Review

Oncopharmacological Perspectives of a Plant Lectin (*Viscum album* Agglutinin-I): Overview of Recent Results from *In vitro* Experiments and *In vivo* Animal Models, and Their Possible Relevance for Clinical Applications

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Introduction

An old goal of natural complementary medical therapy has been to aim at a long-term stimulation of natural resistance in order to restrain cancer progression or improve defective immunological conditions without toxic side effects. Mistletoe extracts were applied to a large number of cancer patients because of their modulatory effect on the natural immune system. By carefully removing lectins, an essential group of components, from mistletoe extracts, a significant reduction of their effectiveness on several cellular immune parameters could be observed *in vivo* (1). That is the reason why, for the last 14 years, biological research of mistletoe extracts has focused on lectins. Meanwhile, the quantitatively dominant lectin, *Viscum album* agglutinin (VAA)-I has become available in a recombinant form (rVAA). Other constituents of plant extracts such as viscotoxins (2,3), poly- and oligosaccharides (4), flavonoids (5,6), chitin-binding mistletoe lectin (7) and arginine have also been investigated in connection with the effects of mistletoe extracts on the host defense. However, little evidence has been found that these substances contribute to the effects of mistletoe *in vivo* (8).

Structural Properties of *Viscum album* Agglutinin (VAA)-I Are Important for the Biological Activity of Mistletoe Plant Extracts

So far, mainly the mistletoe lectins and their sugar-binding B-chain have been considered as responsible for the

immunomodulatory effect of mistletoe extracts (1). Mistletoe lectins are present in all mistletoe extracts in various concentrations. Lectins are sugar-binding proteins that are able to recognize and bind specifically the glycan part of glycoconjugates (such as glycoproteins, glycolipids, oligo- and polysaccharides) (9) (Fig. 1). Lectins are widespread in all living organisms. With regard to their physiological functions, however, there are still numerous uncertainties. An important characteristic property of lectins is their ability to agglutinate erythrocytes *in vitro*. That is why they are frequently called 'agglutinins' (e.g. phytohemagglutinin). For mistletoe lectins, a similar nomenclature is also used: *Viscum album* agglutinin (VAA). The lectins are classified according to their sugar specificity. This classification is based on the monosaccharide that causes the greatest inhibition of the lectin-induced agglutination of erythrocytes or the precipitation of carbohydrate-containing polymers.

With regard to antigenity and chemical structure, there are three similar lectins in mistletoe plants (10,11). The most important and most often investigated lectin in mistletoe extracts is the galactoside-specific VAA-I. As shown in Fig. 1, it consists of a cytotoxic A-chain with a molecular weight of 29 kDa and a carbohydrate-binding B-chain of 34 kDa that is responsible for its immunomodulatory efficacy. VAA-II (according to an alternative nomenclature mistletoe II), with galactoside as well as *N*-acetylgalactosamine specificity, and mistletoe III, with *N*-acetylgalactosamine specificity, could be degradation products of VAA-I in the plants themselves (11–14). At present, the evaluation of mistletoe II and III varies. Some teams have only found two groups of isolectin: galactoside-specific VAA-I and *N*-acetylgalactosamine-specific VAA-II (15). The structural analysis of VAA-I and its physical, chemical and biological characteristics reveal many similarities to the ricin molecule (11,16,17). The A-chain of VAA-I is a potent

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Figure 1. *Viscum album* agglutinin (VAA-I) consists of two chains. The A-chain (white) with a molecular weight of 29 kDa and with *N*-glycosidase activity is a potent ribosomal inactivator. The sugar-binding B-chain (green) with a molecular weight of 34 kDa is responsible for the immunomodulatory effect of the molecule. Red colored parts of the B-chain indicate the sugar-binding receptors. (The picture was kindly provided by Madaus Ag, Germany.)

ribosome inactivator. The carbohydrate-binding B-chain is responsible for the internalization of the lectin molecule (uptake into the cell). The B-chain binds terminal galactoside residues on the cell membrane, preferring certain confirmations (18). It is this chain that brings about the entrance of the whole lectin molecule into the eukaryotic cells. The A-chain, on account of its highly specific enzymatic efficacy, catalytically inhibits protein synthesis in the 28S subunit of rRNA (14,19–21). That is the reason why VAA-I, similarly to ricin, abrin (a lectin from the red seed of *Abrus precatorius*), modeccin and volkensin, belongs to the type II family of ribosome-inactivating proteins (RIPs) with numerous homologous structures (11,22). In addition to the type II RIPs with two chains, a large number of the single-chain type I RIPs, such as gelonin (a glycoprotein from the indian plant *Gelonium multiforum*) or trichosanthin (a cytotoxic protein from the roots of the Chinese drogue *Wangua Trichosantes kirolowii* and *cucumeroides*) have been described (22). They were isolated from a variety of phylogenetically independent plant species so that the RIPs (types I and II) obviously belong to an 'old' evolutionary development. Recently, the primary structure of

VAA-I was analyzed and a strong homology to ricin and abrin was found (23,24). The first cloning experiments for VAA-I were performed by H. Lentzen, J. Eck, A. Bauer and H. Zinke [European Patent, EP 075 1221 B1 (1995)]. Expression studies in *Escherichia coli* allowed the production of the functionally active recombinant A- and B-chains that were linked to an active hololectin. The recombinant VAA (rVAA) showed similar biological activity [cytotoxicity, RIP activity, induction of apoptosis, selective binding, release of cytokines and stimulation of natural killer (NK) function] to that found in the plant extract (VAA-I) (25–27).

Biological Activity of Mistletoe Lectin (VAA-I)

The biological efficacy of mistletoe lectin can be regarded basically as directly cytostatic as well as having an immunomodulatory effect. In cultures of human peripheral mononuclear cells (PBMCs), VAA-I can stimulate cytokine production as well as programmed cell death (apoptosis) in approximately the same concentration as *in vivo* (28–31). These effects are interesting because plant lectins often imitate endogenous lectins which can represent early mechanisms in the elimination of unknown cells showing altered sugar structure at the membrane.

In Vitro Experimental Evidence for Cytotoxic, Cytostatic and Apoptotic Effects

Is VAA-I treatment dose and time dependent in cell cultures?

If eukaryotic cells are incubated for 24 h in the presence of VAA-I, this lectin already causes cytotoxic effects in the picogram range as, for example, in the case of K562 (human erythroleukemia) cells or EL-4 (mouse thymoma) cells (3,28). In cultures of PBMCs. VAA-I also starts to have a cytostatic as well as a cytotoxic effect at concentrations above 10 ng/ml if incubated for 24 h (30). If the incubation time is shorter, this toxic limit is naturally higher. It could also be proved that the growth-inhibiting effect of mistletoe extracts and VAA-I in different cell cultures *in vitro* can be traced back to the induction of programmed cell death (apoptosis) (28,29).

When human peripheral blood lymphocytes (PBLs) were incubated for 24 h with VAA-I at a concentration ranging between 1 μg and 1 ng/ml, the flow cytometric analysis with propidium iodide (PI) in hypotonic buffer solution and the quantitative assessments of DNA fragments with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end-labeling (TUNEL) assay could confirm a dose-dependent VAA-I-induced apoptosis at concentrations above 10 ng/ml (32). Monocytic leukemia (THP-1) cells and thymocytes also showed apoptosis in the presence of VAA-I above 1 ng/ml. A 24 h incubation of PBLs with VAA-I above 10 μg/ml resulted in necrosis. The isolated A-chain caused similar apoptotic effects; the B-chain was ineffective. These results indicate that induction of inhibition of protein synthesis by the A-chain is

responsible for the apoptotic effect of the whole lectin molecule. PBLs showed variable sensitiveness to VAA-I-induced apoptosis: NK, CD19$^+$ > CD8$^+$ > CD4$^+$ cells (26). Activated lymphocytes (CD25$^+$, CD69$^+$ and HLA-DR$^+$ cells) were also more sensitive to lectin-induced apoptosis than non-activated cells (unpublished data). In different lymphocyte populations, selective modulation of Fas antigen by VAA-I indicates that Fas antigen-activated signaling can at least partially play a role in VAA-I-induced apoptosis (26).

What is the pathway of VAA-I-induced apoptosis?

In the early stage of apoptotic cell death, a phospholipid inversion takes place. The phosphatidylserine expression is ascertainable by annexin-V binding. Thus it could be found that VAA-I (100 ng/ml)-induced apoptosis changes into increasing necrosis after 48 h (32). In cultures of U937 promonocytes, VAA-I (30–100 ng/ml) causes an increased cytosolic Ca^{2+} concentration which, among other factors, is a sign of apoptosis (33). In addition, VAA-I could enhance the stimulating effect of histamine (H$_1$) and complement (C5a) on cytosolic Ca^{2+} concentrations that play an accelerating role in the regulation of apoptosis. In Jurkat leukemic T cells, it was demonstrated previously that VAA-I-induced apoptotis was linked to activation of caspase-8 but independent of death receptor signaling (34). Furthermore, mechanistic investigations with the genetically defined p53+/+ and p53−/− murine tumour cell system show that breakdown of the mitochondrial membrane potential and caspase-3 activation occurred in a p53-independent manner on treatment with rVAA. However, the rVAA-induced apoptosis leading to caspase-3-activation requires an apoptosis-associated factor-1 (Apaf-1)-dependent pathway (35). It could be shown that rVAA overcomes a high apoptotic threshold and cooperates with ionizing radiation in tumor cells that lack intact p53 and, therefore, may represent a novel therapeutic approach for the treatment of cancer.

Recently it was found that VAA-I is a potent inducer of human neutrophil apoptosis via caspase-3 activation (36). VAA-I alters the mitochondrial transmembrane potential and increases intracellular levels of reactive oxygen species (ROS). Furthermore, the decrease of the expression of the antiapoptotic Mcl-1 and caspase involvement in the degradation of cytoskeletal paxillin and vimentin proteins in VAA-I-induced neutrophil apoptosis have been described (37). Recently, induction of apoptosis in activated neutrophils by VAA-I above 100 ng/ml concentration and inhibition of lipopolysaccharide (LPS)-induced proinflammatory responses *in vivo* were demonstrated (38). These data may provide further clinical perspectives for future mistletoe therapy.

Not only lectins with RIP activity cause apoptosis. *Griffonia simplicifolia* 1-B$_4$ and wheat germ agglutinin (WGA) stimulate programmed cell death in cultures of different cell lines (39). With regard to the apoptotic effect of lectin–sugar interactions on the cell membrane, the question arises as to whether this is only an *in vitro* phenomenon or whether it has therapeutic relevance. Further *in vivo* animal experiments are necessary to answer these questions.

Investigations of Lectin-induced Gene Expression and Secretion of Proinflammatory Cytokines

Can the proinflammatory cytokine production be influenced?

The results of cytokine research with regard to mistletoe are almost exclusively from *in vitro* data that cannot be directly transferred to *in vivo* situations. *In vivo*, cytokines are active at very low concentrations in a complex network. *In vitro* and *in vivo*, effective immunomodulators can only bring about short-term changes in serum concentrations of cytokines and only to a very small degree (picogram range) (30). The investigation of lectin-induced proinflammatory cytokines was also important because cancer patients often show decreased inflammatory responsiveness. In 24 h culture of PBMCs, low and non-toxic lectin concentrations (with an optimum between 1 and 10 ng/ml) stimulate the release of proinflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α dose dependently. D-Galactose, a monosaccharide with the highest affinity for VAA-I, blocks TNF-α release competitively. Mannose that shows no affinity for VAA-I has no effect in comparable concentrations. These results confirm that sugar–protein interaction mediated by the sugar-binding B-chain is fundamental for the immunomodulating effect of VAA-I. An enhanced expression of TNF-α mRNA was induced in human monocytes and in macrophages from endotoxin-resistant (C3H/HeJ) mice, if these cells had been pre-incubated with VAA-I for 2 h (40). After 24 h incubation of human PBMCs with non-cytotoxic concentrations of VAA-I (10 and 1 ng/ml), the expression of mRNA was measured for a series of cytokines with the help of reverse polymerase chain reaction (rPCR) (31) (Fig. 2). VAA-I induced gene expression of IL-1α and β, IL-6, TNF-α, interferon (IFN)-γ, granulocyte–monocyte colony-stimulating factor (GM-CSF) and IL-10. In contrast, no expression of IL-2 and IL-5 could be found. Non-cytotoxic concentrations of other mistletoe lectins (II and III) also induced increased secretion of proinflammatory cytokines in monocytes isolated from peripheral blood (41).

Which subsets of leukocytes are activated after VAA-I priming in vitro?

So far, the investigation of mistletoe-induced cytokines leads to the assumption that monocytes are the most important site of origin. This hypothesis is supported by the fact (Fig. 3) that monocytes can bind fluorescencently labeled molecules of VAA-I with a considerably higher affinity than lymphocytes (31). Thus lectin–sugar interactions on the cell membrane of monocytes can play an important role in the proinflammatory effect of mistletoe extracts. In cultures of monocytic THP-1 cells, VAA-I increased the concentrations of inositol phosphatase and phosphatidylinositol, indicating lectin-induced signal transduction in monocytes (42). The preferential effect of mistletoe extracts on the natural immune system is not restricted only to monocytes. Granulocytes also show a higher affinity for VAA-I than lymphocytes (31). VAA-I was shown to bind
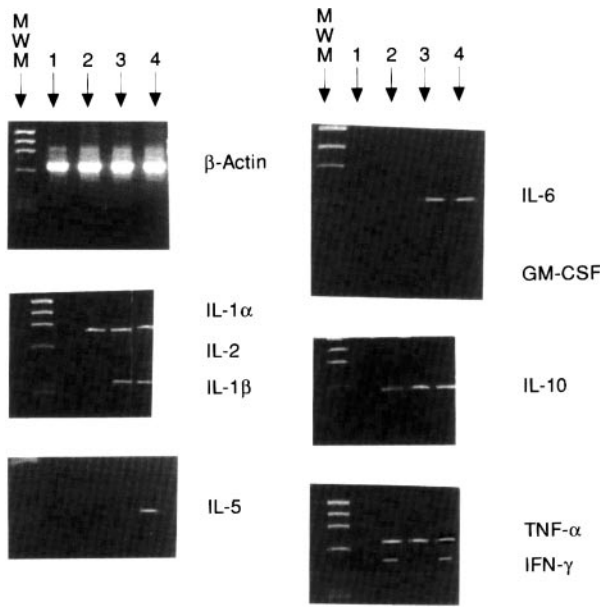
Figure 2. Cytokine gene expression in cultured PBMCs (31). After 24 h of culture in the absence (lane 1) and in the presence of 10 ng/ml (lane 2) or 1 ng/ml (lane 3) VAA-I and 1.5 μg/ml PHA (lane 4), total cellular RNA was extracted, reverse-transcribed and assayed in 35 cycles of PCR in the presence of the indicated pairs of primers. (The figure was kindly provided by S. Karger AG, Switzerland.)

preferentially to terminally α2–6-sialylated neolacto series gangliosides from human granulocytes (43). In cultures of lymphocytes, VAA-I increased the concentration of HLA-DR$^+$ lymphocytes and NK cells and induced gene expression of cytokines (31). When the ED$_{50}$ values of lectin-binding rates of different lymphocyte subpopulations were compared, the following sequence was found: NK, CD19$^+$ > CD8$^+$ > CD4$^+$ (26).

The *in vitro* ability of mistletoe extracts to stimulate proinflammatory cytokines was also used for the biological standardization of medicaments. In a skin model system, VAA-I (0.75–8 ng/ml) given in isolated form or in mistletoe extracts caused increased release of IL-1α and IL-6 dose dependently (44). In a model of multilayered keratinocytes, similar results were found (45). Proinflammatory cytokines play a significant role in the regulation of innate immunity. They can be at least partially responsible for mistletoe-induced immunomodulatory effects.

In addition, another member of the cytokine network, IL-12 that also regulates innate immunity, was investigated. In cultures of PBMCS, VAA-I increased the secretion of total IL-12 and its active p70 form (27). IL-12 is not only important for the well-known control of NK mechanisms, but it also seems to have a key position with regard to the regulation of the balance between cellular and humoral immunity (46) that may be altered as a consequence of many diseases, for instance advanced cancer (46,47). On the other hand, VAA-I could
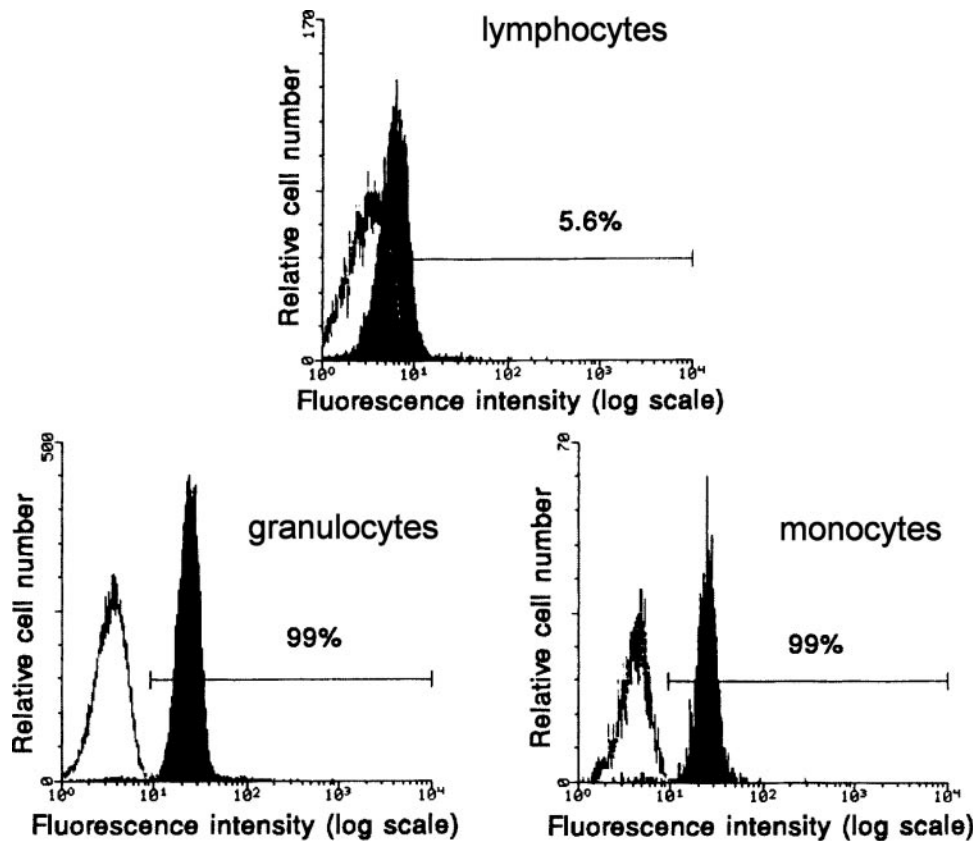

Figure 3. Binding of fluorescein isothiocyanate (FITC)-conjugated VAA-I to different leukocytes in lysed whole blood of one donor as a representative example. Percentages of positively stained lymphocytes, granulocytes and monocytes are shown as a shift towards the right in comparison with fluorescence obtained with control (31). The percentage of fluorescence-shifted cells is given in each histogram. As a negative control, unlabeled VAA-I at 100-fold concentration was used. (The figure was kindly provided by S. Karger AG, Switzerland.)

modulate the IL-15-induced neutrophil responses. A higher concentration of VAA-I (100 ng/ml) was found to reverse the ability of IL-15 to delay neutrophil apoptosis (48). On the basis of these results, *in vivo* model experiments may possibly pave the way for clinical application.

In Vitro Effects of VAA-I on Cellular Parameters of Innate immunity and on Hemopoietic Progenitor Cells of Bone Marrow

More than 15 years ago, the discovery was made that VAA-I and its B-chain stimulate the phagocytotic activity of human leukocytes (49). As mentioned earlier, monocytes and granulocytes show a higher affinity for VAA-I than lymphocytes (31). VAA-I induces a greater release of oxygen radicals from granulocytes than other lectins (50). The influx of Ca^{2+} ions plays a role in the O_2 formation of activated phagocytic cells. It was demonstrated that VAA-I stimulates the uptake of Ca^{2+} into granulocytes. These results support the possibility of a lectin-induced galactoside-specific activation of the biosignalization (51). VAA-I in combination with other cytokines *in vitro* is often more effective than the lectin alone. For example, VAA-I in combination with suboptimal concentrations of IL-2 and IL-12 induced an additive increase of NK cytotoxicity of human PBMCs or rat spleen cells against NK-sensitive target cells (27). These results were confirmed by other investigators (52) who found a synergism between IL-12 and VAA-I in the induction of lymphokine-activated killer (LAK) activity. Lectin-induced enhancement of IL-12 may indicate the selective activation of the Th1 pathway in dendritic cells derived from $CD16^-$ macrophages. In culture of hematopoietic progenitor ($CD34^+$) cells originating from bone marrow, VAA-I in combination with other hematopoietic growth factors [stem cell factor, IL-3, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF) and erythropoetin] also caused significantly increased proliferation in a synergistic manner (53).

In Vivo Effects of Mistletoe Extracts and VAA-I on Cellular Parameters of the Natural Immune System in an Animal Model, Healthy Volunteers and Cancer Patients

Characteristic dose dependency of VAA-I was found in different cell cultures *in vitro* (see above). The number and ratio of circulating lymphocyte subpopulations and their activation markers were investigated using different VAA-I concentrations. With regard to cellular immunological reactions *in vivo*, a bell-shaped dose–response curve of VAA-I and mistletoe extract could be observed (1,54–56) (Fig. 4). A single injection of pure VAA-I (0.25–1 ng/kg) into rabbits dose dependently enhances their temperature as well as the number and phagocytitic activity of granulocytes, the cytotoxic activity of NK cells and the number of large granular lymphocytes (LGLs) in peripheral blood (1). The maximum effect was found at 0.8 ng/kg body weight (1). In humans, the optimal effect was
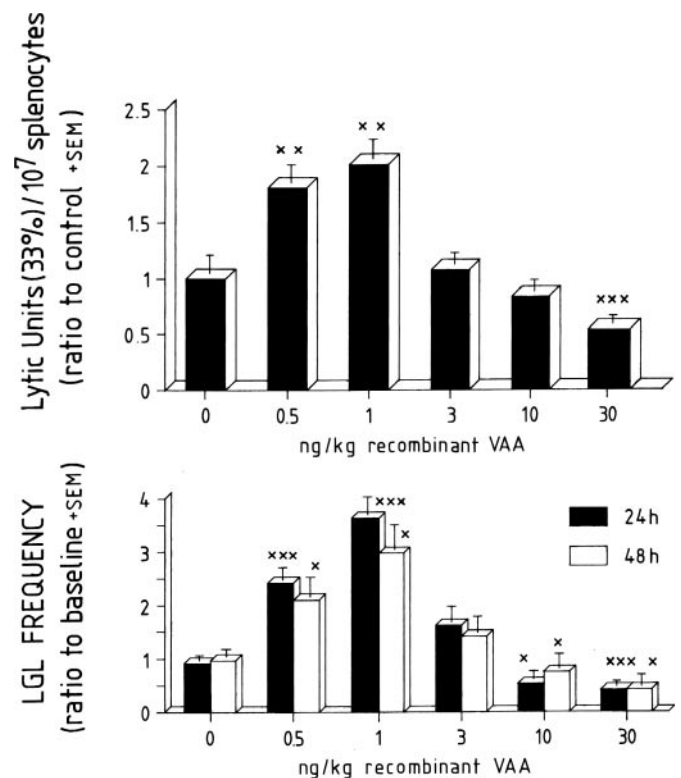

Figure 4. Immunological responses after a single i.v. injection of rVAA in rats (27). Six randomized groups each containing eight animals (Wistar rats) were treated once with placebo or with various doses of rVAA. Blood samples were collected before and 48 h after a single injection. After 2 days, all animals were sacrificed. Upper columns: NK-mediated cytotoxicity of splenocytes against YAC-1 cells (after 48 h) expressed as mean relative increase ($\pm$SEM) in the number of $10^7/LU_{33}$ of each group as ratios to the control. One LU_{33} is defined as the number of lymphocytes required for 33% lysis of 2×10^4 target cells extrapolated from regression line analysis of the specific cytotoxicity of four different effectors: target cell ratios (100:1, 50:1, 25:1 and 10:1). Thereafter, the number of $10^7/LU_{33}$ is calculated. Lower columns: mean relative enhancements ($\pm$SEM) in absolute counts of LGL in peripheral blood expressed as ratios to baseline values. Each value of the treated groups was compared with the control and statistically analyzed (*$P < 0.05$; **$P < 0.01$; ***$P < 0.002$).

within the range of 1 ng VAA-I/kg (57), this dose being far below the toxic limit. The 50% lethal dose (LD_{50}) for mice lies within a few hundred μg/kg (58). Experiments with mistletoe extracts standardized with regard to lectin activity suggest that an immunological stimulation induced by an optimal lectin dose (1 ng VAA-I/kg) can only be repeated after 3 days without therapy (57). In rats, recombinant mistletoe lectin (rVAA) also showed a bell-shaped dose–response relationship when the activity and frequency of NK cells in the blood were investigated after a single injection of various doses (27) (Fig. 4) Similar results were published recently with Argentine mistletoe *Ligaria cuneifolia* applied in a murine model (59).

In the case of cancer patients, subcutaneous injections of mistletoe preparations with a lectin dose of 1 ng VAA-I/kg twice a week led to an elevation of cytotoxic activity and frequency of peripheral NK cells ($CD3^-/CD16^+56^+$) and

LGLs (56). In addition, an increase in peripheral lymphocytes, T cells and Th cells, enhanced expression of CD25$^+$ and HLA-DQ$^+$ activation markers, and increased concentration of acute phase proteins and of complement factor C3 could be observed (60–63). In view of the lack of controlled clinical investigations on the immunomodulating efficacy of VAA-I and mistletoe extracts that were indispensable for further clinical trials, we carried out four randomized crossover double-blind pilot studies with healthy volunteers. For the first and second study, the lectin preparation was isolated from mistletoe extracts. The effect of this concentrated lectin preparation on different lymphocyte subpopulations (CD3$^+$, CD4$^+$, CD8$^+$, CD3$^-$/CD16$^+$56$^+$, CD3$^-$CD25$^+$, CD3$^-$CD69$^+$ and CD3$^-$HLA-DR$^+$) and the cytotoxic activity of NK cells was measured in the peripheral blood of nine and eight persons, respectively.

In contrast to significant lectin-induced increases in the number of lymphocytes and LGLs in animal models, healthy persons did not show any significantly different reactions with regard to the lymphocyte subpopulations mentioned above or NK activity with the same lectin concentration as compared with saline controls (64). However, when comparing mistletoe-induced reactions with the pre-treatment values, the increases in concentration and activity of the NK cells were found to be significant only after lectin application. Because of the considerable intrinsic variations of these parameters after placebo treatment, further randomized crossover double-blind pilot studies with six and eight healthy persons, respectively, were made with a parameter that could be assessed more rapidly following the injection. In addition, VAA-I freshly isolated from the plant was given to diminish the negative effect of a possible lectin instability originating from the commercial extract.

The priming of the granulocytes was tested 5 h after the injections. In both studies, a significant increase in the priming of granulocytes 5 h after the injection of purified VAA-I was found as compared with placebo controls (Table 1). As mentioned above, an immunological stimulation induced by an optimal lectin dose can only be obtained again after 3 days without therapy (57). As a consequence of the results with low doses, the question arises as to whether a regular application of the immunologically active low-dose extracts twice a week can lead to a long-term increase in the cellular parameters of innate immunity. Various independent observations were able to confirm this suggestion (65,66).

Cancer patients often show a correlation between clinical progress, quality of life and responses of the cellular parameters of the natural immune system. Heiny and Beuth assessed the plasma level of β-endorphin together with several immune parameters during the immunologically optimized mistletoe treatment of cancer patients. Significant correlations were found between the β-endorphin level, the mistletoe lectin-induced immunological reactions and the clinical progress (67,68). As an endogenous opioid, β-endorphin levels in plasma correlated with well-being and relief of pain in these patients.

In Vivo **Effect of VAA-I on Proliferation and Apoptosis of Murine Thymocytes**

Can we also detect apoptotic effects of VAA-I in vivo*?*

In a recent study, the short- and long-term *in vivo* effects of VAA-I on thymocyte subpopulations and peripheral T cells were tested using a murine (Balb/c) model (69). The changes of thymocyte subpopulations: CD4$^-$CD8$^-$ double negative (DN), CD4$^+$CD8$^+$ double positive (DP), CD4$^+$ or CD8$^+$ single positive (SP) and mature peripheral T cells were monitored after a single or repeated injections with 1 and 30 ng/kg VAA-I. A single injection of different doses of VAA-I did not cause significant alterations in the absolute thymocyte cell count or in the DN, DP and CD4$^+$ cell number (Table 2). Only the CD8$^+$ thymocyte number increased significantly. In the long-term trial, Balb/c mice were treated with the same doses of VAA-I lectin ± dexamethasone (DX) twice a week for 3 weeks. At 72 h after the last injections, the total thymocyte cell count in the thymus increased significantly after both lectin doses. As demonstrated in Table 2, with the exception of CD4$^+$ cells, all investigated thymocyte subpopulations (DN, DP and CD8$^+$ cells) increased significantly after long-term treatment with 30 ng/kg VAA-I. A dose of 1 ng/kg lectin also caused an increase in all cell populations, but significant growth could be measured only in the CD8$^+$ thymocyte population, indicating that CD8$^+$ thymocytes in both short- and long-term studies were found to be more susceptible to lectin-induced proliferation in the thymus (69).

How does the relationship between glucocorticoids and VAA-I affect murine thymocytes?

Since it is well known that DX causes considerable reduction of the thymocyte count, the effects of VAA-I treatment on short (24 h) and long-term (twice a week, for 3 weeks) DX (1 mg/kg body weight) therapy were also investigated in parallel to the lectin-induced alterations. As expected, DX treatment alone induced a significant reduction in the total number of thymocytes in both cases. This DX-induced reduction of

Table 1. Double-blind crossover studies in healthy volunteers

	First trial (n = 8)		Confirmatory study (n = 6)	
	Placebo	VAA-I	Placebo	VAA-I
Relative increases in priming of PMN				
5 h	0.92 (0.25)	1.94** (0.59)	1.04 (0.31)	3.56** (1.33)
24 h	0.85 (0.29)	1.32* (0.43)	0.8 (0.69)	1.84* (0.88)
LGL/ml				
0 h	656 (163)	540 (173)	639 (205)	555 (204)
24 h	472 (137)	1052* (364)	578 (196)	1131* (395)

Average increases (95% confidence intervals) of oxidative reactions of circulating polymorphonuclear neutrophils (PMNs) were assessed 5 and 24 h after a single injection of 1 ng VAA-I/kg body weight or of salt solution in comparison with pre-treatment values. The absolute number of LGLs in peripheral blood was also tested.
Statistical analysis was carried out between the treated group and the placebo group with the help of a paired *t*-test (*$P < 0.05$; **$P > 0.01$).

Table 2. Effect of a single dose and long-term VAA-I and DX treatment on thymocyte subpopulations $\times 10^6$ (SEM)

Thymus	Control	VAA 1 ng	VAA 30 ng	VAA 1 ng +DX	VAA 30 ng +DX	DX
Single dose						
DN	2.4 (0.4)	3.0 (0.5)	4.7 (0.1)	1.6 (0.4)	1.8 (0.6)	1.2 (0.1)*
DP	117.8 (7.5)	97.1 (1.3)	104.5 (13)	64.4 (14)	55.7 (13.0)	48.1 (5.4)*
CD4$^+$	12.5 (1.4)	12.7 (1.7)	10.5 (1.2)	9.0 (1.7)	8.2 (2.4)	5.4 (0.2)*
CD8$^+$	4.9 (1.3)	8.4 (1.2)*	7.1 (1.7)	7.0 (2.8)	6.9 (2.9)	2.3 (0.03)*
Long-termtreatment						
DN	3.2 (0.7)	3.5 (0.2)	4.7 (0.24)*	4.8 (1.6)#	3.0 (0.8)#	0.9 (0.3)*
DP	48.8 (14.7)	69.7 (15.9)	101 (18.0)*	54.4 (16)#	53.7 (16.7)#	10.2 (1.5)*
CD4$^+$	7.2 (1.4)	9.4 (0.9)	10.6 (2.1)	7.6 (2.0)#	6.3 (1.8)#	2.2 (0.3)*
CD8$^+$	2.3 (0.3)	3.6 (0.08)*	4.4 (0.1)*	3.8 (0.9) #	5.0 (3.0)	0.9 (0.3)*

The average absolute numbers ($\pm$SEM) of double negative (DN), double positive (DP), CD4$^+$ and CD8$^+$ cells are compared after various treatments.
*$P < 0.05$ in comparison with a negative control; #$P < 0.05$ comparing lectin groups with positive control animals that were treated with dexamethasone (DX) alone.

thymocyte cell count was significantly less if DX was injected in combination with VAA-I. As shown in Table 2, all investigated thymocyte subpopulations (DN, DP and SP) showed significant elevation if DX was combined with VAA-I (69).

The apoptosis of the murine thymocytes was detected by flow cytometry using PI and annexin V staining. At 24 h after a single injection of 30 ng/kg VAA-I, CD4$^+$ and CD8$^+$ SP thymocyte subpopulations showed 2- and 1.7-fold enhancements, respectively, in the frequency of apoptosis compared with negative control values. In the long-term trial, only 30 ng/kg VAA-I (72 h after the last injection of a treatment for 3 weeks) caused a significant increase (54%) in the percentage of apoptotic thymocytes.

Modulating the Effect of VAA-I on the Dexamethasone-induced Apoptosis and Glucocorticoid Receptor Level in Balb/c Thymocytes

In another recent study (70), the effect of VAA-I treatment on DX-induced apoptosis of thymocytes in Balb/c mice was tested. The number of early apoptotic cells was detected with annexin V staining while the late apoptotic cells were identified according to their PI incorporation into DNA using flow cytometry. The expression of glucocorticoid receptor (GCR) in DN, DP and CD4 or CD8 SP cell populations was assessed. The additive effect of lectin on DX-induced apoptosis of thymocytes consisted of two different actions of VAA-I and DX. A 1 day treatment with VAA-I caused enhanced apoptosis in SP mature cells, whereas the apoptotic effect of DX was directed mainly towards immature DN and DP cells (62). Treatment with 30 ng/kg VAA-I for 4 days elevated the GCR level (mean fluorescence intensity) in DP thymocytes (70). Lectin treatment for 21 days caused >20% elevation of GCR expression in all thymocyte subpopulations (DN, DP, CD4$^+$ and CD8$^+$). These results suggest that VAA-I may alter the sensitivity of thymocytes to glucocorticoids and this effect may play a role in the bell-shaped dose–response curve of the lectin-induced immunological effects.

Conclusions

With regard to the biological and preclinical research of mistletoe lectin, two essentially different effects must be considered: cytostatic/apoptotic and immunomodulatory effects. Both effects showed a very strong Gaus-type dose dependency. Low doses of VAA-I supported the T-lymphocyte differentiation and maturation, in contrast to the increased VAA-I dose both *in vitro* and *in vivo* in different experimental models. This basic biological effect may support a long-term therapeutic modulation of the natural immune system which is associated with a protective effect in combination with toxic modalities of various therapies and with improved quality of life.

Higher doses of VAA-I with cytostatic/apoptotic effects could suggest new perspectives to modulate the balance between cell growth and programmed cell death therapeutically. In addition, inhibition of proinflammatory responses by higher doses of lectin may provide further clinical perspectives in the future.

It could be shown that VAA overcomes a high apoptotic threshold and cooperates with ionizing radiation in tumor cells that lack intact p53. This may represent a novel therapeutic approach.

At present, it is difficult to judge of the clinical benefit of mistletoe lectin, and in many aspects it is not feasible, but growing evidence (71–81) suggests that VAA-I can improve the clinical situation of patients with a decreased responsiveness of the natural immune system. In addition, further experimental research is required to establish the favorable effect of lectin during the treatment of diseases in which programmed cell death is defective.

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