Modulation of age-related changes in immune functions of protein-deficient senescence-accelerated mice by dietary nucleoside–nucleotide mixture supplementation

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In the present study we examined the immune-enhancing effect of a nucleoside–nucleotide mixture on the non-specific T-cell immune functions of senescence-accelerated mice (SAM) fed on a low-protein diet. The immune functions studied were *in vitro* thymic and splenic cell lymphoproliferative responses to phytohaemagglutinin, lipopolysaccharide and concanavalin A and their production of interleukin-2 (IL-2) and interferon-γ (INF-γ) in response to mitogen stimulation. SAMP8 mice aged 3 and 6 months were used. In each age group, mice were fed on diets containing either 50 g casein/kg, 50 g casein/kg supplemented with 5 g nucleoside–nucleotide mixture/kg or 200 g casein/kg for 3 weeks. The supplemented 3- and 6-month-old mice had higher ($P < 0.05$) thymic and splenic cell counts compared with the low-protein group. In both age groups of mice, concanavalin A induced higher ($P < 0.05$) total thymic and splenic lymphoproliferative responses for the nucleoside–nucleotide mixture-supplemented group compared with the 50 g casein/kg dietary groups. Thymic and splenic production of IL-2 was higher for the 3-month-old mice in both the supplemented and the 200 g casein/kg dietary groups. INF-γ production in the supplemented 3-month-old group and the 6-month-old 200 g casein/kg dietary group was higher ($P < 0.05$) compared with the other groups. Overall the supplemented 3-month-old mice exhibited both higher lymphoproliferative responses and production of cytokines compared with the supplemented 6-month-old mice. The results indicate that early nucleoside–nucleotide mixture supplementation may enhance the immune response in protein-deprived SAMP8 mice.

Nucleoside–nucleotide mixture: Immune function: SAMP8

We have previously demonstrated that dietary sources of nucleosides and nucleotides are important for the maintenance and enhancement of cellular immunity. Nucleotide-free diets (NFD) have been found to suppress T-cell response to mitogens, increase cardiac allograft survival (Van Buren *et al.* 1983); decrease delayed hypersensitivity to chemical and bacterial antigens and xenoantigens (Kulkarni *et al.* 1987); and decrease interleukin-2 (IL-2) production (Van Buren *et al.* 1985). In addition NFD decrease the host resistance to systemic *Staphylococcus aureus* infection (Kulkarni *et al.* 1986; Adjei *et al.* 1993) and *Candida albicans* in mice (Fanslow *et al.* 1988). The effects on the humoral immune system have also been well documented. It has been shown that dietary nucleotides and

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nucleosides increase antibody production, interferon-γ (INF-γ) and B-cell number in both human subjects and mice (Jyonouchi *et al.* 1992; Kemen *et al.* 1992). Thus, one can reasonably speculate that nucleic acids and their components have stimulating effects on the immune system.

The senescence-accelerated mouse (SAM) derived from AKR/J mice is a murine model of accelerated ageing and consists of substrains which are either resistant (SAMR) or prone (SAMP) to rapid senescence (Takeda *et al.* 1981). SAMP mice undergo normal growth and development for about 2 months, after which they show age-related changes in immune characteristics (Takeda *et al.* 1991). Among several abnormal immune activities, SAMP mice show an early decline in T-helper functions leading to an impaired *in vitro* anti-sheep erythrocyte antibody response (Hosokawa *et al.* 1987*a*, *b*; Yoshioka *et al.* 1989).

Although most studies have focused on the cellular bases of impairment of the immune system in SAM mice, no studies to our knowledge have been done on the use of immunomodulatory nutrients to improve the immune system of SAMP mice, which provide a murine model suitable for the study of progressive decline of immune viability with age. In addition to the decline in immune functions, ageing in association with protein malnutrition (PM) leads to further degeneration of immune functions. The present studies were designed to examine the effect of dietary nucleoside–nucleotide mixtures on non-specific T-cell immune responses as a measure of the potential of the mixture to enhance T-helper cell (T_H) function using SAMP8 mice. The immune variable most often studied in age-related decline in immune functions is the ability of lymphocytes to proliferate in response to foreign stimuli, such as antigens or mitogens. Thus we measured lymphocyte response to mitogens and production of IL-2 and INF-γ of SAMP8 mice fed on a low protein diet to simulate the dual conditions of PM and ageing. These two cytokines are indicative of T_H function. We studied these mice because they represent a rapid and easily accessible model of ageing.

MATERIALS AND METHODS

Animals and diets

Two age groups of SAMP8 mice were studied. Specific-pathogen-free 3-month-old and 6-month-old male SAMP8 mice of average weight 22·3 g and 26·8 g respectively, born and raised in our laboratory, were used. The mice were kept in a room with constant temperature ($25 \pm 2°$) and humidity (50–70 %) with a 12 h light period (from 08.00 to 20.00 hours). Mice were maintained on a standard chow diet (Nihon Clear, Osaka, Japan) containing (g/kg) protein 255 and fat 43 during their respective growth periods. At 3 months and 6 months respectively, mice from each age group were divided into three dietary groups and fed on isoenergetic and isonitrogenous experimental diets for 3 weeks. The diets were: nucleic acid-free 50 g casein/kg (low-protein diet, LPD), 50 g casein/kg supplemented with 5 g nucleoside–nucleotide mixture/kg (LPD + NNM) or nucleic acid-free 200 g casein/kg diet (Control). Table 1 shows the composition of the diets. The conditions of the study were based on the results of preliminary studies which indicated that 5 g of this particular mixture/kg (Table 1) and a 3-week period of feeding offered the maximum response. Mice were allowed free access to food and water and body weights were monitored for 3 weeks. Diets and water were renewed every morning between 08.00 and 10.00 hours. Animal care was in compliance with Applicable Guidelines from Ryukyus University Policy on Animal Care and Use. At the end of the period, the following experiments were performed: thymic and splenic lymphocyte responsiveness to a battery of mitogens and production of IL-2 and INF-γ.

Table 1. *Composition of experimental diets (g/kg)*

Ingredient	Diet		
	Control	LPD	LPD + NNM
Casein	200·0	50·0	50·0
Starch	444·0	544·0	543·0
Sucrose	222·0	272·0	272·0
Mineral mixture*	50·0	50·0	50·0
Vitamin mixture†	10·0	10·0	10·0
Cellulose	20·0	20·0	20·0
Maize oil	50·0	50·0	50·0
Glycine	4·0	4·0	–
NNM‡	–	–	5·0

LPD, low-protein diet; NNM, nucleoside–nucleotide mixture.

* Obtained from Oriental Yeast Co., Tokyo, Japan. The composition was as follows (mg/kg): $CaHPO_4.2H_2O$ 7820, $KHPO_4$ 12 800, NaH_2PO_4 4680, NaCl 2330, Ca lactate 17 550, Fe citrate 1590, $MgSO_4$ 3590, $ZnCO_3$ 55, $MnSO_4.6H_2O$ 60, $CuSO_4.5H_2O$ 15, KI 5.

† Obtained from Oriental Yeast Co. The composition was as follows (mg/kg): thiamin HCl 12, riboflavin 40, pyridoxine HCl 8, cyanocobalamin 50, ascorbic acid 300, D-biotin 0·2, pteroylglutamic acid 2, calcium pantothenate 5, *p*-amino-benzoic acid 50, nicotinic acid 60, inositol 60, choline chloride 2000, dl-α tocopherol acetate 50, menadione 52, retinyl acetate 5000 and ergocalciferol 1000.

‡ Obtained from Ohtsuka Phamaceutical Co., Tokushima, Japan. The composition was as follows (g/l): inosine 230, cytidine 210, guanidine monophosphate 2Na 350, uridine 160, thymidine 50.

Cell preparation

Mice were killed by cervical dislocation, thymus and spleen aseptically resected, placed in sterile petri dishes containing RPMI 1640 (Sigma Chemical Co., Sigma, St. Louis, MO, USA), penicillin (100 000 U/1) and streptomycin (50 U/ml) and stored on ice. Single-cell suspensions were obtained by teasing the tissues through 50-mesh stainless steel wire screens with the aid of plastic disposable syringe pistons into ice-cold RPMI 1640. The suspensions were treated with 0·1 M-Tris HCl (pH 7·2) containing 8 g/l Tris NH_4Cl to lyse erythrocytes, and centrifuged at 200 *g* for 5 min at 4°. Cell pellets were then washed three times in RPMI 1640, resuspended in complete RPMI 1640 (containing 20 mM-HEPES, 2 mM-glutamine, 100 μg gentamycin/ml, 100 U streptomycin/ml, and 100 ml heat-inactivated fetal calf serum/l (Sigma), and cell viability determined by trypan blue exclusion. Cell suspensions were enumerated using a haemocytometer and then adjusted appropriately.

Spleen and thymic cells (5×10^5 cells per well) in complete RPMI 1640 ($2{\cdot}5 \times 10^3$ cells/μl) were dispensed into ninety-six-well flat-bottomed Falcon microtitre plates and cultured in triplicate for 30 h at 37° in a humidified 50 ml/l CO_2 incubator in the presence of 5 μg concanavalin A (Con A)/ml (Sigma), 10 μg phytohaemagglutinin (PHA)/ml (Sigma), or 40 μg lipopolysaccharide (LPS)/ml, (Sigma). Cells cultured without mitogens served as negative controls. Lymphocyte blastogenic response was determined by the method of Hansen *et al.* (1989), which is a modification of the method of Mossman (1983). Briefly, to a lymphocyte-containing culture medium in the microplate, 25 μl 3, (4, 5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) (1 mg/ml) was added to each well. After 1 h incubation at 37° in a humidified CO_2 incubator, 100 μl of an extraction buffer was added. After overnight incubation, the absorbances were measured at 570 nm by a Titer-tech (Dynatech, USA) ninety-six-well multi-scanner using the extraction buffer as the blank. The mitogen response was calculated as the mitogen stimulated: spontaneous lymphoproliferative response ratio, and reported as the stimulation index (SI).

Optimal conditions required for application of MTT had been previously determined before the start of the experiment.

Thymic and splenic cell counts

Total thymic and splenic cell counts were determined before lysis. The cells were diluted ten times with Turk solution (1 ml 10 g/l gentian violet and 1 ml 995 g/l acetic acid were diluted to 1 litre with distilled water) and the number was determined by counting them in a haemocytometer.

Interleukin-2 and interferon-γ production

Spleen and thymus cells (5×10^5 cells/well) in complete RPMI 1640 ($2{\cdot}5 \times 10^3$ cells/μl) were dispensed into ninety-six-well flat-bottomed Falcon microtitre plates and cultured in triplicate for 30 h at 37° in a humidified 50 ml/l CO_2 incubator in the presence of 5 μg/ml Con A, as described earlier. At the end of the incubation period, triplicate supernatant fractions were collected, pooled and stored at −80° for IL-2 and INF-γ analysis. IL-2 and INF-γ were quantified with commercial mouse IL-2 and INF-γ ELISA kits (Becton-Dickinson, Maryland, USA), following the manufacturer's instructions. The inter-assay and intra-assay CV were < 10 %. The limits of sensitivity of the assays were < 15 pg/ml for INF-γ and < 3 pg/ml for IL-2.

Chemicals

MTT (Wako Chemical Co., Tokyo, Japan) was dissolved to a concentration of 5 mg/ml in sterile phosphate-buffered saline, pH 7·2, at room temperature. The solution was filter-sterilized (millipore 0·22 μm) and stored at 4° in a dark bottle. SDS and N, N-dimethylformamide (DMF) were obtained from Wako Chemical Co. The composition of the extraction buffer was as follows: 200 g SDS/l was dissolved at 37° in a solution of 500 g/l each of DMF and demineralized water. The pH was adjusted to 4·7 by adding 25 g/l of 800 g/l acetic acid and 25 g/l of 1 M-HCl.

Statistical analysis

Statistical analyses were performed using ANOVA. Duncan's multiple range test was used to determine significant differences among means at $P < 0{\cdot}05$.

RESULTS

Weight gain, tissue weight and cell counts

Table 2 shows the effect of the diets on the body weights of both age groups of mice. At the end of 3 weeks on the diets, in both age groups of mice, the LPD and the LPD + NNM groups had lost weight. Mice on the Control diet had gained weight which was significantly higher ($P < 0{\cdot}05$) compared with the other dietary groups.

Tables 3 and 4 show the splenic and thymic weights as well as the mean total cell counts of both organs of the 3-month-old and 6-month-old mice respectively. In both age groups of mice, the corresponding organ weights were similar among the groups. However, both the LPD + NNM and Control groups had significantly higher ($P < 0{\cdot}05$) mean total spleen and thymus counts than the LPD group in both age groups. A significant feature

Table 2. *Body-weight changes in 3-month-old and 6-month-old mice fed on a low-protein diet (LPD), LPD plus a nucleoside–nucleotide mixture (NNM) or a control diet for 3 weeks**

(Mean values with their standard errors for twelve mice per dietary group)

	Body-weight changes (g)			
Age ...	3 months		6 months	
Dietary group	Mean	SE	Mean	SE
LPD	−2·0[a]	1·14	−2·5[a]	0·97
LPD + NNM	−2·3[a]	2·16	−2·2[a]	0·90
Control	5·7[b]	1·85	3·2[b]	1·22

[a,b] Mean values within a column with unlike superscript letters were significantly different, $P < 0·05$.
* For details of diets, see Table 1.

Table 3. *Organ weights and cell counts in 3-month-old SAMP8 mice fed on a low-protein diet (LPD), LPD plus a nucleoside–nucleotide mixture (NNM) or a control diet for 3 weeks**

(Mean values with their standard errors for twelve mice per dietary group)

	Splenic weight (g)		Total splenic count (cells/ tissue) × 10^6		Thymic weight (g)		Total thymic count (cells/ tissue) × 10^6	
Dietary group	Mean	SE	Mean	SE	Mean	SE	Mean	SE
LPD	0·08	1·01	4·86[a]	0·93	0·02	0·01	4·72[a]	3·45
LPD + NNM	0·09	0·01	9·86[b]	1·20	0·03	0·04	9·89[b]	2·97
Control	0·11	0·10	9·95[b]	1·11	0·03	0·01	9·53[b]	2·44

[a,b] Mean values within a column with unlike superscript letters were significantly different, $P < 0·05$.
* For details of diets, see Table 1.

Table 4. *Organ weights and cell counts in 6-month-old SAMP8 mice fed on a low-protein diet (LPD), LPD plus a nucleoside–nucleotide mixture (NNM) or a control diet for 3 weeks**

(Mean values with their standard errors for twelve mice per dietary group)

	Splenic weight (g)		Total splenic count (cells/ tissue) × 10^6		Thymic weight (g)		Thymic count (cells/ tissue) × 10^6	
Dietary groups	Mean	SE	Mean	SE	Mean	SE	Mean	SE
LPD	0·07	0·01	3·06[a]	1·08	0·01	0·02	4·61[a]	0·72
LPD + NNM	0·09	0·01	8·55[b]	1·63	0·02	0·04	8·63[b]	4·59
Control	0·10	0·10	8·76[b]	1·23	0·02	0·01	8·11[b]	2·40

[a,b] Mean values within a column within unlike superscript letters were significantly different, $P < 0·05$.
* For details of diets, see Table 1.

observed in the LPD + NNM dietary groups of both ages was the presence of numerous small thymic and splenic cells.

Lymphoproliferative responses

Fig. 1 shows the thymic lymphoproliferative responses of 3-month-old (Fig. 1(a)) and 6-month-old (Fig. 1(b)) SAMP8 mice expressed as SI. In both age groups PHA and LPS responses were similar among the dietary groups. With Con A, in both age groups, the LPD + NNM groups exhibited responses which were significantly higher ($P < 0.05$) compared with the LPD groups. Responses were similar between the LPD and the Control groups in both age groups.

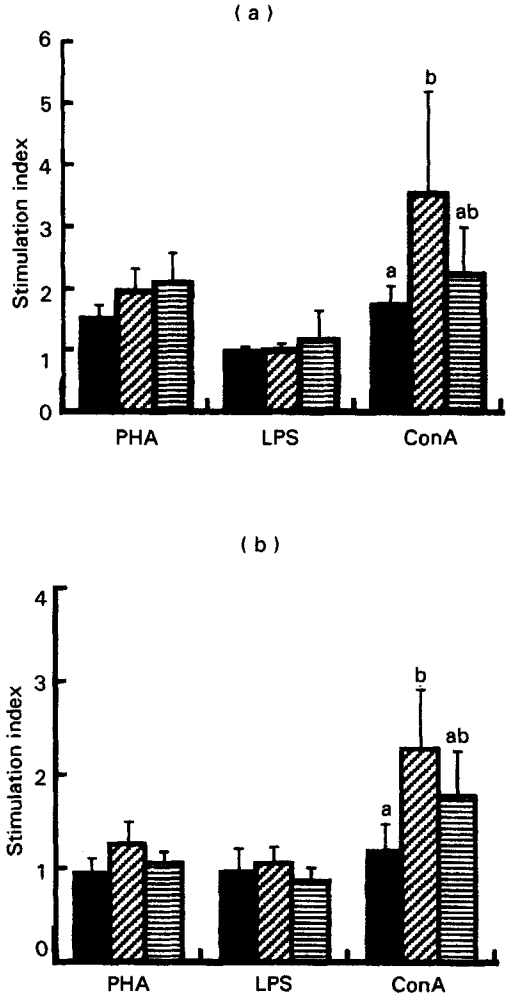

Fig. 1. Thymic stimulation index of (a) 3-month-old and (b) 6-month-old SAMP8 mice fed on a low-protein diet (■), a low-protein diet plus a nucleoside–nucleotide mixture (▨), or a control diet (▤) for 3 weeks. Cell suspensions were cultured with phytohaemagglutinin (PHA), lipopolysaccharide (LPS) or concanavalin A (Con A) for 30 h. For details of procedures, see pp. 796–797. Values are means for twelve mice per dietary group, with their standard errors represented by vertical bars. [a,b] Mean values not sharing a common superscript letter were significantly different, $P < 0.05$.

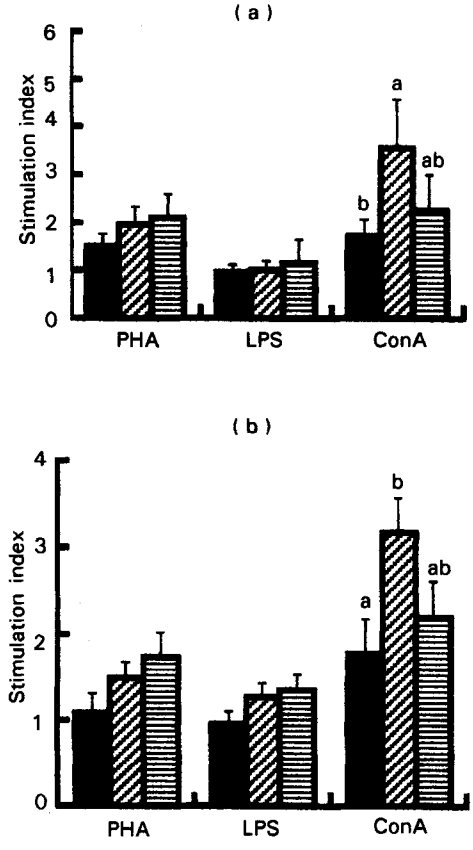

Fig. 2. Splenic stimulation index of (a) 3-month-old and (b) 6-month-old SAMP8 mice fed on a low-protein diet (■), a low-protein diet plus a nucleoside–nucleotide mixture (▨), or a control diet (▤) for 3 weeks. Cell suspensions were cultured with phytohaemagglutinin (PHA), lipopolysaccharide (LPS) or concanavalin A (Con A) for 30 h. For details of procedures, see pp. 796–797. Values are means for twelve mice per dietary group, with their standard errors represented by vertical bars. [a,b]Mean values not sharing a common letter were significantly different, $P < 0.05$.

Fig. 2 shows the splenic lymphoproliferative responses of 3-month-old (2(a)) and 6-month-old (Fig. 2(b)) SAMP8 mice expressed as SI. In both age groups of mice, PHA and LPS exhibited similar responses among the dietary groups. With Con A, the LPD + NNM groups exhibited responses which were higher ($P < 0.05$) than those of the LPD groups. Responses were similar between the LPD and the Control groups in both age groups.

In vitro *cytokine production*

Table 5 shows the results of *in vitro* cytokine production by Con A-stimulated thymic and splenic cells of both age groups of mice. Between the age groups in the LPD + NNM group, the 3-month-old mice showed higher production of both cytokines ($P < 0.05$) compared with the 6-month-old mice. Within the Control group, the 3-month-old mice showed a higher production of IL-2 ($P < 0.05$) in both organs compared with the 6-month-old mice. However, the 6-month-old mice showed a higher production of INF-γ ($P < 0.05$) compared with the 3-month-old mice in both organs. Within the LPD group, production of

Table 5. *In vitro thymic and splenic cell production of interleukin-2 (IL-2) and interferon-γ (INF-γ) (ng/ml) by SAMP8 mice fed on a low-protein diet (LPD), LPD plus a nucleoside–nucleotide mixture (NNM) or a control diet for 3 weeks**

(Mean values with their standard errors for twelve mice per dietary group)

		Thymus				Spleen			
		IL-2		INF-γ		IL-2		INF-γ	
Dietary group	Age (months)	Mean	SE	Mean	SE	Mean	SE	Mean	SE
LPD	3	3·08[a]	1·92	2·41[a]	0·45	4·67[a]	1·44	3·10[a]	1·60
	6	1·17[a]	0·75	2·44[a]	0·79	3·52[a]	1·38	4·19[b]	2·18
LPD + NNM	3	8·58[b]	1·16	7·84[b]	2·33	15·43[b]	3·29	12·06[c]	2·65
	6	4·57[a]	0·77	3·00[a]	0·50	4·54[a]	1·43	7·83[b]	1·57
Control	3	6·36[b]	1·85	3·18[a]	1·16	12·88[b]	2·44	3·72[a]	1·54
	6	1·71[a]	0·56	5·46[b]	1·22	3·34[a]	1·96	10·26[c]	2·35

[a,b,c] Mean values within a column with unlike superscript letters were significantly different, $P < 0·05$.

* For details of diets and procedures, see Table 1 and pp. 796–798.

both cytokines was low and similar between the age groups, except for production of INF-γ by splenocytes which was higher ($P < 0·05$) in the 6-month-old compared with the 3-month-old mice. Between the LPD and LPD + NNM groups, the 3-month-old LPD + NNM group showed significantly higher ($P < 0·05$) production of both cytokines overall. In both organs, the 3-month-old Control group produced more IL-2 ($P < 0·05$), while the 6-month-old Control group produced more INF-γ than the corresponding age groups in the LPD groups.

DISCUSSION

Body-weight loss and lymphoid atrophy are prominent features of protein–energy malnutrition (PEM) (Chandra, 1991), and cellular immunity is most devastated by severe malnutrition (Field *et al.* 1991). The target lymphocyte subpopulation appears to be the T_H cells and is associated with decreased production of lymphokines, such as IL-2 which is necessary for the propagation of normal T-cell mediated immune responses (Chandra, 1983). In the present study this was reflected in the low T-cell responses shown by the LPD group. However, our study utilizing levels of protein intake that resulted in weight loss, showed in these mice that early dietary NNM supplementation in protein deficiency may help in maintaining immune functions as evidenced by the increased productions of IL-2 and INF-γ in the 3-month-old LPD + NNM group. In the 6-month-old mice, although supplementation led to significant increases in the total thymic and splenic cell counts, neither lymphoproliferative response nor the production of IL-2 and INF-γ were significantly different from control levels. This observation is astounding and suggests that despite the enhanced lymphoproliferation, IL-2 and INF-γ-producing cells are dysfunctional or underrepresented at this apparently late stage of supplementation. Whether the defect represents a delay in maturation or a more permanent alteration remains to be elucidated, and whether this defect can be permanently rectified with early and prolonged NNM supplementation is not yet known. Toichi *et al.* (1994) reported that the transfer of purified T-cells from young SAMP mice to "aged" SAMP mice led to restoration of the T-dependent antibody production through normalization of T_H function. The present study showed an improvement in the non-specific T_H function of protein-deficient "aged" mice with dietary NNM supplementation and its importance lies in the

peculiar growth characteristics of these mice which show normal growth for about 2 months and then show an age-related defect in T_H function leading to defective T-dependent antibody response.

Many studies using stimulated splenocytes from mice show an age-related increase in INF-γ production (Heine & Adler, 1977; Saxena *et al.* 1988; Hobbs *et al.* 1993), while decreasing mitogen-induced IL-2 synthesis with advancing age has been described in both human subjects and mice (Thoman & Weigle, 1981, 1985; Negoro *et al.* 1986). In the present studies we also observed an increase in INF-γ production by thymic and splenic cells of the 6-month-old Control mice and by the splenic cells of the LPD groups (Table 5). Increased INF-γ production in ageing has been associated with inhibition of T-cell proliferation (Hobbs *et al.* 1993). This increase in INF-γ production among the 6-month-old mice in the unsupplemented dietary group, together with age, may be the factors responsible for the low lymphoproliferation and IL-2 production observed in these groups. Considering the rather low production of thymic cytokines and splenic IL-2 in the LPD dietary group, it is conceivable that the dietary effect may have been the most important predisposing factor influencing this depression in T-cell function. However, the increased production of both cytokines in the 3-month-old compared with the 6-month-old mice in the LPD + NNM group demonstrate the enhancing effect of early dietary NNM supplementation on IL-2 and INF-γ production.

To our knowledge, this is the first report that has assessed the immunopotentiating effect of NNM on the T-cell lymphoproliferative response to mitogen in SAM mice. These findings suggest that early dietary supplementation with NNM may provide the possibility of modulating the observed decline in T-cell proliferative response with ageing.

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