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MEDIATION OF HUMAN NK-ACTIVITY BY COMPONENTS IN EXTRACTS OF *VISCUM ALBUM*

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Abstract — *Viscum album* extracts (Iscador®) were investigated for their potency to influence NK cytotoxicity *in vitro*. *In vitro* short term cytotoxicity assays (4 h) with human peripheral mononuclear cells (PMNC) and human K 562 tumor cells showed a drastic enhancement of NK cytotoxicity in the presence of *V. album* extracts. The presence of the *V. album* components during tumor cell lysis was essential since pre-incubation of PMNC with *V. album* extract followed by thorough washing did not lead to enhancement of NK cytotoxicity. One responding effector cell was identified as a member of the large granular lymphocyte (LGL) family carrying both Leu 7 and Leu 11 surface markers. Furthermore, monocytes depleted of LGL, but not differentiated macrophages, showed a weak enhancement of their cytolytic activity in the presence of *V. album* extract. Fractionation of *V. album* extracts revealed two active fractions one (C1) with about 3–4000 D and the other (C2) < 1000 D. Both components enhanced NK cytotoxicity of LGL (Leu 7^+, Leu 11^+) as well as of monocytes showing enhancing effects also against moderately NK-sensitive tumor cell lines.

The ability to mediate spontaneous cytotoxicity against a wide variety of tumor cells is a function predominantly expressed by natural killer (NK) cells and monocytes and is found in a wide range of mammalian species. NK cells have been identified as a cell type with large granular lymphocyte (LGL) morphology (Timonen, Saksela, Ranki & Häyry, 1979). The ability to lyse tumor cells spontaneously might play an important role in immune surveillance and offers a potent alternative in immunological protection in all those instances in which T cells do not appear to play an effector role. The major physiological response modifiers of the spontaneous cytotoxicity of NK cells as well as of monocytes, already characterized, are interferons and other lymphokines (Einhorn, Blomgren & Strander, 1978; Trinchieri & Santolini, 1978; Herberman, Ortaldo & Bonnard, 1979; Adams & Hamilton, 1984; Kimber & Moore, 1984; Koff, Fogler, Gutterman & Fidler, 1985). In situations of pathological imbalance of the regulation of spontaneous cytotoxicity other biological response modifiers, i.e. of plant origin, might be of interest. The most promising sources available to screen for such activities are plant extracts with some relevant clinical history.

We investigated preparations of *Viscum album* (mistletoe) which are occasionally used in human adjuvant cancer therapy in Europe (Leroi, 1977; Salzer, 1978; Koch & Voss, 1980; Salzer, 1981). *V. album* extracts were reported to contain some tumor-inhibitory activity (Selawry, Schwartz & Haar, 1959). We now demonstrate that *V. album* extracts strongly increase the cytolytic activity of a LGL subpopulation and to a lower degree cells of a monocyte population when tested in cytotoxicity assays against human tumor cell lines. Furthermore, we identified two components in *V. album* extracts, one with a molecular weight of about 3–4000 D and the other smaller than 1000 D which strongly mediated the spontaneous cytotoxicity of human NK cells against different target cell lines.

EXPERIMENTAL PROCEDURES

Materials

V. album *preparations*. Commercially available preparations (batch 5021 C; Weleda, Schwaebisch Gmuend, FRG) with the trade name Iscador (*Viscum album mali*; 5% solution based on the weight of the fresh plant) were used in all experiments. Iscador is a

mistletoe preparation which is registered in Switzerland. The principal manufactoring step is established by natural fermentation of fresh plant juice. This results in a high content of lactobacilli (Bloksma, van Dijk, Korst & Willer, 1979). For each experiment a fresh ampul stored at 4°C was used. During storage in opened ampules the capacity to enhance NK cytotoxicity was gradually reduced.

Biological response modifiers. Human interleukin 2 (IL2), 100 U/ml, free of lectins, was purchased from Paesel, Frankfurt, FRG. Human α-interferon (IFN) with a specific activity of 4×10^7 U/mg protein was a gift of Dr Bruchelt, Children's Hospital, University Tuebingen, FRG.

Antibodies. The following monoclonal antibodies were used for the characterization of human immune cell populations: OKM1 (null cells and monocytes), OKT 3 (pan-T-cell marker), OKT 4 (helper/inducer T cells) and OKT 8 (suppressor/cytotoxic T cells) were purchased from Ortho Diagnostic Systems, Neckargemuend, FRG; anti-Leu 4 (pan-T-cell marker), anti-Leu 7 (= HNK1; NK cells), anti-Leu 11 (NK cells), anti-Leu 14 (B cells) and anti Leu M3 (monocytes) were obtained from Becton – Dickinson, Heidelberg, FRG.

Tumor cells. The human erythroleukemic cell line K562 (Lozzio & Lozzio, 1975), the human colon tumor cell line HT29 (Fogh & Trempe, 1975), the human mammary tumor cell line MCF-7 and melanoma MEC cell line (both kindly supplied by Dr Sproll and Spindler, Tübingen) were used in the cytotoxicity assay and were grown in RPMI 1640 supplemented with 10% of fetal bovine serum, 10^5 U/ml penicillin and 100 μg/ml streptomycin (RPMI/10% FBS).

Immune cell preparations

Preparation of peripheral mononuclear cells (PMNC). PMNC were obtained from heparinized blood (10 IU/ml) or buffy coat preparations from normal volunteers by Ficoll – Paque (Pharmacia) gradient centrifugation (ϱ = 1.077 g/ml) according to the outlines given by Bøyum (1968). Cells were cultured in RPMI/10% FBS. Experiments were carried out in a humidified atmosphere of air containing 5% CO_2.

Preparation of non-adherent cells (PNAC). PMNC were depleted of monocytes by adherence (60 min, 37°C) on Petri culture dishes (150 × 15 mm) using 40 ml of a cell suspension with 4×10^6 cells/ml. The resulting non-adherent cell population still contained 2 – 4% monocytes determined by surface marker analysis. The cells were subsequently passed through nylon wool columns (Julius, Simpson & Herzenberg, 1973) : 0.6 g of prewashed nylon wool (Fenwal Labs.) was packed into a 10 ml plastic syringe, washed with RPMI/10% FBS at 37°C and loaded with $80 - 100 \times 10^6$ PMNC in 1 ml of culture medium followed by 45 min incubation at 37°C. Elution was performed with 20 ml pre-warmed RPMI/10% FBS at a flow rate of 20 drops/min. The non-adherent cell population contained less than 1% monocytes and B-cells.

Enrichment of NK cells (large granular lymphocytes, LGL). Two procedures were used. In the one-step Percoll gradient centrifugation $50 - 70 \times 10^6$ PNAC in RPMI/10% FBS adjusted to 285 mosmol/kg water were layered onto a cushion of 43.5% Percoll in RPMI/10% FBS also adjusted to 285 mosmol/kg water according to the outlines given by Phillips & Lanier (1985). The low density fraction harvested from the gradient interface after centrifugation at 300 × **g** for 50 min contained essentially all lymphocytes with NK activity (9.4% of input cell number). In the second procedure a discontinuous seven-step density gradient of Percoll as described by Timonen, Ortaldo & Herberman (1981) was used. The gradient was prepared in 15 ml conical Falcon test tubes; 5×10^7 PNAC were layered on top of the gradient followed by centrifugation at 550 × **g** at room temperature for 30 min. The recovery of cells was about 83% and vitality as judged by trypan blue exclusion was > 98% in all fractions except fraction 1 (80%). The cells of fraction 2 (8% of imput) showed the highest content of NK activity and NK cell-associated surface markers.

Enrichment of Null cells. PNAC containing about 80% T-cells were depleted of T-cells by high affinity E-rosetting (Bloom & Babitt, 1985) using the pretreatment of sheep red blood cells with S(2-amino-ethyl)isothiuronium bromide (Urbaniak, White, Barclay, Wood & Kay, 1978) in combination with the procedure of West, Boozer & Herberman (1978). Thereafter, the T-cell content was 20% (OKT 3+).

Preparation of monocytes and monocyte-derived macrophages. Adherent cells were isolated following incubation of 50 ml PMNC (4×10^6 cells/ml) in RPMI/10% FBS at 37°C for 1 h using plastic Petri dishes (150 mm dia) (Fischer, Hubbard & Koren, 1981). The non-adherent cells removed by two gentle washes with PBS containing 5% FBS contained up to 4% monocytes. The adherent cells were removed by gentle scraping with a rubber policeman using PBS containing 0.02% EDTA. The cells recovered

showed 95% viability and contained 75–80% monocytes as judged by staining for α-naphtyl acetate esterase (ANAE) activity (Koski, Poplack & Blaese, 1976).

Monocyte-derived macrophages were obtained from the monocyte population by seeding 5×10^5 cells/200 μl RPMI/10% FBS into a flat-bottomed 96 well microtiter plate and subsequent incubation for 7 days at 37 °C (Cameron, 1984). Thereafter, the loosely adherent cells detached during washing were sucked off (Eggen, 1983). The adherent cells recovered consisted of > 95% $ANAE^+$ cells.

Pretreatment of effector cells. Equal volumes of PMNC (2×10^6 cells/ml) and *V. album* extract with 200, 100 and 20 μg/ml in RPMI/10% FBS were incubated in Petriperm culture dishes (Heraeus, Hanau, FRG) at 37°C with 5% CO_2 in air for various times up to 72 h. Thereafter, the cells were washed thoroughly with PBS. The viability of cells was always > 95%.

Cell surface marker analysis. The PMNC subsets were analyzed for reactivity with monoclonal antibodies by the indirect immunofluorescence method: 50 μl of OKT 3, OKT 4, OKT 8, OKM 1, Leu 4, Leu 7, Leu 11b, Leu M3 or Leu 14 diluted according to the manufacturer's instructions in HBSS/2% FBS/0.1% NaN_3 (HBSS/2% FBS) were added to 50 μl cell suspension (20×10^6 cells/ml, viability > 95%) in HBSS/2% FBS using V-shaped microtiter plates. The plates were incubated for 45 min on ice followed by centrifugation at 300 × g for 5 min. Supernatants were carefully aspirated and after two washings with 100 μl of cold HBSS followed by further incubation on ice with 100 μl of an appropriate dilution of FITC-conjugated rabbit anti-mouse IgG (H + L) (Miles) for 30 min. After two further washings at least 300 cells were scored in a fluorescence microscope.

Complement-mediated cytolytic depletions. Monocytes, PNAC and LGL (1-step Percoll gradient) were reacted on ice with antibodies (anti Leu 7 or anti Leu 11b) at optimal concentrations for 30 min using 10^7 cells in 0.5 ml HBSS/2% FBS. Thereafter, 0.5 ml HBSS/2% FBS was added and the cell suspension centrifuged at 400 × g for 5 min. The sedimented cells were resuspended in 1 ml of rabbit complement (Calbiochem, Hoechst; 1:4 dilution in HBSS/2% FBS) and incubated at 37°C for 1 h under occasional agitation. After incubation the cells were washed three times in PBS, resuspended in RPMI/10% FBS and examined for viability. This procedure was repeated once to assure depletion of the lymphocytes of the designated cell subset. Cells treated with medium and complement alone were used as controls. IF-staining of Leu 7 or Leu 11-depleted cell populations revealed less than 0.5% Leu 7^+ or Leu 11^+ cells.

Cytotoxicity assay

A modified lactic acid dehydrogenase (LDH) release assay for natural cytotoxicity originally described by Korzeniewski & Callewaert (1983) was used in short term (4 h) and long term (18 h) cytotoxicity assays. All cells assayed in this system were transferred into RPMI 1640 medium without phenol red, supplemented with 2% (w/v) bovine serum albumin (RPMI/2% BSA). Assays were carried out in round-bottomed 96 well microtiter plates (Nunc, Wiesbaden) with a final sample volume of 200 μl: 2×10^4 and 4×10^4 K562 cells in 100 μl/well were cocultured with various dilutions of effector cells in 50 μl (resulting in E:T ratios up to 25:1) and with 50 μl of biological response modifiers diluted in RPMI/2% BSA or 50 μl of RPMI/2% BSA alone as effector cell controls including controls of biological modifiers with target cells or effector cells alone. All samples were run in triplicate. At the beginning of the assay the plates were centrifuged for 30 s at 80 × g followed by incubation at 37°C with 5% CO_2 in air for the given assay time. Maximum release of LDH-activity was achieved by addition of 100 μl 2% (w/v) Triton X-100, spontaneous LDH release controled by addition of 100 μl RPMI/2% BSA to 100 μl of target cell suspension. At the end of the assay the microtiter plates were again centrifuged for 2 min at 100 × **g**. Aliquots of cell-free supernatants (100 μl) were transferred into corresponding wells of flatbottomed microtiter plates. Subsequently 100 μl of freshly prepared lactic acid dehydrogenase substrate mixture (5.4×10^{-2} M L(+)lactate, 6.6×10^{-4} M 2p-iodophenyl-3p-nitrophenyl tetrazolium chloride, 2.8×10^{-4} M phenazine methosulfate and 1.3×10^{-3} M NAD in 0.2 M Tris buffer, pH 8.2) were added to each well at 1 s intervals and reaction was allowed to start in the dark. After 8 to 10 min incubation at room temperature the reaction was stopped by adding 50 μl 1 N HCl to each well at the same 1 s intervals. The amount of LDH-release was recorded by a microtiterplate reader (Dynatech) monitoring the absorbance at 490 nm with reference wavelength 630 nm. Percentage cytotoxicity (C) was calculated using the formula:

$$\%C = \frac{E - S}{M - S} \times 100,$$

where E is the experimental LDH-release resulting from coculture of effector and target cells, S is the spontaneous LDH-release resulting from target cell

Table 1. Effects of *V. album* extract on the NK activity of human PMNC from different donors

Donor	Concentration *V. album* extract (μg/ml)	Cytotoxicity* (%)
A	control	15.2 ± 0.8
B	control	20.0 ± 2.4
C	control	18.6 ± 1.7
A	100	45.1 ± 1.2
B	100	55.1 ± 2.1
C	100	44.0 ± 1.3
A	50	44.1 ± 2.0
B	50	45.0 ± 1.6
A	25	40.1 ± 0.6
A	10	28.5 ± 1.1
B	10	25.0 ± 2
C	10	26.3 ± 2
A	5	23.4 ± 0.3
A	1	17.0 ± 1.3
C	1	19.4 ± 2.2
A	0.1	16.8 ± 1.3

*Cytotoxicity was determined in a 4 h cytotoxicity assay using human K562 cells as target cell (E/T = 20). Standard deviations refer to triple determinations.

death alone during assay time and M is the maximum LDH-release from detergent-mediated target cell lysis.

Proliferation assay

The influence of *V. album* extracts on the proliferation of tumor cells and human peripheral mononuclear cells (PMNC) was investigated using the colorimetric method of Mosmann (1983). The cells were incubated in RPMI/10% FBS using population densities of $2 \times 10^4 - 2 \times 10^5$ tumor cells/ml or $2 \times 10^5 - 10^6$ PMNC/ml, *V. album* extract concentrations between 1 and 250 μg/ml and incubation periods up to 3 days.

Fractionation of V. album *extract*

Subfractions of the *V. album* extract were prepared by gel filtration on Biogel P6 columns (1.6 × 90 cm) with a 180 ml bed volume equilibrated with 50 mM Tris / 150 mM NaCl pH 7.0 or water. *V. album* extracts applied to the columns were eluted with the same buffer at a flow rate of 18 ml at room temperature. Thereafter, the fractions were lyophilized and kept at 4°C until use.

RESULTS

Effect of V. album *extracts on cell proliferation* in vitro

The influence of *V. album* extracts on the proliferation of human PMNC as well as of human tumor cells was examined in separate tests with triple determinations using concentrations between 1 and 250 μg *V. album* extract/ml. The incubation of PMNC up to 72 h at 37°C revealed no change of the proliferation rate at any of the *V. album* extract concentrations tested. In some commercial batches of *V. album* extract a slight cytotoxic activity against tumor cells was observed during a 4 h assay. These batches were not used for proliferation and cytotoxicity assays. The other batches showed no influence on the proliferation of K562 tumor cells.

Effect of V. Album *extract on NK cytotoxicity of human PMNC* in vitro

In a first set of experiments the *in vitro* effect of *V. album* extract on the NK activity of human PMNC was examined in a 4 h assay at an effector cell/target cell ratio E/T = 20:1 using varying concentrations of *V. album* extract. To exclude any cytotoxic effects initiated directly by *V. album* extract or its components adequate controls with tumor cells and *V. album* extract samples were run in parallel in each cytotoxicity assay. The assay was started by adding human PMNC and *V. album* extract to tumor cells K562. The increase in NK cytotoxicity dependent on the concentration of *V. album* extract is shown in Table 1 for the PMNC of three different donors. The PMNC of all three donors exhibited an increase in NK cytotoxicity in the presence of *V. album* extract with the strongest effects at the highest *V. album*

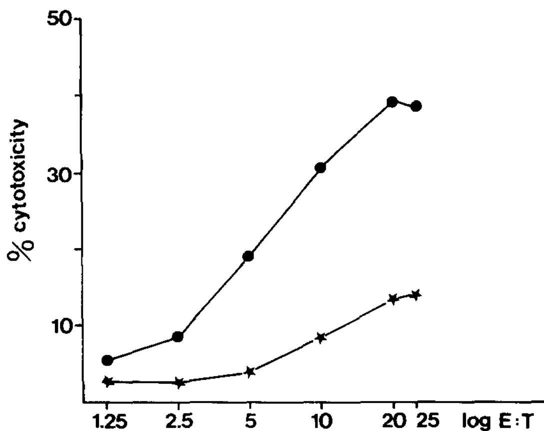

Fig. 1. Effect of *V. album* extract on the NK activity of human PMNC. Cytotoxicity assays (4 h) were carried out at various effector cell/target cell (E/T) ratios (T = 2 × 10^4 K562 cells/well). The standard concentration of *V. album* extract was 50 μg/ml. ● = cytotoxicity in the presence of *V. album* extract; ★ = controls.

extract concentrations. The cytotoxicity activation index (CAI) of the PMNC of 8/11 healthy donors tested under standard conditions (4 h assay; E/T ratio 20:1; *V. album* extract concentration 50 μg/ml) was found to be > 2, and the other 3/11 donors > 1.5. The influence of various E/T ratios on the enhancement of NK cytotoxicity in the presence of *V. album* extracts is shown in Fig. 1. In the semi log plot a linear relation of cytotoxicity was observed for E/T ratios between 2.5 and 20.

A prolonged assay time (18 h) drastically increased the sensitivity of the *V. album* extract-mediated NK cytotoxicity. In the 4 h assay, no significant effects were found at concentrations lower than 5 μg *V. album* extract/ml but in the 18 h assay the enhancing effects observed with 0.1 μg *V. album* extract/ml were only slightly less than those with 50 μg/ml. In Table 2 the increases of NK activity in the presence of various concentrations of *V. album* extract during a 1, 4 and an 18 h assay (triple determinations) are compared for PMNC of a single donor.

A preliminary information on the mechanism of action of *V. album* extracts is offered by the fact that the active components of the *V. album* extract must be present for tumor cell lysis. Preincubation of tumor cells with *V. album* extract for 4 h followed by thorough washing did not render the tumor cells presensitized for the subsequent 4 h cytotoxicity assay with PMNC. Similarly, prolonged preincubation of PMNC with *V. album* extract for 24 h or 72 h followed by thorough washing did neither result in stimulation of NK cells for the subsequent cytotoxicity assay with K562 tumor cells. However, all pre-incubated and washed cell samples were readily accessible to a subsequent enhancement of NK activity in a 4 h assay in the presence of *V. album* extracts (data not shown).

Effect of V. album *extracts on NK cytotoxicity of PMNC sub-populations*

Involvement of adherent cells in *V. album* extract-mediated enhancement of NK activity was investigated with PMNC depleted of adherent cells as well as with isolated monocytes and macrophages. Monocyte-depleted PMNC (PNAC) showed an enhancement of NK activity in the presence of various concentrations of *V. album* extract in a 4 h assay about equivalent to that of the original PMNC sample (Fig. 2) indicating that adherent cells might be only involved to a minor degree in the *V. album* extract-mediated enhancement of NK activity. This assumption was further investigated with isolated monocyte or macrophage populations. Monocytes also showed an enhancement of NK cytotoxicity in the presence of *V. album* extracts when tested in a 18 h cytotoxicity assay, an effect which was scarcely evident in the 4 h cytotoxicity assay. However, differentiated macrophages exhibited no enhancing effects in the presence of *V. album* extract during the 18 h assay, on the contrary, even the spontaneous NK cytotoxicity of differentiated macrophages was blocked in the presence of *V. album* extracts (Fig. 2).

Further subpopulations of human PMNC or PNAC were studied analogously. The surface marker distribution in the cell populations studied are given in Table 3. The LGL and null-cell samples listed were derived from different donors. The *V. album* extract-mediated enhancement of NK activity was highly accumulated in the subpopulations of null cells and LGL (Fig. 2). Identification of the effector cells could be achieved by surface marker-specific, complement-dependent lysis. Depletion of Leu 7^+ or Leu 11^+ cells from plastic non-adherent cells (PNAC) or from the large granular lymphocyte (LGL) fractions abolished the ability to exhibit enhanced NK activity in the presence of *V. album* extract (Fig. 2). This indicated that the effector cells responding to *V. album* extracts must carry both markers, Leu 7 and Leu 11. The question whether the enhancing activity of *V. album* extracts on the NK cytotoxicity of monocytes (Fig. 2) might reside in Leu 7^+/Leu 11^+ cells still present in the monocyte population (Table 3) could be answered by cytotoxicity assays after depletion of Leu 7^+ cells in the monocyte population using two consecutive

Table 2. Effects of *V. album* extract on the NK activity of human PMNC depending on the assay time

Assay time (h)	Concentration *V. album* extract (μg/ml)	Cytotoxicity* (%)
1	control	5.9 ± 0.6
4	control	21.0 ± 0.6
18	control	28.1 ± 0.1
1	100	27.4 ± 1.6
4	100	45.0 ± 1.1
1	50	26.4 ± 0.2
4	50	44.2 ± 1.9
18	50	49.0 ± 2.6
1	25	22.7 ± 2.2
4	25	40.2 ± 0.6
1	10	12.1 ± 1.1
4	10	28.7 ± 1.0
1	5	9.1 ± 1.7
4	5	23.6 ± 0.4
18	5	40.3 ± 2.8
4	1	22.0 ± 1.5
18	1	46.1 ± 1.9
4	0.1	22.1 ± 1.1
18	0.1	41.0 ± 2.8

*Cytotoxicity was determined in 1, 4 and 18 h cytotoxicity assays using human K562 cells as target cells (E/T = 20). Standard deviations refer to triple determinations.

treatments with monoclonal anti Leu 7 antibody and complement. The *V. album* extract-mediated enhancement of NK cytotoxicity of monocytes was partly decreased but still present (Fig. 2) indicating that *V. album* extracts may also enhance NK cytotoxicity of monocytes but to a distinctly lower degree than that of LGL.

Information on whether the Leu 7 and Leu 11 epitopes are directly involved in the *V. album* extract-mediated enhancement of NK cytotoxicity was obtained from cytotoxicity assays in the presence of *V. album* extract and anti Leu 7/anti Leu 11 antibodies but without addition of complement. No significant change of the enhancement of NK cytotoxicity by the *V. album* components could be observed in the presence of anti Leu 7 and anti Leu 11 antibodies.

NK activity-enhancing sub-fractions of V. album *extract*

In order to obtain information on the molecular size of the active component(s) of the *V. album* extract, gel filtration on Biogel P6 was performed. The elution pattern is shown in Fig. 3. Screening of fractions for enhancement of NK cytotoxicity against K562 tumor cells in the 4 h cytotoxicity assay revealed an active fraction, *V. album* C1, in the molecular weight range of the insulin B chain (3300 D) and a second active fraction, *V. album* C2, in the range of salt elution which corresponds to a molecular weight below 1000 D, i.e. below the lower cut-off of Biogel P6 columns. Some inactive components were strongly retarded on Biogel P6 and were eluted after the salt fraction. The elution pattern varied slightly with different batches of *V. album* extract but all batches tested contained the two active fractions.

Both active fractions, *V. album* C1 and C2, were again examined for effector cell specificity using LGL as effector cells in a 4 h cytotoxicity assay. The influence of various E/T ratios on the enhancement of the NK cytotoxicity in the presence of *V. album* extract and its active fractions is shown in Fig. 4. Both active *V. album* fractions behave very similar and exhibit about the same enhancing effects on the NK cytotoxicity of LGL in relation to the varied E/T ratios. Essential differences in the biological function of both active fractions were neither observed in a prolonged cytotoxicity assay with various PMNC subpopulations. The results of a 4 h and an 18 h cytotoxicity assay with human K562

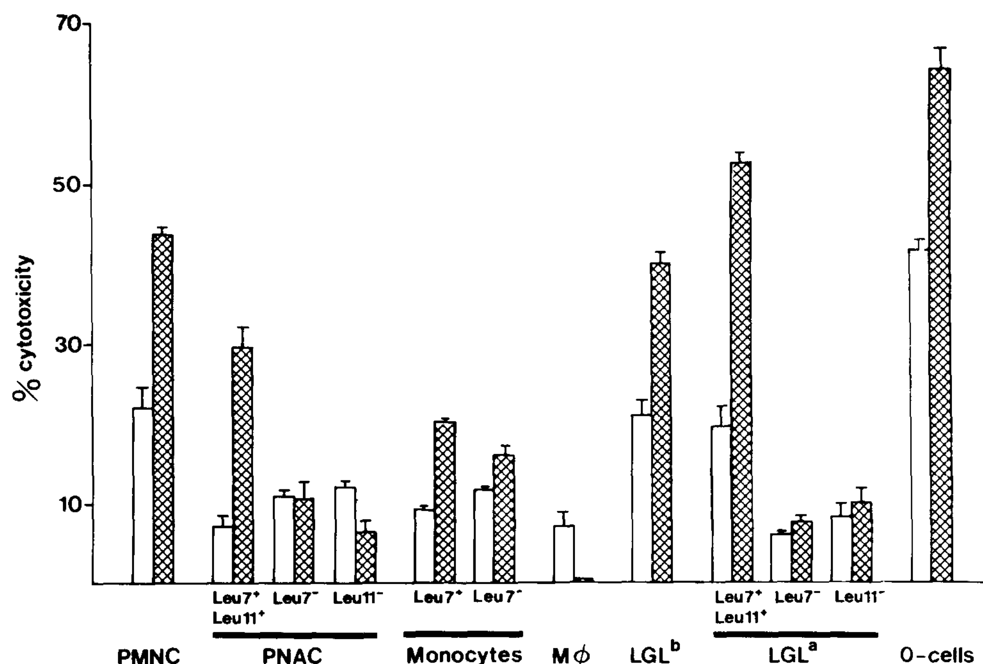

Fig. 2. Effect of *V. album* extract on the NK activity of PMNC and their sub-populations. The individual cell populations were assayed in a 4 h cytotoxicity assay against K562 cells using the following E/T ratios: PMNC (E/T = 25:1), PNAC (E/T = 20:1), monocytes (E/T = 20:1), macrophages (E/T = 10:1), null cells (E/T = 20:1), LGL (E/T = 25:1). The concentration of *V. album* extract was 50 μg/ml. The data of monocyte and macrophage cytotoxicity are given for the 18 h cytotoxicity assay. PNAC, monocytes and LGL were also assayed after depletion of Leu 7^+ cells or Leu 11^+ cells. LGL^a were derived from one-step, LGL^b from seven-step Percoll gradient centrifugation. Open bars represent controls, hatched bars demonstrate cytotoxic effects in the presence of *V. album* extract.

Table 3. Cell surface marker analysis of cell populations used in cytotoxicity assays

Cell population	Cell surface marker (% of total cells)								
	ANAE⁺	Leu M3⁺	OKT3⁺	OKT4⁺	OKT8⁺	Leu 7⁺	Leu 11⁺	OKM1⁺	Leu 14⁺
PMNC	17.3	16.0	63	46.6	36.5	15.7	13.2	21.5	10.3
PNAC	0	0	83	71	43	24	9	—	0
Monocytes	71	75	27	—	—	10.3	9.7	67	—
Macrophages	95	—	—	—	—	—	—	—	—
LGL*	0	—	51	—	—	48	44	—	0
LGL†	0	—	55	—	—	55	54	—	—
Null-Cells	0	—	22	—	—	50.1	48.0	—	0

*Isolated in a one-step Percoll gradient (Phillips & Lanier, 1985).
†Isolated in a seven-step Percoll gradient (Timonen *et al.*, 1981).

tumor cells are summarized in Table 4 and compared with the effects of original *V. album* extract. In addition, data obtained from cytotoxity assays in the presence of α-interferon (IFN) and interleukin 2 (IL-2) are listed for further comparison. The subfractions C1 and C2 of *V. album* extract did not only enhance NK cytotoxicity of LGL, they also enhanced NK cytotoxicity of a monocyte population to a similar degree, both exhibiting improved effects in the 18 h assay. The blocking of the spontaneous cytotoxicity of differentiated macrophages in the presence of *V. album* extract as shown in Fig. 2 was also effected by *V. album* C1 as well as by *V. album* C2 (Table 4, 18 h assay). The comparative data obtained for the enhancement of NK cytotoxicity of PMNC or PNAC by IFN or IL-2 in a 4 h assay were distinctly lower than those obtained in the presence of *V. album* components (Table 4). The data did not

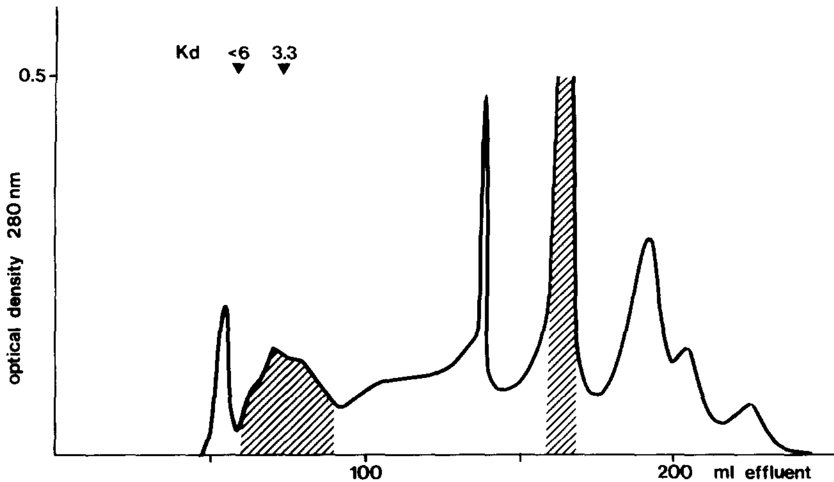

Fig. 3. Fractionation of *V. album* extract on a Biogel P6 column. The column (1.6 × 90 cm) was equilibrated and eluted with 50 mM Tris/150 mM NaCl, pH 7.0. Fractions enhancing NK activity of human PMNC are indicated by hatching; molecular weight markers are given in kD.

change significantly when the effector cells were preincubated with IFN or IL-2 for 18 h (not shown). However, IFN and IL-2 showed improved enhancement of NK cytotoxicity of PNAC in the 18 h cytotoxicity assay but still distinctly lower than that induced by *V. album* components.

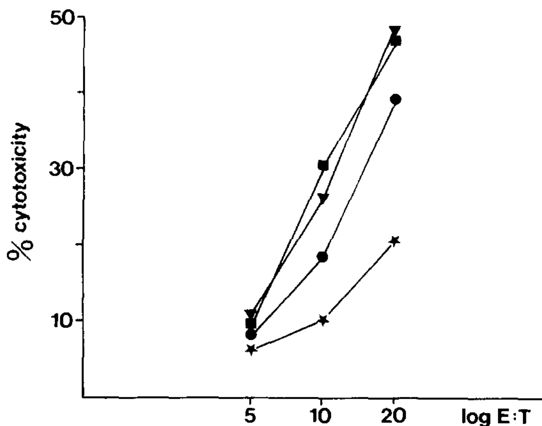

Fig. 4. Effect of *V. album* extract subfractions on NK activity of human LGL. The NK cytotoxicity induced by the active subfractions *V. album* C1 (■) and *V. album* C2 (▲) is compared with that of original *V. album* extract (●) at concentrations of 50 μg/ml and at various E/T ratios in a 4 h cytotoxicity assay (T = 2 × 10^4 K562 cells/well). Controls (★) were performed at the same E/T ratios.

The NK-enhancing capacity of *V. album* components was further investigated with NK-sensitive and moderately NK-sensitive human tumor cell lines using peripheral mononuclear cells (PMNC) of a single donor in a 4 h cytotoxicity assay. The PMNC of this donor showed a different spontaneous cytotoxicity against the selected tumor cell lines: 22% against K562 cells, 29% against MCF-7 mammary carcinoma cells, 3.4% against MEC melanoma and 7.3% against HT29 colon carcinoma cells (Table 5). In the presence of *V. album* extract and its active components the NK cytotoxicity against all cell lines tested was drastically enhanced, especially against the weakly NK-sensitive tumor cell lines MEC and HT29 (Table 5). No essential differences in the biological function of *V. album* components C1 and C2 became evident.

DISCUSSION

The data obtained from the *in vitro* investigations show that at least two components present in *V. album* extracts are able to modify human NK cell cytotoxicity. Enhancement of NK cytotoxicity was observed with all PMNC subpopulations which contained LGL. The effector cell involved must carry both the Leu 7 and the Leu 11 surface marker which identify it as a member of the LGL population (Abo, Miller & Balch, 1984; Lanier, Benike, Phillips & Engleman, 1985). The ability of *V. album* extract components to enhance NK cytotoxicity of LGL was

Table 4. Effects of various biological modifiers on NK cytotoxicity of PMNC and their subpopulations against K562 tumor cells

Assay time	Modifier (conc./ml)	Cytotoxicity of effector cells*				
		PMNC (25:1)†	PNAC (20:1)	LGL (20:1)	Monocytes (20:1)	MØ (10:1)
4 h	control	22.0	7.4	21.0	5.5	—
	V. album extract (50 μg)	43.9	29.7	39.9	8.6	—
	V. album C1 (50 μg)	42.1	31.5	47.6	10.2	—
	V. album C2 (50 μg)	34.5	25.9	48.8	9.4	—
	IL-2 (10 U)	25.0	9.6	—	—	—
	(25 U)	30.0	—	—	—	—
	IFN (200 U)	23.0	8.3	—	—	—
	(2000 U)	27.0	9.1	—	—	—
18 h	control	27.1	14.5	—	9.0	7.1
	V. album extract (100 μg)	38.5	33.8	—	20.4	1.0
	V. album C1 (100 μg)	40.0	38.5	—	23.1	3.2
	V. album C2 (100 μg)	40.9	30.5	—	17.1	4.1
	IL-2 (5 U)	—	24.9	—	—	—
	IFN (500 U)	—	27.9	—	—	—
	(1000 U)	—	24.9	—	—	—

* Given in % cytotoxicity.
† Ratio effector cells/target cells.

abolished after depletion of Leu 7+ cells as well as after depletion of Leu 11+ cells. In addition, it could be demonstrated that *V. album* components also weakly enhanced NK cytotoxicity of monocyte populations more effectively in the 18 h than in the 4 h assay. It could be excluded that this effect was due to LGL frequently present in minor amounts in such populations as reported by Horwitz, Bakke, Abo & Nishiya (1984) and Freundlich, Trinchieri, Perussia & Zurier (1984).

The enhancement of NK cytotoxicity by *V. album* components appeared not to be limited to highly NK-sensitive target cells such as K562 tumor cells. Drastic stimulation of NK cytotoxicity by *V. album* extract and its active component was also obtained against the weakly NK-sensitive human MEC melanoma cell line.

The enhancement of NK cytotoxicity required the presence of active *V. album* components during the cytotoxic event since preincubation of PMNC with *V. album* components followed by washings abolished the enhancing effect. The active *V. album* components possibly mediate the direct interaction of effector cells and target cells. However, it could be

Table 5. Effects of *V. album* components on the NK-cytotoxicity of human PMNC of a single donor against highly and moderately NK-sensitive tumor cell lines

Modifier (μg/ml)		%Cytotoxicity* against			
		K562	MCF 7	MEC	HT 29
Control		21.9 ± 0.5	28.9 ± 2.5	3.4 ± 0.9	7.3 ± 0.5
V. album extract	100	34.3 ± 0.3	—	20.7 ± 2.0	18.5 ± 1.1
	50	36.3 ± 3.1	42.4	15.6	11.7 ± 1.0
	25	32.4 ± 0.6	—	8.0 ± 1.5	9.2 ± 0.4
	10	26.9 ± 0.8	—	4.4	7.5 ± 0.4
	1	15.0 ± 1.9	—	3.2 ± 1.7	7.2 ± 0.6
V.album C1	50	31.4 ± 2.2	46.3	16.1 ± 4.1	11.4 ± 0.9
	10	27.7 ± 1.4	—	6.6	7.1 ± 0.6
V.album C2	50	29.1 ± 0.8	40.6 ± 2.9	12.2	10.8 ± 1.2
	10	23.3 ± 2.8	—	3.7	7.7 ± 0.7

*Cytotoxicity was determined in a 4 h cytotoxicity assay at E/T ratios 20:1.

excluded that the Leu 7 and Leu 11 epitopes on LGL are involved in such a direct interaction of *V. album* components with LGL effector cells and target cells.

The active components C1 and C2 present in *V. album* extracts were both capable of enhancing NK cytotoxicity of Leu 7^{+}/Leu 11^{+} LGL as well as of monocytes in a 4 h assay. Differences in their biological activity are presently not evident except of differences in the stability of the NK-enhancing capacity. Solutions of *V. album* C1 in medium had lost the enhancing capacity completely after 5 days at 4°C whereas solutions of *V. album* C2 maintained full enhancing capacity even after 7 days.

The comparison of the enhancement of NK cytotoxicity mediated by *V. album* components to that by other biological response modifiers, i.e. IFN and IL-2, shows that the *V. album* components are very potent enhancers of NK cytotoxicity possibly exhibiting an entirely different molecular mechanism of activation of tumor cell killing by NK cells. The presence of *V. album* components was essential for tumor cell lysis whereas that of IFN and IL-2 was not. IFN and IL-2 stimulated NK cytotoxicity during preincubation of human PMNC while *V. album* extract was unable to do so. The only reaction step in common might be seen in the cytolytic mechanism thus offering the *V. album* component-induced NK cytotoxicity as a model system to study further details of target cell killing.

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