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Review

Suppression of growth of tumour cell lines *in vitro* and tumours *in vivo* by mistletoe lectins

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Summary. A variety of studies have shown that incubation of different tumour cell lines with mistletoe lectins (MLs) *in vitro* has a marked cytotoxic effect. In the concentration range of low cytotoxicity cell death induced by ML-I is quantitatively due to apoptotic processes. The first events observed being membrane perforation and protusions. Simultaneous treatment of certain tumour cells with MLs rendered them more sensitive to induction of apoptosis by TNFα. The immunomodulatory activity of ML-I was investigated by measuring cytokine release and the results confirmed that cytokine induction by the lectin is regulated at the transcriptional level. ML-I has been shown to potentiate the effect of chemotherapeutic drugs. In addition to an *in vitro* effect a number of workers have demonstrated that MLs suppress tumour growth *in vivo*. Mistletoe lectins have been administered to animals locally to the tumour, systemic, subcutaneously or by the oral route via the diet. In many cases apoptosis was observed in the tumour and instances where complete tumour ablation has occurred have been reported. It has been hypothesized that the anticancer efficacy of tumour necrosis factor-alpha (TNFα) is potentiated by MLs isolated from both European and Korean mistletoe. There is accumulating evidence that both types of MLs are able to induce an anti-angiogenic response in the host suggesting that the anti-metastatic effect observed on a series of tumour cell lines in mice is in part due to an inhibition of tumour-induced angiogenesis and in part due to an induction of apoptosis.

Key words: Mistletoe lectin, Tumour growth, Apoptosis, Anti-angiogenesis, Peyer's patch

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Introduction

During the last ten years advances in the purification of lectins (Eifler et al., 1993; Pfüller, 2000) has led to the isolation of three major lectins from mistletoe extracts (ML-I, ML-II and ML-III). These have now been subject to rigorous study. It has been clearly established that these purified carbohydrate-binding lectins possess cytotoxic properties (Kopp et al., 1993). The best studied mistletoe lectin is ML-I, a type-2 RIP (ribosome-inactivating protein) which is composed of two chains - the A-chain which has N-glycosidase activity (accounting for the property of inactivating ribosomes), and a B-chain possessing galactose-specific binding properties, this subunit being responsible for binding and cellular uptake of the molecule. The individual subunits do not appear to possess cytotoxic properties (Vervecken et al., 2000).

Although cytotoxic properties have been demonstrated *in vitro*, Pusztai et al. (1998) found that feeding purified ML-I in large amounts to rats had no detrimental effects on growth. Plant lectins are known to survive passage through the gut (Pusztai et al. 1990). Both Lavelle et al. (2000-2002) and Ewen et al. (1998) and Pryme et al. (2002, 2004) have presented purified ML-I orally to mice without reporting evidence of any side effects. A growth-stimulatory effect of ML-I has in fact been reported both in rats (Pusztai et al., 1998) and mice (Pryme et al., 2002) where hyperplastic growth of the small intestine has been observed following oral intake of the lectin. In mice a 50% increase in elongation of the crypts was confirmed by histological analysis. It is therefore evident that in addition to the cytotoxic properties of ML-I seen *in vivo*, the lectin can function as a mitogen under *in vivo* conditions.

As well as imparting cytotoxic activity through their type-2 RIP property the mistletoe lectins also have the ability to initiate a profound immunomodulating response (Hajto et al., 1989). Stein et al. (1998) demonstrated a strong initial proliferation of peripheral blood mononuclear cells following the exposure of healthy individuals to a lectin-containing aqueous

mistletoe extract, accompanied by increased TNF and IL-6 production. A less pronounced release of IFN-γ and IL-4 was also observed. In breast carcinoma patients treated with galactose-specific lectin standardized mistletoe extract, Heiny et al. (1998) reported increased ß-endorphin plasma levels, stimulation of T-lymphocytes exhibiting expression of CD25/interleukin-2 receptors and HLA/DR-antigens and enhanced activity of peripheral blood natural killer cells (NK). Baxevanis et al. (1998) also observed enhanced NK activity following the incubation of cultures of PBL for 3 days with ML-I. They reported an expansion and activation of a T-cell population that demonstrated both NK and LAK-like cytotoxicity. ML-I preferentially stimulated and expanded CD8+ T cells which mediated the cytotoxic effect. An activation of PBL with both ML-I and IL-2 resulted in simultaneous induction of T and CD56+ cell-mediated NK and LAK cytotoxicity.

Wenzel-Seifert et al. (1997), in their studies on human U-937 promonocytes, showed that ML-1 induced apoptosis through a signal mechanism that involved intracellular Ca^{2+}. This work indicated an important role of H-1 and C5a receptors in the regulation of the apoptotic process. Büssing et al. (1999) have analysed mitochondrial alterations in human lymphocytes incubated with ML-I and demonstrated generation of reactive oxygen intermediates (ROI) and the induction of expression of newly described mitochondrial membrane proteins Apo2.7. Part of the cytotoxic response thus appears to be a distinct 'death signal' resulting in an induction of Apo2.7 molecules within 24 hr.

Lavelle et al. (2000) studied in mice the mucosal immunogenicity of five plant lectins with different sugar specificities (*Viscum album* (mistletoe lectin 1; ML-1), *Lycospersicum esculentum* (tomato lectin; LEA), *Phaseolus vulgaris* (PHA), *Triticum vulgaris* (wheat germ agglutinin (WGA), and *Ulex europaeus* I (UEA-1). The lectins were administered either by the oral or the intranasal (i.n.) route. The systemic and mucosal antibody responses elicited were compared with those induced by a potent mucosal immunogen (cholera toxin; CT) and a poorly immunogenic protein (ovalbumin; OVA). After three oral or i.n. doses of CT, high levels of specific serum antibodies were detected and specific IgA was present in the serum, saliva, vaginal wash, nasal wash and gut wash. Immunization with OVA elicited low titres of serum IgG but specific IgA was not detected in mucosal secretions. Both oral and i.n. delivery of all five plant lectins investigated stimulated the production of specific serum IgG and IgA antibody after three i. n. or oral doses. Immunization with ML-1 induced high titres of serum IgG and IgA in addition to specific IgA in mucosal secretions. The response to orally delivered ML-1 was comparable to that induced by CT, although a 10-fold higher dose was administered. Lavelle et al. (2001) have demonstrated that ML-I is a strong mucosal adjuvant. A number of plant lectins were investigated as intranasal (i.n.) coadjuvants for a bystander protein, ovalbumin (OVA), where CT was used as a positive control. The co-administration of ML-I or CT together with OVA resulted in production of high titres of OVA-specific serum immunoglobulin G (IgG), in addition to OVA-specific IgA in mucosal secretions. CT and ML-I were also strongly immunogenic, giving rise to high titres of specific serum IgG and specific IgA at mucosal sites. None of the other plant lectins investigated significantly boosted the response to co-administered OVA.

In further studies performed by Lavelle et al. (2002) mistletoe lectins (ML-I, ML-II, ML-III) were compared with CT as adjuvants when delivered nasotracheally together with herpes simplex virus glycoprotein D2 (gD2). All three mistletoe lectins were potent mucosal adjuvants. Co-administration of ML-I, ML-II or ML-III with gD2 led to significantly higher levels of gD2-specific mucosal immunoglobulin A (IgA) and systemic immunoglobulin G (IgG) antibody than when the antigen was delivered alone. The levels of antibodies induced were similar to those generated in mice immunized with gD2 and the potent mucosal adjuvant CT. When ML-1 was administered together with gD2 an enhanced antigen-specific splenic T-cell proliferative response was observed. Interleukin-5 (IL-5), but not interferon-gamma (IFN-gamma), was detected in supernatants from splenocytes stimulated *in vitro* with gD2, which indicated that ML-I enhanced the type 2 T-helper cell (Th2) response to the bystander antigen, gD2. The analysis of gD2- and lectin-specific IgG subclass titres following immunization with gD2 and ML-I, ML-II or ML-III showed a high ratio of IgG1:IgG2a, and this is compatible with the selective induction of Th2-type immune responses. The work of Lavelle and colleagues has thus clearly demonstrated that when delivered by the oral route (three doses) ML-I stimulated the production of specific serum IgG and IgA antibody. The observations being compatible with the selective induction of Th2-type immune responses.

In vitro studies (see below) have been widely used to test the effects of incubating cells with purified lectins. Such studies are quite different to those performed on the intact organism. For example, it has now been established that serum glycoproteins, particularly haptoglobin, but also alpha (1)-acid glycoprotein and transferrin are able to inhibit the apoptosis-inducing properties of ML lectins (Frantz et al., 2000). In addition anti-mistletoe antibodies have been shown to neutralize the cytotoxic effect of mistletoe lectin on peripheral blood mononuclear cells *in vitro* (Stein et al., 1997), and anti-mistletoe lectin antibodies, produced in patients during therapy with an aqueous mistletoe extract, neutralize lectin-induced cytotoxicity *in vitro* (Stettin et al., 1990). These results explain why MLs do not exert harmful effects when they have been administered to animals/patients, by for example, intravenous or subcutaneous injection. It is evident, therefore, that one has to be careful when comparing results from *in vivo* and *in vitro* studies.

Effects of purified mistletoe lectins on tumour cells in culture

As seen in Table 1 reduced growth of a large number of cancer cell lines in the presence of purified mistletoe lectins has been observed *in vitro*. Mockel et al. (1997) examined the effects of ML-I on the human T-cell leukemia line MOLT-4, the monocytic line THP-1 and on human peripheral blood mononuclear cells (PBMC) with regard to general cell viability and induction of apoptosis. Using a sensitive serum-free cytotoxicity assay, the time- and concentration-dependent direct toxicity towards MOLT-4 cells was determined with IC50-values ranging from 20-40 pg/ml (300-600 fmol/l). Investigations on the time course of the toxic effect using selected concentrations of ML-I revealed distinct response curves for concentrations of high, low and intermediate toxicity, respectively. The ratio of apoptotic to viable MOLT-4 cells was determined after treatment with ML-I for 24hr. Apoptosis and cytotoxicity were correlated at low and intermediate concentrations. The data showed that in the concentration range of low cytotoxicity ML-I - induced cell death is quantitatively due to apoptotic processes. The immunomodulatory activity of ML-I was investigated *in vitro* by measuring cytokine release. At concentrations of low cytotoxicity ML-I showed immunostimulatory activity on PBMC and THP-1. RT-PCR with THP-1 cells confirmed that cytokine induction by ML-I is regulated at the transcriptional level. These findings suggest that in the blood cells investigated both apoptosis and cellular signalling are induced by the same concentration range of ML-I. Ribereau-Gayon et al. (1997) also demonstrated an inhibition of Molt 4 cell growth. This occurred with lectin concentrations in the pg/ml range. The first events that were observed were membrane perforation and protusions typical of apoptosis. They showed that ML-III was about 10 times more cytotoxic than ML-I. Treatment of leukemic T- and B-cell lines with ML-I also triggered cell death through the induction of apoptosis (Bantel et al., 1999). A peptide cascade inhibitor was almost completely able to prevent the effect of ML-I. These authors showed that ML-I potentiated the effect of chemotherapeutic drugs.

The cytotoxic activity of ML-I towards the anaplastic glioma cell line (F98) using a three dimensional spheroid model was studied by Lenartz et al. (1998). F98 glioma cell spheroid growth was significantly inhibited after incubation with defined ML-I concentrations of 10 and 100 ng/ml, demonstrating dose dependent cytotoxicity to the lectin.

Using an *in vitro* approach Köteles et al. (1998) have shown that incubation of weakly x-irradiated human lung carcinoma cells (Calu-1) with ML-1, facilitated an apoptotic process in damaged cells but not in non-injured cells. *In vivo* studies on x-irradiated (1 Gy) rabbits resulted in the same effect on damaged cells. The authors came to the conclusion that the combined effects of relatively low doses of ionizing radiation and mistletoe lectin appear to be favourable because of the scavenging effect seen on damaged somatic cells. In their studies on the actions of ML-I, with or without ionizing radiation, Kubasova et al. (1999) demonstrated stress effects of the treatment on cultured Calu-1 cells as judged by altered metabolic processes.

Yoon et al. (1999) isolated cytotoxic lectins (KML-C) from an extract of Korean mistletoe [*Viscum album C*. (coloratum)] by affinity chromatography on a

Table 1. Mistletoe lectins: induction of apoptosis/reduced growth in tumour cell lines *in vitro*.

CELL LINE	REFERENCE
Molt-4 (T-cell leukemia)	Mockel et al. 1997
Molt-4	Ribereau-Gayon et al. 1997
Calu-1 (human lung carcinoma)	Köteles et al. 1998
F-98 anaplastic glioma	Lenartz et al. 1998
Leukemic B- and T-cell lines	Bantel et al. 1999
Human lung carcinoma (Calu-1)	Kubasova et al. 1999
Various human and murine tumour cells	Yoon et al. 1999
HL-60 human leukemia; Human hepatocarcinoma cells	Pae et al. 2000a
HeLa (human cervix carcinoma); MCF-7 breast carcinoma	Pae et al. 2000b
Jurkat T-cells; RAW 264.7; HL-60; DLD-1 cells; human myeloleukemic cells	Park et al. 2000
U-266 plasmacytoma	Stein et al. 2000
Human myeloleukemic U937 cells	Kim et al. 2000
HL-60 human leukemia	Lyu et al. 2001
B16-BL6 melanoma	Park et al. 2001
Human hepatocarcinoma SK-Hep1 cells	Pae et al. 2001a
U937 human monoblastic leukemia	Pae et al. 2001b
Human lung carcinoma (A549)	Siegle et al. 2001
Human hepatocarcinoma (SK-Hep-1, Hep 3B)	Lyu et al. 2002
Human colon cancer HT29	Valentiner et al. 2002
Human A253 cells	Choi et al. 2004
Human melanoma cells	Thies et al. 2005

hydrolysed Sepharose 4B column, and the chemical and biological properties of KML-C were examined, partly by comparing them with a lectin (EML-I) from European mistletoe [*Viscum album* L. (loranthaceae)]. The isolated lectins showed strong cytotoxicity against various human and murine tumour cells, and the cytotoxic activity of KML-C was higher than that of EML-I. Tumour cells treated with KML-C exhibited typical patterns of apoptotic cell death, such as typical morphological changes and DNA fragmentation, and its apoptosis-inducing activity was blocked by addition of Zn^{2+}, an inhibitor of Ca^{2+}/Mg^{2+}-dependent endonucleases, in a dose-dependent manner. These results suggest that KML-C is a novel lectin and that its cytotoxic activity against tumour cells is due to apoptosis mediated by Ca^{2+}/Mg^{2+}-dependent endonucleases. Pae et al. (2000a), using human leukemia HL-60 cells, showed that incubation with various doses of Korean mistletoe lectin (ML-II) resulted in apoptosis. It appeared that activation of PKA or PKC was able to convey protection against apoptosis induced by the lectin. Pae et al. (2000b) have hypothesized that the anticancer efficacy of tumour necrosis factor-alpha (TNFα) may be potentiated by MLs isolated from European or Korean mistletoe. Their studies demonstrated that simultaneous treatment of human cervix carcinoma HeLa or breast carcinoma MCF-7 cells with the lectins rendered them more sensitive to induction of apoptosis by TNF. In studies on cultured human hepatocarcinoma SK-Hep1 cells, using the microculture tetrazolium test, a synergistic effect of ML-I on the *in vitro* cytotoxicity of a clinically important anticancer drug, paclitaxel, was established (Pae et al., 2001a).

Stein et al. (2000) have established that intracellular expression of IL-4 and inhibition of IFNγ production are processes that are involved in the induction of apoptosis in U-266 plasmacytoma cells and T-leukemia cells.

In their search for new approaches in lung cancer treatment, Siegle et al. (2001), using the human lung carcinoma cell line A549, have evaluated the antineoplastic activity of mistletoe lectin alone and in combination with other chemotherapeutic drugs, including doxorubicin, cisplatin and taxol. Cytotoxicity was determined by 5-bromo-2'-deoxyuridine ELISA-assays, drug interaction assessed by the isobologram method and analysis of cell cycle distribution was obtained using flow cytometry. The results showed that for all drug combinations tested the outcome was additive. The combination of lectin and cycloheximide demonstrated strong synergistic effects. Their findings suggest that the simultaneous administration of mistletoe lectin with all anticancer agents tested is advantageous since cytotoxic effects are enhanced. These results may provide interesting new clinical perspectives with regard to the future of mistletoe lectin in cancer therapy.

Treatment of U937 cells (a human monoblastic leukemia cell line) with Korean mistletoe lectin-II caused apoptotic DNA fragmentation, which was preceded by the activation of ERK1/2, p38 MAPK and SAPK/JNK (Pae et al., 2001b). Using HL-60 cells, addition of *Viscum album* L. coloratum agglutinin (VCA) was shown to result in a dose- and time-dependent growth suppression, morphological changes of apoptotic nuclei, and DNA fragmentation characteristic of apoptosis (Lyu et al., 2001). To investigate how caspase-3 activation during VCA-induced apoptosis induced cleavage of PARP, the expression of PARP and the pattern of caspase-3 activation in HL-60 cells was also investigated. The native and processed PARP forms typically seen in apoptotic cells were observed, and a decrease in expression of the 32-kDa form of caspase-3 in a dose-dependent manner was found. The VCA-induced apoptosis was significantly inhibited by a caspase-3 specific inhibitor, z-DEVD-FMK, and the PARP processing and caspase-3 activation were also inhibited by the inhibitor.

In experiments using Korean mistletoe, *Viscum album* var. coloratum, Kim et al. (2000) showed that lectin-II specifically induces apoptotic cell death in cancer cells, but not normal lymphocytes. They observed that when U937 cells were incubated with mistletoe lectin-II, ladder pattern DNA fragmentation was induced and activation of caspase-3, -8, and -9, but not caspase-1 protease, occurred in a time- and dose-dependent manner.

Park et al. (2000) investigated the direct effects of beta-galactoside- and N-acetyl-d-galactosamine-specific mistletoe lectin II in inducing apoptotic death of U937 cells. The induction of apoptotic cell death, characterized by DNA ladder pattern fragmentation, was evident 12 hr after treatment. Consistent with apoptosis a marked increase in phosphotransferase activity of c-Jun N-terminal kinase 1 (JNK1)/stress-activated protein kinase (SAPK) was observed in U937 cells. Catalytic activation of JNK1 induced by mistletoe lectin II was inhibited by the addition of peptide aC-DEVD-CHO, but not by aC-YVAD-CHO. In addition to inducing apoptosis in U937 cells, mistletoe lectin II also induced apoptosis in a variety of cell types including Jurkat T cells, RAW 264.7 cells, HL-60 cells, DLD-1 cells, and primary acute myelocytic leukemic cells.

The effects of Korean mistletoe lectin (*Viscum album* L. coloratum agglutinin, VCA) on proliferation and apoptosis of human hepatoma cells have been studied by Lyu et al. (2002). They observed that VCA induced apoptosis in both SK-Hep-1 (p53-positive) and Hep 3B (p53-negative) cells occurred through p53- and p21-independent pathways. VCA induced apoptosis by down-regulation of Bcl-2 and by up-regulation of Bax functioning upstream of caspase-3 in both cell lines. In addition, down-regulation of telomerase activity was seen in both VCA-treated cell lines. These results provide direct evidence of the anti-tumour potential of this biological response resulting from inhibition of telomerase and a consequent induction of apoptosis. These findings are important regarding therapy with

mistletoe lectins because they show that a telomerase-dependent mechanism can be targeted by VCA in human hepatocarcinoma cells. Their results suggest that the VCA, functioning as a telomerase-inhibitor, can be envisaged as a candidate for enhancing the sensitivity of conventional anticancer drugs.

Based on the knowledge that amphiphilic chemotherapeutic agents can be eliminated out of tumour cells by multidrug resistance glycoprotein1 (MDR-1) leading to therapeutic failure, Valentiner et al. (2002) investigated the cytotoxic effect of mistletoe lectins (MLs) I, II and III on the sensitive human colon cancer cell line HT 29(mdr-), its multidrug resistant variant HT 29 (mdr+), the variant HT 29 (SF1m) transfected with the MDR-1 gene and its sensitive control cell line HT 29 (deltaSF). They analysed both cell proliferation and the lectin binding pattern. Marked quantitative differences with regard to the cytotoxic effect of the three MLs on the different cell lines were observed. All MLs showed the greatest cytotoxicity towards the HT 29 (mdr+) cells, in which multidrug resistance (MDR) was induced by increasing concentrations of a MDR inducing agent. On the other hand MDR-1 and mock-transfected cells showed almost the same sensitivity towards the three MLs as the control cells (HT 29 (mdr-)). Results from FACS analysis showed that the HT 29 (mdr+) cells were those with the highest density of ML binding sites. It was thus evident that the higher sensitivity of HT 29 (mdr+) cells was not caused by the over-expression of MDR-1, but was a result of the general changes in cellular glycosylation that occurred during acquisition of the MDR phenotype.

In their study Choi et al. (2004) observed the induction of apoptotic cell death in VCA-treated A253 cells through activation of caspase-3 and the inhibition of telomerase activity through transcriptional down-regulation of hTERT.

Thies et al. (2005) have recently investigated both the direct and the indirect effects of MLs on the growth of six human melanoma cells *in vitro*. Using the tetrazolium derived reduction (XTT) assay, proliferation of the cell lines was assessed during ML treatment and additionally under the influence of cytokines induced by them (TNF-α, IL-1, IL-6). The results showed that all three MLs inhibited melanoma cell proliferation in a dose-dependent manner starting at very low ML concentrations (0.001-100ng/ml). Of the three lectins ML-I proved to be the most cytotoxic (significant inhibition of ultra-sensitive cell line MV3 at $1x10^{-13}$ ngML-I/ml). Cytokines appeared to have no influence at all on cell proliferation. As a result of incubation with ML-I for 12 h, a significant number of melanoma cells started rounding up, and exhibited cell shrinkage, chromatin condensation and nuclear fragmentation i.e. showed typical signs indicating apoptotic cell death. The study demonstrates the direct anti-proliferative effect of MLs on growth of human melanoma cells in culture.

From the *in vitro* studies discussed above it is evident that the incubation of a wide number of different cancer cell lines with lectins isolated from either European or Korean mistletoe leads to the induction of apoptosis resulting in cell death.

Effects of purified mistletoe lectins on tumours *in vivo*

Local, systemic or subcutaneous administration

In addition to the *in vitro* work described above a number of workers have examined the effects of mistletoe lectins on tumour growth *in vivo* (Table 2). To investigate the *in vivo* cytotoxic efficacy of ML-I, Fischer 344 rats were intracerebrally implanted with F98 glioma cells and subjected to both local and systemic ML-I treatment (Lenartz et al., 1998). Histological and immunohistochemical evaluation showed a reduction in tumour volume for both treatment modalities. The observations were most pronounced and statistically significant after systemic (immunomodulating) administration of the optimal ML-I dosage (1 ng/kg body weight, subcutaneously) and after low dose (10 ng ML-I per application) local treatment. High dose ML-I administration (10 ng/kg body weight; systemically; 100 ng/application, locally) was less effective than low (optimal) dose treatment and apparently the systemic/immunomodulating approach resulted in a greater benefit for glioma bearing rats.

Yoon et al. (1998) have demonstrated the prophylactic effect of lectins (KM-110) from *Viscum album* coloratum, a Korean mistletoe, on tumour metastasis produced by highly metastatic tumour cells, colon 26-M3.1 carcinoma, B16-BL6 melanoma and L5178Y-ML25 lymphoma cells, using experimental models in mice. Intravenous (i.v.) administration of KM-110 (100 mg/mouse) 2 days before tumour inoculation significantly inhibited lung metastasis of B16-BL6 and colon 26-M3.1 cells, and liver and spleen metastasis of L5178Y-ML25 cells. Furthermore, mice given KM-110

Table 2. Mistletoe lectins: reduced tumour growth *in vivo.*

TUMOUR	REFERENCE
Non-Hodgkin lymphoma (mice)	Ewen et al., 1998, 1999, Pryme et al. 1998, 2002, 2004
F-98 glioma (rat)	Lenartz et al. 1998
Colon 26-M3.1 carcinoma (mice); B16-BL6 melanoma (mice); L5178Y-ML25 lymphoma (mice)	Yoon et al. 1998
Lewis lung tumour (mice)	Kubasova et al. 1998
MB49 urinary bladder carcinoma (mice)	Mengs et al. 2000
Malignant glioma (human study*)	Lenartz et al. 2000
B16-BL6 melanoma (mice)	Park et al. 2001

*: prolongation of overall survival rate

(100 mg) 2 days before tumour inoculation showed significantly prolonged survival rates compared with the untreated mice. In a time course analysis of NK activity, i.v. administration of KM-110 (100 mg) significantly augmented NK cytotoxicity to Yac-a tumour cells from 1 to 3 days after KM-110 treatment. Furthermore, depletion of NK by injection of rabbit anti-asialo GM1 serum completely abolished the inhibitory effect of KM-110 on lung metastasis of colon 26-M3.1 cells. These results suggest that KM-110 possesses immunopotentiating activity which enhances the host defence system against tumours, and that its prophylactic effect on tumour metastasis is mediated by NK activation.

A study to investigate the mechanism of anticancer and antimetastatic activity of the purified Korean mistletoe lectin (*Viscum album* L. coloratum agglutinin, VCA) was performed by Park et al. (2001). C57BL6 mice were inoculated with B16-BL6 melanoma cells, treated with VCA and then assessed for survival and metastasis. The induction of apoptosis in B16-BL6 cells by VCA was investigated by studying morphological changes, DNA fragmentation characteristics, and cell cycle analysis. The length of survival of mice was increased and lung metastasis was inhibited by VCA. Treatment of cells with VCA resulted in growth suppression, nuclear morphological changes, DNA fragmentation, and an increased fraction of cells in sub-G1, consistent with apoptosis.

Van Huyen et al. (2002) tested the hypothesis that lectin-containing mistletoe extracts induce endothelial cell death through apoptosis. Mistletoe treated human venous endothelial cells (HUVEC) were studied using morphologic assessment of EC, FACScan analysis after propidium iodine and annexin V labeling, and detection of cleavage of poly(A)DP-ribose polymerase. Apoptosis, assessed by morphologic examination, annexin V labeling, and Western blot analysis for PARP cleavage, was shown to be involved in HUVEC cell death. Van Huyen et al. (2002) conclude that extracts containing mistletoe lectins that induce endothelial apoptosis may explain tumour regression associated with the therapeutic use of such preparations.

There is thus a great deal of evidence clearly demonstrating that mistletoe lectins from various sources are able to induce apoptosis in a wide range of tumour cell types *in vivo*.

Effects of purified mistletoe lectins on tumours *in vivo*

Oral administration

In order to establish whether or not mistletoe lectin (ML-I) could be presented orally without inducing undue side-effects, Pusztai et al. (1998) studied the effects of ML-I on rats. Animals were fed diets containing 67 or 200 mg ML-I/kg body weight for 10 days and various growth parameters were followed. It was noted that although ML-I depressed voluntary feed intake and reduced growth rate, none of the rats were seen to lose weight during the experimental period. With respect to the digestibility of proteins or other dietary components no negative effects were observed. The highest dietary level of lectin tested increased urinary nitrogen loss, causing a reduction in the overall N balance and total body N content. Faecal weight and its N content, though, were not affected. As seen earlier for PHA (Pusztai 1991; Bardocz et al. 1996) dietary ML-I was shown to reduce serum insulin levels. As previously seen for PHA, ML-I induced a dose-dependent hyperplastic growth of the small intestine in rats. Similar hyperplastic growth was also seen in mice (Pryme et al., 2002). A significant increase occurred in plasma TNFα 30 hr after rats were fed a diet containing ML-I, and plasma interleukin-1ß was also elevated. It was thus evident that the release of cytokines, a process that is considered to be an essential step in immunomodulation leading to tumour depression (Mannel et al., 1991), was promoted following the oral presentation of ML-I to rats. The avid binding of ML-I to M cells of Peyers patch observed in the rat small intestine is almost certainly implicated in the observed increase in plasma cytokines.

Pusztai et al. (1998) concluded from their work that ML-I included in the diet fed to rats, even in large amounts, did not produce any detrimental side-effects suggesting that it could be tested for anti-tumour properties in an animal test system. Since impressive effects had been earlier described following the oral

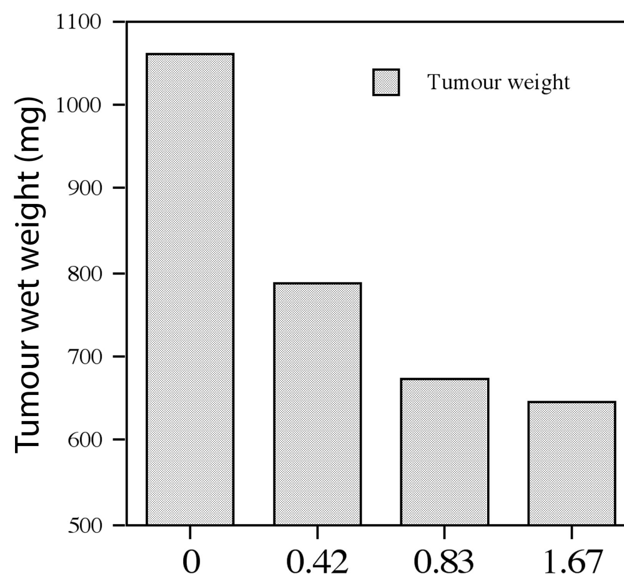
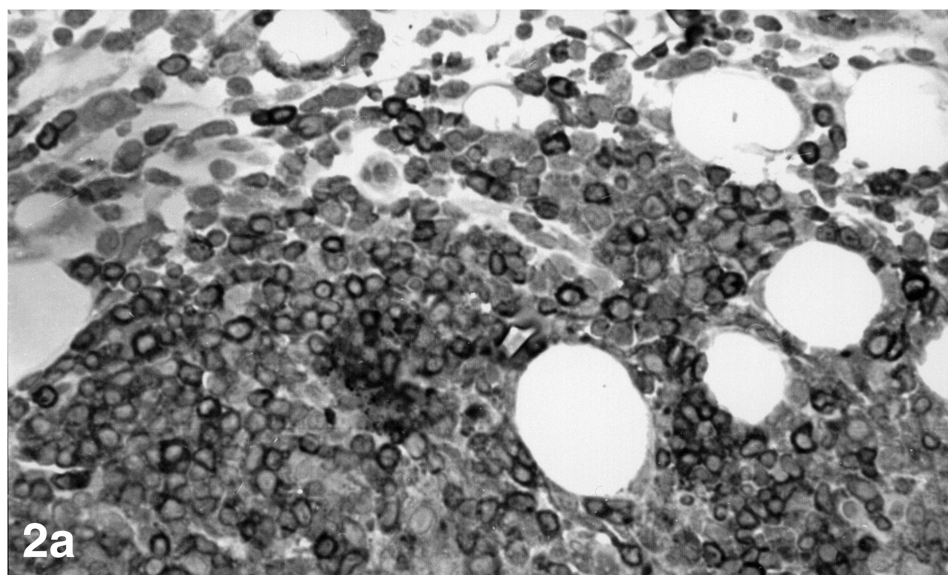


Fig. 1. The mass of NHL tumours in four groups of NMRI mice (5 individuals/group, mean values) after 10 days of growth following the s.c. injection of 2.10^6 NHL cells. One group was fed on an LA diet (0 ML-1) and three groups on diets containing ML-I lectin within the range 0.43-1.67 mg/g diet.

presentation of PHA in reducing growth of a transplantable murine non-Hodgkin lymphoma tumour (Bardocz et al., 1994a,b, 1997; Pryme et al., 1994a,b, 1995, 1996a,d, 1998a,b, 1999a,b, 2000a,b), similar experiments were designed using ML-I. The lectin was incorporated into semi-synthetic diets as for PHA (Pusztai et al., 1998). The effect of feeding mice the LA control diet, or one containing three different concentrations of ML-I lectin are shown in Figure 1 where tumour mass is expressed as a function of the lectin content in the diet. Even at the lowest lectin concentration tested an appreciable effect on tumour growth was evident in that the mass was reduced by about 25%. At the highest concentration of ML-I tested (1.67 mg/g diet) the tumour weight was reduced by approximately 40% (Pryme et al., 1998a). The results indicated that the reduction in tumour growth occurs in a dose-dependent manner.

In earlier experiments PHA was shown to cause hyperplasia of the small intestine and the results have indicated a relationship between the stimulation of "normal" growth and the depressed growth of the tumour, suggesting a competition between the two types of cell proliferation for nutrients and growth factors from a common body pool (Bardocz et al., 1999; Pryme et al., 1999c). Based on tissue mass measurements ML-I was shown to cause hyperplasia of the gut (Pryme et al., 2002), confirmed by histological analysis of the small



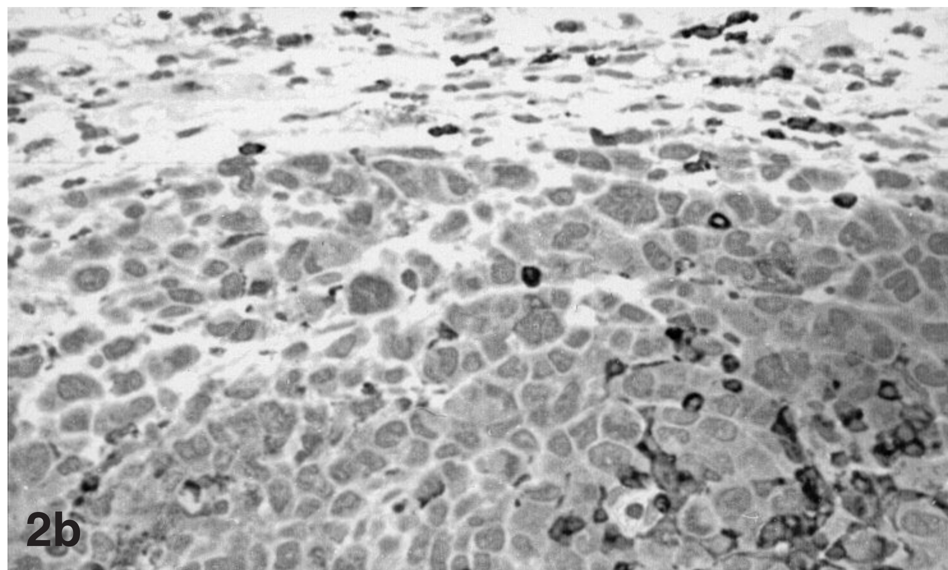


Fig. 2. a. There is increased T lymphocyte infiltration at the leading edge of the transplanted NHL tumour in the ML-1 fed mice (legend to Fig. 1), compared to **(b)** where mice were fed a control (LA) diet. Both preparations were stained with CD3 antibody. x 250

intestine, in a similar manner to that previously observed with PHA (Pryme et al., 1998a).

A clear advantage of providing mistletoe lectins by the oral route is that a large number of the lectin molecules, through their binding to receptors on the gut mucosa, are in due course presented to lymphocytes of Peyers patches and thereby induce a major cytokine response. Within 24 hr of feeding ML-I to mice we have observed a 14% reduction in spleen weight and this has been attributed to a major release of lymphocytes into the blood circulation following increased levels of cytokines caused by their release from stimulated cells in Peyers patches/gut mucosa. We consider it unlikely that such a response would be evoked by the small amounts of lectins that are likely to reach immunostimulatory tissue following the subcutaneous injection of either mistletoe extracts or lectins.

Further detailed studies have been performed to examine the characteristics of the NHL tumour at the microscopic level following the feeding of ML-I-containing diets to mice (Ewen et al., 1998, 1999). An intense lymphoid host response within the NHL tumour was evoked by ML-I (Fig. 2a), compared to a patchy and sparse lymphoid reaction in the control-fed animals (Fig. 2b). Accelerated cellular turnover within the transplanted NHL tumour as a response to oral intake of ML-I was seen as increased numbers of apoptotic cells with an increased area of serpiginous irregular dead cells, and the non-viable cells occupied a two fold increased area in the mice fed the lectin (Ewen et al.,

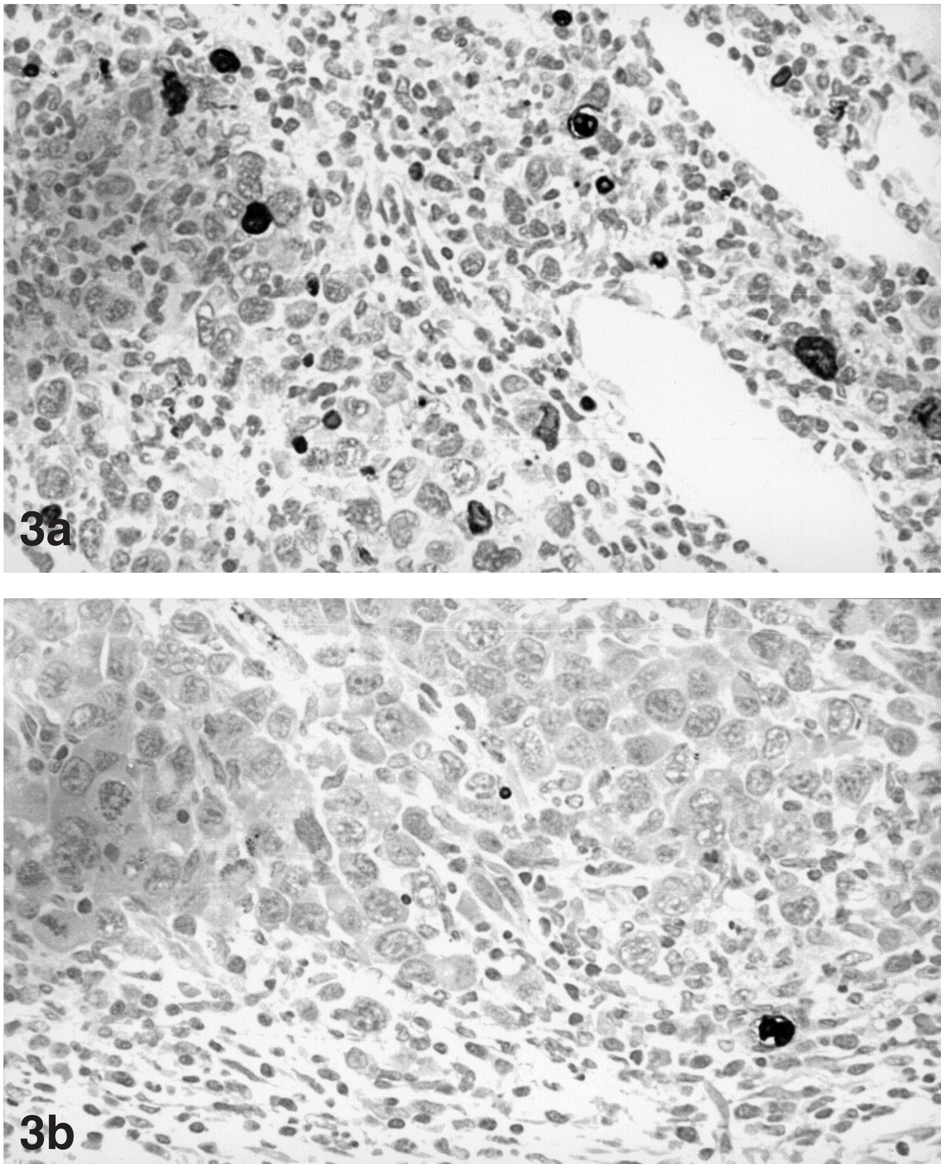


Fig. 3. a. Apoptotic bodies are more numerous throughout the transplanted NHL tumour in the ML-1 fed mice (legend to Fig. 1), compared to **(b)** where mice were fed a control (LA) diet. Apoptoses were visualized by use of the nick end labelling PAP method. x 250

1999). Apoptoses were more numerous in the tumours of mice fed ML-I (Fig. 3a) compared to control diet (Fig. 3b). These were identified around areas of non-viable tumour cells, at the advancing edge of the tumour and within intense lymphoid aggregates. A decrease in the nuclear area of the tumour cells (mean decrease 21%) was observed following feeding ML-I (Pryme et al., 2004). The number of tumour cell mitoses was reduced from 7.4 (control) to 1.7 in tumours from mice with the highest daily intake of ML-I. There was a simultaneous increase in crypt length of the jejunum with increasing intake of ML-I (Ewen et al., 1998; Pryme et al., 2004).

NHL tumours obtained from mice fed control diets or ML-1-supplemented diets were examined at the microscopic level in order to obtain information on their characteristics. Tumours in control mice typically consisted of fairly large blast-type cells and these contained a high number of mitotic figures. In contrast, after feeding ML-1, the tumour cells were smaller in appearance and were arranged in a manner consistent with an increased level of differentiation (Pryme et al., 2002).

In a recent study Pryme et al. (2004) showed that the characteristics of non-Hodgkin lymphoma (NHL) tumours in female NMRI mice fed mistletoe lectin (ML-I)-containing diets were different from those in mice fed control diet. Mice were either fed a lactalbumin (LA)-based control diet or the same diet containing added ML-I that provided up to 10 mg lectin per day. At the highest daily intake (10 mg lectin) the degree of mitotic activity in tumours was reduced by 75% and the nuclear area had diminished by 21%. The overall level of T-lymphocyte infiltration (CD3 positive cells) in tumours from mistletoe lectin fed mice was highly increased. Morphological studies demonstrated a high incidence of apoptotic bodies in NHL tumours obtained from mice fed mistletoe lectin diets. This was consistent with increased lymphocyte infiltration. In 4/15 mice fed a diet containing ML-I for 11 days there was no longer evidence of viable tumour. The results provided clear evidence that ML-I exerts powerful anti-tumour effects when included in the diet.

Kubasova et al. (1998) have demonstrated that administration of ML-1 (either presented orally or given by subcutaneous injection) reduced the number of lung metastases in mice injected intramuscularly with $5x10^5$ Lewis lung tumour cells. When fed on a diet containing ML-I at a concentration of 70 micrograms/kg the total number of metastases on the lung surface was reduced by about 75% during a period of 19 days.

A study was performed by Mengs et al. (2000) to investigate the effects of a locally applied aqueous mistletoe extract (AME) on the growth of urinary bladder carcinoma MB49 in an orthotopic murine model. On day 1 of the experiment a total of $4x10^4$ tumour cells was implanted into the bladder of female C57BL/6J mice which were then randomly allocated into three groups of 13 mice in each group. From day 11 onwards, AME (equivalent to concentrations of 30 or 300 ng bioactive mistletoe lectin (ML)/ml) was given intravesically 3 days a week for a period of 4 consecutive weeks. 39% of the mice in the control group survived the scheduled study period in comparison to 69% and 85% in the groups treated with 30 or 300 ng ML/ml, respectively. Visible solid bladder tumours were seen at necropsy in 80% of the surviving control animals, while only 56% and 18% had tumours in the respective treated groups. The differences were statistically significant at the high concentration in comparison to controls ($p<0.05$), whereas a non-significant effect was observed with respect to the formation of multiple metastases (40% in controls versus 33% and 18% in the treated groups). Mengs et al. (2000) concluded that under their experimental conditions AME showed antitumoural activity due to the cytotoxic properties of the mistletoe lectins.

Experiments with purified ML-III showed that feeding of the lectin to mice bearing established NHL tumours was extremely effective in reducing further tumour growth (Pryme et al., 2002). No effect was observed on the dry weight of liver, kidneys or gastrocnemius muscle. Taken together the results demonstrate that ML lectins induce powerful anti-cancer effects when provided by the oral route.

Anti-angiogenic effects of mistletoe lectins

Yoon et al. (1995) suggested that the anti-metastatic effect of an extract of Korean mistletoe on a series of tumour cell lines in mice was in part due to an inhibition of tumour-induced angiogenesis. They examined the inhibitory effect of an aqueous extract (KM-110) from *Viscum album* coloratum, a Korean mistletoe, on tumour metastasis produced by highly metastatic murine tumour cells: B16-BL6 melanoma, colon 26-M3.1 carcinoma and L5178Y-ML25 lymphoma cells. Intravenous (i.v.) administration of KM-110 (100 micrograms/mouse) 1 day after tumour inoculation significantly inhibited lung metastasis of both B16-BL6 and colon 26-M3.1 cells. A therapeutic effect was also observed on liver and spleen metastasis of L5178Y-ML25 lymphoma cells. Interestingly, in spontaneous metastasis of B16-BL6 melanoma cells, multiple administration of KM-110 into tumour-bearing mice resulted in significant inhibition of lung metastasis, as well as a suppression of the growth of the primary tumour. *In vivo* analysis for tumour-induced angiogenesis showed that i.v. administration of KM-110 inhibited the number of blood vessels oriented towards the tumour mass. The results of Yoon et al. (1995) suggested that the extract of Korean mistletoe reduces tumour metastasis by inhibition of tumour-promoted angiogenesis by inducing TNFα production.

In their studies designed to investigate the mechanism of anticancer and antimetastatic activity of the purified Korean mistletoe lectin (*Viscum album* L. coloratum agglutinin, VCA), Park et al. (2001) inoculated C57BL6 mice with B16-BL6 melanoma cells and then treated the mice with VCA. The anti-

angiogenic activity of VCA was measured by the choriallantoic membrane assay where vessel growth induced by fat emulsion was decreased. The results suggested that VCA inhibited tumour growth and metastasis by two mechanisms: 1) an anti-angiogenic effect, 2) by increasing the level of apoptosis.

In control fed mice or mice fed PHA the NHL tumour was characterised as having an extremely well-developed capillary system giving it an extremely bloody appearance upon dissection of sacrificed animals. In comparison the NHL tumours removed from ML-I fed mice were white in appearance and thus characterised as having a poorly developed vascularisation. Morphological studies on sections derived from NHL tumours that developed in mice fed ML-I showed a greatly reduced incidence of tumour vascularisation (Fig. 4a) compared to mice not fed the lectin (Fig. 4b). These observations can be interpreted as an indication that one of the responses to oral ML-I is an induction of the production of anti-angiogenic factors. These results corroborated well with the initial "naked eye" observations of the NHL tumours mentioned above. Importantly, as a result of feeding ML-I the NHL tumour was histologically completely ablated in 6 of a total of 25 mice. An ablation of the NHL tumour was not observed when mice were fed PHA.

There is thus accumulating evidence that both European and Korean mistletoe lectins are able to induce

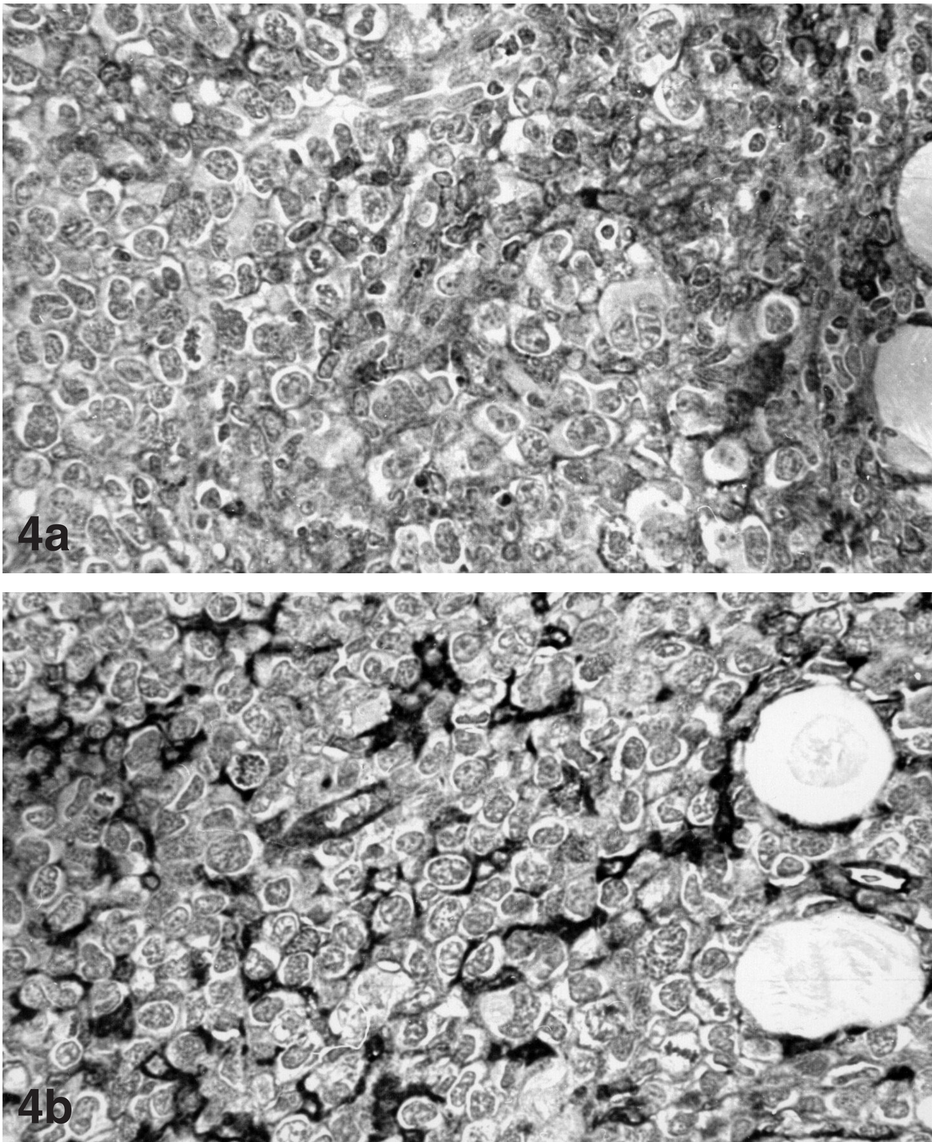


Fig. 4. a. Binding sites in tumour vessel endothelial cells are less numerous in the NHL tumour of ML-1 fed mice ((legend to Fig. 1), compared to **(b)** where mice were fed a control (LA) diet. Visualisation of blood capillary endothelium by immunocytochemistry where dewaxed sections were first reacted with ML-1 lectin followed by exposure to ML-1 antibody. x 250

an anti-angiogenic response in the host. The significance of these observations will have to await the results of further experimentation.

Purified mistletoe lectin in clinical trials

Lenartz et al. (2000) have performed a clinical trial where the effects of ML-I on patients with malignant glioma were studied. All the patients enrolled into the trial were subjected to internationally recommended oncologic standard treatment (neurosurgery, radiation, basic clinical care according to protocol and indication) and randomly divided into two groups: a treatment group (receiving additional complementary immunotherapy with a galactoside-specific lectin from mistletoe, ML-1) and a control group (without additional complementary treatment). An evaluation of relapse free/overall survival was performed after a follow-up time of 50 months. Non-stratified analysis of all the patients in the treatment group showed a non-relevant prolongation of relapse-free intervals/overall survival time. An analysis of stratified stage III/IV glioma patients, however, demonstrated the following: 1) a tendency for a prolongation of relapse-free survival for patients of the treatment group (17.43±8.2 months) vs. the control group (10.45±3.9 months), 2) a statistically significant (BRESLOW p=0.035) prolongation of the overall survival for the treatment group (20.05±3.5 months) as compared to the control group (9.90±2.1 months). These promising data indicate the possible potential of complementary immunotherapy with ML-I in addition to standard oncologic treatment.

In a recent study Elsasser-Beile et al (2005) reported results from a phase I/II clinical trial where a mistletoe extract standardized for lectin content was administered intravesically to 30 patients with superficial urothelial bladder carcinoma. About 4 weeks after transurethral resection each patient received 6 instillations, each retained in the bladder for 2 hours (mistletoe lectin concentrations between 10 and 5,000 ng/ml), at weekly intervals. Clinical follow-up consisted of cystoscopy, cytology and random biopsies. The tolerability of intravesically administered mistletoe lectins was good at all concentrations applied. None of the patients showed local or systemic side effects according to WHO classification 1-4. The authors conclude that this form of treatment could be a potential alternative to adjuvant therapy for superficial bladder cancer.

Anti-tumoural properties of recombinant mistletoe lectin

The anti-cancer potential of recombinant mistletoe lectin (rML) produced in bacteria on the growth of SoTu3 human ovarian cancer cells injected intraperitoneally into SCID mice has been studied by Schumacher et al. (2000). Only 10% animals in the control group had survived eighty-four days after inoculation and were tumour free. In the experimental group, however, where the mice had received 500 ng/kg rML daily, 65% survived. At the end of the experiment, furthermore, no tumour cells could be identified in the peritoneum.

Schaffrath et al. (2001) studied the antitumoural and immunostimulating properties of rViscumin (recombinant mistletoe lectin) in two mouse tumour models. After Balb/c mice were given intravenous inoculations of RAW-117-P or L-1 sarcoma cells, rViscumin was injected s.c. at non-toxic doses ranging from 0.3 to 150 ng rViscumin/kg. One set of experiments was designed to investigate the survival of rViscumin-treated animals. Other experiments were carried out to analyze the effect of rViscumin treatment on the number of tumour colonies in infiltrated lungs (RAW-117P cells) or liver (L-1 cells). They also studied the activation of immune cell subsets. After treatment with rViscumin an overall prolonged survival time was observed and after administration of certain rViscumin doses a decrease in number of tumour colonies was observed. Immunophenotyping of the peripheral leukocytes obtained from treated mice revealed increased numbers of T-lymphocytes, pan-NK cells and activated monocytes. The results gave a clear indication that rViscumin has antineoplastic properties and suggest that it might be a promising candidate in cancer therapy.

Elsasser-Beile et al. (2001) performed a study to determine the effect of recombinant, galactoside specific mistletoe lectin (rML) on chemically induced tumour development in the urinary bladder of rats. To induce tumours, rats were treated with four biweekly 1.5 mg doses of N-methyl-N-nitrosourea (NMU) intravesically. A control group received no treatment. The four therapy groups also received rML twice weekly according to one of the following instillation regimens: 1) 30 ng rML per instillation from week 8 to week 13, 2) 150 ng rML per instillation from week 8 to week, 3) 30 ng rML per instillation from week 14 to week 19, and 4) 150 ng rML per instillation from week 14 to week 19. The rats were asphyxiated at week 21 and the urinary bladders excised *in toto* and examined histopathologically. By week 21 both atypical hyperplasia and neoplastic transformation was observed in 82% of the animals in the control group. In contrast, in all four cohorts that were treated with rML, significantly lower rates of atypical hyperplasia and neoplastic transformation were seen (Group 1, 50%; Group 2, 52%; Group 3, 45%; and Group 4, 42%). The data provide evidence suggesting that rML has an inhibitory effect on experimental urothelial carcinogenesis.

Aviscumine is an *Escherichia coli*-derived recombinant type II ribosome-inactivating protein which exhibits potent antitumour activity both *in vitro* and *in vivo* (Schoffski et al., 2004). It is the recombinant counterpart of natural mistletoe lectin-I. Their study was designed to determine the safety profile, dose-limiting toxicity (DLT) and maximum tolerated dose (MTD) of the intravenous (i.v.) administration of aviscumine in cancer patients. The pharmacokinetics of Aviscumine

were evaluated and plasma cytokine and anti-aviscumine antibody induction was monitored after administration. Aviscumine was given twice weekly as a 1 h central i.v. infusion (10 to 6400 ng/kg) in patients (forty-one fully eligible patients: 19 male and 22 female, with a median age of 56 years, range 37-74) all with advanced, refractory progressive, solid malignant tumours (including colorectal, ovarian, renal cell and breast) and had not earlier been exposed to natural mistletoe preparations. The median number of cycles was two (range one to eight). Common clinical toxicities in cycle 1 were fatigue, fever, nausea, vomiting and allergic reactions. Fatigue grade 3 was dose limiting in one of six patients at 4000 ng/kg and reversible grade 3 liver toxicity (elevation in alkaline phosphatase, transaminases and/or gamma-glutamyltransferase) occurred in one of 10 patients at 4800 ng/kg and in two of five patients at 6400 ng/kg. The pharmacokinetic evaluation revealed a short alpha half-life of 13 min and linear kinetics on dose levels ≥ 1600 ng/kg. Aviscumine stimulated the immune system with a release of cytokines such as interleukin (IL)-1beta, IL-6 and interferon-gamma, and induced the formation of immunoglobulin (Ig) G- and/or IgM-anti-aviscumine antibodies (clinical relevance uncertain). A recommended dose for further clinical trials was calculated as 5600 ng/kg twice weekly. Because of the short half-life of the recombinant protein observed in the trial, the authors suggest that exploration of prolonged infusion schedules of aviscumine is required.

Conclusions

The observations indicate that the oral administration of lectins derived from mistletoe or the kidney bean (PHA), result in different biological effects following binding to their respective sets of receptors in the small intestine. This would in turn imply that the binding of different lectins to different cell surface carbohydrate receptors in the small intestine can result in evoking diverse biological responses. Sharma et al. (1996) have shown that lectins which possess different receptor binding specificities have quite different patterns of binding to cells of the follicle-associated epithelium, villus epithelium and gut-associated lymphoid tissue.

Based on the observations described above we suggest the following sequence of events after the oral intake of mistletoe lectins: 1) MLs bind strongly to the gut mucosa, 2) binding of MLs to lymphocytes of Peyers patches, 3) limited endocytosis of MLs, 4) stimulation of cytokine release into the blood, 5) activation and release of splenic lymphocytes, activation of NK and macrophages, 6) production and release of anti-angiogenic factors, 7) reduced tumour vascularisation, 8) reduced availability of nutrients for tumour growth and oxygen supplies decline, 9) cytotoxic effects exerted on tumour cells, 10) induction of apoptosis resulting in tumour cell death ultimately leading to a reduction in tumour mass.

In summary the extremely promising results obtained using both European and Korean mistletoe lectins on tumour cells *in vitro* and tumours *in vivo* suggest that the use of these molecules should be attractive in establishing a novel form of cancer treatment. Taking into consideration the situation of the cancer patient the oral ingestion of an anti-cancer preparation with an apparent lack of side-effects, would provide an excellent alternative to conventional anti-cancer treatment such as chemotherapy which is very often associated with major discomfort. A combinatorial regime should be considered in which oral mistletoe lectins are given as an adjuvant as soon as possible after a biopsy-proven tumour has been diagnosed, and then continued during, and after, chemotherapy or radiotherapy.

Mistletoe preparations have the unfortunate reputation of being toxic. This is due to the presence of molecules in the mistletoe plant that can cause acute nausea when ingested. These molecules can be easily removed from mistletoe extracts enabling a lectin-enriched preparation to be taken orally without causing any undue side-effects. Hall et al. (1986) reviewed data on a total of 318 cases of mistletoe ingestion received by the FDA poison control case reporting system between 1978 and 1983 (n=177) and 1984 (n=141). The majority remained asymptomatic and no deaths were recorded. Krenzelok et al. (1997) examined the outcome of 1,754 cases of accidental intake of mistletoe and did not find any signs of toxicity. It is thus evident that mistletoe has an unfounded reputation of being classified as toxic following oral intake. Indeed when presented orally to rats ML-1 was shown to have a growth stimulatory role on the crypt cells of the small intestine in rats and mice (Pusztai et al., 1998; Pryme et al., 2002). It is evident that many who have voiced derogatory opinions on the oral use of mistletoe lectin-containing preparations should re-examine their standpoint.

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