled tubulin was antigenic. To investigate the nature of this limited binding, experiments were initially carried out in which iodinated purified tubulin was fractionated on a Sepharose 4B column and the binding of fractionated tubulin to excess goat serum was determined. The major proportion of antibody binding occurred to fractions in the region of the void volume (molecular weights greater than 1 × 106) while most of the iodinated tubulin (acid-precipitable counts per min) eluted in fractions corresponding to the molecular weight of native 6 S tubulin (results not shown). Since all fractions contained only tubulin and antibody was in sufficient excess to allow binding to all tubulin, these data suggested that the antiserum bound preferentially to oligomeric classes of iodinated tubulin larger than 6 S.

The specificity of the goat antiserum for binding to polymeric tubulin was investigated further by preparing iodinated

![Fig. 1. SDS gel electrophoresis of purified tubulin. Tubulin was purified from porcine brain microtubule protein by two cycles of polymerization-depolymerization in Pipes/GTP buffer and chromatography on DEAE-Sephadex A-50. Purified tubulin (20 μg) was analyzed on a 7.5% polyacrylamide-SDS slab gel (18, 19) stained with 0.025% Coomassie blue (20) and scanned at 590 nm.](http://www.jbc.org/)

![Fig. 2. Goat serum dilution curve with iodinated tubulin as antigen. Iodinated purified tubulin (2 ng/sample) was incubated with dilutions of goat serum (1/16 to 1/4096) in saline/borate buffer containing albumin. Results are presented as the radioactivity bound by immune serum (●), preimmune serum (○), or buffer alone (---). In this experiment, immune serum at saturation was bound as a complex with approximately 30% of the iodinated tubulin.](http://www.jbc.org/)
Carried out in which tubulin centrifuged at 50,000 g for 2 h was used as competitor. Under these conditions, tubulin competed (50% level) at a protein amount 1000-fold greater than fixed microtubules (data not shown). These data indicate that the results presented in Fig. 4 represent a minimum estimate of the difference in binding of these two forms.

The possibility that the observed difference in affinity of the goat antiserum for the two tubulin preparations was due to the presence of microtubule-associated proteins on the microtubules has been investigated in preliminary experiments. Microtubules were assembled from purified tubulin in low concentrations, or to the presence of antibodies with low affinities for monomeric tubulin, similar experiments were carried out in which purified tubulin, predominantly in the 6 S form, and unlabeled, glutaraldehyde cross-linked microtubules were used as competitors for binding of the iodinated microtubules to the goat antiserum. As shown in Fig. 4, samples of sheared, fixed microtubules competed at amounts of protein that were at least 100 times lower than the amounts of monomeric tubulin needed to compete at the 50% level. Since the residual competition at high concentrations of monomeric tubulin might be due to either an increased number of oligomers at these protein concentrations, or to the presence of antibodies with low affinities for monomeric tubulin, similar experiments were carried out in which tubulin centrifuged at 50,000 \( \times \) g for 2 h was used as competitor. Under these conditions, tubulin competed (50% level) at a protein amount 1000-fold greater than fixed microtubules (data not shown). These data indicate that the results presented in Fig. 4 represent a minimum estimate of the difference in binding of these two forms.

Antiserum dilution assays were carried out over a wide range (1/4 to 1/260,000) with trace amounts (2 ng) of iodinated microtubules (Fig. 3). In contrast to the results obtained using iodinated tubulin (see Fig. 2), a high proportion of binding (88% of the trichloroacetic acid-precipitable counts) was observed using labeled microtubules as the antigen. This high level of binding was consistent with the previous data, suggesting preferential binding of oligomeric forms of tubulin by the goat antiserum. However, since other interpretations were possible, experiments were carried out in which purified tubulin, predominantly in the 6 S form, and unlabeled, glutaraldehyde cross-linked microtubules were used as competitors for binding of the iodinated microtubules to the goat antiserum. As shown in Fig. 4, samples of sheared, fixed microtubules competed at amounts of protein that were at least 100 times lower than the amounts of monomeric tubulin needed to compete at the 50% level. Since the residual competition at high concentrations of monomeric tubulin might be due to either an increased number of oligomers at these protein concentrations, or to the presence of antibodies with low affinities for monomeric tubulin, similar experiments were carried out in which tubulin centrifuged at 50,000 \( \times \) g for 2 h was used as competitor. Under these conditions, tubulin competed (50% level) at a protein amount 1000-fold greater than fixed microtubules (data not shown). These data indicate that the results presented in Fig. 4 represent a minimum estimate of the difference in binding of these two forms.

The possibility that the observed difference in affinity of the goat antiserum for the two tubulin preparations was due to the presence of microtubule-associated proteins on the microtubules has been investigated in preliminary experiments. Microtubules were assembled from purified tubulin alone using glycerol-containing buffer (17) and competition experiments carried out with the microtubules and with tubulin predominantly in monomeric form. Again, intact microtubules were found to compete at protein concentrations which are significantly lower than subunit tubulin. These experiments demonstrate that the goat antiserum can be used at dilutions which selectively bind to polymeric tubulin. These data are currently being extended to determine the affinity of the goat serum for various size ranges of tubulin, and to explore the use of the serum for studying specific tubulin conformations. In addition, since the goat antiserum will bind to glutaraldehyde-fixed microtubules, it should be useful as a probe for microtubules using immunochemical techniques at both the light and electron microscopic level (27).

In order to establish a radioimmunoassay in which total levels of tubulin could be measured independent of conformation or aggregation state, it was necessary to obtain antisera which would bind to all tubulin quantitatively. Several antisera raised against SDS-treated tubulin were therefore screened for binding to purified porcine brain tubulin. One antiserum raised in a rabbit using purified T. pyriformis tubulin in SDS sample buffer as an immunogen (gift of S. Guttman and M. A. Gorovsky) bound both SDS treated and native porcine tubulin. As shown in Fig. 5, an antibody dilution curve obtained using iodinated porcine brain tubulin showed the characteristic sigmoidal shape. However, in contrast to the goat serum (see Fig. 2), the rabbit serum bound maximally with approximately 90% of the iodinated subunit tubulin. Similar binding was also observed with the antibody if the iodinated brain tubulin was first treated with SDS. Therefore, the rabbit serum has similar titers and extent of binding regardless of the aggregation or denaturation state of the tubulin.

Because tubulin was observed to bind efficiently to the rabbit serum, even if denatured with SDS, it is possible to develop a radiolmunoassay for total tubulin. The results of a representative experiment, in which native purified tubulin was used as a competitor, are shown in Fig. 6. Competition was observed over a range of tubulin from 10 ng to 10 \( \mu \)g/sample. In a separate experiment, protein samples from T. pyriformis ciliary axonemes, L. pictus sperm axonemes, and mouse neuroblastoma extracts were used as competitors and competition was observed within a similar range of protein concentrations.
tubulin (1 ng) was incubated with dilutions of rabbit serum (1/4 to 1/4096) in saline/borate buffer containing albumin as described under "Experimental Procedures." Immune serum, preimmune serum, buffer, --. In a separate experiment, iodinated purified tubulin treated with 2% SDS, in 0.0625 M Tris, pH 6.8, was dialyzed for 18 h against saline/borate buffer without albumin. Trace amounts of protein (2 ng) were incubated (22°C) with dilutions (1/4, 1/32, 1/128) of rabbit serum in saline/borate buffer containing 0.5% Triton N-101 and albumin. The Protein A adsorbent was then incubated with the mixture, and samples were processed as outlined previously except all operations were at 22°C. Binding to immune serum, T. Results are presented as the percentage bound of the total trichloroacetic acid-precipitable antigen in each tube.

Fig. 5. Antibody dilution curve: rabbit serum. Iodinated purified tubulin (1 ng) was incubated with dilutions of rabbit serum (1/4 to 1/4096) in saline/borate buffer containing albumin as described under "Experimental Procedures." Immune serum, preimmune serum, buffer, --. In a separate experiment, iodinated purified tubulin treated with 2% SDS, in 0.0625 M Tris, pH 6.8, was dialyzed for 18 h against saline/borate buffer without albumin. Trace amounts of protein (2 ng) were incubated (22°C) with dilutions (1/4, 1/32, 1/128) of rabbit serum in saline/borate buffer containing 0.5% Triton N-101 and albumin. The Protein A adsorbent was then incubated with the mixture, and samples were processed as outlined previously except all operations were at 22°C. Binding to immune serum, X. Results are presented as the percentage bound of the total trichloroacetic acid-precipitable antigen in each tube.

values. Since this rabbit antiserum binds tubulin maximally regardless of species origin or molecular form, it should be possible to assay total tubulin pools in cellular extracts isolated under a variety of conditions and from a number of species.

Although a number of radioimmunoassays have been published for tubulin, the data presented here indicate the need for caution in interpreting results of such assays for tubulin or other self-associating proteins. Variations in the extent of antibody binding to tubulin have been reported (8, 13), and often such differences can be attributed to the labeling procedure used or incubation damage (28). However, as we demonstrated, these different levels of binding may also be due to a variety of monomeric or oligomeric species in solution for which the antiserum has different affinities. Since solution conditions used for the majority of tubulin radioimmunoassays are not those in which tubulin association reactions have been defined, controls are needed to ensure that assays, run under conditions where less than maximum binding can be demonstrated, are adequately documented with respect to species which bind to and compete for the antibody.

The antisera characterized are currently being used to develop assays for measuring the total tubulin pool and polymeric tubulin in clonal lines of neuroblastoma cells at various stages of neurite differentiation and the cell cycle. Using the Protein A adsorbent assay system, equivalent binding levels have been obtained in saline/borate buffer or in buffer containing nonionic detergents (Nonidet P-40, Triton X-100); this method should therefore be readily applicable for quantitation of fractions, such as insoluble or membrane-bound tubulin, which are extracted with these detergents. Assays have also been carried out in microtubule stabilization media, demonstrating the feasibility of using this method for quantitation of fractions derived from stabilized cell preparations. By combining the unique properties of the antisera with various preparative techniques, it is anticipated that it will be possible to have a reliable, sensitive assay for various states of tubulin within cells and thereby facilitate studies on the biological regulation of tubulin distribution.

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L Van De Water, 3rd and J R Olmsted


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