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Mistletoe extract-induced effects on immunocompetent cells: *in vitro* studies

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Cytotoxic as well as immunomodulatory effects of mistletoe extracts and their components have been described and seem to depend upon the host tree, the manufacturing process and the composition of the different components present in the extracts. *In vitro* studies showed that a fermented mistletoe extract derived from *Viscum album* L. grown on pine trees was less cytotoxic to peripheral blood mononuclear cells (PBMC) than other preparations. This finding could be related to its very low content of mistletoe lectins. Furthermore, this extract stimulated PBMC from healthy and especially allergic donors who had never received any mistletoe treatment. By analysing these *in vitro* reactions, an involvement of CD4+ T helper cells and CD14+ monocytes/macrophages was observed, suggesting an interaction of the specific and non-specific immune system. In the supernatants of stimulated PBMC from healthy individuals, type 1- (interferon-γ and interleukin-2) and type 2- (interleukin-4 and interleukin-5) associated cytokines were detected in about 20%. In patients with colorectal tumours, however, type 1-associated cytokines were found with a significantly reduced frequency, suggesting a functional impairment of certain immunocompetent cells in these patients. These studies may help to evaluate properties of the natural and the specific immune system.

Keywords: Mistletoe, stimulation of immune system, T helper cells, monocytes/macrophages, T helper-1 cytokines, T helper-2 cytokines.

Introduction

A large number of *in vitro* and *in vivo* studies have been carried out in recent years on the cytotoxic and immunomodulatory effects of mistletoe extracts used for adjuvant cancer treatment. Different components of these extracts are thought to be responsible for these effects [1–8]. However, there are also studies that have tried to show that only mistletoe lectin-I is responsible for the beneficial immunomodulatory effects [9].

Our present studies strong evidence that other non-lectin components may be responsible for the effects observed in the *in vitro* and *in vivo* studies.

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Cytotoxic and stimulatory effects of mistletoe extracts on peripheral blood mononuclear cells from untreated individuals *in vitro*

In order to evaluate the cytotoxic effect of different mistletoe extracts, peripheral blood mononuclear cells (PBMC) from healthy donors were incubated with six commercially available preparations: aqueous (*Helixor*) or fermented (Iscador) extracts and vesicular juices (*Abnobaviscum*; micelles) derived from *Viscum album* L. grown on apple trees or on pine trees. A dose-dependent inhibition of the proliferation could be detected by measuring ^{3}H-thymidine incorporation. However, the fermented mistletoe extract Iscador Pini (IP) exerted cytotoxic effects only at very high concentrations (10 000 µg/ml, referred to the weight of fresh plant). By Western blotting, these findings could be related to the very low amount of lectins, since this extract contained no mistletoe lectin-I and only very faint bands of mistletoe lectin-II or mistletoe lectin-III were detectable in contrast to pronounced lectin bands in the other five extracts [10].

Analysing the stimulatory potency of these six extracts to PBMC it became evident that only IP led to a strong mitogenesis in the cell cultures of untreated allergic individuals both in the presence and in the absence of anti-lectin antibodies which neutralized the extract's cytotoxicity [10]. These data suggested that components other than the lectins were responsible for the observed effects. Further immunological characterization of this extract revealed that only 10–20% of PBMC from normal controls, but about 65% from allergic individuals, proliferated strongly in response to IP *in vitro*. An antigen present in this extract, therefore, might be able to detect an allergic disposition in untreated individuals and to indicate that a kind of natural immunity towards archaic antigens of mistletoe extracts is present, especially in allergic individuals. This natural reactivity could be demonstrated on the cellular level, while on the humoral level no antimistletoe lectin-I or anti-IP antibodies of the

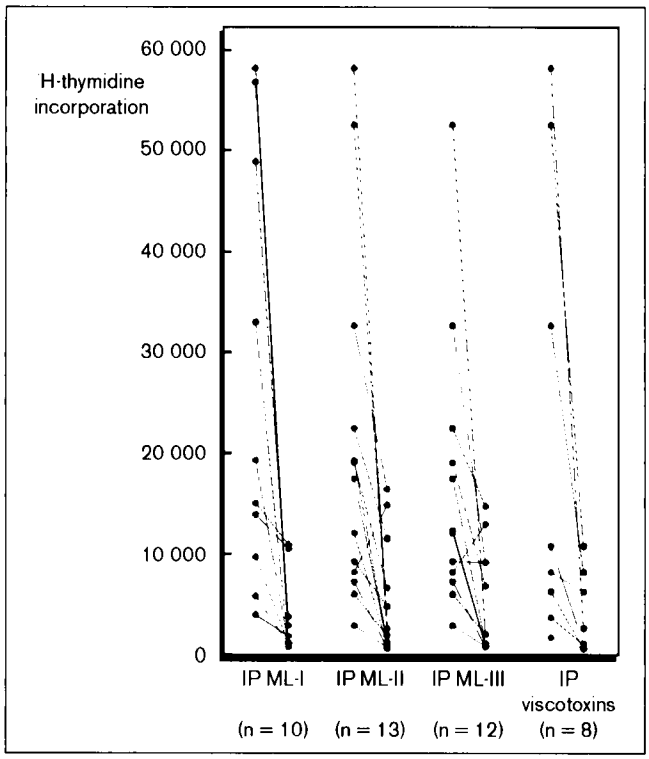

Figure 1. Stimulatory activity of *Iscador* Pini (IP), mistletoe lectins (ML-I, -II and -III) and viscotoxins on peripheral blood mononuclear cells of untreated healthy controls measured by ^{3}H-thymidine incorporation in autologous plasma in 7-day cultures. ML-II and ML-III were a kind gift from Dr Pfüller (Witten, Germany) and the viscotoxin fraction was kindly provided by Dr Schaller (Hiscia, Arlesheim, Switzerland).

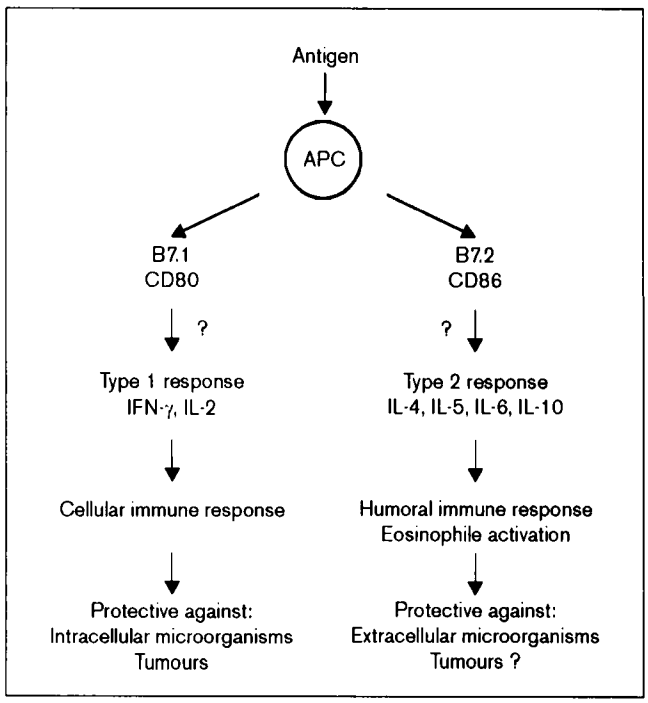

Figure 2. A possible role of the different co-stimulatory signals B7.1 (CD80) and B7.2 (CD86) for directing immune reactions towards a type 1 or type 2 response. APC, antigen presenting cell; IFN, interferon; IL, interleukin.

immunoglobulin G type could be found in untreated subjects [10].

Immunological characterization of the stimulatory antigen

To verify the hypothesis that a non-lectin component has to be responsible for the stimulation, the effects of a 'mistletoe lectin-I standardized' extract (*Eurixor*), the isolated lectins mistletoe lectin-I (Sigma, St Louis, USA), mistletoe lectin-II and mistletoe lectin-III (kindly provided by Prof. Pfüller, Witten, Germany) as well as a crude fraction of viscotoxins isolated from IP (a kind gift from the Forschungsinstitut Hiscia, Arlesheim, Switzerland) on PBMC of healthy and allergic individuals were analysed. As shown in Fig. 1, none of these substances, with only a few exceptions, were able to induce a similar ^{3}H-thymidine incorporation to that of IP. There were, however, great individual variations [11].

Endotoxins and the bacteria used for the fermentation process (*Lactobacillus plantarum*, kindly provided by Dr Werner, Forschungsinstitut Hiscia) could be excluded as the stimulatory antigen.

To characterize the relevant antigen more precisely, kinetic and flow cytometric studies were performed. Since in the previous analyses the reactivity was demonstrated in untreated individuals, the antigenic structure should behave like a primary 'non-recall' antigen (e.g. keyhole limpet haemocyanin), which stimulates naive cells. IP indeed showed the kinetics of a primary antigen, and flow cytometric analyses using the surface markers CD45RA for naive cells and CD45RO for memory/effector cells are also in favour of the concept of an interaction with naive cells. Thus, in the cell cultures, the proportion of naive cells decreased while that of memory/effector cells and that of cells of the transitional stage expressing both molecules increased due to IP stimulation [12].

Characterization of cell populations involved in the cellular response to Iscador Pini

The data described above allowed the conclusion that naive cells could be activated by a non-lectin-associated antigen present in IP because untreated individuals showed a strong stimulatory response to an antigen which behaves like a primary antigen.

Table 1. Frequency of *Iscador*Pini-induced cytokine profiles (%) in the supernatants of peripheral blood mononuclear cells from healthy controls, allergic individuals and tumour patients cultivated for 6 days in autologous plasma

Individuals	Type 1 reaction IFN-γ/IL-2	Type 2 reaction IL-4/IL-5	Type 0 reaction IFN-γ/IL-2 + IL-4/IL-5	Monocyte-associated reactivity TNF-α/IL-6
Healthy controls (n = 23)	22	17	4	96
Allergic individuals (n = 16)	13	25	0	100
Tumour patients				
Breast cancer (n = 20)	15	15	0	100
Colorectal tumours (n = 22)	5*	9	0	100

IFN, interferon; IL, interleukin. *P < 0.05, versus healthy controls by Mann-Whitney (Wilcoxon) two sample statistics.

Flow cytometric analyses of the cell populations involved in the stimulatory response to this mistletoe extract showed that CD4+ T cells and CD14+ monocytes were predominantly activated. Thus, an increased proportion of CD4+ T-helper cells expressed the activation marker CD25 (interleukin-2 receptor) and an increased proportion of monocytes expressed the human leukocyte antigen (HLA)-DR molecules, probably suggesting a better capacity for antigen presentation. B cells (CD19), T-suppressor cells/cytotoxic T cells (CD8) and natural killer cells (CD56) were only partially activated by IP [12].

Preliminary studies on the mistletoe extract-induced expression of co-stimulatory signals (CD80, CD86 [13]) as well as their respective receptors (CD28, CTLA-4) further confirm that interaction of antigen presenting cell (APC) with T-helper cells occur in this *in vitro* model (Stein and Berg, unpublished data, 1996). Detailed analyses of the involved T-helper cell subpopulations using flow cytometry are not yet possible, since no specific surface markers for the different subsets are known. However, it is proposed that the expression of CD80 on the APC seems to favour a type 1 response and the expression of CD86 a type 2-response (Fig. 2). The existence of these basic effector type 1 and type 2 subpopulations is now well accepted and precise characterization can only be performed by measuring the different cytokines they secrete [14,15].

Cytokine release in the supernatants of mistletoe extract-stimulated peripheral blood mononuclear cells

Flow cytometric data suggesting the activation of monocytes as well as T-helper cells were confirmed by measuring the release of the monocyte- (tumour necrosis factor-α and interleukin-6), type 1- (interferon-γ and interleukin-2) and type 2- (interleukin-4 and interleukin-5) related cytokines in the supernatants of IP-stimulated cultures of PBMC from healthy and allergic individuals. Thus, tumour necrosis factor-α and interleukin-6 were released in almost all cell cultures, while type 1- or type 2-associated cytokines were found in about 20% in healthy controls (Table 1). In the allergic individuals the type 2-response was, as expected, more pronounced. PBMC from tumour patients, however, secreted significant lesser amounts of tumour necrosis factor-α after IP stimulation and, in patients suffering from colorectal tumours, type 1-associated cytokines were also released in a significantly reduced frequency when compared to healthy controls [16]. These data suggest that IP functions as an indicator antigen for the activity of PBMC. Our data fit with reports in the literature detecting a reduced function of the monocyte/macrophage- and the type 1-associated immune system in tumour patients [17–19].

Conclusions

Our studies on the effects of mistletoe extracts on the immune system suggest that a non-lectin, non-viscotoxin-associated antigen present in the mistletoe extract IP induces a strong lymphocyte proliferation, especially in allergic but also in normal individuals, and thus probably indicates an allergic disposition in unexposed individuals. The involvement of T-helper cells and monocytes which could be demonstrated by flow cytometric analyses of the expression of activation markers and co-stimulatory signals provide evidence for a specific interaction of T-helper cells and antigen-presenting cells in this model. The non-lectin-, non-viscotoxin-associated release of monocyte/macrophage-associated cytokines and, to a lesser extent, the type 1- and type 2-related cytokines into the supernatants of the stimulated cell cultures could be demonstrated.

The relevance of these findings might be that mistletoe extracts favour a type 1 or a type 2 response that mainly mediate cellular or humoral immune reactions. From preliminary studies it has become evident that during mistletoe therapy a switch from a type 1 towards a type 2 response or vice versa may occur. Thus the individual condition of the patient may have a strong influence on the therapeutic efficacy of these extracts.

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