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Species-dependent immunological differences between vertebrate brain tubulins

(radioimmunoassay/iodination/coprecipitation)

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ABSTRACT The antigenic similarities and differences between highly purified brain tubulins from lamb, mouse, and chick embryo have been examined using rabbit antisera prepared against each of these tubulins. These antisera are capable of binding ^{125}I-labeled tubulin in homologous or heterologous combinations, demonstrating immunological similarity between the tubulins. However, there are quantitative differences in the maximum amount of binding observed. Differences between the tubulins were further resolved by radioimmunoassays, comparing the ability of each of the tubulins to inhibit the binding of each ^{125}I-labeled tubulin to each antiserum. Competition curves generated for all possible combinations revealed quantitative immunological differences between the tubulins that imply different densities of shared antigenic determinants on all three tubulins and a unique determinant on the chick tubulin molecule.

Microtubules are dynamic organelles that are present in all eukaryotic cells and participate in a variety of cellular activities (1). The major structural protein of microtubules is tubulin, a heterodimer composed of two closely related polypeptides, α and β tubulins (2–4). There is abundant evidence that tubulin is a highly conserved molecule, but there is also increasing evidence suggesting differences between tubulins from different species and from different classes (flagellar, mitotic, and ciliary) of microtubules. The conservative nature of tubulins is best demonstrated in the comparison of the sequence of the first 25 amino acids of the NH_2-terminal end of α and β tubulins from embryonic chick brain microtubules and central pair microtubules of sea urchin sperm tails (5), and by comparisons of amino acid compositions of tubulins from several other sources (2, 5, 6). In addition, antibodies raised against different classes of tubulins show a high degree of crossreactivity with tubulins from various sources. For example, antibodies generated against outer doublet flagellar tubulins of sea urchin sperm tails crossreact with mitotic spindle microtubules and ciliary microtubules, as well as with outer doublet tubulins from other species (7–9). Antibodies against brain cytoplasmic tubulin have also been shown to crossreact with microtubules in a number of different tissue culture cell types, by immunofluorescence (10).

The conservative nature of tubulins is consistent with the fundamental involvement of microtubules in essential cellular functions. However, differences between tubulins may be crucial to microtubule regulation for different functions in different species and different cell types. Differences have been noted in electrophoretic mobility of tubulin subunits derived from the three classes of microtubules (11, 12) and in the quantitative immunoprecipitation of tubulins from different species (7, 13). Published immunological data suggest differences between tubulins from different classes of microtubules and species differences between flagellar outer doublet tubulins.

The present study has quantitatively analyzed the purified cytoplasmic brain tubulins from three different vertebrate species by radioimmunoassay. The results demonstrate that although heterologous combinations of tubulin and antisera will interact, there are clear quantitative differences in the ability of the various radiolabeled tubulins to bind to the antibodies and in the ability of the various unlabeled tubulins to compete with the different labeled tubulins for antibody binding sites.

MATERIALS AND METHODS

Tubulin Purification. Tubulin was isolated and purified from lamb brain, mouse brain, and 14-day embryo chick brain by two cycles of polymerization in 0.1 M 1,4-piperazinediethanesulfonic acid (Pipes) (pH 6.8), containing 1 mM GTP, 1 mM $MgCl_2$, 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate (EGTA), and 50% glycerol, followed by chromatography on a column of phosphocellulose (14, 15). Purity was assessed by electrophoresis on sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels (16).

Immunization and Antigen Iodination. Antisera to lamb, mouse, and chick brain tubulins were produced by immunizing rabbits with purified antigen that had been partially crosslinked with glutaraldehyde (17). Rabbits were injected subcutaneously with 2 mg of antigen in complete Freund's adjuvant, followed at 2-week intervals by boosters in incomplete Freund's adjuvant. Each antiserum discussed in this paper was collected from a single rabbit.

Purified tubulins were radiolabeled with ^{125}I through the iodinated imidoester, methyl-3,5-diiodo-*p*-hydroxybenzimidate, by a modification of the technique of Wood *et al.* (18). Ten microliters of a 20 mM solution of methyl-*p*-hydroxybenzimidate·HCl in 50 mM sodium borate (pH 8.5) was mixed with 10 μl of a 40 mM chloramine T solution, 10 μl of a 40 mM NaI solution, and 10 μl of ^{125}I ($\sim$1 mCi) and allowed to react at room temperature for 15 min. The reaction was stopped by the addition of 1 μl of 1 M 2-mercaptoethanol, and the iodinated imidoester was precipitated by neutralizing with 1 M acetic acid ($\sim$5 μl). The iodinated imidoester was collected by centrifugation at 10,000 × *g* for 5 min, and the pellet was dissolved in 25 μl of 50 mM sodium borate (pH 8.5). Approximately 25 μg of purified tubulin was added to the iodinated imidoester, and the mixture was reacted for 24 hr at room temperature. Unbound iodinated imidoester was then removed from the solution by dialysis against borate-buffered saline (pH 8.0), and the iodinated protein was diluted to 10 ml with borate-buffered saline

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Abbreviations: ^{125}I-tubulin, ^{125}I-labeled tubulin; $NaDodSO_4$, sodium dodecyl sulfate.

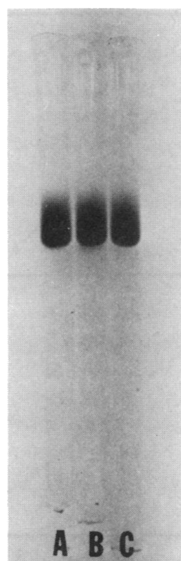

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis of purified tubulin from (*A*) mouse brain, (*B*) lamb brain, and (*C*) chick brain. One hundred micrograms of each protein was electrophoresed. Gels were stained with Coomassie blue. Only the 55,000-dalton tubulin subunit is visible on these stained gels.

containing bovine serum albumin (5 mg/ml) and stored at −70°. The specific activity of this preparation was about 4000 cpm/25 ng of tubulin. In one experiment, lamb brain tubulin was iodinated with lactoperoxidase (19) to a specific activity of about 100,000 cpm/0.5 ng of tubulin.

Coprecipitation and Radioimmunoassay. The ability of various antisera to bind various ^{125}I-labeled tubulins (^{125}I-tubulins) was determined by coprecipitation tests in which identical amounts of labeled tubulin were incubated with increasing amounts of rabbit antiserum, and then sufficient goat anti-rabbit IgG was added to precipitate all rabbit IgG. Precipitates were collected by centrifugation, and the pellet and supernate radioactivity was determined in a gamma-well scintillation counter. The results were corrected for nonspecific binding to nonimmune rabbit serum and instrument background, and expressed as percentage of total trichloroacetic acid-precipitable counts specifically bound by the antiserum. This indirect precipitation test does not rely on the formation of precipitating tubulin–anti-tubulin complexes.

The procedures for indirect radioimmunoassay of brain tubulin have been published (20). Briefly, a quantitative competition or inhibition curve was generated by competition between increasing amounts of unlabeled and ^{125}I-labeled tubulin for a constant, limited number of antibody binding sites. The maximum or control amount of labeled tubulin bound by the antiserum is the amount bound in the absence of unlabeled tubulin. The data in the inhibition curves are expressed as the percentage of the uninhibited control specifically precipitated compared to the amount of unlabeled tubulin in the assay. As in the coprecipitation tests, the data were corrected for background counts, nonspecific binding, and radioactivity that is not precipitable by trichloroacetic acid. Again, the assay does not depend on the formation of precipitating tubulin–anti-tubulin complexes.

Protein Determination and Electrophoresis. Protein concentrations were determined by the method of Lowry *et al.* (21). NaDodSO$_4$/polyacrylamide gel electrophoresis was by the method of Shapiro *et al.* (16), with 7.5% gels (0.1% NaDodSO$_4$), and gels were stained with Coomassie blue.

Table 1. Maximum percent of labeled tubulin specifically bound to antisera

	Maximum percent bound*†		
^{125}I-Tubulin	Chick antiserum	Lamb antiserum	Mouse antiserum
Chick	89 (50)	44 (50)	74 (50)
Lamb	68 (30)	32 (75)	56 (30)
Mouse	81 (20)	67 (75)	91 (30)

* Microliters of antiserum required is in parentheses.

† The percentages are shown from a single iodination series. Exact percentages vary from iodination to iodination, but the pattern is always the same.

RESULTS AND DISCUSSION

Tubulin purity and antigenicity

Brain tubulins purified by two cycles of polymerization and chromatography on phosphocellulose are free of microtubule-associated proteins, including the high-molecular-weight proteins and the tau factors implicated in tubulin assembly (14, 15, 22). They are at least 98–99% pure, as determined by electrophoresis on NaDodSO$_4$/polyacrylamide gels (Fig. 1). No polypeptides other than the 55,000-dalton tubulin monomer can be seen on the stained gels of 100 μg of purified chick, mouse, or lamb brain tubulin. Thus, any individual contaminant(s) would have to represent only a minor fraction of the total protein or have a molecular weight of about 55,000 and copurify with tubulin. Furthermore, antisera raised against these tubulin preparations will bind the bulk of the protein that can be iodinated in identical preparations (Table 1). This suggests an antiserum to tubulin since it is unlikely that a minor contaminant would be labeled while tubulin remained unlabeled. The fact that the bulk of ^{125}I-protein can be bound by the antisera is therefore consistent with the presence of antibodies against tubulin and a high degree of tubulin purity in our preparations, and inconsistent with the presence of nontubulin antigenic contaminants (of similar or different molecular weight) in the same preparations. Finally, tubulin–anti-tubulin interactions are unaffected by the presence of actin and other proteins (20), including microtubule-associated proteins (data not shown). Thus, we are confident that we are dealing with tubulin–anti-tubulin interactions.

Tubulin differences by coprecipitation and radioimmunoassay competition

The antisera raised against lamb, chick, and mouse tubulins do not form precipitating antigen–antibody complexes with highly purified tubulins. Therefore, the immunological relationships between the tubulins could not be analyzed by quantitative precipitin tests. The more sensitive radioimmunoassay and coprecipitation test using goat anti-rabbit IgG to precipitate all the tubulin–anti-tubulin complexes allows the comparisons to be made because all complexes are collected for analysis.

Coprecipitation tests were conducted on the different tubulins to determine the ability of the various antisera to bind each of the ^{125}I-tubulins. For each determination, 25 ng of purified lamb, chick, or mouse brain ^{125}I-tubulin was incubated with increasing amounts of each antiserum. All data for each ^{125}I-tubulin preparation were collected on the same day. The results in Table 1 show maximum percent of each labeled tubulin bound by each antiserum. Antisera against chick and mouse tubulins bound their homologous tubulins best, while antiserum against lamb brain tubulin bound lamb ^{125}I-tubulin least well. This result seems not to be simply a function of the

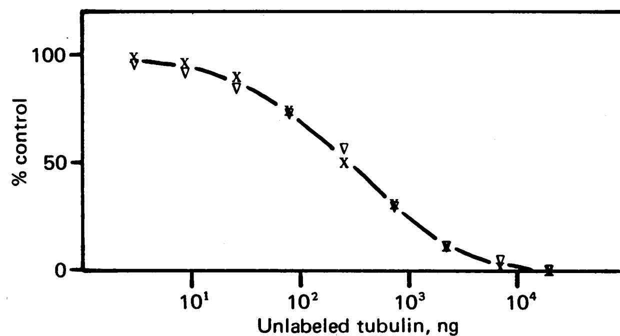

FIG. 2. Quantitative radioimmunoassay. Curves show the ability of increasing concentrations of unlabeled chick brain tubulin to inhibit the binding of ^{125}I-tubulin labeled with lactoperoxidase (X) or iodinated imidoester (∇) to antiserum against chick brain tubulin. The competition curves superimpose, demonstrating that the method of iodination does not alter the assay.

lamb ^{125}I-tubulin preparation, since the antisera against both chick and mouse brain tubulins bind higher percentages of the lamb ^{125}I-tubulin. These coprecipitation tests demonstrate immunological crossreactivity between the various tubulins, but also show that the degree of crossreactivity is quantitatively distinguishable.

To further explore these immunological relationships, we generated inhibition curves for all possible combinations of antiserum, ^{125}I-tubulin, and unlabeled tubulin. A typical inhibition or competition curve is shown in Fig. 2. Increasing amounts of unlabeled tubulin produced increasing inhibition of ^{125}I-tubulin binding. Fig. 2 compares chick brain tubulin iodinated with lactoperoxidase with that labeled with iodinated imidoester for ability to compete with unlabeled chick brain tubulin for antibody binding sites in a chick tubulin antiserum. All data points in such experiments were run in triplicate. The average standard error for all data points in this experiment is 0.11%. The two curves superimpose, demonstrating that the method of iodination does not alter the competition results. This is particularly interesting since lactoperoxidase labels tyrosine residues while iodinated imidoester labels via lysine residues.

The ability of a given unlabeled tubulin to compete against a different ^{125}I-tubulin for antibody sites reveals the presence of shared antigenic determinants on the molecules. Quantitative differences in the amounts of different unlabeled tubulins required for similar competition curves implies that the shared determinants are present in different densities. The quantity of unlabeled tubulin producing 50% inhibition of control binding can be used to illustrate differences in different competition curves: when those amounts are identical, the curves superimpose (as in Fig. 2), and when the 50% inhibition concentrations are different, the curves are different.

The 50% inhibition of control binding data for the 27 different combinations of antiserum, ^{125}I-tubulin, and unlabeled tubulin are shown in Table 2. The data are expressed as the amount of each unlabeled tubulin required for 50% inhibition, relative to the most effective unlabeled competitor (normalized to 1.0), for each combination of antiserum and ^{125}I-tubulin.

The results show that lamb, chick, and mouse brain tubulins have shared antigenic determinants, since they are all capable of inhibiting the binding of any of the ^{125}I-tubulins to antisera against lamb and mouse brain tubulins. However, the fact that there are demonstrable differences in competition efficiency indicates immunological differences among these tubulins. Such differences can be explained by subtle differences in the shared determinants, by identical determinants present in different densities on the three tubulins, or by a combination of the two.

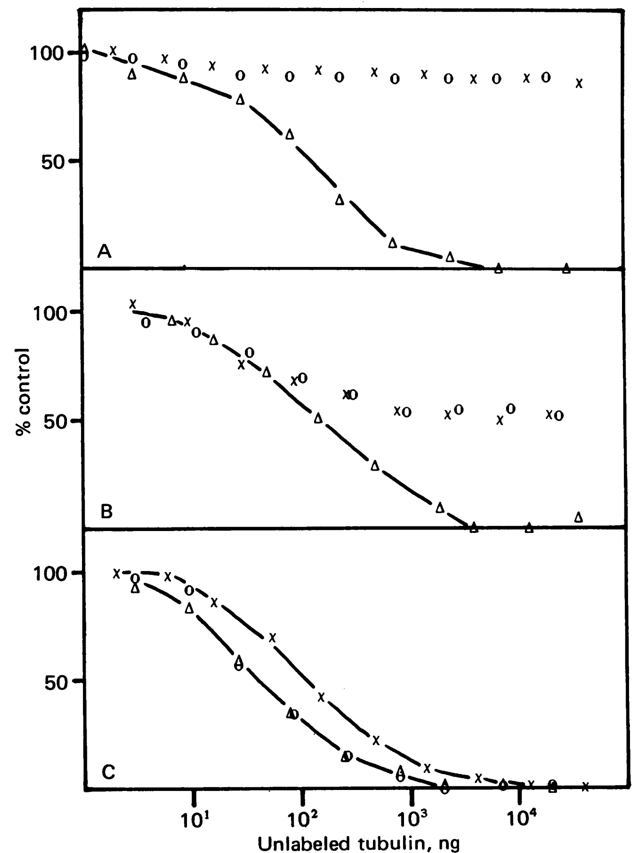

FIG. 3. Immunological differences among lamb, mouse, and chick tubulins resolved by radioimmunoassay. Inhibition curves generated by competition between unlabeled tubulins and chick ^{125}I-tubulin (*A*) using antiserum against chick brain tubulin (rabbit no. 147); (*B*) using another antiserum against chick brain tubulin (rabbit no. 149); and (*C*) using antiserum against mouse brain tubulin (rabbit no. 15). Unlabeled competitor tubulins were from lamb brain (X), mouse brain (O), and chick brain (Δ). Note that lamb and mouse tubulins fail to inhibit the binding of chick ^{125}I-tubulin to homologous antiserum (*A*). The same kind of competition test using antiserum from a different rabbit (*B*) shows a higher degree of crossreactivity, but the same inability of lamb and mouse tubulins to completely inhibit chick ^{125}I-tubulin binding to homologous antiserum. However, all three unlabeled tubulins will inhibit the binding of chick ^{125}I-tubulin to antiserum against mouse brain tubulin (*C*).

For chick brain tubulin, however, the data suggest the presence of a unique antigenic determinant in addition to those shared with mouse and lamb tubulins. Thus, lamb and mouse brain tubulin fail to inhibit the binding of chick ^{125}I-tubulin to its homologous antiserum completely, or even by 50%, even at concentrations of 30,000 ng in the radioimmunoassay (Table 2; Fig. 3). A similar result is obtained using antiserum against chick brain tubulin raised in a different rabbit (Fig. 3), suggesting that the result is due to the chick tubulin itself rather than to some idiosyncrasy of an individual rabbit. Although binding of chick tubulin to this second antiserum is not completely inhibited by lamb or mouse tubulin, there is significant inhibition observed (40–50%). Whether this means that the "unique" determinant has some structural similarity to shared determinants or that the second antiserum has higher concentrations of antibodies against shared determinants is unclear. This unique determinant is clearly in addition to shared determinants since both mouse and lamb brain tubulins will

Table 2. Species-dependent differences in brain tubulins as resolved by radioimmunoassay (RIA)

Anti-tubulin antiserum*	^{125}I-Tubulin in RIA	Relative amounts unlabeled tubulin required for 50% inhibition in RIA			Least amount unlabeled tubulin giving 50% inhibition	
		Lamb	Chick	Mouse	Source	ng
Lamb (As 13)	Lamb†	2.67	1.56	1.00	Mouse	45
	Chick	3.00	2.07	1.00	Mouse	150
	Mouse	1.44	1.00	1.00	Mouse	170
Mouse (As 15)	Lamb	2.09	1.09	1.00	Mouse	110
	Chick	3.33	1.00	1.00	Mouse	45
	Mouse	3.36	1.76	1.00	Mouse	125
Chick (As 147)	Lamb	1.14	1.00	1.24	Chick	210
	Chick	‡	1.00	‡	Chick	160
	Mouse	2.75	2.50	1.00	Mouse	40

* Antiserum (As) is from rabbit no. 13, 15, or 147.

† This lamb tubulin was iodinated by the lactoperoxidase method (19).

‡ Neither lamb nor mouse brain tubulin will inhibit the binding of chick brain ^{125}I-tubulin to this homologous antiserum (As 147) by 50%.

compete with chick ^{125}I-tubulin for binding to antiserum against lamb (Table 2) or mouse tubulin (Table 2; Fig. 3).

Species-dependent differences in purified proteins can be sensitively discriminated by immunological techniques. The power of such techniques has been best demonstrated by studies on vertebrate cytochromes *c* (23, 24). The radioimmunoassay experiments on tubulin competition reported here reveal species differences among lamb, chick, and mouse brain tubulins. The experimental system resolves nonprecipitating antigen–antibody complexes as well as precipitating ones. The importance of recognizing nonprecipitating complexes has been previously illustrated by work on cytochromes *c* (25) and α lactalbumins (26). This resolution is essential since purified tubulin elicits only poorly precipitating or nonprecipitating antibodies in our experience. The three tubulins differ from one another quantitatively, apparently possessing different densities of shared antigenic determinants. The number of different shared (or subtly altered) determinants involved is not revealed in our system. Our quantitative results are likely a function of both shared determinant densities on the tubulins and concentrations of antibodies against different shared determinants in each antiserum. However, chick brain tubulin appears to possess at least one unique antigenic determinant not shared by the lamb and mouse tubulins. The significance of the immunological differences between brain tubulins is not yet understood, but may underlie regulatory differences and/or evolutionary relationships. These immunological probes offer potentially important tools for analysis of tissue-specific differences in cytoplasmic tubulin molecules as well as species-specific ones.

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