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(54) **Titre :** ANTICORPS MONOCLONAL CONTRE LES AGENTS CHELANTS COMPLEXES ET NON COMPLEXES, POUR ELIMINER LES METAUX LOURDS DES SOLUTIONS AQUEUSES ET POUR ANALYSE
(54) **Title:** MONOCLONAL ANTIBODY AGAINST COMPLEXED AND NONCOMPLEXED COMPLEXING AGENTS FOR REMOVING HEAVY METALS FROM AQUEOUS SOLUTIONS AND FOR ANALYSIS

(57) **Abrégé/Abstract:**

The invention relates to monoclonal antibodies (mAB) which bind to water-soluble complexones such as ethylenediamine tetraacetate (EDTA) or diethylenetriamine pentaacetate (DTPA) with high specificity and avidity and retain their high avidity and specificity for these complexones after complexing of EDTA or DTPA with metal ions. These mAB can therefore be used, e.g. coupled on filters or other supports, for removing toxic heavy metals which are complexed with EDTA or DTPA. Furthermore, these mAB are suitable as components of immunoassays (RIA, ELISA etc.) for the quantitative determination of EDTA or DTPA in aqueous solutions.



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Abstract

A monoclonal antibody against complexed and noncomplexed complexing agents for removing heavy metals from aqueous solutions and for analysis

The invention relates to monoclonal antibodies (mAB) which bind to water-soluble complexones such as ethylenediamine tetraacetate (EDTA) or diethylenetriamine pentaacetate (DTPA) with high specificity and avidity and retain their high avidity and specificity for these complexones after complexing of EDTA or DTPA with metal ions. These mAB can therefore be used, e.g. coupled on filters or other supports, for removing toxic heavy metals which are complexed with EDTA or DTPA. Furthermore, these mAB are suitable as components of immunoassays (RIA, ELISA etc.) for the quantitative determination of EDTA or DTPA in aqueous solutions.

Description

- 5 A monoclonal antibody against complexed and noncomplexed
complexing agents for removing heavy metals from aqueous
solutions and for analysis

10 The invention relates to monoclonal antibodies (mAB)
which bind to water-soluble complexones such as ethy-
lenediamine tetraacetate (EDTA) or diethylenetriamine
pentaacetate (DTPA) with high specificity and avidity and
retain their high avidity and specificity for these
15 complexones after complexing of EDTA or DTPA with metal
ions. These mAB can therefore be used, e.g. coupled on
filters or other supports, for removing toxic heavy
metals which are complexed with EDTA or DTPA. Further-
more, these mAB are suitable as components of immuno-
assays (RIA, ELISA etc.) for the quantitative determina-
20 tion of EDTA or DTPA in aqueous solutions.

Heavy metals which are linked to complexones such as EDTA
are a serious danger in the field of drinking water
supply. For this reason, the fast, exact and quantitative
determination of the concentration of EDTA is of as great
25 an importance as the removal of EDTA metal complexes from
waste waters or drinking water.

In our project for obtaining mAB against hydrophilic
complexones for the purpose of tumor therapy, we have
surprisingly been successful in generating mAB which have
30 a high avidity against both noncomplexed and metal-
complexed EDTA or DTPA.

The mAB were prepared as follows. As is explained indi-
vidually in the examples, isothiocyanatobenzyl-DTPA was
coupled to human serum albumin and complexed with yttrium
35 ions as hapten. This hapten-"carrier"-complex was

subsequently used for immunizing suitable mice and the spleens of the mice with the highest anti-DTPA antibody titers were fused with a suitable myeloma cell line. The resulting hybridomas were tested in the specific enzyme-linked immunoassay (ELISA) described below.

The ELISA comprised a solid phase which was loaded with a solution containing human serum albumin (HSA)-benzyl-DTPA. The supernatant which was to be tested and contained the mAB was preincubated with free complexone or the metal ion complex thereof, and its binding to the specific solid phase was measured. For this purpose, an enzyme amplification system which is coupled to an anti-mouse-immunoglobulin antibody was used. The details of this method are described in Examples 2 and 3.

By means of this test system, mAB which have the properties described in Table I were obtained.

These mAB, in contrast to many other anti-DTPA/EDTA mAB, do not bind to normal human tissue as was determined on cryopreserved tissues by means of the APAAP technique (Cordell et al., J. Histochem. Cytochem. 32: 219, 1984). It is thus possible to employ these mAB in vivo in the field of diagnosis and therapy.

The complexones DTPA and EDTA in noncomplexed and in complexed form (Example 4) were used as competitors. Additionally, the structurally related compounds trans-aconitic acid and 1,2-diaminoethane were used as inhibitors (see Tab. I). The excess amounts of competitor presented in Tab. I, which lead to a 50% inhibition of the mAB antigen binding, show that the mAB listed have a strong binding to both noncomplexed EDTA or DTPA and EDTA or DTPA complexed with metal ions. These characteristics make it possible to use the mAB, e.g. coupled on filters or other solid supports, for removing both noncomplexed EDTA or DTPA and EDTA-metal ion or DTPA-metal ion complexes from liquids. Precipitation reactions of

mAB-/EDTA-metal ion complexes are likewise suitable for removing the toxic metal ions.

5 A particularly suitable mAB for this use is mAB BW 2050/535 whose interaction with EDTA and the complexes thereof is particularly strong.

10 The invention therefore relates to an mAB with a high affinity against those EDTA or DTPA complexed with metal ions and noncomplexed EDTA or DTPA, to a process for the preparation thereof and to the use thereof as component of a diagnostic or therapeutic, or for the removal of toxic metal ions.

The invention is furthermore described in more detail in the examples and contained in the patent claims.

Example 1:

15 Preparation of EDTA- or DTPA-specific mAB

15 Isothiocyanatobenzyl-DTPA was, as hapten, covalently coupled to human serum albumin (HSA) as carrier according to the method described by N.W. Brechbiel et al., Inorganic Chemistry 25: 2772-2781 (1986) with a degree of
20 derivatization of 19 benzyl-DTPA molecules per HSA molecule. 20 µg of this hapten-carrier complex, into which unlabeled YCl₃ was complexed, were injected s.c. into BALB/c mice on day 0 with Freund's adjuvant, on day 7 and day 14 with incomplete Freund's adjuvant and on day
25 21 with PBS. On day 24 the spleens of the mice with the highest anti-DTPA antibody titers were fused with the SP2/0-Ag14 myeloma cell line (Shulman et al., Nature 276: 269 (1976)). Resulting hybridomas were tested for the production of mAB with high affinity in a specific ELISA
30 (Example 2).

Example 2:Quantitative inhibition ELISA for mAB by DTPA or EDTA complexes

5 Divisible 96-well polystyrene microtiter plates (U-shape) type B, supplied by Nunc, No. 4-60445 were employed as support material. The assay was carried out according to the following protocol.

- 10 (1) 50 μ l of Y benzyl-DTPA-HSA 19-conjugate with a concentration of 1 μ g of conjugate per ml of PBS, pH 7.2, are pipetted per well and incubated overnight at room temperature (RT)
- 15 (2) The supernatant is aspirated off and washing is carried out 3x using 0.05 M tris-citrate buffer, pH 7.4 (wash solution 1); (1x washing $\hat{=}$ introduce 250 μ l of wash solution per well, leave to stand for 2 min, aspirate off).
- 20 (3) If the microtiter plate is not required directly, it is left standing (opening down) on cellulose at RT overnight. Then the plate is heat-sealed in films together with balance desiccators (supplied by Gaplast, Postfach 529, 8100 Garmisch-Partenkirchen). Under these conditions, the plates can be kept for at least 8 weeks at +4°C.
- 25 (4) 250 μ l of blocking solution per well are applied and incubated at 37°C for 30 min.
- (5) During the blocking, the preincubation of the diluted hybridoma supernatant with the competitor is carried out (see Example 3 or Example 6).
- 30 (6) 50 μ l of the hybridoma supernatants which are to be tested and have been appropriately prediluted and preincubated are applied per well and incubated at room temperature for 30 min.
- (7) Washing is subsequently carried out 3x using wash solution 2.
- 35 (8) Then 50 μ l of goat anti-mouse-IgG₁ antibody which has been diluted 1:500 in blocking solution and is labeled with alkaline phosphatase are applied per

well and incubated at room temperature for 30 min.

(9) Washing is then carried out 3x using wash solution for Enzygnost^R (Behringwerke AG).

(10) Subsequently, 50 μ l of 0.1 mM NADP are added.

5 (11) Incubation is then carried out at room temperature for 30 min.

(12) During the incubation with NADP, the amplifier system is prepared as follows:

10 per plate are added 2 parts of p-iodo-nitrotetrazolium violet (INT) and 1 part of PBS, pH 7.2, furthermore 1 part of diaphorase, and 1 part of ADH is furthermore pipetted into the mixture.

(13) 50 μ l of amplifier system are added per well.

15 (14) In the case of a clear change in color from transparent to red, the reaction is stopped by 100 μ l of a 0.1 N H₂SO₄ solution per well.

20 (15) The extinctions are measured at 492 nm in the TITERTEK^R MULTISCAN. 50 μ l of NADP together with 50 μ l of amplifier solution and 100 μ l of 0.1 N H₂SO₄ are employed as blank value.

The following reagents were used:

NADP	- supplied by Sigma, order no. N-0505
INT	- supplied by Sigma, order no. I-8377
ADH	- supplied by Sigma, order no. A-3263
25 DIAPHORASE	- supplied by Sigma, order no. D-2381
Wash solution 2	- supplied by Behring, order no. OSEW96, contains Tween/PBS

30 Blocking solution: a 3% casein solution is prepared in PBS, pH 7.2, by adding casein and stirring for 30 minutes, and the pH is adjusted to 7.4. Then particles are centrifuged off at 4000 rpm for 10 min.

Goat anti-mouse-IgG₁ antibody which has been diluted and is labeled with alkaline phosphatase (supplied by Southern Biotechnology Associates, Cat. No. 1080-04)

Preparation of 0.1 mM NADP:

Dissolve 7.65 mg of NADP in 100 ml of 20 mM Tris, 0.1 mM MgSO_4 , pH 9.5; the solution can be stored for several months at -20°C .

- 5 Preparation of INT (p-iodonitrotetrazolium violet):
Dilute 2.5 mg/ml of 30% ethanol in an ultrasonic bath; always prepare freshly.

Preparation of diaphorase:

- 10 1 mg of diaphorase/ml of PBS, pH 7.2, is stored in portions at -20°C .

Preparation of alcohol dehydrogenase:

0.5 mg of ADH/ml of PBS, pH 7.2, is stored in portions at -20°C .

Example 3:

- 15 Preincubation of the hybridoma supernatant with the competitor

The mouse-IgG concentration in the hybridoma supernatants can be determined by means of commercially available quantitative ELISA systems and is state of the art.

- 20 With reference to the determination of the concentration in the ELISA, the hybridoma supernatants are diluted to 1.25 $\mu\text{g/ml}$ in PBS without Ca^{++} and Mg^{++} .

Conversion of grams into moles: $150,000 \text{ g} = 1 \text{ mole of mAB}$
 $1.25 \mu\text{g} = 8.33 \times 10^{-12} \text{ mole}$

- 25 In order to have a 1+1 ratio of mAB and inhibitor, 10 μl of inhibitor with a concentration of $8.33 \times 10^{-12} \text{ mole/200 } \mu\text{l}$ which had been increased a factor of 5 were added to 50 μl of hybridoma supernatant with a concentration of $8.33 \times 10^{-12} \text{ mole/ml}$.

5 The hybridoma supernatant is incubated for 30 min with a 100,000 fold, 50,000 fold, 10,000 fold, 5,000 fold, 1,000 fold and 100 fold excess of competitor at room temperature. 50 μ l thereof are pipetted into the ELISA (see Example 2, item 6).

Example 4:

Generation of the DTPA or EDTA complexes

10 The complex constant of DTPA or EDTA in relation to the metal ions shown in Table I is extremely high so that a complete saturation is to be expected in the case of an equimolar mixture of DTPA or EDTA with these metal ions. The appropriate metal ions were therefore incubated with the DTPA or EDTA in a 3 fold molar excess. As an example, 170 μ l of a 10 mM cadmium sulfate solution in double-
15 distilled water (see Example 5) were incubated at room temperature with 30 μ l of a 0.028 molar DTPA stock solution in double-distilled water for 5 min. Admixing 10 μ l of this competitor solution to the hybridoma supernatant leads to a 100,000 fold excess of competitor
20 over the mAB contained in the hybridoma supernatant. Lower ratios of competitor to mAB were achieved by diluting the competitor solution in the particular salt ion solution according to the desired molar excess (see Example 3).

25 Example 5:

Source and relevant physicochemical parameters of the metal ions used

10 mmolar solutions of the following metal ions were prepared in double-distilled water:

30 Manganese chloride MW 161.88
supplied by Merck, No. 5934 Ionic radius of Mn: 80 pm

Cadmium sulfate MW 256.5
supplied by Riedel de Haen
No. 31145

Ionic radius of Cd: 97 pm

5 Zinc chloride MW 136.28
supplied by Merck, No. 8816

Ionic radius of Zn: 74 pm

Copper sulfate MW 159.61
supplied by Riedel de Haen
No. 31294

Ionic radius of Cu: 96 pm

10 Yttrium chloride MW 303.36
supplied by Aldrich
No. 20,491-9

Ionic radius of Y: 92 pm

15 Lead(II) nitrate MW 331.20
supplied by Riedel de Haen
No. 31137
pm

Ionic radius of Pb: 120

Example 6:

Competitive enzyme immunoassay for determining the EDTA
or DTPA concentration in aqueous solvents

20 In the quantitative inhibition ELISA described in Example
2, an aqueous sample (50 μ l) having an unknown EDTA
content to be determined is added instead of the defined
amounts of competitor (lines 26-28, step 5). The assay is
further carried out as described in Example 2, step 6-15.
25 The reduction of the extinction is proportional to the
concentration of EDTA or DTPA in the unknown sample.
After establishing a calibration plot by adding defined
EDTA or DTPA concentrations and appropriate predilution
of the unknown sample to be tested, the EDTA or DTPA con-
centration in the unknown sample can be determined
30 quantitatively.

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5 Example 7:Description of the particularly suitable mAB BW 2050/535

10 The V_H and V_L genes of mAB BW 2050/535 have been cloned according to the method described by Orlandi et al. (Proc. Natl. Acad. Sci. U.S.A. 86, 3833 (1989)) and were then sequenced. The nucleotide sequence of the V_H gene of mAB BW 2050/535 is depicted in Table II, and of the V_L gene in Table III. The sequence of the V_H gene
15 comprises the coding sequence for amino acids 4 through 110 of the mature protein. The sequence of the V_L gene comprises the coding sequence for amino acids 3 through 106 of the mature protein.
The amino acids are designated in three letter code.

20

Table 1

Quantitative inhibition assay of mAB by DTPA or EDTA complexes

Molar excess of competitor, which leads to 50% inhibition of binding to the solid phase antigen.

MAK-Nr.	DTPA-Y	DTPA	DTPA-Mn	DTPA-Cd	DTPA-Zn	DTPA-Cu	DTPA-Pb	1,2- diaminoethane
2050/174	10^4	10^3	10^2	10^2	5×10^3	5×10^3	10^3	no inhibition up to 10^5
2050/531	5×10^4	10^3	10^2	10^2	5×10^3	5×10^3	5×10^3	"
2050/532	5×10^4	10^3	10^2	10^2	5×10^3	5×10^3	5×10^3	"
2050/534	5×10^4	10^3	10^2	10^2	5×10^3	5×10^3	5×10^3	"
2050/535	10^4	10^2	10^2	10^2	10^3	10^3	10^3	"

Table I - continued

mAb no.	trans-aconitic acid	EDTA-Y	EDTA	EDTA-Mn	EDTA-Cd	EDTA-Zn	EDTA-Cu	EDTA-Pb
2050/174	no inhibition up to 10 ⁵	10 ²	10 ³	10 ³	10 ³	10 ³	10 ³	5x10 ³
2050/531	"	10 ³	10 ³	10 ³	10 ³	10 ³	10 ²	10 ⁵
2050/532	"	10 ²	10 ³	10 ³	10 ³	10 ²	5x10 ³	10 ⁵
2050/534	"	10 ²	10 ³	10 ²	10 ²	10 ³	10 ³	5x10 ³
2050/535	"	10 ²	10 ²	10 ²	10 ²	10 ²	10 ²	10 ²

Table II:

1	/	1	31	/	11														
ctg	cag	cag	tct	ggg	gca	gag	ctt	gtg	aag	cca	ggg	gcc	tca	gtc	aag	ttg	tcc	tgc	aca
leu	gln	gln	ser	gly	ala	gln	leu	val	lys	pro	gly	ala	ser	val	lys	leu	ser	cys	thr
61	/	21								91	/	31							
gct	tct	ggc	tta	aac	att	aaa	gac	acc	tat	ata	aac	tgg	gtg	aag	cag	agg	cct	gaa	cag
ala	ser	gly	leu	asn	ile	lys	asp	thr	tyr	ile	asn	trp	val	lys	gln	arg	pro	glu	gln
121	/	41								151	/	51							
ggc	ctg	gag	tgg	att	gga	agg	att	ggt	cct	gcg	aat	ggt	aat	act	aaa	tat	gac	cag	aag
gly	leu	glu	trp	ile	gly	arg	ile	gly	pro	ala	asn	gly	asn	thr	lys	tyr	asp	pro	lys
181	/	61								211	/	71							
ttc	cag	ggc	aag	gac	act	tta	aca	gca	gac	aca	tcc	tcc	aac	aca	gcc	tac	cta	caa	ctc
phe	gln	gly	lys	ala	thr	leu	thr	ala	asp	thr	ser	ser	asn	thr	ala	tyr	leu	gln	leu
241	/	81								271	/	91							
agc	agc	ctg	aca	tct	gag	gac	act	gcc	gtc	tat	tac	tgt	tct	aga	aga	tgg	ttc	tct	ctt
ser	ser	leu	thr	ser	glu	asp	thr	ala	val	tyr	tyr	cys	ser	arg	arg	trp	phe	phe	leu
301	/	101																	
tac	tgg	ggc	caa	ggg	acc	acg	gtc	acc											
tyr	trp	gly	gln	gly	thr	thr	val	thr											

Table III:

2050/535 VI.

1	/	1	31	/	11	
cag	ctg	act	cag	gaa	tct	gca
gln	leu	thr	gln	ser	ala	leu
61	/	21	91	/	31	
cgc	tca	agt	act	gga	gct	gca
arg	ser	ser	thr	gln	ala	val
121	/	41	151	/	51	
gat	cat	tta	ttc	act	ggt	cta
asp	his	leu	phe	thr	gln	leu
181	/	61	211	/	71	
aga	ttc	tca	ggc	tcc	ctg	att
arg	phe	ser	gln	ser	leu	ile
241	/	81	271	/	91	
gag	gat	gag	gca	ata	tat	ttc
glu	asp	glu	ala	ile	tyr	phe
301	/	101				
ggg	acc	aag	ctg	aag	atc	
gly	thr	lys	leu	lys	ile	

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE
PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A monoclonal antibody (mAB) which has a high
affinity and reacts with about the same specificity
and avidity against EDTA or DTPA in the complexed or
noncomplexed state.
5
2. An mAB as claimed in claim 1, wherein isothio-
cyanatobenzyl-DTPA or isothiocyanatobenzyl-EDTA, as
hapten, coupled to human serum albumin (HSA) is used
as "carrier" for immunization.
- 10 3. An mAB as claimed in claim 2, which is isolated from
the resulting hybridomas by means of a competition
ELISA with HSA-benzyl-DTPA or HSA-benzyl-EDTA as
solid phase.
- 15 4. A process for the preparation of an mAB as claimed
in claim 1 to claim 3, which comprises using iso-
thiocyanatobenzyl-DTPA or isothiocyanatobenzyl-EDTA
coupled to HSA for immunization.
- 20 5. The process as claimed in claim 4, wherein an mAB
with a high affinity is isolated by means of a
competition ELISA with HSA-benzyl-DPTA or HSA-
benzyl-EDTA as solid phase.
6. The use of an mAB as claimed in claim 1 to claim 3
for purifying aqueous liquids contaminated with
heavy metal ions.
- 25 7. A filter material which contains mAB as claimed in
claim 1 to claim 3.
8. The use of an mAB as claimed in claim 1 to claim 3
for the quantitative determination of the EDTA or
DTPA concentration in aqueous solutions by means of
immunoassays.
30

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9. The use of an mAB as claimed in claim 1, claim 2 or claim 3 in an immunoassay.
10. A process for the quantitative determination of EDTA or DTPA in aqueous solutions, which comprises using
5 an mAB prepared as claimed in claim 1 or claim 2, preferably in the form of a suitable immunoassay.
11. An mAB as claimed in claim 1, wherein the V_H and V_L genes possess the nucleotide sequences described in Table II and Table III.