[CANCER RESEARCH 49, 4803–4808, September 1, 1989]

Modulatory Potency of the β-Galactoside-specific Lectin from Mistletoe Extract (Iscador) on the Host Defense System *in Vivo* in Rabbits and Patients[1]

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ABSTRACT

Proprietary extract of mistletoe (Iscador) that has federal approval for clinical application can exhibit immunomodulatory capacity. However, the nature of this responsible substance has still remained elusive. To validate the hypothesis that specific lectin-carbohydrate interactions at least participate in eliciting immunomodulation, the modulatory efficiency of the major β-galactoside-specific mistletoe lectin (ML I) from the clinically applied extract on selected immunological parameters was monitored "*in vivo*" in rabbits. Injections of nontoxic doses of the purified lectin or even only of its carbohydrate-binding subunit (0.25–1.0 ng/kg) into rabbits yielded significant increases in natural killer cytotoxicity, frequency of large granular lymphocytes, and phagocytic activity of granulocytes. In the clinically relevant situation, changes in these parameters were also determined in cancer patients after extract (Iscador) injection s.c. as well as i.v., emphasizing the potential relevance of the lectin. Comparative analyses of the changes in the selected parameters following injection of extract with normal lectin content as well as of extract, selectively depleted of lectin, into healthy volunteers corroborated this inference. Lectin depletion by affinity chromatography was highly specific and did not affect any other substance in the extract. Remarkably, contamination by endotoxin has been rigorously excluded in each applied specimen. These results encourage detailed elucidation of lectin action on various parts of the tumor defense system "*in vitro*" with the long range goal of achieving progress in the treatment of cancer through immunological strategies, exploring selective mediatory lectin-carbohydrate interactions.

INTRODUCTION

Increasing evidence suggests that saccharide determinants present on cell surface glycoconjugates serve as important binding sites for specific factors, exerting modulatory influences within the immune system (1–3). Such mediatory factors with carbohydrate specificity may be potentially exploitable for therapeutically beneficial manipulation of the host's ability to attack malignant cells. In the search for adequate substances, plant extracts provide a readily available and rich source. The proprietary extract of mistletoe (Iscador) is one illustrative example (4). It has already found approval for clinical application in tumor therapy in Europe, although optimal application schedules and rigorous standardization protocols are still lacking. This is due to the fact that the precise nature of the effective agent in the complex mixture is, up to now, elusive. Besides other substance classes, the extract of mistletoe has been shown to contain three types of proteins with specificity to the carbohydrate part of the glycoconjugates, the lectins ML I, ML II, and ML III (5–8). Previous "*in vitro*" measurements on PMN[3] cells had raised the possibility that these lectins may well be able to influence the functions of PMN cells (5, 9). Hypothetically ascribing these lectins in the commercially available extract significant functional roles in tumor defense by immunomodulation, we have thus consequently tested the efficiency of the purified proteins with respect to modulation of selected immunological parameters in rabbits for this report. These parameters comprised cytotoxicity of NK cells, frequency of LGL and phagocytic activity of PMN leukocytes. Absence of influence of endotoxin contamination was strictly ascertained. To gain clinically relevant data that can additionally corroborate any conclusions from the initial animal experiments, the same set of parameters was monitored in two groups, namely cancer patients after treatment with defined quantities of lectin in the complete extract and healthy volunteers after injection of extract containing lectin and, notably, of extract, selectively and completely depleted of lectin without altering any other extract characteristic. Therefore, our hypothesis of the importance of the presence of lectins in the extract was attempted to be substantiated independently in different sets of experimental series with the purified proteins and with the extract, especially after removal of the supposedly decisive components.

MATERIALS AND METHODS

Purification of Lectins from Mistletoe Extracts (Iscador). Affinity matrices for lectin purification were prepared by coupling carbohydrates to divinyl sulfone-activated Sepharose 4B and their capacity was determined as described (10). Binding of lectin to the affinity ligand on the matrix was accomplished batchwise by incubation of mistletoe extract (Iscador QuFrF batch number 166) in a sample rotator overnight at 4°C. The slurry was then filled into a column (4 ml resin for 3 ml extract). The amount of resin was chosen to exceed by far the lectin content in the extracts to ensure complete lectin depletion of the extract. Following incubation with lactose-Sepharose 4B, the flow through was similarly treated with D-galactosamine-Sepharose 4B and the lectins were eluted by specific sugar, and additionally, 0.2 M glycine (pH 2.6) in the case of the second column, as described (5, 7). The last eluant was immediately neutralized to avoid lectin inactivation. All eluants were then extensively dialyzed against water to remove sugar and salt and analytical procedures were performed as described (11). Separation of lectin subunits was achieved by *in situ* cleavage of the disulfide bond with β-mercaptoethanol and chromatographic separation.

Preparation of Polyclonal Antibody to Mistletoe Lectin. When considering the lectin as antigen, it is imperative to realize that parts of the protein part as well as of the carbohydrate part of the glycoprotein may serve as antigenic epitopes. To raise specific polyclonal antibodies in rabbits to the protein part of the purified lectin periodate oxidation of the carbohydrate chains of the lectin was performed at a concentration of 2 mM sodium periodate for 10 min at 4°C. Residual periodate was quenched by addition of 100 μl glycerol. Following repeated cycles of concentrating the solution by ultrafiltration and dilution with phosphate-buffered saline (20 mM, pH 7.2) reactive dialdehydes in the carbohydrate chains of the lectin were reduced by addition of 20 mM $NaBH_4$ for 20 min at 4°C. After removal of the reagent by dialysis, the lectin was further treated with 2% formaldehyde for 3 days at room temperature. Excess reagents were again removed and immunization was initiated by an intradermal injection of 50 μg periodate-oxidized,

Received 3/30/89; accepted 5/26/89.

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[1] This study was supported in part by a grant from the BMFT program on alternative methods for cancer therapy.

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[3] The abbreviations used are: PMN, polymorphonuclear; NK, natural killer; LGL, large granular lymphocytes; ELLA, enzyme-linked lectin assay; LD_{50}, dosage level resulting in 50% lethality; LAL, limulus amoebocyte lysate; CL, chemiluminescence; PBS, phosphate-buffered saline.

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formaldehyde-treated lectin in ABM solution (antibody multiplier, Linaris GmbH, Bettingen, FRG) according to the instructions of the manufacturer. At 3-week intervals, three booster injections were given, and postboost bleeding was taken 1 week later. The IgG fraction of serum was purified by chromatography on protein A-Sepharose 4B (Pharmacia, Freiburg, FRG). Ouchterlony double diffusion, immunoblotting, and immunospotting (both with the indicator conjugate goat anti-rabbit peroxidase) for ascertaining the antibody specificity were performed with the IgG fraction of preimmune serum and serum after injections as described (12).

Quantification of the Lectin Content in Mistletoe Extract (Iscador) by Application of an Optimized ELLA. Quantitative determination of the lectin content was carried out with an optimized ELLA technique, based on lectin binding to an immobilized ligand (asialofetuin) and subsequent binding of specific antibody to the bound lectin, essentially as described (13). The specific binding of rabbit antibodies was quantitatively assessed using goat anti-rabbit peroxidase, the subsequent generation of a colored product from the substrate phenylenediamine hydrochloride and measurement in an enzyme-linked immunosorbent assay plate reader at 492 nm.

Animals. Twelve New Zealand rabbits, divided into two groups of six female and six male animals with an individual age in the range of 5–14 weeks, obtained from Madörin AG (Füllinsdorf, Switzerland), were included into the *in vivo* study of immunomodulatory potency of the purified lectin (ML I). The injected dose was well below the LD_{50} level, determined in mice in RCC AG (Itingen, Switzerland). The weight of the rabbits varied between 3.4 and 4.8 kg, the average is 4.2 kg (±0.31). All the assays were performed at least in triplicate, referring to separate, not sex-matched measurements on different rabbits.

Healthy Volunteers and Patients. Four healthy female subjects with an average age of 53.5 years (±12) were tested to compare the immunomodulatory effects of Iscador either containing the regular lectin content or being selectively depleted of lectin by one cycle of affinity chromatography. For studies on patients, only regular Iscador was given. Herein, immunological parameters of 14 patients with histologically documented, severely disseminated breast cancer were studied after a single i.v. injection of fermented Iscador. The age of the female patients varied between 38 and 68 years (average ± SD = 54.9 ± 10.7 years). Another group of 17 patients, similarly suffering from histologically documented, disseminated breast cancer, received a single s.c. injection of unfermented iscador and monitoring of immunological parameters was similarly carried out. The age distribution of this group, receiving a s.c. instead of an i.v. injection, was between 32 and 58 years (average ± SD = 45.4 ± 8.1 years). We strictly ensured that all patients gave their informed consent and also agreed to blood samples, being taken for immunological investigations.

Therapeuticals. New Zealand rabbits were monitored after a single i.v. injection of biochemically purified lectin (ML I) or its subunits, derived from the clinically applied extract. Within the schedule of the i.v. injections, 14 patients received the commercially available fermented Iscador MSF (batch number 7F-7365) which was given in a 250-ml saline infusion. The four female healthy volunteers and the group of 17 patients received their single injection of unfermented, commercially available Iscador QuFrF (batch number 166) or lectin-depleted Iscador s.c. To ensure complete biochemical removal of lectin from the extract, the size of the columns exceeded by far the immunologically determined lectin quantity. However, additional quantitative measurements were carried out to verify complete absence of lectin after specific removal by affinity chromatography.

Quantitative Determination of Endotoxin Contamination. All batches, especially after biochemical processing including purified lectin and its subunits, were regularly checked with a quantitative kinetic LAL microtiter assay, to rigorously exclude endotoxin contamination. This LAL assay, which is able to determine sample-related interferences, was regularly carried out at Heidelberg University (FRG) by Dr. K. B. Becker and Dr. R. Urbaschek as described in detail elsewhere (14).

Assay of NK Cytotoxicity and Frequency of LGL. The applied methods were carried out, as described in detail previously (15, 16). Briefly, mononuclear cells were isolated by Ficoll-Hypaque gradient. To assess cellular cytotoxicity in correlation to the number of effector cells, a fixed amount of K_{562} target cells, namely 2.5×10^3 cells, was added to effector cells at different concentration to yield final effector to target cell ratios of 50:1, 25:1, 10:1, as well as 5:1 in a final volume of 200 μl. 2 μCi [*methyl*-^{3}H]thymidine (specific activity, 25 Ci/mmol; Amersham, England) was added to the wells immediately after the preparation of the cell suspension. Subsequently to an incubation for 18–20 h the cells were harvested and the [^{3}H]thymidine incorporation was quantified in a liquid scintillation counter. The spontaneous incorporation of label referred to the activity of the target cells. The percentage of specific inhibition (P_i), exerted by the cytolytic activity of the effector cells, was calculated from the following formula:

$$P_i = \left(1 - \frac{\text{Test incorporation}}{\text{Spontaneous incorporation}}\right) \times 100.$$

All assays were performed in triplicate. [^{3}H]Thymidine incorporation into the effector cells was also similarly measured. Since this value was negligibly low, this factor could be consciously disregarded. The number of effector cells yielding a specific inhibition of 33% (P_i-33) was calculated using a semilogarithmic scale. These P_i-33 values were used as a valuable means of comparing the various experimental results. In addition to functional measurements morphological examinations were carried out by light microscopy to assess the frequencies of lymphocytes, LGL cells, monocytes, and granulocytes that were present in the mononuclear cell population isolated for the NK assays. A correction factor was obtained which accounted for cells other than lymphocytes. The frequency of LGL in peripheral blood was determined by morphological examinations in leukocyte concentrates (15).

CL Assay of PMN Leukocytes. The assay was carried out as described elsewhere (17) with slight modifications. In detail, PMN leukocytes from human peripheral blood were isolated by Percoll gradients (first step: 71/55%, second step: 63/55%). The solutions were centrifuged for 20 min and for 10 min, respectively, PMN leukocytes were then washed in isotonic saline and resuspended in PBS containing albumin (1 g/liter) and glucose (1 g/liter) at a cell density of 3×10^5 cells/ml. 200 μl of this PMN leukocyte cell suspension were preincubated together with 1 ml PBS and 100 μl Luminol solution (0.2 mmol/liter) for 10 min at 37°C. The reaction was initiated by addition of 40 μl opsonized zymosan (50 g/liter) obtained from the same batch for each series of the examinations to ensure valid comparison. CL was measured in a liquid scintillation counter. The assays were performed in triplicates and the average of the maximum values of CL, which were usually reached between 20 and 60 min, was taken as a measure of phagocytosis activity. PMN leukocytes from blood of rabbits were separated employing NH_4Cl hemolysis. In detail, 500 μl of heparinized blood were incubated with 10 ml ice-cold NH_4Cl (8.3 g/liter NH_4Cl, 37 mg/liter Na_2EDTA, 1 g/liter $KHCO_3$) for 5 min. After centrifugation (5 min, 200 × *g*) the cells were washed and adjusted to cell concentration of 3×10^5 granulocytes per milliliter. The CL was measured for these cells exactly, as described above.

Statistical Evaluation. All data were evaluated by the employment of Student's *t* test. A value of $P < 0.05$ was considered significant and a value of $P \geq 0.05$ nonsignificant.

RESULTS

Lectin Purification and Its Quantitative Determination. A prerequisite for meaningful studies to conclusively elucidate the relationship of defined extract components and their influence on immunological parameters, favorable to the prognosis of therapy, is the reproducible purification and standardization of the supposedly crucial substance. Application of affinity chromatography on a support with immobilized lactose to the commercially available and proprietary mistletoe extract (Iscador), itself used in cancer therapy, yielded predominantly one type of protein, the β-galactoside-specific lectin ML I. It is composed of two subunits with different functional domains, the toxic subunit with an apparent molecular weight of 29,000, termed A, and the carbohydrate-binding subunit with an appar-

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ent molecular weight of 34,000, termed B (Fig. 1). Chromatographically separated subunits revealed no cross-contamination at the level of the highly sensitive silver staining procedure (Fig. 1). Compared to the major lectin only amounts of less than 15% of total lectin quantity were due to the presence of the closely related lectins ML II and ML III, purified by additional affinity chromatography on a support with immobilized galactosamine (not shown).

Quantitative determination of the lectin content was based on application of specific antibodies, raised against ML I. Its carbohydrate chains, generally the main immunological epitopes, had been destroyed by preceding periodate treatment to restrict antibody binding to antigenic determinants, established by peptide sequences. To focus evaluation of lectin to functionally active, not just immunologically reactive lectins in the extract, binding of the lectins to a natural glycoprotein as ligand, immobilized on microtiter plates, preceded the immunological detection. This assay enabled reliable determination of lectin concentrations in original or concentrated extracts (Fig. 2).

Effect of Lectin on the Activity of NK Cells and the Frequency of LGL in Rabbits. In the first step to assess the potential significance of the lectin for immunomodulation the effect of injection of purified lectin from extract on immunological parameters in a group of 12 rabbits was monitored. The injected individual doses were five to six orders of magnitude below the LD_{50} value of ML I. Initial measurements on the dependence of immunological effects and amount of injected lectin revealed that 0.8 ng/kg ML I and 0.4 ng/kg carbohydrate-binding B chain gave the highest response. NK cytotoxicity and frequency of LGL in peripheral blood of the rabbits were significantly enhanced between 24 and 72 h following a single i.v. injection of the optimal dose of ML I (Fig. 3, *A* and *B*) and, notably, its carbohydrate-binding B chain (Fig. 3, *C* and *D*). Injection of saline in control experiments was found to be ineffective to lead to any parameter changes.

Since the results in Fig. 3 appeared to indicate that the lectin may influence both the NK activity and the number of LGL, additional experiments were carried out with varying ratios of effector/target cells to further corroborate this assumption. Following injection of various doses of lectin or its carbohydrate-binding B chain, the experimentally determined P_i-33 value, referring to the proportion of effector cells that resulted in an inhibition of [^{3}H]thymidine incorporation into target cells of 33% (see "Materials and Methods"), should exhibit similar changes in relation to NK activity and LGL frequency, if only the cell number can be influenced by the lectin. The results demonstrated no such clear correlation, emphasizing modulatory effects on effector cell number and their reactivity (Fig. 4). With respect to the specificity of the molecular interaction, initiating these reactions, it is remarkable that the A chain of ML I that has no capacity to selectively bind to cellular glycoconjugates in contrast to the B chain completely failed to affect the NK activity at a concentration of 0.4 ng/kg, where the carbohydrate-binding B chain was effectively enhancing the NK-mediated defense.

In addition to the lectin ML I, quantitatively less pronounced effects on these parameters were detectable for the closely related minor lectin species ML II and ML III, given at a suitable concentration in comparison to ML I (Fig. 5).

Effect of Lectins from Mistletoe Extracts on Temperature. Besides responses to lectin injection on the cellular level monitoring of lectin-dependent effects was extended to other parameters. The increases in NK activity and LGL frequency in blood of rabbits following the i.v. injections of purified lectins were preceded by several signs of an acute phase response in the first 24 h, such as neutrophilia with a shift to juvenile form, as described in detail later, and small, but significant elevation of temperature. We thus at first determined the extent of elevation of temperature, characteristic for acute phase reactions, accompanying the changes at the cellular level. In the first 48 h after i.v. lectin injections the following maximum increases (±SE) in temperature (relative to baseline values obtained from averages of temperature measured during 24 h before treatment) were observed: 0.8 (±0.2)°C after 0.8 ng/kg ML I; 0.63 (±0.15)°C

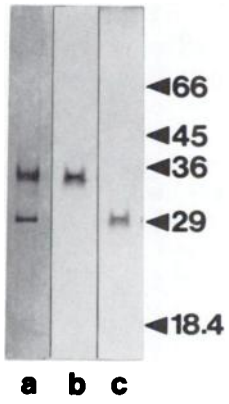

Fig. 1. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate of mistletoe lectin I and its subunits from Iscador(R) after purification by affinity chromatography (*lane a*) and reductive cleavage of the disulfide bond and chromatographic separation (*lane b*, β-galactoside-binding subunit; *lane c*: toxic subunit). Standards for molecular weight designation, indicated by *arrowheads*, are: bovine serum albumin (M_r 66,000), egg albumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000), and β-lactoglobin (M_r 18,400).

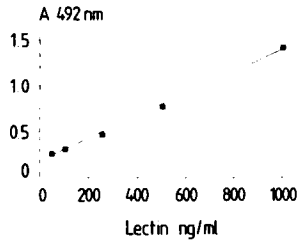

Fig. 2. Quantitative determination of ML I by an optimized ELLA. Wells were coated with 100 μl of asialofetuin (0.1 mg/ml in PBS). The dilutions of anti-ML I and horseradish peroxidase-secondary antibody, applied following incubation with lectin-containing solution were 1:20,000, and 1:2,500, respectively.

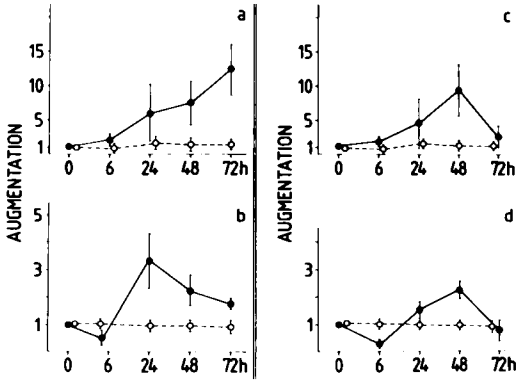

Fig. 3. Influence of injection of optimal doses of mistletoe lectin ML I (*a*, *b*) and its carbohydrate-binding subunit B (*c*, *d*) on two immunological parameters of rabbits. Monitoring of relative values of NK activity (*a*, *c*), measured functionally, and LGL frequency (*b*, *d*), determined morphologically, was performed at various intervals after a single i.v. injection of 0.8 ng/kg ML I (*a*, *b*) and 0.4 ng/kg B chain (*c*, *d*). The results after lectin injections are shown with closed circles (●). Results of control experiments are displayed with open circles (○). All assays within the group of 12 animals are performed in triplicate. Mean values ± SE are shown.

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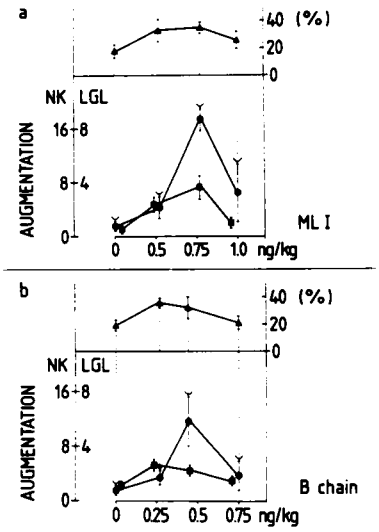

Fig. 4. Maximum increases in NK activity (●) and LGL frequency (■) observed in blood of rabbits between 24 and 72 h following a single i.v. injection of various doses of ML I (*a*) and its B chain (*b*). In addition the frequency of LGL among the effector lymphocyte population (▲) investigated functionally against K_{562} target cells is also shown. All assays were performed in triplicate. Mean values ± SE are shown.

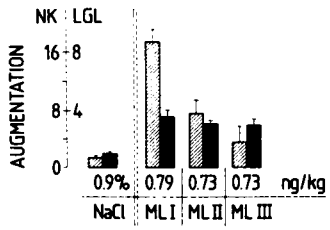

Fig. 5. Influence of i.v. injections of mistletoe lectins ML I, II, and III on two immunological parameters in rabbits. Maximum increases in NK-cytotoxicity (▨) and LGL frequency (■) observed between 24 or 72 h after a single i.v. injection of ML I, II, and III into rabbits. As negative control isotonic saline was given. The assays were performed in triplicate. Mean values ± SE are shown.

after 0.4 ng/kg B chain; 0.6 (±0.2)°C after 0.73 ng/kg ML II; and 0.43 (±0.08)°C after 0.73 ng/kg ML III. Each value for temperature elevation was found to be statistically significant ($P < 0.05$). The increases occurred mainly immediately after the i.v. injection. The maximum elevations of temperature correlated with responses concerning NK activity as well as LGL frequency, when the same doses of ML (0.8 ng/kg) and B chain (0.4 ng/kg), respectively, were given. It is noteworthy that the toxic A chain that has no carbohydrate-binding domain failed to induce temperature changes at the same dose, which was found to be optimal for the B chain (0.4 ng/kg). A similar lack to initiate immunomodulation had been noted for the NK activity in the preceding section. The influence of endotoxin contamination, suggestive as a cause for acute phase reactions, was thoroughly ruled out in each case with the highly sensitive kinetic LAL assay. The following limits can be given for the individual batches following biochemical procedures: in ML I <0.0002 ng/kg, in ML II <0.0028 ng/kg, in ML III <0.0005 ng/kg and in B chain preparations <0.00004 ng/kg of endotoxin.

Comparison of Effects of Isolated Mistletoe Lectin I and Mistletoe Extracts on Several Immunological Parameters of Rabbits and Breast Cancer Patients. Any tests of the isolated lectin in clinical trials is at present precluded. Only the extract has federal approval for clinical application. Consequently, we chose to provide two independent lines of evidence to address the question, whether the changes observed after lectin injections into rabbits are of clinical relevance. Firstly, we measured the course of alterations in immunological parameters after i.v. and s.c. injection of mistletoe extracts into cancer patients. Secondly, immunological parameter changes were evaluated after s.c. injection of complete and lectin-depleted extracts into healthy volunteers, as described later. The effective lectin concentration of the applied extract batch for this series of experiments on cancer patients was carefully determined by the ELLA technique. Fourteen patients with breast cancer received a single i.v. infusion of Iscador (MSF 7F 7365). The mean dosage (±SD) was 0.33 (±0.07) mg/kg plant substance, which corresponded to 1.65 (±0.35) ng/kg ML I. Endotoxin contamination was determined to be <0.001 ng/kg/ml. In comparison between rabbits, i.v. receiving the optimal dose of purified ML I, and cancer patients, i.v. receiving mistletoe extract, qualitatively similar profile changes were measurable (Fig. 6, *A* and *B*). Nearly indistinguishable changes were noted for phagocytic activity and frequency of LGL. Moreover after both injections a neutrophilia ($P < 0.10$; $P < 0.005$) with a shift to the juvenile forms was observed (Fig. 6, *A* and *B*). Concomitantly, a slight elevation in the number of lymphocytes occurred ($P < 0.19$; $P < 0.05$).

In a second set of experiments 17 patients with breast cancer received a single sc. injection of Iscador (QuFrF 166). The mean dosage was 0.17 mg/kg plant substance, which corresponded to 12 ng/kg ML I. Endotoxin contamination was determined to be <0.045 ng/kg. As shown in Fig. 6*C*, the injections caused the same pattern of alterations for the measured cell responses. The count of PMN leukocytes increased 6 and 24 h after the injection 1.36- and 1.38-fold ($P < 0.0025$; $P < 0.05$), the phagocytic activity of PMN leukocytes was augmented by a factor of 1.38 and 1.35 ($P < 0.0025$; $P < 0.0025$). The frequency of LGL was enhanced only after 24 h 1.52-fold ($P < 0.05$). Overall, the efficiency of immunomodulation within this protocol of s.c. injection of a higher amount of lectin was found to be reduced in comparison to the aforementioned schedule.

Effect of Unfermented Mistletoe Extract (Iscador QuFrF 166) before and after Lectin Depletion on Immunological Parameters of Healthy Volunteers. To establish further evidence for the role

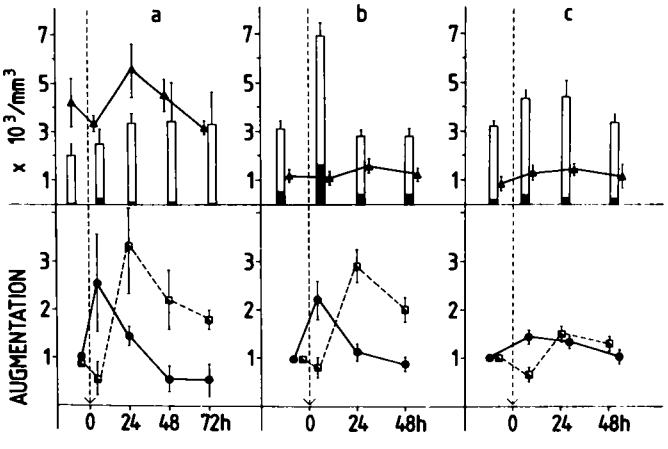

Fig. 6. Comparison of several immunological parameters after the following injections: *a*, three rabbits received a single i.v. injection of 0.8 ng/kg mistletoe lectin ML I; *b*, 14 patients with breast cancer were investigated after a single i.v. injection of 0.33 mg/kg mistletoe extract which corresponds to 1.65 ng/kg mistleton lectin ML I; *c*, 17 patients with breast cancer received 0.17 mg/kg plant substance s.c., which corresponds to 12 ng/kg mistletoe lectin ML I. Assessment of the following parameters at various intervals after the injections comprised: absolute number of segmented (□) and young neutrophils (■), as well as of lymphocytes (▲) in 1 μl blood. Relative values of CL response of PMN leukocytes (●) and frequency of LGL in 1 μl blood (◪). Mean values ± SE are shown.

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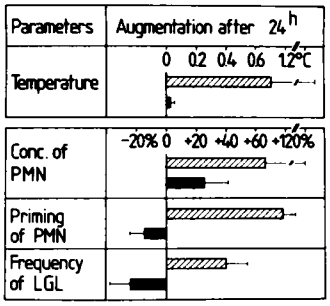

Fig. 7. Differences in several parameters observed in four healthy female volunteers 24 h after a single s.c. injection of Iscador (QuFrF 166) with the same dose of plant substance (0.045 mg/kg). Lectin was either present at a concentration of 3.1 ng/kg (▨) or completely and selectively removed by affinity depletion (■). Mean values ± SE are shown.

of lectins with respect to the immunomodulatory efficiency of Iscador, ML I, II, and III were completely removed from the QuFrF 166 preparation by chromatographic depletion without any further alteration in the extract composition. This absence of a certain compound, achieved by a specific process, caused significant differences in the course of parameter changes observed in four female healthy volunteers 24 h after a single s.c. injection of complete Iscador as well as lectin-depleted Iscador. To allow meaningful comparison the same dose of plant substance (0.045 mg/kg) was given. Its only difference was the presence of lectin (3.1 ng/kg ML I) or its absence. Again, endotoxin contamination was determined to be <0.015 ng/kg, consequently it cannot affect these results. Each parameter (elevation in temperature, count of PMN, CL responsiveness of PMN, and frequency of LGL) differed considerably between the two groups (Fig. 7). In addition, after the injection of Iscador, which did not contain lectin, decreases in priming of PMN and frequency of LGL were found (Fig. 7). This cytostatic effect may be related to the presence of viscotoxins that are present in a dose of 22 ng/kg, as established by high-performance liquid chromatographic analysis.[4]

DISCUSSION

Plant extracts with phenomenologically indicated clinical benefit offer valuable sources to detect and define otherwise undetectable classes of substances with importance for immunomodulation in cancer therapy. Inevitably, the supposedly effective isolated substances of the extract should prove their favorable effect in subsequent *in vivo* studies. This rational prompted our initial investigation on lectin I of mistletoe extract.

The present results indicate that small and nontoxic doses of mistletoe lectin ML I are able to activate tumoricidal effector mechanisms. As is well known, many types of effector cells participate in cytolytic activity against tumors. These include not only T-lymphocytes, but also granulocytes, activated macrophages, NK cells, and lymphokine-activated killer cells (18). NK cells are of particular interest because they apparently play an important role in immune surveillance against primary tumors and in defense against tumor spread (19–22). Our present study demonstrates that ML I and, even more remarkable, its carbohydrate-binding B chain alone significantly increased the activity and recruitment of NK cells in peripheral blood of rabbits. The absence of detailed knowledge on the molecular mechanisms for these enhancements notwithstanding, it can be suggested that ML I plays a decisive role in the immunomodulatory effects described following therapy with mistletoe extract (4, 7, 23, 24). The animal studies with purified lectin, mimicking parameter changes after administration of the complete extract to cancer patients, and the effects of complete lectin depletion of extracts on selected immunological parameters in healthy volunteers underscored this conclusion. The removal of ML I from unfermented Iscador resulted only in toxic effects in healthy volunteers who were injected with such lectin-free preparations. This residual toxicity may be related to viscotoxins which are toxic polypeptides with low molecular weight and some data indicate that they can act cytolytically by damage of cell membrane at high concentration levels (25). Thus, we cannot at present rule out a synergism between the lectin ML I and viscotoxins in the complete extract. Indeed, when taking the complete extract, an enhancement in NK cytotoxicity against K_{562} cell "*in vitro*" in the presence of fractions with low molecular weight from mistletoe extracts had been reported (26). It may indicate that NK cells activated by ML I "*in vivo*" may be more effective against tumor targets which have already been acted on by viscotoxin. In addition, viscotoxins had been suggested to increase cell-mediated cytotoxicity by inhibition of suppressor cell activity (27).

The difference of responsiveness in tumor patients and healthy persons warrants emphasis. When healthy persons received a single s.c. injection of mistletoe extract (Iscador QuFrF 166) that was nearly four times less the dose than that of cancer patients, it was still able to induce similar immune responses. This quantitative difference betweeen the two groups suggests that cancer patients may exhibit less sensitivity to lectin. This observation may be related to a depression in efficiency of the natural host defense system observed in tumor patients (28–31). However, an inhibitory effect of circulating anti-ML I antibody as a result of previous treatment with mistletoe extracts may also play a role in this diminished sensitivity of patients.

In conclusion, our results from "*in vivo*" studies provide evidence for immunomodulatory potency of the β-galactoside-specific plant lectin ML I, a constituent of a clinically approved plant extract. The presented data encourage detailed delineation of the molecular trigger mechanisms that apparently are initiated by specific protein (lectin)-carbohydrate interactions by in-depth "*in vitro*" measurements, *e.g.*, assessment of lectin-dependent effects on the release of cytokines in appropriate test system. Such measurements are in progress.

[4] Kohlhase, VfK Arlesheim, Switzerland, personal communication.

REFERENCES

1. Gabius, H. J. Endogenous lectins in tumors and the immune system. Cancer Invest., *5:* 39–46, 1987.
2. Gabius, H. J. Tumorlectinology: at the intersection of carbohydrate chemistry, biochemistry, cell biology and oncology. Angew. Chem. Int. Ed. Engl., *27:* 1267–1276, 1988.
3. Sharon, N. Carbohydrates as recognition determinants in phagocytosis and in lectin-mediated killing of target cells. Biol. Cell., *51:* 239–246, 1984.
4. Hajto, T. Immunomodulatory effects of Iscador: a Viscum album preparation. Oncology, *43*(Suppl. 1): 51–65, 1986.
5. Franz, H. Mistletoe lectins and their A and B chains. Oncology, *43*(Suppl. 1): 23–34, 1986.
6. Holtskog, R., Sandvig, K., and Olsnes, S. Characterization of a toxic lectin in Iscador(R), a mistletoe preparation with alleged cancerostatic properties. Oncology, *45:* 172–179, 1988.
7. Luther, P., Theise, H., Chatterjee, B., Karduck, D., and Uhlenbruck, G. The lectin from Viscum album L. Isolation, characterization, properties and structure. Int. J. Biochem., *11:* 429–435, 1980.
8. Olsnes, S., Stirpe, F., Sandvig, K., and Pihl, A. Isolation and characterization of viscumin, a toxic lectin from Viscum album L. (mistletoe). J. Biol. Chem., *257:* 13263–13270, 1982.
9. Metzner, G., Franz, H., Kindt, A., Fahlbusch, B., and Süss, J. The *in vitro* activity of lectin I from mistletoe (ML I) and its solated A and B chains on

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functions of macrophages and polymorphonuclear cells. Immunobiology, *169:* 461–471, 1985.
10. Gabius, H. J., and Engelhardt, R. Interaction of mannose-binding proteins with different types of immobilized ligands. J. Chromatogr., *391:* 452–456, 1987.
11. Gabius, H. J., and Engelhardt, R. Sugar receptors of different types in human metastases to lung and liver. Tumor Biol., *9:* 21–36, 1988.
12. Gabius, H. J., Engelhardt, R., Schröder, F. R., and Cramer, F. Evolutionary aspects of accuracy of phenylalanyl-tRNA synthetase: accuracy of fungal and animal mitochondrial enzymes and their relationship to their cytoplasmic and a procaryotic enzyme. Biochemistry, *22:* 5306–5315, 1983.
13. Vang, O., Larsen, P., and Bog-Hansen, T. C. A new quantitative and highly specific assay for lectin-binding activity. *In:* T. C. Bog-Hansen, F. van Driessche (eds.), Lectin-Biology, Biochemistry and Clinical Biochemistry, Vol. V, pp. 637–644. Berlin: Walter de Gruyter, 1986.
14. Ditter, B., Becker, K. B., Urbaschek, R., and Urbaschek, B. Quantitativer Endotoxin-Nachweis. Automatisierter, kinetischer Limulus-Amöbozyten-Lysat-Mikrotiter-Test mit Messung probenabhängiger Interferenzen. Arzneim.-Forsch., *5:* 681–687, 1983.
15. Hajto, T., and Lanzrein, C. Frequencies of large granular lymphocytes in peripheral blood of healthy persons and breast cancer patients. Cancer Immunol. Immunother., *16:* 65–66, 1983.
16. Hajto, T., and Hostanska, K. Effect of "*in vivo*" hyperthermia on human natural killer cells. Clin. Trials J., *22:* 514–520, 1985.
17. Hajto, T., and Hostanska, K. An investigation of the ability of Viscum album-activated granulocytes to regulate natural killer cells: "*in vivo.*" Clin. Trials J., *23:* 345–358, 1986.
18. Urban, J. L., and Schreiber, H. Host-tumor interactions in immunosurveillance against cancer. *In:* J. M. Cruse, R. E. Lewis (eds.), Immunomodulation of Neoplasia, pp. 17–68. Basel: Karger, 1988.
19. Hanna, N. Expression of metastatic potential of tumor cells in young nude mice is correlated with low levels of natural killer cell-mediated cytotoxicity. Int. J. Cancer, *26:* 675–680, 1980.
20. Herbermann, R. B., and Ortaldo, J. R. Natural killer cells. Their role in defenses against disease. Science (Wash. DC), *214:* 24–30, 1981.
21. Ojo, E. Positive correlation between the levels of natural killer cells and *in vivo* resistance to syngeneic tumour transplants as influenced by various routes of administration of *Corynebacterium parvum* bacteria. Cell. Immunol., *45:* 182–187, 1979.
22. Talmagde, J. E., Meyers, K. M., Prieur, D. J., and Starkey, J. R. Role of NK cells in tumor growth and metastasis in "beige" mice. Nature (Lond.), *284:* 622–624, 1980.
23. Bloksma, N., Schmiermann, P., de Reuver, M., van Dijk, H., and Willers, J. Stimulation of humoral and cellular immunity by Viscum preparations. Planta Medica, *46:* 221–227, 1982.
24. Rentea, R., Lyon, E., and Hunter, R. Biologic properties of Iscador: a Viscum album preparation. Lab. Invest., *44:* 43–48, 1981.
25. Franz, H., Kindt, A., Eifler, R., Ziska, P., Benndorf, R., and Junghahn, J. Differences in toxicity and antigenicity between mistletoe lectin I and Viscotoxin A3. Biomed. Biochem. Acta, *42:* K21–K25, 1983.
26. Hamprecht, K., Handgretinger, R., Voetsch, W., and Anderer, F. A. Mediation of human NK-activity by components in extracts of Viscum album. Int. J. Immunopharmacol., *9:* 199–209, 1987.
27. Wagner, H., Jordan, E., and Zänker, K. S. Cell-mediated and direct cytotoxicity of purified ingredients of Viscum album. J. Cancer Res. Clin. Oncol. Suppl. D-Ther., *37:* 53, 1987.
28. Boetcher, D. A., and Leonard, E. J. Abnormal monocyte chemotactic response in cancer patients. J. Natl. Cancer Inst., *52:* 1091–1099, 1974.
29. Mullen, C. A., Urban, J. L., Van Waes, C., Rowley, D. A., and Schreiber, H. Multiple cancers: tumor burden permits the outgrowth of other cancers. J. Exp. Med., *162:* 1665–1682, 1985.
30. Siepl, C., Bodmer, S., Frei, K., MacDonald, H. R., De Martin, R., Hofer, E., and Fontana, A. The glioblastoma derived T cell suppressor factor/transforming growth factor—2 inhibits T cell growth without affecting the interaction of interleukin 2 with its receptor. Eur. J. Immunol., *18:* 593–600, 1988.
31. Snyderman, R., Seigler, H. F., and Meadows, L. Abnormalities of monocyte chemotaxis in patients with melanoma: effects of immunotherapy and tumor removal. J. Natl. Cancer Inst., *58:* 37–41, 1977.

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