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Acknowledgement—The authors would like to acknowledge the support of the Cancer Research Campaign and Sterling Winthrop Inc., U.S.A., which has enabled this work to be carried out.

Pergamon

European Journal of Cancer Vol. 30A, No. 12, pp. 1836–1841, 1994
Elsevier Science Ltd
Printed in Great Britain
0959–8049/94 $7.00+0.00

0959-8049(94)00299-1

Viscum album L. Extracts Reduce Sister Chromatid Exchanges in Cultured Peripheral Blood Mononuclear Cells

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Increasing concentrations of *Viscum album* L. extracts were shown to significantly reduce sister chromatid exchange (SCE) frequency of phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) of healthy individuals. This decrease of SCE could not be explained either by changes in lymphocyte subpopulations, by cytostatic effects of the drug or by accelerated proliferation of PHA-stimulated PBMC. Currently, no other cells tested have shown this effect. One therapeutic effect of these anti-mutagenic drugs could be a stabilisation of mononuclear blood cell DNA.

Key words: sister chromatid exchange, SCE, mistletoe extracts, *Viscum album* L., DNA, anti-mutagenic, peripheral blood mononuclear cells
Eur J Cancer, Vol. 30A, No. 12, pp. 1836–1841, 1994

INTRODUCTION

IT IS generally agreed that *Viscum album* L. (mistletoe) preparations may play a beneficial role during and after conventional cancer therapy [1]. Explanations of these effects usually postulate an enhanced immune reactivity or immune reconstitution with reappearance of anti-tumour activity. Notwithstanding the crucial and controversally discussed role of the immune system in tumour surveillance and defence, the reconstitution of a therapeutically altered immune system may be of substantial benefit for the patient.

Numerous drugs are able to produce DNA damage in proliferating cells. Sister chromatid exchange frequencies (SCE) are considered to be sensitive and specific indicators of cytogenetic damage and mutagenicity [2–5]. SCE represent the interchange of DNA replication products at apparently homologous loci. These exchanges presumably involve DNA breakage and reunion [6]. SCE are known to be induced by substances that interfere with DNA metabolism or repair, or by drugs that produce crosslinks to the DNA, such as the alkylating agent cyclophosphamide and its metabolites [7].

Mistletoe pharmaceutical preparations are being used in cancer treatment. Cytotoxic effects on both cultured tumour cells and human lymphocytes have been shown [8–10]. In addition, *Viscum album* extracts are reported to stimulate natural killer (NK) cells [11, 12], phagocytosis [11] and the production of cytokines from peripheral blood mononuclear cells (PBMC) [13–15].

Various substances with cytotoxic activity on cultured cells have been isolated from extracts of *Viscum album* L., such as viscotoxins, lectins and alkaloids (reviewed in [16]). Viscotoxins inhibit DNA synthesis by binding to nucleic acid and cause cell death by damaging the cell membrane. The mistletoe lectins, which are believed to represent the main biological activity in the drug, inhibit protein synthesis by inactivating the 60S subunit of the ribosomes. The mechanism of cytotoxicity of mistletoe alkaloids is still unclear. The specific activity of *Viscum album* L. preparations is dependent on the proportions of the components and on the dose of administration.

The aim of this study was to investigate whether *Viscum album* L. preparations affect DNA and so alter SCE frequencies of cultured PBMC of healthy individuals.

MATERIALS AND METHODS

Mistletoe preparations

For our investigations, we used the *Viscum album* L. preparation Helixor Abietis (Helixor^R A), which is produced from mistletoes living on fir trees, and Helixor Pini (Helixor^R P), from mistletoes living on pine trees. The aqueous solutions of the whole fresh plants were kindly provided by Helixor Heilmittel GmbH & Co. (Rosenfeld, F.R.G.).

SCE analysis of PBMC

Peripheral whole blood was taken from 10 healthy non-smokers, 5 female and 5 male. The mean age was 25 years.

For SCE analysis, we used a modified fluorescence-plus-Giemsa technique [17–19]. Heparinised peripheral blood (1.5 ml) was given to 9 ml of chromosome medium B (Biochrom KG, Berlin, F.R.G.), which contained 2.5 μg/ml phytohaemagglutinin (PHA). After addition of the thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU; Sigma, Deisenhofen, F.R.G.), at a final concentration of 5 μg/ml, the cultures were incubated with 1.43, 7.15 and 14.3 μg Helixor A or Helixor P per ml culture medium. Related to body weight and body fluid, these drug concentrations correspond approximately to the dose which cancer patients receive daily by subcutaneous injection. Samples without added *Viscum album* L. extracts served as controls. The cells were cultured in the dark for 70 h at 37°C. Subsequently, colcemide (Demecolcine; Biochrome KG, Berlin, F.R.G.) was added and the incubation was continued for an additional 2 h. The cells were then suspended in a hypotonic buffer with 0.075 mol/l potassium chloride and fixed on slides with acetic acid/methanol (1:4). The slides were stained with Hoechst 33258 (0.5 μg/ml), exposed to UV light (254 nm) for 2 h, incubated for 1 h in 2 × SSC (sodium chloride-trisodium citrate) buffer at 60°C and counterstained with Giemsa.

For scoring SCE (Figure 1), at least 20 M2 metaphases for each blood sample were recorded. To achieve stronger criteria for possible differences, only one SCE per chromosome was evaluated. SCE frequency was given in mean SCE number per 46 chromosomes ± S.D. Statistical analysis was made by Wilcoxon sign rank test. Differences were considered significant when the probability of the measured differences occurring by chance was less than 5%.

Mitotic indices were determined by counting the first (M1), second (M2) and third (M3) division per 1000 cells. Numbers of metaphases were recorded as means ± S.D.

Proliferation assays

Lymphocyte proliferation was measured by [^{3}H]thymidine incorporation by heparinised peripheral blood lymphocytes, taken from five healthy test persons. Cells were separated by Ficoll–Hypaque sedimentation and cultures were established using 2×10^6 cells/ml. Mistletoe preparation Helixor A or Helixor P was added at concentrations of 10 μg/ml and 100 μg/ml, respectively. Controls without added mitogen were used for each culture. After incubation for 96 h at 37°C, cultures were pulsed with 5 μCi/ml tritiated-thymidine ([^{3}H]thymidine). After an additional 16 h of culture, harvested cells were washed and scintillation fluid was added. Incorporation of [^{3}H]thymidine was determined in counts per minute (cpm) on a LKB 1219 Rackbeta liquid scintillation counter. Proliferation was measured by stimulation index ($SI = cpm_{test}/cpm_{basal}$).

Surface markers

Differentiation of leucocyte and lymphocyte subpopulations was accomplished by immunofluorescent staining and flow cytometry analysis. Heparinised peripheral blood (1.5 ml) from five healthy individuals was added to 9 ml of chromosome medium B. Helixor A, at concentrations of 10 and 100 μg/ml culture medium, was added. Identical samples without mistletoe extract served as controls. Cells were cultured for 72 h at 37°C before 15 μl of the monoclonal antibody (anti-CD3-, anti-CD19-, anti-CD20-, anti-CD4-, anti-CD8-, anti-CD45-, anti-CD14-, anti-CD16-, anti-CD56-, anti-CD25) were added to 100 μl of leucocytes. Further preparation of cells and analysis in the FACScan flow cytometer (Becton Dickinson, Heidelberg, F.R.G.) was performed according to the instructions of the manufacturer.

RESULTS

SCE frequency and mitotic indices of PBMC after administration of Helixor A

Untreated PHA-stimulated PBMC that served as controls had an average SCE rate of 4.63 SCE/metaphase. The addition of Helixor A resulted in a dose-dependent decrease of SCE to a mean rate of 3.22 SCE/metaphase after administration of 14.3 μg/ml (Table 1, Figure 2a). Compared to the controls, the decrease of SCE was statistically significant at concentrations of 7.15 and 14.3 μg/ml ($P < 0.0001$). This decrease was not dependent on an increasing cytostatic effect of Helixor A. The

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Revised 19 May 1994; accepted 9 June 1994.

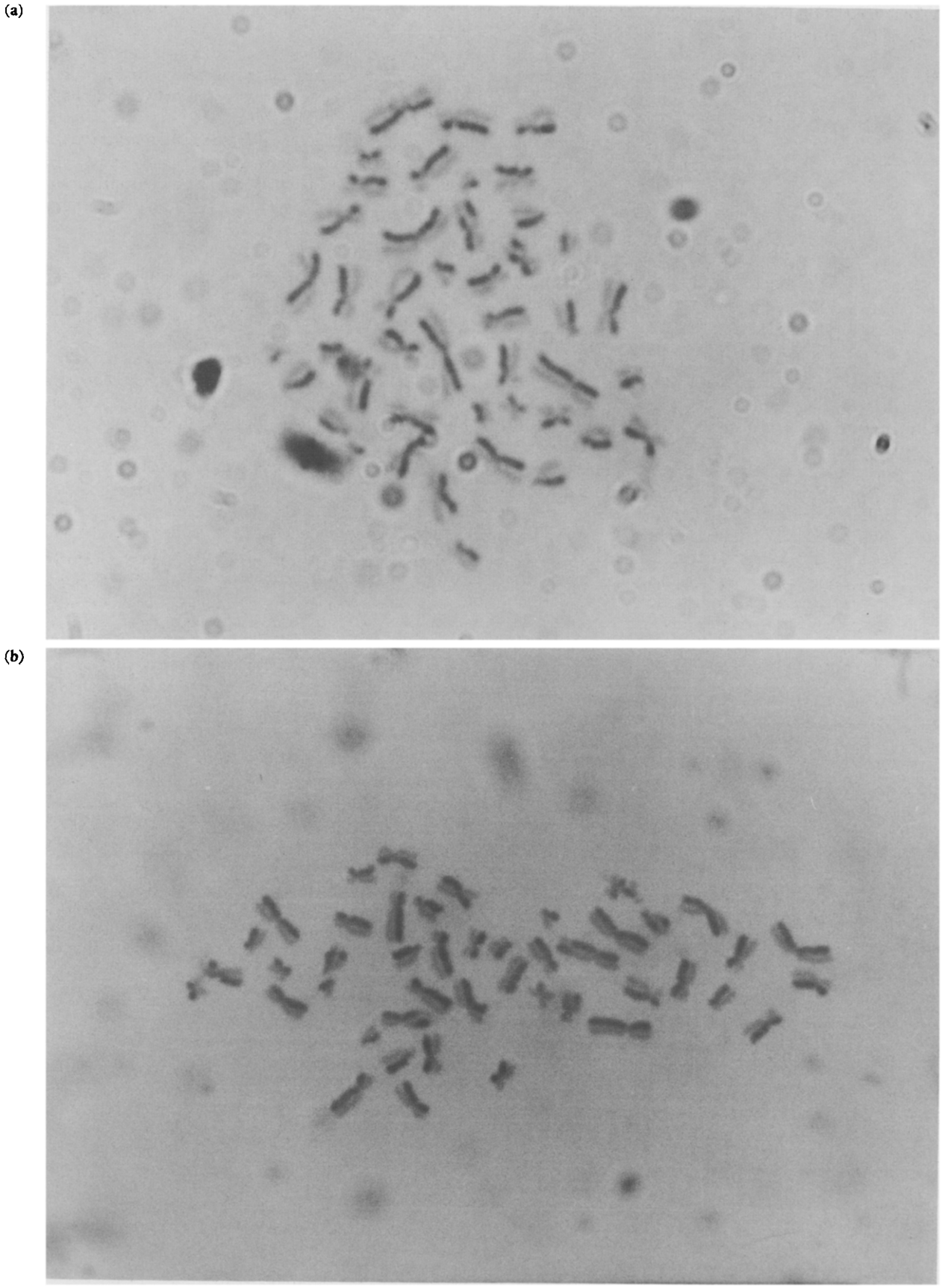

Figure 1. Sister chromatid exchanges of peripheral blood mononuclear cells (a) after the addition of *Viscum album* L. and (b) without *Viscum album* L. M2 metaphases after two rounds of replication in the presence of BrdU and staining with the FPG technique. Bifilarly substituted DNA stains less intensely with Giemsa than the unifilarly substituted sister chromatid.

Table 1. Sister chromatid exchanges of phytohaemagglutinin-stimulated peripheral blood mononuclear cells after addition of Helixor A and Helixor P

	SCE/metaphase (μg/ml)			
	0.0	1.43	7.15	14.3
Helixor A				
1	4.9 ± 1.81	3.7 ± 1.82	3.1 ± 1.44	3.4 ± 2.05
2	4.3 ± 2.41	3.6 ± 1.83	3.1 ± 1.77	3.5 ± 1.84
3	3.9 ± 1.44	3.2 ± 1.42	3.0 ± 0.82*	2.8 ± 1.69
4	4.0 ± 1.79	3.9 ± 1.78	3.4 ± 1.61	3.7 ± 1.94
5	4.6 ± 3.25	3.7 ± 1.49	3.5 ± 1.79	3.2 ± 1.43
6	4.3 ± 1.92	4.2 ± 1.25	4.2 ± 1.93	3.0 ± 1.72
7	4.5 ± 1.95	3.7 ± 1.48	2.9 ± 1.56	3.0 ± 1.34
8	4.5 ± 2.05	3.8 ± 2.21	4.3 ± 1.54	3.3 ± 2.18
9	5.7 ± 2.67	4.5 ± 2.30	3.6 ± 1.92	2.9 ± 1.30
10	5.3 ± 2.16	4.9 ± 1.60	3.7 ± 1.73	3.4 ± 1.45
Mean†	4.63 ± 0.56	3.90 ± 0.49	3.57 ± 0.45	3.22 ± 0.29
Helixor P				
1	4.9 ± 1.81	4.5 ± 2.28	3.6 ± 1.90	2.85 ± 1.42
2	4.3 ± 2.41	4.0 ± 2.15	3.8 ± 1.62	3.06 ± 1.32
3	3.9 ± 1.44	3.7 ± 1.87	3.2 ± 1.21	2.28 ± 1.61
4	4.0 ± 1.79	3.6 ± 1.40	3.5 ± 1.64	3.06 ± 1.33*
5	4.1 ± 1.44	3.2 ± 1.32	3.3 ± 1.59	2.58 ± 1.37
6	4.3 ± 1.92	3.4 ± 1.40	3.2 ± 1.32	2.84 ± 1.35
7	4.5 ± 1.95	4.4 ± 1.39	3.0 ± 1.60	2.97 ± 1.37
8	4.5 ± 2.05	4.4 ± 1.91	3.9 ± 1.63	3.64 ± 1.57
9	5.7 ± 2.67	4.1 ± 1.60	3.3 ± 1.55	3.11 ± 1.43
10	5.3 ± 2.16	4.7 ± 1.32	3.9 ± 1.44	4.11 ± 2.22*
Mean†	4.58 ± 0.59	3.99 ± 0.51	3.47 ± 0.31	3.05 ± 0.51

Results are given as mean of at least 20 metaphases ± S.D. * Value did not meet the statistical requirements (<20 M2 metaphases). † Calculation to two decimal places.

mitotic index of healthy controls showed an average 3.4 M1, 8.5 M2 and 11.4 M3 metaphases. This PHA-induced acceleration was only slightly altered by different Helixor A doses and, in no case, significantly reduced or elevated (Figure 3a).

SCE frequency and mitotic indices of PBMC after administration of Helixor P

A similar reduction of the SCE frequency was obtained using Helixor P. Again, a statistically significant decrease of SCE ($P < 0.0001$) at the higher concentrations of 7.15 and 14.3 μg/ml was shown (Table 1, Figure 2b).

A significant increase in proliferation was not observed with preparation P, although higher numbers of M2 and M3 metaphases were seen at some concentrations compared to the controls.

Proliferation assay

PHA-stimulated lymphocytes of five healthy persons were treated with increasing concentrations of Helixor A and Helixor P or Helixor M (Figure 4). Relative to controls, administration of 10 μg/ml Helixor A led to a SI of 0.98 ± 0.30, and after administration of 100 μg/ml, to a SI of 0.83 ± 0.27. The [^{3}H]thymidine uptake of Helixor A-treated lymphocytes remained almost stable, and no significant cytostatic effects could be observed. Helixor P gave a SI of 0.85 ± 0.19 at a concentration of 10 μg/ml, but the high concentration of 100 μg/ml led to a reduced SI of 0.49 ± 0.30.

Leucocyte and lymphocyte subpopulations after administration of Helixor A

After addition of 0.0, 10 or 100 μg/ml Helixor A to PHA-stimulated PBMC and incubation for 72 h, expression of various cell surface antigens was recorded in a FACScan flow cytometer. The relative levels of monocytes and lymphocytes, as well as that of the lymphocyte populations (T lymphocytes, low affinity interleukin-2R+ (CD25+), T lymphocytes, B lymphocytes, NK cells) remained almost stable when increasing concentrations of mistletoe extracts were applied (Table 2).

DISCUSSION

The BrdU-induced SCE frequency of PBMC decreased by adding *Viscum album* L. extracts at increasing concentrations. Currently, only PBMC, no other proliferating cells tested, such as amniotic fluid cells [20], have this clear anti-mutagenic effect at therapeutical doses of the drug.

It could be argued that the decreased SCE frequencies are due to cytostatic effects, controversially discussed in the literature [8, 16, 21]. However, the low doses of *Viscum album* L. extracts

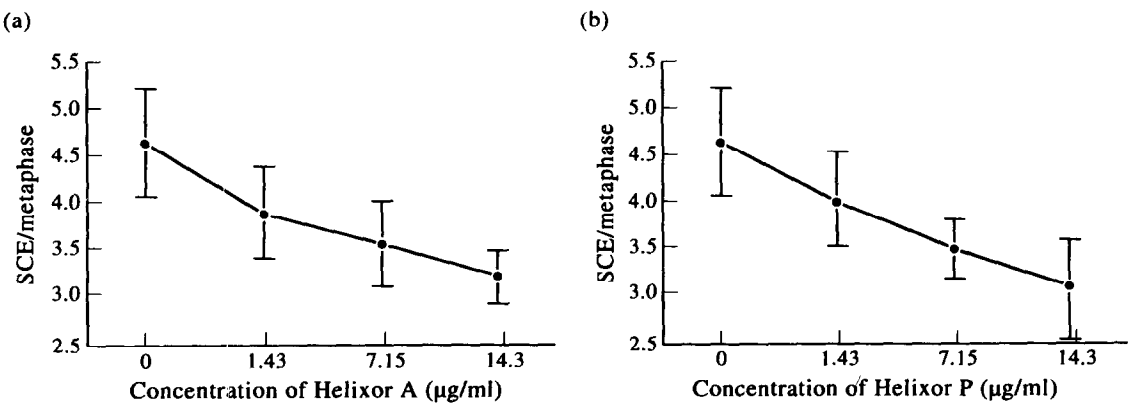

Figure 2. Sister chromatid exchanges of phytohaemagglutinin-stimulated peripheral blood mononuclear cells after treatment with mistletoe preparation (a) Helixor A or (b) Helixor P.

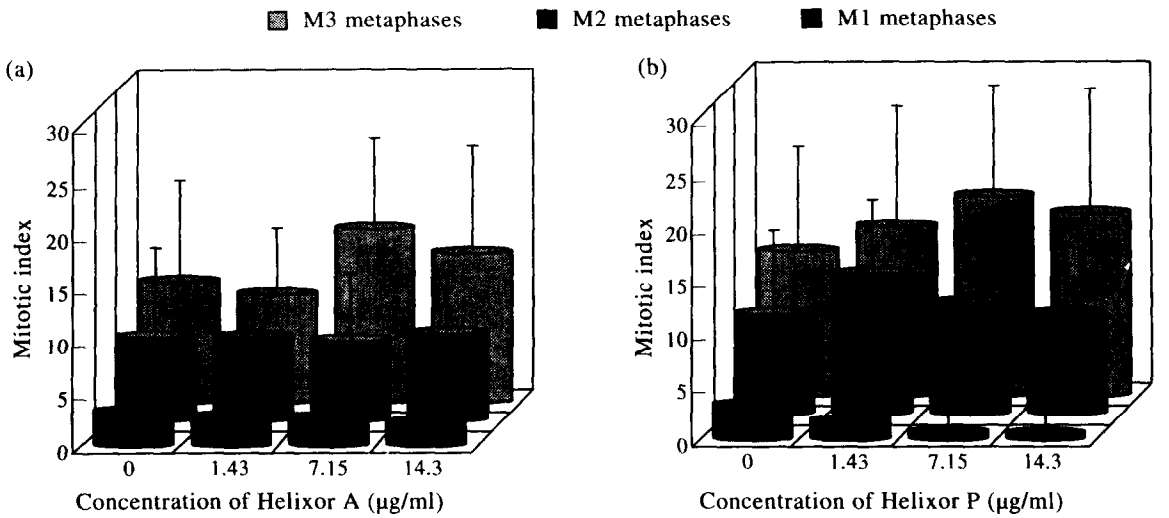

Figure 3. Mitotic indices of phytohaemagglutinin-stimulated peripheral blood mononuclear cells after treatment with preparation (a) Helixor A or (b) Helixor P.

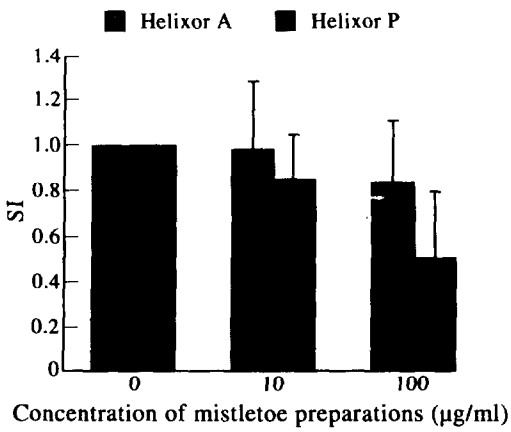

Figure 4. [^{3}H]Thymidine uptake of phytohaemagglutinin-stimulated lymphocytes after addition of different *Viscum album* L. extracts. SI, stimulation index = [$cpm_{(test)}/cpm_{(basal)}$].

Table 2. Differentiation of phytohaemagglutinin-stimulated peripheral blood mononuclear cells after addition of Helixor A (n = 5)

	Helixor A (μg/ml)		
	0	10	100
Lymphocytes*	56.2 ± 9.70	70.2 ± 9.36	54.0 ± 8.28
Monocytes*	4.2 ± 1.48	4.2 ± 0.84	3.4 ± 2.30
CD3+ T-cells†	75.4 ± 11.76	79.2 ± 9.65	81.6 ± 8.02
CD4/CD8 ratio	1.7 ± 0.58	1.9 ± 0.92	1.7 ± 0.54
CD20+ B-cells†	7.4 ± 3.05	8.4 ± 2.41	7.8 ± 3.03
NK cells†‡	4.4 ± 2.70	6.4 ± 3.21	5.2 ± 2.59
Interleukin-2R+/T cells§	84.6 ± 2.97	84.6 ± 7.86	87.6 ± 4.28

* Values are given in % ± S.D. of gated leucocytes. † Values are given in % ± S.D. of gated lymphocytes. ‡ NK cells = natural killer cells with CD16+CD56+CD3− cell surface antigens. § Value is given in % ± S.D. of CD3+ T lymphocytes.

used in our experiments gave no evidence for remarkable alteration of cell proliferation (Figures 3, 4). Cytostatic effects were only observable after administration of high concentrations of Helixor P (100 μg/ml), but not of Helixor A at any concentration tested.

As shown by others [22], B and T lymphocytes differ with regard to proliferation rates and SCE. B-cell cultures demonstrate lower SCE rates at faster proliferation rates than T lymphocytes. In our study, T lymphocytes remained the predominating population with a stable number of their regulatory subpopulations (Table 2). In addition, stable numbers of B-cells and monocytes, independent of drug concentration, were found.

Thus, we suggest that this effect of DNA stabilisation at therapeutical drug concentrations could counteract the negative effects of chemotherapy or radiation by protecting PBMC DNA itself or by enhancing DNA repair. This seems to be in agreement with the findings of others, such as those of Kovacs and colleagues [23], who observed reduced [^{3}H]thymidine incorporation of UV-damaged lymphocytes in breast cancer patients before treatment and an increase in incorporation after administration of mistletoe extracts. *Viscum album* L. extracts were also shown to exert a radioprotective effect on whole-body gamma-irradiated mice, producing an increased survival rate [24], and were found to reduce the leucocytopenia produced by radiation and cyclophosphamide treatment in animals [25].

More complex processes of cell–cell interaction seem to account for this SCE reduction. Purified lectin from *Viscum album* was shown to induce a release of tumour necrosis factor-α (TNF-α) [14, 15], of interleukin-1 (IL-1) and IL-6 [14], and of interferon-γ (IFN-γ) [15] by PBMC. The cytokines IL-2, IFN-γ and TNF have been shown to increase cell proliferation and SCE [26–28], whereas IFN-α and IFN-β [29, 30] reduce SCE frequencies. Therefore, the DNA protective effect of the *Viscum album* L. preparations may have some similarities with the lipopolysaccharide-induced radioprotection of immune cells (reviewed in [31]), that is, treatment with *Viscum album* L. extracts could be the initial step in a cascade of sequentially interacting events or substances (cytokines), leading to DNA protection and SCE reduction.

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