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Immunology

Antibodies to tubulin in normal nonimmunized animals

(natural antibodies/peroxidase-labeled antibodies/auto-antibodies/immunoadsorbents)

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ABSTRACT **Sera of normal nonimmunized rabbits, pigs, calves, and humans contain tubulin-reactive antibodies. Usually, low amounts of antibodies against tubulin of the IgG class (2.5–4 mg/100 ml of serum from nonimmunized animals) were isolated. Anti-tubulin antibodies were also produced by injecting pig tubulin in complete Freund's adjuvant into rabbits. Slightly higher amounts of anti-tubulin antibody were isolated from sera of immunized rabbits (7 mg/100 ml of serum). The cytoplasmic network of microtubules of Tcc 36 mouse cells in culture was not clearly stained by natural anti-tubulin antibodies, but dense staining of the centrosphere was observed. In contrast, induced anti-tubulin antibodies densely stained cytoplasmic microtubular networks. Vinblastine-induced tubulin paracrystals were equally stained by natural and induced anti-tubulin antibodies.**

The occurrence of natural antibodies directed against various substances is known and has been reviewed by Boyden (1). The case of blood group antigens is particularly well established (2). Recently, natural autoantibodies directed against brain antigens common to several species (3) and against thymus cells (4) have been found in the sera of nonimmunized mice. Isolation of carbohydrate-specific immunoglobulin from the serum of healthy animals of various species, using insolubilized bovine fetuin, has also been recently reported (5). In this last study it was pointed out that the low amount of anti-carbohydrate antibodies which was found in the serum of nonimmunized animals did not significantly increase after immunization with the corresponding antigen. The above studies dealt with antigens whose main characteristics were their presence in many species of animals and their poor immunogenicity. Tubulin seems to belong to this class of antigen. Indeed, the primary structure of tubulin is apparently very similar in various species (6), and only low antibody titers are found in the sera of animals injected with this molecule (7). These antisera allowed a fine visualization of a cellular cytoplasmic network by indirect immunofluorescence (7–9). We have recently prepared antisera against tubulin in order to check the possible interaction of cell surface receptors with microtubules, a possibility that was suggested to us by our previous experiments (10). Testing the antisera obtained with purified pig brain tubulin, by peroxidase immunocytological technics, we made the following observations: (*i*) Low dilutions of the sera were needed in order to obtain a satisfactory staining of the cells. (*ii*) Some cellular fibers were visible, but a background staining prevented detailed observation. (*iii*) Similar pictures were obtained when either whole antisera or their immunoglobulin (Ig) fractions were employed. (*iv*) Similar pictures were noted with sera or Ig fractions from normal nonimmunized rabbits, provided that an adequate dilution was used. These observations prompted us to search for the occurrence of natural anti-tubulin antibodies in the sera of various animal species. The most direct approach to this problem was to isolate antibodies from the sera of normal or immunized animals on immunoadsorbent columns. For comparison, we used a fetuin immunoadsorbent.

MATERIALS AND METHODS

Antigen Source. Pig brain tubulin was a gift of D. Pantaloni (Laboratoire d'Enzymologie, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France). It was prepared according to the method of Shelansky *et al.* (11) with an additional cycle of polymerization–depolymerization. The purity, estimated by sodium dodecyl sulfate gel electrophoresis, was at least 90%. To test the specificity of sera and isolated antibodies, absorptions were carried out with 100% pure tubulin isolated according to the method of Weingarten *et al.* (12). Fetuin (type II) was purchased from Sigma Chemical Co., St. Louis, MO.

Serum Source. Normal rabbit sera obtained from several nonimmunized apparently healthy animals were pooled. Normal human serum and umbilical cord serum were obtained from the Centre National de la Transfusion Sanguine (6, Rue Alexandre-Cabanel, 75015 Paris, France). The normal pig serum, the anti-lysozyme pig serum, and the calf serum were a gift from J. J. Metzger (Institut National de la Recherche Agronomique, Grignon, France). We used the serum of newborn piglets and 14-day-old germ-free piglets, obtained by hysterectomy. These were gifts from R. Ducluzeau (Institut National de la Recherche Agronomique, Jouy-en-Josas, France). Fetal calf serum was purchased from Gibco-Biocult, Scotland. Rabbit antiserum to tubulin was prepared following procedures similar to those described by others, which involve insolubilization of tubulin by glutaraldehyde (8).

Preparation of Immunoadsorbents. Tubulin and fetuin were coupled to glutaraldehyde-activated 3% polyacrylamide/4% agarose gels (AcA 3.4) following procedures described elsewhere (13). One milligram of tubulin was bound per 1 ml of AcA 3.4 beads and a column of 32 ml of beads was used. For fetuin, 0.7 mg was bound per 1 ml of AcA 3.4 beads and a column of 30 ml of beads was used.

Isolation of Antibodies from Normal and Immune Sera. The following procedure was used: The insolubilized tubulin or fetuin was equilibrated with 0.01 M potassium phosphate buffer, pH 7.4/0.15 M NaCl (phosphate-buffered saline). The serum to be tested was passed through the column at room temperature, at a flow rate of 20 ml/hr. The column was then brought to 4° and extensively washed with phosphate-buffered saline (flow rate: 20 ml/hr). When the absorbance of the effluent at 280 nm was lower than 0.005, the antibodies were eluted at 4° with 0.2 M glycine-HCl buffer at pH 2.8. The eluted material was immediately brought to pH 6.8 with 1 M HK_2PO_4 and concentrated to 1 mg/ml on Amicon cells with a Diaflo PM10 membrane. It was then dialyzed against phos-

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Abbreviation: AcA 3.4, 3% polyacrylamide/4% agarose.

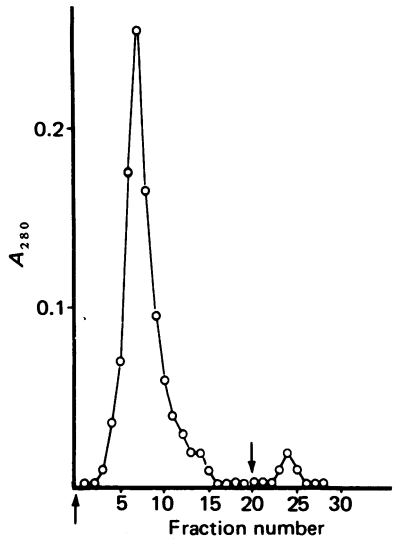

FIG. 1. Purification of antibodies from normal rabbit serum on insolubilized tubulin. One hundred milliliters of a pool of normal rabbit sera was run over the insolubilized tubulin (32 ml of beads, 1 mg of tubulin per ml) at a flow rate of 20 ml/hr at room temperature. After extensive washing of the column at 4° with phosphate-buffered saline, the antibodies were eluted with 0.2 M glycine·HCl buffer at pH 2.8 (first arrow) and 0.2 M glycine·HCl buffer at pH 2.2 (second arrow).

phate-buffered saline, centrifuged (10,000 × *g* for 10 min), and stored at 4° before use. The column was further washed with 0.2 M glycine·HCl at pH 2.2, and neutralized with 0.1 M HK_2PO_4. When the pH of the effluent was 7.4, the column was finally extensively washed in phosphate-buffered saline.

Analysis of Isolated Antibodies. The isolated antibodies were analyzed by double immunodiffusion (14) and by immunoelectrophoresis (15) using monkey antiserum to rabbit serum proteins, rabbit antiserum to human serum proteins, rabbit antiserum to pig serum proteins, and rabbit antiserum to calf serum proteins. The following monospecific antisera were also used: sheep antiserum to human IgG and IgM, sheep antiserum to the constant-region fragment of IgG heavy chain (Fc γ) from rabbits, and rabbit antiserum to pig Fc γ.

Immunocytochemical Labeling with the Isolated Antibodies. *Cell cultures:* The cells used to test the specificity of the isolated antibodies were mouse epithelioma cells (Tcc 36 cells) cultured in the laboratory of J. C. Guillon (Institut Pasteur, Paris, France). Cells were maintained in culture in Dulbecco's medium containing 5% fetal calf serum and antibiotics in 90% air/10% CO_2 atmosphere. Tubulin paracrystals were induced in the cytoplasm of the cells by adding 10 μM vinblastine in the culture medium for 1 hr at 37° before fixation of the cells.

Fixation procedure: The cells were washed twice in Hanks' balanced salt solution, air-dried, and treated for 30 min in absolute acetone at −20°.

Incubation with isolated antibodies: Fixed cells were incubated for 1 hr at room temperature with isolated antibodies in a humidified atmosphere. The concentration of antibodies ranged from 200 μg/ml to 500 μg/ml. After two washes of 3 min in phosphate-buffered saline, the cells were further incubated for 1 hr at room temperature with sheep or rabbit peroxidase-labeled antibody directed against the first antibody. The concentration of the peroxidase-labeled antibody was 125 μg/ml. These antibodies were prepared as previously described (16). After two washes of 3 min in phosphate-buffered saline, the peroxidase activity was revealed by the method of Graham and Karnovsky (17). The cell preparations were finally washed with distilled water, mounted in glycerin jelly (Difco Laboratory), and examined under the microscope (Leitz Orthoplan).

Table 1. Milligrams of protein isolated from 100 ml of various sera on tubulin and fetuin immunoadsorbents

Serum origin	Adsorbent* Tubulin insolubilized on AcA 3.4	Fetuin insolubilized on AcA 3.4	Unsubstituted AcA 3.4
Rabbit anti-tubulin	7.6		
Normal rabbit (pool I)	2.5	1.8	1
Normal rabbit (pool II)	4.1	1.4	0.8
Human	2.7		
Human umbilical cord	2.8		
Normal pig	11.4		
Lysozyme-immunized pig	32.0		2.8
Germ-free piglet	2.0		
Neonatal germ-free piglet	1.6		
Calf	7.0		
Fetal calf	2.0		

* AcA 3.4, 3% polyacrylamide/4% agarose beads.

RESULTS

Isolation of Antibodies. When normal rabbit sera were passed through insolubilized tubulin, the elution profile shown in Fig. 1 was obtained. The amounts of protein isolated from sera of various origins are given in Table 1; we have also reported for comparison the values obtained when sera were passed through columns of untreated AcA 3.4 or columns of insolubilized fetuin. The amount of protein retained on insolubilized tubulin from sera of nonimmunized animals was significantly higher than that obtained by passage of the same sera on untreated AcA 3.4 or even on insolubilized fetuin. All figures are expressed in mg/100 ml of sera. The higher level of proteins retained on insolubilized tubulin was obtained from rabbit (4.1 mg), pig (11.4 mg), and calf (7 mg) sera. In the case of pig sera, one has to note the low amount of material that was recovered from sera of neonatal and 14-day-old germ-free piglets (1.6 and 2.0 mg, respectively). A low amount of material was also recovered from fetal calf serum (2.0 mg). On the other hand, a relatively high amount of material (32 mg) was recovered from the serum of one pig hyper-immunized with lysozyme. In the case of human sera, the same amount of material was retained on insolubilized tubulin from either adult (2.7 mg) or umbilical cord sera (2.8 mg). It is noteworthy that immunization of rabbits with tubulin increases only moderately the amount of isolated material (7.6 mg in the case of tubulin-immunized rabbit instead of 2.5 or 4.1 mg of nonimmunized rabbits). The amount of material retained on insolubilized fetuin from rabbit serum was 1.4 to 1.8 mg/100 ml of serum and was comparable to values reported by Sela *et al.* (5).

Specificity of Antibodies Purified on Insolubilized Tubulin and Fetuin. In a first attempt to determine the specificity of antibody purification, we tested the crossreactivity of normal sera with insolubilized tubulin and fetuin. An aliquot of a pool of rabbit sera was applied to a column of insolubilized fetuin and the adsorbed material was eluted with glycine·HCl, pH 2.8. The nonabsorbed material (whole serum) was re-applied on the same column twice. The whole serum obtained after three runs on insolubilized fetuin was then applied to a column of insolubilized tubulin and the adsorbed material was eluted with glycine·HCl, pH 2.8. The amount of eluted material obtained in this way was compared to the amount obtained when the same pool of sera was directly run on a tubulin column.

Table 2. Specificity of binding on insolubilized tubulin and fetuin

Rabbit serum	Exp.	Protein isolated,* mg/100 ml: Insolubilized fetuin 1st	2nd	3rd	Insolubilized tubulin 1st	2nd	3rd	Insolubilized fetuin 1st
Pool I	1†	—	—	—	2.5			
	2	1.8	0.6	0.6	2.5			
Pool II	1‡				—	—	—	1.4
	2				4.1	1.6	0.6	1.4

* The sequence of the various runs on the two immunoadsorbents is obtained by reading the table from left to right.
† In this experiment serum was passed through only tubulin immunoadsorbent.
‡ In this experiment serum was passed through only fetuin immunoadsorbent.

An identical experiment was performed with another pool of rabbit sera but in the reverse order of immunoadsorbtions; the sera were run three times on insolubilized tubulin and then run on insoluble fetuin. The results were compared to those obtained with a run on insoluble fetuin alone.

The results are summarized in Table 2. (*i*) A significant amount of protein was isolated on either column, only in the first elution; a low amount of material was recovered during the following elutions. (*ii*) Three passages of a serum on one antigen failed to inhibit the binding of proteins from this serum to the other antigen.

Immunochemical Characterization of the Isolated Antibodies. In order to define the nature of the material isolated on insolubilized tubulin and fetuin, immunodiffusion and immunoelectrophoresis tests were carried out. Fig. 2 shows an example of the immunoelectrophoretic patterns obtained with the antibody isolated from the serum of a rabbit immunized with tubulin and from the serum of a normal nonimmunized rabbit.

Table 3 summarizes the nature of the antibodies isolated from various sera using either insolubilized tubulin or fetuin. The following observations were made: (*i*) Proteins eluted from adult sera always included immunoglobulins belonging to the IgG class. In the case of the preparations obtained by passage of rabbit serum over insolubilized fetuin, IgM was also detected. (*ii*) Albumin was frequently found in various preparations. This is a constituent known to be present in preparations of antibodies purified on immunoadsorbents (5, 18). (*iii*) A low amount of protein was isolated from germ-free piglet and fetal calf sera, which was not of Ig nature. However, the material isolated from germ-free piglets seemed to share antigenic determinants with IgG molecules.

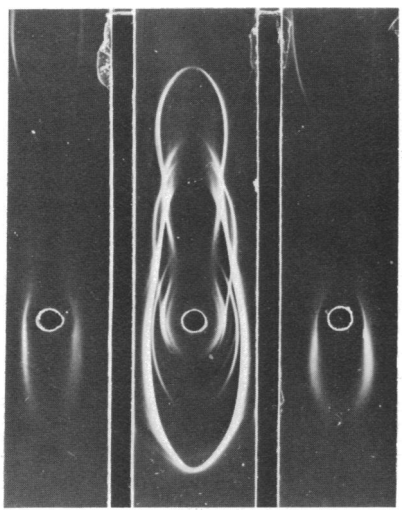

FIG. 2. Immunoelectrophoretic patterns of isolated anti-tubulin antibodies. (Left well) Anti-tubulin isolated from immune rabbit serum. (Right well) Natural antibody isolated from normal rabbit serum. (Center well) Whole rabbit serum. Both troughs were filled with monkey anti-rabbit serum proteins.

Table 3. Immunochemical characterization of the isolated antibodies

Serum origin	Nature* of protein isolated on: Insolubilized tubulin	Insolubilized fetuin
Rabbit anti-tubulin	IgG	—
Normal rabbit (pool I or II)	IgG	IgG + IgM
Human	IgG	—
Human umbilical cord	IgG	—
Normal pig	IgG	—
Lysozyme-immunized pig	IgG	—
Germ-free piglet	Not Ig	—
Neonatal germ-free piglet	Not Ig	—
Calf	IgG	—
Fetal calf	Not Ig	—

* All the preparations contained traces of albumin.

Attempts to obtain precipitin lines in double immunodiffusion with isolated induced or natural antibodies and tubulin were unsuccessful.

Immunocytochemical Characterization of Isolated Antibodies. To test the specificity of the isolated IgG, we took advantage of the characteristic structural organization of tubulin in well-spread cultured cells, and of the characteristic shape of tubulin paracrystals which are present in the cytoplasm of vinblastine-treated cells. This allowed us to make an unambiguous check of the specificity of antibodies by immunocytochemistry.

The following observations were made: (*i*) The IgG isolated from normal rabbit serum on insolubilized tubulin displayed a good staining of the centrosphere (Fig. 3*B*). However, when this staining was compared to that in the pictures obtained with IgG isolated from tubulin-immunized rabbits (Fig. 3*A*), it appeared that the cytoplasmic network did not show up correctly. With both types of serum, the vinblastine-induced tubulin paracrystals were heavily stained, while the surrounding cytoplasm was quite negative (Fig. 3*C*). (*ii*) Absorption of antibodies obtained from normal or immunized rabbits with soluble 100% pure tubulin led to complete inhibition of the cellular labeling. Staining of tubulin paracrystals was equally inhibited by absorption of antibodies with soluble tubulin (Fig. 3*D*). (*iii*) The Ig molecules isolated from nonimmunized rabbits on AcA 3.4 fetuin displayed a very weak and diffuse staining, not confined to tubulin structures of the cells. Moreover, when the cells were treated with vinblastine, this pattern of labeling was not affected, and the paracrystals were not stained by these antibodies.

The absorption of natural anti-tubulin antibodies by soluble tubulin was considered to be specific on the basis of the two following experiments: (*i*) Tubulin did not inhibit the detection of immunoglobulins in plasmocytes when purified anti-immunoglobulin antibodies were used. (*ii*) Tubulin did not inhibit the weak and diffuse labeling of cells obtained with Ig purified on the insolubilized fetuin.

Table 4 summarizes the results obtained with various sera, concerning their immunocytological staining. It can be noted that: (*i*) Negative results were obtained only in the case of the

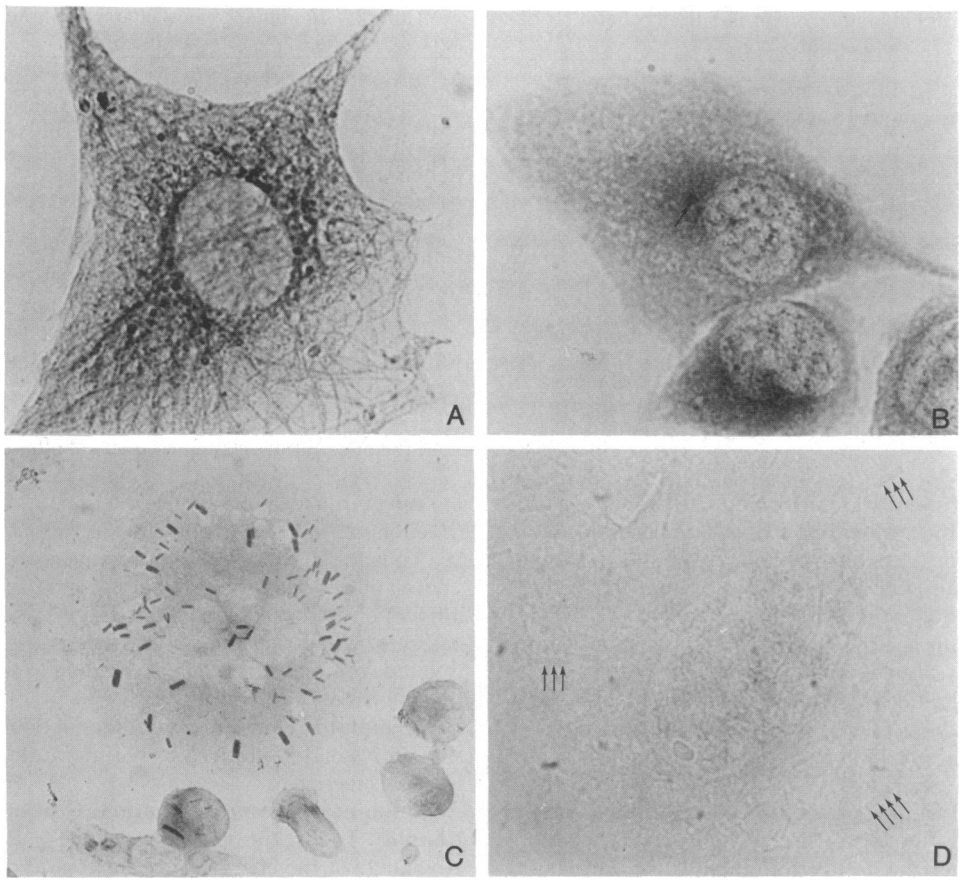

FIG. 3. Staining of normal and vinblastine-treated mouse cells with natural and induced anti-tubulin antibodies. Normal cells were stained with IgG isolated from tubulin-immunized rabbit antiserum (*A*) or from nonimmunized rabbit serum (*B*). Vinblastine-treated cells stained with IgG isolated from nonimmunized rabbit serum are shown in *C; D* shows vinblastine-treated cells that were stained with the same IgG as in *C* but absorbed with pure tubulin before use. Unstained tubulin paracrystals can be recognized (arrows). (*A* and *B*, ×600; *C*, ×380; *D*, ×960.)

material isolated from germ-free piglet serum. (*ii*) Although some natural anti-tubulin antibodies from the species tested displayed a rather diffuse staining of the cells, the same antibodies stained the tubulin paracrystals heavily. Both types of staining were always inhibited after absorption of the antibodies with soluble pure tubulin.

DISCUSSION

The results of the various tests we have carried out in this study indicate that tubulin-reactive antibodies are present in the serum of nonimmunized animals and man. This raises a technical problem concerning the current use of fluorescein-tagged gamma globulin fractions of antisera against various antigens for their immunocytologic demonstration in cells or tissues, because natural anti-tubulin antibodies and perhaps other natural antibodies could be present in such fractions. Therefore, the use of purified antibodies or of absorbed gamma globulin fractions is recommended to avoid unexpected staining.

The specificity and the nature of the natural antibodies that we have isolated in this work are established from the following observations: (*i*) The amount of material retained on insolubilized tubulin was significantly higher than the amount of material retained on AcA 3.4 alone. (*ii*) Preabsorption of a given serum by three passages on insolubilized fetuin did not inhibit the binding of IgG of this serum to insolubilized tubulin. The inverse situation was also true. (*iii*) The isolated material belongs generally to the IgG class of globulins. One can note also that a low amount of material was retained on the insolubilized tubulin from sera of germ-free piglets, which are known to contain very low concentrations of Ig (19). In fact, this material did not stain the cells. However, the component isolated from germ-free piglets seemed to share some antigenic determinants with pig IgG. Porter (20) has reported that the serum of newborn piglets was almost entirely deficient in immunoglobulins, but he found a component sharing antigenic determinants with IgG molecules that was present in concentrations less than 50

Table 4. Immunocytochemical characterization of isolated antibodies

	Cellular staining	
Serum origin	Normal cells	Vinblastine-treated cells
Rabbit anti-tubulin	Positive cytoplasmic network	Positive tubulin paracrystals
Normal rabbit (pool I or II)	Positive centrosphere with diffuse cytoplasmic staining	Positive tubulin paracrystals
Human	Positive centrosphere with cytoplasmic staining	Positive tubulin paracrystals
Human umbilical cord	Diffuse cytoplasmic staining	Positive tubulin paracrystals
Normal pig	Diffuse cytoplasmic staining	Positive tubulin paracrystals
Germ-free piglet	Unstained cells	Unstained paracrystals
Neonatal germ-free piglet	Unstained cells	Unstained paracrystals

μg/ml. Our present observation is therefore intriguing and needs further investigation. (*iv*) Isolated induced or natural anti-tubulin antibodies gave generally comparable but not identical immunocytochemical labeling patterns. Tubulin paracrystals in vinblastine-treated cells were heavily stained by both induced and natural antibodies; while the surrounding cytoplasm was completely negative. Absorption of the isolated antibodies with pure tubulin led to an inhibition of the staining of normal and vinblastine-treated cells. IgG molecules isolated on insolubilized fetuin gave a diffuse cellular staining that was not comparable with anti-tubulin staining, and they did not stain the tubulin paracrystals. These antibodies were not absorbed by soluble tubulin.

The main difference observed between natural and induced anti-tubulin antibodies lies in the pattern of staining of the cellular network of microtubules: natural antibodies clearly stained the centrosphere, but did not allow a good visualization of the thin cytoplasmic network revealed by induced anti-tubulin antibodies.

There are at least two possibilities for this difference in cytoplasmic staining by anti-tubulin antibodies: (*i*) Natural antibodies may recognize some antigenic determinants on the tubulin molecules, which are hidden in microtubules. In tubulin molecules present in the centrosphere and in paracrystals, these determinants would remain accessible to these antibodies. (*ii*) Natural antibodies may recognize very few determinants. This would not allow the visualization of microtubules in light microscopy. It appears therefore that fewer antigenic determinants of tubulin molecules may be involved in the induction of natural anti-tubulin antibodies compared to those involved in the induction of antibodies by injection of tubulin into the animal.

Nevertheless, it can be said that natural antibodies from various animal species, including pig, recognize specifically some antigenic determinants related to pig brain tubulin and to tubulin of cultured mouse cells. Therefore, the natural antibodies isolated seem to recognize some interspecies antigenic determinants present on tubulin molecules. Such antibodies might be considered as "auto-antibodies." That such antibodies are present only in low amounts in the various sera tested would fit well with this conclusion. One can note also that immunization of several rabbits with tubulin did not lead to an important increase in anti-tubulin concentration in their serum. These observations recall the results reported by Sela *et al.* (5) on natural anti-carbohydrate antibodies. In this case also, antibodies could be considered as auto-antibodies, because they interacted with glycopeptides present at the surface of various cells, including lymphocytes. It seems, therefore, that the tolerance of animals to self-constituents is not absolute, and the synthesis of auto-antibodies appears to be tightly regulated. This is in accord with the finding that cells capable of producing antibodies against self-constituents are present in healthy animals, and that their expression is revealed under certain experimental conditions (21, 22). The pathological states in which auto-antibodies are observed (for reviews see refs. 23 and 24) might correspond to a disregulation of the system. An example of this might be the case in acute chronic hepatitis, where a large amount of anti-actin antibodies is present in the serum of patients (25). Indeed, preliminary results in our laboratory suggest that in the serum of healthy animals anti-actin antibodies are also present, but in low concentration. Moreover, immunization of such animals with actin did not lead to a large increase in the antibody concentration in the serum of the animals.

All these results are in agreement with the hypothesis that auto-antibody synthesis is an intrinsic characteristic of the immune system (26). A complex network of regulation, perhaps involving T-cell function (21), could be responsible for the control of auto-antibody synthesis. An intriguing question is the potential physiological functions of auto-antibodies. A role in the cleaning up of the organism of self-antigen excess has been proposed by Grabar (26); auto-antibodies may be involved in the control of cellular differentiation and cell growth control as well. With this reasoning it is noteworthy that injection of anti-idiotypic antibodies in mouse (27) and rabbit (28) can induce a complete suppression of the synthesis of the corresponding idiotype.

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