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Biochemical, histochemical and cell biological investigations on the actions of mistletoe lectins I, II and III with human breast cancer cell lines*

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Cytotoxicity of the mistletoe lectins I, II and III towards six human breast cancer cell lines was assessed using the Mossman assay. In addition, binding of the three mistletoe lectins to the separated membrane glycoproteins of these cell lines, the binding and uptake of these lectins into the cells in tissue culture and the binding of the lectins to histological preparations of these cell lines were analysed. The results indicate that there are quantitative differences concerning the toxicity of these three lectins towards the different cell lines. Furthermore, the lectin binding pattern in the cell lines differed. In Western blots, several membrane glycoproteins were labelled by the lectins. Our results indicate subtle differences between the three lectins with regard to the parameters mentioned above; however, the toxicity of all three lectins from mistletoe is so similar that they all seem suitable for the construction of immunotoxins.

Keywords: Breast cancer, cell membrane glycoproteins, cytotoxicity, mistletoe lectins

Introduction

Mistletoe extracts have been widely used for several decades in cancer treatment [1]. Several potentially therapeutically active substances have been discovered in these extracts, but in recent years research has focused on lectins as the most potent biomodulators present in mistletoe extracts [2]. Three mistletoe lectins, I, II and III (ML-I, II, III) have been isolated. These consist of two chains, the A-chains being the toxophores, and the B-chains possessing the carbohydrate binding sites. The major sugar specificities of these lectins are defined as follows: ML-I is D-galactose (Gal) specific [3, 4]; ML-II is Gal and *N*-acetyl-D-galactosamine (GalNAc) specific; and ML-III is GalNAc specific [5, 6].

In relation to (breast) cancer research and treatment, these lectins are of interest for several reasons. 1) Binding of ML-III to histological sections of breast cancer seems to be of predictive value for disease outcome in premenopausal women. Patients whose tumours bound this lectin had a worse prognosis (shorter disease free interval) than those whose tumours did not stain with ML-III in an indirect immunoperoxidase technique indicating that the sugar residues are associated with a biological process relevant to metastasis [7]. 2) The administration of mistletoe lectin containing extracts is widespread despite the lack of convincing clinical studies demonstrating the efficacy of this treatment [8]. However, several studies have shown that immunological parameters of cancer patients can be changed favourably by the application of mistletoe extracts containing MLs [9–12]. 3) The A-chains of mistletoe lectins are toxophoric proteins which inactivate ribosomes in the same way as the A-chain of ricin does [13, 14] and therefore A-chains have been used to construct immunotoxins; these are superior to ricin immunotoxins since the ML-A chain toxin is 80-fold more active than the corresponding conjugate with ricin A-chain toxin [15, 16].

The potential application of these lectins as anti-tumour agents is indicated; however, no systematic investigations on the interaction of the MLs with human breast cancer cell lines have been reported. We therefore addressed the following questions. 1) What is the relative toxicity of these lectins towards human breast cancer cells? Since the MLs have different carbohydrate binding properties and the B-chain in ricin facilitates the entry of the A-chain into the tumour cells [17], differences in relative toxicity as reported for other ribosomal

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*Dedication: This work is dedicated to one of the discoverers (amongst many other important contributions) of *Helix pomatia* agglutinin, which plays an important role in metastasis research, Professor Dr G. Uhlenbruck on the occassion of his 65th birthday.

inactivating proteins [14] might be expected. This is of interest since ML shows a differential binding to normal and neoplastic cells and it has been speculated that this differential binding leads to a killing of metastatic tumour cells [18]. The testing of all three MLs is desirable since all three lectins have been detected in clinically applied mistletoe extracts [19]. 2) Which are the glycoproteins involved in the binding of MLs to breast cancer cell lines? 3) How do the three Mls react histochemically with the different cell lines? Can any correlations be drawn between the histochemical results and the results obtained by the other techniques? The latter question arises since lectin binding in tissue sections may be different from lectin binding *in vivo* [20] and hence the assumption that histochemical results are indicative of the results from other techniques has to be tested for each lectin separately.

Materials and methods

Lectin preparation

MLs were isolated from air-dried ground plant material from mistletoe grown on locust tree and acer by using affinity chromatography after preliminary separation of the protein fraction by batch treatment with ion exchangers. Briefly, 1 kg plant material was stirred with 4 l 0.1 M acetic acid. Using filtration and centrifugation, the unsolubilized materials were pelleted down and the supernatant adjusted to pH 4.0 and 14 g SP-Sephadex P50 added. The proteins were eluted by a TRIS-NaCl gradient-buffer (pH 8.0) and applied to a lactosyl-Sepharose-4B column. The column was washed with elution buffer A (0.1 M Tris/HCl-buffer, pH 8.0, 0.5 M NaCl) until the absorption (E_{280}) in the eluate was less than 0.01. ML-II and ML-III were eluted by elution buffer B (0.1 M Tris/HCl, pH 8.0, 0.015 M Gal, 0.15 M NaCl). Thereafter elution buffer C (0.1 M Tris/HCl, pH 8.0, 0.2 M Gal, 0.15 M NaCl) was used for the elution of ML-I. The solutions were concentrated by ultrafiltration (PM 30, Amicon, Danvers MA, USA) and the protein content was determined with the Lowry method using bovine serum albumin as a standard.

ML-II and III were further separated by ion exchange chromatography using FPLC (Pharmacia-LKB, Freiburg, FRG). Filtered (0.2 µm pore size) lectin mixture was applied to a Mono S column (Pharmacia, HR 10/10) equilibrated with 0.015 M citrate buffer, pH 4.2. The Mls were eluted with a linear NaCl gradient from 0 to 0.5 M NaCl in 0.015 citrate buffer, pH 4.2 (from 0 to 150 ml). Three fractions were collected according to increasing retention times in the following order: ML-II, ML-III and ML-I. The purity of the lectins was assayed by SDS-PAGE according to Laemmli (for further details see [21]).

Finally the lectins were precipitated by ammonium sulfate and stored as suspensions in 2.67 M ammonium sulfate at 4°C. Prior to usage, the MLs in the ammonium sulfate suspension were pelleted (10 000 g, 5 min) and resuspended in the appropriate buffer (see below).

Cell culture and cell cytotoxicity assay

The following human breast cancer cell lines were obtained from the American Type Tissue Culture Collection and from the European Collection of Animal Cell Cultures (both through Public Health Laboratory Services, Porton Down, Salisbury, UK): BT 20; BT 549; MCF 7; HS578T; HBL 100 and T47D (Table 1). They were maintained under standard tissue culture conditions using humidified air supplemented with 5% CO_2. The cell culture media and the additives were all supplied by Sigma (Poole, Dorset) and were as follows: minimal essential medium supplemented with non-essential amino acids and 1 mM sodium pyruvate for BT20 and MCF7, RPMI-1640 supplemented with 24 IU l^{-1} insulin and 100 mM oxaloacetic acid for BT549 and T47D, and Dulbecco's minimal essential medium supplemented with 24 IU l^{-1} insulin and 100 mM oxaloacetic acid for HS578T and McCoys medium for HBL100. For maintenance culture, 10% fetal calf serum (Imperial, Andover, Hampshire) plus 1% penicillin/streptomycin and 1% fungizone were added to the media.

For the cytotoxicity assay, the cells were growth arrested for 48 h in serum free medium prior to the experiment, dispersed by trypsinization and plated at 1 µg DNA per ml into 96 well microtitre plates with four different lectin concentrations ranging from 0.1 to 100 ng ml^{-1} dissolved in the cell line specific cell culture media as given. Cell culture medium alone served as the control. The incubation was stopped at 72 h and cell viability was assessed by the Mossman cytotoxicity assay [22] by adding 100 µl nitroblue tetrazolium substrate to each well for 4 h, removing the supernatant and adding 200 µl solubilizer (20% SDS w/v dissolved in a 1: 1 N,N-dimethylformamide: H_2O), and reading the absorption at 580 nm in an Argos 300 96 well microtitre plate reader. The data from seven experiments were subjected to the non-parametric Wilcoxon signed rank-test using Statview on an Apple Macintosh microcomputer and were considered significant if $p < 0.05$.

Lectin binding and uptake studies

For labelling purposes, 10 mg of each lectin were dissolved in 1 ml $NaHCO_3$ (0.1 m, pH 8.5) and 0.85 mg 5(6)-carboxyfluorescein-N-hydroxysuccinimideester (FLUOS, Boehringer Mannheim, FRG) dissolved in 850 µl dimethylsulfoxide were added. ML-I was incubated for 4 h in this solution while MLs II and III were incubated for 2 h with constant shaking. The conjugate was separated from the free FLUOS by Sephadex G 100 superfine chromatography in the NaHCO3 buffer. The labelled lectin was stored in ammonium sulphate (as above). The fluorescein to protein ratio used was 2.5 for all three lectins.

For lectin binding and uptake studies live cells were cultured in small Petri dishes and incubated with FLUOS labelled MLs (50 µg lectin per ml diluted in 0.1 M phosphate buffered saline (PBS), pH 7.4) for 1 h either at 4°C or at 37°C in the tissue culture incubator. After washing with PBS, the cells were fixed with 4% formalin and viewed under an Olympus

BH2 fluorescence microscope. Additionally, formalin-fixed cells grown on coverslip and also formalin-fixed/wax embedded cells were sectioned and incubated with the FLUOS-labelled MLs (50 µg lectin per ml; for details of fluorescence technique see [23]). The formalin-fixed and wax-embedded cells were sectioned and treated with/without trypsin [0.1% trypsin type II, 0.1% $CaCl_2$, both (w/v) obtained from Sigma, Poole, Dorset for 15 min at 37°C]. Inhibition experiments were carried out using the formalin fixed and wax embedded cells by incubating the sections with the lectins dissolved in 0.3 M of the appropriate inhibitory sugar (ML-I: Gal; ML-II, ML-III: GalNac). In addition, 0.3 M xylose was used as a non-specific control sugar. PBS alone instead of lectins was used for the control of autofluorescence.

The results of the lectin binding studies were semiquantitatively evaluated ranging from 0 = no binding to +++ = very intense binding. In addition, the percentage of cells reacting are indicated if less than 100%.

Analysis of ML binding glycoproteins

The cells were cultured as above, washed in PBS and cells from five large tissue culture flasks (250 cm2) were harvested by the use of a rubber policeman and disrupted using a Parr cell disruption bomb (700 psi, 15 min). Cell membranes were isolated by ultracentrifugation (100 000 g, 1 h) and the protein content in the pellet was determined using the BioRad D/C protein assay using bovine gamma globulin as a standard. The membrane proteins were separated by SDS-PAGE (25 µg protein per slot) in a mini protean II chamber (BioRad, Hemel Hempstead, Herts). After transfer onto nitrocellulose, biotinylated MLs were applied and the lectin binding visualized using an indirect immunoperoxidase method [24]. The lectins were biotinylated using the long-arm NHS-spacer obtained from Vector Laboratories (Peterborough, Cambs) according to [25]. The blotted nitrocellulose sheets were incubated for 1 h at 37°C in a 0.1 M PBS solution supplemented with 0.04% Tween 20 and 3% saline (PTS). The nitrocellulose sheets were incubated overnight at 4°C with the lectins (ML-I: 0.016 mg ml–1; ML-II: 0.035 mg ml–1; ML-III: 0.00625 mg ml–1). After two washes in PTS for 5 min each, the lectin binding sites were revealed using an avidin-biotin peroxidase complex kit (ABC-kit, Vector Laboratories, Peterborough, Cambs) and diaminobenzidine and H2O2. For comparison, Coomassie Brilliant Blue staining was performed [24]. Concentrated human milk and membrane isolates from the human colon cancer cell line HT29 were run in parallel since the lectin binding characteristics of both samples are established as a standard in our group.

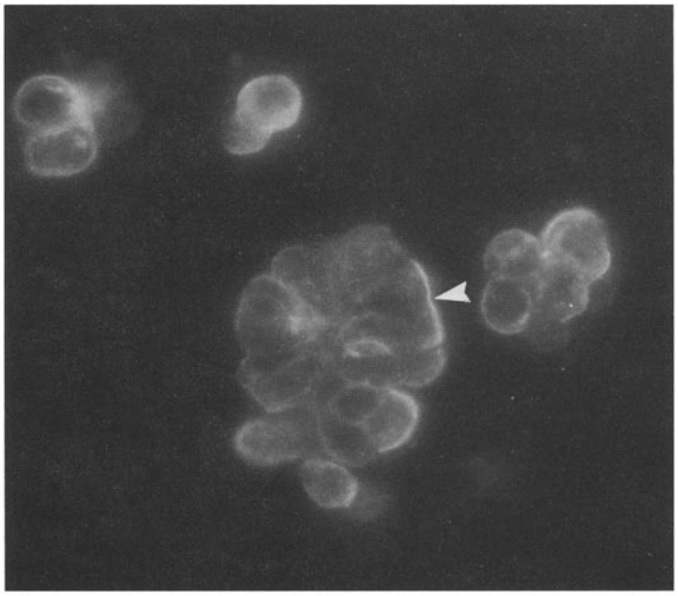

Figure 1. ML-II binding to wax embedded and trypsinized MCF 7 cells. Note the intensive labelling of the apical cell membrane (arrowhead) which is particular to this cell line (× 1800).

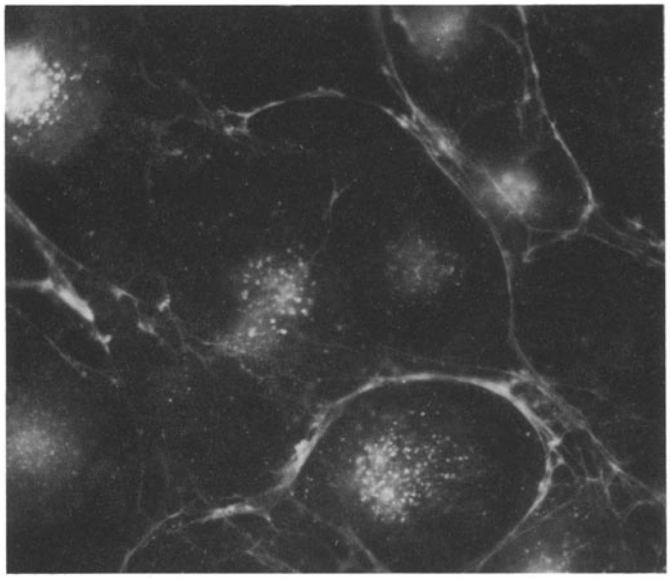

Figure 2. ML-II binding and incorporation into living HS578T cells at 37°C. Note the intracytoplasmic uptake granules in the centre of the cells and the labelling of the cell membrane and possibly of glycoconjugates deposited between the cells (× 1800).

Results

Cell cytotoxicity assay

The results of the cytotoxicity assays for ML-I to ML-III are summarized in Tables 2–4. ML-I became toxic for the cells at 100 ng ml^{-1} (see Table 2). The least toxic effect was seen in T47D (64% of control) and the most toxic effect was seen in HBL100 (14% of control). ML-II was already toxic for several cell lines at 10 ng ml^{-1}, namely for BT20 (30% of control) where it was most toxic, while with BT549 (65% of control) and T47D (65% of control) it was less toxic (see Table 3). At

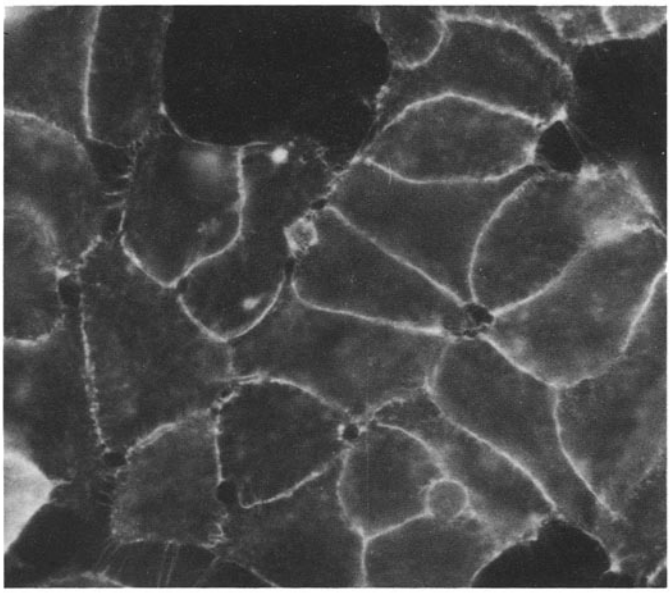

Figure 3. ML-II binding to live T47D cells at 4°C. Note that the binding is restricted to the cell membrane and the extracellular space and no granular uptake as in Fig. 2 can be seen (× 1800).

100 ng ml^{-1} ML-II was toxic to all cell lines. As with ML-I, T47D (64% of control) was the most ML-II resistant cell line while HBL100 was the most susceptible cell line (12% of control). At 10 ng ml^{-1} ML-III had a toxic effect on only one cell line, namely on T47D. At 100 ng ml^{-1} all cell lines were affected by ML-III, BT20 was the most susceptible cell line (30% of control) while HS578T was the most resistant (70% of control, see Table 4).

Lectin binding and uptake studies

The lectin binding and uptake studies are summarized in Table 5. The results concerning lectin binding under different conditions are consistent. ML-I and ML-III bound less intensively than ML-II to the cells of all cell lines in culture (both at 37° and 4°C), to cells fixed with formalin and to cells fixed and wax embedded except for HS578T which stained equally well with all three lectins in the wax embedded preparation. When using wax embedded cells, trypsinization proved useful to uncover lectin binding sites (see for example MCF 7, Fig. 1). The binding pattern of the lectins was markedly different: cells incubated at 37°C showed granular uptake of the lectin glycoprotein complexes into the cells (see Fig. 2). This did not happen when the cells were incubated at 4°C where lectin binding was evenly distributed along the cell membranes and extracellular matrix (Fig. 3). Most of the cells of the different cell lines reacted uniformly with the lectins, however, a microheterogeneity was detected with MCF7 and with BT549.

ML-I reactivity towards all wax embedded cell lines was competely inhibited by Gal, but no inhibition occurred with xylose. ML-II fluorescence was completely inhibited by GalNAc while xylose had no inhibitory effect. Xylose did not inhibit ML-III and complete inhibition could not be achieved using GalNAc although a substantial reduction of fluorescence intensity was observed.

Table 1. Growth characteristics and origin of the human breast epithelial cell lines.

Cell line	*Origin of cell lines*
BT20	Derived from primary tumour, an adenocarcinoma of the breast
BT549	Derived from infiltrating ductal carcinoma, primary tumour had metastasized to local lymph nodes
HBL 100	Breast milk from nursing mother, part of SV40 genome integrated in this line
HS578T	Derived from breast cancer
MCF7	Derived from pleural effusion from breast cancer, estrogen sensitive
T47D	Pleural effusion of an infiltrating ductal carcinoma

Lectin binding to isolated membrane glycoproteins

Many membrane protein bands were stained by Coomassie Brilliant Blue in the SDS-gels (Data not shown). On the Western blots, all three lectins bound to a restricted number of membrane glycoproteins (Figs 4–6). In general, ML-I and ML-II labelled membrane glycoproteins in both the high and low molecular weight range, while ML-III preferentially labelled membrane glycoproteins in the high molecular weight range. Using human milk proteins as a control, it is remarkable that ML-I and -II stained only one milk glycoprotein, while ML-III showed a broader labelling pattern.

Discussion

The present study has analysed the interaction of MLs-I, -II and -III with human breast cancer cells at the cell biological, biochemical and histochemical level. The histochemical analysis demonstrated the lectin binding sites *in vivo* and *in situ*, the cytotoxicity assay showed the subsequent actions of the lectins upon cell division and the Western blot analysis revealed the membrane glycoproteins possibly responsible for the binding and uptake of the lectins into the cells. Part of the discussion aims to summarize and combine the results from these three different methodological approaches in order to form a unified picture on the interactions of the three MLs with human breast cancer cells.

The first aim of this study was to evaluate the relative toxicity of the three MLs. While ML-I started to show toxic effects only at 100 ng ml^{-1} in all cell lines, ML-III was toxic for T47D at 10 ng ml^{-1} and ML-II was toxic for BT20, BT549 and T47D at 10 ng ml^{-1}. The dose needed to exhibit a toxic effect on breast cancer cells (at least 10 ng ml^{-1}) is therefore at least 40 times higher than the dose of ML-I (0.25 ng ml^{-1}) needed to activate lymphocytes in cell culture [11]. In the same study it

Table 2. The effect of ML-I on several human breast cancer cell lines as measured in the Mossman assay. The percentage of the control (pure cell culture medium instead of lectin) is indicated, the standard deviation is given in brackets. If statistically significant differences were observed in the non-parametric Wilcoxon signed rank test ($p < 0.05$), the p values are also given.

Cell lines	*Control*	*ML-I*			
		0.1 ng ml^{-1}	*1 ng ml^{-1}*	*10 ng ml^{-1}*	*100 ng ml^{-1}*
BT20	100 (± 4.86)	101 (± 0.83)	94.3 (± 2.40)	96.7 (± 1.10)	29.7 (± 0.33) $p = 0.0273$
BT549	100 (± 4.28)	100 (± 0.90)	100 (± 2.08)	103 (± 4.54)	41.3 (± 0.3) $p = 0.0273$
HBL100	100 (± 0.03)	96.8 (± 4.78)	91.1 (± 4.98)	95.1 (± 7.36)	13.8 (± 0.55) $p = 0.0108$
HS578T	100 (± 0.07)	107 (± 2.64)	105 (± 5.84)	129 (± 8.50)	38.3 (± 1.30) p = 0.0108
MCF7	100 (± 8.84)	103 (± 0.52)	104 (± 1.72)	87.3 (± 3.10)	49.0 (± 0.50) $p = 0.0464$
T47D	100 (± 8.98)	94.7 (± 3.22)	77.9 (± 1.68)	73.6 (± 7.14)	63.8 (± 2.65) $p = 0.0431$

Table 3. – The effect of ML-II on several human breast cancer cell lines as measured in the Mossman assay. The percentage of the control (pure cell culture medium instead of lectin) is indicated, the standard deviation is given in brackets. If statistically significant differences were observed in the non-parametric Wilcoxon signed-rank test ($p < 0.05$), the p values are also given.

Cell lines	*Control*	*ML-II*			
		0.1 ng ml^{-1}	*1 ng ml^{-1}*	*10 ng ml^{-1}*	*100 ng ml^{-1}*
BT20	100 (± 4.86)	100 (± 2.67)	93.8 (± 4.96)	29.9 (± 0.38) $p = 0.0277$	28.6 (± 0.20) $p = 0.0277$
BT549	100 (± 4.28)	102 (± 0.80)	103 (± 1 .33)	65.0 (± 1.69) $p = 0.0464$	42.6 (± 1.00) $p = 0.0277$
HBL100	100 (± 0.03)	92.0 (± 8.24)	92.5 (± 2.03)	93.0 (± 3.35)	12.3 (± 1.56) $p = 0.0108$
HS578T	100 (± 0.07)	107 (± 1.78)	116 (± 0.98)	114 (± 1.80)	27.4 (± 2.88) $p = 0.0108$
MCF7	100 (± 8.84)	106 (± 1.38)	104 (± 0.76)	88.4 (± 2.10)	46.9 (± 1.13) $p = 0.0464$
T47D	100 (± 8.98)	91.3 (± 1.10)	78.1 (± 0.40)	64.3 (± 0.70) $p = 0.0431$	64.4 (± 2.20) $p = 0.0431$

was shown that immunostimulatory effects could be observed after giving patients suffering from advanced breast cancer 1 ng ML-I per kg body weight for 4 weeks. The doses influencing the immune system in humans *in vivo* is orders of magnitude below the doses which had toxic effects on human breast cancer cells *in vitro*. It therefore seems reasonable to attribute any therapeutic effects of MLs to their immunostimulatory role; however, the dosage differences inducing an immuno-stimulatory effect *in vitro* (0.25 ng ML-I per ml) and *in vivo* (1 ng ML-I per kg body weight) in the study mentioned above [11]

Table 4. – The effect of ML-III on several human breast cancer cell lines as measured in the Mossman assay. The percentage of the control (pure cell culture medium instead of lectin) is indicated, the standard deviation is given in brackets. If statistically significant differences were observed in the non-parametric Wilcoxon signed-rank test ($p < 0.05$), the p values are also given.

Cell lines	*Control*	*ML-III*			
		0.1 ng ml^{-1}	*1 ng ml^{-1}*	*10 ng ml^{-1}*	*100 ng ml^{-1}*
BT20	100 (± 4.86)	92.6 (± 4.76)	93.3 (± 3.18)	91.1 (± 3.92)	29.7 (± 0.75) $p = 0.0277$
BT549	100 (± 4.28)	98.7 (± 1.05)	95.0 (± 4.06)	105 (± 3.61)	47.3 (± 1.85) $p = 0.0273$
HBL100	100 (± 0.03)	101 (± 4.46)	93.4 (± 1.98)	99.4 (± 1.75)	52.4 (± 1.71) $p = 0.0108$
HS578T	100 (± 0.07)	116 (± 2.72)	116 (± 2.95)	76.6 (± 19.59)	70.2 (± 6.86) $p = 0.0108$
MCF7	100 (± 8.84)	108 (± 2.04)	104 (± 0.58)	69.0 (± 2.21)	45.6 (± 2.00) $p = 0.0277$
T47D	100 (± 8.98)	89.1 (± 1.63)	70.5 (± 0.58)	59.3 (± 2.10) $p = 0.0430$	62.4 (± 1.53) $p = 0.0430$

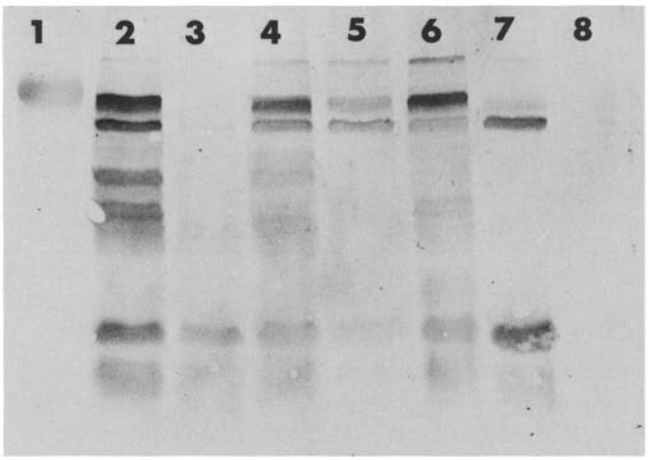

Figure 4. ML-I binding to isolated membrane glycoproteins and human milk. Lane 1: human milk; lane 2: T47D; lane 3: MCF7; lane 4: HT29; lane 5: BT549; lane 6: BT20; lane 7: HBL100; lane 8: HS578T. Several membrane glycoproteins of the cell lines T47D, HT29 and BT20 are stained with ML-I, while the binding pattern of the other cell lines is more restricted. Only one major milk glycoprotein, lactoferrin, MW 86 kDa, is labelled.

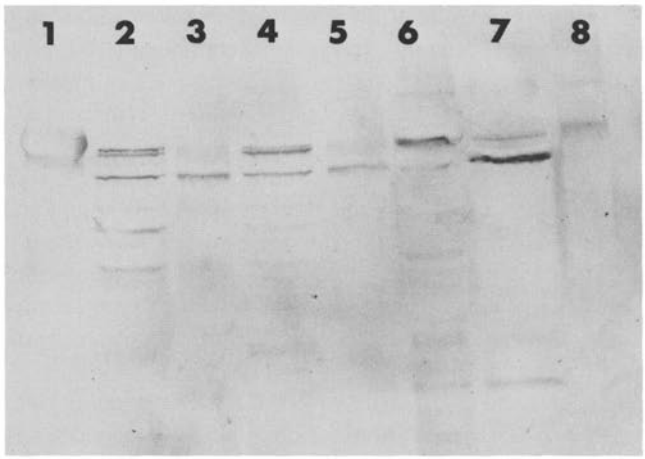

Figure 5. ML-II binding to isolated membrane glycoproteins and human milk.
For legend see Fig. 4. Note the similarity of the ML-II binding pattern in comparison to that observed with ML-I.

are very significant and the comparison of toxic effects of MLs *in vitro* as compared to *in vivo* warrants further investigation.

In BT20, BT549, MCF7 and T47D no major differences in cytotoxicity between the three MLs could be detected. While the first two cell lines were established from primary tumours, the latter two cell lines were derived from malignant pleural effusions of breast cancer patients (Table 1). This finding could indicate that the relation of Gal/GalNAc containing glycoconjugates in primary and secondary breast cancer remains constant. This is in accord with *in situ* findings which indicate that breast cancer metastases to the brain are positive for the GalNAc specific lectin *Helix pomatia* agglutinin (HPA) which is indicative of metastasis in the primary tumour [26]. Our findings may therefore indicate that the expression of Gal/GalNAc residues *in situ* and *in vitro* are the same or at least very similar.

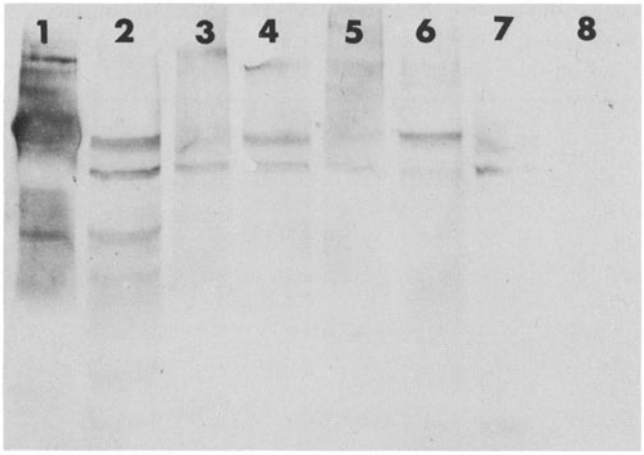

Figure 6. ML-III binding to isolated membrane glycoproteins and human milk. For legend see Fig. 4. Binding of this lectin to membrane glycoproteins from breast cancer cells seems to be more restricted in comparison to the other two lectins while more milk glycoproteins react with ML-III than with ML-I and II.

Table 5 – Binding of mistletoe lectins I, II and III to living human breast cancer cells at 37°C and 4°C, to formalin fixed and to formalin-fixed and wax embedded cells. Note that two different techniques were used in the formalin-fixed and wax-embedded preparations. 0 = no staining, (+) very weak staining, + = weak staining, ++ = intense staining and +++ = very intense staining. If less than 100% of the cells were stained, the percentage of cells stained is indicated before the intensity of staining.

	BT20	*BT549*	*HBL100*	*HS578T*	*MCF7*	*T47D*
Living 37°C						
ML-I	+	+ – ++	(+)	+	0	+
ML-II	++	++	+	++	50% (+)	++
ML-III	+	(+) – +	(+)	+	0	+
Living 4°C						
ML-I	+	+	(+)	(+)	0	+
ML-II	++	++	+	+	(+)	++
ML-III	+	+	(+)	(+)	0	(+)
Fixed						
ML-I	0	(+)	0	0	0	+
ML-II	+	++	(+)	+	0	++
ML-III	0	+	0	0	(+)	+
Fixed, wax embedded FITC						
ML-I	+	+	0	++	(+)[a]	+
ML-II	++	20% +	0	++	+[a]	++
ML-III	+	10% (+)	0	++	0[a]	(+)
Fixed, wax embedded trypsinized FITC						
ML-I	++	++	(+)	+++	+[a]	++
ML-II	+++	20% ++	+	+++	++[a]	+++
ML-III	++	10%+	0	+++	(+)[a]	+

[a] In MCF7 a stronger apical staining was observed in some of the cells.

The second aim of the study was to analyse the ML binding glycoproteins. It is well established that carbohydrate residues are limited to the extracellular surface of the cell membrane and that cross-linking of membrane glycoproteins leads to the internalization of lectin-glycoprotein complexes [27]. Hence all the glycoproteins recognized by the three MLs can serve as potential and likely binding partners for internalization of lectin-glycoprotein complexes. The toxicity of these three lectins was in general very similar despite their different binding patterns to membrane glycoproteins, indicating that all Gal and GalNAc carrying membrane glycoproteins were internalized and processed in a similar fashion. Hence the choice of a particular membrane glycoprotein as a carrier for immunotoxins does not seem to be critical as long as these molecules are internalized.

The third aim of the study was to analyse the binding pattern of Mls in histochemical preparations and the correlation of this binding pattern with the toxicological experiments. In the histochemical experiments a differential lectin binding pattern was observed: ML-I and ML-III show in general a less intensive reactivity towards the cell lines than ML-II. This fits well with the known carbohydrate specificities of the three MLs, with ML-II (Gal, GalNAc) having overlapping specificities with both ML-I (Gal) and ML-III (GalNAc) [5, 6]. Both Gal and GalNAc residues are commonly found in mucin-type glycoproteins which can also be found as part of the membrane proteins in epithelia [28]. Our *in vitro* demonstration of Gal and GalNAc residues in breast cancer cells is mirrored by the *in situ* demonstration of these carbohydrate residues in breast cancer [29] indicating that the expression of these carbohydrate residues is stable *in vitro*. Fixation resulted in a loss of lectin binding sites in some but not all cell lines, while fixation and wax embedding resulted both in an enhancement and loss of binding sites depending on the cell line. Fixation, wax embedding and sectioning has a complex influence on the lectin binding sites of cells: glycolipids as potential lectin binding sites are lost due to alcohol and chloroform treatment during the wax embedding process (probably accounting for some of the losses of MLs binding sites in HBL100) while lectin binding sites on internal membranes are more easily exposed as a result of sectioning (probably explaining the gain of MLs binding sites for HS578T). A comparable influence of fixation, processing and microheterogeneity with respect to lectin binding was found in clinical human breast cancer material [30] again indicating the comparability of cell culture with *in situ* work. The results of the lectin binding in histochemical preparations show the same pattern – ML-I and ML-III having almost identical binding characteristics and ML-II being different (as in the cytotoxicity assays) with regard to the cell – lines BT20, BT 549 and HS578T (see Tables 2–4) indicating that binding and internalization of the lectins is a prerequisite for their toxic effects. Furthermore, MCF 7 and HBL100 did not bind or only

very weakly bound ML-I and III (Table 5) and were the cell lines which were relatively resistant to MLs since only the highest concentration used (100 ng MLs per ml) showed cytotoxic effects, again indicating the correlation between binding, uptake and cytotoxic effects. However, there was one exception namely HS578T which bound and internalized the MLs relatively well but only showed toxic effects at the highest concentrations used. This indicates that mechanisms other than binding and internalization might play a role in determining the cytotoxicity of the MLs. Despite this one exception it is remarkable that the correlation between cytotoxicity on the one hand and binding and uptake on the other is so consistent since the concentrations of lectins used for both experiments (0.1 to 100 ng MLs per ml for cytotoxicity and 50 µg MLs per ml for fluorescence imaging) differed so widely. This indicates that only a minor fraction of the bound and internalized lectins is needed to influence the cellular metabolism which explains the high toxicity of the MLs. Since the differences in toxicity are most likely to be due to the lectin and not the toxophore part of the three MLs, the toxophores of all three Mls seem to be suitable for the construction of immunotoxins. An immunotoxin using a CD5 monoclonal antibody and the toxophore of ML-I has already been proven to be superior to an immunotoxin using the same CD5 antibody and the ricin A-chain [15] and hence further investigations using ML toxophores for immunotargeting purposes seem warranted. Studies using monoclonal antibodies directed against other antigens such as the CD5 linked to the toxophoric chains of MLs are already under way in our laboratories to prove the effectiveness of this toxin.

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