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Modulation of cytotoxicity and enhancement of cytokine release induced by *Viscum album* L. extracts or mistletoe lectins

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Cytotoxic effects of *Viscum album* L. (mistletoe) extracts and mistletoe lectins were studied by light and electron microscopy. The first events observed were membrane perforation and protrusions typical for apoptosis. Inhibition of Molt 4 cell growth was obtained with lectin concentrations in the pg/ml range as long as cells were cultured in serum-free medium. Under this condition, mistletoe lectin-III was about 10 times more cytotoxic than mistletoe lectin-I ; mistletoe lectin-II was in between. Lectin cytotoxicity was modulated by human serum from donors who had never been treated with mistletoe preparations and lectin-specific carbohydrates, added at the mmol/l range, particularly D-galactose (or β-lactose) for mistletoe lectin-I and *N*-acetyl-galactosamine for mistletoe lectin-II and -III. In addition, at subtoxic concentrations, mistletoe lectin-I , -II and -III enhanced the production of cytokines (tumour necrosis factor-α, interleukin-1α) by isolated human monocytes. The experimental results are discussed in relation to the treatment of cancer patients administered with mistletoe extracts.

Keywords: Mistletoe lectins, cytotoxicity, cytokine release, monocyte.

Introduction

Viscum album L. (mistletoe) preparations and mistletoe lectins are cytotoxic for a large variety of tumour cells [1,2]. It was originally believed that this effect was due to a non-specific cell necrosis but, later, mistletoe was shown to contain several lectins which have a highly specific mechanism of cytotoxic activity [3].

Electron microscopy observation of mistletoe extract cytotoxicity

Several years ago, we observed that an extract from *Viscum album* produced lysis in cells from hepatoma tissue culture (HTC) from the rat [2]. The images obtained by scanning-electron microscopy show membrane perforation after only 1 h, later cytoplasm dissolution and finally cell death (Fig. 1). Concentrations of extract were high and were not representative of *in vivo* activity. We observed by transmission electron microscopy the formation of protrusions on the cell membrane. These protrusions are an indication that, under our experimental conditions, cell death occurs through apoptosis, as demonstrated recently by Büssing *et al.* [4,5].

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Cell specificity toward cytotoxicity of mistletoe extracts

Human leukaemia Molt 4 cells, derived from a childhood T cell leukaemia, grow in suspension and are commonly used for the study of cytotoxicity, especially for quantification. Damaged cells can be observed after 24 h in the presence of a mistletoe extract [6]. Molt 4 cells are about 100 times more sensitive to the extracts than HTC cells [2] while another cell line, K562, is about 1000 times more sensitive (Urech K, personal communication, 1996). Large differences in cytotoxicity toward fibroblastic cell lines were also found [2]. These data indicate a cell type specificity for mistletoe extract cytotoxicity.

Comparison of the cytotoxic effects of mistletoe lectin-I , -II and -III

Cytotoxicity of mistletoe extracts is mostly related to their lectin content [6–8]. We tested the three purified mistletoe lectins, kindly provided by Dr Pfüller (University Witten-Herdecke, Germany) [9]. Dose-effect studies on Molt 4 cells show a strong reduction in cell viability. Concentrations which maintain the number of viable cells during the 3 days of the experiment were 0.3 ng/ml for mistletoe lectin-I , 0.03 ng/ml for mistletoe lectin-III and between these two values for mistletoe lectin-II. Thus, mistletoe lectin-III is about 10 times more cytotoxic than mistletoe lectin-I on Molt 4 cells [10].

Cytotoxicity is probably the most reproducible biological activity of these lectins, at least if it is determined under appropriate culture conditions. Cytotoxicity can be used as a basis for bioassays of mistletoe lectins [11].

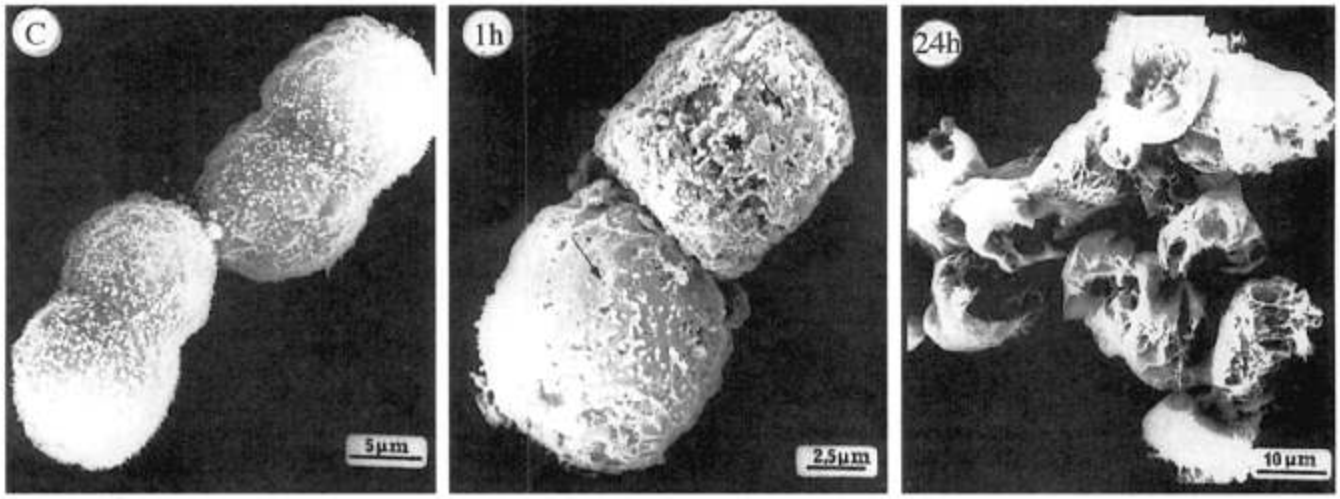

Figure 1. Scanning electron micrographs of hepatoma tissue culture cells incubated with fermented oak mistletoe preparation (*Iscador* Qu). The concentration corresponded to the extract of 1 mg fresh plant per ml cell culture medium. C, control cells; 1h, cells after 1 h treatment; 24h, cells after 24 h treatment at the same extract concentration.

Mechanism of toxicity of mistletoe lectin-I , -II and -III

Mistletoe lectins consist of two glycosylated polypeptide chains, termed A and B, covalently linked through a disulphide bridge [12]. Mistletoe lectins are known to inhibit protein synthesis through a specific mechanism: (1) binding of the mistletoe lectin-I B chain to a cell membrane galactoside; (2) endocytosis of the whole lectin; and (3) inhibition of protein synthesis at the ribosome level by the A chain through *N*-glycosidase activity [7,13]. This ribosomal degradation would be sufficient to lead to cell death.

Recently, it has been shown that the toxic effects of mistletoe extracts and mistletoe lectins may occur through apoptosis [4,5]. It is not yet clear if apoptosis takes place independently of protein synthesis inhibition or not. Comparison of the data from different laboratories suggests that apoptosis probably develops in the presence of lectin concentrations higher than those needed for protein synthesis inhibition [4,5,10,14] but additional investigation is needed to clarify this question.

For the expression of their cytotoxicity, mistletoe lectins must first bind to complex galactosides from the cell membrane and then penetrate into the cell. Therefore, it should be possible to control mistletoe lectin toxicity by a modulation of their binding on the cell surface. The following experiments were designed to obtain a modulation of the lectin cytotoxicity by various glycoproteins and galactosides.

Modulation of lectin cytotoxicity by serum glycoproteins

A modulation of the cytotoxicity of the three mistletoe lectins by exogenous carbohydrates was not possible as long as cells were grown in the usual culture medium containing 10% foetal calf serum. Therefore, we adapted Molt 4 cells to serum-free culture conditions (Ultraculture medium; Boehringer Ingelheim Bioproducts, Gagny, France), and observed that the cytotoxicity of the lectins strongly increased [14,15]. Moreover, using human serum from healthy donors, mistletoe lectin-I is highly cytotoxic in the absence of serum while, in the presence of 10% human serum, cytotoxicity is much lower (Fig. 2). For mistletoe lectin-II and -III, a similar effect with human serum was observed [14]. We conclude that serum contains glycoproteins which reduce the toxic effect of the lectins [14,15].

Quantification of the decrease of lectin cytotoxicity by human serum

The effect of human serum is summarized in Table 1 which indicates the lectin concentrations inhibiting cell viability by 50% (IC_{50}). In the absence of serum, the IC_{50} of the three lectins are in the pg/ml range, but mistletoe lectin-III is more cytotoxic than mistletoe lectin-I and -II . In the presence of serum, the IC_{50} values are increased 10–36-fold for the three lectins, indicating a potent modulation of their activity by serum glycoproteins. It has to be emphasized here that this effect of human serum can explain the lack of toxicity of mistletoe preparations injected into cancer patients.

Effect of blood derivatives on lectin cytotoxicity

Different blood derivatives, used in hospital as medication, were tested for their ability to reduce lectin cytotoxicity (Fig. 3). Partially purified serum albumin was quite active at reducing mistletoe lectin-I cytotoxicity, but in

Table 1. Effect of human serum on the cytotoxicity of mistletoe lectins.

	ML-I	ML-II	ML-III
IC_{50} without human serum	0.07	0.06	0.009
IC_{50} with human serum 10%	0.7	2.2	0.3
Ratio	10	36	30

ML, mistletoe lectin; IC_{50}, concentration inhibiting cell viability by 50%.

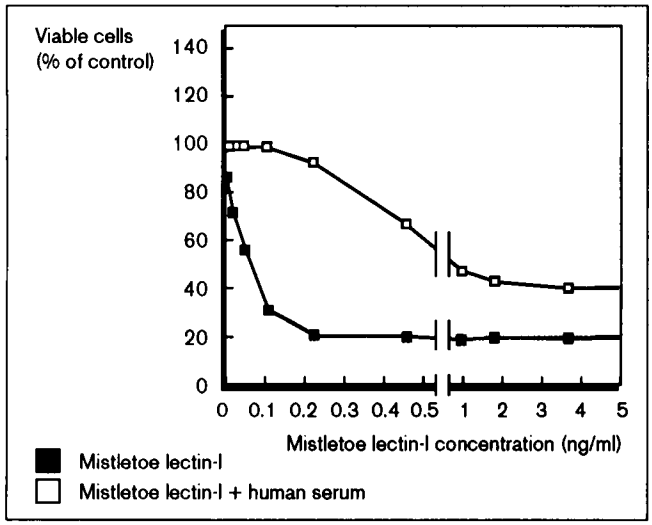

Figure 2. Effect of human serum on the cytotoxicity of mistletoe lectin-I incubated with Molt 4 cells. The serum was diluted 10-fold in the culture medium.

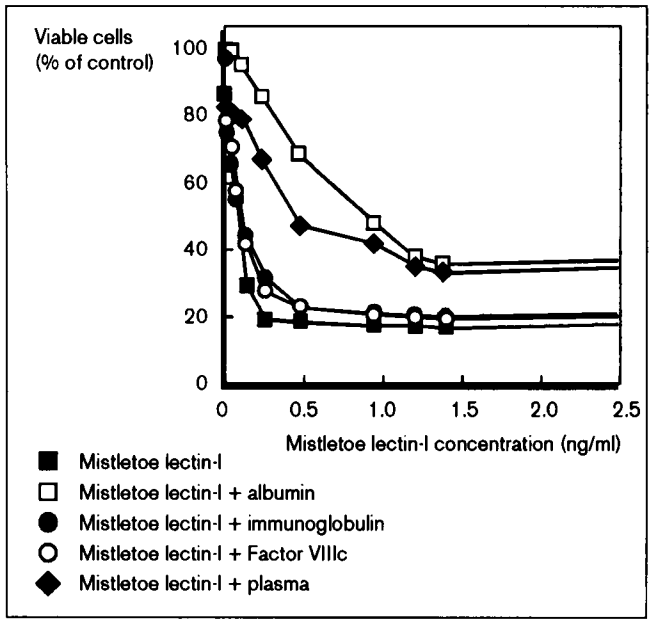

Figure 3. Effects of various blood derivatives on the cytotoxicity of mistletoe lectin-I incubated with Molt 4 cells. These derivatives were in solutions prepared for clinical use and corresponded to partially purified substances. They were diluted 10-fold in culture medium.

Table 2. Concentrations (mmol/l) of D-galactose, *N*-acetyl-galatosamine and β-lactose which reduce the cytotoxicity of mistletoe lectins (ML) by 50%.

	ML-I	ML-II	ML-III
D-Galactose	12	32	40
N-acetyl-galactosamine	30	5	2
β-Lactose	7	48	15

another experiment highly purified human albumin was found to be inactive. We therefore suggest that the partially purified albumin contains glycoproteins able to bind to the lectin. Immunoglobulins and Factor VIII had no effect, and plasma decreased the cytotoxicity of mistletoe lectin-I in a similar way to that of serum.

Modulation of lectin cytotoxicity by carbohydrates

Using Molt 4 cells grown in serum-free medium, it became possible to modulate the lectin cytotoxicity with carbohydrates such as D-galactose, *N*-acetyl-galactosamine or β-lactose. The amount of lectin was adjusted to obtain a cell viability of about 20% (Fig. 4). Addition of D-galactose allowed cell viability to be restored to more than 80% with mistletoe lectin-I , but less with mistletoe lectin-II and -III. In contrast, in the presence of *N*-acetyl-galactosamine, mistletoe lectin-I is the lectin that is most influenced with mistletoe lectin-III being the lowest. β-Lactose, a dimer galactose-glucose, has a similar but stronger effect than D-galactose.

Quantification of the effect of the three galactosides can be obtained by determination of the sugar concentration necessary to reduce the lectin cytotoxicity by 50% (Table 2). In the case of mistletoe lectin-I , 12 mmol/l D-galactose or 7 mmol/l β-lactose were enough to produce this effect, while for mistletoe lectin-II and -III only 2–5 mmol/l *N*-acetyl-galactosamine were required. We therefore confirmed that known from *in vitro* binding studies [16], namely mistletoe lectin-I is mostly specific to D-galactose, and mistletoe lectin-II and mistletoe lectin-III are mostly specific to *N*-acetyl-galactosamine [16]. However, cytotoxicity of mistletoe lectin-II was not modulated by D-galactose or β-lactose as was observed in the *in vitro* binding experiments [16].

Inhibition of cytotoxicity by lectin antibodies

Lectins are known to be immunogenic and we found antilectin antibodies in the serum of rabbits administered with lectins [8]. These antibodies produce a strong inhibition of the cytotoxicity at any lectin concentration [8].

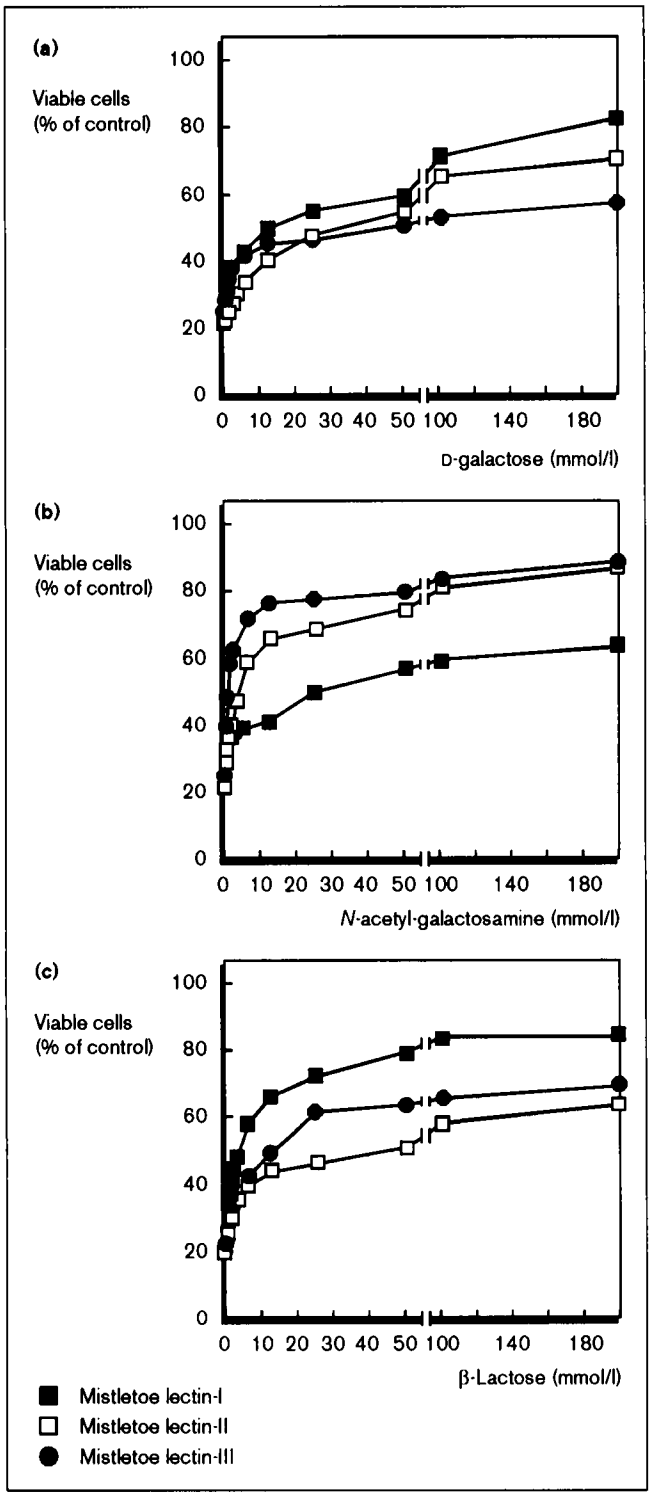

Figure 4. Effects of **(a)** D-galactose, **(b)** *N*-acetyl-galactosamine and **(c)** β-lactose on the cytotoxicity of mistletoe lectin-I, -II and -III incubated with Molt 4 cells.

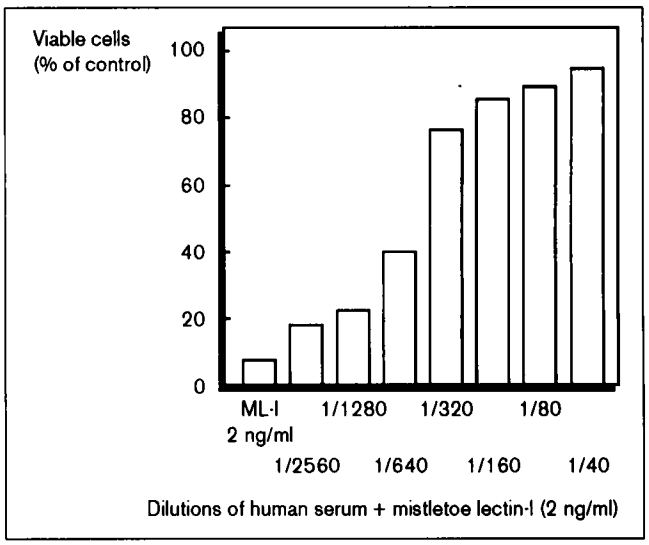

Figure 5. Protection of mistletoe lectin (ML)-I cytotoxicity on Molt 4 cells by serum from a patient treated with *Iscador*.

It is to be expected that antilectin antibodies are present in serum from patients treated with mistletoe extracts. We showed inhibition of the cytotoxicity of the extracts with serum from these patients [17]. The lowest serum dilution (1/40) produces an almost total neutralization of the toxic effect of mistletoe lectin-I (Fig. 5). Antilectin antibodies appear to be glycoproteins which influence the cytotoxicity of mistletoe lectins to the greatest extent by producing a complete toxicity inhibition. We can determine, for a certain amount of added lectin, an antibody titre in the serum of patients for the serum dilution which re-establishes 50% cell viability. For example, in the case of the sample described in Fig. 5, the titre is about 500. Antimistletoe lectin-I antibodies were also found by Stettin *et al.* [18] in the sera of patients treated with mistletoe preparations.

Stimulation of cytokine release by mistletoe lectins

At concentrations far below those inducing toxic activity, mistletoe lectins may stimulate cytokine production by monocytes. We investigated the effects of the three lectins on the release of cytokines by isolated human monocytes from healthy donors [19]. The release of tumour necrosis factor-α is shown as an example in Fig. 6. Interferon (plus lipopolysaccharide) was used as a reference. Lectin concentrations 10 000 times smaller than toxic doses produced a stimulation of tumour necrosis factor-α release markedly above control values. The effects of the three lectins were different, depending on

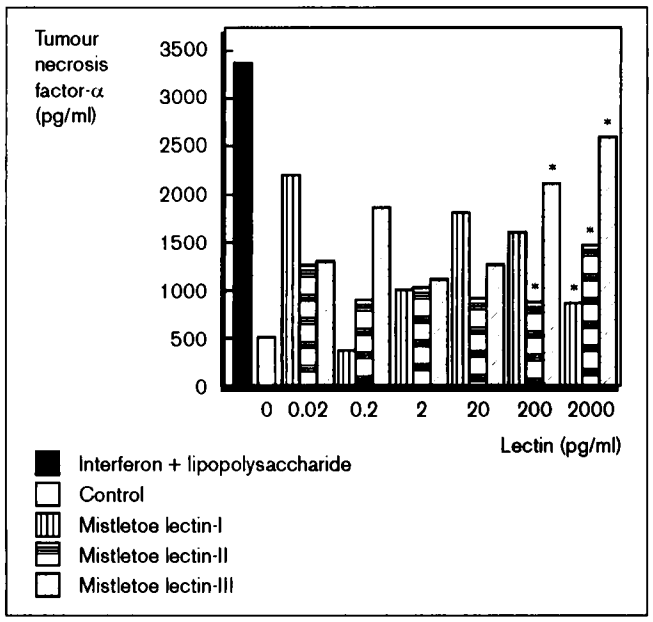

Figure 6. Effects of mistletoe lectin-I, -II and -III on tumour necrosis factor-α production by monocytes from healthy human donors. *Lectin was cytotoxic, with more than 80% mortality at the end of the 18 h incubation period.

their concentration and the donor [16]. It seems that, at low lectin concentrations, the binding of a few lectin molecules to a limited number of monocytes may be followed by a cytokine cascade in surrounding cells.

The low concentrations of mistletoe lectins needed to enhance cytokine production may be easily obtained *in vivo* with the clinically used mistletoe preparations standardized at 250 ng/ml of total lectins and injected at the tumour site [20]. In human cancer, monocytes and macrophages are accumulated around the tumour. A stimulation of cytokine production by these cells, particularly of tumour necrosis factor-α, may help to inhibit tumour growth.

Conclusions

The studies presented in this review indicate that human serum strongly reduces the cytotoxicity of mistletoe lectins and mistletoe extracts. Modulation of the mistletoe lectin cytotoxicity can be obtained through exogenous carbohydrates only when Molt 4 cells are grown in serum-free medium. On the other hand, serum from patients treated with mistletoe may totally inhibit the cytotoxicity of lectins; antilectin antibodies are responsible for this effect. Finally, far below toxic concentrations, mistletoe lectins stimulate the production of cytokines from human monocytes.

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