

Rat Monoclonal Antitubulin Antibodies Derived by Using a New Nonsecreting Rat Cell Line

J. V. KILMARTIN, B. WRIGHT, and C. MILSTEIN

Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, England

ABSTRACT Hybrid myeloma cell lines secreting monoclonal antibodies to tubulin have been prepared using rat myelomas and spleen cells from rats immunized with yeast tubulin. A comparison between the results obtained with the rat myeloma Y3-Ag 1.2.3., which secretes a light chain, and a new line, YB2/O, which does not, shows that they are both excellent parental lines and that the second produces hybrids with no myeloma chain components.

The antitubulin antibodies in the serum of rats bearing two of the hybrid myeloma tumors gave titers of up to $1:10^6$ from which large amounts of monoclonal antibodies could be easily purified. They recognized tubulin from yeast as well as from birds and mammals. The two antibodies gave clear immunofluorescent staining of yeast mitotic spindles as well as the interphase microtubule network of tissue culture cells. Some difference in the pattern of immunofluorescence staining of yeast cells and nuclei was observed between the two antibodies. The purified antibodies could be conjugated to colloidal gold particles and used for direct labeling of yeast microtubules for electron microscopy.

Antibodies to tubulin play an important role in investigating the structure and function of microtubules in cells. For instance, immunofluorescence with antitubulin antibodies has shown that, in addition to their role in the mitotic spindle, microtubules are a basic component of the cytoskeleton of the interphase cell and are long structures extending from the centrosphere just outside the nucleus to the plasma membrane (25). In addition, immunofluorescence is a rapid method for checking the response of microtubules in lysed cell models to various stimuli, such as drugs (34) or calcium ions (30).

The structural information from immunofluorescence has been extended further by electron microscopy, either directly in negatively stained whole mounts (26, 35) or indirectly in thin sections after labeling second antibodies with peroxidase (7, 8) or gold particles (9). Radioimmune assays of amounts of tubulin in cells have been useful in, for example, determining whether formation of microtubules in neuroblastoma cells during neurite extension occurs as a response to tubulin synthesis, or activation of a pre-existing tubulin pool (16). Antibodies can be made specific for the α or β subunits of tubulin, and used to identify these subunits in SDS gels (31). The use of these antitubulin antibodies could be extended further to purify microtubule-containing organelles.

Unfortunately, tubulin is usually a poor antigen even after repeated doses and, apart from a few exceptional cases (20, 33), the antisera have low titers, in the region of 1:200 (1, 15, 22, 23), indicating a combination of low affinity and low concen-

tration of antibodies. Affinity purification of the antibody (12) yields only 0.1 mg/75 ml serum (11) or 0.1–1 mg/10 ml serum (2), adequate for indirect immunofluorescence but insufficient for preparative work. A solution to this problem is to derive hybrid myeloma lines (19) secreting appropriate antibodies against tubulin in order to prepare monoclonal antibodies in unlimited quantities. In addition to their preparative uses, such antibodies used either singly or in mixtures would be more convenient reagents for immunofluorescence than the monospecific antisera currently available in some laboratories.

Since the antitubulin monoclonal antibodies are ultimately to be used for preparative purposes, hybrids between rat cells and a rat myeloma rather than mouse \times mouse hybrids is a better choice because of the larger volume of serum and ascitic fluid of tumor-bearing animals.

At present, the rat myeloma Y3-Ag 1.2.3. is being used in such fusions (13). This line is very suitable but has the disadvantage of secreting a light chain. The hybrids express both the antibody and the myeloma light chain, giving rise to what we refer to as HLK antibodies. These are mixtures of molecules containing the myeloma (K) and antibody (L) light chains in different combinations, namely H_2L_2 , H_2K_2 (usually inactive) and LH_2K (usually monovalent) (6, 19).

To avoid this complication, a nonsecreting rat myeloma (YB2/O) was prepared, and its derivation and use are described here. Hybrids secreting antitubulin antibody were prepared using both myeloma lines Y3-Ag 1.2.3. and YB2/O. Both

proved equally suitable for production of antitubulin monoclonal antibodies. As expected, the hybrids produced by YB2/O did not express the myeloma light chain.

MATERIALS AND METHODS

Tissue Culture

Dulbecco's modified Eagle's medium (DME) (Gibco-Biocult, Glasgow, Scotland) was supplemented with sodium pyruvate and penicillin/streptomycin. The batches of fetal calf serum (FCS) (Sera Lab., Crawley Down, England) were selected to sustain best growth at limiting cell dilution in the absence of feeder cells. The atmospheric environment was 10% CO₂-air, and the NaHCO₃ content adjusted accordingly. Radioactive cultures for the chemical identification of immunoglobulin chains were prepared as described in Galfre and Milstein (14).

Derivation of the Rat Myeloma Cell Line YB2/O

This is a derivative of the hybrid myeloma YB2/3HL (21). The cells, grown in DME with 10% FCS, were cloned in soft agar. Clones were isolated and treated for the production of rat Ig by indirect agglutination of sheep red blood cells which had been CrCl₃-coupled to sheep anti-rat Ig (14). From 116 clones tested, three were selected as being negative or weak positives.

Immunoglobulin synthesis in either the secreted or intracellular fractions was investigated by [¹⁴C]lysine incorporation followed by SDS gel electrophoresis of the spent medium (for secreted Ig) and of the sheep anti-rat immunoglobulin precipitable material in total cell lysates (for intracellular Ig). One clone contained both heavy and light chains intracellularly but seemed to have lost the capacity to secrete the Ig. The second synthesized and secreted light chains only, and the third did not secrete but synthesized heavy chains. This third clone was selected and 43 subclones were isolated. The presence of intracellular heavy chains was tested by fluorescence microscopy, using rabbit FITC-anti-rat-Ig. It was found that the majority of the subclones (21) were negative, nine were positives, and 12 were either mixed clones or uncertain. The negative clones were further checked for their growth characteristics, and for absence of intracellular Ig chains, by [¹⁴C]lysine intracellular incorporation into antibody-precipitable material. After further subclonings including an azaguanine resistant step, the clone YB2/3HL.P2.G11.16Ag.20 was selected. This will be referred to as YB2/O. It dies within 3 d in HAT medium, and, before use, it was adapted to grow in 2.5% fetal calf serum. Cloning and other experimental procedures were carried out as described in (14).

The line YB2/O is a nonproducer variant of a hybrid myeloma originating from a fusion between AO cells and LOU cells. Tumors are grown in (LOU × AO)F₁ hybrids. When injected into LOU inbred strain animals, tumors are sometimes rejected. Recently, we have been able to adapt the tumor by serial passage into animals, and a derivative called YBO has been prepared (C. Milstein and B. Wright, unpublished results) which is not rejected by LOU rats. It suitability for the derivation of hybrid myelomas is being tested.

Immunization Schedule

Two LOU rats (originally obtained from the Institute of Cancer, Catholic University, Louvain, Belgium) were injected intraperitoneally with 20 μg of yeast tubulin (5 mg/ml in 0.1 M PIPES pH 6.9, 0.1 mM MgCl₂, 1 mM GTP) purified by one cycle of assembly-disassembly (18) emulsified in Freund's complete adjuvant. They were boosted with similar doses in incomplete adjuvant at days 22 and 53. At day 29 the titer of the antiserum determined by the solid phase indirect binding assay was measured and the rat giving the better apparent serum titer of about 1:1,000 was given an intravenous dose of 20 μg yeast tubulin at day 89 and used for the fusion experiment.

Fusion and Derivation of Hybrids

4 d after the intravenous injection of yeast tubulin, the hyperimmunized rat was sacrificed and the spleen removed. Preparation of spleen cells and the protocol for fusion were as described by Galfre and Milstein (14). The myeloma cells were taken from steady-state growing spinner cultures, and this seems quite important for good yields of hybrids. Two independent fusions with 10⁶ spleen cells each, and with 6 × 10⁷ each of YB2/O and Y3/Ag 1.2.3. were prepared in parallel. Each fused population was distributed in 2-ml well Linbro plates (Flow Laboratories, Ltd., Irvine, Scotland) containing a feeder layer of irradiated 3T3 fibroblasts as follows: 36 wells, each containing 1/50th of the total fused cells, 12 wells each containing 1/100th, 12 wells with 1/200th, and 36 wells with 1/400th of the total fused cells. After 24 h of culture in DME containing 20% of FCS, one-half of the medium was replaced with medium containing hypoxanthine,

aminopterin, and thymidine. The operation was repeated on the two subsequent days, and then every 2 d. After ~20 d, the presence of antibody to tubulin was tested by the binding assay described below.

Binding Assay

25 μl of yeast or chick brain tubulin (50 μg/ml in 0.1 M PIPES, pH 6.9, 0.1 mM MgCl₂, 1 mM GTP) was added to each well of a 96-well microtiter plate (Sterilin Ltd., Teddington, England), and left overnight at 4°C. Chick brain tubulin was prepared as described by Dentler et al. (10) and purified on a DEAE Sephadex A-50 column (24). The tubulin was removed from the well and replaced by 100 μl 10 mg/ml bovine serum albumin (Armour, Eastbourne, England), 0.05 M sodium phosphate, pH 7.4, 0.15 M NaCl, 1 mg/ml NaN₃ (BSA-PBS), and the plate was left at room temperature for 1 h. The wells were washed twice with 100 μl of BSA-PBS, and 25 μl of cell supernatant or antiserum was added and left for 1 h at 4°C. The wells were again washed twice with 100 μl of BSA-PBS, and 25 μl of ~100,000 cpm of I¹²⁵-(Fab)₂ sheep anti-rat Ig (36) in BSA-PBS was added. The sheep anti-rat Ig antibody was purified by affinity chromatography of antiserum from a sheep immunized with rat IgG, the absorbent was the rat IgG₂ myeloma protein S208 (28). The wells were washed four times with 100 μl of BSA-PBS, and the bound I¹²⁵ counts were eluted with 2N NaOH and counted in a γ counter.

Fluorescence Microscopy

Yeast spheroplasts were prepared by digestion of log phase *Saccharomyces uvarum* (NCYC 74) with 1.2 mg/ml mutanase (Novo, Windsor, England) and 0.6 mg/ml Zymolyase 5000 (Miles Laboratories, Slough, England) in 1.1 M sorbitol at 10⁹ cells/ml for 2 h at 30°C. Yeast nuclei were prepared as described by Rozijn and Tonino (29). CHO-K₁ and 3T3 cells were incubated on an 8-well Flow Laboratories slide at 37°C overnight, and fixed in glutaraldehyde/NaBH₄ (8, 35) or in MeOH for 6 min and acetone for 30 s at -20°C. Yeast spheroplasts and nuclei were placed in an 8-well slide coated with 1 mg/ml polylysine (400,000 mol wt; Sigma Chemical Co., St. Louis, MO), and fixed for 10 min at room temperature with 3.7% formaldehyde or 1% glutaraldehyde in 1.1 M sorbitol for spheroplasts or, for nuclei, in 1.8 M sucrose nuclear buffer (29). This was followed by fixation in MeOH and acetone or NaBH₄ treatment as above. Antibody was added for 1 h at 20°C, then the wells were washed three times with BSA-PBS, and second antibody was then added. This was for nuclei FITC-anti rat IgG (Miles, Slough, England) and for cells the same antiserum purified by affinity chromatography on Sepharose 4B coupled with YL1/2 IgG. After 1 h at 20°C the wells were washed three times with BSA-PBS and mounted in 90% glycerol. Slides were observed by epifluorescence using a Zeiss standard WL microscope equipped with a HBO 50 W high pressure Hg lamp.

Cloning of Hybrids

Cloning was attempted starting ~1 mo after fusion. Plates containing 10,000, 3,000, 1,000, and 300 cells were seeded on semi-solid agar in the absence of feeder cells as described by Galfre and Milstein (14). Clones were picked when they were of ~100-500 cells and transferred to 2-ml culture wells. When the microculture was nearly confluent, the presence of antitubulin antibody was tested by the binding assay. Clones retained were the positive ones which originated from agar dishes with the lowest density. When positive cultures were from high density plates, the picked material ("agar plugs") may contain multiple clones. The step was not considered a cloning step, but if the cultures were positive they were retained as better adapted for cloning.

The most valuable clones were subcloned once again before final selection of a permanent stock clone. Frozen samples were prepared at different stages by the usual method (14).

Large-scale Preparation of Monoclonal Antibody

Large quantities of monoclonal antibody were prepared by growing tumours in rats by subcutaneous injection of 5 × 10⁷ cells of the selected hybrid clone. Serum from the animal which was killed was used to purify IgG. The Y3/Ag 1.2.3. myeloma is of LOU origin and the spleen was from a LOU rat. Tumors from them were usually grown in inbred LOU rats.

Since YB2/O is itself a hybrid originating from a fusion between LOU myeloma (Y3/Ag 1.2.3.) and AO spleen cells, tumors derived from the YB2/O fusion were grown in (LOU × AO) F₁ hybrids. We have observed that hybrid myelomas prepared with YB2/O can be adapted to grow in LOU rats.

In some experiments, tumors were grown ascitic, by intraperitoneal injection of 5 × 10⁷ cells into rats which 2 wk previously had been injected with 0.5 ml of pristane.

Purification of IgG from Serum/Ascites

YL1/2 or YOL1/34 IgG was precipitated from serum or ascites by 50% ammonium sulphate, and, after dialysis against 0.01 M sodium phosphate, pH 7.0, was collected as the breakthrough peak after chromatography on DE-52 (Whatman Ltd., Maidenhead, England) equilibrated with the same buffer. The yield of IgG was 10 mg/ml serum.

Conjugation of IgGs to 200 Å Colloidal Gold Particles

YOL1/34 and YL1/2 IgGs were conjugated to 200 Å colloidal gold particles at pH 9.0, as described by De May et al. (9), except that 10 mg/ml BSA adjusted to pH 7.0 with NaOH was added 2 min after the IgG followed by 0.15 M NaCl. The antibodies conjugated to the 200 Å colloidal gold particles did not penetrate the intact nuclei, so nuclei were lysed in 0.1 mM MgCl₂ and separated on a sucrose gradient at 180,000 g for 90 min at 4°C. The gradient consisted of equal volumes of 1.25, 1.5, 1.75, 2.0, and 2.5 M sucrose in 0.01 M bis-tris/Cl pH 6.5, 0.1 mM MgCl₂. The fraction banding at the 2.0/2.5 M interface was pelleted at 130,000 g, fixed in glutaraldehyde/NaBH₄ and reacted with the conjugated antibodies overnight at 25°C.

RESULTS

Fusion with both cell lines was satisfactory, although the Y3 line was more efficient in this particular comparative experiment (Table I). Since considerable variations in fusion efficiency are common, we do not attach much significance to this individual result. In spite of the difference in fusion efficiency, the number of cultures which gave positive binding was comparable for both experiments.

SDS gel electrophoresis of the secreted Ig chains in fusion cultures prepared with YB2/O showed that the light chains often had different mobilities (Fig. 1). No myeloma light chain component was detected which would have appeared as a common light chain band in addition to the various ones present in Fig. 1.

The cultures which contained higher antitubulin activity were cloned, and active clones were recovered between 1 and 2 mo after fusion. At that stage the original cultures were still positive in all cases, which simplified the recovery of active clones. Among those clones which grew after picking, the majority were antitubulin secretors (Table II). The most glaring exception was YOL1/34, where only 3 out of 18 clones were positive. The analysis of the immunoglobulin secreted by the uncloned culture gave as the major component an IgM, while the cloned product was an IgG antitubulin (Fig. 1). Clearly, the culture contained at least two clones and it was not difficult to isolate the positive one in spite of being a minor component. The second cloning step gave a very high proportion of posi-

TABLE I
Comparison between Two Rat Myeloma Lines as Parentals for Hybrid Myelomas

No. of cells per culture	No. of cultures	Parental myeloma			
		YB2/O		Y3/Ag 1.2.3.	
		Growing cultures	Antitubulin*	Growing cultures	Antitubulin*
2 × 10 ⁶	36	34	4	36	4
1 × 10 ⁶	12	6	1	12	—
0.5 × 10 ⁶	12	3	—	12	1
0.25 × 10 ⁶	36	9	—	20	1

* Binding to tubulin-coated plates was three times or more than inactive supernatants or to uncoated plates.

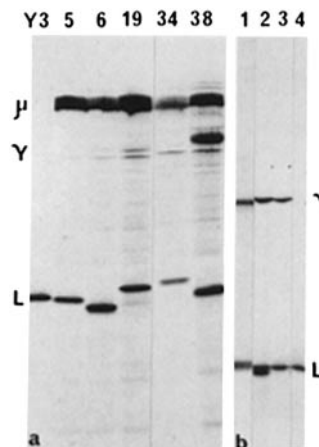


FIGURE 1 Autoradiographs of the secreted products of hybrid myeloma cultures. (a) 10% SDS gel electrophoresis of selected supernatants from individual cultures (YOL1/5, 6, 19, 34, and 38) from the YB2/O fusion prior to cloning and incubated with [¹⁴C]lysine. (b) 7% SDS gel electrophoresis of similar supernatants from: lane 1 cloned YOL1/34; lane 2 cloned YL1/2 HKL; lane 3 YL1/2 HK; and lane 4 Y3. μ and γ represent the mobilities of the immunoglobulin heavy chains, L the mobility of the light chains.

tives (Table II). The results show an overall stability of expressed chains which was comparable for both sets of fusions.

Characterisation of the Antitubulin Monoclonal Antibodies

The results of the binding of the antitubulin monoclonal antibodies to yeast and brain tubulin are shown in Table III. There are clearly two classes of antibodies, those that bind to both yeast and chick brain tubulin, and those that preferentially bind to yeast tubulin. Other positive hybrids (not included in Table II) were isolated, but their antibodies had anomalous properties; for example, high binding was observed in the absence of antigen or they bound to antigens other than tubulin. One antibody bound well to tubulin, actin and myosin. These antibodies were not studied further.

The binding assay for screening used soluble tubulin, so would select for antibodies binding to most parts of the tubulin molecule. This type of binding assay was used because it was the fastest available. However, since one of the proposed uses of antibodies was purification of microtubule-containing organelles, it was essential to demonstrate binding to the outside of the microtubule. To select for such antibodies, we tested those supernatants which were positives in the binding assay for their capacity to give immunofluorescent staining of microtubules. Of all the supernatants, only YL1/2 and YOL1/34 gave an unequivocal immunofluorescent staining of both yeast mitotic spindles and the interphase microtubule network in CHO and 3T3 cells. Thus, these two clones were selected for large-scale production of immunoglobulin in rats.

The serum of rats bearing the hybrid myeloma tumour YL1/2 or YOL1/34 gave titers in the binding assay of between 1:10⁵ and 1:10⁶. Both were purified from the sera, and their DEAE cellulose chromatographic behavior and SDS gel electrophoretic analysis of the reduced chains gave results in agreement with the radioactive experiments (Fig. 1), suggesting that the antibodies were of the IgG class. A measure of the binding constant of YL1/2 IgG was obtained by immunoprecipitation of MAP-free I¹²⁵ brain tubulin (10) using sheep anti-rat IgG as precipitant at monoclonal antibody concentrations from 80 μ g/ml to 80 ng/ml. This gave a value for the apparent dissociation constant of 50 nM, assuming a valency of one for both the HLK antibody and the antigen. The possibility that this value is affected by a shift in the equilibrium caused by the binding of the sheep anti-rat IgG is not excluded.

Indirect immunofluorescence using YL1/2 and YOL1/34

TABLE II
Recovery of Antitubulin Clones

Cloning step	Positive clones/Total tested							
	Y3 hybrids				YB2/O hybrids			
	YL1/2	YL1/13	YL1/19	YL1/26	YOL1/5	YOL1/19	YOL1/23	YOL1/38
First	13/13	6/14	6/6	3/3*	6/6	9/10	3/18	9/9
Second	42/48‡	—	—	8/8	22/23	17/22	12/12	11/11

* One of these became negative 3 wk after first test.

‡ Analysis of the secreted material showed that of the six negative clones, three had lost the heavy chain, and three had lost the antibody light chain (e.g., Fig. 1). Both variants have been kept.

TABLE III
Results of Binding Studies

	cpm bound by cell supernatants after last cloning step							
	Y3 hybrids				YB2/O hybrids			
	YL1/2	YL1/13	YL1/19	YL1/26	YOL1/5	YOL1/19	YOL1/34	YOL1/38
Yeast tubulin	15,000	2,000	2,000	8,000	3,000	1,000	15,000	2,000
Chick brain tubulin	8,000	60	3,000	250	120	1,100	10,000	300
No tubulin	190	60	120	60	120	70	70	120
Immunofluorescent staining of yeast and CHO or 3T3 microtubules	+	—	—	—	—	—	+	—

IgGs showed that both stained the interphase network of microtubules in 3T3 (Fig. 2a and b) and Chinese hamster ovary (CHO) cells (data not shown) similarly. However, their staining of yeast spheroplasts isolated from log phase cells was slightly different, in that YL1/2 IgG gave a diffuse cytoplasmic staining (Fig. 2c and d). Fig. 2e shows that by lowering the IgG concentration by a factor of ten the diffuse cytoplasmic staining of YL1/2 decreases and the spindle staining is now seen to be similar to YOL1/34 IgG. Although antitubulin immunofluorescence of yeast cells has not been reported before, the staining pattern observed with YOL1/34 seems to fit previous results obtained with the electron microscope (3). Mitotic spindles of various length are observed, presumably depending on what phase of the cell cycle the spheroplast was in. Other microtubules, presumably cytoplasmic, which project from either end of the spindle, were also observed.

An inactive derivative YL1/2HK was detected as a subclone during the subcloning of the YL1/2HLK line (Fig. 1). The spent medium of this line was negative in the binding assay. The IgG, which retains the antitubulin heavy chain but has its light chain replaced by the myeloma light chain, was isolated from serum ascites, and was negative in immunofluorescence of yeast (Fig. 2f) and tissue culture cells (data not shown).

There were also differences in indirect immunofluorescent staining of yeast nuclei by both antibodies (Fig. 3). During isolation of the nuclei, most of the spindles break down. Short microtubules remain attached to the spindle pole bodies which would stain as a dot by immunofluorescence. YOL1/34 IgG gives the expected pattern of staining, either a single dot per nucleus or more rarely a double dot or an occasional complete spindle (Fig. 3a). In contrast, YL1/2 IgG gave multiple dot staining (Fig. 3b) though when the IgG concentration was lowered to 0.8 μ g/ml the staining was the same as with YOL1/34 (data not shown). The multiple dot staining of YL1/2 IgG correlates with the presence of intranuclear tubular complexes

(J. V. Kilmartin and C. Milstein, manuscript in preparation) previously described by Peterson et al. (27). When these complexes are removed by preparing crude nuclear envelopes, the multidot staining disappears and both antibodies now give a similar pattern of a single or double dot per nuclear ghost.

The specificity of the YL1/2 and YOL1/34 IgGs was further confirmed in the electron microscope after their conjugation to ~200 Å colloidal gold particles (9) and reaction with crude nuclear envelopes, the conjugated antibodies did not penetrate intact nuclei. Specific reaction with microtubules attached to yeast spindle pole bodies is clearly seen (Fig. 4). This indicates that the dots seen in the immunofluorescent staining of the crude nuclear envelopes does represent binding of YOL1/34 and YL1/2 IgG to yeast microtubules attached to the spindle pole bodies. It is very interesting to note that the size of the gold particles greatly impairs the ability of the attached antibody molecules to penetrate the tightly packed microtubules close to the spindle pole body.

Both YL1/2 and YOL1/34 monoclonal antibodies seem to bind the α subunit of tubulin specifically, since they stain that subunit in pig and partially purified *Physarum* tubulin after transfer of the proteins from SDS gels to nitrocellulose sheets (4).

DISCUSSION

Quite apart from the advantages offered by rat \times rat fusions in terms of large-scale production of monoclonal antibody, the rat myeloma lines which we have used in this paper seem to possess other valuable properties. The proportion of hybrids which express spleen immunoglobulin chains is considerably higher than in mouse \times mouse hybrids (5). It was suggested that this was due to a higher stability of rat hybrids. The implication in practical terms, that the rat line was potentially better than the mouse, is justified by the results presented here. No problem of loss of cultures before cloning was encountered

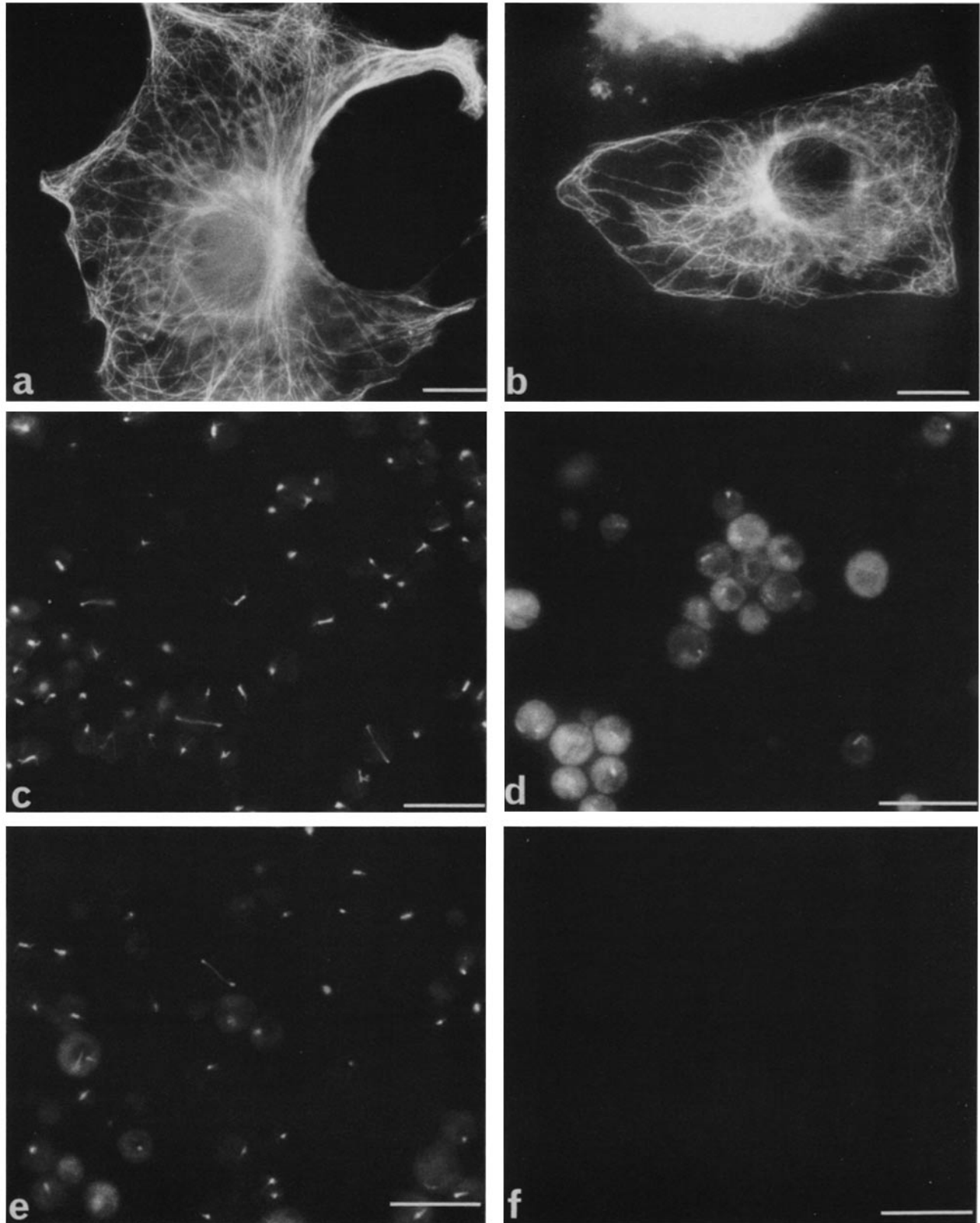


FIGURE 2 Indirect immunofluorescent staining of cells with the monoclonal antitubulin IgG's. 3T3 cells stained with (a) YOL1/34 IgG (30 $\mu\text{g}/\text{ml}$) or (b) YL1/2 IgG (70 $\mu\text{g}/\text{ml}$). Yeast spheroplasts stained with (c) YOL1/34 IgG (30 $\mu\text{g}/\text{ml}$), (d) YL1/2 IgG (25 $\mu\text{g}/\text{ml}$), (e) YL1/2 IgG (2.5 $\mu\text{g}/\text{ml}$), and (f) YL1/2 HK IgG (40 $\mu\text{g}/\text{ml}$). Bar, 10 μm . (a) $\times 1,110$. (b) $\times 1,270$. (c) $\times 1,430$. (d) 1,730. (e) $\times 1,580$. (f) $\times 1,630$.

but, instead, a stability, even at early stages, which we have not normally observed with mouse fusions. The results are, however, to be taken with caution. They are limited, and only long-

term experience can give a more reliable picture.

No significant difference between YB2/O and Y3/Ag 1.2.3. was observed. Perhaps the hybridization efficiency of YB2/O

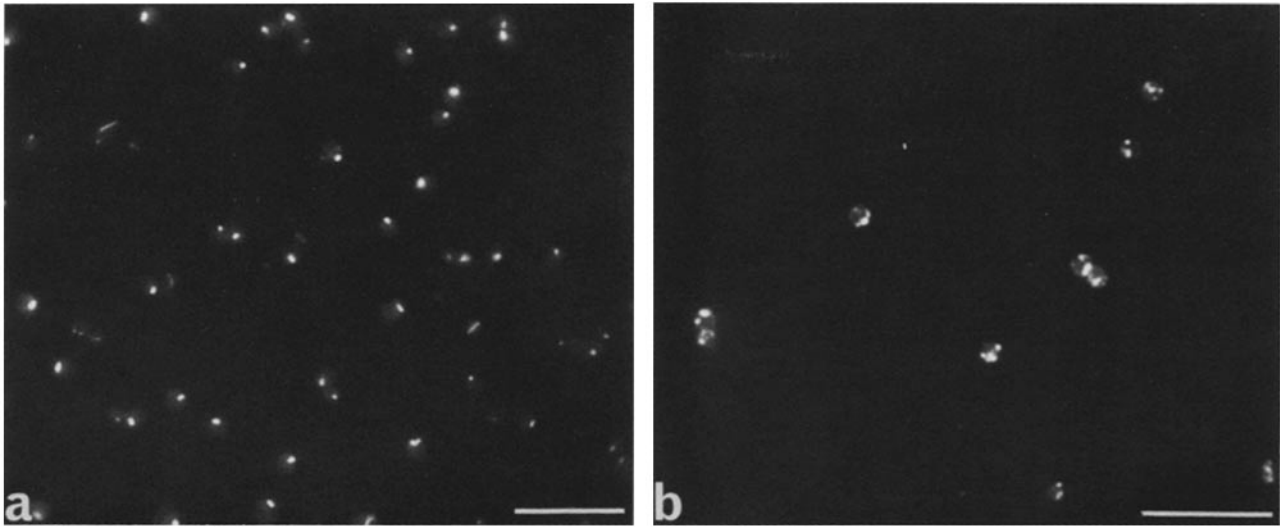


FIGURE 3 Indirect immunofluorescent staining of yeast nuclei with (a) YOL/34 IgG (30 µg/ml) or (b) YL1/2 IgG (25 µg/ml). Bar, 10 µm. (a) $\times 1,460$. (b) $\times 1,730$.

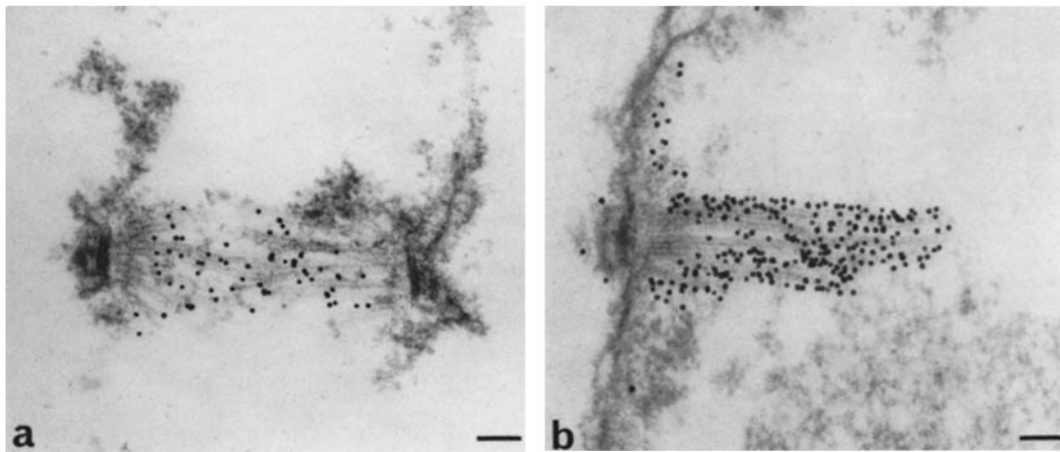


FIGURE 4 Electron micrographs of thin sections of a crude yeast nuclear envelope preparation showing yeast microtubules attached to spindle pole bodies and labeled with (a) YOL1/34 IgG or (b) YL1/2 IgG conjugated to 200 Å colloidal gold particles. Bar, 0.1 µm. $\times 57,000$.

is a little lower than that of Y3/Ag 1.2.3., but experience shows that generally it improves with further use. At any event, the efficiency was sufficient, and the total number of active clones was not significantly different. Apart from the absence of a myeloma light chain, YB2/O has some other advantages over Y3/Ag 1.2.3. The cells are slightly larger, more rounded, and less adherent to the culture vessel. In addition, they are easier to clone in soft agar because the hybrid clones derived are tighter, decreasing the risk of cross-contamination with neighboring clones.

We have concentrated our attention on two derivatives, YL1/2 and YOL1/34. When the first fusion wells were assayed, the binding activity of these two clones was only slightly less than that observed in Table III after cloning. This was also found for the other positive hybrids. Thus, if a fusion produces only weakly positive hybrids, then it may be better to avoid lengthy cloning attempts, unless some of the clones have particularly interesting properties. A better investment of time would be to improve the immunizations and to attempt to increase the titer of the antisera in other animals followed by more fusions. Of course, this comment is valid only for cases like this one when the search is for high affinity antibodies to

a major antigenic component. In other circumstances, a different strategy may be more adequate (14).

The good yield of active clones indicates that immunization with yeast tubulin may have been a good choice. It is possible that using yeast tubulin as an antigen is a better way to prepare antibody to mammalian tubulin, because induction of a strong immune response could require multiple immunogenic determinants. The antiserum prepared from rats which had tumours of YL1/2 or YOL1/34 cells had very high titers of between $1:10^5$ and $1:10^6$, compared to the titer of $\sim 1:1,000$ of the original antiserum after two tubulin injections. This low titer is slightly higher than that of most conventional rabbit antitubulin sera (1, 15, 22). The purified IgGs of YL1/2 and YOL1/34 display overall similar properties to the rabbit monospecific antitubulin antibodies described to date (2). For example, they stain the interphase microtubule network of tissue culture cells at similar concentrations. YOL1/34 IgG is perhaps more suitable for general immunofluorescence work, since it gives clear microtubule staining in yeast and tissue culture cells.

The reason for the diffuse cytoplasmic immunofluorescent staining observed in yeast spheroplasts with YL1/2 IgG as compared with YOL1/34 is not clear. It is unlikely to be due

to a fixation artifact because it was not observed in the HK control and it was observed with glutaraldehyde, formaldehyde and methanol fixation. It is possible that in yeast spheroplasts there are different forms of tubulin antigens, and the equilibrium constant of YL1/2 and YOL1/34 for the structural variants are different. Diffuse cytoplasmic staining with monospecific antitubulin has also been observed in *Dictyostelium* (32). Whatever the reason, this difference as well as the multidot staining of nuclei (Fig. 3 b) given by the different monoclonal antibodies is a likely long-term asset for many purposes. It remains to be seen whether for general use specific mixtures to mimic polyclonal reagents will be more convenient or better than selected monoclonal antibodies.

We are currently investigating the potential of these monoclonal antibodies in purifying microtubule-containing organelles, particularly in yeast spindle pole bodies with half-spindles attached, using the methods developed by Ito and Palade (17) with conventional antisera.

We thank J. Fogg, J. M. Jarvis, and P. Wright for skilled assistance and Mark Furth for help with the immunofluorescence method.

Received for publication 9 November 1981, and in revised form 2 February 1982.

REFERENCES

- Bhattacharyya, B., and J. Wolff. 1975. Membrane-bound tubulin in brain and thyroid tissue. *J. Biol. Chem.* 250:7639-7646.
- Brinkley, B. R., S. H. Fistel, J. M. Marcum, and R. L. Pardue. 1980. Microtubules in cultured cells; indirect immunofluorescent staining with tubulin antibody. *Int. Rev. Cytol.* 63:59-95.
- Byers, B., and L. Goetsch. 1974. Duplication of the spindle plaques and integration of the yeast cell cycle. *Cold Spring Harbor Symp. Quant. Biol.* 38:123-131.
- Chang, M. T., J. V. Kilmartin, and W. F. Dove. 1981. Monoclonal antibodies directed against yeast tubulin: cross-reaction to tubulin from pig and *Physarum polycephalum* and subunit specificity. *J. Cell. Biol.* 91(2, Pt. 2):333a (Abstr.).
- Clark, M. R., and C. Milstein. 1981. Expression of spleen cell immunoglobulin phenotype in hybrids with myeloma cell lines. *Somatic Cell Genet.* 7:657-666.
- Cotton, R. G. H., and C. Milstein. 1973. Fusion of two immunoglobulin producing myeloma cells. *Nature (Lond.)* 244:42-43.
- De Brabander, M., J. De May, M. Jonian, and S. Genens. 1977. Immunocytochemical visualization of microtubules and tubulin at the light and electron microscopic level. *J. Cell Sci.* 28:283-301.
- De May, J., M. Moremans, G. Genens, R. Muydens, H. Van Belle, and M. De Brabander. 1980. Immunocytochemical evidence for the association of calmodulin with microtubules of the mitotic apparatus. In *Microtubules and Microtubule Inhibitors*. M. De Brabander, and J. De May, editors. Elsevier/North Holland Biomedical Press, Amsterdam. 227-241.
- De May, J., M. Moeremans, G. Genens, R. Nuydens, and M. De Brabander. 1981. High resolution light and electron microscopic localization of tubulin with the IGS (immunogold staining) method. *Cell Biology International Reports*. 5:889-899.
- Dentler, W. L., S. Granett, and J. L. Rosenbaum. 1975. Ultrastructural localization of the high molecular weight proteins associated with in vitro-assembled brain microtubules. *J. Cell Biol.* 65:237-241.
- Eckert, B. S., and J. A. Snyder. 1978. Combined immunofluorescence and high-voltage electron microscopy of cultured mammalian cells, using an antibody that binds to glutaraldehyde-treated tubulin. *Proc. Natl. Acad. Sci. U. S. A.* 75:334-338.
- Fuller, G. M., B. R. Brinkley, and J. M. Boughter. 1975. Immunofluorescence of mitotic spindles by using monospecific antibody against bovine brain tubulin. *Science (Wash. D. C.)* 187:948-950.
- Galfre, G., C. Milstein, and B. Wright. 1979. Rat x rat hybrid myelomas and a monoclonal anti-Fd portion of mouse IgG. *Nature (Lond.)* 277:131-133.
- Galfre, G., and C. Milstein. 1981. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol.* 73(part B):1-46.
- Gozas, I., U. Z. Littauer, B. Geiger, and S. Fuchs. 1977. Immunochemical determination of tubulin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 73:109-114.
- Hiller, G., and K. Weber. 1978. Radioimmunoassay for tubulin: a quantitative comparison of the tubulin content of different established tissue culture cell lines and tissues. *Cell.* 14:795-804.
- Ito, A., and G.E. Palade. 1978. Presence of NADPH-cytochrome P₄₅₀ reductase in rat liver Golgi membranes. Evidence obtained by immunoadsorption methods. *J. Cell Biol.* 79:590-597.
- Kilmartin, J.V. 1981. Purification of yeast tubulin by self-assembly *in vitro*. *Biochemistry.* 20:3629-3633.
- Kohler, G., and C. Milstein. 1976. Derivation of specific antibody-producing tissue culture and tumour lines by cell fusion. *Eur. J. Immunol.* 6:511-519.
- Kowit, J. D., and C. Fulton. 1974. Purification and properties of flagellar outer doublet tubulin from *Naegleria gruberi* and a radioimmune assay for tubulin. *J. Biol. Chem.* 249:3638-3646.
- Lachmann, P. J., R. G. Oldroyd, C. Milstein, and B. W. Wright. 1980. Three rat monoclonal antibodies to human C3. *Immunology.* 41:503-515.
- Le Guern, C., P. Pradelles, F. Dray, C. Jeantet, and F. Gros. 1977. Radioimmunoassay for tubulin detection. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 84:97-100.
- Morgan, J. L., C. R. Holladay, and B. S. Spooner. 1978. Species-dependent immunological differences between vertebrate brain tubulins. *Proc. Natl. Acad. Sci. U. S. A.* 75:1414-1417.
- Murphy, D. B., R. B. Vallee, and G. G. Borisy. 1977. Identity and polymerization-stimulating activity of the nontubulin proteins associated with microtubules. *Biochemistry.* 16:2598-2605.
- Osborn, M., and K. Weber. 1976. Cytoplasmic microtubules in tissue culture cells appear to grow from an organising structure towards the plasma membrane. *Proc. Natl. Acad. Sci. U. S. A.* 73:867-871.
- Osborn, M., R. E. Webster, and K. Weber. 1978. Individual microtubules viewed by immunofluorescence and electron microscopy in the same PtK2 cell. *J. Cell Biol.* 77:R27-R34.
- Peterson, J. B., R. H. Gray, and H. Ris. 1972. Meiotic spindle plaques in *Saccharomyces cerevisiae*. *J. Cell Biol.* 53:837-841.
- Querijnjean, P., H. Bazin, A. Beckers, C. Deckens, J. F. Heremans, and C. Milstein. 1972. Transplantable immunoglobulin secreting tumours in rats. *Eur. J. Biochem.* 31:354-359.
- Rozijn, Th.H., and G. J. M. Tonino. 1964. Studies on the yeast nucleus. I. The isolation of nuclei. *Biochim. Biophys. Acta* 91:105-112.
- Schliwa, M., U. Enteneuer, J. C. Bulinski, and J. G. Izant. 1981. Calcium lability of cytoplasmic microtubules and its modulation by microtubule-associated proteins. *Proc. Natl. Acad. Sci. U. S. A.* 78:1037-1041.
- Silflow, C. D., and J. L. Rosenbaum. 1981. Multiple α and β tubulin genes in *Chlamydomonas* and regulation of tubulin mRNA levels after flagellation. *Cell.* 24:81-88.
- Unger, E., S. Rubino, T. Weinert, and P. Cappuccinelli. 1979. Immunofluorescence of the tubulin system in cellular slime molds. *FEBS (Fed. Eur. Microbiol. Soc.) Lett.* 6:317-320.
- Van De Water III, L., and J. B. Olmsted. 1978. Quantitation and characterization of antibody binding to tubulin. *J. Biol. Chem.* 253:5980-5984.
- Weber, K., J. Wheland, and W. Herzog. 1976. Griseofulvin interacts with microtubules both *in vivo* and *in vitro*. *J. Mol. Biol.* 102:817-829.
- Weber, K., P. C. Rathke, and M. Osborn. 1978. Cytoplasmic microtubular images in glutaraldehyde fixed tissue culture cells viewed by electron microscopy and by immunofluorescence microscopy. *Proc. Natl. Acad. Sci. U. S. A.* 75:1820-1824.
- Williams, A. F. 1977. Differentiation antigens of the lymphocyte cell surface. *Contemp. Top. Mol. Immunol.* 6:83-116.