Dr. Hajtò Tibor:

Immunomodulatory effects of a plant lectin, Viscum album agglutinin-I (VAA-I).

Thesis

PTE-ÀOK Doktori Iskola B139 “Az Immunològia alapjai” alprogram

Alprogram vezetö: Prof. Dr. Németh Péter

Témavezetö: Dr. Berki Timea

PTE-ÀOK, Immunològiai és Biotechnologiai Intézet

P é c s

2003

Abbreviations I.

VAA-I	Viscum album agglutinin-I
LGL	Large granular lymphocytes
PBMC	Peripheral mononuclear cells
MHC	Major histocompatibility complex
NK cells	Natural killer cells
PMN	Polymorphonuclear leukocytes
M	Macrophages
DC	Dentric cells
TAM	Tumor associated macrophages
ADCC	Antibody dependent cell-mediated cytotoxicity
TH	T helper cells
IL	Interleukin
IFN	Interferon
TNF	Tumor necrosis factor
mo1DC	From macrophage 1 originated dentric cells
GC	Glucocorticoid
GCR	Glucocorticoid receptor
DX	Dexamethasone

Contents:

II.

Abstract

Natural (innate) immunity exhibits impaired basic activity and balance in many clinical situations but until recently the research into its important role in host defense mechanisms was particularly concerned with malignant tumors. In tumor patients, a great number of cellular parameters of the natural immune system show decreased activity which are correlated with the progression of the disease. Therefore immunomodulatory treatments should aim at a regular improvement of the innate immune functions and in this manner support the effect of other therapy modalities. Research into a plant lectin, Viscum album agglutinin (VAA)-I is a good example of this aim. VAA-I belongs to the type II ribosome inactivating proteins and it has been shown to induce both cytotostatic/apoptotic and immunomodulatory effects in cultures of various eukaryotic cells in vitro. In 24h culture of human peripheral lymphocytes, a dose-dependent VAA-I-induced apoptosis between 10 ng/ml and 1 μg/ml lectin concentration was established. After 24h incubation of peripheral blood mononuclear cells [PBMC] with non-cytotoxic concentrations (1 ng/ml -10ng/ml), VAA-I induced mRNA expression and enhanced secretions of proinflammatory cytokines. It was also found that the binding of FITC-conjugated VAA-I to monocytes and granulocytes were higher than to lymphocytes. Natural killer (NK)-cells were stimulated by VAA-I in vitro in an additive manner which was enhanced by combining it with IL-2 and IL-12. In cultures of PBMC and bone marrow CD34+ cells, coincubation of VAA-I with various hematopoietic growth factors induced a dose-dependent increase in clonogenic growth. In vivo, VAA-I is also able to stimulate the cellular parameters of natural immunity with a bell-shaped curve of efficacy. Studies in animal models confirm that application of 0.5-3 ng/kg VAA-I twice a week effectively sustains elevation in number and activity of peripheral blood NK cells. In healthy persons, these parameters often exhibit high intrinsic fluctuations, but blind crossover studies reveal an optimal lectin dose of about 0.5 and 1 ng/kg bw suggesting a potential use of

mistleoe lectin as a modulator of the natural immune system. Selective apoptotic effect of VAA-I may represent a novel approach for pharmacological manipulation of balance between cell growth and programmed death using mistletoe lectin. Recent studies demonstrate that VAA-I is able to modulate the maturation of thymocytes in vivo. In Balb/c mice, the total cell count of thymocytes showed significant increase after repeated injections of 1 or 30 ng/kg VAA-I and an elevated percentage of apoptotic cells was also found. Single positive (SP) thymocytes revealed higher increases in lectin-induced apoptosis than double negative (DN) or double positive (DP) cells. In addition, both lectin doses significantly inhibited the dexamethasone (DX)-induced reduction of all thymocyte subpopulations investigated. In addition, an additive effect of lectin on DX-induced apoptosis of thymocytes was also observed, VAA-I and DX having different activities. Treatment with 30 ng/kg VAA-I for four days elevated the GCR level (mean fluorescence intensity) in DP thymocytes. In conclusion, it can be established that VAA-I is an effetive immunomodulator but judgment of a possible clinical benefit of these results requires further preclinical and clinical research.

1.) Introduction

1.1. Several new aspects in research of natural (innate) immunity: why is it important for clinical research?

An old goal of the medical therapy of various clinical situations in which the immune system is defective has been to aim at a long-term stimulation of natural resistance and thus to attain a clinical benefit without toxic side effects. Since experimental and clinical medicine has often had difficulty to define the exact role of natural host defense mechanisms in a great number of diseases, these issues were often dealt with under the so-called “natural medicine”. However, the immunomodulatory treatments must not be a part of “alternative medicine”.

1.1.1. Definitions of natural immunity

It is well known that natural immunity is an essential part of the first and last line of host defense which can be rapidly mobilized and that the neuroendocrine system is an important

regulator of its activity [1]. Many years ago, the members of the natural immune system, such as polymorphonuclear neutrophils (PMNs), macrophages, natural killer (NK) cells, gamma-delta T lymphocytes, dendritic cells and CD5+ B lymphocytes were defined as cells that they can recognize cells lacking the surface expression of major histocompatibility complex (MHC)-class I molecules and can act non-specifically against various microorganisms and tumor cells. However, it was often forgotten that one of the most important characteristics of the natural immune system is a continuous regulation of the "basic" activity of their effector cells which may possibly prime these cells to determine their reactivity to "foreign" agents. The neuroendocrine system is regularly responsible for their responsiveness but various clinical situations can also induce a diminished basic activity of the natural immune system. For example, viral infections are able to inhibit several components of innate immunity [2] resulting in a viral persistence due to evasion or inactivation of its effector mechanisms. Surgical interventions and anesthesia can also lead to impaired functions of natural immunity [3-8]. Until recently, the role of altered mechanisms of innate immunity was studied particularly in relation to malignant tumors.

1.1.2. Natural immune system plays an essential role in influencing the immune responses towards a protective antitumor activity.

1.1.2.1. Pathological alterations in the functional activity of T cells infiltrating the tumor mass

T-cell chauvinism was so widespread [9] that the antitumor potential of natural effector cells continues to receive insufficient attention, and researchers have not yet fully considered the possibility of exploiting their functions as effective weapons against cancer. Growing evidence suggest that the tumor microenvironment is able to alter the functional activity of T cells infiltrating the tumor mass [10]. The interaction between the specific and general inflammatory reaction and its relation to prognosis of cancer patients was often studied. It was shown that although a large specific anti-tumor response reflected by T cells, protects against

distant metastases, their effect on patient survival and local recurrence are less important than the effects of the nonspecific inflammatory response [11]. In addition, defects in the major histocompatibility complex (MHC) class I antigen-processing machinery (APM) have been described in tumors of different histology. Murine data suggest that defects in the MHC class II APM might also be associated with malignant transformation of human cells [12]. Malignant transformation of melanocytes may also be associated with changes in the expression of the major histocompatibility complex (MHC) HLA class I antigens [13]. HLA expression is frequently altered in tumors compared to the tissue from which they originate. Given the central role of MHC products in the restriction of T-cell recognition, regulation of tumor HLA expression might be a strategy for the evasion of immune surveillance by the malignant cells as these HLA defects regularly increase with invasion or progression of tumors [14]. Thus, the abnormalities of the antigen-processing machinery in tumor cells can contribute to their escape from recognition by T cells.

1.1.2.2. Role of nonspecific inflammatory effector cells in anti-cancer reactions

Natural killer (NK) cells, the most evolutionary developed effector cells of innate immunity, are implicated in various host defense reactions. In contrast to T lymphocytes, NK cells can sense whether cells have lost the surface expression of major histocompatibility complex (MHC)-class I molecules. In recent years there has been remarkable progress in our understanding of the molecular mechanism regulating natural killer (NK) cell function [15]. The discovery of MHC-class I-specific inhibitory receptors clarified the basis for this discrimination and elucidated the nature of the 'off' signal. However, the receptors responsible for the 'on' signal during natural cytotoxicity remained mysterious. [15]. Recently, an apoptosis-inducing ligand was described which is a type II membrane protein belonging to the TNF family and plays a critical role in the NK cell-mediated suppression of tumor growth [16].

It is generally accepted that NK cells are implicated in immune surveillance against tumor development but the sensitivity of tumor cells to NK cytotoxicity is different. The spectrum of the NK susceptibility to various tumor targets is in many cases greater if NK cells are activated to a higher degree. Among the numerous regulating factors IL-2 and IL-12 appear to be the most important NK stimulators [17-18].

Although in most solid cancer types the emphasis is placed on the presence of T lymphocytes as a specific anti-cancer reaction [19-21], there is also a role for the nonspecific inflammatory reaction since the presence of NK cells [22-23], macrophages [24-26], mast cells [27-29], neutrophilic and eosinophilic granulocytes [28-31] as well as dentritic cells [32] have prognostic value in various solid cancer types. In a clinical study, the interaction between the specific and general inflammatory reaction and its relation to the prognosis of patients with rectal cancer was evaluated. It was observed that although specific anti-tumor responses mediated by T cells protect against distant metastases, their effects on patient survival and local recurrence are less important than the effects of the nonspecific inflammatory response [11]. Consequently, it was thought that the peritumoral presence of the nonspecific infiltrate is mainly involved in local control of the tumor process, as elucidated by the clear relation between inflammatory reaction and local recurrence. In addition, the nonspecific infiltrate is indirectly linked to the prevention of distant metastases by interactions with the specific response, as well as by the improved local control [11]. Results in animal models are in agreement with these finding showing that T-cells are unnecessary or only play a minor role in Cop rat resistance to hepatocarcinogenesis and that the nude parental strain is also likely to be resistant to the growth of preneoplastic liver lesions [33]. In some studies in humans, infiltration of tumors by leukocytes has been associated with a favorable prognosis [34-36]. Polymorphonuclear neutrophils (PMNs) are the most abundant circulating blood leukocytes. They provide the first-line of defense against infection and are potent effectors of inflammation. Nevertheless, various studies have suggested that PMNs are active in

immunosurveillance against several tumors [37-40]. Tumor destruction on the part of activated PMNs is achieved through their release of a variety of factors (cytokines, enzymes, chlorinated oxidants, etc) whose effects include direct tumor killing, extracellular lysis, inhibition of angiogenesis and activation of other reactive cells, resulting in NK cell and antibody-dependent cytotoxicity [41]. A further mechanism of PMN-mediated tumor cell killing is antibody-dependent cell-mediated cytotoxicity (ADCC) [42-43]. Granulocyte-macrophage-CSF (GM-CSF) augments the normal PMN ADCC of melanoma, neuroblastoma, and colorectal cancer cells [44-46]. The crucial importance of PMNs was made clear when their selective depletion abolished the rIL-12-induced antitumor effect [41]. Whether PMNs also play a role in IL-12 therapy of human cancer has still to be determined.

1.1.2.3. Impaired basic functions (priming) of natural immune system in tumor disease.

There is growing evidence that the basic activity of various effector cells of innate immunity diminishes in parallel with the progression of the tumor disease This tumor-induced reduction of priming of phagocytic cells is not clearly understood. A great number of experiments have tried to find an answer to this phenomenon. For example, an absolute requirement of Mac-1 for FcR-mediated PMN cytotoxicity toward tumor targets is well documented. Mac-1 receptor, an important activation marker, shows a diminished expression on phagocytic and NK cells due to a tumor-induced immune suppression. Mac-1 (-/-) PMNs exhibit defective spreading on Ab-coated targets, impaired formation of immunologic synapses, and lack of tumor cytolysis [47]. When the effect of malignant glioma cells on the maturation of dendritic cells (DCs) was investigated, it was found that IL- 12 production by DCs was inhibited by coculture with glioma cells. However, proinflammatory stimulation restored the production of IL- 12 by both human and mouse DCs. These data suggest that tumor cells can suppress the maturation of DCs [48]. Abnormal differentiation of DCs is an important factor in tumor escape from immune-system control. A close association between expression of linker histone H1(0) and DC differentiation in vitro has been found. DC production in H1(0) -deficient mice

was decreased significantly [49]. Tumor-derived factors considerably reduced H1(0) expression in hematopoietic progenitor cells. Thus, H1(0) histone may be an important factor in normal DC differentiation and tumor-derived factors may inhibit DC differentiation by affecting H1(0) expression [49].

Soluble factors produced or induced by malignant cells can be responsible for depressed basic activity of the natural immune system. In a clinical trial, the functional properties of mononuclear phagocytes isolated from ascitic fluid from patients with peritoneal carcinomatosis (PC) was investigated and compared with peripheral monocytes of patients and healthy control volunteers [50]. The results of this study showed that, contrary to peripheral blood monocytes where phagocytic function was not altered, tumor-associated macrophages (TAM) had impaired phagocytic activity. Moreover, dilutions of crude supernatant from short-term cultures of these peritoneal cells caused a significant, dose dependent inhibition in phagocytosis of control peripheral blood monocytes and peritoneal macrophages (PEM), indicating that a soluble factor(s) plays a prominent role in this phenomenon. In addition, it was also found that TAM produce fourfold lower levels of NO than PEM from control subjects [50]. These data support the hypothesis that depressed TAM function may contribute to the mechanisms of tumor escape from immune destruction. Figure 1 and 2 demonstrating some examples originate from previous results of the author [117-118].

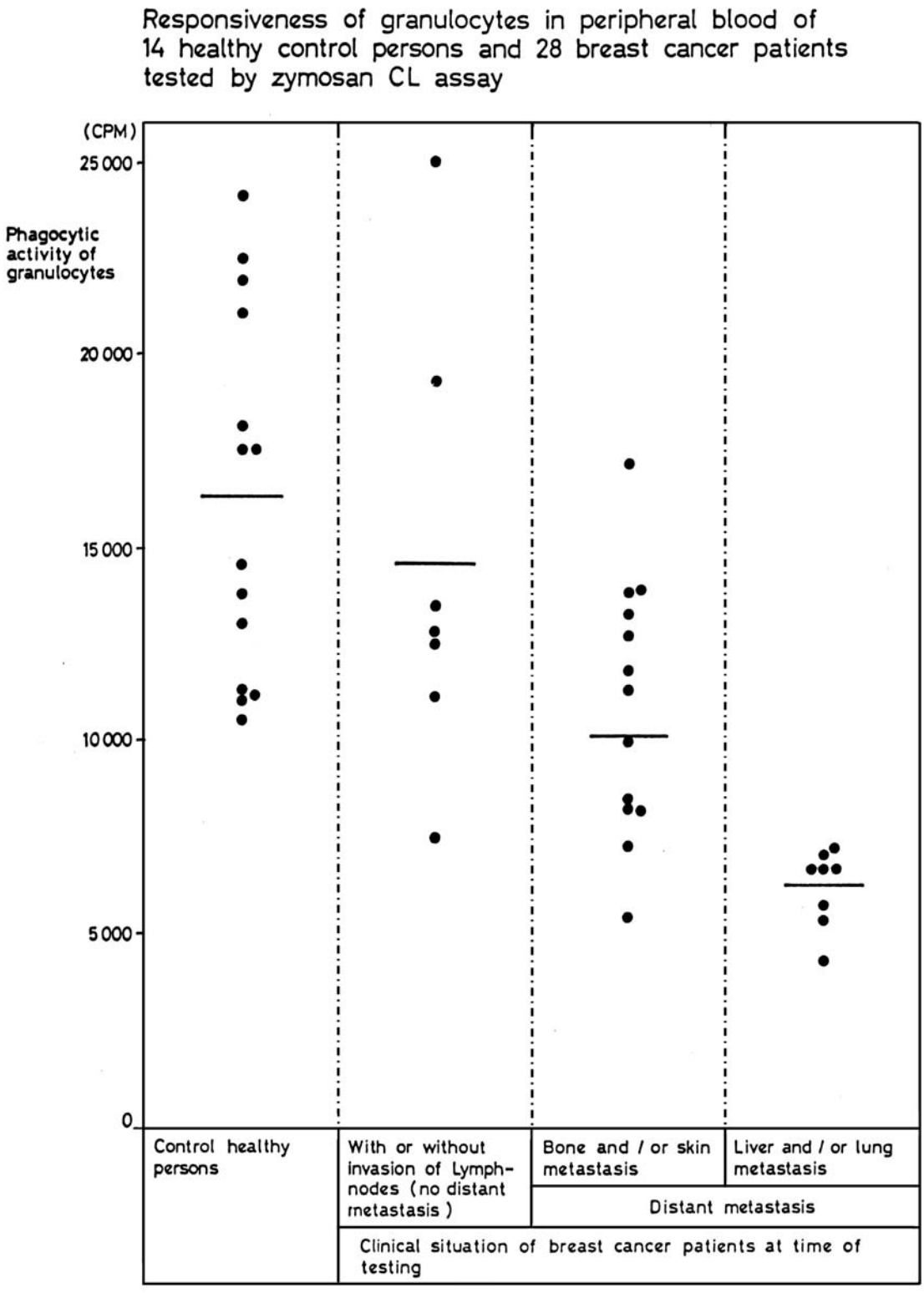


Figure 1. Priming of granulocytes in peripheral blood of 14 healthy control persons and 28 breast cancer patients tested by chemiluminescence assay during zymosan phagocytosis. The values are expressed in counts per minute (cpm). Each point represents one person and the bare represent the mean values for each group. The examinations of the patients were carried out during therapy-free intervals [118].

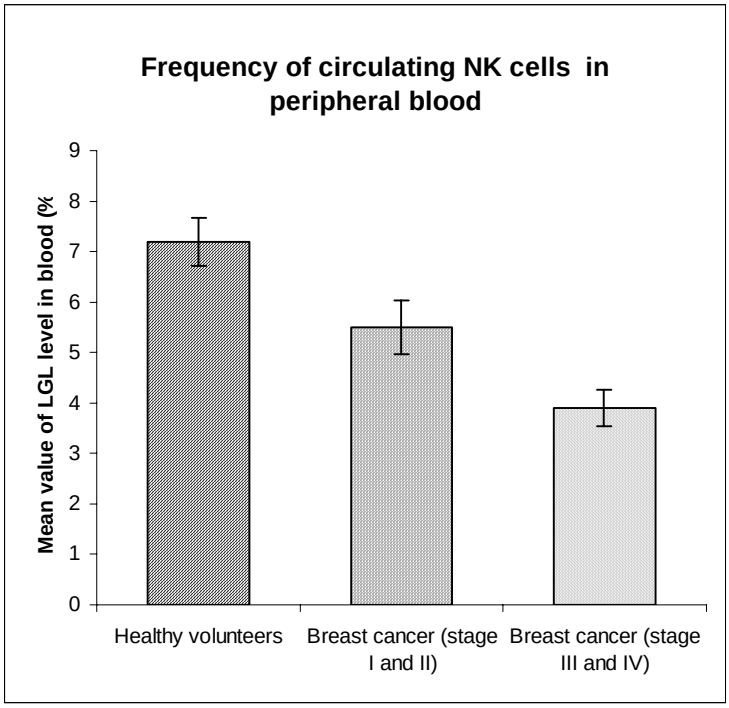


Figure 2. Mean frequency of circulating NK (Large granular lymphocytes = LGL) cells in blood of 56 healthy persons, 31 breast cancer patients with stage I or II disease and 42 patients with stage III or IV disease. The differences between the subgroups are significant [117].

1.1.2.4. A modulation of the natural immune system is still not an established treatment in cancer immunotherapy and some undesired side effects must also be taken into consideration. It is generally accepted that interactions between the immune system and malignant cells play an important role in tumorigenesis. Failure of the immune system can represent mechanisms by which tumor cells escape from immune-mediated rejection. Many of these mechanisms are now known on a cellular and molecular level [51]. Despite this knowledge, cancer immunotherapy is still not an established treatment in the clinic. It is also well established that cancer is a progressive disease, occurring in a series of well-defined steps, arising typically as a consequence of activating mutations (oncogenes) or deactivating mutations (tumor suppressor genes) in proliferating cells.

An uninterrupted, induced activation of innate immunity as biomodulatory tumor therapy is not always beneficial or feasible. Persistent infections within the host induce chronic inflammation. Leukocytes and other phagocytic cells induce DNA damage in proliferating cells through their generation of reactive oxygen and nitrogen species that are produced normally by these cells in order to fight infection [52]. Unfortunately, these react to form peroxynitrite, a mutagenic agent [52]. Therefore a permanent inflammatory response can have dual effects on the progression of the cancer. On one hand, inflammatory cells are prognostically a good sign, probably by maintaining control due to elimination of tumor cells, while on the other hand overproduction of reactive species, cytokines and growth factors can provide a growth stimulating microenvironment for the cells. Another problem that arises is that each inflammatory stimulation initiates cascade mechanisms inducing counter-regulatory responses which can attribute to an permanent alteration of the homeostatic immune balance. It was established that natural immune cells, such as PMNs [53], macrophages [54]or dentritic cells [55] can also modulate the balance between humoral and cell-mediated immunity by contributing to the promotion of a TH1 or TH2 response. Two distinct macrophage populations (M1 and M2) regulate $CD4^+$ T cells. M1 and M2 cells exert opposite effects on Th-cell development. M1 cells generate IL-12 which facilitates the development of Th1 cells, whereas M2 cells generate IL-10 which facilitates the generation of Th2 cells. Th1 cells release IFN-gamma, which stimulates M1 cells and inhibits M2 cells, whereas Th2-produced IL-10 inhibits M1 cells and promotes the generation of M2 cells. Through the release of IL-1 and TNF-alpha, M1 functions as a proinflammatory macrophage, whereas M2 cells, by virtue of IL-10 production, act as anti-inflammatory macrophages [54]. Interestingly, tumor patients can have up to 40% more M2 peripheral monocytes which in healthy individuals represent only 10% of blood monocytes [55]. If DCs derived from M1 CD16(-) monocytes or from M2 CD16(+) monocytes (mo1DC and mo2DC) were compared, similar differences were found [55]. CD16(-) mo1DC cells stimulated with proinflammatory agents expressed increased

levels of IL-12 p40 mRNA and secreted greater amounts of IL-12 p70 than CD16(+) mo2DC, whereas levels of transforming growth factor-beta1 mRNA were higher in CD16(+) mo2DC. Moreover, CD4(+) T cells stimulated with CD16(+) mo2DC secreted increased amounts of IL-4 compared to those stimulated by CD16(-) mo1DC [55]. These data demonstrate that both moDCs are not equivalent, suggesting either that they reach different stages of maturation during culture or that the original monocytes belong to cell lineages with a distinctly different ability to differentiate.

Therapeutic modulation of innate immunity requires a long-term support of M1/Th1 functions which are responsible for the basic activity of cellular host defense. However, it must be taken into consideration that persistent stimulation of M1/Th1 lineage is not possible and therefore persistent inflammatory reactions often lead to an alteration of balance towards M2 development. Furthermore, if the immonomodulatory therapy is not optimized, it can cause undesirable side effects.

The study of the plant lectin, Viscum album agglutinin (VAA)-I represents a new model to look for an answer to these problems.

1.2. A review of previous research with the plant lectin (VAA-I) as effective biomodulator.

1.2.1. Mistletoe extracts have been used as immunomodulators for more than 70 years.

Mistletoe extracts have been given to a large number of cancer patients because of their modulatory effect on the natural immune system. By carefully removing lectins, an essential component of mistletoe extracts, a significant reduction of their effectiveness on several cellular immune parameters could be observed in vivo [56]. That is the reason why, for the last fourteen years, the biological research of mistletoe extracts has focused on lectins. The quantitatively dominant lectin, Viscum Album Agglutinin (VAA) - I is also now available in a recombinant form (rVA A). Other components of the plant extracts such as viscotoxins [57-58], poly- and oligosaccharides [59], flavonoids [60-61], membrane lipids [62] and arginin [63] have also been discussed in connexion with the effects of mistletoe extracts on the host

defense. So far, however, little evidence has been found that support the contribution of these substances to the effects of mistletoe in vivo.

1.2.2. Structural properties of Viscum album agglutinin (VAA)-I as active substance in mistletoe extracts.

So far, mainly the mistletoe lectins and their sugar-binding B-chain have been considered to be responsible for the immunomodulatory effect of mistletoe extracts [56]. In all mistletoe extracts, mistletoe lectins are present in various concentrations. Lectins are sugar-binding proteins that are able to recognize and bind the glycan part of glycoconjugates (such as glycoproteins, glycolipids, oligo- and polysaccharides) specifically [64]. Lectins are widespread in all living organisms. However, with regard to their physiological functions, much is still unknown. An important characteristic property of lectins is their ability to agglutinate erythrocytes in vitro. That is why they are frequently called "agglutinins“, (for example phythemagglutinin). For mistletoe lectins, a similar nomenclature is also used i.e. Viscum album agglutinin (VAA). The lectins are classified according to their sugar specificity. This classification is based on the monosaccharide that can cause the greatest effect in the lectin-induced agglutination of erythrocytes or in precipitation of carbohydrate containing polymers.

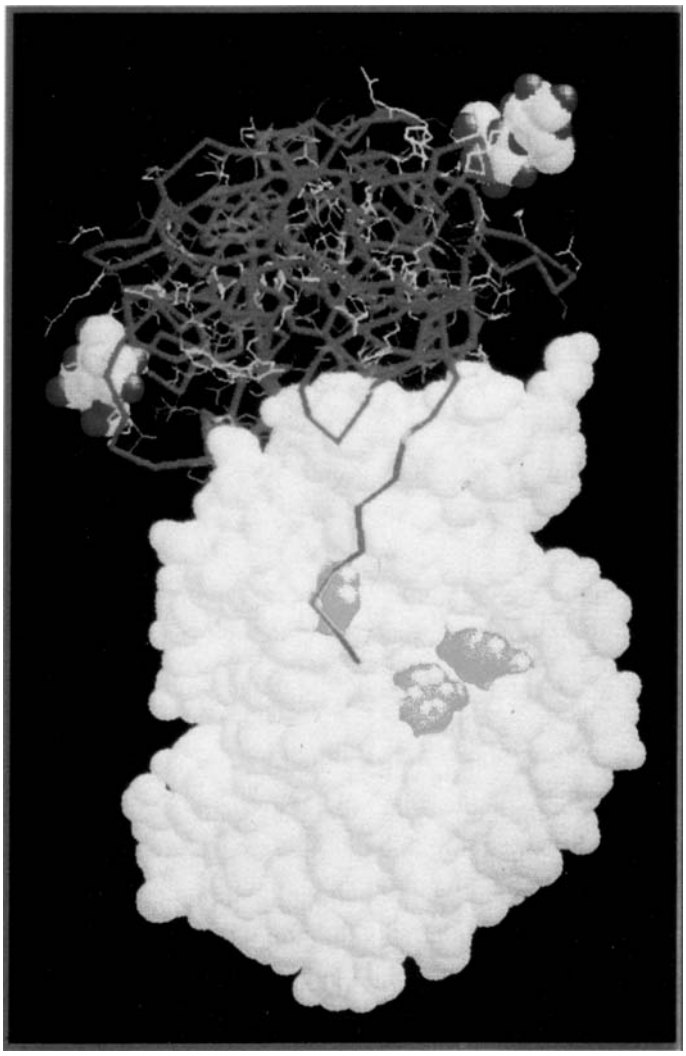


Figure 3. The structure of VAA-I. The bright part represents the ribosome inactivator A chain with enzymatic active site. The dark part shows the B chain with two galactoside-binding receptors which is responsible for its immunomodulatory efficacy (A kind gift from Madaus AG, Cologne, Germany).

With regard to antigenity and chemical structure, there are three similar lectins in mistletoe plants [65-66]. The most important and most often investigated lectin in mistletoe extracts is the galactoside-specific VAA-I. It consists of a cytotoxic A-chain with a molecular weight of 29 kd and a carbohydrate binding B-chain of 34 kd that is responsible for its immuno-modulatory efficacy. VAA-II, or mistletoe II, with galactoside as well as N-acetylgalactosamine specificity and mistletoe III with N-acetylgalactosamine specificity could also be degradation products of VAA-I in the plants themselves [66-69]. At present, the evaluations of mistletoe II and III are not in agreement. So far, some teams have only found

two groups of isolectin: galactoside-specific VAA-I and N-acetylgalactosamin-specific VAA-II [70]. The structural analysis of VAA-I and its physical, chemical as well as biological characteristics reveal many similarities with the ricin molecule [66, 71-72]. The A-chain of VAA-I is a potent 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 [73]. It is this chain that brings about the entrance of the whole lectin molecule into eukaryotic cells. The A-chain, on account of its highly specific enzymatic efficacy, catalytically inhibits protein synthesis in the 28S subunit of rRNA [69, 74-76]. That is the reason why VAA-I, similar to ricin, abrin (lectin from the red seed of abrus precatorius), modeccin and volkensin, belongs to the type II family of ribosome inactivating proteins with numerous homologous structures [66, 77]. In addition to the type II of RIP with two chains, a large number of the one-chained type I of RIP, such as gelonin (glycoprotein from the indian plant: gelonium multiforum) or trichosanthin (cytotoxic protein from the roots of the chinese drogue: wangua trichosantes kirolowii and cucumeroides) have been described [77]. They were isolated from a variety of phylogenetically independent plant species so that the RIPs (type I and II) obviously belong to an early evolutionary development.

During the last few years, the primary structure of VAA-I has been analyzed. A strong homology to ricin and abrin was found [78-79]. The first cloning experiments for VAA-I were made by H. Lentzen, J. Eck, A. Bauer and H. Zinke [European Patent, EP 075 1221 B1 (1995)]. Expression in E. Coli resulted in 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 NK function) to the plant extract (VAA-I) [80-82].

1.2.3. Biological activity of mistletoe lectin (VAA-I)

From its biological activity, mistletoe lectin can be regarded as a direct cytostatic as well as an immunomodulatory agent. In cultures of human peripheral mononuclear cells (PBMC), VAA-I stimulates cytokine production as well as programmed cell death (apoptosis) at the same concentration [83-86]. These effects are interesting because plant lectins often imitate endogenous lectins which may represent old primitive mechanisms in that they eliminate cells which show altered sugar structure on the membrane.

1.2.3.1. Cytotoxic, cytostatic and apoptotic effects of VAA-I in vitro.

If eucaryotic cells are incubated for 24 h in the presence of VAA-I, this lectin can be cytotoxic in the picogram range as for example in K562 (human erythroleukemia) cells or of EL-4 (mouse thymoma) cells [83-84]. In cultures of human peripheral mononuclear cells (PBMC), VAA-I starts to have a cytostatic as well as cytotoxic effect above 10 ng/ml if incubated for 24h [85]. If incubation is shorter this toxic concentration is of course, higher. It could also be proved that the growth inhibiting effect of mistletoe extracts and VAA-I in different cell cultures in vitro is due to the induction of programmed cell death (apoptosis) [83-84]. When human peripheral lymphocytes (PBL) were incubated for 24 h with VAA-I in a concentration ranging between 1ng and 1 μg/ml, flow cytometric analysis with propdium 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 confirmed that VAA-I induced a dose-dependent apoptosis above 10 ng/ml concentration [87]. Monocytic leukemia (THP-1) cells and thymocytes also showed apoptosis in presence of VAA-I above 1 ng/ml concentrations. 24h incubation of PBL with VAA-I above 10 μg/ml caused necrosis [87]. The isolated A-chain caused similar apoptotic effects, the B chain was ineffective [87]. These results indicate that for the apoptotic effect of the whole lectin molecule, the A chain, which induces inhibition of protein synthesis, is responsible. PBL showed variable sensitiveness to VAA-I induced apoptosis: NK, $CD19^+$ > $CD8^+$ > $CD4^+$ cells [81]. Activated lymphocytes ($CD25^+$, $CD69^+$ and $HLA\text{-}DR^+$ 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 signalling can at least partially play a role in VAA-I-induced apoptosis [81]. In the early stage of apoptotic cell death, a phospholipid inversion takes place. The phosphatidylserin expression is ascertainable by Annexin-V binding. Thus it was found that VAA-I (100ng/ml) – induced apoptosis changes into increasing necrosis after 48h. In cultures of U937 promonocytes, VAA-I (30-100 ng/ml) causes increased cytosolic Ca^{2+} which, among other things, is a sign of apoptosis [88].In addition, VAA-I could enhance the stimulating effect of histamine (H_1) and complement (C5a) on cytosolic Ca^{2+} concentration that play an accelerating role in the regulation of apoptosis [88]. It is also suggested that caspase-3 is involved, at least in part in the apoptotic effect of VAA-I [89].

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 various cell lines [90]. With regard to the apoptotic effect of lectin-sugar interactions on the cell membrane, the question arises whether this is only an in vitro phenomenon or whether it has therapeutical relevance? Further preclinical and clinical research is necessary to answer these questions. The recent in vivo trials will reported in chapter 3.

1.2.3.2.Lectin-induced gene expression and secretion of proinflammatory cytokines in vitro.

The investigation of the effect of mistletoe extracts on the cytokine network can also be of great significance because it allows a better interpretation of its immunomodulatory efficacy. The results of cytokine research with regard to mistletoe have been obtained almost exclusively from in vitro data that cannot be directly applied to in vivo situations. In vivo cytokines are active in very low concentrations in a complex network. In vivo effective immunomodulators, such as mistletoe extracts standardized with regard to lectin content can only bring about short-term changes in serum concentrations of cytokines and only to a very small degree (picogramme range) [85]. Proinflammatory cytokines, such as interlekin (IL)-1,

tumor necrosis factor (TNF)-α and IL-6 in physiological concentrations can be antitumoral, antiviral, antimicrobial, antiautoimmune and radioprotective and can play an important role in regulation of innate immunity, cell growth, cell differentiation and in the regulation of neurovegetative functions [91]. However, higher and pathological levels of the proinflammatory cytokines can have dual effects which result in more risk than benefit [91].

In 24h culture of PBMC, low and non-toxic lectin concentrations (with an optimum between 1 and 10ng/ml) stimulate the release of IL-1, IL-6 and TNF–α dose-dependently [85]. D-galactose, a monosaccharide with the highest affinity to VAA-I, blocks TNF-α release competitively [85]. Mannose that shows no affinity to VAA-I, has no effect in a comparable concentration [85]. These results confirm that sugar-protein interaction mediated by the sugar-binding B-chain is fundamental to the immunomodulating effect of VAA-I. An enhanced expression of TNF-α mRNA was induced in human monocytes and in macrophages originating from endotoxin resistant (C3H/HeJ) mice, if these cells had been incubated with VAA-I for two hours [92]. After 24h incubation of human PBMC with non-cytotoxic concentrations of VAA-I (1 ng/ml and 10 ng/ml) the expression of mRNA was measured for a series of cytokines by the reverse polymerase chain reaction (rPCR) [86]. VAA-I induced gene expression of IL-1α and β, IL-6, TNF-α, interferon (IFN)-γ and granulocyte-monocyte colony stimulating factor (GM-CSF) but no expression of IL-2 and IL-5 could be found [86]. Non-cytotoxic concentrations of other mistletoe lectins (II-III) could also induce increased secretion of proinflammatory cytokines in monocytes isolated from peripheral blood [93]. So far, the investigation of mistletoe-induced cytokines leads to the asumption that monocytes are the most important site of origin. This hypothesis seems to be supported by the fact that monocytes can bind fluorescent labelled VAA-I molecules with a considerably higher affinity than lymphocytes [86]. Thus lectin-sugar interactions on the cell membrane of monocytes can play an important role in the proinflammatory effect of VAA-I. In cultures of monocytic THP-1 cells, VAA-I increased the concentrations of inositol phosphatase and

phosphatidylinositol indicating lectin-iduced signal transduction in monocytes [94]. The preferential effect of mistletoe extracts on the natural immune system is not restricted to monocytes. Granulocytes, also, show a higher affinity for VAA-I than lymphocytes [86]. In cultures of lymphocytes, VAA-I increased the concentration of HLA-DR^+ lymphocytes and NK cells and induced gene expression of cytokines [86]. When the ED_{50} values of lectin binding rates of different lymphocyte subpopulations were compared with each other, the following sequence was found: NK, $CD19^+ > CD8^+ > CD4^+$ [81].

The in vitro ability of mistletoe lectin to stimulate proinflammatory cytokines has also been used for the biological standardization of medicaments. In a skin model system, VAA-I (0.75 – 8 ng/ml) given in isolated form or as a mistletoe extract caused a dose-dependent increase in the release of IL-1α and IL-6 [95]. Proinflammatory cytokines play a significant role in the regulation of natural immunity. They can be responsible at least partially for mistletoe-induced immunomodulatory effects.

In addition, another member of the cytokine network, IL-12 that also regulates natural immunity, was investigated. In cultures of PBMC, VAA-I increased the secretion of total IL-12 and its active p70 form [82]. It is well known that I L-12 is important for the adjustment of natural killer mechanisms. In addition, it seems that it also plays a key role with regard to the regulation of the balance between cellular and humoral immunity which can be altered as a consequence of many diseases, for instance advanced cancer [96-97]. On account of such interactions this research is of particular clinical significance.

1.2.3.3. In vitro effects of VAA-I on cellular parameters of natural immunity and on hematopoietic progenitor cells of bone marrow.

More than 15 years ago, the discovery was made that VAA-I and its B-chain stimulate the phagocytic activity of human leukocytes [98]. As mentioned earlier, monocytes and granulocytes show a higher affinity to VAA-I than lymphocytes [86]. VAA-I induces a higher release of oxygen radicals from granulocytes than other lectins [99]. The influx of Ca^{2+} ions

plays a role in the O_2 formation of activated phagocytic cells. It was demonstrated that VAA-I stimulate the uptake of Ca^{2+} into granulocytes. These results support the possibility of a lectin-induced galactoside-specific activation of the biosignalling [100]. In vitro VAA-I in combination with other cytokines is often more efficient 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 PBMC or rat spleen cells against NK sensitive target cells [82]. These results were confirmed by other investigators [101] who have found synergism between IL-12 and VAA-I in the induction of lymphokine -activated killer (LAK) activity. In cultures of hematopoietic progenitor ($CD34^+$) cells from bone marrow, VAA-I in combination with other hematopoietic growth factors (stem cell factor, IL-3, G-CSF, M-CSF and erythropoetin) caused significantly increased proliferation also on synergism [102].

1.2.3.4. In vivo effects of mistletoe extracts and VAA-I on cellular parameters of natural immune system in animal model, healthy volunteers and cancer patients.

With regard to cellular immunological reactions in vivo a bell-shaped dose – response

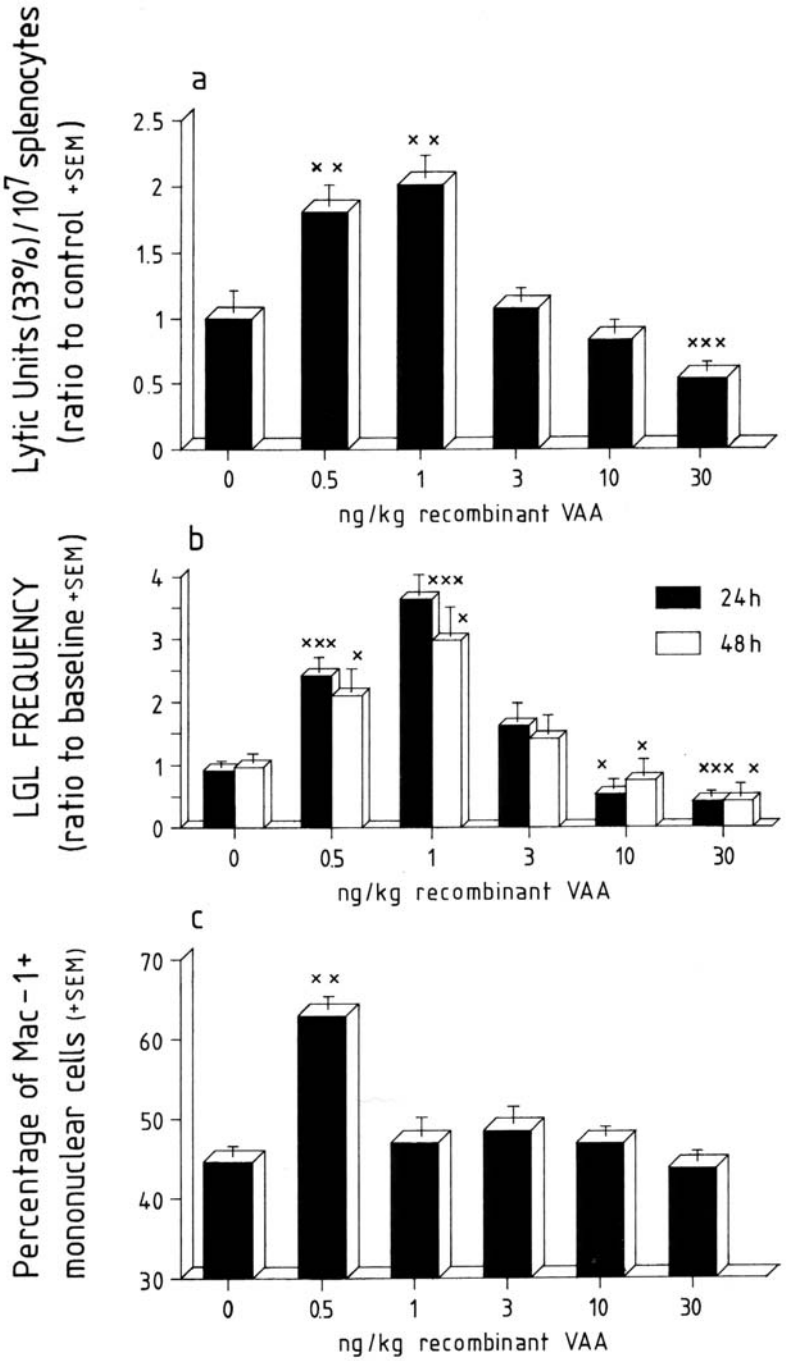


Figure 4. Immunological responses after a single intravenous injection of recombinant lectin (rVAA) in rats. Six randomized groups each containing 8 animals were treated once with placebo or with various doses of rVAA. Blood samples were collected before, 24h and 48h after a single injection. After two days all animals were sacrificed. **a**. NK-mediated cytotoxicity of splenocytes against YAC-1 cells expressed in mean relative augmentations [82]. **b**. Mean relative enhancement in absolute count of LGL (circulating NK cells) in blood expressed in ratios to baseline values. **c**. Mean percentage of CD11b+ (MAC-1+) mononuclear cells (monocytes and NK cells) in peripheral blood after 48h. Each value of treated groups was compared with control and statistically analyzed. *$p<0.05$; **$p<0.01$; ***$p<0.002$ [82].

relationship of VAA-I and mistletoe extract could be observed [56, 103-105]. A single injection of pure VAA-I (0.25-1 ng/kg) into rabbits enhances dose-dependently 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 (LGL) in peripheral blood [56]. The maximum effect was found at 0.8 ng/kg bw [56]. In humans, the optimal effect was within the range of 1 ng VAA-I/kg [106], a dose far below the toxic limit. The 50% lethal dose (LD_{50}) for mice lies in the region of a few hundred μg/kg [107]. Experiments with mistletoe extracts standardized in regard to lectin activity suggest that an immunological stimulation induced by an optimal lectin dose (1 ng VAA-I /kg) can only be initiated again after three days without therapy [106]. In rats, recombinant mistletoe lectin (rVAA) also shows a bell-shaped dose-respone relationship when activity and frequency of NK cells in the blood were investigated after a single injection (Fig.4)[82]. 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 ($CD3^-/CD16^+56^+$) and LGL cells (105). Increased numbers of peripheral lymphocytes, T cells and Th cells, enhanced expression of $CD25^+$ and HLA-DQ^+ activation markers, as well as higher concentrations of acute phase proteins and complement factor C3 could also be observed [108-111]. As there were no controlled clinical investigations of the immunomodulating efficacy of VAA-I respectively mistletoe extracts, we performed four randomized crossover double blind pilot studies with healthy volunteers. For the first and second study a lectin concentrate 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 in the peripheral blood of 9 resp. 8 persons was tested. In contrast to the significant lectin-induced increases in the number of lymphocytes and LGL cells in animal models, healthy volunteers did not show any significantly different reactions with regard to the lymphocyte

subpopulations mentioned above or NK activity after the same lectin concentrate or salt solution [112]. However, when comparing mistletoe-induced reactions with the pretreatment values, the elevations in concentration and activity of the NK cells were found to be significant only after the lectin concentrate [112]. Because of the considerable intrinsic fluctuations of these parameters after placebo treatment, further randomized crossover double blind pilot studies with 6 resp. 8 healthy persons were made using a parameter that could be assessed more rapidly following the injection and freshly isolated plant VAA-I was given to

Table 1. Double blind cross over studies in healthy volunteers

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

Average increases (95% confidence intervals) of oxidative reactions of circulating polymorphonuclear neutrophils (PMN) were assessed 5h and 24h after a single injection of 1 ng VAA-I/kg body weight or of salt solution compared with pretreatment values. The absolute number of LGL cells in peripheral blood was also tested. Statistical analysis was carried out between the treated group and the placebo group with the help of paired t test (*$p<0.05$; **$p>0.01$) [112].

diminish the negative effect of a possible lectin instability in the commercial extract. The priming of the granulocytes was tested five hours after the injections. In both studies

significant increases in the priming of granulocytes 5h after the injection of purified VAA-I was found as compared to placebo controls (table 1) [112].

As mentioned above, an immunological stimulation induced by an optimal lectin dose can only be obtained again after three days without therapy [106]. As a consequence of the results with low doses, the question arises as to whether a regular application of the immunologically active low-dosed extracts twice a week can lead to a long-term increase in the cellular parameters of natural immunity. Various independent observations were able to confirm this suggestion [113-114].

Cancer patients often show a correalation between clinical progress, quality of life and responses of 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 [115-116]. As an endogenous opioid, β-endorphin level in plasma correlated with well-being and relief of pain [116].

1.3.Recent in vivo studies of mistletoe lectin-induced effect on murine thymocytes.

1.3.1. In vivo effect of VAA-I on proliferation and apoptosis of murine thymocytes.

Because proinflammatory and apoptosis-inducing activity of VAA-I has been shown to occur in vitro in similar concentrations, it was interesting to investigate the in vivo effect of VAA-I on proliferation and apoptosis of murine thymocytes. For this reason, we have recently examined the short- and long-term in vivo effects of VAA-I on thymocyte subpopulations and peripheral T cells in a murine (Balb/c) model [119]. 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 was monitored after a either a single or repeated injections with 1 ng/kg and 30 ng/kg VAA-I.

1.3.2. Galactoside-specific misletoe lectin modulates the dexamethasone-induced apoptosis and glucocorticoid receptor level in Balb/c thymocytes.

Glucocorticoids (GC) play an important role in treatment of malignant diseases [126] and in the therapy of a great number of other disorders e.g. autoimmune diseases, rheumatological diseases, patients with tissue transplants etc. [127-128]. Since mature and immature T lymphocytes, important mediators of the immune response are highly influenced by GC therapy [128], investigation of the GC sensitivity of thymocytes is of clinical interest. It is known that T cells undergo rigorous selection processes in the thymus to prevent those with either autoreactive or nonfunctional T-cell receptors (TCRs) from entering the periphery [129-130]. GC hormones are also produced by the thymic epithelial cells [131] and are believed to play a role in T cell development and selection [132], although their precise mode of action is controversial. The difference in glucocorticoid sensitivity of mature and immature subpopulations of thymocytes can play an important role in both positive and negative selection steps depending on TCR-mediated signal [133]. It was also shown that among the thymocyte subpopulations, the most GC-sensitive double positive (DP) thymocytes have the lowest GCR level [134]. GCR-deficient thymocytes were resistant to dexamethasone (DX)-induced apoptosis, confirming the absence of glucocorticoid responsiveness [135]. However the absence of genomic GCR action had no impact on thymocyte development either in vivo or in vitro since T cell differentiation, including positive and negative selection, was normal as judged by the development of CD4+CD8+, αβTCR+CD4+, and αβ TCR+CD8+ thymocytes [136]. These results question whether there is a relationship between GCR expression and GC hormone action in different cell populations [137].

In our first study [119] we showed that in vivo administration of a low dose (30 ng/kg body weight) of galactoside-specific plant lectin, VAA-I influenced the thymocyte development and inhibited the Dexamethsaone (DX)- induced reduction of thymocyte cell number. Enhanced proliferation and selection of immature cell populations was the cause of the

elevated total thymocyte cell number [119]. In our second study the effect of VAA-I on DX-induced apoptosis and GCR expression of thymocytes was investigated in the same animal model. An additive effect on the total number of apoptotic cells with a higher GCR expression in the surviving cells was detected. VAA-I, that can cause an enhanced proliferation of immature cells, induced increased apoptosis of the mature single positive (SP) cell populations in contrast to DX which had the opposite effect [138].

1.4. Principle designs of the present investigation

1.4.1. Further examination of immunomodulatory effects of lectin (VAA-I).

1.4.2. Determination of lectin-induced proliferation and apoptosis of CD4+ and CD8+ mature- as well as of DN and DP immature thymocytes in vivo.

1.4.3. Investigation of the protective effect of VAA-I on GC-induced reduction of thymocyte number.

1.4.4. Study of the additive effect of VAA-I on GC-induced apoptosis in various thymocyte subpopulations.

1.4.5. Examination of effect of lectin on GCR expression of thymocytes

1.4.6. New interpretation of bell-shaped dose response curve of lectin efficacy on the basis of the present results.

1.4.7. Further promotion of lectin standardization and reproducibility for preclinical and clinical research.

2. Material and methods

2.1.Investigation of effect of VAA-I on proliferation and apoptosis of murine thymocytes.

As previously described [119], VAA-I was prepared from fresh aqueous extracts of the leaves and stems of mistletoe plants and purified on lactosylated agarose columns [82]. Endotoxin contamination in the samples used was less than 0.5 pg/ml, as determined by quantitative kinetic LAL assay. Three groups of three Balb/c mice were treated subcutaneously with placebo (phosphate-buffered saline) or with VAA-I and/or with DX using the following

doses: 1 or 30 ng/kg VAA-I alone, 1 or 30 ng/kg VAA-I combined with 1 mg/kg DX. As positive control, 1 mg /kg DX was given alone. In the first study, all animals were sacrificed 24h after one subcutaneous injection. In the second trial the subcutaneous injections were administered twice a week for three weeks. 72h after the last injection, all animals were sacrificed.

Thymus glands were removed and placed in ice-cold PBS. Thymus tissue was homogenized in a glass/glass homogenizer and the suspension filtered through a nylon mesh filter. The thymocytes were washed in PBS and the total cell number and viability were determined by counting in a hemocytometer using the trypan blue dye exclusion test. 1 x 10^6 thymocytes in 100 µl binding buffer (PBS/ 0,1%NaN_3/0,1%BSA) were labeled for the expression of CD4 CD8 for 30 min on ice. After 2 washing steps in PBS, the cells were stored in 500 µl 0,1%PFA/PBS buffer until flow cytometric analysis. Apoptosis and cellular DNA content were analysed after fixation with 4%PFA. Samples were kept in saponin buffer (0,1%saponin/0,1%BSA/0,1%NaN3 in PBS) and incubated with 100µg/ml of DNase-free RNase (Sigma) followed by 50µg/ml of propidium iodide (PI SIGMA P 4170) for 30 min at room temperature. The samples were analyzed in a FACS Calibur flow cytometer (Becton Dickinson, San Jose CA), using the CellQuest software. Thymocytes were gated on forward and side scatter plots according to their size and granularity. The gate determined by the untreated thymocyte sample was used for all further measurements. Thymocytes were gated according to their CD4 and/or CD8 fluorescence. To determine the apoptotic cell number using the detection of the DNA content of the cells, PI incorporation into DNA was examined in FL-2 channel of the flow cytometer using a linear amplification. In addition, Annexin V binding to membrane phosphatidylserine of various thymocyte subpopulations was also measured. The results were analyzed with Student's t test and U test according to Wilcoxon, Mann and Whitney using the Statgraphics statistical package for IBM-compatible computers.

2.2. Investigation of *dexamethasone-induced apoptosis and glucocorticoid receptor level in Balb/c thymocytes.*

VAA-I was prepared as described above (2.1). Dexamethasone (Oradexon, Organon) was purchased from N.V. Organon Oss Holland in ampoules containing 5 mg/ml. Apoptosis was detected with Annexin-V- FITC (Pharmingen) and propidium iodide (SIGMA P 4170). Anti-mouse-CD4-PE and anti-CD8-CyChr antibodies were purchased from Pharmingen, anti-GCR-FITC was prepared in our laboratory [139] and used for intracellular staining.

Balb/c mice (6 weeks old, body weight approximately 20 g +/-10%) were assigned at random to six groups of 3 animals each in both studies. Three animals/group were treated either subcutaneously with placebo (phosphate-buffered saline) or with VAA-I and/or with dexamethasone (DX) at the following doses: 1 or 30 ng/kg VAA-I alone, 1 or 30 ng/kg VAA-I combined with 1 or 2 mg/kg DX. The subcutaneous injections were administered twice a week. All animals were sacrificed after 24h, four days or three weeks.

The thymus glands were removed and placed on ice-cold PBS. Thymus tissue was homogenized in a glass/glass homogenizer and the resulting suspension was filtered through a nylon mesh filter. 1 x 10^6 thymocytes in 100 µl binding buffer (PBS/ 0,1%NaN_3/0,1%BSA) were labeled for the expression of CD4 and CD8 for 30 min on ice. After 2 washing steps in PBS, the cells were fixed with 4% paraformaldehyde (PFA)/PBS for 20 min, washed 2x in PBS and stained in saponin buffer (0,1% saponin, 0,1% NaN_3, 0,1% BSA) for intracellular GCR expression After 30 min incubation on ice, the cells were washed 2x in saponin buffer, 1x in binding buffer and stored in 500 µl 0,1%PFA/PBS buffer until flow cytometric analysis. For the detection of early apoptosis, cells were labeled with Annexin V-FITC for 15 min in Annexin buffer after CD4/CD8 staining [140]. For determination of late apoptosis, cells were fixed with 4%PFA. Samples were kept in saponin buffer and incubated with 100µg/ml of DNase-free RNase (Sigma) followed by 50µg/ml of propidium iodide (PI, SIGMA P 4170) for 30 min at room temperature. The cells were protected from light, prior to flow cytometric

analysis. The samples were analyzed in a FACS Calibur flow cytometer (Becton Dickinson, San Jose CA), using the Cell Quest software. Thymocytes were gated on forward and side scatter (FSC/SSC) plots according to their size and granularity. The gate determined by the untreated thymocyte sample was used for all further measurements. To determine the expression of GCR positive cells in double negative, double positive and CD4 or CD8 single positive populations two parameter dot plots showing cell surface CD4/8 staining were first created from the previous gate. Thymocytes were gated according to their CD4 and/or CD8 fluorescence and these populations were separately analyzed for GCR-FITC log fluorescence (FL-1). The fluorescence intensity of GCR staining was compared in different thymocyte subpopulations by overlaying the FL-1 histograms. To determine the total apoptotic cell number the PI incorporation into DNA was measured in FL-2 channel of the flow cytometer using a linear amplification. In addition, the early apoptotic cells after CD4/CD8 staining were estimated by Annexin V-FITC binding to membrane phosphatidylserine molecules of various thymocyte subpopulations. Statistical analysis was carried out as described above (2.1).

3. Results

3.1. Effect of VAA-I on thymocyte proliferation

In the first trial, double negative (DN), double positive (DP), CD4+ and CD8+ single positive (SP) cells in the thymus as well as CD4+ and CD8+ cells in peripheral blood of Balb/c mice were investigated 24h after a single lectin (VAA-I) injection. As shown in the first three columns of Figure 5A, a single injection of different doses of VAA-I did not cause significant alterations in the total thymocyte cell count or in the DN, DP and CD4+ cell number (Table 2A). Only the CD8+ thymocyte number revealed a significant ($p<0.0\ 25$) increase (70%) 24h after 1ng/kg VAA-I and 44% after 30ng/kg ($p>0.05$). Therefore, the CD4+/CD8+ ratio in thymus 24h after the two different lectin doses fell 39% and 38% ($p<0.01$; $p<0.01$ respectively) (Fig.6C, Table 2A). However, at the same time the CD4+/CD8+ ratio in

peripheral blood, elevated 74% after 1ng/kg VAA-I and 55% following 30ng /kg d ($p<0.005$; $p<0.01$) as illustrated in Fig. 6A.

In the second long-term trial, Balb/c mice were treated with the same doses of VAA-I lectin + DX twice a week for three weeks. 72h after the last injections the same investigations as after 24h were carried out. As the first three columns of Figure 5B show, the total thymocyte cell count in the thymus increased significantly after both lectin doses ($p<0.05$ and $p<0.025$, respectively). As demonstrated in Table 2B, with the exception of CD4+ cells all investigated thymocyte subpopulations (DN, DP and CD8+ cells) revealed significant elevation after long-term treatment with 30ng/kg VAA-I ($p<0.01$; $p<0.05$ and $p<0.0025$, respectively). 1ng/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.

3.2. VAA-I inhibited the Dexamethasone-induced thymocyte reduction

Since it is well known that DX causes considerable reduction in the thymocyte count parallel to the lectin induced alterations, we also investigated the effects of VAA-I treatment on short (24h) and long-term (twice a week, for three weeks) DX (1mg/kg BW) therapy. As expected, DX treatment alone in both cases induced significant reduction in total number of thymocytes ($p<0.05$) (4^{th} columns of Fig. 5A and 5B). This DX-induced reduction of thymocyte cell count was significantly less if DX was injected in combination with VAA-I (5^{th} and 6^{th} columns of Fig. 5A and 5B). As shown in Table 2B, all investigated thymocyte subpopulations (DN, DP, SP) showed significant elevation if DX was combined with VAA-I for three weeks.

Table 2.

A.Effect of single dose VAA-I and DX treatment on thymocyte subpopulations x 10^6 (SEM)

Thymus	**Ctrl**	**VAA 1ng**	**VAA 30ng**	**VAA 1ng +DX**	**VAA 30ng +DX**	**DX**
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)

B.Effect of long-term VAA-I and DX treatments on thymocyte subpopulations x 10^6 (SEM)

Thymus	**Ctrl**	**VAA 1ng**	**VAA 30ng**	**VAA 1ng +DX**	**VAA 30ng +DX**	**DX**
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)

Table 2. The average absolute numbers (+SEM) of double negative (DN), double positive (DP), CD4+ and CD8+ cells compared after various treatments. (*) indicate that in comparison with negative control a p value < 0.05 and ($^{\#}$) relates to statistical significance ($p<0.05$) when comparing lectin groups with positive control animals that were treated with dexamethasone (DX) alone.

3.3 Effect of VAA-I on apoptosis of murine thymocytes in vivo

In the first trial, 24h after a single injection of either 1ng/kg or 30 ng/kg VAA-I the percentage of apoptotic cells rose 1.9-fold and 2.2-fold ($p<0.07$ and $p<0.05$, respectively; see Fig 7A). This increase in apoptosis was higher than that caused by 1mg/kg DX treatment, suggesting a possible additive effect of VAA-I on DX. To investigate the initial phase of apoptosis in a more sensitive manner, the phosphatidylserine expression on the surface of thymocyte subpopulations by Annexin V binding was also determined. As shown in Fig 8, 24h after a single injection of 30 ng/kg VAA-I, CD4+ and CD8+ SP thymocyte subpopulations showed 2 fold and 1.7 fold enhancement in the frequency of apoptosis compared to negative control values ($p<0.05$ and $p<0.01$, respectively). In the second trial, only 30 ng/kg VAA-I (72h after the last injection of a treatment for three weeks) caused (see Fig. 7B) a significant increase (54%) in percentage of apoptotic thymocytes ($p<0.01$). The elevated number of DN and DP thymocytes showing increased apoptosis induced by VAA-I may be a reflection of the more rapid proliferation and enhanced maturation (positive and negative selection) of these immature cells resulting in increased number of mature SP cells.

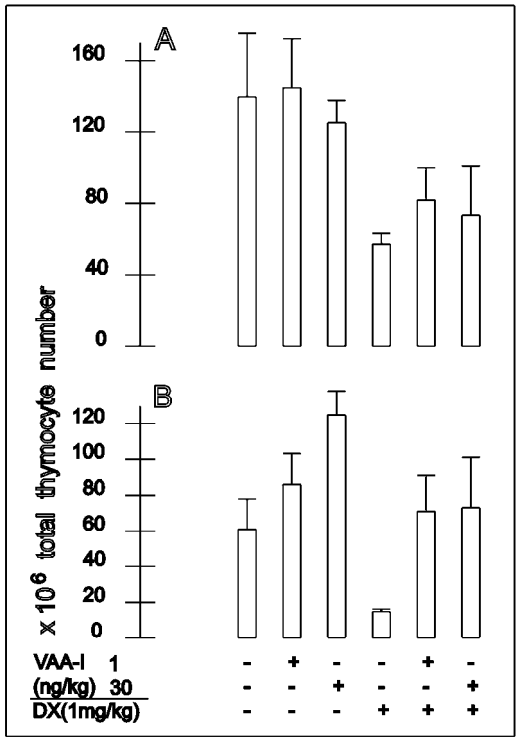


Figure 5. Effect of VAA-I on thymocyte proliferation.
Total numbers of thymocytes in Balb/c mice after treatment with 1 and 30 ng/kg lectin (VAA-I) and/or Dexamethasone (DX). The experiments were carried out either 24h after a single subcutaneous injection (5A) or 72h after the last injection of treatment twice a week for three weeks (5B). Each value represents the average of three animals (+SEM). The results of various subpopulations and statistical analysis are shown in table 2.

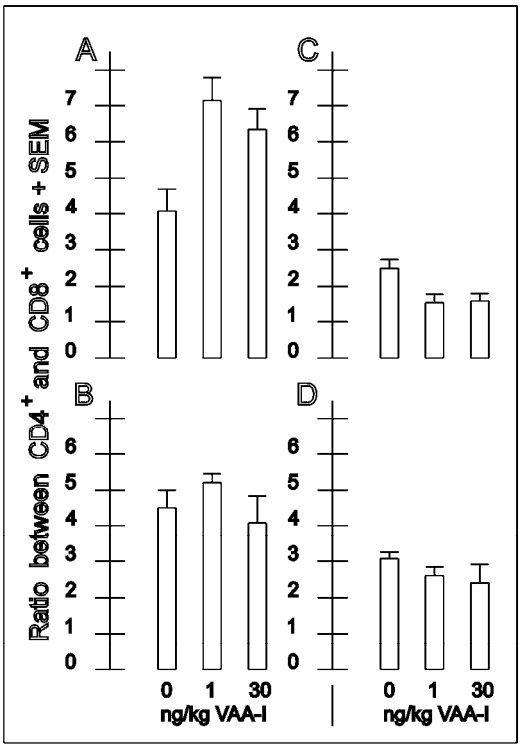


Figure 6. Effect of VAA-I on ratio between CD4+ and CD8+ cells.
The mean ratios (+SEM) between CD4+ and CD8+ cells are shown in peripheral blood and in thymus 24h after a single injection (6A and 6C) or 72h after the last injection of a three weeks treatment (6B and 6D). 24h after a single injection of both lectin doses this ratio significantly decreased (6C) in thymus ($p<0.01$; $p<0.01$) and increased (2A) in blood ($p<0.01$; $p<0.05$). After three weeks (6B and 6D) no significant differences between treated and control groups were found.

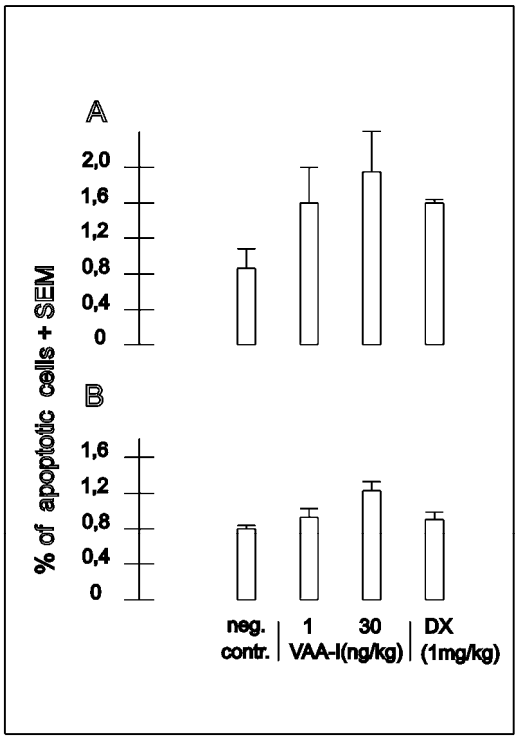


Figure 7. Effect of VAA-I on thymocyte apoptosis
Mean percentage (+SEM) of apoptotic cells in thymus 24h after a single injection of lectin (7A) or 72h after the last injection of treatment twice a week for three weeks treatment (7B). 30 ng/kg lectin induced significant enhancement in both trials ($p<0.05$; $p<0.01$).

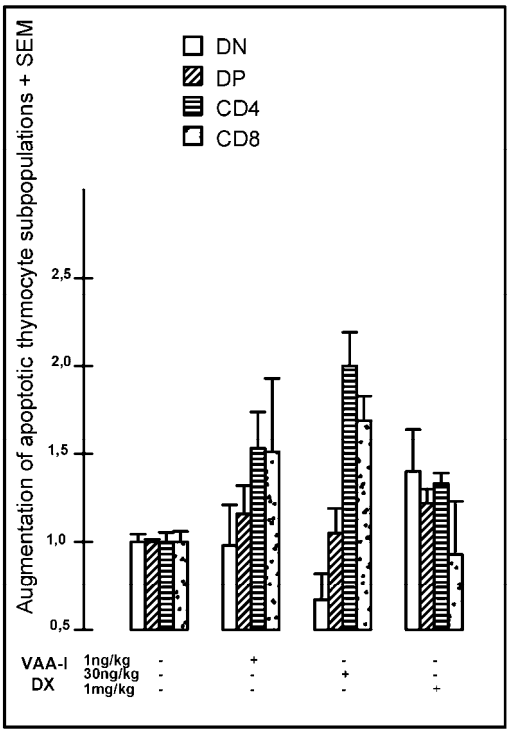


Figure 8. Effect of VAA-I on apoptosis of thymocyte subpopulations.
Mean augmentation [(experimental value / negative control value) + SEM] of apoptotic thymocyte subpopulations 24h after asingle injection of VAA-I or DX. All investigations were carried out with Annexin V staining of mouse thymocytes. 30 ng/kg lectin induced significant increases in apoptosis of CD4+ and CD8+ SP subpopulation ($p<0.05$ and $p<0.01$).

3.4. Effect of VAA-I on DX-induced thymocyte apoptosis

Effects of a single injection of 30 ng/kg VAA-I alone or in combination with 1 mg/kg DX on the percentage of apoptotic cells in various thymocyte subpopulations were investigated. As shown in Fig 9, detection of early apoptosis in thymocte subpopulations by Annexin V-binding revealed that VAA-I alone induced significant increases in apoptosis of SP (CD4 and CD8+) subpopulations. However, there was a small difference between the augmentation of apoptosis in SP cells caused by the lectin alone or in combination with DX: 2.0fold and 2.1fold increases of CD4+ cells; 1.69fold and 1.82fold elevations of CD8+ cells (Fig 9). Interestingly the lectin caused diminished apoptosis in DN cell populations, while in the DP

cell population no significant alteration could be observed. DX alone induced enhanced apoptosis in DN, DP and CD4+ cell populations (P= 0,09, P<0,05 and P<0,01 respectively). Combined treatment induced similar effects to those induced by DX alone on immature cell groups.

After four days or three weeks, the percentage of late apoptotic cells in total thymocyte populations was investigated by PI incorporation into DNA. The combination of VAA-I given on day 0 and day 3 with continuous DX therapy for four days caused enhanced apoptosis of total thymocyte populations (23+/-3.1%) compared to the DX treatment alone (15,1%+/-1.7; p<0.05) (data not shown). As illustrated in Fig.10, 72 hours following the last administration of treatment with 30 ng/kg VAA-I alone (twice a week for three weeks) 54% enhancement (p<0.01) of total apoptotic thymocytes was detected while the 3 weeks treatment with DX alone caused only an increase of 13% (p>0.05). Combined treatment (lectin injections twice a week together with DX) resulted in an additive increase (2-fold) in percentage of apoptotic thymocytes (p<0.05).

3.5. Effect of VAA-I on the glucocorticoid receptor (GCR) level in thymocyte subpopulations

The previously described lectin-induced proliferation of immature thymocyte subpopulations which effectively inhibited the DX induced depletion of the thymus and its apoptotic effects on mature thymocytes (present trial) raised the question whether VAA-I influences the expression of intracellular GCR level in thymocyte subpopulations. 24h after a single injection of VAA-I, the GCR mean fluorescence intensity (expression) in thymocyte subpopulations did not alter significantly (data not shown). Treatment with 30 ng /kg VAA-I for four days caused a 2.5fold increase in GCR mean fluorescence intensity of DP thymocytes (p<0.05) but only a 1,17fold elevation of DN thymocytes (p>0.05) (Fig. 11). The third group of Balb/c mice was treated with 30 ng/kg VAA-I twice a week for three weeks. 72h after the last injections the same investigations as after 24h and four days were carried out. The GCR expression (mean fluorescence intensity) of thymocytes increased similarly in all

subpopulations: 25% GCR of DN, 23% of DP, 27% of CD4+ and 20% of CD8+ cells (see Fig. 12; p values >0.05).

Figure 9. Effect of VAA-I on apoptosis of thymocyte subpopulations. The bars represent the apoptosis index [(percentage of Annexin positive thymocytes in treated mice / percentage of Annexin positive thymocytes in untreated control) + SEM] of thymocyte subpopulations 24h after a single injection of VAA-I and/or DX. 30 ng/kg lectin alone and/or in combination with DX induced significant increases in apoptosis of CD4+ and CD8+ SP subpopulations (*$p<0.025$; **$p<0.01$; $^{+}p<0.001$ and $^{++}p<0.025$, respectively). DX alone caused elevation of apoptosis in DN, DP and CD4 SP thymocytes ($^{\#}p<0.09$; $^{\#\#}p<0.05$, $^{\#\#\#}p<0,01$).

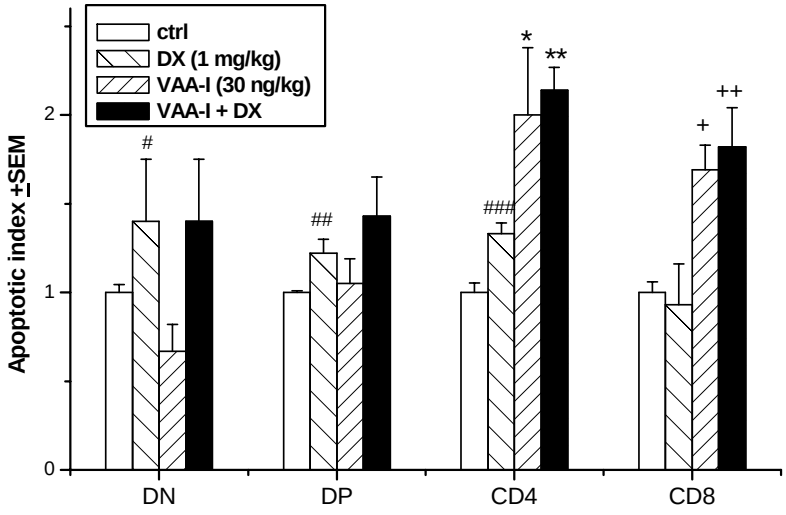


Figure 10. Effect of repeated VAA-I treatment on DX-induced thymocyte apoptosis. The apoptotic index (+SEM) of all thymocytes 72h after the last injection of repeated (3 weeks) treatment with 30ng/kg VAA-I and / or 1 mg/kg Dexamethasone are illustrated. VAA-I both alone and in combination with DX induced significant enhancements in the number of apoptotic cells (*$p<0.01$ and **$p<0.05$).

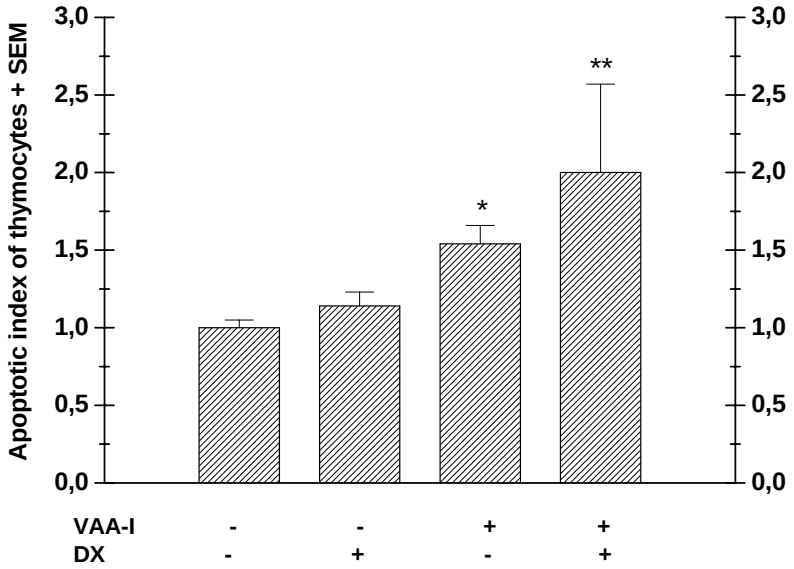


Figure 11. Effect of VAA-I and DX on the GCR level of thymocytes after a 4 day combined treatment. Bars represent the GCR mean fluoresecence intensities (± SEM) in DP and DN thymocytes. The treatment induced significant elevations of GCR expression in DP thymocytes (*p<0.05).

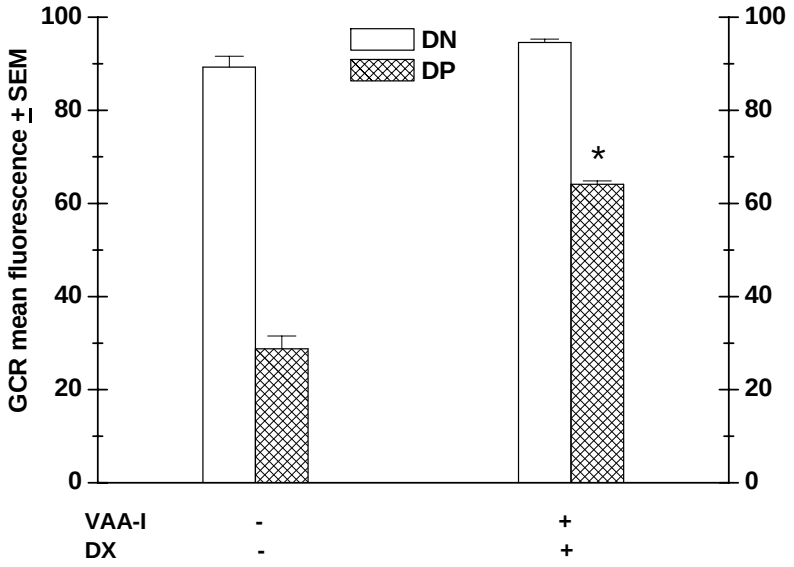
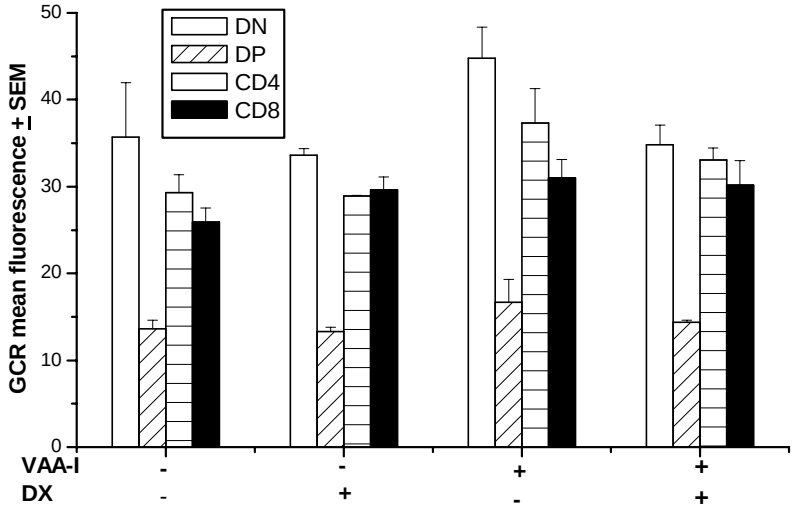


Figure 12. Effect of 3 weeks VAA-I and / or DX treatment on the GCR level of thymocyte subpopulations. Mean fluorescence intensities of GCR staining (±SEM) in DN, DP, CD4 and CD8 thymocytes are shown. 72 hours after the repeated VAA-I treatment no significant alteration in GCR expression (p values >0.05) could be observed.



4. Discussion

Our studies showed that in agreement with previous in vitro findings [81, 87], VAA-I can act in vivo on the regulation of thymus function inducing both proliferation and apoptosis of thymocytes. The short- and long-term effects of VAA-I on thymus proliferation revealed responses of both mature and immature cells. These effects at least in part may be related to the proinflammatory activity of VAA-I [82] since IL-1β, IL-6, TNFα and IFN□ are known to play an important role in proliferation and apoptosis of thymocytes [120-125]. Whether or not there is a direct effect of the lectin on the thymocytes or whether it is a consequence of the lectin-induced proinflammatory cytokines has yet to be determined.

VAA-I was also found to modulate protein synthesis and to induce apoptosis in various eukaryotic cells in culture. The apoptotic effect of VAA-I is dose-dependent and involves caspase-3 at least in part [89]. In vitro VAA-I can induce a characteristic programmed cell death in concentrations between 10 and 1000 ng/ml after 24h in various cells of immune origin [81, 89]. In the present in vivo study, a dose as low as 1ng/kg VAA-I appears to be more effective during DX therapy in reconstitution of lymphoid tissues than 30 ng/kg. However, the higher lectin dose (30 ng/kg bw) was more effective in enhancing the

proliferation rate and percentage of apoptotic thymocytes. This observation indicates that VAA-I induced apoptosis may be a consequence of the enhanced selection steps in contrast to the direct apoptosis induced by DX in immature thymocytes. Our finding, that long-term VAA-I can inhibit the DX-induced reduction in thymocyte number, may indicate the importance of studying the effect of lectin on the glucocorticoid treatment of various diseases. Further investigations are necessary to clarify this question.

Immunosuppression due to the inhibition of expression of a wide variety of activation-induced gene products is perhaps the best known biological effect of GCs on peripheral T cells [128, 133]. In the thymus, GCs are also potent inducers of apoptosis, and even GC concentrations achieved during a stress response can cause the death of CD4+CD8+ (DP) thymocytes [135]. Endogenous GCs produced by the thymic epithelial cells are important in the positive selection of DP thymocytes which interestingly express the lowest GCR level [134]. In contrast an increased GCR gene dosage in mice has been shown to enhance the glucocorticoid-induced apoptosis of thymocytes [141]. These effects underscore the importance of a tight regulation of GCR expression in the the control of physiological and pathological processes.

As described in chapter 3, VAA-I induced a dose-dependent increase in numbers of both immature and mature thymocyte subpopulations [119]. Furthermore, it has been also observed that VAA-I is able to modulate the circulating pool of certain lymphocyte subpopulations [82, 114]. There was a significant difference between the lower doses (between 0.5 and 1 ng/kg) which enhance the lymphocyte count and the higher amounts (over 30 ng/kg) which reduce it [82]. The homeostatic regulation that controls total thymocyte and peripheral lymphocyte numbers is not clearly understood. However, transgenic mice with reduced GCR expression show increased thymocyte and T-cell numbers [142]. Thus, it is very probable that physiological GC and GCR levels are directly involved in controlling the size of both thymocyte and T-cell pools [132]. For these reasons, the lectin-induced enhancement of GCR

levels and its additive effect on DX-induced apoptosis of thymocytes may contribute towards a better understanding of the different effects of VAA-I given in low (0.5-1 ng/kg) as compared to higher (30 ng/kg) doses [143]. In previous studies, VAA-I has been shown to be effective with a bell-shaped dose response curve not only on numbers of circulating lymphocytes [56, 144] but also on several cellular parameters of the natural immune system [145-146]. In addition, the lower and more immunomodulatory active dose (1 ng/kg) of VAA-I has been found to be more protective against the DX–induced reduction of total thymocyte number than the higher one (30 ng/kg). In contrast, the higher dose (30 ng/kg) of VAA-I exhibited a stronger apoptotic effect on thymocytes than the lower ones [119]. Therefore for the present study, 30 ng/kg lectin was selected to investigate its effect on DX-induced apoptosis in various thymocyte subpopulations. This dosage of VAA-I also induced an enhanced proliferation of thymocytes with a parallel increase in number of apoptotic cells which may reflect an enhanced maturation (positive selection) caused by the lectin treatment. The present results indicate that the apoptotic effects of DX and VAA-I on thymocyte subpopulations differ: DX is more effective in immature DP thymocytes while VAA-I induces enhanced apoptotosis mainly in mature SP (CD4+ and CD8+) subpopulations. Consequently, the significant additive effect on the total number of apoptotic thymocytes (see Fig 2) may indicate that VAA-I and DX act in different ways and is associated with some protective effect against the DX-induced reduction in total cell count. Therefore an additive effect of VAA-I on GC hormone therapy may be beneficial if the higher circulating level of GC results in a lower sensitivity or resistance.

Because of the bell-shaped dose response curve of VAA-I, GCs are also known to induce apoptosis in thymocytes at high concentrations. At lower concentrations, GCs antagonize TCR-mediated deletional signals and allow survival of thymocytes [133]. Therefore further investigations are necessary to clarify whether the lectin-induced augmentation of GCR levels

may also be involved in its bell-shaped dose-response curve of immunomodulatory or apoptotic effects.

5. Conclusions and pharmacological perspectives

5.1. Although a number of various clinical situations can lead to impaired functions of natural immunity, the change most investigated is the tumor-induced suppression of innate host defense mechanisms. A therapeutic modulation of basic activity and balance of the natural immune system is desirable in many clinical situation. A plant lectin, VAA-I may possibility achieve this objective. With regard to the biological and preclinical research of mistletoe lectin, two essentially different effects must be considered: cytostatic / apoptotic and immunomodulatory activity.

5.2. Low , immunomodulatory doses of VAA-I could support a long -term therapeutic modulation of the natural immune system which is associated with a protective effect. This treatment could be combined with the toxic modalities of various therapies and also improve the quality of life.

5.3 Higher doses of VAA-I with cytostatic / apoptotic effects could suggest new perspectives of how to modulate the balance between cell growth and programmed cell death therapeutically.

5.4. At present, judgment of clinical benefits of an immunologically optimized mistletoe lectin is difficult and in many aspects is not feasible. However, growing evidence [147-154] suggests that VAA-I is able to improve the clinical situation of patients with a decreased responsiveness of their natural immune system. Determination of sugar-binding lectin activity by an enzyme linked lectin assay enabled a clinically useful standardization of the immunomodulatory capacity of mistletoe extracts. In a multi-centre clinical study, 689 breast cancer patients were treated with extracts containing optimal lectin doses (1 ng VAA-I/kg twice a week) and the side effects of oncological basic therapy modalities (operation,

chemotherapy, irradiation and hormone therapy) were compared with a control group (n=470). VAA-I significantly reduced the frequency of nausea, problems with appetite, depression, tiredness and insomnia [155]. In addition, a beneficial effect on local recurrence was also established supporting a possible enhanced basic activity of the natural immune system.

5.5. Recent studies demonstrate that VAA-I is able to modulate the maturation of thymocytes in vivo. The total cell count of thymocytes showed significant increases after repeated injections of immunomodulatory doses of VAA-I and an elevated percentage of apoptotic cells was also found. Single positive (SP) thymocytes revealed higher increaes in lectin-induced apoptosis than double negative (DN) or double positive (DP) cells. Similarly, small lectin doses significantly inhibited the dexamethasone (DX)-induced reduction of all thymocyte subpopulations investigated. These results indicate that during a glucocorticoid treatment, VAA-I may be an effective immunoprotective biomodulator by diminishing the undesired side effects on cellular immunity and immune balance.

5.6. An additive effect of lectin on DX-induced apoptosis of thymocytes was also observed which consisted of two different actions of VAA-I and DX. A lectin-induced enhancement of the glucocorticoid receptor level may be also involved. The additive effect of VAA-I on glucocorticoid (GC) hormone therapy may be beneficial if the higher circulating level of GC causes a lower sensitivity or resistance. Evaluation of this possible clinical benefit requires further preclinical and clinical research. In addition, further studies are required to establish the favorable effect of lectin during the treatment of diseases in which programmed cell death is defective.

6.1 References originated from the author

56**. Hajto** T, Hostanska K, Gabius H-J: Modulatory potency of the β-galactoside-specific lectin from mistletoe extract (Iscador) on the host defense system in vivo in rabbits and patients. Cancer Res 1989; **49**: 4803-8.

81. Hostanska K**, Hajto** T, Fischer J, Lentzen H, Mengs U, Weber K, Saller R: Selective modulation of phosphatitylserine expression on various subpopulations of human peripheral blood lymphocytes by a plant lectin, Viscum album agglutinin VAA-I and its recombinant form (rVAA) in vitro. Cancer Detect Prevent 1998; **22 Supp 1**: 89.

82**. Hajto** T, Hostanska K, Weber K, Zinke H, Fischer J, Mengs U, Lentzen H, Saller R: Effect of a recombinant lectin, Viscum album agglutinin (rVAA) on secretion of interleukin-12 in cultured human peripheral blood mononuclear cells and on NK cell-mediated cytotoxicity of rat splenocytes in vitro and in in vivo. Nat Immun 1998; **16**:34-46.

85. **Hajto** T, Hostanska K, Frei K, Rordorf C, Gabius H-J: 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 misletoe extract. Cancer Res 1990; **50**:3322-6.

86. Hostanska K, **Hajto** T, Spagnoli G, 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.

87. Hostanska K, **Hajto** T, Weber K, Fischer J, Lentzen, H., Sütterlin B, Saller R. A natural immunity activating plant lectin, Vscum album agglutinin-I (VAA-I) inducesapoptosis in human lymphocytes, monocytes, monocytic THP-1 cells and murine thymocytes. Nat Immun 1996-97; **15**:295-311.

89. Savoie A, Lavastre V, Pelletier M, **Hajto** T, Hostanska K, Girard D: Activation of human neutrophils by the plant lectin Viscum album agglutinin-I: modulation of de novo protein synthesis and evidence that caspases are involved in induction of apoptosis. J Leukoc Biol 2000**; 68**: 845-853.

102. Vehmeyer K, **Hajto** T, Hostanska K, Könermann S, Lösert H, Saller R, Wörmann B:Lectin-induced increase in clonogenic growth of hematopoietic progenitor cells. Eur J Hematol 1998; **60**: 16-20.

105. **Hajto** T, Hostanska K, Herrmann R: Immunomodulatory potency of mistletoe lectins in cancer patients. Results of a dose finding study. Allergy (Suppl.) 1993; **48**:548.

106. **Hajto** T, Hostanska K, Gabius H-J: Zytokine als Lektin-induzierte Mediatoren in der Misteltherapie. Therapeutikon 1990; **4**: 136-145.

112. **Hajto** T, Hostanska K, Fischer J and Lentzen H: Investigations of cellular parameters to establish the response of a biomodulator: galactoside-specific lectin from Viscum album plant extract. Phytomedicine 1996; **3**:129-137.

113. **Hajto** T, Hostanska K, Steinberg F, Gabius H-J: Galactoside-specific lectin from clinically applied mistletoe extract reduces tumor growth by augmentation of host defense system. Blut, 1990; **61**: 164.

114. **Hajto** T, Hostanska K, Fornalski M, Kirsch A: Antitumorale Aktivität des immunmodulatorisch wirkenden Beta-galaktosidspezifischen Mistellektins bei der klinischen Anwendung von Mistelextrakten (Iscador). Dtsch Zschr Onkol 1991; **23**:1-6.

117. **Hajto** T, Lanzrein C.: Frequency of large granular lymphocytes in peripheral blood of healthy persons and breast cancer patients. Cancer Immunol Immunother 1983; **16**: 65-66.

118. **Hajto** T, Hostanska K.: An investigation of the ability of Viscum album-activated granulocytes to regulate natural killer cells in vivo. Clin Trial 1986; **23**: 345-358.

119. **Hajtó** T, Berki T, Boldizsár F, Németh P: Galactoside-specific plant lectin, Viscum album agglutinin-I induces enhanced proliferation and apoptosis of murine thymocytes in vivo. Immunol Letters 2003; **86**: 23-27.

138. **Hajtó** T, Berki T, Pálinkás L, Boldizsár F, Nagy G, Németh P: Galactoside-specific misletoe lectin modulate the dexamethasone-induced apoptosis and glucocorticoid receptor level in Balb/c thymocytes. In Vivo 2003; **17**/2 in press.

147. Weber K, Mengs U, Schwarz T, **Hajto** T, Hostanska K, Allen TR, Weyhenmeyer R, Lentzen H: Effects of a standardized mistletoe preparation on metastatic B16 melanoma colonization in murine lungs. Arznem Forsch 1998; **48**: 497-502.

154. Kovacs E, **Hajto** T, Hostanska K: Improvement of DNA repair in lymphocytes of breast cancer patients treated with Viscum album extract (Iscador). Eur J Cancer 1991; **27**: 1672 - 1676.

6.2. References originated from other authors

1. Berczi I, Bertok L, Chow DA.: Natural immunity and neuroimmune host defense. Ann N Y Acad Sci 2000; **917**:248-57.

2. Woodworth CD.: HPV innate immunity. Front Biosci 2002; **7**:d2058-71

3. Beilin B, Shavit Y, Cohn S, Kedar E.: Narcotic-induced suppression of natural killer cell activity in ventilated and nonventilated rats. Clin Immunol Immunopathol. 1992; **64**:173-6.

4. Beilin B, Shavit Y, Hart J, Mordashov B, Cohn S, Notti I, Bessler H. Effects of anesthesia based on large versus small doses of fentanyl on natural killer cell cytotoxicity in the perioperative period. Anesth Analg. 1996; **82**: 492-7.

5. Mealy K, O'Farrelly C, Stephens R, Feighery C. Impaired neutrophil function during anesthesia and surgery is due to serum factors. J Surg Res. 1987; **43**: 393-7.

6.Griffith JP, Everitt NJ, Lancaster F, Boylston A, Richards SJ, Scott CS, Benson EA, Sue-Ling HM, McMahon MJ.: Influence of laparoscopic and conventional cholecystectomy upon cell-mediated immunity. Br J Surg. 1995; **82**: 677-80.

7. Faist E, Ertel W, Salmen B, Weiler A, Ressel C, Bolla K, Heberer G.: The immune-enhancing effect of perioperative thymopentin administration in elderly patients undergoing major surgery. Arch Surg. 1988;**123:** 1449-53.

8. MacLean LD.: Delayed type hypersensitivity testing in surgical patients. Surg Gynecol Obstet. 1988;**166:** 285-93.

9. Houghton AN, Lloyd KO. Stuck in the MUC on the long and winding road. Nat Med. 1998;**4**: 270-271.

10. Blohm U, Roth E, Brommer K, Dumrese T, Rosenthal FM, Pircher Corrie AM. Marijnen H.: Lack of effector cell function and altered tetramer binding of tumor-infiltrating lymphocytes. J Immunol 2002; **169**: 5522-30

11. Iris D. Nagtegaal, , Elma Klein Kranenbarg, Adri Mulder-Stapel, Jo Hermans, Cornelis JH. van de Velde and J Han JM.. van Krieken: Local and distant recurrences in rectal cancer patients are predicted by the n**on**specific immune response; specific immune response has only a systemic effect - a histopathological and immunohistochemical study. BMC Cancer. 2001; **1**: 7

12. Seliger B, Maeurer MJ, Ferrone S. Antigen-processing machinery breakdown and tumor growth.Immunol Today 2000;**21**: 455-64

13. Ferrone S, Marincola FM..: Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. Immunol Today 1995; **16** :487-94

14. Garrido F, Cabrera T, Concha A, Glew S, Ruiz-Cabello F, Stern PL.: Natural history of HLA expression during tumour development. Immunol Today 1993;**14**: 491-9

15. Moretta L, Biassoni R, Bottino C, Mingari MC, Moretta A.: Natural killer cells: a mystery no more. Scand J Immunol 2002; **55**: 229-32

16. Takeda K, Smyth MJ, Cretney E, Hayakawa Y, Yamaguchi N, Yagita H, Okumura K.: Involvement of tumor necrosis factor-related apoptosis-inducing ligand in NK cell-mediated and IFN-gamma-dependent suppression of subcutaneous tumor growth. Cell Immunol 2001; **214**: 194-200.

17. Mariani E, Meneghetti A, Tarozzi A, Cattini L, Facchini A.: Interleukin-12 induces efficient lysis of natural killer-sensitive and natural killer-resistant human osteosarcoma cells: the synergistic effect of interleukin-2. Scand J Immunol 2000; **51**: 618-25

18. Okuno K, Jinnai H, Lee YS, Kaneda K, Yasutomi M.: Interleukin-12 augments the liver-associated immunity and reduces liver metastases. Hepatogastroenterology 1996; **43**: 1196-1202.

19. Dolcetti R, Viel A, Doglioni C, Russo A, Guidoboni M, Capozzi E, et al.: High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability Am J Pathol 1999; **54**: 18051813.

20. Goedegebuure PS, Eberlein, TJ.: The role of CD4+ tumor-infiltrating lymphocytes in human solid tumors Immunol Res 1995; **14**: 119-131.

21. Ropponen, KM, Eskelinen, MJ, Lipponen, PK, Alhava, E, & Kosma, VM.: Prognostic value of tumour-infiltrating lymphocytes (TILs) in colorectal cancer. J Pathol 1997; **182**: 318-324.

22. Espi A, Arenas J, Garcia-Granero E, Marti E, Lledo S.: Relationship of curative surgery on natural killer cell activity in colorectal cancer. Dis Colon Rectum 1996; **39**: 429-434.

23. Coca S, Perez-Piqueras J, Martinez D, Colmenarejo A, Saez MA, Vallejo C et al.: The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma Cancer 1997; **79**: 2320-2328.

24. Skinner JM, Jarvis LR, Whitehead R.: The cellular response to human colonic neoplasms: macrophage numbers J Pathol 1983; **139**: 97-103.

25.Higgins CA, Hatton WJ, McKerr G, Harvey D, Carson J, Hannigan BM: Macrophages and apoptotic cells in human colorectal tumours. Biologicals 1996; **24**: 329-332.

26. Cameron DJ, O'Brien P.: Cytotoxicity of cancer patient's macrophages for tumor cells Cancer 1982; **50**: 498-502.

27. Lachter J, Stein M, Lichtig C, Eidelman S, Munichor M.: Mast cells in colorectal neoplasias and premalignant disorders. Dis Colon Rectum 1995; **38**: 290-293.

28. Fisher ER, Paik SM, Rockette H, Jones J, Caplan R, Fisher B.: Prognostic significance of eosinophils and mast cells in rectal cancer. Hum Pathol 1989; **20**: 159-163.

29. Nielsen HJ, Hansen U, Christensen IJ, Reimert CM, Brünner N, Moesgaard, F, et al. : Independent prognostic value of eosinophil and mast cell infiltration in colorectal cancer tissue. J Pathol 1999; **189**: 487- 495.

30. Pretlow TP, Keith EF, Cryar AK, Bartolucci AA, Pitts AM, Pretlow TG, et al. : Eosinophil infiltration of human colonic carcinomas as a prognostic indicator. Cancer research 1983; **43**: 2997-3000.

31. Satomi A, Murakami S, Ishida K, Hashimoto T, Sonoda, M..: Significance of increased neutrophils in patients with advanced colorectal cancer. Acta Oncol 1995; **34**: 69-73.

32. Chapoval AI, Tamada K, Chen L.: In vitro growth inhibition of a broad spectrum of tumor cell lines by activated human dendritic cells. Blood 2000; **95**: 2346-51

33. Wood GA, Korkola JE, Archer MC.: Resistance of Copenhagen rats to hepatocarcino-genesis does not involve T-cell immunity. Carcinogenesis 2001; **22**: 357-9

34. Hamlin IME.: Possible host resistance in carcinoma of the breast: a histological study. Br J Cancer. 1968; **22:** 383-401.

35. Black MM, Barclay THC, Hankey BF.: Prognosis in breast cancer utilizing histologic characteristics of the primary tumor. Cancer. 1975; **36**: 2048-2055.

36. Bassler R, Dittman AM, Dittrich M.: Mononuclear stromal reactions in mammary carcinoma, with reference to medullary carcinomas with a lymphoid infiltrate. Virchows Arch A Pathol Anat Histol. 1981; **393**: 75-91.

37. Lichtenstein A.: Granulocytes as possible effectors of tumor immunity. In: Oettgen HF, ed. Human Cancer Immunology. Vol 1. Philadelphia, PA: Saunders; 1990: 731-747.

38. Midorikawa Y, Yamashita T, Sendo F.) Modulation of the immune response to transplanted tumors in rats by selective depletion of neutrophils in vivo using a monoclonal antibody: abrogation of specific transplantation resistance to chemical carcinogen-induced syngeneic tumors by selective depletion of neutrophils in vivo. Cancer Res. 1990; **50**: 6243-6247.

39. Matsumoto Y, Saiki I, Murata J, Okuyama H, Tamura M, Azuma I.: Recombinant human granulocyte colony-stimulating factor inhibits the metastasis of hematogenous and non-hematogenous tumors in mice. Int J Cancer. 1991; **49**: 444-449.

40. Colombo MP, Ferrari G, Stoppacciaro A, et al.: Granulocyte colony-stimulating factor gene transfer suppresses tumorigenicity of a murine adenocarcinoma in vivo. J Exp Med. 1991; **173**: 889-897.

41. Di Carlo E, Forni G, Lollini PL, Colombo MP, Modesti A, Musiani P.: The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood, 2001; **97**: 339-345.

42. Kindzelskii AL, Petty HR.: Early membrane rupture events during neutrophil-mediated antibody-dependent tumor cell cytolysis. J Immunol. 1999; **162**: 3188-3192.

43. Heijnen IAFM, Rijks LJM, Schiel A et al.: Generation of HER-2/neu-specific cytotoxic neutrophils in vivo: efficient arming of neutrophils by combined administration of granulocyte colony-stimulating factor and Fc receptor I bispecific antibodies. J Immunol. 1997; **159**: 5629-5639.

44. Kushner BH, Cheung NV.: Absolute requirement of CD11/CD18 adhesion molecules, FcRII, and the phosphatidylinositol-linked FcRIII for monoclonal antibody-mediated neutrophil antihuman tumor cytoxicity. Blood. 1992; **79**: 1484-1490.

45. Kushner BH, Cheung HKV.: GM-CSF enhances 3F8 monoclonal-dependent cellular cytotoxicity against human melanoma and neuroblastoma. Blood. 1989; **73**: 1936-1941.

46. Ragnhammar P, Frödin JE, Trotta PP, Mellestedt H. Cytotoxicity of white blood cells activated by granulocyte-colony-stimulating factor, granulocyte-macrophage-colony-stimulating factor and macrophage-colony-stimulating factor against tumor cells in the presence of various monoclonal antibodies. Cancer Immunol Immunother. 1994; **39**: 254-262.

47. Van Spriel AB, Leusen JH, van Egmond M, Dijkman HB, Assmann KJ, Mayadas TN, van de Winkel JG.: Mac-1 (CD11b/CD18) is essential for Fc receptor-mediated neutrophil cytotoxicity and immunologic synapse formation. Blood; **97**: 2478-86.

48. Kikuchi T, Abe T, Ohno T.: Effects of glioma cells on maturation of dendritic cells. J Neurooncol 2002; **58**: 125-30.

49. Gabrilovich DI, Cheng P, Fan Y, Yu B, Nikitina E, Sirotkin A, Shurin M, Oyama T, Adachi Y, Nadaf S, Carbone DP, Skoultchi AI. H1(0) histone and differentiation of dendritic cells. A molecular target for tumor-derived factors. J Leukoc Biol 2002; **72**: 285-96.

50. Baskic D, Acimovic L, Samardzic G, Vujanovic NL, Arsenijevic NN.: Blood monocytes and tumor-associated macrophages in human cancer: differences in activation levels. Neoplasma 2001; **48**: 169-74.

51. Igney FH, Krammer PH.: Immune escape of tumors: apoptosis resistance and tumor counterattack . J Leukoc Biol, 2002; **71**: 907 - 920.

52..Maeda H, Akaike T.: Nitric oxide and oxygen radicals in infection, inflammation, and cancer. Biochemistry 1998; **63**: 854-865.

53. Romani L, Bistoni F, Puccetti P.: Initiation of T-helper cell immunity to Candida albicans by IL-12: the role of neutrophils. Chem Immunol. 1997; **68**: 110-135.

54. Murray JS.: How the MHC selects Th1/Th2 immunity. Immunol Today 1998; **19**:157-63.

55. Sanchez-Torres C, Garcia-Romo GS, Cornejo-Cortes MA, Rivas-Carvalho A, Sanchez-Schmitz G.: CD16+ and CD16- human blood monocyte subsets differentiate in vitro to dendritic cells with different abilities to stimulate CD4+ T cells. Int Immunol 2001; **13**: 1571-81.

57. Samuelsson G, Pettersson B: Separation of viscotoxins from the European mistletoe Viscum album L (Loranthacea) by chromatography on sulfoethyl sephadex. Acta Chem Cand 1970; **24**: 27751-2756.

58. Urech K, Schaller G, Ziska P, Giannattasio M: Comparative study on the cytotoxiceffect of viscotoxin and mistletoe lectin on tumor cells in culture. Phytother Res1995; **9**: 49-55.

59. Müller EA, Anderer FA: A Viscum album oligosaccharide activating human natural cytotoxicity is an interferon-gamma inducer. Cancer Immunol Immunother 1990; **32**:221-227.

60. Sakurai A, Okumara Y: Chemical studies on the mistletoe. The structure of Taxillusin, a new flavinoid glycoside isolation from Taxillus kaempferi. Bull Chem Soc Jpn 1983; **56**: 542-544.

61. Becker H, Exner J: Vergleichende Untersuchungen von Misteln verschiedener Wirtsbäume an Hand der Flavonoide und Phenylcarbonsäuren. Z Pflanzenphysiol 1980; **97**:417-428.

62. Scheffler A, Musielski H, Scherr R: Synergismus zwischen Lektinen und Vesikeln in Viscum album L. 1995; Dtsch Zschr Onkol 27:72-75.

63. Vester F, Mai W: Zur Kenntnis der Inhaltsstoffe von Viscum album. Freie Aminosäuren. Hoppe-Seyler`s Z Physiol Chem 1980; **322**:273-277.

64. Sharon N: Carbohydrates as recognition determinants in phagocytosis and in lectin-mediated killing of target cells. Biol Cell 1984; **51**:239-246.

65. Ziska P, Franz H: Determination of lectin contents in commercial mistletoe preparations for cancer therapy using the ELISA technique. In: Bog Hansen TC, Breborowicz J (Eds): Lectins, Vol IV, Berlin, Walter de Gruyter & Co, 1985, pp 473-480.

66. 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 Drug. 1992; **3**: 507-511.

67. Olsnes S, Stirpe F, Sandvig K, Pihl A: Isolation and characterization of viscumin, a toxic lectin from Viscum album L. (Mistletoe). J Biol Chem 1982; **257**: 13263-70.

68. Holtskog R, Sandvig K, Olsnes S: Characterization of a toxic lectin in Iscador, a mistletoe preparation with alleged cancerostatic properties. Oncology 1988; **45**:172-179.

69. Franz H: Mistletoe lectins and their A and B chains. Oncology 1986; **43**: 23-34.

70. Samtleben R, Kiefer M, Luther P: Characterization of the different lectins from Viscum album (mistletoe) and their structural relationship with agglutinins from Abrus precatorius

and Ricinus communis. In: Bog Hansen TC, Breborowicz J (Eds): Lectins, Vol IV, Berlin, Walter de Gruyter & Co, 1985, pp 617-626.

71. Bushueva TL, Tonevitsky AG: Similarity of protein conformation at low pH and high temperature observed for B-chains of two plant toxins: ricin and mistletoe lectin I. FEB 1988; **229**:119-122.

72.Luther P, Uhlenbruck G, Reutgen H, Samtleben R, Sehrt I, Ribereau-Gayon G: Are lectins of Viscum album interesting tools in lung diseases? A review of recent results. Z Erkr. Atmungsorgane 1986; **166**:247 - 56.

73. Lee RT, Gabius H-J, Lee YC: The sugar-combining area of the galactose-specific toxic lectin of mistletoe extends beyond the terminal sugar residue: comparison with homologous toxic lectin, ricin. Carbohydr Res 1994; **254**: 269-276.

74. 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.

75. Sandvig K, Olsnes S: Entry of the toxic proteins abrin, modeccin ricin and diphtheria toxin into cell. II. Effect of pH, metabolic inhibitors, and ionophores and evidence for toxin penetration from endocytotic vesicles. J Biol Chem 1982; **257**: 7504-13.

76. Wiedlocha A, Sandvig K, Walzel H, Radzikowsky C, Olsnes S: Internalization and action of an immunotoxin containing mistletoe lectin A-chain. Cancer Res 1991; **51**:916-920.

77. Stirpe F, Barbieri L, Batelli MG, Soria M, Lappi DA: Ribosome-inactivating proteins from plants: present status and future prospects. Bio Technology 1992; **10**:405-412.

78. Soler MH, Stoeva S, Schwamborn C, Wilhelm S, Stiefel T, Voelter W: Complete amino acid sequence of the A chain of mistletoe lectin 1. FEBS Lett 1996; **399**:153-157.

79. Soler MH, Stoeva S, Voelter W: Complete fluid acid sequence of the B chain of mistletoe lectin I. Biochem Biophys Res Commun 1998; **246**:596-601.

80. Zinke H, Eck J, Langer M, Möckel B, Baur A, Lentzen H: Molecular cloning of the Viscum album L (mistletoe) gene for ML-1 and characterization of the recombinant protein. Phytomedicine 1996; **3 Suppl 1**: 25.

83. Janssen O, Scheffler A, Kabelitz D: In vitro effects of mistletoe extracts and mistletoe lectins. Cytotoxicity towards tumor cells due to the induction of programmed cell death (apoptosis). Drug Res 1993; **34**: 1221-1227

84. 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 mistletoe lectins. Cancer Letters 1996; **99**:59-72.

88. Wenzel-Seifert K, Lentzen H, Seifert R: In U-937 peomonocytes, mistletoe lectin I increases basal [Ca2+]i, enhances histamin H1 - and complement C5a-receptor-mediatedrises in [Ca2+]i, and induces cell death. Naunyn-Schmiedeberg`s Arch Pharmacol 1997; **355**: 190-197.

90. Kim M, Rao MV, Tweardy DJ, Prakash M, Galili U, Gorelik E: Lectin-induced apoptosis of tumor cells. Glycobiology 1993; 3:447-453.

91. Jacob CO.: Tumor necrosis factor alpha in autoimmunity: pretty girl or old witch ? Immunol Today 1992; **13:** 122-125.

92. Männel DN, Becker H, Gundt A, Kist A, Franz H: Induction of tumor necrosis factor expression by a lectin from Viscum album. Cancer Immunol Immunother 1991; **33**: 177-182.

93. Ribereau-Gayon G, Dumont S, Müller 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**:33-8.

94. Walzel H, Bremer H, Gabius H-J: Lectin-induced alterations in the level ofphospholipids, inositol phosphates, and phosphoproteins. In: Gabius H-J, Gabius S (eds):Lectins and Glycobiology. Berlin, Springer Verlag, 1993; pp.357-361.

95. Joller PW, Menrad JM, Schwarz T, Pfüller U, Parnham MJ, Weyhenmeyer R, Lentzen H:Stimulation of cytokine production via a special standardized mistletoe preparation in an in vitro human skin bioassay. Drug Res 1996; **46**:649-53.

96. Manetti R, Parronchi P, Guidizi MG, Piccinni MP, Maggi E, Trinchieri G, Romagnani S: Natural killer cell stimulating factor (IL-12) induce T helper type (Th1)- specific immune responses and inhibits the development of IL-4-producing Th cells. J Exp Med 1993; **177**: 1199-1204..

97. Huang-M, Stolina-M, Sharma-S, Mao-JT, Zhu-L, Miller-PW, Wollman-J, Herschman-H, Dubinett-SM: Non-small cell lung cancer cyclooxygenase-2-dependent regulation of ytokine balance in lymphocytes and macrophages: up-regulation of interleukin 10 and down-regulation of interleukin 12 production. Cancer-Res 1998; **58**: 1208-16.

98. Metzner G, Franz H, Kindt A, Fahlbusch B, Süss J: The in vitro activity of lectinfrom mistletoe (ML-I) and its isolated A and B chains on functions of macrophages and polymorphonuclear cells. Immunobiol 1985; **169**:461-471.

99. Timoshenko AV, Gabius HJ: Efficient induction of superoxide release from human neutrophils by the galactoside-specific lectin from Viscum album. Biol Chem Hoppe-Seyler1993; **374**: 237-243.

100. Wenzel-Seifert K, Krautwurst D, Lentzen H, Seifert R: Concavalin A and mistletoelectin I differentially activate cation entry and exocytosis in human neutrophils: lectins may activate multiple subtypes of cation channels. J Leuko Biol 1996; **60**: 345-355.

101. Baxevanis CN, Voutsas LF, Soler MH, Gritzapis AD, Tsitsilonis OE, Stoeva S, Voelter W, Arsenis P, Papamichail M: Mistletoe lectin I-induced effects on human cytotoxic lymphocytes. Synergism with IL-2 in the induction of enhanced LAK cytotoxicity. Immunopharm Immunotox 1998; **20**: 355-372.

103. Beuth J, Ko HL, Tunggal L, Buss G, Jeljaszewicz J, Steuer MK, Pulverer G: Immunaktive Wirkung von Mistellektin 1 in Abhängigkeit von der Dosierung. Aerzneim Forsch 1994; **44**: 1255-8.

104. Beuth J, Stoffel B, Ko HL, Buss G, Tunggal L, Pulverer G: Immunaktive Wirkung verschiedener Mistellektin-1 Dosierungen in Mammakarzinompatientinennen. Arzneim Forsch 1995; **45**: 505-507.

107. Ziska P, Gelbin M, Franz H: Interaction of mistletoe lectins ML-I, ML-II, and ML-III with carbohydrates. In: Van Driessche E, Franz H, Beeckmans R, Pfüller U, Kallikorm A, Bog-Hansen TC, (Eds) Lectins: Biology Biochemistry, Clinical Biochemistry, Berlin, Walter de Gruyter & Co, 1993; **8**: 10-13

108.Beuth J, Ko HL, Gabius HJ, Burrichter H, Oette K, Pulverer G: Behaviour of lymphocyte subsets and expression of activation markers in response to immunotherapy with galactoside-specific lectin from mistletoe in breast cancer patients. Clin Investig 1992; **70**: 658-661.

109. Beuth J, Gabius HJ, Steuer MK, Geisel J, Steuer M, Ko HL, Pulverer G: Effect of mistletoe lectin therapy on serum level of defined serum proteins (acute phaseproteins) in tumor patients. Med Klin 1993; **88**: 287-90.

110. Beuth J, Ko HL, Tunggal L, Geisel J, Pulverer G: Comparative studies on the immunoactive action of galactoside-specific mistletoe lectin. Pure substance comparedto the standardized extract. Arznem Forsch 1993: **43**:166-9.

111. Mayer H, Steppkes R, Roth R, Richter C-P: Mistelextrakte zur Immunmodulation bei Tumorpatienten. Pharm Zeitung 1996; **141**:11-23.

115. Heiny BM, Beuth J: Mistletoe extract standardized for the galactoside-specific lectin (ML-1) induces beta-endorphin release and immunopotentiation in breast cancer patients.Anticancer Res 1994; **14**:1339-1342.

116. Heiny BM, Albrecht V, Beuth J: Correlation of immune cell activities and beta-endorphin release in breast carcinoma patients treated with galactose-specific lectin standardized mistletoe extract. Anticancer Res 1998; **18**:583-6.

120. Deman, J., Van Meurs, M., Claassen, E., Humblet, C., Bonvier, J. and Defresne, M., In vivo expression of interleukin-1 beta (IL-1 beta), IL-2, IL-4 IL-6, tumor necrosis factor-alpha and interferon-gamma in the fetal murine thymus. Immunology 1996; **89**: 152-157.

121. Von Patay, B., Loppnow, H., Feindt, J., Kurz, B. and Mentlein, R., Chatecholamines and lipopolysaccharide synergistically induce the release of interleukin-6 from thymic epithel cells. J. Neuroimmunol. 1998; **86**: 182-189.

122. Pezzano M, Philip D, Stephenson S, Li Y, Reid V, Maitta R, Guyden JC.: Positive selection by thymic nurse cells requires IL-1 beta and is associated with an increased Bcl-2 expression. Cell. Immunol. 1996; **169**; 174-84.

123. Durkin, H.G. and Waksman, B.H., Thymus and tolerance. Is regulation the major function of the thymus? Immunol. Rev. 2001; **182**: 33-57.

124. Artico, M., Cavallotti, C., Camerini, M. and Cavallotti, D., Interleukin 1 beta as stimulator of the rat thymus. Cytokine 2001; **15**: 261-265.

125. Baseta, J.G. and Stutman, O., TNF regulates thymocyte production by apoptosis and proliferation of the triple negative (CD3-CD4-CD8-) subset. J. Immunol. 2000; **165**: 2621-30.

126.Fryer CJ, Kinyamu HK, Rogatsky I, Garabedian MJ and Archer TK: Selective activation of the glucocorticoid receptor by steroid antagonists in human breast cancer and osteosarcoma cells. J Biol Chem 2000; **275**: 17771-17777.

127. Morand E: Corticosteroids in the treatment of rheumatologic diseases. Curr Opin Rheumatol 1997; **9**: 200-205.

128. Boumpas DT, Chrousos GP, Wilder RL, Cupps TR and Balow JE: Glucocorticoid therapy for immune-mediated diseases: basic and clinical correlates. Ann Intern Med 1993; **119**: 1198-1208.

129. Surh CD and Sprent J: T-cell apoptosis detected in situ during positive and negative selection in the thymus. Nature 1994; **372**: 100-103.

130. Jameson SC and Bevan M: T-cell selection. Curr Opin Immunol 1998; **10**: 214-227.

131. Pazirandeh A, Xue Y, Rafter I, Sjövall J, Jondal M and Okret S: Paracrine glucocorticoid activity produced by mouse thymic epithelial cells. FASEB J 1999; **13**: 893-900.

132. Vacchio MS, Ashwell JD and King LB: A positive role for thymus-derived steroids in formation of the T-cell repertoire. Ann NY Acad Sci 1998; **840**: 317-27.

133. Baus E, Andris F, Dubois PM, Urbain J and Leo O. Dexamethasone inhibits the early steps of antigen receptor signaling in activated T lymphocytes. J Immunol 1996; **156**: 4555-4561.

134. Berki T, Pálinkás L, Boldizsár F and Németh P: Glucocorticoid (GC) sensitivity and GC receptor expression differ in thymocyte subpopulations. Int Immunol 2002; **14**: 463-469,.

135. Ashwell JD, Lu FW and Vacchio MS. Glucocorticoids in T cell development and function Annu Rev Immunol 2000; **18**: 309-45.

136. Purton JF, Boyd RL, Cole TJ and Godfrey DI. Intrathymic T cell development and selection proceeds normally in the absence of glucocorticoid receptor signaling. Immunity 2000; **13**: 179-86.

137. Miller AH, Spencer RL, Pearce BD, Pisell TL, Azrieli Y, Tanapat P, Moday H, Rhee R and McEwen BS: Glucocorticoid receptors are differentially expressed in the cells and tissues of the immune system. Cell Immunol 1998; **186**: 45-54.

139. Berki T, Kumanovics G, Kumanovics A, Falus A, Ujhelyi E and Nemeth P. Production and flow-cytometric application of a monoclonal anti-glucocorticoid receptor antibody. J Immunol Methods 1998; **14**: 19-27.

140. Vermes I, Haanen C, Steffens-Nakken H and Reutelingsperger C. A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on erly apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 1995; **184**: 39-46.

141. Reichardt HM, Umland T, Bauer A, Kretz O and Schutz G. Mice with an increased glucocorticoid receptor gene dosage show enhanced resistance to stress and endotoxic shock. Mol Cell Biol 2000; **20**: 9009-17.

142. Pazirandeh A, Xue Y, Prestegaard T, Jondal M and Okret S. Effects of altered glucocorticoid sensitivity in the T cell lineage on thymocyte and T cell homeostasis. FASEB J 2002; **16** :727-9.

143. Mengs U, Göthel D and Leng-Peschlow E. Mistletoe extracts standardized to mistletoe lectins in oncology: review on current status of preclinical research. Anticancer Res. 2002; **22**: 1399-1408.

144. Heiny BM, Beuth J: Mistletoe extract standardized for the galactoside-specific lectin (ML-1) induces beta-endorphin release and immunopotentiation in breast cancer patients. Anticancer Res , 1994; **14**: 1339-1342.

145. Heiny BM, Albrecht V, Beuth J: Correlation of immune cell activities and beta-endorphinrelease in breast carcinoma patients treated with galactose-specific lectin standardized mistletoe extract. Anticancer Res 1998; **18**: 583-6.

146. Beuth J, Ko HL, Gabius H-J, Pulverer G: Influence of treatment with the immuno-modulatory effective dose of the beta-galactoside-specific lectin from mistletoe on tumor colonization in BALB/c-mice for two experimantal model system. In vivo 1991; **5**: 29-32.

148. Lenartz D; Dott U, Menzel J, Schierholz JM, Beuth J: Survival of glioma patients after complementary treatment with galactoside-specific lectin from mistletoe. Anticancer Res 2000; **20**: 2073-2076.

149. Heiny BM, Beuth J: Das Lektin der Mistel als Immunmodulator: Effektorwirkung auf β-Endorphin- und Zytokinfreisetzung bei Mammakarzinompatientinnen. Dtsch Zschr Onkol 1994; **26**: 103-108 .

150. Lenartz D, Stoffel B, Menzel J, Beuth J: Immunoprotective activity of the galactoside-specific lectin from mistletoe after tumor destructive therapy in glioma patients. AnticancerRes 1996; **16**: 3799-802.

151. Beuth J, Ko HL, Tunggal L, Buss G, Jeljaszewicz J, Steuer MK, Pulverer G: Immunoprotective activity of the galactoside-specific mistletoe lectin in cortisone-treated BALB/c-mice. In vivo 1994; **8**: 989-92.

152. Weber K, Mengs U, Schwarz T, Becker H, Lentzen H: Stimulation of neutropoiesis by a special standardized mistletoe preparation after cyclophosphamide chemotherapy in mice.Arzeim Forsch 1996; **46**:1174-1178.

153. Stoffel B, Beuth J, Pulverer G: Effect of immunomodulation with galactoside-specific mistletoe lectin on experimental listerosis. Zentralbl Bakteriol 1996; **284**: 439-42.

155. Schumacher K, Schneider B, Reich G, Stiefel T, Stoll G, Bock PR, Hanisch J, Beuth J.: Postoperative komplementäre Therapie des primären Mammakarzinoms mit lektinnormiertem Mistelextrakt. Eine epidmiologische, kontrollierte, multizentrische retrolektive Kohortensudie. 2002; **3**: 10-21.