Biological Effects of Natural and Recombinant Mistletoe Lectin and an Aqueous Mistletoe Extract on Human Monocytes and Lymphocytes In Vitro

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Mistletoe lectin is thought to constitute the active principle in extract preparations from mistletoe, which are widely used as immunomodulators in adjuvant tumor therapy. However, no study exists which compares the immunological potency of different well-defined mistletoe lectin preparations on human immune cells. Therefore, in the present study the biological effects of an aqueous mistletoe extract, standardized for mistletoe lectin I (eML), the isolated natural mistletoe lectin (nML), and the recombinant form of this lectin (rML) on human peripheral blood monocytes and lymphocytes were compared with respect to cell viability and cytokine induction. After 48-hr incubation of peripheral blood mononuclear cells (PBMC) with rML, nML, and eML, a continuous concentration-dependent decrease in cell viability was found with an IC_{50} of about 3 ng/ml for rML and nML and 10 ng/ml for eML, respectively. This effect also was seen when isolated lymphocytes and monocytes were separately incubated with the lectin preparations. After incubation of PBMC and isolated monocytes of 5/10 blood donors with eML, an increase in cell viability was found at lectin concentrations between 10 and 1,000 pg/ml. This

effect was not seen with the pure lectin preparations nML and rML. After 48-hr incubation of PBMC with rML, nML, and eML, induction of IL-1- β , TNF- α , IL-2, IL-6, and IL-10 but not IFN- γ was measured. For IL-1- β it could be shown that cytokine induction took place at a broad lectin concentration range (0.1–100 ng/ml). Cytokine levels varied greatly in the PBMC cultures of the different blood donors. When monocytes were separately incubated with eML, nML, and rML for 48 hr, high levels of IL-1- β were found. In contrast, in cultures of separated lymphocytes from the same donors only a minimal production of IL-1- β and no production of IFN- γ was found after incubation with rML, nML, and eML. It is concluded that there are quantitative differences in the immunomodulatory effects of the mistletoe lectin preparations on human monocytes and lymphocytes. Therefore, measurement of cell viability and cytokine induction may be a diagnostic laboratory tool to determine the immunological potency of various mistletoe preparations and may help to clarify the clinical benefit of therapies with these substances. J. Clin. Lab. Anal. 14:255–259, 2000. © 2000 Wiley-Liss, Inc.

Key words: mistletoe lectin; cytokine induction; immunomodulation; cytotoxicity; human lymphocytes and monocytes

For more than 70 years, mistletoe extracts (*Viscum album* L.) have been widely used as an alternative treatment in cancer therapy (1). Although abundant literature is accessible reporting beneficial effects of commercial mistletoe extracts on cancer patients, randomized controlled clinical trials have so far been lacking and no study has been presented that unequivocally documents the anticancer potential of these preparations (2,3). Consequently, the clinical application of mistletoe preparations must be considered as a therapy modality with unproven antitumor efficacy (4). Reliable evaluation of the effects of extract preparations is seriously hampered by the fact that their composition is complex and markedly depends on the different methods of preparation. Therefore, the use of standardized extracts or defined substances, which can be reproducibly tested in vitro, has been repeatedly postulated.

Among the numerous constituents of mistletoe extracts, the galactoside-specific mistletoe lectin I has been identified as the biologically most active substance. Not only does it possess potent toxicity but it also triggers immunomodulatory responses (5–7). It consists of two types of chains, linked by a disulfide bond. The B chain confers the lectin activity to the dimer, whereas the A chain acts as a ribosome inactivator. It catalyzes the selective hydrolysis of the N-glycosidic bond at the adenine-4324 residue in the eukaryotic 28 S ribosomal

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RNA, resulting in inhibition of protein biosynthesis and toxicity of the holotoxin (8). The structure of the A chain has been determined (9), and its homology with other ricin-like plant toxins has been demonstrated (10–12).

Prompted by the necessity of evaluating defined mistletoe preparations for clinical trials, the present study was designed to determine and compare the cytotoxic and immunomodulatory effects of a standardized mistletoe extract and pure mistletoe lectin in its natural and recombinant form on the immune cells of different healthy blood donors.

MATERIALS AND METHODS

Mistletoe Lectin and Antiserum

Recombinant mistletoe lectin (rML; batch no. 161296) was provided by Madaus AG (Köln, Germany). For preparation of rML, the A and B chains were expressed separately in *Escherichia coli* yielding insoluble inclusion bodies, which were dissolved in 6 M Gu-HCl and refolded to soluble protein. By renaturation and association of A and B chains, a disulfide-linked bioactive protein was generated with a MW of 55 kDa and 98.2% purity (13,14).

Natural mistletoe lectin (nML; batch no. 171194) was provided by Prof. Dr. U. Pfüller, University Witten/Herdecke, Germany. The nML was isolated from mistletoe through extraction of the dried cut plant with diluted acetic acid according to the method described by Eifler et al. (15). The nML was separated from other proteins by affinity chromatography on lactosyl Sepharose precipitated by ammonium sulfate and stored as suspension at 2–8°C.

The aqueous mistletoe extract (eML; batch no. 511126) was provided by Madaus AG. The content of active (galactose-specific) mistletoe lectin was 408 µg/ml. Fresh plant material of *Viscum album* L. without lignified twigs and without berries grown on poplar was extracted with water and filtrated to yield a sterile extract, which was stored at 2–8°C.

Anti-mistletoe lectin antiserum from goat was obtained from Prof. Dr. U. Pfüller. The serum contains antibodies against all mistletoe lectin isoforms.

Blood Donors

A group of 10 healthy subjects (laboratory staff and relatives) aged between 20 and 64 years served as blood donors. None of them was receiving concurrent drug treatment.

Isolation of PBMC, Monocytes, and Lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by centrifugation on a Ficoll Paque (Pharmacia, Uppsala, Sweden) density gradient. Mononuclear cells were collected from the gradient interphase, washed twice, counted, and checked for viability using trypan blue dye.

Untouched lymphocytes and monocytes from the PBMC were isolated with the monocyte isolation kit and the pan T

cell isolation kit (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Cell Cultures

Cells were cultured in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 50 U/ml penicillin (Biochrom), 50 µg/ml streptomycin (Biochrom), and 8% autologous plasma. Incubation of the cell cultures was performed at 37°C in a humidified atmosphere of 5% CO₂. For determination of viability, cells were cultured in 96-well culture plates (Nunc, Wiesbaden, Germany) at 1×10^5 cells in 250 µl medium. After 48 hr of culture, the viability test was prepared directly in these plates. For testing cytokine induction, cells were cultured in polystyrol tubes at 2×10^5 cells in 500 µl medium. After 48 hr of culture, the supernatant was removed to be assayed for cytokine levels.

Determination of Cytokines

Enzyme-linked immunoassays (ELISA) were applied for qualitative and quantitative determinations of the cytokines in the culture supernatants as described previously (16). These tests were based on the sandwich principle and performed in one step. Recombinant human cytokines were used as standards.

For determination of tumor necrosis factor α (TNF-α), the coating antibody was a monoclonal mouse-anti-human-TNF-α antibody (clone 6b) and the second, a peroxidase (pod)-coupled polyclonal rabbit-anti-human TNF-α antibody. (This ELISA had an assay range of 20–500 pg TNF-α/ml.)

In the interleukin 1-β (IL-1-β) ELISA, the first immobilized antibody was a polyclonal goat-anti-human-IL-1-β antibody. For the detection of bound IL-1-β, the pod-linked Fab-fragment of a polyclonal goat-anti-human-IL-1-β antibody was used (assay range of this ELISA was 100–1,000 pg IL-1-β/ml).

For determination of IL-2, microtiter plates were coated with two monoclonal mouse anti-human IL-2 antibodies (clone 3D5 and clone 7B1) and a third pod-linked monoclonal mouse anti-human IL-2 antibody (clone 13A6) was used for detection of bound IL-2 (assay range: 50–1,000 pg/ml IL-2).

For the IL-10 ELISA, microtiter plates were coated with the monoclonal mouse-anti-human-IL-10 antibody (clone ilt-3) and a second pod-linked monoclonal, mouse-anti-human-IL-10 antibody (clone ilt-9) was used for detection of bound IL-10 (assay range: 50–500 pg IL-10/ml).

For determination of the dimeric interferon-γ (IFN-γ), the same monoclonal mouse-anti-human-IFN-γ antibody (clone 69) was used for coating and detection (assay range: 50–1,000 pg IFN-γ/ml).

In the IL-6 test, the first immobilized antibody was a monoclonal mouse anti-human IL-6 antibody (clone 16). For the detection of bound IL-6, a pod-linked sheep-anti-human-IL-6 antibody was used (assay range: 100–1,000 pg IL-6/ml).

Determination of Cell Viability

The formation of a soluble formazan dye from the tetrazolium salt WST-I (Boehringer Mannheim, Germany) was used to determine the viability of PBMC as well as isolated monocytes and lymphocytes after treatment with the lectins. All supernatant except 90 μ l was removed from each well of the culture plate and 10 μ l WST-solution was added. The amount of dye, which is proportional to the amount of viable cells, was assayed at 450 nm in a microplate reader (Flow) immediately after addition and 60 min later.

RESULTS

Determination of Cell Viability in Cultures of PBMC and Isolated Monocytes and Lymphocytes

The direct cytotoxic action of rML, nML, and eML on human PBMC as well as on isolated lymphocytes and monocytes was determined using the sensitive colorimetric WST assay, based on the activity of mitochondrial dehydrogenases (17). The amount of soluble formazan dye formed correlates to both the cell numbers and the viability of the cells. In an attempt to simulate *in vivo* conditions, cells were cultured in medium supplemented with 8% autologous plasma. When the mistletoe lectin preparations were added to the PBMC cultures of 10 healthy donors at concentrations ranging from 0.3 pg/ml to 200 ng/ml, a dose-dependent decrease in cell viability was observed with rML, nML, as well as with eML. The IC_{50} was about 3 ng/ml for rML and nML and 10 ng/ml for eML.

Incubation of PBMC with eML showed an increase in cell viability in the cultures of 5/10 blood donors at concentrations between 10 and 1,000 pg/ml (Fig. 1). This effect was not seen with the pure lectins nML and rML and could not be inhibited with goat-anti-mistletoe lectin antiserum. Therefore it is suggested that this cell proliferation is due to a non-lectin component in the extract (data not shown).

Fig. 1. Concentration-dependent reduction of PBMC viability by eML (■), nML (▲), and rML (○) in a cell culture system supplemented with 8% autologous serum. Median values of 10 blood donors are shown.

After 48-hr incubation of isolated monocytes and lymphocytes with rML, nML, and eML a similar dose-dependent decrease in cell viability was seen as compared to the PBMC cultures. Cytotoxicity of the lectin preparations was somewhat stronger toward monocytes than lymphocytes (Fig. 2). An increase in cell viability at eML concentrations between 10 and 100 pg/ml was seen only in the monocyte but not in the lymphocyte cultures.

Measurement of Cytokine Production in Cultures of PBMC and Isolated Monocytes and Lymphocytes

After incubation of PBMC cultures with 1 and 10 ng/ml rML, nML, and eML for 48 hr, a measurable induction of the cytokines IL-1- β , TNF- α , IL-6, IL-10, and IL-2 but not IFN- γ was found. Cytokine levels varied in a broad range in the PBMC cultures of the different blood donors. Data are summarized in Table 1.

By measurement of the IL-1- β levels it could be shown that cytokine induction took place at a broad lectin concentration range (0.1–100 ng/ml). Peak IL-1- β levels were found at lectin concentrations between 1 and 100 ng/ml, the same range where a maximal decrease in cell viability was also seen (Fig. 3).

When monocytes were separately incubated with eML, nML, and rML for 48 hr, the highest levels of IL-1- β were found at lectin concentrations between 1 and 100 ng/ml (Fig. 4). In contrast, only a minimal production of IL-1- β and no production of IFN- γ was found in the lymphocyte cultures of the same blood donors after incubation with rML, nML, and eML.

DISCUSSION

There are several studies on the immunomodulative and cytotoxic activity of various mistletoe extracts (18–25). How-

Fig. 2. Concentration-dependent reduction of the viability of PBMC and isolated monocytes and lymphocytes of the same blood donor after incubation with eML at different concentrations (■) PBMC, (▲) monocytes, and (○) lymphocytes.

TABLE 1. Median cytokine levels in PBMC cultures of 10 blood donors stimulated with 1 and 10 ng/ml eML, nML, or rML for 48 hr

		IL-1- β (pg/ml)	TNF- α (pg/ml)	IL-2 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)
eML	10 ng/ml	2,640	312	55	78,445	0
	1 ng/ml	1,792	300	11	73,760	124
nML	10 ng/ml	1,264	156	51	25,630	0
	1 ng/ml	328	401	44	47,131	10
rML	10 ng/ml	316	143	31	17,390	0
	1 ng/ml	254	167	179	63,101	42

ever, to our knowledge, this is the first report that correlates the immunomodulatory effects of a standardized extract to those of the natural mistletoe lectin and its (non-glycosylated) recombinant form in a dose-dependent manner.

To simulate the *in vivo* situation, PBMC as well as the isolated lymphocytes and monocytes were incubated in a cell culture system supplemented with autologous plasma. This system has been previously shown to be optimal for cytokine induction (16). Additionally, a highly sensitive and reproducible method for cytotoxicity determination was used (17).

The current quantitative data provide evidence that there is no general difference between the natural and the recombinant forms of mistletoe lectin I with respect to cytotoxic effects on human PBMC. The similar IC_{50} values for nML and rML indicate that glycosylation does not affect cytotoxicity. The IC_{50} values of our study are clearly higher than those published for serum-free conditions. However, it is well known that cytotoxicity is reduced when medium is supplemented with serum (23). The cell death after lectin treatment has been shown to be quantitatively due to apoptosis at concentrations below 10 ng/ml in the 24-hr treatment interval (22).

In contrast to rML and nML, a different curve shape was observed after incubation of PBMC and isolated monocytes with eML. With this extract an increase in cell viability was

Fig. 3. Release of IL-1- β from PBMC after 48-hr treatment with eML (■), nML (▲), and rML (○). Human peripheral blood mononuclear cells were cultured with 8% autologous serum, and the concentrations of IL-1- β were measured in the culture supernatants by ELISA. Median values of 10 blood donors are shown.

Fig. 4. Release of IL-1- β after 48-hr treatment of PBMC (■), isolated monocytes (▲), and lymphocytes (○) of the same blood donor with eML.

seen at low concentrations, which seems to be due to a non-lectin component. This is in accordance with the data published by other authors showing leukocyte proliferation after incubation with an extract containing only a very small amount of mistletoe lectins (26,27). However, cell proliferation after incubation of PBMC with eML was seen only in the cultures of 5/10 blood donors, which may indicate a different response of the immune cells of different persons to the extract.

After incubation of PBMC with rML, nML, and eML not only cytotoxicity but also cytokine induction was measured at a comparable lectin concentration range. We found induction of the cytokines IL-1- β , TNF- α , IL-6, IL-2, and IL-10 but not of IFN- γ . The measured cytokine levels were higher after induction with eML and nML compared to rML and varied in a broad range in the PBMC cultures of the different blood donors. Induction of these cytokines also was found in the cultures of isolated monocytes but only to a minimal degree in the cultures of lymphocytes. This is in accordance with published data showing that mistletoe lectin binds to a much higher degree to monocytes than to lymphocytes. This different binding affinity has been related to a different cytokine-inducing effect (24,28). Additionally, it has been shown that a lymphocyte population did not respond to nML without costimulation (23). In contrast to our results, induction of IFN- γ by mistletoe extract has been demonstrated by Stein and Berg (29). However, in their study a fermented mistletoe extract with a very low content of lectins was used, and therefore IFN- γ induction may be due to a non-lectin component.

In summary, it can be stated that there is a quantitative difference between the biological effects of the tested mistletoe preparations on human immune cells, which can be determined *in vitro*. Measurement of cell viability and cytokine induction in PBMC cultures of cancer patients may be a diagnostic tool to determine the immunological potency of various mistletoe extracts and thus may help to clarify their role in adjuvant cancer therapy.

REFERENCES

1. Kleijnen J, Knipschild P. Mistletoe treatment for cancer. Review of controlled trials in humans. *Phytomedicine* 1994;1:255–260.
2. Hauser SP. Unproven methods in oncology. *Eur J Cancer* 1991;27:1549–1551.
3. Hauser SP. Unproven methods in cancer treatment. *Curr Opin Oncol* 1993;5:646–654.
4. Gabius HJ. Concepts of tumor lectinology. *Cancer Invest* 1997;15:454–464.
5. Gabius HJ, Gabius S, Joshi SS, et al. From ill-defined extracts to the immunomodulatory lectin: will there be a reason for oncological application of mistletoe? *Planta Med* 1994;60:2–7.
6. Bocci V. Mistletoe (*Viscum album*) lectins as cytokine inducers and immunoadjuvant in tumor therapy: a review. *J Biol Regul Homeostatic Agents* 1993;7:1–6.
7. Heiny BM, Beuth J. Mistletoe extract standardized for the galactose-specific lectin (ML-1) induces β -endorphin release and immunopotential in breast cancer patients. *Anticancer Res* 1994;14:1339–1342.
8. Endo Y, Tsurugi K, Franz H. The site of action of the A chain of mistletoe lectin I on eukaryotic ribosomes. The RNA *N*-glycosidase activity of the protein. *FEBS Lett* 1988;231:378–380.
9. Soler MH, Stoeva S, Schwamborn C, Wilhelm S, Stiefel T, Voelter W. Complete amino acid sequence of the A chain of mistletoe lectin I. *FEBS Lett* 1996;399:153–157.
10. Dietrich JB, Ribereau-Gayon G, Jung ML, Franz H, Beck JP, Anton R. Identity of the N-terminal sequences of the three A chains of mistletoe (*Viscum album* L.) lectins: homology with ricin-like plant toxins and single-chain ribosome-inhibiting proteins. *Anticancer Drugs* 1992;3:507–511.
11. Eschenburg S, Krauspenhaar R, Mikhailov A, Stoeva S, Betzel C, Voelter W. Primary structure and molecular modeling of mistletoe lectin I from *Viscum album*. *Biochem Biophys Res Commun* 1998;247:367–372.
12. Lee RT, Gabius HJ, Lee YC. The sugar-combining area of the galactose-specific toxic lectin of mistletoe extends beyond the terminal sugar residue: comparison with a homologous toxic lectin, ricin. *Carbohydr Res* 1994;254:269–276.
13. Eck J, Langer M, Möckel B, Witthohn K, Zinke H, Lentzen H. Characterization of recombinant and plant-derived mistletoe lectin and their B chains. *Eur J Biochem* 1999;265:788–797.
14. Eck J, Langer M, Möckel B, et al. Cloning of the mistletoe lectin gene and characterization of the recombinant A chain. *Eur J Biochem* 1999;264:775–784.
15. Eifler R, Pfüller K, Göckeritz W, Pfüller U. Improved procedures for isolation and standardization of mistletoe lectins and their subunits: lectin pattern of the European mistletoe. In: Basu J, Kundu P, Chakrabarti P, editors. *Lectins: biology, biochemistry, clinical biochemistry*, Vol. 9. New Delhi: M/S Willey Eastern Limited; 1993. p 144–151.
16. Elsässer-Beile U, von Kleist S, Gallati H. Evaluation of a test system for measuring cytokine production in human whole blood cell cultures. *J Immunol Methods* 1991;139:191–195.
17. Ishiyama M, Tomingaga H, Shiga M, Sasamoto K, Ohkura Y, Ueno K. A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biol Pharm Bull* 1996;19:1518–1520.
18. Jung ML, Baudino S, Ribereau-Gayon G, Beck JP. Characterization of cytotoxic proteins from mistletoe (*Viscum album* L.). *Cancer Lett* 1990;51:103–108.
19. Büssing A, Suzart K, Bergmann J, Pfüller U, Schietzel M, Schweizer K. Induction of apoptosis in human lymphocytes treated with *Viscum album* L. is mediated by the mistletoe lectins. *Cancer Lett* 1996;99:59–72.
20. Ribereau-Gayon G, Dumont S, Muller C, Jung ML, Poindron P, Anton R. Mistletoe lectins I, II and III induce the production of cytokines by cultured human monocytes. *Cancer Lett* 1996;109:33–38.
21. Ribereau-Gayon G, Jung ML, Frantz M, Anton R. Modulation of cytotoxicity and enhancement of cytokine release induced by *Viscum album* L. extracts or mistletoe lectins. *Anticancer Drugs* 1997;8:S3–S8.
22. Hostanska K, Hajto T, Weber K, et al. A natural immunity-activating plant lectin, *Viscum album* agglutinin-I, induces apoptosis in human lymphocytes, monocytes, monocytic THP-1 cells, and murine thymocytes. *Nat Immun* 1996;15:295–311.
23. Möckel B, Schwarz T, Zinke H, Eck J, Langer M, Lentzen H. Effects of mistletoe lectin I on human blood cell lines and peripheral blood cells. Cytotoxicity, apoptosis, and induction of cytokines. *Arzneim-Forsch/Drug Res* 1997;47:1145–1151.
24. Hostanska K, Hajto T, Spagnoli GC, Fischer J, Lentzen H, Herrmann R. A plant lectin derived from *Viscum album* induces cytokine gene expression and protein production in cultures of human peripheral blood mononuclear cells. *Nat Immun* 1995;14:295–304.
25. Hajto T, Hostanska K, Frei K, Rordorf C, Gabius HJ. Increased secretion of tumor necrosis factor α , interleukin 1, and interleukin 6 by human mononuclear cells exposed to β -galactoside-specific lectin from clinically applied mistletoe extract. *Cancer Res* 1990;50:3322–3326.
26. Stein G, Berg PA. Non-lectin component in a fermented extract from *Viscum album* L. grown on pines induces proliferation of lymphocytes from healthy and allergic individuals in vitro. *Eur J Clin Pharmacol* 1994;47:33–38.
27. Stein GM, Berg PA. Evaluation of the stimulatory activity of a fermented mistletoe lectin-I free mistletoe extract on T-helper cells and monocytes in healthy individuals in vitro. *Arzneim-Forsch* 1996;46:635–639.
28. Hajto T, Hostanska K, Fischer J, Saller R. Immunomodulatory effects of *Viscum album* agglutinin-I on natural immunity. *Anticancer Drugs* 1997;8:43–46.
29. Stein GM, Berg PA. Mistletoe extract-induced effects on immunocompetent cells: in vitro studies. *Anticancer Drugs* 1997;8:S39–S42.