Antisera against electrophoretically purified tubulin stimulate colchicine-binding activity
(chick brain/Ouchterlony/immunofluorescence)

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ABSTRACT Several rabbit antisera have been prepared against reduced and alkylated, electrophoretically purified tubulin isolated from chick brain. These antisera give a single precipitin line in Ouchterlony double diffusion plates when tested against partially purified tubulin, and label specifically microtubule- and tubulin-containing structures, such as mitotic spindles, cilia, and vinblastine-induced crystals, in a variety of cells. The same antisera also display the unique ability to stimulate the colchicine-binding activity of tubulin preparations from chick brain and Chinese hamster ovary tissue culture cells. This specific stimulation of colchicine-binding activity is also obtained with the gamma globulin fractions purified by ammonium sulfate precipitation of these antisera.

Intensive effort has been directed at characterizing the distribution, ultrastructure, and mode of action of microtubules; their assembly from tubulin subunits; and their response to various agents, for example, temperature, pressure, and the plant alkaloids colchicine and vinblastine (1–5). Recent efforts have centered upon the raising of specific antisera (6–12) so that microtubule structure can be studied immunologically. The antisera have been prepared in different ways against intact microtubules or tubulin, using for immunization preparations having various degrees of purity obtained from a number of sources, including vinblastine-induced paracrystals, flagellar outer doublets, and tubulin isolated from brain. By the fluorescein-labeled antibody technique, the antisera have been observed to stain specifically microtubule- and tubulin-containing structures such as vinblastine paracrystals (6), mitotic spindles (6, 7, 10), and fibrous networks in the cytoplasm (11). Whether or not these antisera affect the rather specific binding of colchicine to tubulin (3, 4) has not been reported.

We report here the preparation and initial characterization of antisera produced against electrophoretically purified, reduced, and alkylated tubulin from chick brain. These antisera precipitate native tubulin and label microtubule-containing structures in a variety of species. Moreover, they show an interesting interaction with the colchicine-binding protein from chick brain and Chinese hamster ovary (CHO) cells.

MATERIALS AND METHODS

Antigen. Tubulin was extracted from brains of 14-day-old chick embryos using the polymerization-depolymerization method of Shelanski et al. (13). The tubulin obtained was reduced and alkylated (14) and further purified by electrophoresis on 7.5% preparative polyacrylamide gels in 0.1% sodium dodecyl sulfate. After the positions of the α and β tubulin bands were located by scanning the gels at 280 nm, they were cut out and the tubulin was eluted and used for immunization. About 30% of the original amount placed on the preparative gels was recovered. When the recovered tubulin was tested by analytical gel electrophoresis, only the two tubulin bands were detected.

Antisera. Each of several rabbits was injected subcutaneously with a total of 1 mg of the reduced-alkylated, electrophoretically purified tubulin in complete Freund's adjuvant, over a period of 6 weeks. A detailed method for preparing the antisera will be published elsewhere (manuscript submitted for publication). The antisera obtained were tested by Ouchterlony double diffusion in 1% agarose gels at 4° for 24 hr. Control sera were obtained from the same rabbits prior to immunization.

Cells. Mouse embryo fibroblasts, HeLa cells, and CHO cells were grown on coverslips in α-minimal Eagle's medium and 10% fetal calf serum (Flow Laboratories). To induce crystal formation, 10 μg/ml of vinblastine was added to the HeLa cell cultures for a 4-hr period prior to fixation. Ciliated cells were obtained by scraping the epithelium from 19-day-old chick tracheas.

Immunofluorescent Studies. Cells were fixed in phosphate-buffered formaldehyde for 30 min at room temperature, washed, and air-dried. They were covered with antiserum against tubulin diluted 1:10 and left for 45 min at room temperature in a humidified chamber. After several washes, the cells were treated for 30 min with fluorescein-labeled goat anti-rabbit IgG (Hyland Laboratories, F/P molar ratio 4:6) diluted 1:5 and washed again. All preparations were mounted in 50% glycerol in phosphate buffer (pH 7.4) and examined in a Zeiss UV microscope.

Immunoglobulin Preparations. Gamma globulin fractions of the sera were prepared as follows by adding 1 volume of 100% saturated ammonium sulfate to 2 volumes of whole serum. Precipitation was allowed to proceed for 30 min at 4°. The precipitate was collected by centrifugation at 3000 X g for 30 min and the pellet redissolved in a volume of phosphate-buffered saline equal to the original serum volume. The globulin was dialyzed overnight at 4° against 1 liter of phosphate-buffered saline before use. In some cases, the ammonium sulfate precipitation was repeated twice more to ensure a purer fraction.

Preparation of CHO Cell Supernatants. CHO cells were grown in suspension in α-minimal Eagle's medium and 10% fetal calf serum. Cells in exponential phase of growth (approximately 3 X 10⁶ cells per ml) were collected by centrifugation, washed in an equal volume of cold phosphate-buffered saline, resuspended in 2-(N-morpholino)ethanesulfonic acid (Mes) containing 0.1% Triton X-100 (Rohm and Haas), and hand-homogenized with 75 strokes in a glass Dounce
obtained in polymerization-depolymerization diffusion our acid measurement mined by chick brain tisirum. colchicine in assayed phate-buffered saline, at beled properd and dilution and M aliquots those to chick chicine stream cific activity thoxy-3H\textsuperscript{3H}colchicine binding assays.

The total of each measure Activity Assay. Colchicine-Binding Tests using the indirect fluorescein-labeled antibody technique. (a) Ouchterlony double diffusion plate showing the precipitin line between the well containing 50 \mu g of antiserum against tubulin (A) and that containing 100 \mu g of partially purified chick brain tubulin (T). (b) A mouse embryo fibroblastic undergoing mitosis with a clearly stained mitotic spindle. (c) Ciliated cell from the chick trachea epithelium, showing the stained ciliated border in the apical end. (d) HeLa cell treated with vinblastine, showing staining of the induced paracrystals.

homogenizer. The homogenate was centrifuged at 15,000 \times g for 15 min, and the supernatant collected for the colchicine-binding assays.

Colchicine-Binding Activity Assay. Radioactive [methoxy-\textsuperscript{3H}]colchicine in benzene:ethanol with an initial specific activity of 5 Ci/mmol was obtained from New England Nuclear. The solvent was removed in the dark under a stream of nitrogen gas and the drug redissolved in phosphate-buffered saline with an amount of unlabeled colchicine (Sigma Chemical Co.) to give an appropriate final colchicine concentration.

To measure the amount of colchicine-binding activity in chick brain or CHO intracellular material, methods similar to those described by Weisenberg et al. (15) were used. Duplicate aliquots (10–50 \mu l) of tubulin in Mes were added to an equal volume of the antiserum or solutions of IgG of appropriate dilution and were incubated for 18 hr at 4°. Labeled colchicine was added to a final concentration of 2 \times 10^{-5} M (2 Ci/mmol) and binding allowed to occur for 2.5 hr at 37°C. The total of each reaction volume was applied to a 2.5-cm DE81 paper disc (Whatman) prewetted with phosphate-buffered saline, and the amount of bound colchicine was assayed as previously described (16). Colchicine-binding activity was expressed as a percent of the amount of bound colchicine in tubulin samples incubated in the absence of antiserum.

Protein concentrations of the immunoglobulin solutions, chick brain tubulin, and CHO cell supernatants were determined by the method of Lowry et al. (17) and by a turbidity measurement on a solution containing 10% trichloroacetic acid (18).

RESULTS

When our antisera were examined by Ouchterlony double diffusion analysis against tubulin purified by two cycles of polymerization-depolymerization in vitro (13), only one strong precipitin line was observed (Fig. 1a). The antisera obtained were also able to precipitate native tubulin in solu-

tion (data not shown). The antisera against tubulin thus appear to recognize native tubulin, even though they were prepared against tubulin denatured by reduction-alkylation and eluted from sodium dodecyl sulfate-polyacrylamide gels. The fact that denaturation often allows one to raise antisera against proteins that are otherwise poor immunogens is well known. Furthermore, Lazarrides and Weber (19), in preparing antiserum to actin, demonstrated that sodium dodecyl sulfate purification of a protein lessens the possibilities of contamination by proteins of other molecular weight.

By using the indirect fluorescein-labeled antibody technique, the antisera against tubulin were used to examine cells from a number of different species. Specific fluorescence of the mitotic spindles of mouse embryo fibroblasts (Fig. 1b) and CHO cells (not shown), and of the ciliated border of epithelial cells from chick trachea (Fig. 1c) was observed. Vinblastine-induced paracrystals in HeLa cells were also stained by this technique (Fig. 1d). These examples demonstrate that the antibody produced against highly purified chick brain tubulin crossreacts with microtubule- and tubulin-containing structures in a number of different species. Moreover, they support the concept of the universality and highly conserved nature of tubulin structure (1–5, 7).

Since colchicine is thought to bind specifically to tubulin dimers, it was of interest to determine whether or not the antisera produced against tubulin interfere with this binding. Fig. 2 shows that when tubulin treated with various concentrations of the antisera was incubated with [\textsuperscript{3H}]colchicine in an in vitro binding assay, colchicine-binding activity increased with increasing concentrations of antiserum. A plateau was reached at a serum dilution of about 1:20, when colchicine binding was stimulated almost 2-fold. No stimulation was observed with any of the control sera obtained from rabbits before immunization, nor do any of the sera, assayed under these conditions, contain colchicine-binding activity. The fact that our antisera perturb the binding of colchicine to tubulin, albeit in an unexpected manner (stimulation rather than inhibition), provides additional support
Cell binding was consistent with colchicine-binding in our ecule of colchicine-binding of tubulin. Antisera or pre-immunized control sera at various dilutions in phosphate-buffered saline were incubated overnight at 4°C with equal volumes of purified chick brain tubulin, after which their colchicine-binding activity was measured at 37°C, as described in Materials and Methods. Colchicine-binding activity (100%) was taken as the amount of colchicine bound by a sample of tubulin that had not been treated by serum, which usually bound about 10^3 cpm of [3H]colchicine. Antiserum from immunized rabbit α; control serum from the same rabbit before immunization. 

FIG. 2. Stimulation of colchicine-binding activity by antiserum against tubulin. Antisera or pre-immunized control sera at various dilutions in phosphate-buffered saline were incubated overnight at 4°C with equal volumes of purified chick brain tubulin, after which their colchicine-binding activity was measured at 37°C, as described in Materials and Methods. Colchicine-binding activity (100%) was taken as the amount of colchicine bound by a sample of tubulin that had not been treated by serum, which usually bound about 10^3 cpm of [3H]colchicine. Antiserum from immunized rabbit α; control serum from the same rabbit before immunization.

for the presence of an antibody specific for the tubulin molecule in our antisera.

From Fig. 2 it can be observed that a small decrease in colchicine-binding activity occurs with pre-immunized control sera and antisera against tubulin. Because this inhibition of colchicine-binding was present in both sera, it may be due to some nonspecific interaction of serum components with the colchicine-binding assay.

To further establish that the stimulation of colchicine binding was consistent with the presence of a specific antibody, and in an attempt to remove the inhibitory component(s) present in both normal and immune sera, immunoglobulin fractions were prepared by ammonium sulfate precipitation (see Materials and Methods). Fig. 3 indicates that the anti-tubulin globulin again stimulates colchicine-binding activity. However, immunoglobulin from both the antisera and pre-immunized control serum still display about equal amounts of inhibitory activity at the lower dilutions. That this stimulation of colchicine-binding activity occurs only in globulin fractions obtained from antisera against tubulin is shown in Table 1. Pre-immunized sera and antisera from animals injected with antigens unrelated to tubulin, including antisera to actin prepared by sodium dodecyl sulfate electrophoresis (preliminary observations), do not show any stimulatory activity.

FIG. 3. Stimulation of colchicine-binding activity by anti-tubulin immunoglobulin. Globulin fractions were prepared as described in Materials and Methods. The colchicine-binding activity of chick brain tubulin in the presence of various dilutions of globulins was measured as described in legend of Fig. 2. Each point is the average of duplicate samples from two independent experiments. Anti-tubulin globulin α; pre-immunized serum globulin O.

Table 1. Colchicine-binding activity of tubulin preparations treated with various immunoglobulin fractions

<table>
<thead>
<tr>
<th>Immunoglobulin fractions tested</th>
<th>Immunoglobulin added mg/ml</th>
<th>10^2</th>
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<tbody>
<tr>
<td>With chick brain tubulin</td>
<td>% Colchicine-binding activity ± standard deviation</td>
<td>10</td>
</tr>
<tr>
<td>AS-1</td>
<td>68 ± 5</td>
<td>165 ± 2</td>
</tr>
<tr>
<td>NS-1</td>
<td>67 ± 5</td>
<td>70 ± 10</td>
</tr>
<tr>
<td>AS-2</td>
<td>89 ± 2</td>
<td>161 ± 3</td>
</tr>
<tr>
<td>NS-2</td>
<td>88 ± 8</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>AS-7</td>
<td>99 ± 10</td>
<td>99 ± 10</td>
</tr>
<tr>
<td>NS-7</td>
<td>93 ± 10</td>
<td>102 ± 3</td>
</tr>
</tbody>
</table>

With CHO cell supernatants

<table>
<thead>
<tr>
<th>Immune globulin fractions tested</th>
<th>10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-1</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>NS-1</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>AS-2</td>
<td>125 ± 5</td>
</tr>
<tr>
<td>NS-2</td>
<td>100 ± 2</td>
</tr>
</tbody>
</table>

AS-1 and AS-2 are anti-tubulin globulin fractions from two different rabbits; NS-1 and NS-2 are globulin fractions from the corresponding pre-immunized control sera; AS-7 is globulin from an antisera to mouse hemoglobin; NS-7 is the globulin from the corresponding control serum.

Table 1. Colchicine-binding activity of tubulin preparations treated with various immunoglobulin fractions

served with our antisera and their globulin fractions is not due to a simple stabilization of the colchicine-binding sites of tubulin, but raises the intriguing possibility that the interaction of antibody with tubulin perturbs the conformation of tubulin molecules such that additional colchicine-binding sites, not previously detected, are now exposed.

**DISCUSSION**

In this report we describe the preparation and characterization of antisera against electrophoretically purified chick brain tubulin. As demonstrated previously for the purification of actin (19), the use of tubulin prepared by this method greatly reduces the possibility of contamination by a number of other polypeptides which have been observed to copurify with tubulin prepared by other techniques (7, 9, 13). This technique of preparation, after a preliminary denaturation by reduction-alkylation, assures the use of a highly purified antigen for injection. Our antisera share properties in common with other antisera prepared against microtubule proteins previously reported, namely, (i) they form a single precipitin line in Ouchterlony double diffusion plates against purified tubulin and (ii) they label specifically a variety of microtubule- and tubulin-containing structures in cells from several different species. However, unlike other reported antisera to microtubule proteins, our antisera against tubulin appear to stimulate the colchicine-binding activity in tubulin preparations. Since this stimulatory activity is also present in the gamma globulin fractions of the antisera, it is likely due to the interaction of a specific antibody with the tubulin molecule, although verification of this point will rest on the purification of the specific anti-tubulin immunoglobulin from the total globulin fractions used here. While the nature of this interaction is not understood at present, it is possible that just as antibodies against various enzymes have been observed to increase the activity of the enzymes by causing conformational changes when the antibody binds to the enzyme (21–25), so antibody in our antisera may alter the colchicine-binding site in a similar way.

The antisera against tubulin described in this report have been useful in the detection of microtubule- and tubulin-containing structures in cells and should make possible study of the effects of various stimuli on microtubules and their distribution. Since the antisera against tubulin have been shown to affect the colchicine-binding activity in tubulin preparations, we have demonstrated the usefulness of the colchicine-binding assay in the detection of a probable specific antibody to the tubulins themselves. Furthermore, the globulins of these antisera should be useful also as molecular probes to study the colchicine-binding sites of the microtubule proteins.

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