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Full Papers

Effects of mistletoe (*Viscum album* L.) extracts on cultured tumor cells[1]

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Summary. Bacterially fermented mistletoe preparations (BFMP) were tested on rat hepatoma tissue culture (HTC) cells and human leukemia Molt 4 cells. A dose-dependent inhibition of the growth rate of the cells was observed. For both cell lines, cytostatic concentrations, expressed in weight of fresh plant, were 0.5 mg/ml culture medium for oak BFMP and 1 mg/ml for apple tree BFMP. However, the action of the two preparations was markedly different on each cell line. Non-viable HTC cells were not stained by trypan blue while non-viable Molt 4 cells were fully colored by this reagent. A lysis of cellular membranes of HTC cells was observed by electron microscopy. Furthermore, oak BFMP inhibited the growth of virus transformed 3T3-SV40 cells more than that of non-transformed 3T3 cells. In contrast to BFMP, non-fermented extracts and a purified mistletoe lectin showed a greater inhibition of the growth of Molt 4 cells than of HTC cells. Samples withdrawn at different times during fermentation gradually lost their inhibitory effect on the growth of Molt 4 cells while their action on HTC cells increased up to the 4th day of fermentation. These results are discussed in relation to the cytotoxic substances of mistletoe already characterized.
Key words. Mistletoe extract; cytotoxic substances; hepatoma tissue culture; human leukemia cells.

Introduction

Bacterially fermented aqueous extracts of mistletoe have a cytostatic effect on animal tumoral cells in culture[2]. They inhibit the growth of plant roots[3] and plant tumoral tissue in culture[4]. In mice inoculated with tumor cells from different experimental cell lines (Ehrlich ascites carcinoma, Sarcoma 180 and Lewis lung carcinoma) the survival time of treated animals is equal to or higher than that of mice treated with 5-fluoro-uracil, a well known anti-tumoral agent[2]. Moreover, the extracts stimulate humoral and cellular immunity in mice[5,6]. A marked increase of the weight of the thymus, corresponding to a higher proliferation rate of cortical thymocytes, is also observed and this effect is reversible[7].

Several cytostatic or cytotoxic proteins have been characterized in mistletoe, particularly three glycoproteins classified as lectins[8–11] (mol.wt 115 kD, 60 kD and 50 kD), four viscotoxins[12,13], (basic polypeptides of mol.wt around 5 kD) and 10 other basic proteins, the so-called Vester protein complex (VP 16)[14–16] which has potent cancerostatic and immunomodulatory properties. Moreover, the presence of cytotoxic alkaloids[2] and immunomodulatory polysaccharides[17] has been described. The amount of lectins in bacterially fermented preparations is 5–10 times lower than in non-fermented preparations[18]. It is not known whether lectins are metabolized during bacterial fermentation or slowly degraded by storage. Viscotoxins are quite resistant to degradation[19]. The VP 16 complex loses a large part of its biological activity upon storage[16].

In this paper we describe the inhibitory effect of bacterially fermented aqueous extracts of mistletoe on the growth of cultured cells in vitro and their effects on cell morphology and ultrastructure. The results are compared with those obtained with non-fermented extracts of mistletoe and with samples taken at various times during the fermentation process.

Materials and methods

Extracts of mistletoe and mistletoe lectins. For some experiments an extract of apple tree mistletoe was prepared as follows[20]. Leaves and small stems (10 g) were chopped into slices of approximately 1 mm and frozen in liquid nitrogen. The frozen material was ground into a fine powder in a Waring blender placed in a well-ventilated hood for protection from dust. Then a solution containing 0.1 M lactose and 10 mM N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid (HEPES), pH 7.0, was added up to 100 ml and the resulting suspension slowly stirred at 4 °C for 24 h. Bacterial fermentation of the crude extract was performed with a suspension of Lactobacillus plantarum (10^5 bacteria/ml) in air at 30 °C without stirring. Before adding the bacteria and after different periods of time, 10 ml samples of the fermentation mixture were withdrawn and stored at −20 °C. After thawing, the homogenates were centrifuged at 10,000 × g for 5 min. The concentration of the clear supernatants corresponded to an extract of 100 mg of the fresh plant per ml solution. All other mistletoe fermented preparations (BFMP) were obtained from the Hiscia Institute[1] and corresponded to 50 mg of the fresh plant per ml solution. A sample of *Viscum album* lectin (VAA 1) was a gift from Dr Luther, Forschungsinstitut für Lungenkrankheiten und Tuberkulose, Abteilung Klinische Immunologie, 1115–Berlin (GDR).

Rat hepatoma tissue culture (HTC) cells. These cells are derived from clone 7288 of a Morris rat hepatoma[21]. They were cultured in suspension, in 75 ml of SWIM's S77 medium (Gibco) enriched with 10% newborn calf serum (Gibco), at 37 °C in air with constant stirring. At the start, cultures were diluted with fresh medium and adjusted at 10^5 cells per ml. The mistletoe extracts were added at various concentrations. Cell growth was measured every 24 h by counting the cells with a Neubauer microcytometer and cell viability was evaluated using the trypan blue exclusion test[22]. Experiments were repeated several times.

Human leukemia Molt 4 cells. This cell line is derived from a child T-cell leukemia[23] and was kindly provided by Dr F. Mechelke, University of Hohenheim, D–7000 Stuttgart 70 (FRG). Cells were cultured in suspension in RPMI 1640 medium (Flow) enriched with 10% fetal calf serum previously heated at 56 °C for 30 min, in 5% CO_2, 95% air, at 37 °C. Assays of the mistletoe extracts were carried out as described for HTC cells using a suspension of 10^6 cells in a 10 ml volume of culture medium.

Mouse 3T3 cells and SV40 – transformed 3T3 cells. The 3T3 cells[24] constitute an established line of non-tumoral mouse fibroblast. They were grown as monolayers in 25 cm^2 Falcon culture flasks containing 5 ml MEM medium (Gibco) supplemented with 0.3% glucose and 10% newborn calf serum. The SV40-3T3 cells[25] are viral-transformed, not contact-inhibited, 3T3 cells. They were grown under the same condition as the non-transformed cells. For the tests, mistletoe extracts were added to the medium 24 h after cell plating. After 48-h incubation, the cells were dispersed with 0.5 ml of a mixture of trypsin 0.5 g/l and EDTA 0.2 g/l (Gibco). Cells were then counted as for HTC cells. All the cultured cells were normally observed without further treatment, using a Reichert phase-contrast microscope.

Electron microscopy. Samples of HTC cells, incubated with oak BFMP for various periods of time, were fixed by 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h at 25 °C. After having been washed 3 times in phosphate buffer by centrifugation at 800 × g for 5 min, cells were post-fixed by 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h at 25 °C, washed again by centrifugation and dehydrated. For transmission electron microscopy, cells were embedded in an epoxy resin 'M araldite'. Ultra-thin sections were stained[26] and then examined with a Philips EM 301 S electron microscope. For scanning electron microscopy, cells post-fixed with osmium tetroxide as above were spread on glass slides and submitted to the critical-point drying technique[27]. Then they were coated with a gold-palladium mixture and examined with a Cameca MEB 07 electron microscope.

Results

Effects of BFMP on HTC cells. Both oak and apple tree BFMP produced a dose-dependent inhibition of cell growth (fig. 1). Cytostatic effects were obtained at concentrations corresponding respectively to 0.5 and 1 mg of the fresh plant per ml culture medium. At lower concentrations the growth of the cells was partly inhibited and at higher concentrations toxic effects were observed. It is noteworthy that at all concentrations tested no more than 3% trypan blue stained cells could be observed, probably because of the very rapid lysis of the dead cells. Under phase-contrast microscopy, control HTC cells (fig. 2a) showed a well-defined, refringent membrane

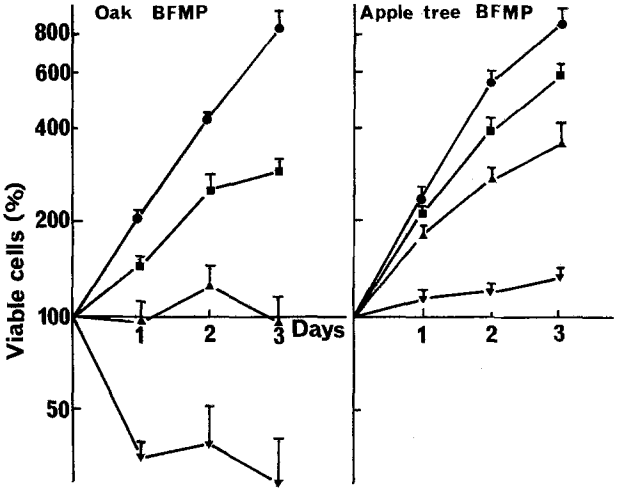


Figure 1. Effects of oak and apple tree BFMP on the growth of HTC cells. ●, control; ■, 0.25 mg/ml; ▲, 0.5 mg/ml; ▼, 1 mg/ml; Concentrations are in mg of fresh plant per ml culture medium. Each point is the mean ± SE of 3 experiments.

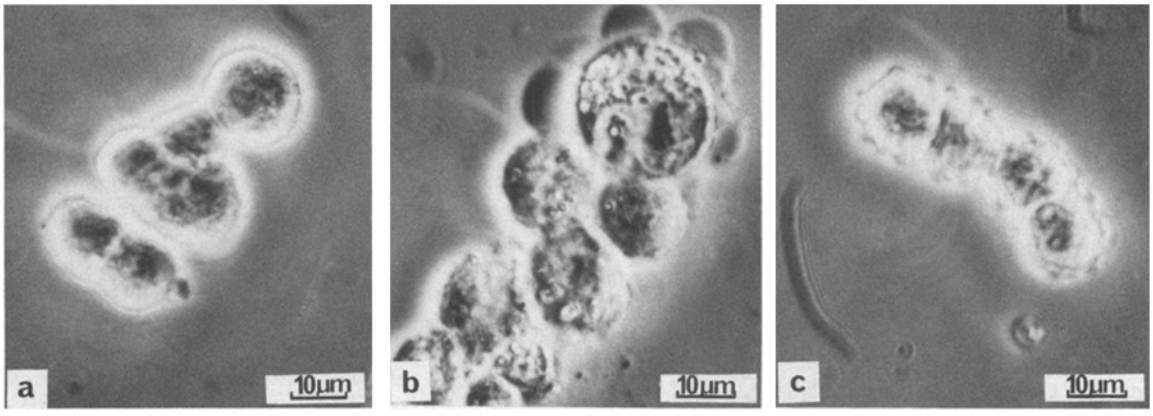


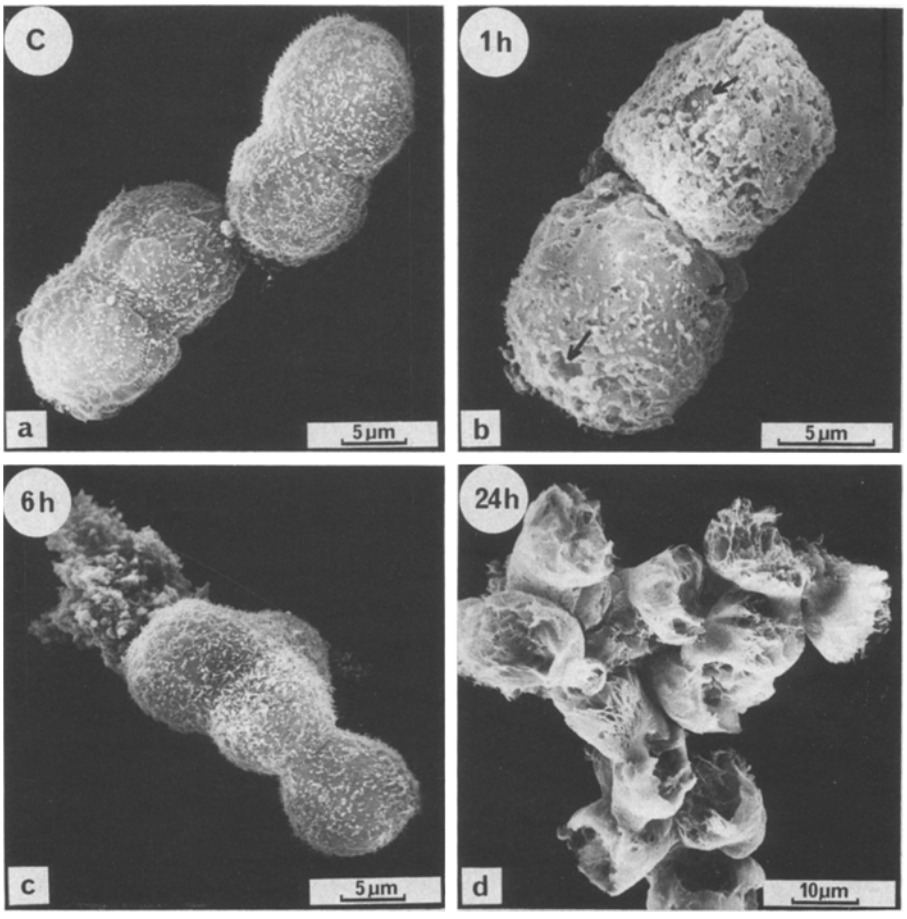
Figure 2. Phase-contrast micrographs of HTC cells incubated with oak and apple tree BFMP. a, control; b, oak BFMP 1 mg/ml, for 24 h; c, apple tree BFMP 1 mg/ml, for 24 h.

while that of cells incubated with oak BFMP (fig. 2b) or apple tree BFMP (fig. 2c) appeared much less refringent. Protrusions developed on the treated cells indicating marked alterations of their membranes. As with the study on cell growth, the action of oak BFMP appeared more effective than that of apple tree BFMP.

Figure 3. Scanning electron micrographs of HTC cells incubated with oak BFMP. a, control; b, 1 mg/ml for 1 h; c, 1 mg/ml for 6 h; d, 1 mg/ml for 24 h. Arrows indicate perforations of the cell membrane.

Studies by scanning electron microscopy (fig. 3) showed that the incubation of HTC cells for only 1 h with oak BFMP 1 mg/ml, produced significant modifications of the cell surface. The microvillosities, well observed on control cells (fig. 3a), partly disappeared and a disintegration of the membrane was observed in some places (fig. 3b). After a 6-h contact with oak BFMP (fig. 3c) cells were either completely lysed, or only slightly altered. Statistical examination of the micrographs showed that approximately 20% of the cells appeared to be lysed. After 24 h (fig. 3d) almost all the cells were lysed.

Studies by transmission electron microscopy (fig. 4) indicated that the ultrastructure of HTC cells was strongly altered by oak BFMP. The membranes were



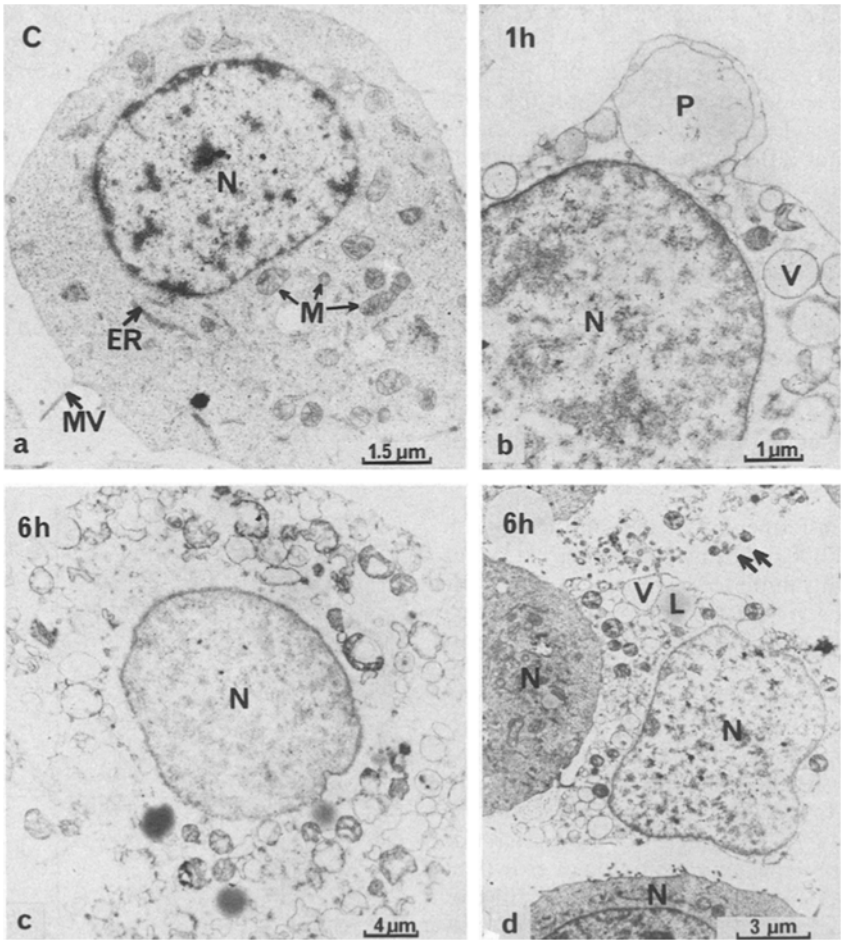


Figure 4. Transmission electron micrographs of HTC cells incubated with oak BFMP. a, control; b, 1 mg/ml for 1 h; c, 1 mg/ml for 6 h. N, nucleus; M, mitochondrion; MV, microvillosity; ER, endoplasmic reticulum; V, cytosolic vesicle; L, lipidic vesicle; P, protrusion. Arrows indicate the presence of cellular debris.

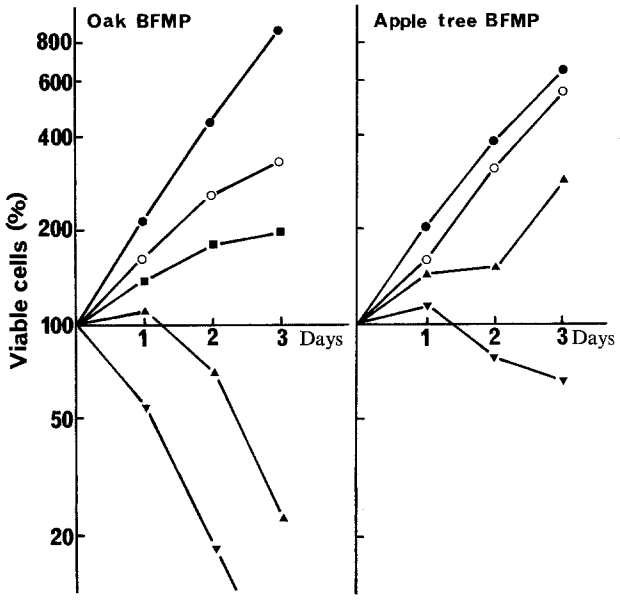


Figure 5. Effects of oak and apple tree BFMP on the growth of Molt 4 cells. ●, control; ○, 0.1 mg/ml; ■, 0.25 mg/ml; ▲, 0.5 mg/ml; ▼, 1 mg/ml. For other details see 'Materials and methods'.

progressively disrupted, as shown in figure 4b for 1-h incubation and in figure 4c for 6-h incubation. Numerous protrusions formed on the cell membranes and vesicles appeared in the cytoplasm. Nuclei were less dense than in control cells (fig. 4a). After 24 h all the cells were completely disrupted.

Effect of BFMP on Molt 4 cells. Oak and apple tree BFMP inhibited the growth of Molt 4 cells at doses of the same order of magnitude as in HTC cells (fig. 5). The inhibition was also dose-dependent. However, in contrast to HTC cells, dead Molt 4 cells could be visualized by trypan blue coloration. Only at the end of the incubation period was a cytolysis observed.

Effects of oak BFMP on 3T3 cells and SV40-transformed 3T3 cells. A first set of experiments (fig. 6) was performed with cells at their maximum division rate. For this purpose the culture was started with 8×10^5 cells per 25 cm^2 culture flask, and oak BFMP was added 24 h later. Under these conditions, the estimated concentrations of oak BFMP which inhibited cell growth by 50% were 0.5 mg/ml for SV40-transformed 3T3 cells and 2 mg/ml for non-transformed fibroblasts.

In another series of experiments, oak BFMP was tested at high cell concentrations (3.5×10^6 per 25 cm^2 culture flask) which lead to contact inhibition in 3T3 cultures, but not in SV40-3T3 cultures. Under these conditions, oak BFMP at 2 mg/ml, was almost ineffective on 3T3 cells while all SV40-transformed cells were dead after a 48-h incubation period.

Progression of the effects of a crude extract of mistletoe during the bacterial fermentation. In order to establish a comparison between fermented and non-fermented preparations of mistletoe, crude extracts from apple tree mistletoe were prepared as described under Methods. Samples were withdrawn at different times during fermentation and their activities on HTC and Molt 4 cells were tested (fig. 7). The concentrations used to measure the inhibitory effect of these preparations were 0.05 mg/ml of culture medium on HTC cells and 0.02 mg/ml on Molt 4 cells. Allowing for these differences of concentration, it is estimated from figure 7 that crude extracts produce cytostatic effects at a concentration approximately 10 times lower on Molt 4 cells than on HTC cells. Moreover, figure 7 shows that the inhibition of Molt 4 cell growth decreased as the fermentation progressed, while that of HTC cells increased during the first 4 days of fermentation.

The effects of the non-fermented extract on the two cell lines were considerably reduced by heating the extract at 56 °C for 1 h, and totally inhibited by heating at 100 °C for 1 h. However, the activity of the completely fermented extract on HTC cells was unchanged after heating at 100 °C for 1 h.

Effect of a mistletoe lectin on HTC and Molt 4 cell growth. The difference in activity between the crude and fermented extracts of mistletoe on HTC cells and Molt 4 cells led us to investigate the action on the same cells of a purified mistletoe lectin, which is known to be a cytotoxic component of the plant[9, 10]. In figure 8 it is shown that this lectin produced a dose-dependent inhibition of growth of HTC and Molt 4 cells. Cytostatic effects were obtained at approximately 500 ng/ml culture medium on HTC cells and only 5 ng/ml on Molt 4 cells. Thus mistletoe lectin appeared to be particularly toxic to these leukemic cells.

Discussion

The data presented show that oak and apple tree BFMP produced a dose-dependent inhibition of the growth of cultured rat hepatoma cells. Oak BFMP was twice as potent as apple tree BFMP. After treatment of HTC cells with oak and apple tree BFMP, protrusions developed on the membranes and vesicles appeared in the cytoplasm; these could be observed by phase-contrast and electron microscopy. These modifications of cell morphology and ultrastructure led to a disintegration of the cellular membranes and a rapid lysis of the cells. Lysis did not appear at the same time on all cells suggesting that it occurred at a specific step of the cell cycle. Due to this strong cytolytic effect of BFMP, dead cells were probably not able to accumulate the trypan blue dye and could therefore not be revealed by this reagent.

Oak and apple tree BFMP also produced dose-dependent inhibition of the growth of human leukemia Molt 4 cells

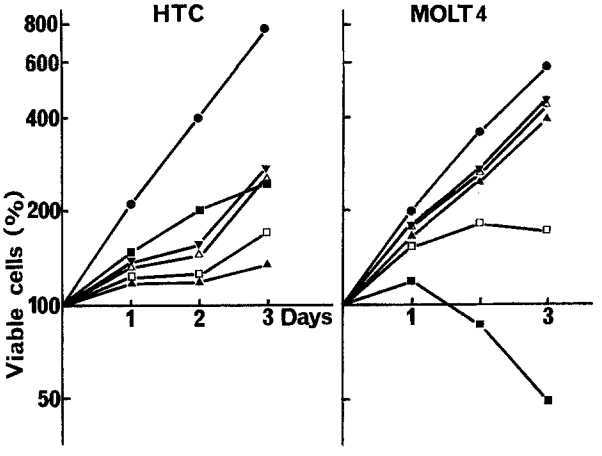


Figure 7. Progression during bacterial fermentation, of the effects of an apple tree mistletoe crude extract on the growth of HTC and Molt 4 cells. ●, control; ■, non-fermented extract; □, 1 day fermentation; ▲, 4 days fermentation; △, 8 days fermentation; ▼, 14 days fermentation. Concentrations of the extracts on HTC cells, 0.05 mg/ml; on Molt 4 cells, 0.02 mg/ml. For other details see 'Materials and methods'.

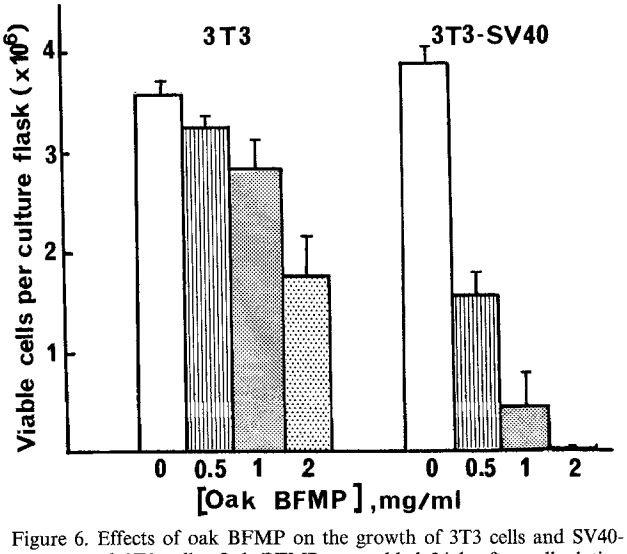


Figure 6. Effects of oak BFMP on the growth of 3T3 cells and SV40-transformed 3T3 cells. Oak BFMP was added 24 h after cell plating (8×10^5 cells per culture flask). The incubation time was 48 h. Each value is the mean ± SE of 3 experiments. For other details see 'Materials and methods'.

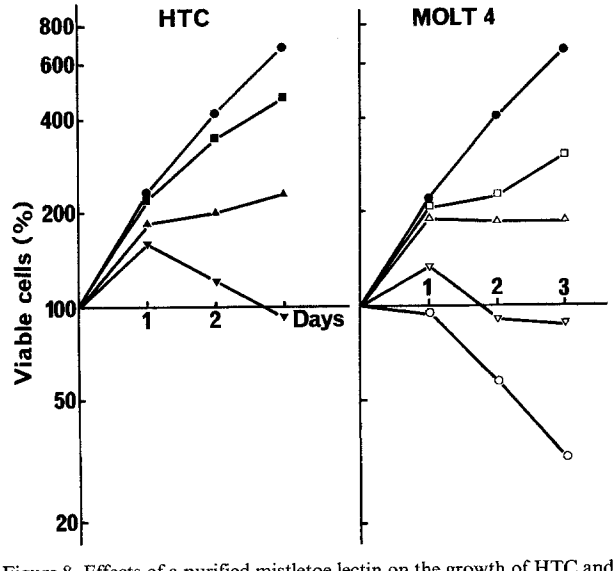


Figure 8. Effects of a purified mistletoe lectin on the growth of HTC and Molt 4 cells. ●, control; □, 1 ng protein/ml; △, 2 ng/ml; ▽, 5 ng/ml; ○, 10 ng/ml; ■, 20 ng/ml; ▲, 100 ng/ml; ▼, 500 ng/ml. For other details see 'Materials and methods'.

at a similar dose range as with HTC cells. Observations of these cells by phase-contrast microscopy showed that dead cells were fully colored by trypan blue, without notable cytolysis, suggesting a different primary effect of BFMP on this cell type as compared to HTC cells.

The cell ultrastructure effects of BFMP may be a secondary event produced after a more specific interaction of active proteins with cell metabolism. For instance, the protein complex VP 16 which inhibits DNA and RNA synthesis in cell cultures[28-30] at a concentration of 0.1 µg per ml may be present in the tested sample of BFMP at a similar concentration[16]. Moreover, mistletoe lectin inhibits protein synthesis by inactivating ribosomes catalytically[20].

In order to investigate the specificity of BFMP for tumoral cells versus non-tumoral cells the effect of oak BFMP was tested on two closely related cell lines, 3T3 cells and SV40-transformed 3T3 cells. The inhibitory effect of BFMP on cells in their logarithmic growth phase was much higher on the SV40-transformed cells than on the non-transformed cells, whatever the concentration tested. At high cell density, when the growth of 3T3 cells was contact inhibited, the differential effect was even more pronounced. This observation favors the hypothesis that the cytotoxic effect of BFMP might be stronger on malignant cells than on normal non-cycling cells. A similar specificity has been establish for VP 16 suggesting that these proteins are present in BFMP.

The significant decrease of activity against Molt 4 cells, observed during a 14-day bacterial fermentation of a crude extract, indicated that some effective substances were transformed. The high sensitivity of Molt 4 cells to lectins suggested that these substances were mainly lectins. This is in agreement with the small amount of lectins found in BFMP[18]. The evolution of activity of the extracts on HTC cells, which were weakly sensitive to lectins, was different. First, non-fermented extracts inhibited the growth of HTC cells 10 times less than that of Molt 4 cells. Second, 4-day fermented extracts were more active than non-fermented ones. New substances active against HTC cells might be formed from lectins by the bacterial metabolism. They could be responsible for the marked cytolytic effect of BFMP.

The effects of non-fermented extracts on Molt 4 cells were found to be thermolabile, as are lectins[9]. On the contrary the effects of fermented extracts on HTC cells were heat-stable, as are viscotoxins[19]. Therefore, it is proposed that the activities of a typical mistletoe extract should be tested on both cell lines in order to estimate the biological activities of the two class of components.

1 The bacterially fermented mistletoe preparations, named BFMP in the text, were obtained from the Hiscia Institute, CH-4144 Arlesheim, Switzerland, under the name of Iscador. For oak BFMP, mistletoe was from *Quercus petraea* Liebl. and *Quercus robur* L.; for apple tree BFMP from *Malus domestica* Borkh.

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