

DEMONSTRATION OF ANTIBODIES TO THE CHITIN-BINDING MISTLETOE LECTIN (CBML) IN TUMOR PATIENTS BEFORE AND DURING THERAPY WITH AN AQUEOUS MISTLETOE EXTRACT

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Abstract: Mistletoe extracts exert immunomodulatory properties *in vivo* and *in vitro*, and these effects have been related mainly to mistletoe lectin 1 (ML-1). Recently, a new chitin-binding mistletoe lectin (cbML) has been isolated and structurally characterized in these extracts. Aim of the present study was, therefore, to evaluate whether this cbML also affects immunocompetent cells and can for instance activate B-cells to produce anti-cbML-specific antibodies.

Sera from patients with different tumors who were treated with the mistletoe extract ABNOBAviscum Mali (AM) 4 for at least 18 weeks were analysed before therapy and after 3, 6, 9, 12, 18, and 24 weeks. Sera were tested by ELISA against ML-1, -3, and cbML, isolated from a single mistletoe plant collected from an apple tree (*Malus domestica*).

Eight of the 26 patients (31%) had IgG anti-cbML antibodies already before therapy, while only four had anti-ML-1 and -3 antibodies. Of the 18 anti-cbML negative patients before therapy 54% developed these antibodies during therapy, and there was a significant increase in anti-cbML antibody titers. In contrast, anti-ML-1 or -3-antibodies developed in almost 100% of the 25 patients being negative before therapy.

These data indicate that cbML can induce immunological responses in patients treated with mistletoe extracts, although it seems to have lower antigenicity. Interestingly, anti-cbML antibodies can be observed in a low incidence also in individuals, not having yet received mistletoe therapy.

Key words: mistletoe-therapy; antibodies; chitin-binding mistletoe lectin

Abbreviations: BSA: bovine serum albumin; cbML: chitin-binding mistletoe lectin; ELISA: enzyme linked immuno sorbent assay; ML: mistletoe lectin; NK-cells: natural killer cells; OD: optical density; PBS: phosphate buffered saline

INTRODUCTION

Extracts of the mistletoe plant *Viscum album* L. are quite frequently used in human cancer patients as ad-

ditive therapy. Several components have been meanwhile isolated from these extracts, such as amino acids, flavonoids, viscotoxins, poly- and oligosaccharides and lectins [11, 12, 35]. In recent years, the main interest has focused on mistletoe lectins (ML)-1, ML-2, and ML-3 because of their cytotoxic and immunomodulatory properties [3, 4, 9, 10, 13-15, 21, 23, 29, 32]. In each of the three lectins a cytotoxic protein A chain is linked via a disulfide bridge to a carbohydrate-binding protein B chain [7, 8]. Detailed primary and three-dimensional structures, including their glycosylation sites and oligosaccharide residues, have been reported for ML-1 [19, 24, 25, 34]. Recently, a new class of mistletoe lectins specifically binding to chitin (cbML) was detected; several members of this peptide family were isolated, and their primary structures determined. The main isomer consists of two identical polypeptide chains, each of 49 amino acid residues length. The two cysteine- and glycine-rich chains are linked by an intermolecular disulfide bond. The protein shows striking sequence homology with hevein and other proteins with hevein-like domains [2, 5, 18, 22, 26, 33, 36]. On the basis of NMR data of hevein from *Hevea brasiliensis* [2], a three-dimensional model of cbML could be generated, exhibiting as secondary structural elements one α -helix and four short β -sheets. The high-affinity chitin-binding site as well as the four intramolecular disulfide bonds are highly conserved.

In patients receiving mistletoe therapy, the production of antibodies against ML-1 as well as against viscotoxins is induced, and cells of the natural immune system (NK-cells, monocytes/macrophages, granulocytes) as well as T-lymphocytes are activated as shown by an enhanced proliferation and cytokine production [1, 10, 16, 17, 20, 23, 28, 30-32]. In the present study we will show that this kind of therapy also induces immunological reactions towards cbML, as evidenced by an induction of anti-cbML antibodies. It is, however, of interest, that some of the tumor patients had antibodies against cbML already prior to mistletoe treatment indicating that there may exist some kind of 'natural immunity' or cross reactivity towards this highly conserved protein.

PATIENTS AND METHODS

PATIENTS

Sera from 26 patients with different tumours (breast cancer $n = 8$, colon cancer $n = 3$, prostate cancer $n = 2$, renal cell carcinoma $n = 2$, basalioma $n = 2$, others $n = 9$) being treated with a vesicular mistletoe extract, were included in this study (provided by Dr. von Laue, Niefern-Öschelbronn) [16]. All patients had been treated for at least 18 weeks receiving 3x1 ampoules of AB-NOBAviscum Mali 4 (0.2 mg plant extract) subcutaneously three times per week. None of them had a previous viscum treatment, and all patients were without chemotherapy or radiation for at least eight months. Clinical data from these patients are presented in Table 1.

The stock solution of ABNOBAviscum Mali 4, ABNOBAviscum Mali 2 (20mg), contains 1,5-3 μ g/ml cbML, 6 μ g/ml ML-1 and ML-3, and about 70 μ g/ml viscotoxins.

Sera were obtained from all 26 patients before mistletoe therapy, from 20-23 of them after 3, 6, 9, 12, 18 and 24 weeks, and from 7 even after 31 weeks. Most patients developed local reactions and flue-like symptoms; severe side effects were not observed in any of the patients. The study was performed according to the Helsinki Declaration guidelines, and all patients gave written informed consent to participate in this study.

As controls, sera from 38 healthy blood donors were used in whom clinically and biochemically there was no evidence for any inflammatory process, but tumour had not been excluded with certainty. Sera from these individuals were kindly provided by Prof. Dr. D. Wernet, Department of Transfusion, University of Tübingen, Germany.

ANTIGENS

Material was obtained from a single mistletoe plant collected from an apple tree (*Malus domestica* Bang [Rosacea]) in the middle of September 1999. The plant was freed from the berries and stored at -20°C until use. The mistletoe lectins ML-1, ML-3 and cbML were isolated from the total mistletoe extract and purified as recently described [33]. The isoform cbML3 of cbML was used as antigen in further tests.

METHODS FOR DETECTION OF ANTIBODIES

Antibodies against mistletoe lectin 1 (ML-1) were determined by ELISA as recently described [22], and the same procedure was used for the detection of antibodies against ML-3 and cbML. Briefly, microtitre plates (Maxisorp, Nunc, Denmark) were coated with the antigens in a concentration of 1 μ g/ml, dissolved in hydrogen carbonate buffer (pH 9.6) over night at 4°C . Plates were then washed three times with phosphate buffered saline (PBS), pH 7.4, containing 1% bovine serum albumin (BSA; Sigma, St. Louis, USA) and incubated with this buffer for 60 min at room temperature in order to block free binding sites. After discarding the buffer, sera of the patients were added in duplicates at a dilution of 1:400 for the detection of IgG-antibodies

(90 min at room temperature). The plates were washed again three times with PBS containing 0.5% BSA and 0.2% Triton X 100 (Sigma, St. Louis, USA) and then incubated with monovalent peroxidase-conjugated anti-human IgG antibodies from goat (DIANOVA, Hamburg, Germany) for 60 min at room temperature. Reactivity of the antibodies was visualized by adding ortho-phenylenediamine as substrate (0.5 mg/ml in citrate buffer, pH 5.0) and read at 495 nm in an ELISA reader. Results are given as optical density (OD)x1000.

Optimal antigen, serum and anti-serum concentrations were determined by serial dilutions. In each further test, three sera with high, medium, and low antibody titres as well as a negative control were used as standard sera.

Normal values were determined testing sera from the 38 healthy blood donors against the three antigens. Mean values plus double the standard deviation of the OD measured with these sera were defined as upper limit of the normal range. Normal values for anti-ML-1 were calculated as $\text{OD} < 400$, for anti-ML-3 as $\text{OD} < 300$, and for anti-cbML as $\text{OD} < 500$.

ABSORPTION STUDIES

Sera from three individuals, one showing anti-cbML antibodies already before treatment with mistletoe extract and two developing the antibodies during therapy were incubated with cbML coupled to chitin beads [36] three times for 24h at 4°C . The sera before absorption and the supernatants of the beads after absorption were retested by ELISA against cbML as well as ML-1 and ML-3.

STATISTICAL ANALYSIS

Statistical analysis was performed using the nonparametric tests of Wilcoxon and Mann-Whitney. P values of less than 0.05 were considered as significant.

RESULTS

ANTI-ML-ANTIBODIES OF THE IGG-ISOTYPE

Sera from 8 of the 26 patients (31%) had anti-cbML antibodies of the IgG-type already before therapy with mistletoe extract (Table 2a). During the treatment period of up to 24 weeks, the incidence of anti-cbML increased to 65%. In contrast, only four of the sera (15%) reacted before therapy with ML-1 and only one serum (4%) with ML-3, but after 24 weeks 70-90% had become positive (Table 2b).

Of the 38 sera from healthy blood donors, none reacted with ML-1 or ML-3 and two with cbML (5%).

Within the group of patients being anti-cbML negative before therapy, the incidence of anti-cbML antibodies increased up to 54% during therapy until week 24 (Table 2), and there was a significant increase in antibody titres (Fig. 1a). In the group of patients being anti-cbML positive before therapy, antibody titres did not significantly change during therapy (Fig. 1b); nevertheless, some fluctuations were observed: three patients became transiently negative during therapy but

Table 1. Clinical data from the 26 tumour patients who have been treated with ABNOBAvis-cum Mali 4.

Patient number	Age*)	sex	kind of tumour	stage	Follow up time of anti-body determination (weeks)
1	71	m	multiple metastasis, unknown primary tumour	multiple metastasis	24
2	47	f	breast cancer	pT1bN0M0	24
3	59	f	breast cancer	PT1cN0M0, G1-2	24
4	59	m	basalioma	local	24
5	67	m	hepatocellular carcinoma	metastasis	18
6	73	f	colon cancer	pT3N0M0G2-3	>31
7	39	f	cervix cancer	pT1bG2N0M0	>31
8	57	f	breast cancer	pT1cG1/2N1bcM0	24
9	61	m	vocal cord leukoplakia	local	24
10	60	f	colon cancer	carcinoma in situ	24
11	57	m	renal cell carcinoma	multiple metastasis	>31
12	64	f	urinary bladder carcinoma	grade II, no metastasis	>31
13	58	f	breast cancer	sarcoma phylloides, no metastasis	24
14	68	f	gallbladder carcinoma	pT4N2M1	18
15	81	f	colon cancer	pT4pN3G3N1M1	24
16	67	f	breast cancer	pT1cG2N0M0	24
17	44	f	basalioma	local	>31
18	74	m	prostate cancer	pT3G2NxMx	18
19	72	m	renal cell cancer	pT2G2pN0(0/3)pMx	18
20	60	f	breast cancer	T2N1(2/15)M0	24
21	69	m	multiple myeloma		24
22	73	m	stomach cancer	pT2G3pN0M0	>31
23	32	m	synovialioma	no metastasis	18
24	48	f	breast cancer	pT2N1(11/19)M0G3	24
25	56	m	prostate cancer	pT2cG1bN0M0	>31
26	76	f	breast cancer	pT1cN0Mo	18

*) at entry into the study

T0: Carcinoma in situ; T1: tumour diameter less than 2cm, not fixed; T2: tumour not extending beyond the organ; T3: tumour extending beyond the organ; T4: tumour infiltrates surrounding tissue.

N0: regional lymph nodes not demonstrable; N1: demonstrable regional lymph nodes, not fixed; N2: distant lymph nodes involved, not fixed; N3: clinically palpable lymph nodes are fixed, metastases suspected.

M0: no evidence of distant metastasis; M1-M3: ascending degrees of metastatic involvement of the host including distant nodes. p: after surgery

in week 24, 86% still had anti-cbML antibodies in their blood (Table 2a).

The titres of antibodies against ML-1 and ML-3 increased significantly during the treatment period (Fig. 2).

With respect to the course of anti-cbML antibody development during therapy, three major patterns could be observed: patients, with negative antibody titers before and positive ones during therapy (n=11), patients with negative antibody titers before and during therapy (n = 7), and patients with positive antibody titers before and during therapy (n = 8) (Fig. 3a-

c). In all these patients, there was no correlation to the presence or absence of anti-ML-1 or anti-ML-3 antibodies (Fig. 3).

ABSORPTION STUDIES

Sera from three individuals had been incubated with cbML coupled to chitin beads and were retested by ELISA against cbML as well as ML-1 and ML-3. While the antibody reactivity to cbML had been completely abolished by this procedure in two and partially in one serum, the reactivity towards ML-1 and ML-3 was not altered (data not shown).

Table 2a. Incidence and development of anti-cbML antibodies of the IgG-isotype in 26 tumor patients before and during mistletoe therapy¹⁾

Week of treatment	number patients anti-cbML antibody positive/number tested (percent)		
	anti-cbML negative before therapy (n = 18) ²⁾	anti-cbML positive before therapy (n = 8) ²⁾	total
3	3/16 (19)	4/6 (67)	7/22 (32)
6	5/17 (29)	4/5 (80)	9/22 (41)
9	7/16 (44)	5/6 (83)	12/22 (55)
12	7/16 (44)	6/6 (100)	13/22 (59)
18	5/15 (33)	5/7 (71)	10/22 (45)
24	7/13 (54)	6/7 (86)	13/20 (65)

¹⁾ sera from two of the 38 healthy blood donors reacted against cbML (5%)
²⁾ at different time points sera of different numbers of patients were available

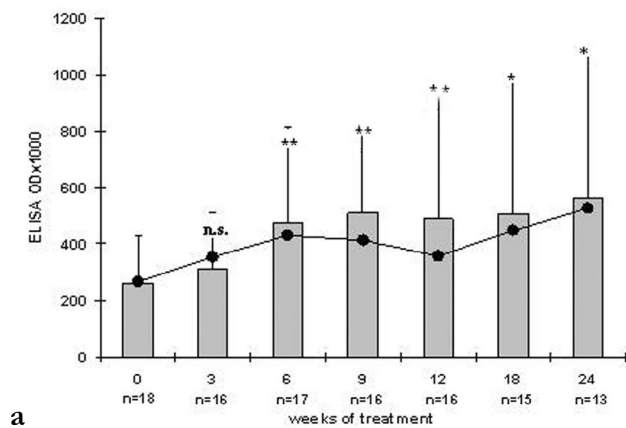
Table 2b. Incidence and development of anti-ML-1 and ML-3 antibodies of the IgG-isotype in 26 tumor patients before and during mistletoe therapy^{1, 2)}

Week of treatment	anti-ML-1	anti-ML-3
	number positive/number tested (percent)	
0 (before treatment)	4/26 (15)	1/26 (4)
3	11/22 (50)	5/22 (23)
6	20/22 (91)	13/22 (59)
9	22/23 (96)	17/23 (73)
12	20/21 (95)	16/21 (77)
18	19/21 (86)	16/21 (75)
24	18/20 (90)	14/20 (70)

¹⁾ sera from none of the 38 healthy blood donors reacted with ML-1 or ML-3
²⁾ Sera from seven patients could be analysed in week 31; all of them were anti-ML-1 and six (86%) anti-ML-3 positive.

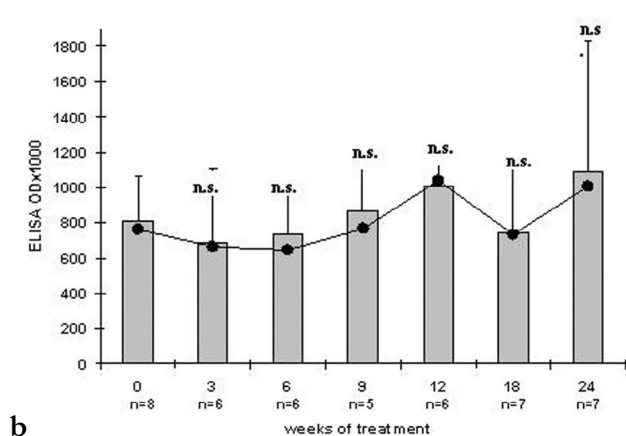
Fig. 1. Development of anti-cbML antibody titres in sera from 26 tumor patients treated with mistletoe extract being either anti-cbML negative (a) or positive (b) before therapy (week 0). Mean and standard deviation as well as the median (•) are given. * p<0.05; ** p<0.01; *** p<0.001

anti-cbML negative before treatment (n=18)



a

anti-cbML positive before treatment (n=8)



b

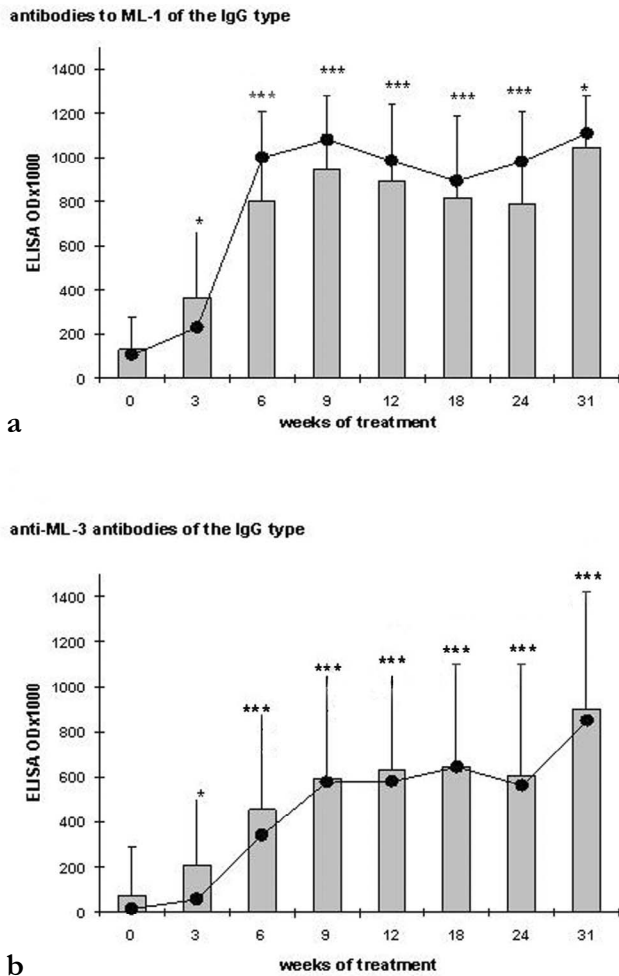


Fig. 2. Development of anti-ML-1 (a) and anti-ML-3 antibody titres (b) in 26 tumor patients treated with mistletoe extract. Mean (grey bars) and standard deviation as well as the median (•) are given. * p<0.05; ** p<0.01; *** p<0.001

DISCUSSION

There are many studies investigating the immunomodulatory properties of different components of mistletoe extracts *in vivo* and *in vitro*, and it was shown that especially ML-1, but also ML-2, ML-3 and viscotoxins, present in these extracts, activate immunocompetent cells of the innate as well as the specific immune system [3, 4, 6, 9, 10, 13-17, 20, 21, 23, 27-32]. Recently, a further component has been detected in these extracts, namely a chitin-binding lectin, synonymous to cbML [22, 33, 36]. In the present study we confirmed previous data on the production of anti-ML-1 and -3 antibodies of the IgG-type in tumour patients treated with mistletoe extracts [31, 32], and also antibodies of the IgE-type developed in 27%. However, it became evident that cbML can also provoke a specific immune response as shown by the induction of anti-cbML antibodies of the IgG-type. Only one individual developed anti-cbML antibodies of the IgE-type (not shown). CbML exists in three isoforms, cbML1, cbML2, and cbML3, which are all composed of two protein chains of 48-49 amino acid residues, linked by an intermole-

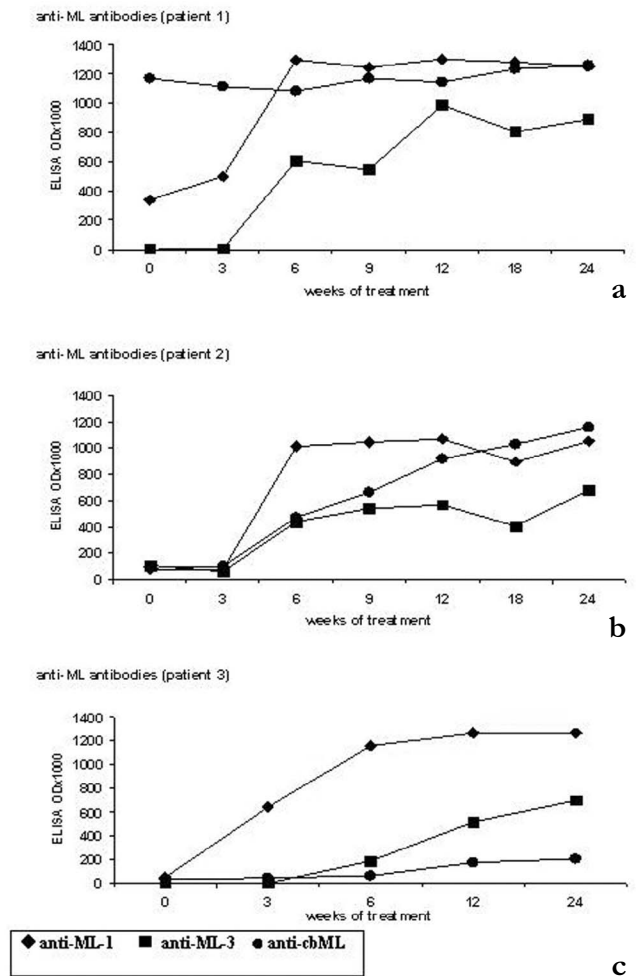


Fig. 3. Different courses of anti-cbML antibody titres and correlation with anti-ML-1 and anti-ML-3 in tumor patients treated with mistletoe extract exemplified for three patients. (a) the patient had anti-cbML antibodies already before therapy, and these remained strongly positive during the treatment period. In contrast, anti-ML-1 and -3 antibodies were negative before therapy and increased during treatment. (b) The patient had no anti-ML-1, -3 or -cbML antibodies before therapy, all three kinds of antibodies became positive during mistletoe extract exposure. (c) The patient had no anti-ML-1, -3 or -cbML antibodies before therapy; during treatment only anti-ML-1 and anti-ML-3 antibodies became positive while anti-cbML remained negative. Normal values for anti-ML-1: OD<400, anti-ML-3: OD<300, anti-cbML: OD<500.

cular disulfide bond (20). CbML1 consists of two identical truncated polypeptide chains (1-48), cbML2 is a heterodimer, containing one truncated (1-48) and one full length chain (1-49), and cbML3 is composed of two full-length chains (1-49). In the present study we used only the cbML3 presuming that the antigenicity of the three isoforms may be similar. Cross reactivity of anti-cbML antibodies with ML-1, -2 or -3 can be excluded due to their complete different structures. Furthermore, absorption of anti-cbML positive sera with cbML coupled to chitin beads abolished the anti-cbML activity but had no effect on anti-ML-1 or -3 titers. The fact, that we did not observe any correla-

tion between the presence of anti-cbML and anti-ML-1 or -3 antibodies also excludes cross reactivity, and indicates that the cbML antigen preparation was not contaminated with ML-1/3 epitopes or *vice versa*.

Interestingly, anti-cbML antibodies have been detected in about one third of tumour patients who have not yet been exposed to those extracts, while anti-ML-1 and -ML-3 antibodies occur only in the sera of patients having already received mistletoe extract therapy. We suggest, that there may exist some kind of 'natural immunity' towards this highly conserved protein. This is not surprising considering the fact that cbML shows striking sequence homology (55%) with hevein and other proteins with hevein-like domains which are present almost ubiquitously in natural rubber latex, bananas, avocados, wound-induced gene products of potato, antimicrobial peptides or antifungal proteins [33]. Indeed, preliminary data indicate that anti-cbML antibodies in sera from tumour patients being anti-cbML positive already before mistletoe therapy reacted also with recombinant hevein (kindly provided by Dr. Monika Raulf-Heimsoth, Berufsgenossenschaftliches Forschungsinstitut für Arbeitsmedizin, Bochum, Germany) [unpublished observation], while anti-cbML antibodies developing during therapy did not react with this recombinant antigen. This may indicate that cbML consists of at least two epitopes, one recognized by 'natural' or cross-reacting anti-cbML antibodies, and the other by 'therapy-specific' anti-cbML antibodies. The suspected existence of a 'natural immunity' against cbML is substantiated by our preliminary data showing that cbML induces a strong proliferative response of lymphocytes from probands who had never contact with mistletoe extracts [unpublished data]. However, this hypothesis does not explain why 31% of tumour patients but only 5% of healthy controls ($p < 0.05$) had antibodies against this postulated 'cross-reactive' epitope. One could assume, that these anti-cbML antibodies are induced by the tumour disease itself or even in chronic inflammatory diseases in general, but this has to be proven in further studies.

In conclusion, the presented data again indicate that not only ML-1 – as quite frequently propagated – but also other components in the mistletoe extracts exert immunogenic properties. The postulated beneficial effect of these extracts may, therefore, depend upon the composition, distribution and concentration of the single compounds and not on just one antigen. The study provides, however, an interesting new aspect indicating that at least some of the mistletoe components may contain also epitopes which are highly conserved and recognized either by the natural immune system or by memory T- and B-cells. Whether the reactivity towards those epitopes has any clinical and therapeutic relevance or may be even related to the tumour disease itself has to be analysed in further studies.

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