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ENHANCEMENT OF HUMAN NATURAL KILLER CELL ACTIVITY BY MODIFIED ARABINOXYLANE FROM RICE BRAN (MGN-3)

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Summary: *Arabinoxylane from rice bran (MGN-3) was examined for its augmentory effect on human NK (NK) cell activity* in vivo *and* in vitro. *Twenty-four individuals were given MGN-3 orally at three different concentrations: 15, 30 and 45 mg/kg/day for 2 months. Peripheral blood lymphocyte-NK cell activity was tested by ^{51}Cr release assay against K562 and Raji tumor cells at 1 week, 1 month and 2 months post-treatment and results were compared with baseline NK activity. Treatment with MGN-3 enhanced NK activity against K562 tumor cells at all concentrations used. In a dose-dependent manner, MGN-3 at 15 mg/kg/day increased NK activity after 1 month posttreatment (twofold over control value), while significant induction of NK activity at 30 mg/kg/day was detected as early as 1 week posttreatment (three times control value). NK cell activity continued to increase with continuation of treatment and peaked (fivefold) at 2 months (end of treatment period). Increasing the concentration to 45 mg/kg/day showed similar trends in NK activity, however the magnitude in values was higher than for 30 mg/kg/day. After discontinuation of treatment, NK activity declined and returned to baseline value (14 lytic units) at 1 month. Enhanced NK activity was associated with an increase in the cytotoxic reactivity against the resistant Raji cell line. MGN-3 at 45 mg/kg/day showed a significant increase in NK activity after 1 week (eightfold) and peaked at 2 months posttreatment (27 times that of baseline). Culture of peripheral blood lymphocytes (PBL) with MGN-3 for 16 h demonstrated a 1.3 to 1.5 times increase in NK activity over control value. The mechanism by which MGN-3 increases NK activity was examined and showed no change in cluster of differentiation (CD)16$^+$ and CD56$^+$ CD3$^-$ of MGN-3-activated NK cells as compared with baseline value; a fourfold increase in the binding capacity of NK to tumor cell targets as compared with baseline value; and a significant increase in the production of interferon-γ (340-580 pg/ml) postculture of PBL with MGN-3 at concentrations of 25-100 µg/ml. Thus, MGN-3 seems to act as a potent immunomodulator causing augmentation of NK cell activity, and with the absence of notable side-effects, MGN-3 could be used as a new biological response modifier (BRM) having possible therapeutic effects against cancer.*

Introduction

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There is an increasing body of evidence implicating the NK phenomenon as a discrete subpop-

ulation of lymphocytes capable of mediating lysis of a variety of tumor target cells regardless of major histocompatibility components (1-5). For example, it has been shown that patients with Chediak-Highashi syndrome who display selective natural killer (NK) deficiency are also prone to develop lymphoproliferative disorders (6); defective NK cell activity predisposes patients to develop lymphomas (7); genetic abnormalities associated with impaired NK activity may predispose animals to develop lymphoid malignancies (8, 9); adoptive transfer of NK clone to beige mice provided resistance to radiation-induced thymic leukemia (10); and data from our laboratory show that female mice that have lower NK activity compared with males are also more susceptible to tumor development than males (2). These data taken together provide compelling evidence supporting the role of NK cells in host surveillance.

The various immunological functions of NK cells make them prime candidates as therapeutic agents. Interleukin-2 (IL-2) has been shown to boost NK activity in peripheral blood, both *in vitro* and *in vivo*. These activated NK cells have broader antitumor cytolytic capabilities, including lysis of fresh, uncultured, human tumor cells as well as a wide variety of tumor cell lines (11, 12). Activated NK cells are defined as lymphokine activated killer (LAK) cells. Together, IL-2 and LAK cells have been used as adoptive immunotherapy against cancer (13). However, although IL-2 has been reported to have promising results when administered to patients with advanced malignancies (14-16), the overall clinical success of IL-2 has been limited due to its severe side-effects.

In this study, we tested the ability of a new immunomodulator called MGN-3, an enzymatically modified arabinoxylane from rice bran with no notable side-effects, to enhance human NK cell activity both *in vivo* and *in vitro*.

Materials and methods

Human subjects and treatment with MGN-3. Twenty four healthy control subjects (15 females and nine males) participated in this study. Subjects ranged in age from 20-46 years, with a mean age of 34 years. They had not ingested any medications or vitamins for at least 2 weeks prior to their participation. Moreover, the subjects did not have a history of chronic diseases. During the course of the experiment that extended for 2 months, the women were not menstruating nor taking oral contraceptives – both of which can affect the level of NK cell activity.

Subjects were divided into three groups of eight individuals and were given MGN-3 orally for 2 months. Group 1, group 2 and group 3 recieved MGN-3 in doses of 15 mg/kg/day, 30 mg/kg/day and 45 mg/kg/day, respectively. Twenty ml of blood was drawn from each individual before treatment (day zero) and at different intervals posttreatment – 1 week, 1 month and 2 months.

MGN-3. MGN-3 is an arabinoxylane from rice bran, a polysaccharide that contains β1,4-xylopyronase hemicellulose, that has been enzymatically treated with an extract from *hyphomycetes mycelia* and was prepared in 500 mg tablets (Daiwa Pharmaceutical Co., Ltd., Tokyo, Japan). Fig. 1 shows the main chemical structure of MGN-3.
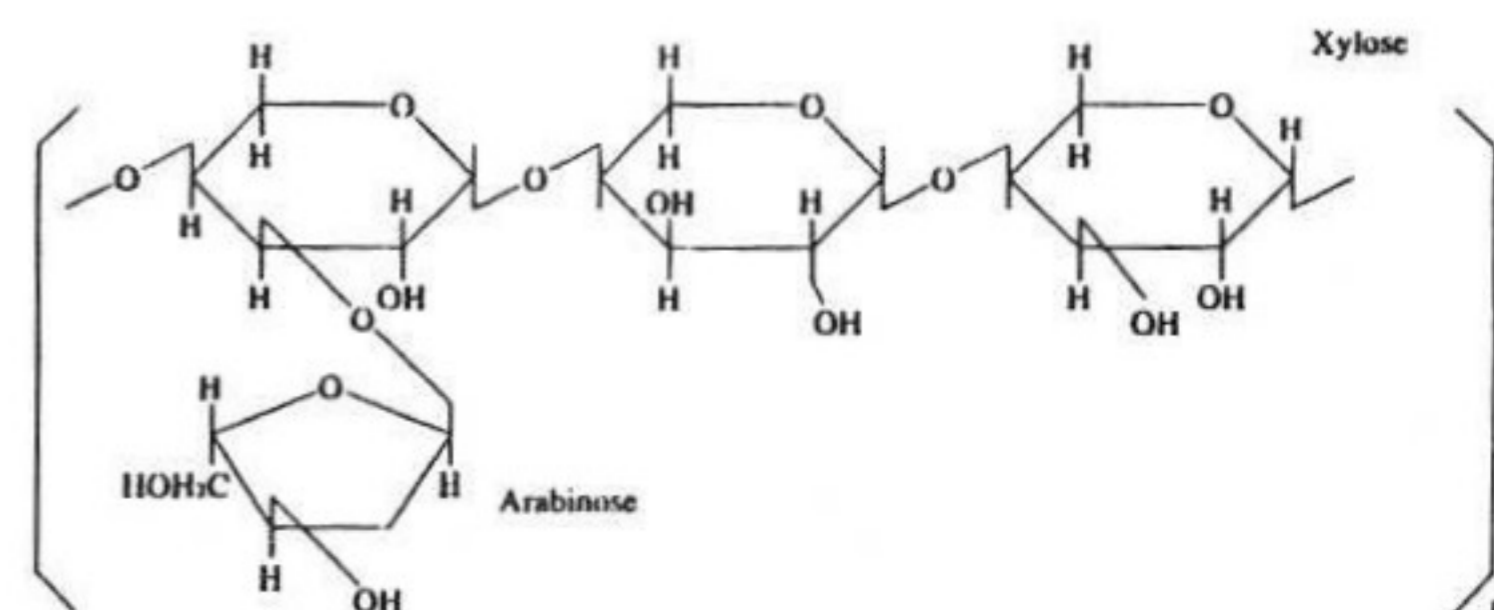
Fig. 1 Main chemical structure of MGN-3. It is an arabinoxylane with a xylose in its main chain and an arabinose polymer in its side chain.

Complete medium. Complete medium consisted of RPMI-1640 supplemented with 10% fetal calf serum and 1% antibiotic (100 U penicillin and 100 µg/ml streptomycin).

Tumor cell lines. Two human tumor cell lines were used as targets: sensitive K562, an erythroleukemic cell line, and resistant Raji, a Burkett cell lymphoma.

Preparation of peripheral blood lymphocytes (PBLs). PBLs were prepared from fresh heparinized peripheral venous blood by Ficoll-Hypaque density gradient centrifugation. Cells were washed three times with Hanks balanced salt solution and resuspended to 10×10^6 cells/ml in complete medium.

Culture of PBLs with MGN-3. PBLs from six healthy control subjects were adjusted to 1×10^6 cells/ml in complete medium and cultured with MGN-3 at concentrations of 25 and 100 µg/ml for 16 h. PBLs were then washed twice and examined for NK activity at effector:target (E:T) ratios of 100:1.

^{51}Cr-release assay for measuring NK activity. NK activity was measured by a standard 4-h ^{51}Cr-release assay. Briefly, 1×10^4 ^{51}Cr-labeled tumor target cells in 0.1 mL complete medium were added to different wells of a 96-well microtiter plate. Effector cells were then pipetted into quadruplicate wells to give E:T ratios of 12:1, 25:1, 50:1 and 100:1. After a 4-h incubation (37 °C), the plates were centrifuged (1,400 rpm for 5 min) and 0.1 mL of supernatant from each well was collected and counted in a gamma counter (Beckmann G50, Beckmann Instruments).

The percentages of isotope released were calculated by the following formula:

$$\% \text{ lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

Spontaneous release from target cells was no more than 8-10% of total release. Total release was measured by adding 0.1 mL Triton X-100 (Sigma Chemical Co.) to designated wells. Lytic units were calculated from effector titration curves with one lytic unit defined as the number of effector cells required to achieve 30% lysis for K562 and 8% lysis for Raji cells. This serial performance of NK cytotoxic assay was based on criteria for a reproducible NK test and was intended to help minimize associated errors (17).

NK subpopulations. NK cell subset enumeration was carried out with PBLs from individuals for baseline and after 1 month treatment with MGN-3. A single laser flow cytometer (Epics Profile, Coulter Epics, Inc., Hialeah, FL), which discriminates forward and right-angle light scatter, as well as two colors, was used with a software package (Quad Stat, Coulter). Mononuclear cell populations were determined by two-color direct immunofluorescence, by using a whole-blood staining technique with the appropriate monclonal antibody and flow cytometry (18). Fluorescein isothiocyanate- (FITC, CD3⁻FITC), or phycerythrin (PE,CD56-PE)-conjugated monclonal antibodies (Coulter Immunology) were selected for the determination of NK cell subsets. To monitor lymphocyte markers, bitmaps were set on the lymphocyte population of the forward-angle light scatter versus a 90° light scatter histogram. The percentage of positively stained cells for each marker, as well as the percentage of double-stained lymphocyte positive for the respective surface markers, were determined.

Effector:target cell conjugate assay. The capacity of NK effector cells from different treatment conditions to form conjugates with K562 targets was measured as previously described (19). Briefly, 1 x 10^5 lymphocytes were incubated with 1 x 10^6 K562 target cells in 1.0 mL complete medium in 12 x 75 mm glass tubes, pelleted at 130 G for 5 min and incubated for 1 h at 4 °C. The pellets were gently resuspended and cytocentrifuged. Smears were prepared using a cytospin cytocentrifuge (Shandon Instruments) and stained with Giemsa. The percentage of conjugates was determined by counting 200 lymphocytes (bound and free) in triplicate samples.

Interferon-γ production. Peripheral blood mononuclear cells from five individuals were collected, adjusted to 10 x 10^6 cells/ml, and cultured with MGN-3 at different concentrations (0, 5, 50 and 100 μg/ml) for 16 h. Culture supernatants were then collected and analyzed for interferon (IFN)-γ using enzyme-linked immunoabsorbent assay.

Blood chemistry. Five individuals were given MGN-3 (45 mg/kg/day) for 1 month. At day zero and the end of treatment, 5 ml of blood was drawn from each subject for blood chemistry analysis using Panel 20 which includes liver enzymes (serum glutamic-oxaloacetic transaminase [SGOT], and serum pyruvic-oxaloacetic transaminase [SGPT]).

Statistical analysis. The statistical analysis software (SAS) procedure of analysis of variance was used to examine the effects before and after treatment with MGN-3 in different determinations; the effect of changing the ratios between E:T cells; and the interaction of the two effects.

Results

Augmentation of NK activity by ingestion of MGN-3.
Results of NK activity enhancement by ingestion of MGN-3 were expressed with respect to dose-response and time-course of changes in NK activity; E:T ratios; quantification of NK cells; and percentage of conjugate formation.

To test the reproduceability of the results, the present study was repeated with the same individuals after 4-6 month intervals, and by assaying NK function at different E:T ratios (12:1, 25:1, 50:1 and 100:1). These results were similar with regard to enhancement of NK function by MGN-3 (second trial data not shown).

Effect against K562. Dose-range and time-course of NK activation by MGN-3 was examined. Fig. 2 shows the augmenting effect of MGN-3 on NK cell activity against K562 tumor cells (activity expressed as number of lytic units). MGN-3 at a dose of 15 mg/kg/day showed no changes at 1 week as compared with baseline values, however a twofold increase in NK cytotoxicity was detected after 1 month of treatment. Increasing the dose to 30 mg/kg/day resulted in a significant enhancement of NK activity (310% over baseline) that was detected as early as 1 week. The activity of NK cells continued to increase with continuation of treatment. The peak response was observed at the end of the treatment period (2 months) where NK activity increased fivefold (68.2 lytic units in comparison with 13.6 lytic units for baseline). Increasing the dose to 45 mg/kg/day demonstrated a similar increasing trend in NK activity but the values were higher in magnitude than those for 30 mg/kg/day. Discontinuation of treatment resulted in a decline of NK activity and at 1 month, NK activity returned to baseline. Fig. 3 shows NK cell activity against K562 at different E:T ratios. All subjects demonstrated enhance-
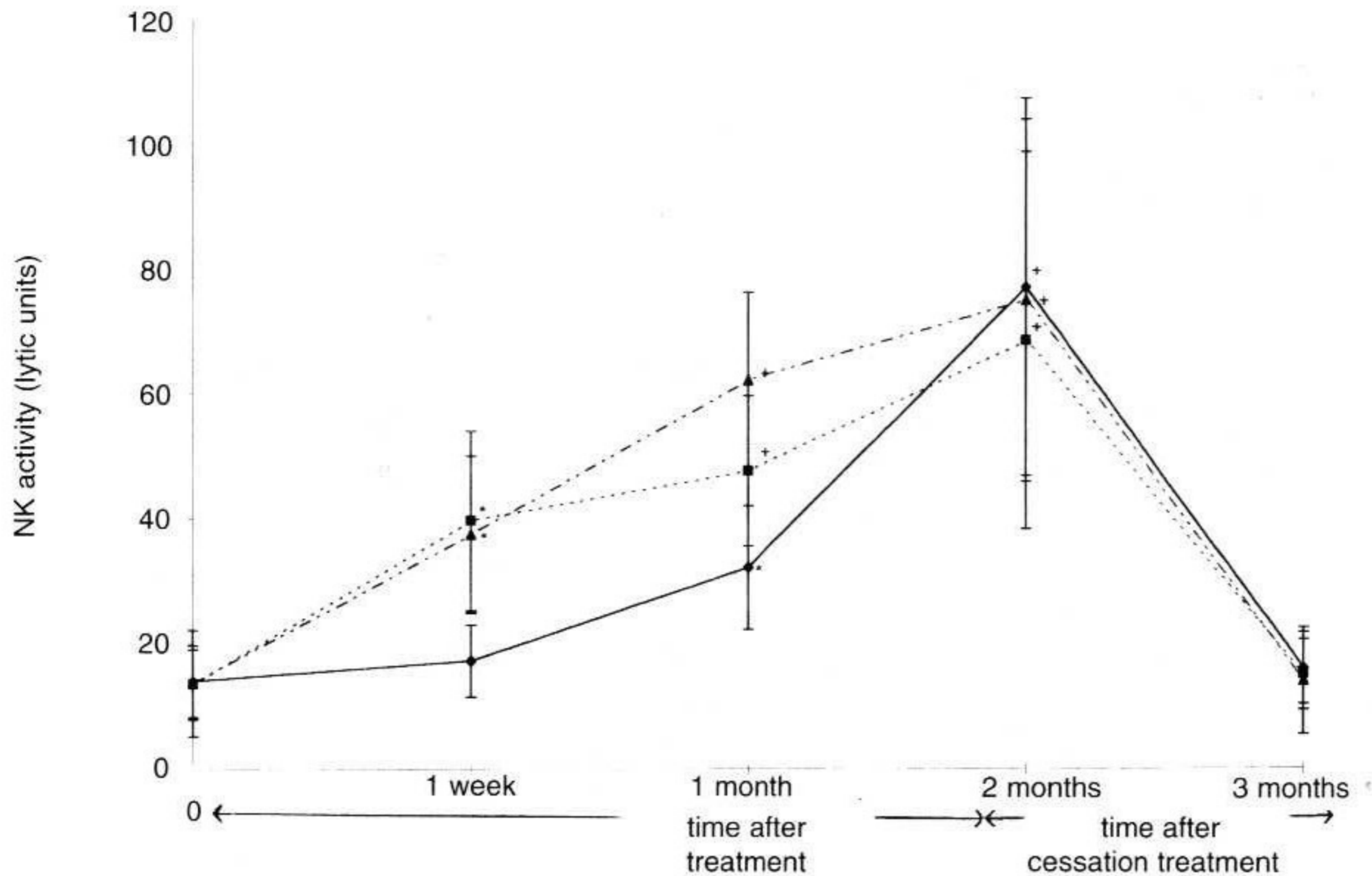
Fig. 2 Dose range and time course of natural killer (NK) cell activation by MGN-3 against K562 tumor cells. Activity is expressed as number of lytic units at 30%. MGN-3 at 15 mg/kg/day (●), 30 mg/kg/day (◆) and 45 mg/kg/day (▲). NK activity was examined at baseline zero day, 1 week, 1 month, 2 months and 3 months. Mean ±SD of eight different individuals in each dose. †$p<0.001$

ment in their NK activity, however, there was a differential response among different individuals toward the augmentory function by MGN-3. An increase in activity was detected for all E:T ratios, but the level of enhancement was higher at lower E:T ratios (12:1, 25:1) than at higher ratios (50:1, 100:1).

Effect against Raji cells. Fig. 4 summarizes NK activation against Raji cells. The augmentory effect of MGN-3 was examined at 45 mg/kg/day taken for 2 months. Activated NK cells were able to kill Raji cells as early as 1 week (7.4 lytic units as compared with 0.9 lytic units for baseline). Activity continued to increase and peaked at 2 months after treatment (24.6 lytic units). After discontinuation of treatment, NK activity gradually declined to 10.9 lytic units at 2 weeks and 6.3 lytic units after 1

month. Enhancement of NK activity was detected at all E:T ratios (Fig. 5).

Quantification of total NK cells. Flow cytometry was used to analyze changes in the total NK cells cultured with MGN-3 (at all doses). Table I demonstrates that treatment with MGN-3 at different concentrations had no significant effect on the percentages of total NK cell population as identified by $CD56^+$ $CD3^-$ and $CD16^{+-}$ monoclonal antibodies, respectively.

Percent conjugates. The binding capacity of NK cells to K562 tumor targets was examined after 1-month treatment (45 mg/kg/day) with MGN-3. Figs. 6 and 7 show that the percentage of conjugate formation increased significantly posttreatment (38.5%), compared with baseline (9.4%).
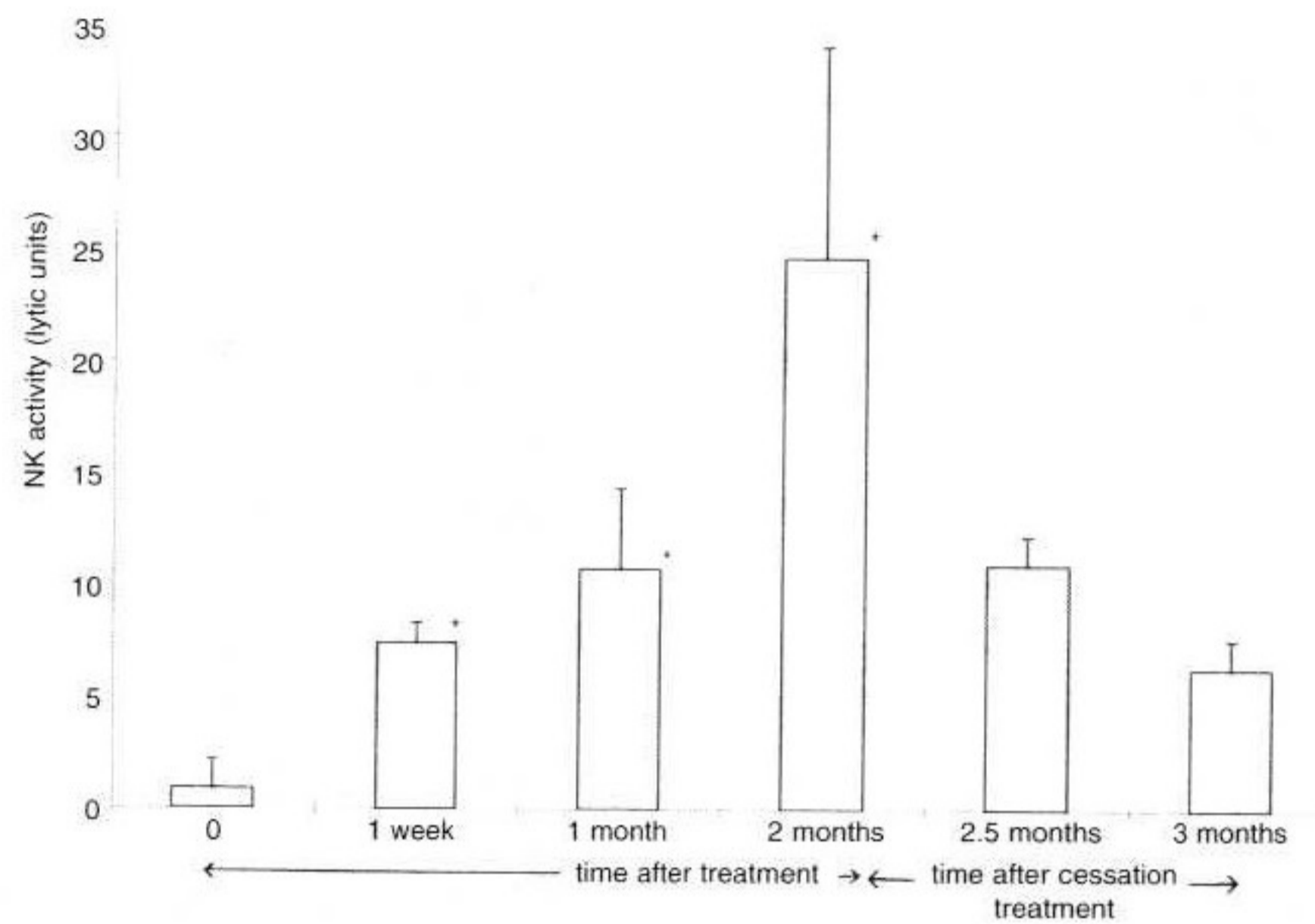
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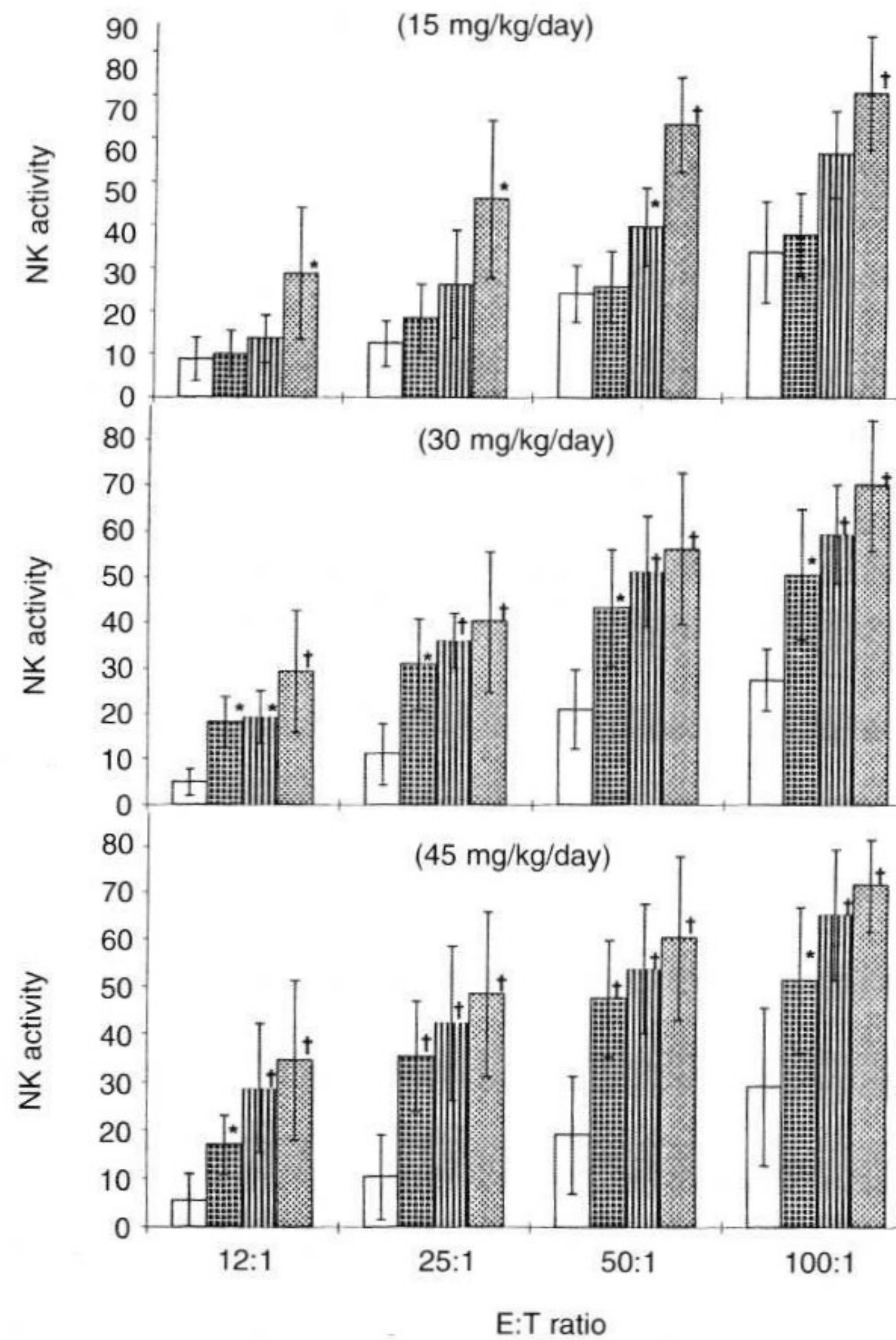
Fig. 3 Natural killer (NK) cell activation by MGN-3 against K562 at different effector:target (E:T) ratios. MGN-3 at 15, 30 and 45 mg/kg/day. NK activity was examined at baseline, 1 week, 1 month, 2 months and 3 months posttreatment. Mean ±SD of eight different individuals in each dose. *$p<0.005$, †$p<0.001$

In vitro *studies*

NK activity post culture with MGN-3. Culture of PBL with MGN-3 for 16 h resulted in an enhancement of NK cell activity that was dose-dependent. MGN-3 at a concentration of 25 μg/mL increased NK activity by 130%. NK activity was further enhanced (150%) when the MGN-3 concentration was increased to 100 μg/mL (Fig. 8).

Production of IFN-γ. Fig. 9 summarizes results of the effect of MGN-3 on the production of IFN-γ. PBL cultured without MGN-3 showed little IFN-γ. MGN-3 treatment resulted in a significant increase in IFN-γ production that was dose-dependent. Treated PBL at MGN-3 concentrations of 25, 50 and 100 μg/ml demonstrated 340, 390 and 580 pg/ml of IFN-γ production, respectively.

Fig. 4 Time course of natural killer (NK) activation by MGN-3 (45 mg/kg/day) against Raji cells. Activity was expressed as number of lytic units at 8% NK activity at baseline (zero day), 1 week, 1 month, 2 months, 2.5 months and 3 months. Mean ± SD of eight different individuals. †$p<0.001$
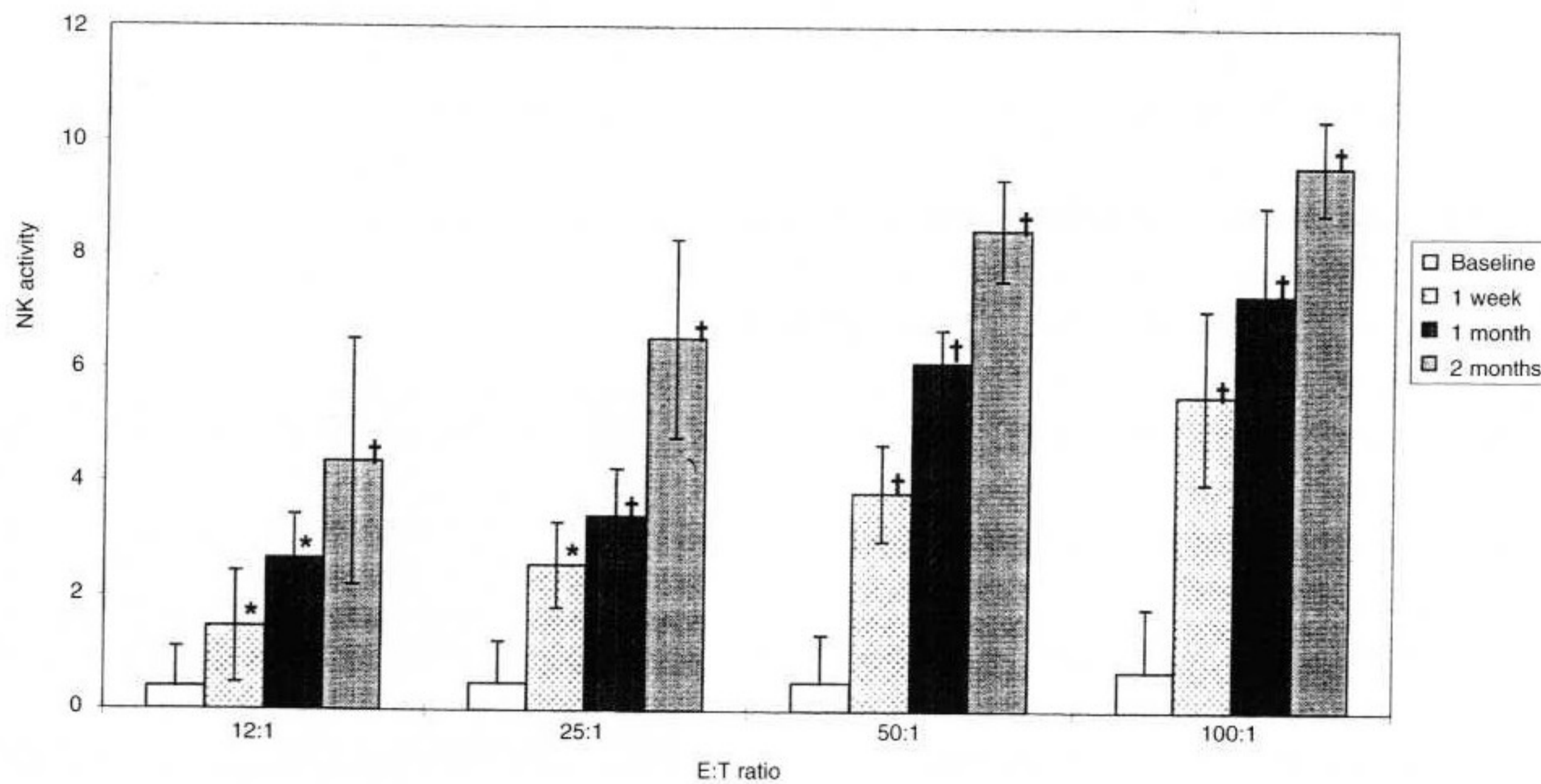

Fig. 5 Action of MGN-3 on natural killer (NK) activity against Raji cells at different effector:target ratios. Activity was examined at baseline, 1 week, 1 month and 2 months. Mean ±SD of eight different individuals. *$p<0.005$, †$p<0.001$

Discussion

In this study a new biological response modifier, MGN-3, was examined for its ability to enhance human NK cell activity *in vivo* and *in vitro*. The results showed that MGN-3 is a potent biological response modifier (BRM) as manifested by significant induction of NK cytotoxicity upon MGN-3 ingestion – the augmentory action was detected not only against sensitive K562 targets, but also
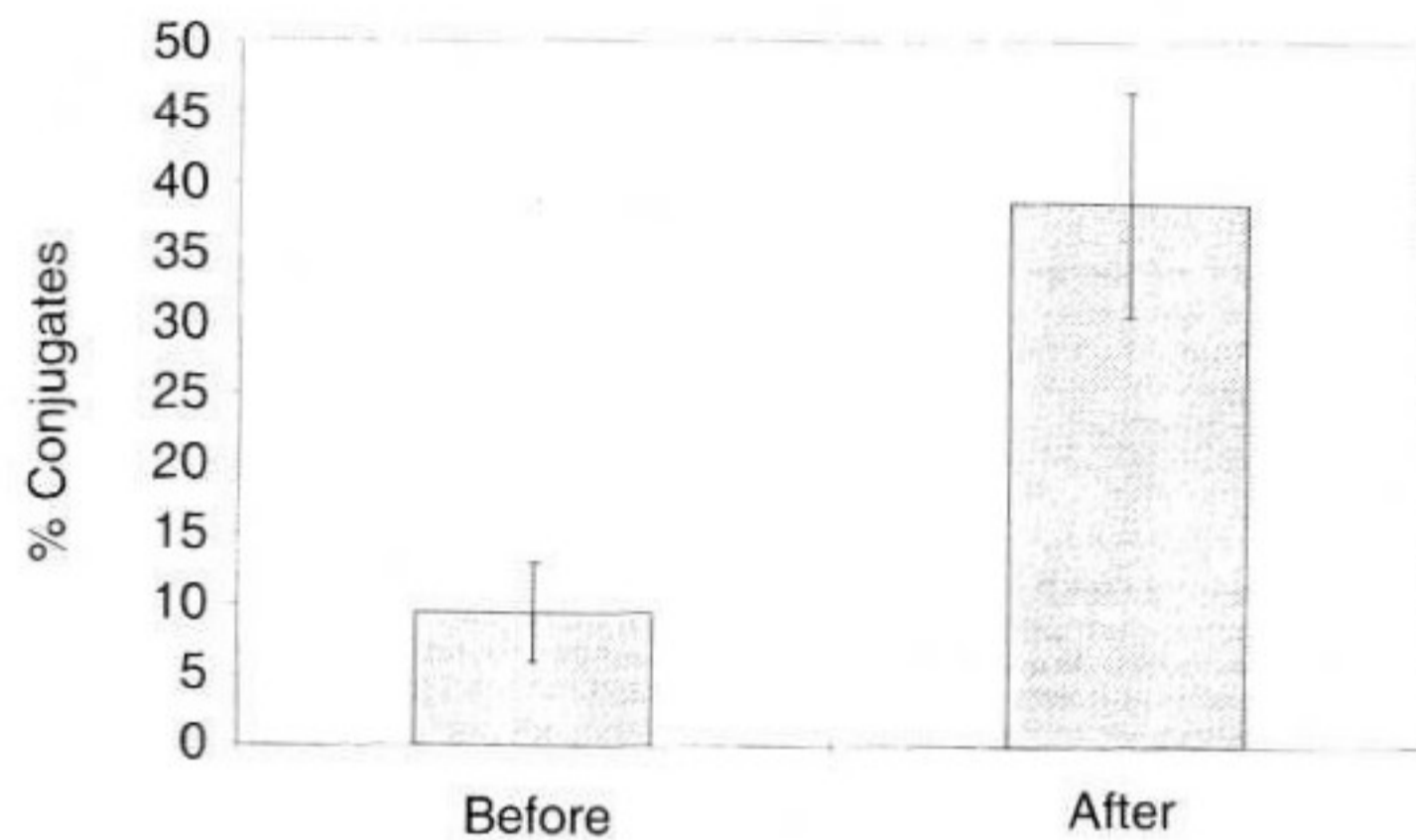
Fig. 6 Percentage of conjugate formation between natural killer (NK) cells and K562 target cells. Conjugate formation was examined at baseline (zero day) and at 1 month, posttreatment. Mean ±SD of five different individuals. *$p<0.005$

against cell lines known to be highly resistant to NK activity, such as Raji, a Burkitt lymphoma, and Daudi, another Burkitt cell line (data not shown). That MGN-3 potent biological response modification is also shown by the decline of NK activity after discontinuation of treatment, as well as an increase in NK activity postculture with MGN-3 for 16 h. The immunomodulatory function of MGN-3 depended on two factors. Firstly, the concentration. As shown in Figs. 2 and 3, MGN-3 increases NK activity in a dose-dependent manner. MGN-3 at 15 mg/kg/day demonstrated an increase in NK cytotoxicity against K562 tumor cells after 1-month treatment, while higher concentrations of 30 and 45 mg/kg/day resulted in a significant induction of NK activity after 1-week treatment. Activity continued to increase and peaked at 2 months. Secondly, the

Table I *Total NK cells posttreatment with MGN-3*

| Dose mg/kg/day	Baseline		1 month after treatment	
	CD56$^+$/CD3$^-$	CD16$^+$	CD56$^+$/CD3$^-$	CD16$^+$
15	4.7 ± 3.1	not tested	4.3 ± 2.4	not tested
30	6.4 ± 1.8	12.2 ± 1.7	8.2 ± 3.6	12 ± 5.3
45	4.2 ± 2.6	10 ± 2	6.4 ± 2.4	9 ± 2.4

augmentory function of MGN-3 is differential among subjects.

The type of MGN-3-activated killer cells is not fully defined, but it may be similar to the IL-2-induced LAK cell phenomenon with respect to heterogeneity. Both types of cells are associated with increased conjugate formation between peripheral blood mononuclear cells and tumor targets (12). Consequently, various effector cells may be involved. While NK cells may represent a major component of MGN-3-activated cells, as seen in increased lysis of the classical NK-sensitive K562, lysis of resistant tumor cell lines (Raji and Daudi) may involve either NK cells and/or other effector cells having anti-cancer activity such as cytotoxic T-lymphocytes. Another similarity between the two induced cell types (MGN-3 and IL-2) is the upregulation of CD25$^+$ and CD69$^+$ receptors (20).

The mechanism by which MGN-3 boosts NK activity appears to be via its ability to induce IFN production. Most agents that can activate NK cells appear to be acting by their ability to induce, either *in vivo* or *in vitro*, IFN production (21). Several bacte-
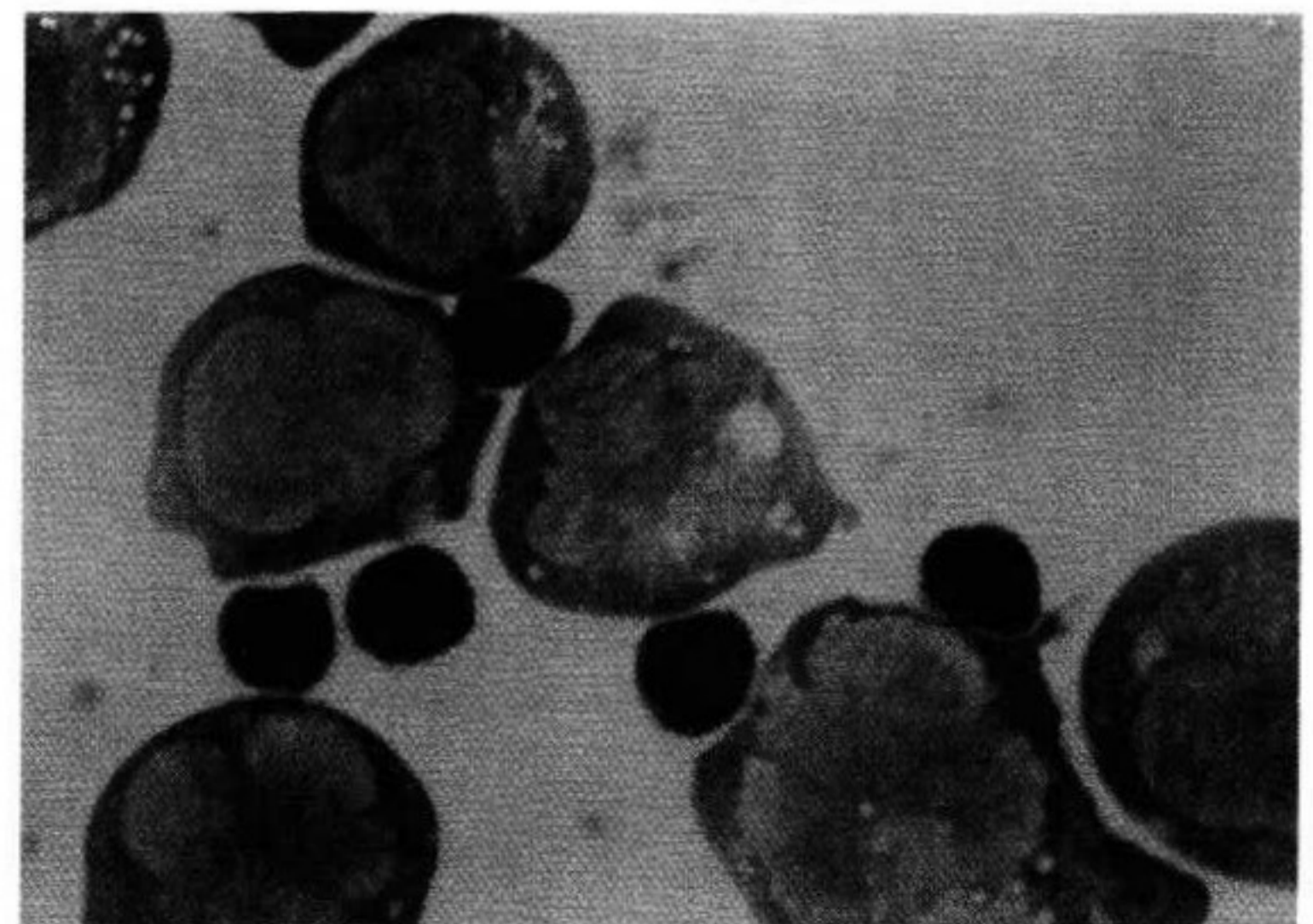
Fig. 7 Natural killer (NK) effector-tumor targets conjugate formation. Peripheral blood lymphocyte from an individual treated with MGN-3 (45 mg/kg/day) for 1 month and cultured with K562. Note increased binding of NK cells to tumor cells and 1 NK cell binding to 3 tumor cells. Giemsa stained.
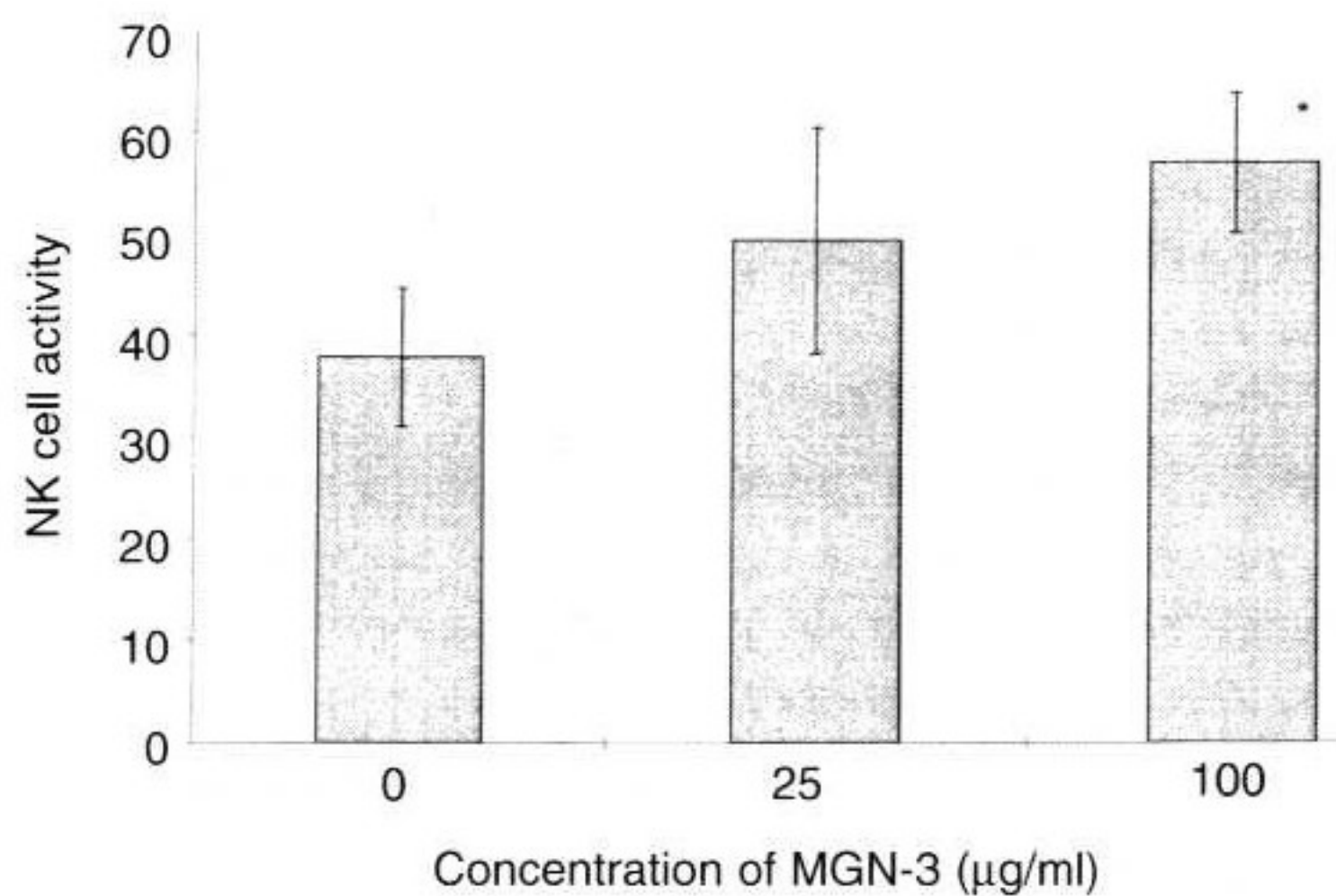
Fig. 8 *In vitro* effect of MGN-3 on natural killer (NK) activity. PBL were cultured for 16 h with MGN-3 at two different concentrations; 25 and 100 µg/ml. NK activity examined at 100:1. Mean ±SD of five different individuals in each concentration. *$p<0.005$
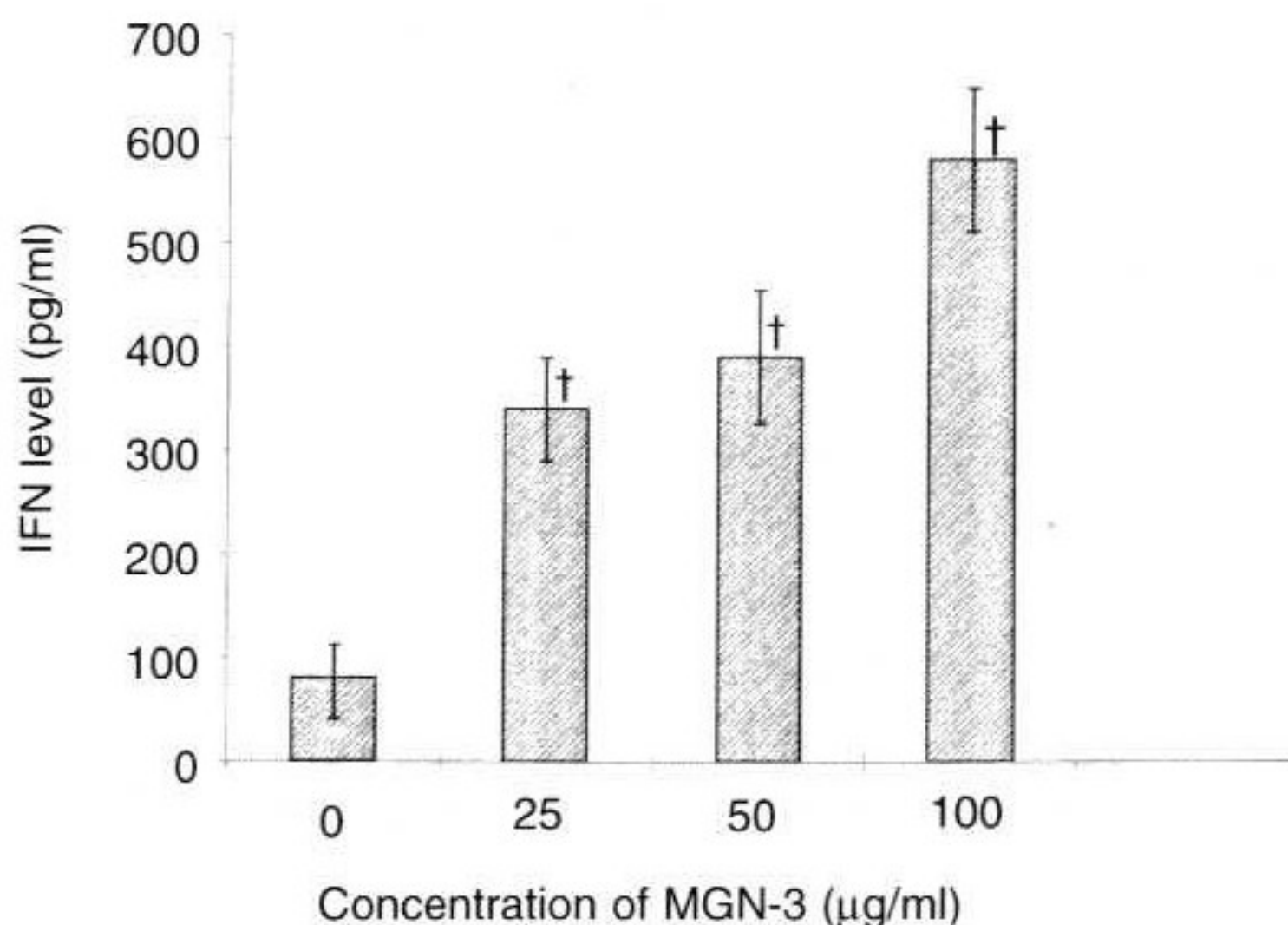
Fig. 9 *In vitro* action of MGN-3 on interferon (IFN)-γ production. PBL were cultured with MGN-3 at different concentrations: 25, 50 and 100 µg/ml, mean ±SD of five different individuals in each concentration. †$p<0.001$

rial immunomodulators, such as *bacilli* Calmette-Guérin and *Corynebacterium parvum*, have been found to induce IFN and augment NK activity (22). Among the IFN stimulators studied so far, bacteria appear to have specificity for induction in large granular lymphocytes (LGLs) alone (23). Other biological

agents can induce rapid IFN production from LGLs and it is the production of IFN which produces the self-activation of NK activity in LGLs. Our work shows that MGN-3 (25-100 µg/ml) induces PBL IFN-γ production (340-580 pg/ml) in culture. This suggests that MGN-3 enhances IFN production, which in turn may augment NK activity. Our work also shows the ability of MGN-3 to stimulate the production of other cytokines such as TNF-α (20).

It is possible that the increase in NK cell activity from ingestion of MGN-3 may be due to an increase in the activity per cell and not due to an increase in the actual NK cell number. This was confirmed by noting that lower E:T ratios (12:1 and 25:1) achieved a maximum induction in NK activity (in comparison with 100:1), while flow cytometry analysis showed no significant changes in total NK cells after treatment (compared with baseline values). The results also showed an increase in the binding capacity of MGN-3-cultured effector cells to tumor targets (four-fold). The increased number of NK cells in conjugates with no increase in NK cell numbers in PBLs after treatment suggests that MGN-3 increases the binding capacity of NK cells as well as other cell populations to tumor targets. It is well established that the NK-tumor cell interaction proceeds through several discrete stages (24) including E:T cell recognition and binding; triggering and activation of the NK cells; release of the granules from NK cells and binding to receptor sites on the tumor cell surface; and tumor target cell death. This binding process is a key event in the activation of NK cells.

The anticancer activity of natural BRMs may involve activation of different arms of the immune system. For example, killed bacteria (*Corynebacterium parvum* and *Bacilli* Calmette-Guérin) may involve NK activation (25-28); lectins from plants such as *griffonia simplicflial*-ß4 isolectin may involve activation of macrophage (29); bitter melon protein may involve neutrophil activation (30); and lentinan isolated from edible mushroom, *Lentius edodes*

(Berk) Sing. possesses augmentory effects on NK and killer T-cell activity (31-35). With respect to MGN-3, activated cells are mainly NK cells although cytotoxic T-lymphocytes may be involved in the activation process. Extracted hemicellulose from rice bran fiber has known unique biological effects; for example, α-glucan from rice bran shows potent antitumor activity in mice (36), and arabinose and xylose from rice bran fiber shows defensive effects against *bis*(n-tributyltin) oxide-induced thymic atrophy in rats (37). Unprocessed rice bran fiber and cholestyramine have been observed to increase peripheral blood leukocytes in humans (38). The product used in this study is a modified arabinoxylane from rice bran. Modification occurs by enzymatic treatment with an extract from *Hyphomycetes mycelia*, which shows a high augmentory effect on human NK activity *in vivo* and *in vitro,* as well as in mice and rats (data not shown). MGN-3 was examined for toxicity using blood chemistry analysis utilizing Panel 20 which includes liver enzymes (SGOT and SGPT). After 1-month treatment, no abnormalities were detected for these parameters as compared with baseline. We conclude that the high augmentory effect of MGN-3 and the absence of notable side-effects make this material a promising immunotherapeutic agent for treating cancer patients. Preliminary studies in this regard are very encouraging. NK immunomodulatory function by MGN-3 was detected in 27 patients afflicted with different types of malignancies (39), additionally, the relationship between the immunomodulatory and anticancer properties of MGN-3 was assayed in five patients with breast cancer (40).

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