Inhibition of microtubule assembly in vitro by anti-tubulin monoclonal antibodies

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Five monoclonal antibodies against N-terminal domains of α- or β-tubulin were tested for their ability to interfere with the in vitro formation of microtubules. Although all the antibodies exhibited similar association constants for immobilized tubulin, they differed in their inhibitory effect on microtubule assembly. For the most potent antibody, TU-13, the antibody/tubulin molar ratio of about 1:320 was sufficient for a 50% inhibition. The data indicate that the surface regions of N-terminal domains of tubulin are involved in the formation of microtubules.

Tubulin domain; Microtubule protein; Microtubule assembly; Monoclonal antibody

1. INTRODUCTION

Tubulin, the major component of microtubules, consists of two subunits (α and β) with a molecular weight of 50 000 each [1], that can be subdivided into N-terminal and C-terminal structural domains [2]. Although a model arrangement of these domains within a tubulin heterodimer has been proposed [3], there is little information on the participation of tubulin domain surfaces in the microtubule assembly. One possible approach to the structural and functional mapping of the tubulin molecule is the application of domain-specific antibodies [4]. Here were report the differential inhibitory effect of mAbs against N-terminal domains of tubulin subunits on in vitro assembly of microtubules.

2. MATERIAL AND METHODS

2.1. *Protein preparation*

MTP was isolated from porcine brain according to [5] and the microtubule sediment resulting from the second reassembly cycle was dissolved in 20 mM MES, pH 6.4, 80 mM NaCl, 0.5 mM $MgCl_2$, 1 mM EGTA (buffer A). The preparation contained more than 85% of tubulin as assessed by SDS-PAGE [6] and densitometric scanning of the gel. Tubulin was separated from the associated proteins as described in [7]. Protein concentration was determined according to [8] using bovine serum albumin as a standard.

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Abbreviations: (m)Ab(s), (monoclonal)antibody(ies); MES, 2-(*N*-morpholino)ethanesulphonic acid: MTP, microtubule protein; SDS-PAGE, polyacrylamide gel electrophoresis with sodium dodecylsulphate; SwAM, swine antibody against mouse immunoglobulin

2.2. *Antibodies*

Mouse mAbs TU-02, TU-03, TU-04, TU-07, TU-09 against the α-subunit of tubulin, and mAbs TU-06, TU-13 against the β-tubulin have been previously described [9,10]. The mouse mAb TEC-01 against the carbohydrate epitope of teratocarcinoma stem cells [11] was used as a negative control. All mAbs belonged to the IgM class. The mAbs were purified from ascitic fluid [9], dialyzed against phosphate-buffered saline, pH 7.2 and centrifuged at 100 000 × *g* for 20 min at 4°C. The mAb purity attained was higher than 80%.

2.3. *Turbidimetric measurements*

The microtubule assembly was monitored by turbidimetric measurements [12] at 360 nm and 37°C in 0.5 cm cuvettes using the spectrophotometer SPECORD UV VIS with temperature-controlled cuvette holder (Carl Zeiss, Jena, GDR). The assembly was initiated by an addition of cold (4°C) MTP and GTP solutions to the prewarmed (37°C) mAbs at various concentrations (method I) [13]. The resulting assembly mixture contained 1.5–1.8 $mg \cdot ml^{-1}$ MTP in 10 mM MES, 105 mM NaCl, 5 mM Na_2HPO_4, 0.25 mM $MgCl_2$, 0.5 mM EGTA, 0.36 mM GTP. To distinguish between the turbidity caused by microtubule formation and that caused by non-microtubular aggregates, the turbidity drop after 5 min cold (4°C) incubation was recorded (method II). Electron microscopy [14] verified that the microtubules completely disassembled after cold incubation. To test the microtubule-destroying action of mAbs, these were added to preformed microtubules and the mixtures were incubated for 20 min at 37°C.

2.4. *Sedimentation assay*

Microtubules were prepared by polymerizing purified tubulin (0.5 $mg \cdot ml^{-1}$) for 30 min at 37°C in buffer A supplemented with 10 μM taxol, 1 mM GTP. After adding of mAbs (1:10 mAb/tubulin molar ratio) the microtubules were incubated 30 min at 37°C and pelleted at 200 000 × *g* for 30 min at 25°C through a cushion of 4 M glycerol in buffer A. The protein content of the pellet was determined on 12.5% SDS-PAGE.

2.5. *Solid phase radiometric assay*

Measurements of the reactivity of various mAbs with tubulin were performed as in [15] using Terasaki microplates coated with tubulin and ^{125}I-labelled SwAM (10^5 cpm per well, sp. act. approximately 370 $kBq \cdot \mu g^{-1}$).

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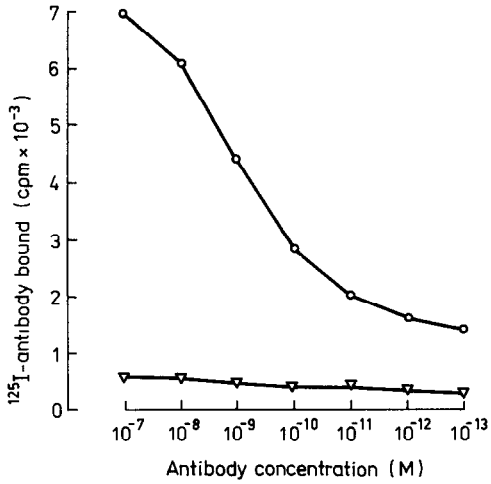

Fig.1. Binding of antibodies to immobilized tubulin as revealed by solid phase binding assay [15] using ^{125}I-labelled SwAM (10^5cpm/well), O—O, TU-02; ▽—▽, TEC-01.

Table 1

Effect of antibodies on microtubule assembly as assessed by turbidimetric measurements

Antibody	Concentration of mAbs (nM) causing 50% inhibition	
	Method I	Method II
TU-02	170	145
TU-03	50	50
TU-04	>330	>330
TU-06	150	n.m.
TU-09	>360	>360
TU-13	35	40
TEC-01	>350	>350

The extent of MTP assembly (concentration of tubulin approximately 12–14 μM) was determined either by $(\Delta A/\Delta A_C)\cdot 100\%$ (Method I) or by $(\Delta A_{cold}/\Delta A_{cold,C})\cdot 100\%$ (Method II). For explanation of the symbols see fig. 2; n.m., not measurable because of strong increase in turbidity at 4°C.

3. RESULTS

In the solid phase radiometric assay, all anti-tubulin mAbs gave titers similar to those shown for TU-02 in fig.1. The results indicate no substantial differences in the association constants between the mAbs and the tubulin. The TU-02 inhibited polymerization of MTP in vitro as can be inferred from the results of turbidimetric measurements; i.e., the lag phase of assembly was slightly prolonged and the extent of assembly *(ΔA) was reduced. After cold incubation turbidity* (ΔA_{cold}) did not drop completely to the initial basal level (fig.2), indicating the formation of a few nonmicrotubular aggregates. The assembly inhibition was concentration-dependent. In a similar way, microtubule assembly was also suppressed by the mAbs TU-03, TU-06 and TU-13 (table 1). An incubation (up to 30 min at 4°C) of mAbs with MTP prior to the assembly did not change the inhibitory action. The microtubule formation was not significantly inhibited by the mAbs TU-04, TU-09 and TEC-01, while the mAb TU-07 precipitated MTP. The mAbs which exhibited an inhibitory effect also partially disrupted the preformed steady-state microtubules. However, the disrupting activity was weaker than the inhibitory effect. The sedimentation assay using taxol-stabilized microtubules and the mAb TU-13, which had been found to possess the strongest inhibitory power, showed an almost undetectable binding of the mAb to the microtubules contrary to the mAb TU-07 (fig.3).

4. DISCUSSION

The influence of N-terminal tubulin domains on the process of microtubule assembly in vitro was studied by means of the mAbs TU-02, TU-03 and TU-09 binding to the N-terminal domain of α-subunit, as well as the TU-06 binding to the N-terminal domain of β-subunit [9]. The epitope recognized by the mAb TU-13 is also

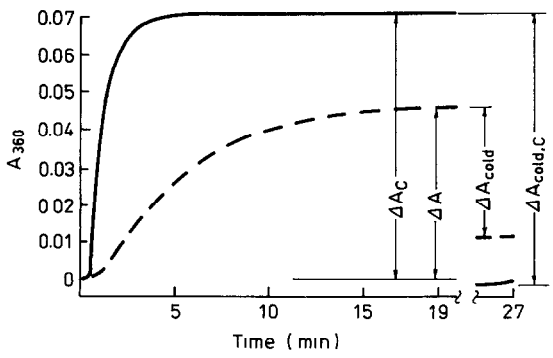

Fig.2. Time course of microtubule polymerization in the absence (——) and presence (– – –) of TU-02 (47 nM). 0–20 min, incubation at 37°C; 20–27 min, incubation at 0°C. Assembly extent at 37°C in the absence (control, ΔA_C) or presence (ΔA) of TU-02. Drop in the assembly extent at 0°C in the absence (control, $\Delta A_{cold,C}$) or presence (ΔA_{cold}) of mAb.

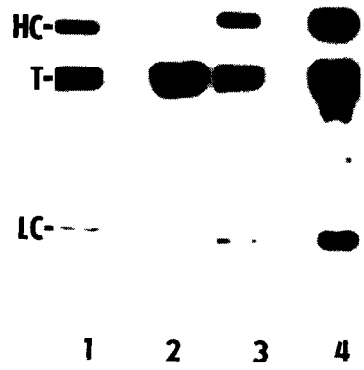

Fig.3. SDS-PAGE analysis of the binding of anti-tubulin antibodies to taxol-stabilized microtubules. Antibodies TU-13 (lanes 1,2) and TU-07 (lanes 3,4) were incubated with taxol-microtubules and pelleted through the 4 M glycerol cushion. Lanes 1,3, mixtures before centrifugation; lanes 2,4, corresponding pellets; HC, IgM heavy chain; LC, IgM light chain; T, tubulin.

located in the N-terminal domain of β-tubulin [unpublished]. The mAbs TU-04 and TU-07 against C-terminal domain of α-subunit and against conformational epitope of α-tubulin, respectively, were used as a control [9]. Our data show that some mAbs against the N-terminal domains of tubulin subunits can strongly inhibit the microtubule assembly. The results of the solid phase radiometric assay indicated that the differential inhibition effect of mAbs was not due to differences in the association constants between the mAbs and tubulin.

Whereas some polyclonal anti-tubulin Abs or their F_{ab} fragments are known to be able to inhibit microtubule assembly in vitro at the 1:1 Ab/tubulin molar ration [2], mAbs tested so far had reportedly no effect on microtubule assembly in vitro [16]. Some of the mAbs tested here do inhibit the microtubule formation even at substoichiometric concentrations e.g. 50% inhibition by TU-13 at a mAb-tubulin molar ratio of approximately 1:320. In the experiments measuring the association of TU-13 with taxol-stabilized microtubules we were unable to detect the binding of TU-13 even at higher mAb concentrations. To explain the inhibition mechanism, it could be assumed that the active mAbs block the microtubule formation by binding to the microtubule ends. Since steady-state microtubules reveal remarkable length fluctuations at both ends [17], such a capping phenomenon could also explain the disruption of preformed microtubules. Microinjection experiments could reveal whether or not the mAbs influence the stability of microtubules also in vivo. Reversible disruption of cytoplasmic microtubules in living cells by high affinity polyclonal antibody to β-tubulin has already been described [18].

In summary, we described for the first time an inhibitory effect of domain-specific mAbs on microtubule assembly in vitro. Our data suggest that surface regions of N-terminal domains of both tubulin subunits are involved in the formation of microtubule structures. Some of the target epitopes recognized by the inhibitory antibodies could be essential for tubulin polymerization.

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