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Enhancement of Natural Killer Cell Activity of Aged Mice by Modified Arabinoxylan from Rice Bran (BioBran/MGN-3)

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Abstract

The present study is aimed to examine the possibility of enhancement of natural killer (NK) cell activity in aged C57BL/6 and C3H mice using BioBran/MGN-3, a modified arabinoxylan from rice bran. Intraperitoneal injection of BioBran/MGN-3 (10 $mgkg^{-1}$ per day) caused a remarkable increase in the peritoneal NK activity as early as 2 days (35.2 lytic units), and the level remained elevated through day 14. The control aged mice had a level of 5.8 lytic units. Enhancement in NK activity was associated with an increase in both the binding capacity of NK cells to tumour targets and in the granular content as measured by BLT-esterase activity. Treatment did not alter the percentage of peritoneal NK cells. Data showed that peritoneal macrophages inhibit NK activity. In conclusion, BioBran/MGN-3 enhances murine NK activity of aged mice and may be useful for enhancing NK function in aged humans.

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Introduction

Immunodeficiency of ageing is manifested in terms of increased infection, cancer and autoimmunity. Immunodeficiency is apparently a multifactorial problem, where the loss of immunologic vigour plays a major role in the above-mentioned diseases. For example, the number of white blood cell subpopulations does not change appreciably with age, however qualitative changes in the leukocytes do appear to occur with age (Itoh et al. 1982; Albright & Albright 1983; Ghoneum et al. 1991a, b; Miller 1996; Pawelec et al. 1997, 1998; Greeley et al 2001; Rafi et al. 2003). Several studies have shown that natural killer (NK) cells, the activity of which resides in large granular lymphocytes, are important in the natural resistance against tumour (Lotzova 1984; Moretta et al. 2002), viral (Leong et al. 1998; Biron et al. 1999) and bacterial infection (Ghoneum et al. 2003). The functional significance of these cells has attracted considerable interest for its possible role in host defence against cancer (Herberman 1983, 2002; Wu & Lanier 2003).

Our earlier studies (Ghoneum et al. 1987, 1989, 1991a, b) and those of others (Itoh et al. 1982; Albright & Albright 1983) clearly demonstrated an age-association defect in NK activity. Therefore, several attempts have been made to augment NK activity during ageing. Whithin the last two decades, extraordinary emphasis has been placed on biological response modifiers (BRMs) as anticancer agents: interleukin-2 (Konjevic et al. 2003), interferon-γ (Appasamy et al. 1994; Allavena et al. 1998), killed streptococcal preparations (OK432) (Kurosawa et al. 1996), *Corynebacterium parvum* (Ghoneum et al. 1987), and bacilli Calmette-Guerin (Mizutani & Yoshida 1994). However, the clinical use of these BRMs has been severely limited because of their *in vivo* cytotoxicity. In this study, we examined the effect of a food supplement BioBran/MGN-3, for possible enhancement of NK cell activity in aged mice BioBran/MGN-3 is an arabinoxylan from rice bran that has been modified by carbohydrate hydrolysing enzymes from shiitake mushrooms (Ghoneum 1998a). We have previously reported that BioBran/MGN-3 enhances NK cell activity in healthy humans (Ghoneum 1998b), and increases the production of TNF-α by human peripheral blood lymphocytes (Ghoneum & Jewett 2000). It also sensitizes human leukaemia cells to death recepter (CD95) induced apoptosis (Ghoneum & Gollapudi 2003). In a double-blind study, Tazawa et al. (2003) found a prophylactic effect with the use of BioBran/MGN-3 against the common cold syndrome. In addition, BioBran/MGN-3 accelerated the protection against severe loss of bodyweight of mice due to treatment with the chemotherapeutic agent cisplatin (Jacoby et al. 2001; Endo & Kambayashi 2003). In this study, we tested the ability of BioBran/MGN-3 to enhance NK cell activity in aged mice, and to elucidate the possible mechanism that underlies the BioBran/MGN-3 effect.

Materials and Methods

1. Animals and materials

The mice used in this study were the inbred strain of aged C57BL/6 and C3H female mice (18 months old) and were purchased from the Animal Laboratory, University of California, Berkeley, CA. Mice were housed five per cage and were permitted free access to water and food; they were accommodated for 1 week before experiments.

Complete medium (CM) consisted of RPMI-1640 supplemented with 10% fetal calf serum. 100 units penicillin and 100 μ g streptomycin.

Target cells were the YAC-1 cell line, a Moloney leukaemia virus-induced mouse T-cell lymphoma of A/Sn mice origin, which was maintained in CM at a starting density of 3×10^5 cells mL^{-1}.

BioBran/MGN-3 is an arabinoxylan extracted from rice bran that is treated enzymatically with an extract from shiitake mushrooms; it contains polysaccharides (β1, 3-glucan and activated hemicellulose). BioBran /MGN-3 was provided by Daiwa Pharmaceuticals Co., Ltd, Tokyo, Japan.

2. *In vivo* treatment protocol

BioBran/MGN-3 was orally administered to two groups of mice daily in a volume of 0.1 mL at a concentration of 10 $mgmL^{-1}$ of BioBran/MGN-3 (dry weight) in water or by intraperitoneal injection in a volume of 0.1 mL at the same concentration of BioBran/MGN-3 in phosphate-buffered saline. The does of 1 mg of BioBran/MGN-3 per mouse was calculated from that recommended for humans (50 $mgkg^{-1}$). Control mice were given saline solution alone. At 2, 5 and 14 days after treatment, the peritoneal and splenic NK activity, as well as the cellularity of these tissues, were examined.

3. Preparation of peritoneal exudate cells (PECs), splenic NK cells and bone marrow NK cells

At 2, 5 and 14 days after treatment with BioBran/MGN-3, mice were killed by cervical dislocation, and PECs were isolated as follows: 5 mL of HBSS was injected intraperitoneally, the abdomen was massaged and 90% of the injected volume was recovered. Cell viability was 95%, as determined by the trypan-blue exclusion test. Spleens were removed, teased in CM, and contaminating erythrocytes were lysed with distilled water. Splenic lymphocytes were pelleted, and washed three times with HBSS. Bone marrow cells were flushed out from femur and tibia and washed in HBSS.

4. Measuring NK activity by the ^{51}Cr-released assay

The ^{51}Cr-released assay was used to determine NK cell activity in the peritoneum, spleen and bone marrow. In brief, 5×10^6 YAC-1 cells were labelled with 100 $\mu CI^{51}CrO_4$ solution (New England Nuclear, Boston, MA) for 1h at 27℃. Cells were washed four times with HBSS and resuspended in CM at 1×10^5 cells mL^{-1}. YAC-1 cells (1×10^4) were then pipetted into each well of 96-well round-bottomed Linbro plates. Effector cells were pipetted to quadruplicate wells to give effector/target cells ratios of 25 : 1, 50 : 1 and 100 : 1. The plates were incubated at 37℃ for 4h, then centrifuged at 1000 g for 5 min, and 0.1 mL of supernatant from each well was collected and counted in a gamma counter. The percentage of isotope released was calculated by the following formula:

$$\text{Lysis (\%)} = \frac{\text{(experimental release} - \text{spontaneous release)}}{\text{(total release} - \text{spontaneous release)}} \times 100$$

where spontaneous release is cpm from YAC-1 incubated in CM without effector cells and total release is cpm from YAC-1 incubated in Triton X-100. Results were expressed in lytic units (LU)/10^7, with 1LU being the number of effector cells required to lyse 5% and 15% of YAC-1 targets by peritoneal NK (P-NK) and splenic NK cells, respectively.

5. Flow cytometry

PECs were prepared from saline-treated and BioBran/MGN-3-treated mice. The percentage of peritoneal NK (P-NK) cells was demonstrated by their expression of NK 1.1 marker, and determined by flow cytom-

etry. Cells were first gated with forward and side scatter characteristics, and then the percentage of surface marker positive cells was measured. The monoclonal antibodies used were anti-NK 1.1 (Pharmingen, San Diego, CA).

6. BLT-esterase release assay

N-a-CBZ-L-Lysine thiobenzyl ester (BLT)-esterase activity was examined according to the procedure of Green & Shaw (1979) with little modification. In brief, PECs (3×10^6 cells mL^{-1}, 100 μL/well), from BioBran/MGN-3- and saline-treated aged mice were incubated with YAC-1 target cells (9×10^6 cells mL^{-1}, 100 μL/well) for 4h at 37℃ in RPMI 1640, in V-bottomed 96-microwell plates. The relative amount of secreted BLT-esterase activity was determined by incubating triplicate samples of supernatant (100 μL) and reaction buffer (100 μL) for 30 min at 37℃ in 96-well ELISA plates. The reaction buffer contained 0.1M Tris-HCl, pH 8.1, 0.2 mM DTNB, and 0.2 mM BLT (Sigma, St Louis, MO). The colour was allowed to develop for 40 min. Optical density readings were taken at 405 nm using an ELISA plate reader.

7. Morphological assessment of NK granularity

The effect of BioBran/MGN-3 on the level of granularity of P-NK cells was examined in cytospin preparations as previously described (Itoh et al. 1982). P-NK cells were purified as follows: macrophages and B-cells were removed by sequential incubation in CM in 150-cm^2 flasks for 1h at 37℃ and on nylon wool columns. Non-adherent mononuclear cells from nylon wool columns were fractionated by overlaying onto Percoll discontinuous gradients. For morphological assessment of NK granularity, P-NK cells (10^5 mL^{-1}) from BioBran/MGN-3- and saline-treated aged mice were centrifuged on slides at 200 g for 5 min using a cytospin cytocentrifuge (Shandon Southern Inst., Sewickley, PA, USA). Slides were air dried, fixed in 100% MeOH for 5 min, and then stained with 4% Giemsa solution for 15 min.

8. Percentage conjugate formation

The percentage of conjugate formation between P-NK cells and YAC-1 cells was examined in cytospin preparations (Itoh et al. 1982). In brief, peritoneal non-adherent cells (10^5 mL^{-1}) from BioBran/MGN-3 and saline-treated aged mice were mixed with YAC-1 cells (10^6 cells mL^{-1}) at 4℃ for 1h. The cells were centrifuged on slides at 200 g for 5 min using a cytospin cytocentrifuge and stained with Giemsa as previously described. The percentage of NK cells binding to YAC-1 cells within 200 cells was calculated in triplicate samples.

9. Suppressive effect of peritoneal macrophage (P-Mϕ) on P-NK cell activity

A set of experiments was carried out to examine the suppressive effect of P-Mϕ on P-NK cell activity. C57BL/6 mice were injected intraperitoneally with BioBran/MGN-3 for 5 days and the natural cytotoxicity by P-Mϕ, PECs, and PECs depleted of P-Mϕ (non-adherent cells) was examined separately by ^{51}Cr-released assay.

10. Admixture of P-Mϕ added to the non-adherent cells

To further confirm the suppressive effect of P-Mϕ, an admixture of equal numbers of P-Mϕ and non-adherent cells was examined for NK activity. The admixture contained cells at an effector/target ratio of 50 : 1. Results were compared with non-adherent cells alone.

Table 1 *In vivo* affect of MGN-3 on tissue cellularity

Strain of mice	Time after MGN-3 treatment (days)	Spleci cells ($\times 10^6$)		Peritoneal cells ($\times 10^6$)	
		MGN-3	Control	MGN-3	Control
C57BL/6	2	60±1** (150%)	40±2.3	9.4±1* (470%)	2.0±0.08
	5	61±0.08** (145.2%)	42±0.08	9.7±0.08 (461.9%)	2.1±0.08
	14	85.5±1.75** (192%)	44.5±2.1	8.5±1.05* (404.7%)	2.1±0.5
C3H	5	70±2.5** (166.7%)	42±3.7	6.8±0.4* (453.3%)	1.5±0.3

The percentage induction is given in parentheses.
Data represent the mean percentage of total peritoneal and splenic cells from C57BL/6 and C3H mice after intraperitoneal treatment with MGN-3.
Means±s.e.m of six mice at each time point were examined separately.
*P<0.01. **P<0.025, significantly different compared with control saline-treated aged mice.

11. BioBran/MGN-3 treatment by oesophageal tubing

Another set of experiments was carried out to examine splenic NK activity C57BL/6 mice. The mice were given BioBran/MGN-3 through oesophageal tubing at a concentration of 10 mg in 100 μL distilled water/mouse. After 14 days, the mice were killed and examined for splenic NK activity.

12. *In vitro* effect of BioBran/MGN-3 on NK activity

Splenic cells (1×10^6 cells mL^{-1}) from aged C57BL/6 mice were cultured with BioBran/MGN-3 at concentrations of 25 and 100 $\mu g mL^{-1}$. At 16h after treatment, cells were washed twice in CM and were examined for NK activity.

13. Statistical analysis

A two-tailed Student's *t*-test was used to determine the degree of significance between NK cell activity of control and BioBran/MGN-3-treated mice.

Results

1. Peritoneal and splenic cellularity

Results of the effect of intraperitoneal treatment with BioBran/MGN-3 on peritoneal and splenic cellularity are shown in **Table 1**. BioBran/MGN-3 significantly increased peritoneal cellularity (404-470% of control) in C57BL/6 and C3H mice. The effect was noted as early as 2 days, and remained elevated through day 14. An increase in splenic cellularity in both strains of mice was also noted but to a lesser extent (145-192% of control).

2. NK activity after intraperitoneal injection

Results in **Table 2** show that intraperitoneal injection with BioBran/MGN-3 resulted in enhancement in P-NK activity. This was detected as early as 2 days (35.2 LU), and the level remained elevated through Day 14 as compared with saline-treated aged mice. A similar pattern of increase was observed with the P-

Table 2 Effect of intraperitoneal injection of MGN-3 on natural killer (NK) cell activity in the peritoneal cavity (PC) and spleen

Strain of mice	Treatment	Day of assay	NK activity							
			PC				Spleen			
			25: 1	50: 1	100: 1	LU 5%	25: 1	50: 1	100: 1	LU 15%
C57BL/6	Saline	2	1.1	1.5	2.5	5.8	4.1	9.3	14.3	9.6
	MGN-3	2	4.0	7.1	9.2	35.2*	2.5	8.2	11.9	9.0
	Saline	5	0.8	1.7	2.4	6.2	5.1	8.0	10.3	7.7
	MGN-3	5	3.9	8.1	11.2	37.0*	6.1	9.2	14.0	9.2
	Saline	14	1.3	2.2	3.0	6.6	5.3	8.3	14.4	9.1
	MGN-3	14	4.4	6.7	8.8	34.0*	5.5	7.8	12.5	8.5
C3H	Saline	5	0.1	0.3	1.0	5.0	3.8	8.4	13.0	9.1
	MGN-3	5	1.2	2.4	7.5	24.1*	4.5	10.5	14.5	10.0

MGN-3 was injected into C57BL/6 and C3H mice.
Pooled lymphoid cells were prepared from the peritoneal cavity or spleens of saline-or MGN-3-treated mice.
Cells were harvested at 2, 5 and 14 days after treatment.
NK activity was assayed in a 4-h Cr-release assay against YAC-1 target cells at effector/target cell ratios of 25 : 1, 50 : 1 and 100 : 1 and expressed as the number of lytic units (LU).
*P<0.01, significantly different compared with saline-treated mice.

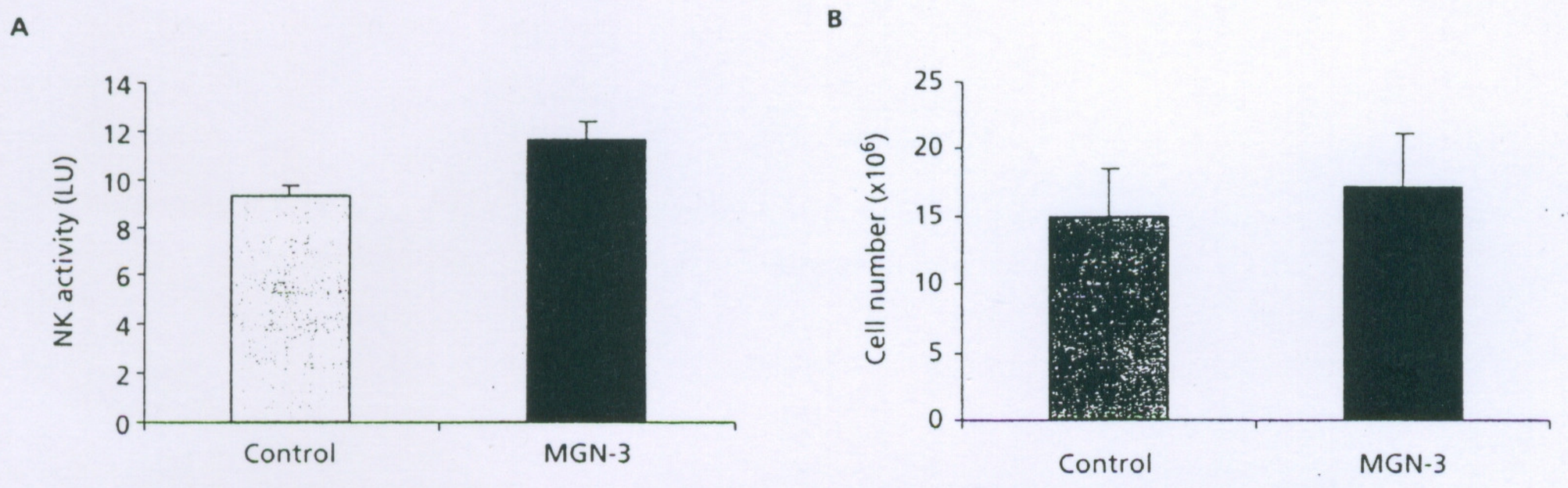

Figure 1 *In vivo* action of MGN-3 on bone marrow natural killer (NK) cell activity.
A : NK activity in C57BL/6 mice was examined 14 days after intraperitoneal treatment with MGN-3.
Activity of NK cells was measured by 4-h Cr-release assay at an effector/target ratio of 100 : 1.
B : The cell number of bone marrow was also examined.
Data are mean ± s.e.m of four mice examined separately.

NK activity of C3H mice after injection with BioBran/MGN-3. However, intraperitoneal treatment with BioBran/MGN-3 did not increase splenic NK cell activity at 2, 5 or 14 days after treatment in either strain of mice (**Table 2**).

3. Bone marrow NK activity

Bone marrow NK activity was examined in C57BL/6 mice at 14 days after intraperitoneal injection with

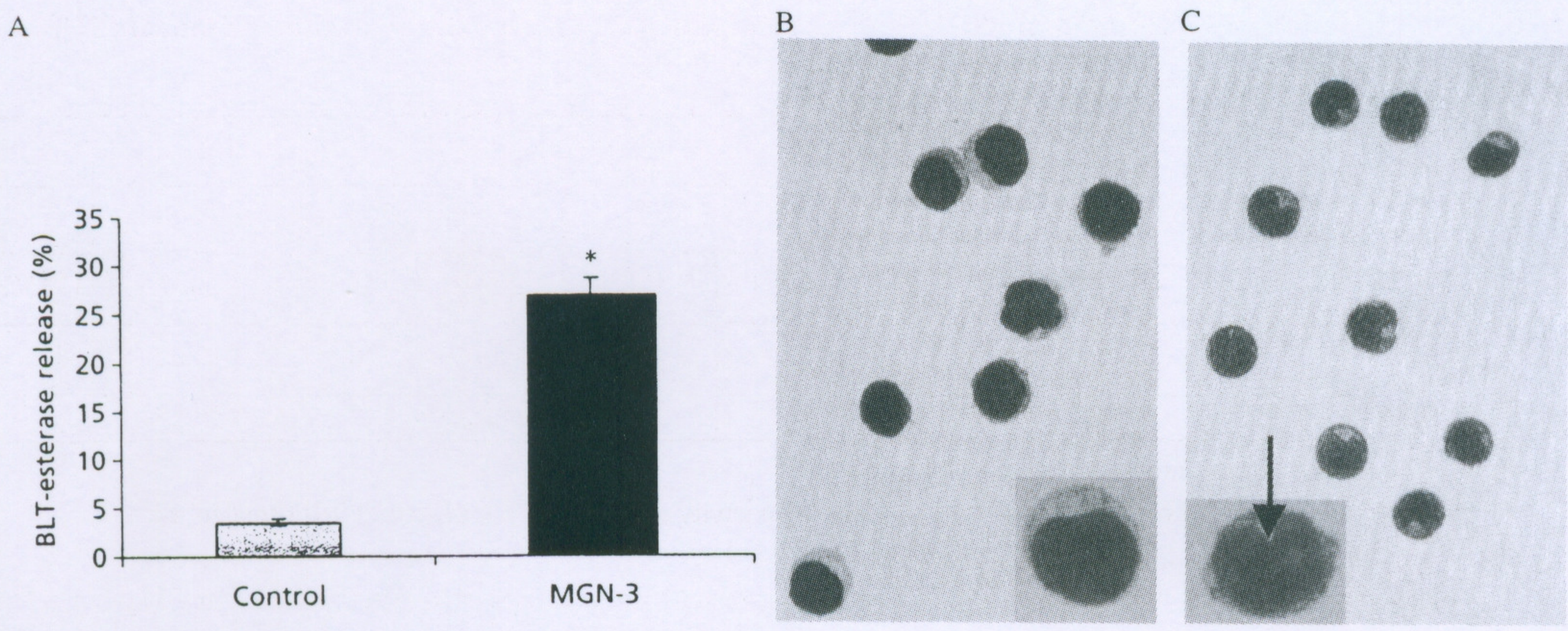

Figure 3

A : *In vivo* action of MGN-3 on BLT-esterase activity.
BLT-esterase activity was examined in C57BL/6 mice injected intraperitoneally with MGN-3 and saline.
Data are mean ± s.e.m of three experiments.
*$P<0.01$, significantly different compared with control saline-treated aged mice.
B : Cytocentrifuge preparation of peritoneal NK (P-NK) cells of saline-treated mice.
P-NK cells were used in a purified from using Percoll discontinuous gradients.
Notice the high nuclear cytoplasmic ratio and absence of granules in control aged mice.
C : Preparation of NK cells after MGN-3 treatment.
Notice the high granularity of NK cells.
Notice one NK cell with an arrow pointing to the granules.
(Giemsa stain, ×740.)

6. Percentage conjugate formation

The effect of BioBran/MGN-3 on the binding capacity of P-NK cells to YAC-1 cells was examined in cytospin preparations. As shown in **Figure 2**, BioBran/MGN-3-treated P-NK cells demonstrated 26% conjugate formation as compared with saline-treated P-NK cells (13%); this represents a 2-fold increase.

7. Granularity of P-NK cells

BioBran/MGN-3-treated P-NK cells demonstrated a remarkable increase in the BLT-esterase activity. **Figure 3** shows an increase in the BLT-esterase activity at 5 days as compared with saline-treated mice. This observation was further confirmed in cytospin preparations. **Figure 3B**, **C** shows NK cells from aged mice with a low or absent granular content, yet BioBran/MGN-3-treated mice at 5 days demonstrated an increase in NK cell granular content.

8. Suppressive effect of P-Mϕ on P-NK cell activity

The suppressive effect of P-Mϕ on P-NK cell activity after treatment with BioBran/MGN-3 was examined. **Figure 4A** shows that P-Mϕ alone sowed an undetectable level of antitumour activity. On the other hand, natural cytotoxicity by BioBran/MGN-3-treated PEC showed 7%, while PEC depleted of P-Mϕ (the non-adherent cells) demonstrated a further increase to 16%. An admixture of P-Mϕ and non-adherent cells resulted in a significant inhibition of NK activity exhibited by non-adherent cells (33%) as compared with non-adherent cells alone (**Figure 4B**).

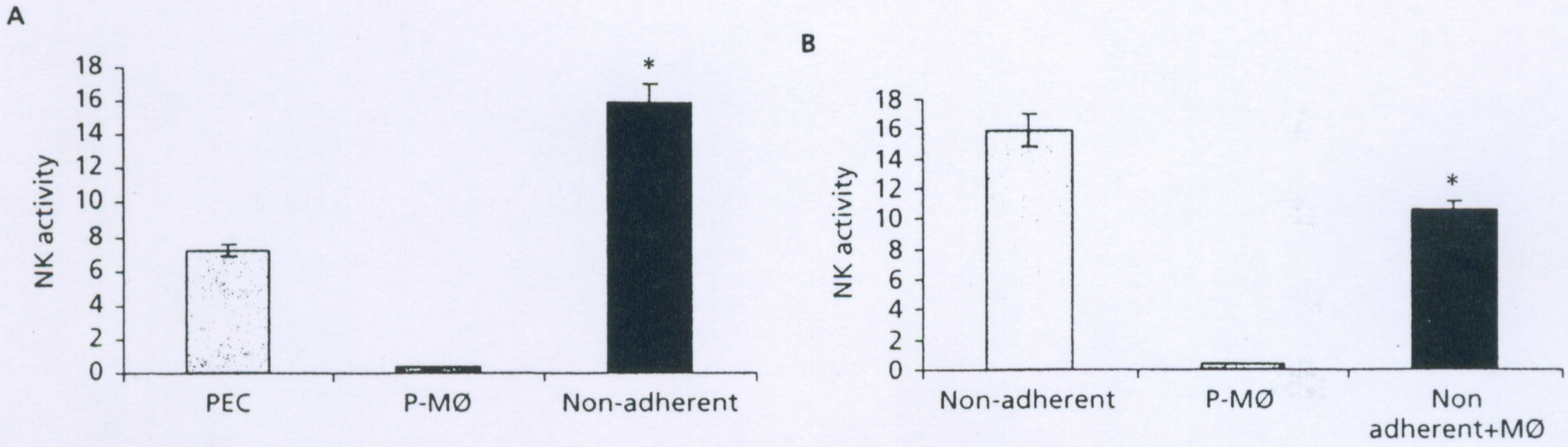

Figure 4 *In vivo* effect of MGN-3 on peritoneal exudate cells (PECs), macrophages (P-Mϕ), and non-adherent cells.

A : Aged C57BL/6 mice were injected intraperitoneally with MGN-3 for 5days and natural killer (NK) activity was examined at an effector/target ratio of 50 : 1 (A)

*P<0.025, compared with PECs.

B : Admixture of P-Mϕ to non-adherent cells from MGN-3-treated aged mice.

NK activuty was examined at an effector/target ratio of 50 : 1.

*P<0.025, significantly different compared with non-adherent cells alone.

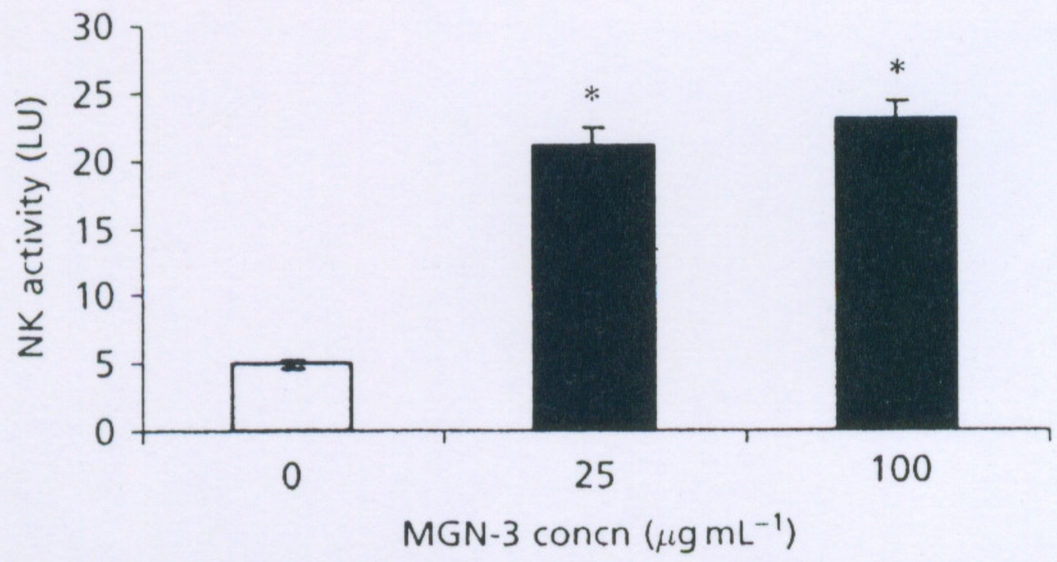

Figure 5 *In vitro* action of MGN-3 on natural killer (NK) cell activity.

Splenic lymphcytes from aged C57BL/6 mice were cultured for 16h in the presence or absence of MGN-3.

Activity was measured by 4-h Cr-release assay and expressed as number of lytic units (LU).

Data are mean ± s.e.m of three experiments.

*P<0.01, significantly different compared with control untreated cells.

9. *In vitro* action of BioBran/MGN-3 on NK activity

Culture of lymphocytes from aged C57BL/6 mice with BioBran/MGN-3 for 16h resulted in a significant induction of NK activity as follows: 21 and 23 LU at a concentration of 25 and 100 μgmL^{-1}, respectively, as compared with control untreated cells (**Figure 5**).

Discussion

In the present study, we demonstrated that BioBran/MGN-3 enhances murine NK activity in aged mice both *in vivo* and *in vitro*. The increase in NK activity was not due to simple intercompartmental redistribution of NK cells, but rather to an actual increase in the activity of the effector cells. This was evidenced

by examining the NK activity and the cell number of other lymphoid organs. Intraperitoneal injection with BioBran/MGN-3 did not significantly alter NK activity in the spleen or bone marrow, despite an increase in cellularity.

The mechanism by which BioBran/MGN-3 enhances murine NK activity may involve enhancing both the binding and the lethal hit. The study showed an increase in the percentage of conjugates of NK cells to their tumour targets after treatment with BioBran/MGN-3, which may be attributed to an increase in the expression of adhesion molecule (ICAM-1) on NK cells (Ghoneum & Jewett 2000) or other cell surface markers, for example LFA-1. The increase in conjugation after treatment with BioBran/MGN-3 may also suggest involvement of other effector cytotoxic cells such as CTL killing YAC-1 targets, since the percentage of NK cells did not alter with BioBran/MGN-3. The study also indicated an increase in the activity of the granule lytic BLT-esterase. It has been reported that aging is associated with a decline in the percentage of murine NK cells that carry granules (Ghoneum et al. 1989), and in the perforin expression in aged human NK cells (Rukavina et al. 1998).

Data demonstrated that P-Mϕ exhibit an inhibitory effect on NK cells. This was indicated by the ability of BioBran/MGN-3 to significantly increase the activity of P-NK cells that had been depleted of Mϕ. Earlier studies by Irimajiri et al. (1985) suggested that the active adherent suppressor cells, possibly macrophages, are responsible for the suppression of aged murine NK cell activity. We do not know the mechanism by which P-Mϕ suppress P-NK activity after treatment with BioBran/MGN-3, but it may involve increased production of H_2O_2 (Kono et al. 1996).

The increase in P-NK activity and in PECs is due to the direct effect of BioBran/MGN-3; the possibility that repeated injections of BioBran/MGN-3 caused any persistent peritoneal inflammation was excluded since control mice were repeatedly subjected to injections of saline solutions without the increase in P-NK activity or in PECs. The immunomodulatory effect of BioBran/MGN-3 was further confirmed by *in vitro* studies of a co-culture of BioBran/MGN-3 with NK cells for 16h. The increase in both splenic and peritoneal cellularity was a consistent finding in both strains of mice. The reason for this finding is not clear but it could be attributed to cell proliferation in these compartments. We have previously reported a significant increase in human T- and B-cell proliferation after ingestion of BioBran/MGN-3 (Ghoneum 1998a). Further studies are needed to clarify this point.

The inability of BioBran/MGN-3 to enhance splenic NK activity *in vivo* is not fully understood, but it could be attributed to the dilution of NK cells in the spleen due to an expansion of other cell populations. As shown in **Table 1**, splenic cellularity in BioBran/MGN-3-treated mice was increased. It has been reported that NK cells in different tissues display a differential response towards the enhancing effect of other BRMs. For example, treatment with *Candida albicans* remarkably inceased P-NK activity without an effect on splenic NK activity (Marconi et al. 1985). On the other hand, introducing BioBran/MGN-3 into the mice by oesophageal tubing increased splenic NK activity (200% as compared with control mice). This suggests that the enhancement of splenic NK cell activity depends on how this agent is introduced into the body.

Conclusions

The results of the present study show that intraperitoneal injection and oral treatment with BioBran/MGN-3 resulted in a significant enhancement of murine peritoneal and splenic NK cell activity, respectively.

The increase in NK activity was associated with an increased level of NK granularity. We conclude that BioBran/MGN-3 has the ability to enhance NK activity in aged mice and may be useful for enhancing the NK function in ageing humans.

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