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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

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Mistletoe lectin (ML) I increases the production of cytokines by mononuclear cells and has been proposed as a useful biological response modifier in the treatment of cancer. Two other lectins, ML II and ML III, have been identified in mistletoe. We report that the N-terminal sequences of the three A chains of ML I, ML II and ML III are identical, and have interesting homology with the N-terminal sequences of the A chain of ricin-like toxins and of single-chain ribosome-inhibiting proteins. In addition, the three mistletoe lectins inhibit the growth of the human tumor cell line Molt 4, ML III being the most potent, followed by ML II and ML I. This inhibition is suppressed by addition of rabbit anti-ML I antibodies to the cultured cells. The data obtained suggest that the three lectins have amino acid sequences which show extensive homology and exert very similar biological effects. They may be derived from the same precursor.

Key words: Lectins, mistletoe, toxins.

Introduction

Mistletoe contains three toxic lectins, ML I, ML II and ML III. ML I is mainly specific for D-galactose, ML III for *N*-acetylgalactosamine and ML II for both sugars.[1–5] Recent publications show that ML I increases the production of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6 by mononuclear cells,[6,7] and that a bacterially fermented mistletoe preparation increases the production of these cytokines in the serum of cancer patients.[8] It has been proposed that the biological activity of mistletoe preparations is due to ML I.[6,8] However, these preparations contain other lectins, particularly ML II and ML III, which are not identical with ML I.[9–11] ML II and ML III have molecular weights smaller than ML I (63 000 for ML I, 60 000 for ML II and 50 000 for ML III). Like ML I, the two other lectins are constituted of two chains, A and B, covalently linked by a disulfide bridge.[2–5]

The mechanism of the toxic effect of ML I is identical with that of ricin from *Ricinum communis*, i.e. first, binding of the lectin by its B chain to the cell surface through interaction with sugars[12] and, second, endocytosis of the lectin into the cell followed by enzymatic inhibition of ribosomes by the A chain.[13] Moreover, other plant heterodimeric toxins, such as abrin from *Abrus precatorius*,[14] modeccin from *Modecca digitata*[15] and also the single-chain ribosome-inhibiting proteins, such as pokeweed antiviral protein (PAP II) from summer leaves of *Phytolacca americana*,[16] dodecandrin from *Phytolacca dodecandra*[14] and trichosanthin from *Tricosantheskirilowii maxim*,[17] inhibit protein synthesis by a mechanism identical with that of the A chain from ricin.[14]

In this paper we present studies concerning: (i) the cytotoxicity of the three MLs on a human tumoral cell line, and (ii) the relationship between the primary structures of the N-terminal of the mistletoe lectins A chains and that of various toxins with a related mechanism of action.

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Materials and methods

Lectins

Lectins ML I, ML II and ML III were purified as previously described.[1,18] Rabbit anti-ML I polyclonal antibodies were prepared as indicated elsewhere[1] and were partially purified by ammonium sulfate precipitation.

Protein concentration determinations

Protein concentrations were determined with the Coomassie blue dye–protein assay performed on microplates.[19]

Human leukemia Molt 4 cells and cytotoxicity tests

The Molt 4 cell line is derived from a child T cell leukemia.[20] Culture of these cells and cytotoxicity tests were performed as described elsewhere.[21] In brief, cell growth was determined by counting cells under a light microscope with a hemacytometer. Viable cells were those which, under the microscope, appear to have normal membranes and remain unstained in the presence of trypan blue. For a more precise determination of cytotoxicity the inhibition of [^{3}H]thymidine incorporation into the cells was also determined.[11]

Protein sequencing

The A and B chains of the three different MLs were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and then electroblotted onto polyvinylidene difluoride membranes.[22]

Picomole quantities of ML A chains were then sequenced in a gas-phase sequencer (Applied Biosystems 470 A). All the sequences were compared by eye alignment to detect homologies with the published sequences of other toxins. We take highly conservative changes to be those which occur by a single base change in the gene and which are chemically very similar. Examples include glutamic acid and glutamine, leucine and isoleucine, leucine and valine, valine and phenylalanine, etc.

Results

Inhibition of Molt 4 cell growth by ML I, ML II and ML III, and protection against the cytotoxicity by anti-ML I antibodies

Figure 1 shows the toxic and inhibitory effects of MLs on cell growth determined by counting cells as indicated in Materials and methods. At the highest concentrations used, the three lectins were toxic, ML III being the most toxic, followed by ML II and ML I. The concentrations of lectins leading to a more or less constant number of viable cells during the 3 days of culture were estimated from the data shown in Figure 1. They were 0.3 ng/ml for ML I, 0.04 ng/ml for ML II and 0.03 ng/ml for ML III. At all concentrations tested, the toxic effects of these lectins were inhibited by addition to the culture medium of anti-ML I antibodies at a dilution of 1/1000. The measurement of [^{3}H]thymidine incorporation into the cells (Figure 2) gave similar results to those obtained by counting cells. A 50% inhibition of [^{3}H]thymidine incorporation was obtained with 0.06 ng/ml for ML I, 0.015 ng/ml for ML II and 0.010 ng/ml for ML III.

N-terminal sequencing of the A chains of ML I, ML II and ML III

The N-terminal sequences of the three A chains of ML I, ML II and ML III, shown in Figure 3, were found to be identical. For comparison, the published sequences of various toxins with a mechanism of action identical with that of the A chain of ricin or ML I are also shown in Figure 3. An empty space has been introduced at position 9 to maximize the similarity of the sequence of the A chain of MLs to that of the other toxins. Amino acids in outline letters, at positions 14, 18, 21, 25 and 26, are identical in at least seven of the nine sequences shown. Amino acids in bold letters are identical in less than seven sequences or highly conserved.

Discussion

The above results indicate that the A chains of the three lectins ML I, ML II and ML III have an identical N-terminal sequence. In addition, the three lectins inhibit the growth of the human tumor cell line Molt 4, ML III being the most potent, followed by ML II and ML I. This inhibition is suppressed

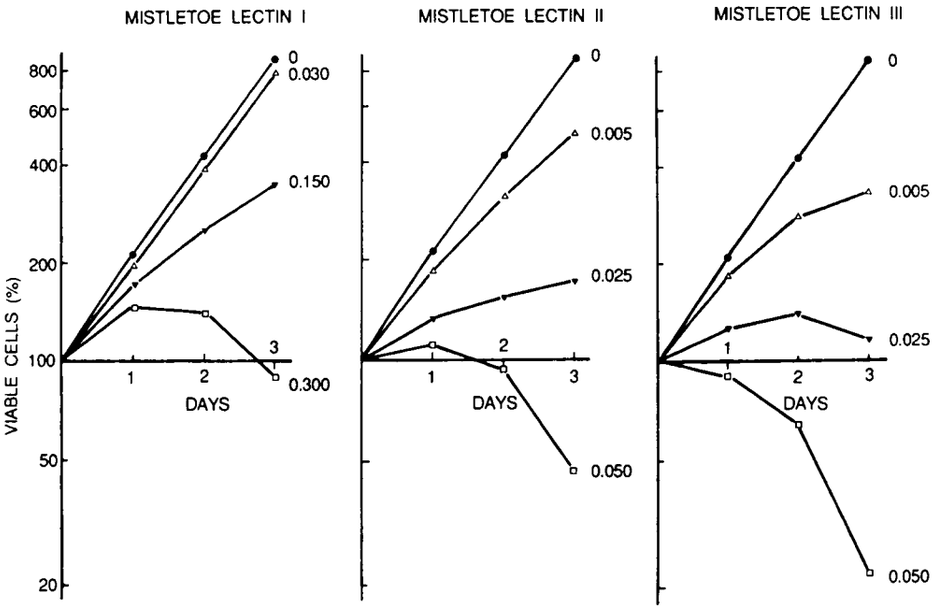

Figure 1. Effect of ML I, ML II and ML III on the growth of Molt 4 cells. Growth of cells was determined by counting the cells with a hemacytometer as indicated in Materials and methods. Concentrations of lectins indicated near each growth curve are in nanograms per milliliter. Addition of anti-ML I antibodies (dilution 1/1000) protected the cells totally against the toxic effects of the three lectins (data not shown).

by addition of rabbit anti-ML I antibodies to the cultured cells. These observations suggest that the three lectins, although not being identical, have amino acid sequences which show extensive homology and exert very similar biological effects. In a previous paper[23] the toxic effects of ML I on Molt 4 cells were used to develop a bioassay of lectins. The present data show that in such assays ML I, ML II and ML III cannot be differentiated by polyclonal antibodies against ML I nor, probably, by polyclonal antibodies against the other two lectins.

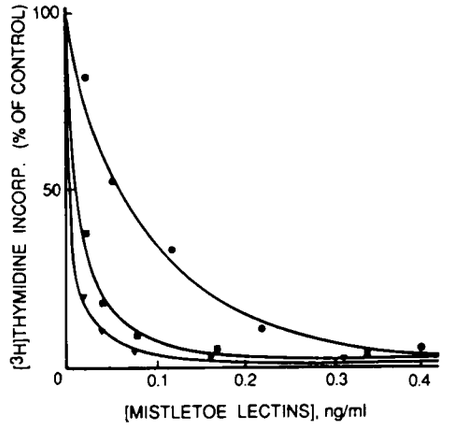

Figure 2. Inhibition of [^{3}H]thymidine incorporation into Molt 4 cells by ML I (●), ML II (■) and ML III (▼). See Materials and methods for details.

ML II and ML III have a molecular weight slightly smaller than that of ML I. Therefore, the formation of ML II and ML III may be the result of a proteolytic degradation of ML I. Another possibility would be molecular heterogeneity of the sugar moiety of the molecule leading to anomalous behavior of the lectins on sodium dodecylsulfate gel electrophoresis.[24] In this respect, it is interesting to note that in mistletoe extracts we found a protein of a molecular weight of 45 000[23] which binds to anti-ML I antibodies and which, following reduction with mercaptoethanol, appeared to have a molecular weight of about 50 000. We proposed that this protein may represent a precursor of either the A or the B chain of ML I.[23]

Recently, the N-terminal sequences of two isomers of the A chain (A_1 and A_2) of ML I have been reported[25] with slight differences between the two sequences. In the present study there was no attempt to isolate lectin isomers. We found a leucine at position 4 (Figure 3) instead of an isoleucine in

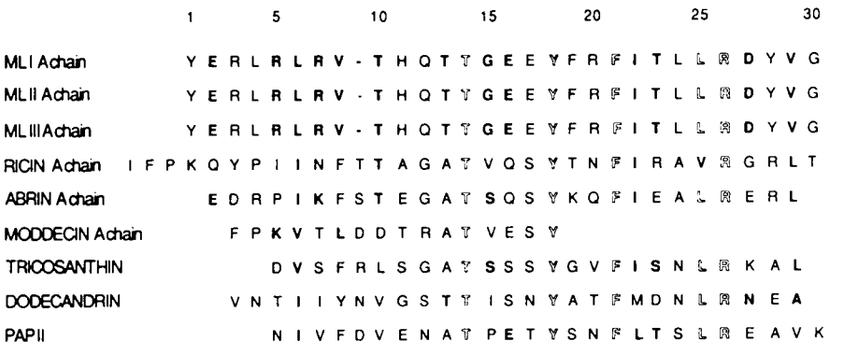

Figure 3. N-terminal sequences of the A chains from ML I, ML II and ML III, and comparison with those of ricin, abrin, moddecin, tricosanthin, dodecandrin and PAP II.[14–17] One empty space at position 9 of the sequences of ML I, ML II and ML III has been added to maximize their similarity with those of the other toxins. Outline letters indicate residues which are identical in at least seven of the nine sequences shown. Bold letters indicate amino acids which are identical in less than seven sequences or highly conserved.

the A_1 chain and a glutamic acid at position 16 instead of an aspartic acid in the A_2 chain. All other amino acids are identical in both studies. The differences might be related to the experimental procedure as PTH-amino acids of these two pairs are not always fully separated by HPLC. However, our results were confirmed when the determination was repeated. It is worth noting that the presence of the sugar moities did not present any problems, neither for SDS–PAGE and blotting, nor for sequencing. Actually, 30 amino acids were identified in a single run, demonstrating the efficiency of this simple method in the case of glycoproteins, and only picomole amounts were needed.

The comparison of the N-terminal sequences of the MLs with other toxins, whether heterodimeric lectins such as ricin, abrin and modeccin or single-chain ribosome-inhibiting proteins like tricosanthin, dodecandrin and PAP-II indicates the presence of several highly conserved groups of amino acids. It is therefore likely that not only ML I but also ML II and ML III are true members of the few toxic lectins which inhibit specifically ribosomal activity. It would be of great interest to investigate the potential immunostimulating effect of ML II and ML III as well as that of the 45 000 Da protein related to mistletoe lectins.

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