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Age-related enhancement of tumor necrosis factor (TNF) production in mice

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Abstract

We investigated age-related changes in the production of TNF at the cellular level using immunocompetent peritoneal and spleen cells from C3H/He mice of various ages. The density of cultured peritoneal macrophages and spleen cells required for TNF production was at least 5×10^5 cells/dish. The optimal concentration of OK-432 for 24-h culture of peritoneal macrophages (1×10^6 cells) and spleen cells (1×10^7 cells) was 0.5 and 0.1 KE/ml, respectively. Among peritoneal cells, adherent macrophages were the major TNF-producing cells, whilst nonadherent T or B cells alone did not produce TNF after stimulation with OK-432. In the case of spleen cells, T or B cells were involved in the production of TNF when cultured with a few adherent cells in the presence of OK-432. However, T or B cells alone failed to produce TNF. Production of TNF by peritoneal macrophages from both male and female mice increased significantly with aging. In contrast, although TNF production by spleen cells tended to increase with aging, no significant change was noted. The total number of peritoneal and spleen cells, respectively increased up to about 18 months after birth with B cells being principally responsible for this age-related increase. We previously reported that systemic production of TNF increases with aging. The present study of TNF production at the cellular level in mice indicated (1) that TNF production per macrophage increased with aging, and (2) that the number of T and B cells involved in the production of TNF in the presence of macrophages also increased at least up to middle age.

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However the mechanism involved and the physiological significance of this increase in macrophage TNF production remain to be elucidated.

Keywords: Tumor necrosis factor (TNF) production; Aging; A streptococcus-derived immunomodulator (OK-432)

1. Introduction

Tumor necrosis factor (TNF) has a wide variety of physiological actions as well as a necrotizing effect on tumors. These physiological actions include an activation of vascular endothelial cells [1], an immunopotentiating effect on immunocompetent cells [2–5], a cytotoxic effect on vascular endothelial cells [6], an antiviral effect [7,8], a cachectin-like effect [9,10], and activity as an inflammatory mediator [11–13]. Hence, TNF is considered to play an important role in the general regulation of the immune system.

Most immunological functions progressively decline with advancing age [14,15]. The age-associated decline mainly occurs in the T-dependent immune system, which is considered to be affected by the age-related physiological thymic involution [16]. Cellular senescence in the T-cell lineage reflecting the proiferative limit of the mammalian cell proposed by the programmed senescence theory [17] may also be responsible as a major cause of the age-associated functional decline of the T cell [18]. One of the important age-associated changes in the T-cell compartment is an increase in the ratio of memory T cells to naive cells [15,16]. The increase in the memory cell ratio may lead to an increase in the production of some lymphokines [15, 19, 20]. Thus, some immune functions appear to augment with age and the immune system may undergo a complex remodelling rather than an unidirectional deterioration [21].

TNF plays an important role in immune regulation as mentioned above and its involvement in changes of immune function with aging is an interesting subject, but the age-related changes of TNF production are still unclear. Accordingly, we previously investigated the changes of TNF production with aging by treating mice with OK-432, a hemolytic streptococcus-derived immunomodulator [22]. We found that TNF production increased with aging in both male and female mice. However, the precise cellular mechanism involved remained to be determined.

In the present study, we therefore obtained peritoneal and spleen cells from young, mature, and old mice, and compared TNF production among macrophages, T cells, and B cells to investigate age-related changes of TNF production at the cellular level.

2. Materials and methods

2.1. Experimental animals

Eight-week-old C3H/HeSlc mice were purchased from SLC Co. (Hamamatsu,

Shizuoka, Japan) and kept in the animal room of our department. At 24–29, 13–17, and 2 months old, the mice were used as the old, mature, and young groups, respectively. In this study, unless otherwise stated, the age of mice was 2 months old. Mice were housed in cages disinfected with sodium hypochloride and 70% ethanol, with bedding of autoclaved soft chips and artificial lighting from 7:00 a.m. to 7:00 p.m. at a room temperature of 24–26°C. The animals were given commercially available pellet food and tap water ad libitum.

2.2. L-M cells

TNF activity was determined by bioassay using TNF-sensitive L-M cells. L-M cells were cultured in Eagles MEM (Nissui Pharmaceutical Co., Tokyo, Japan) containing 5% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a CO_2 incubator under humidified 5% CO_2-95% air at 37°C, and were used in the logarithmic growth phase.

2.3. OK-432

Induction of TNF production in immunocompetent mouse cells was assessed using OK-432 (Picibanil, a lyophilized product of a penicillin-treated attenuated Streptococcus type A Su strain) supplied by Chugai Pharmaceutical Co. (Tokyo, Japan). The dose of OK-432 was expressed as KE units, with 1 KE being equivalent to 0.1 mg of lyophilized bacteria by weight. OK-432 was suspended at a concentration of 10 KE/ml in phosphate buffered-saline (PBS), and an appropriate volume of the suspension was added to cultures.

2.4. Preparation of peritoneal cells and peritoneal macrophages

Six to ten milliliters of ice-chilled RPMI-1640 medium (Nissui Pharmaceutical Co.) containing 5% FCS was injected intraperitoneally into mice killed by exsanguination. Then the abdomen was massaged for about 3 min and the peritoneal fluid was collected. This fluid was centrifuged twice at 1000 rev./min and for 5 min, and the resultant cell pellet was suspended in complete medium (RPMI-1640 medium containing 5% FCS, 2 mM L-glutamine, and an appropriate amount of sodium hydrogen carbonate) for use as the peritoneal cell suspension. The number of F4/80-positive cells per milliliter of suspension was counted, and suspensions containing an appropriate number of these cells were placed in 3.5-cm Petri dishes (# 3001, Falcon, Becton Dickinson Labware, Lincoln Park New Jersey, USA). The total volume was made up to 1 ml and incubation was performed at 37°C for 2 h. Then each dish was washed 3 times with RPMI-1640 medium (warmed to 37°C) containing 5% FCS to remove nonadherent cells. Adherent cells remaining in the dishes were double-stained with a-naphthyl acetate esterase and naphthol-AS-D-chloroacetate esterase to identify macrophages. More than 90% of the adherent cells were shown to be macrophages. The washings were combined and centrifuged, the supernatant was discarded, and the resultant pellet was suspended in complete medium for use as nonadherent cells. A portion of the non-adherent cells were

double-stained with esterases in the manner mentioned above, and less than 5% of these cells were found to be macrophages.

2.5. Preparation of unfractionated spleen cells or spleen T and B cells

The spleen was gently teased apart with a stainless mesh and forceps to obtain a spleen cell suspension. This suspension was treated with 1.66% NH_4Cl to remove red blood cells and centrifuged. The resultant pellet was then suspended in complete medium to obtain an unfractionated spleen cell suspension. An aliquot of the spleen cell suspension was applied to a nylon column according to the method of Julius et al. [23] to obtain nonadherent cells. These cells were then applied to a Sephadex G-10 column according to the method of Ly & Mishell [24] to obtain nonadherent cells, which were used as spleen T cells. Flowcytometry showed that more than 85% of these spleen T cells were positive for Thy.1 and less than 5% were positive for surface Ig.

Another aliquot of the spleen cell suspension was treated with anti-mouse Thy.1.2 antibody (Meiji Health Science Co. Odawara, Kanagawa, Japan) and rabbit serum complement, after which they were applied to a Sephadex G-10 column to obtain nonadherent cells, for use as spleen B cells. More than 90% of these B cells were positive for surface Ig and less than 5% were positive for Thy.1.

2.6. Flowcytometry

To 100 μl of the peritoneal cell suspension or the spleen cell suspension at a concentration of 1×10^7 cells/ml was added 10 μl of an appropriately diluted FITC-labeled anti-mouse Thy.1.2 monoclonal antibody (Meiji Health Science Co.), an FITC-labeled anti-mouse Ig (IgM + IgG) antibody (Tago Inc., Burlingame California, USA), or an FITC-labeled anti-F4/80 monoclonal antibody (Dainippon Pharmaceutical Co., Osaka, Osaka, Japan). After stirring, the suspension was allowed to stand on ice for 20 min for staining. Then the cells were washed twice with PBS containing 2% FCS and examined by flowcytometry using a FACScan (Becton Dickinson Immunocytometry System, Mountain View, California, USA). The results were analyzed using LYSIS and PAINT-A-GATE.

2.7. Induction of TNF production by OK-432

Peritoneal or spleen cells at various concentrations were suspended in RPMI-1640 medium containing 5% FCS, 2 mM L-glutamine, and an appropriate amount of sodium hydrogen carbonate (complete medium) and placed in 3.5-cm Petri dishes. OK-432 was added to the dishes at various concentrations, the final volume of medium was made up to 1 ml and incubation was done in a CO_2 incubator under a humidified 5% CO_2 atmosphere at 37°C for 24 h. The supernatant of each culture was collected by centrifugation at 1000 rev./min for 5 min and the TNF activity was determined.

2.8. Measurement of TNF activity

TNF activity in the culture supernatants was determined by a bioassay using TNF-sensitive L-M cells. Since L-M cells are sensitive to both TNF-α and TNF-β

the total activity was determined. Culture supernatant samples were serially diluted two-fold with MEM containing 1% FCS, and 100-μl aliquots were placed in a 96-well flat-bottomed microculture plate (# 3072 Falcon, Becton Dickinson Labware Lincoln Park, New Jersey, USA). Then L-M cells were suspended at a concentration of 2×10^5 /ml in MEM containing 2 μg/ml actinomycin D (Sigma Chemical Co., St. Louis, Missouri, USA) and 100 μl each of the suspension was added to wells containing the diluted supernatants. The plate was incubated in a humidified 5% CO_2 atmosphere at 37°C for 24 h, and then the number of viable cells in each well was determined by colorimetry. A well containing only 100 μl of MEM with 1% FCS was used as the control. Mouse recombinant TNF-α (Genzyme Co., Cambridge, Massachusetts, USA) was used as the TNF standard to draw a calibration curve for each plate, and the measured values of TNF in the supernatants were normalized. The TNF concentration in each supernatant was expressed as units/ml, with the concentration at which the absorbance of a well was 50% of the control level being defined as 1 U/ml.

2.9. Determination of cell viability by MTT colorimetry

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma Chemical Co.) is reduced by enzymes of the respiratory chain in the mitochondrial inner membrane of living cells to form MTT formazan and the amount of the formazan product is proportional to the number of viable cells [25]. Ten microliters of MTT dissolved in PBS at a concentration of 5 mg/ml was added to each well of a 96-well microplate containing L-M cells, and incubated for 2 h. Then the medium from each well was discarded and 100 μl of dimethyl sulfoxide (DMSO) was added to each of the wells to completely dissolve the MTT formazan product. Immediately afterwards, the plate was examined with a Titertek Multiscan plate reader (Flow Laboratories, McLean, Varginia, USA), and the formazan content of each well was determined by colorimetry based on the difference in absorbance at 540 and 620 nm.

2.10. Statistical analysis

Experimental data were expressed as the mean $\pm$ S.E., and statistical analysis was performed by Student's t-test and ANOVA, with $P < 0.05$ indicating statistical significance.

3. Results

3.1. Optimal OK-432 concentration for induction of TNF production by peritoneal macrophages

To determine the optimal concentration of OK-432 for induction of TNF production by peritoneal macrophages, OK-432 dissolved in RPMI-1640 supplemented with 5% FCS and added to Petri dishes containing F4/80-positive peritoneal macrophages (1×10^6/dish) from young male mice at doses of 0.125, 0.25, 0.5, 1.0, and 1.5 KE. Then the total volume of medium was made up to 1 ml, and TNF production was observed. A culture without OK-432 served as the control. As shown in Fig. 1, TNF production was highest at a concentration of 0.5 KE/ml.

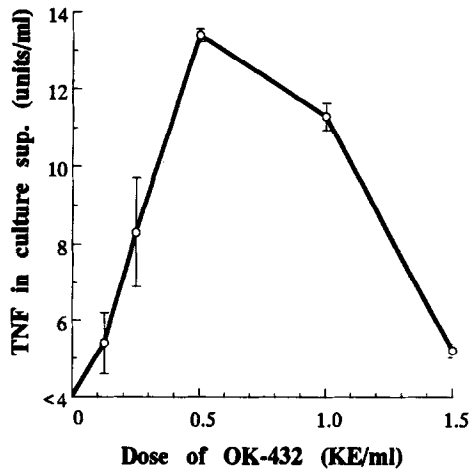

Fig. 1. TNF production by adherent peritoneal cells in response to various concentrations of OK-432. Adherent peritoneal cells (1×10^6/dish) were incubated with various concentrations of OK-432 for 24 h and the TNF activity in the culture supernatant was determined. Points and bars indicate the mean value and the standard error of tripliate cultures, respectively.

3.2. Optimal OK-432 concentration for induction of TNF production by spleen cells

To determine the optimal concentration of OK-432 for induction of TNF production by spleen cells, OK-432 was dissolved in RPMI-1640 supplemented with 5% FCS and added to culture dishes containing spleen cells (1×10^7/dish) from young male mice at doses of 1×10^{-4}, 1×10^{-3}, 1×10^{-2}, 1×10^{-1}, and 1.0 KE. Then the total volume of medium was made up to 1 ml and TNF production

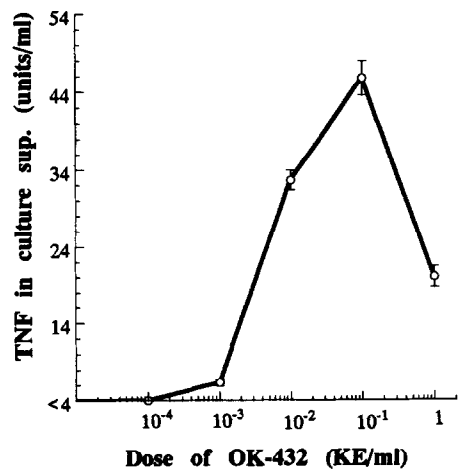

Fig. 2. TNF production by spleen cells in response to various concentrations of OK-432. Unfractionated spleen cells (1×10^7/dish) were incubated with various concentrations of OK-432 for 24 h. See legend for Fig. 1.

Table 1
TNF production by unfractionated peritoneal cells (PC) non-adherent peritoneal cells, and adherent peritoneal cells (macrophges) from C3H/He mice

No. of cells ($\times 10^4$/dish)	TNF activity in culture supernatant (unit/ml)[a]		
	PC	Non-adherent cells	Macrophages
10	<4[b]	<4	<4
50	17.6 ± 1.3	<4	9.2 ± 0.4
100	26.6 ± 1.6	<4	15.0 ± 0.9
150	51.1 ± 2.7	<4	24.7 ± 2.6

[a]values represent the mean $\pm$ SE of TNF activity in supernatants from three mice.
[b]TNF activity was not detected.

was observed. A culture without OK-432 served as the control. As shown in Fig. 2, TNF production was highest at a concentration of 1×10^{-1} KE/ml.

3.3. Dependence of TNF production on cell density

To assess the relationship between cell density and TNF production, 1×10^5, 5×10^5, 10×10^5, and 15×10^5 unfractionated peritoneal cells or peritoneal cells containing these respective numbers of F4/80-positive cells were placed in dishes and incubated for 2 h. Then nonadherent cells were recovered from cultures containing the adjusted numbers of F4/80-positive cells, and 1×10^5, 5×10^5, 10×10^5, and 15×10^5 of these nonadherent cells were placed into other dishes. Thus, three sets of cultures were prepared, i.e. unfractionated peritoneal cells, adherent peritoneal cells, and nonadherent cells. OK-432 (0.5 KE/ml) was added to each dish and TNF production was observed. As shown in Table 1, the nonadherent cells failed to produce TNF at any cell density. The unfractionated and adherent peritoneal cells produced TNF at cell densities of 5×10^5 per dish or more, and TNF production increased at higher cell densities. The adherent cells among the peritoneal cells were found to be responsible for TNF production. On the basis of these results, TNF production was investigated at a density of at least 1×10^6 cells/dish in the present study.

3.4. Effect of preincubation on TNF production by spleen cells

Since culture for 2 h was needed to separate adherent and non-adherent peritoneal and spleen cells, the effect of 2-h preincubation on TNF production was assessed. Spleen cells (3×10^6 cell) were incubated in a Petri dish for 2 h with OK-432 at 0.1 KE/dish to assess the effect of preincubation on TNF production. As shown in Table 2, TNF production was higher when spleen cells were preincubated than without preincubation. Accordingly, cells were cultured for 2 h before the addition of OK-432 in all experiments, including comparison between adherent and unfractionated cells.

Table 2
Effect of preincubation on TNF production by unfractionated spleen cells before stimulation with OK-432

Preincubation (h)	TNF activity in culture supernatant (U/ml)[a]
0	21.4 ± 1.0
2	68.4 ± 2.7[b]

[a]3 × 10^6 unfractionated spleen cells were preincubated for 2 h and then were cultured with OK-432 for 24 h. Values represent the mean ± SE of TNF activity in triplicate cultures.
[b]Difference between the two groups was statistically significant ($P < 0.05$).

3.5. Cell cooperation in the TNF production by spleen cells

To identify the TNF-producing spleen cells, dishes containing 3 × 10^6 unfractionated spleen cells, spleen T and B cells, adherent cells were prepared by removing nonadherent cells after 2-h preincubation of 3 × 10^6 unfractionated spleen cells, and the corresponding adherent cells plus 3 × 10^6 spleen T or B cells were prepared. Then OK-432 (0.1 KE/ml) was added to each dish and TNF production was assessed. As shown in Fig. 3, TNF was not detected in cultures of T or B cells alone or cultures of a few adherent cells alone. However, cultures containing 3 × 10^6 unfractionated spleen cells and a few adherent cells plus 3 × 10^6 T or B cells produced TNF.

3.6. Age-related changes of TNF production by peritoneal macrophages and spleen cells

To investigate age-related changes in TNF production, groups of 6 mice aged 2 months old (young group), 12 months old (mature group), and 24 months old (old

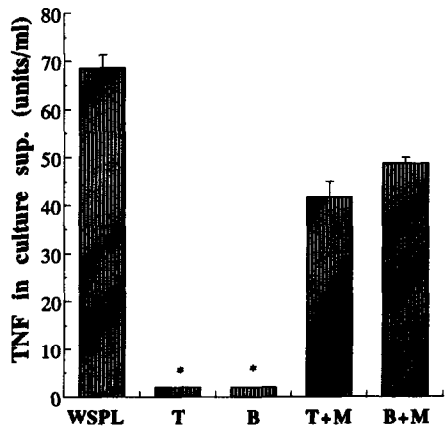

Fig. 3. TNF production by unfractionated (WSPL) and fractionated spleen cells. T cells (T), B cells (B), T cells + adherent spleen cells (T + M) and B cells + adherent spleen cells (B + M) were stimulated with 0.1 KE/ml of OK-432. *TNF activity was not detected (< 4 U/ml). Columns and bars indicate the mean value and standard error of triplicate cultures respectively. TNF activity was not detected in the culture of adherent cells alone.

Table 3
Age-associated changes of TNF production by peritoneal macrophages and spleen cells from C3H/He mice

Cells cultured	Sex	TNF activity in culture supernatant (units/ml)[a]		
		Young	Mature	Aged
Peritoneal macrophages	Male	16.9 ± 0.5	24.8 ± 2.5	37.0 ± 4.2*
	Female	13.3 ± 1.2	24.3 ± 3.6	29.1 ± 4.0*
Spleen cells	Male	42.8 ± 3.2	42.1 ± 12.8	50.5 ± 9.3
	Female	37.8 ± 4.5	38.5 ± 7.8	48.2 ± 12.3

[a]Values represent the mean ± SE of TNF activity in supernatants from 6 mice Young, mature, and aged mice were 2 12 and mid 24 months old, respectively. *$P < 0.05$ by one-way ANOVA.

group) were studied. F4/80-positive peritoneal macrophages (1×10^6) and spleen cells (1×10^7) obtained from each mouse were placed into culture dishes, and OK-432 (0.5 or 0.1 KE/ml) was added to each dish to observe TNF production. TNF production by peritoneal macrophages increased significantly with aging in both male and female mice (Table 3). However, TNF production by spleen cells only tended to increase with aging in both male and female mice, and no significant change was noted.

3.7. Age-related changes of mouse peritoneal cells

Male mice aged 2, 13, 17, and 29 months and females aged 2, 13 and 17 months were used to determine the proportions of T cells B cells, and macrophages as well as their absolute numbers in mouse peritoneal cells at each age. The age-related changes in the proportion of each cell type are presented in Fig. 4. In both male and female mice, the proportion of T and B cells increased with aging from 2 to 17 months, whilst the proportion of macrophages tended to decrease. In male mice aged 29 months no increase in the proportion of B cells or decrease of macrophages was observed as compared with 17 months old.

With regard to the absolute numbers of each type of peritoneal cells, the total number of cells and the number of T and B cell increased markedly from 2 months to 13 months of age in both male and female mice, and this trend subsequently became less marked (Fig. 5). In male mice aged 29 months, the number of T cells showed a slight increase, but the total number of peritoneal cells and that of B cells showed a decrease. The number of macrophages increased from 2 to 13 months in male mice, but no changes were noted at the other ages.

3.8. Age-related changes of spleen cells

Male mice aged 2, 13, 17, and 29 months and female mice aged 2 13, and 17 months were used to determine the proportions of T cells, B cells, and macrophages as well as their numbers in the spleen. Age-related changes in the proportion of the respective cells are presented in Fig. 6. In both male and female mice, the proportions of T and B cells tended to increase slightly from 2 to 17 months. In

male mice aged 29 months, the proportion of T cells still showed an increase, whilst that of B cell had decreased marginally. The proportion of macrophages showed no age-related changes.

With regard to age-related changes in the absolute number of cells, the total number of spleen cells, T cells, and B cells increased from 2 to 17 months in both male and female mice (Fig. 7). In male mice aged 29 months, the total number of spleen cells and B cells decreased compared with that at 17 months but the number of macrophages showed no age-related changes.

4. Discussion

In the present study, peritoneal cells and spleen cells were cultured in the presence of OK-432 at the optimal concentration to induce TNF production, since the optimal conentrations for each type of cells did not change with cell donor age at least up to the mature age in preliminary experiments. Non-adherent peritoneal cells alone or spleen T and B cells alone failed to produce TNF, but spleen T or B cells cultured with a few adherent cells yielded TNF as did cultures of adherent peritoneal cells alone. These results indicate that macrophages were principally responsible for TNF production and that both T and B cells were involved in the TNF production after stimulation with OK-432 in the presence of macrophages. It seems that the TNF activity detected in supernatants of the co-cultivation of a few spleen adherent cells and T (or B) cells was produced by T (or B) cells, because the adherent cells alone of the same dose as in the co-cultivation did not produce

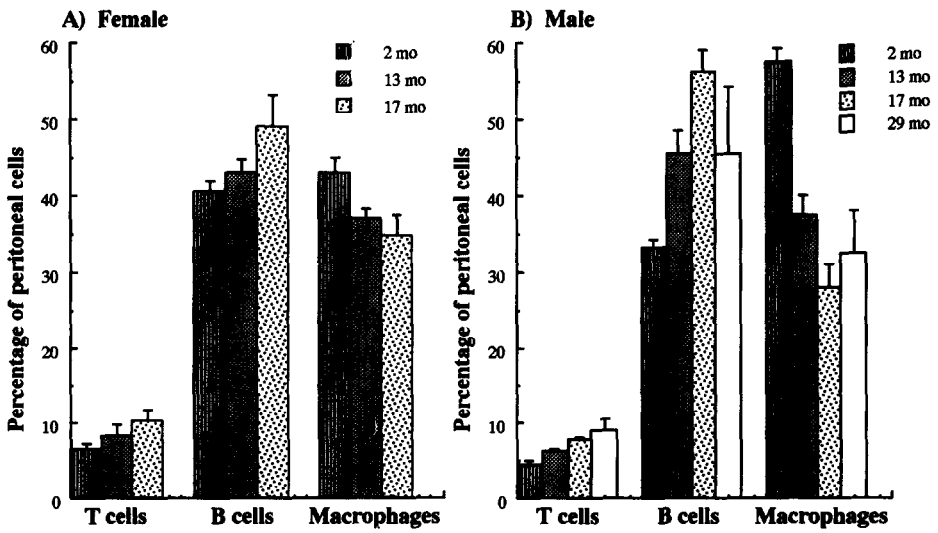

Fig. 4. Age-associated changes in the composition of peritoneal cells in male and female C3H/He mice. The percentages of T cells, B cells, and macrophages in the peritoneal cells of female and male C3H/He mice from three and four different age groups, respectively, were analyzed by flowcytometry. Columns and bars represent the mean value and stansard error for three mice.

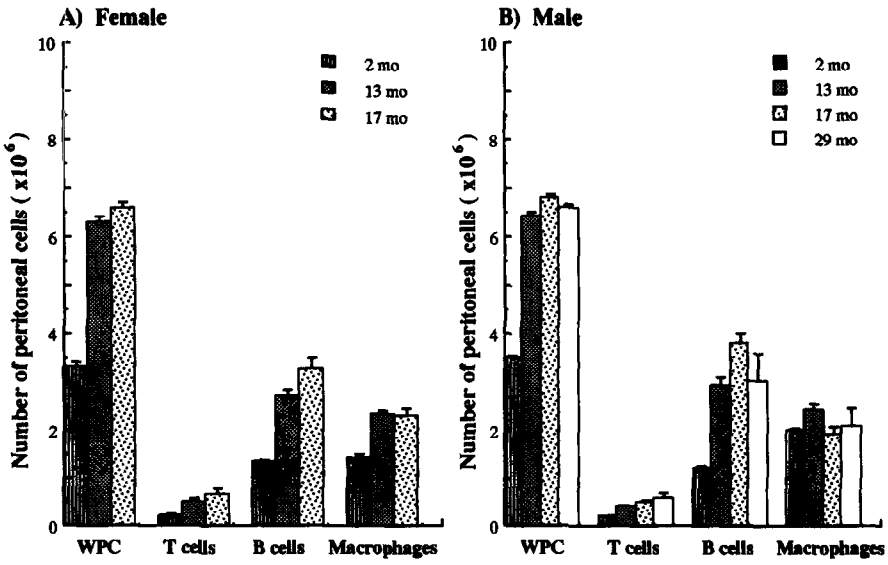

Fig. 5. Effect of aging on the recovery of unfractionated peritoneal cells (WPC), T cells, B cells, and macrophages from the peritoneal cavities of male and female C3H/He mice. The number of WPC, T cells, B cells, and macrophages obtained from the peritoneal cavity was counted by flowcytometry in three and four different age groups of female and male C3H/He mice respectively.

detectable amount of TNF. It is likely that, after stimulation with OK-432, macrophages produce not only TNF-α but other cytokines like interleukin-1 (IL-1) which may activate the antigen-stimulated B cells to enhance TNF-β production. Furthermore, macrophages may also process OK-432 antigens for presentation to antigen-specific T cells for activation to produce TNF-β.

However, it is equally possible that the presented OK-432 antigens may activate the antigen-specific T cells to produce cytokines like IL-1 and interferon-γ (IFN-γ) as well as TNF-β, which reversely enhance the TNF production by the macrophage. In our present results, TNF production by co-culture of T or B cells with macrophages was lower than that by unfractionated spleen cells. This suggested that T cells, B cells, and macrophages cooperatively form the most effective cytokine network for promoting TNF production.

The optimal OK-432 concentrations were different between the cultures of peritoneal macrophages and of spleen cells. That for peritoneal macrophages was about 5 times higher than for spleen cells. The reason why the optimal concentrations were different between the two cultures is not clear. Yet, major differences between the two cultures was the proportion of macrophage incubated. More than 90% of the peritoneal adherent cells cultured were macrophage, while only a few percent of the spleen cells were. Thus, the following possibilities are conceivable: (1) a much higher density of macrophages in the peritoneal cell culture might produce some inhibitory factor for TNF production such as prostaglandin E2 [26, 27], IL-6 [28], and IL-10 [29 30], or (2) some enhancing factors like IFN-γ and IL-1 might be

produced in the spleen cell culture, amplifing the magnitude of TNF producing response as discussed above.

When 2-h pre-incubation was performed before the addition of OK-432 to allow adherence of macrophages, TNF production by peritoneal cells and spleen cells became higher. In contrast when unfractionated peritoneal cells did not undergo preincubation, TNF was not detected at any density from 1×10^5 to 15×10^5 cells/dish (data not shown). However, when preincubation was performed, TNF was detected at densities of 5×10^5 to 15×10^5 cells/dish. These results suggest that adherence of macrophages stimulates the first step of activation that leads to a higher response to OK-432 stimulation.

In a previous study [22], we observed that *in vivo* TNF production stimulated by OK-432 increased with aging. There are two possible mechanisms explaining enhanced TNF production with aging: (1) the production per cell increases or (2) the absolute number of TNF-producing cells increases. The major TNF-producing cell is the macrophage, so resident peritoneal macrophages were investigated in the present study. It was found that TNF production per cell increased with increasing age of the donor, suggesting that mechanism (1) is more likely. In addition, our present results indicate that the number of peritoneal cells or spleen T and B cells increased with age at least up to 18 months after birth, suggesting that the increase of T and B cells in addition to macrophages may contribute partly to the age-related increase in TNF production at the systemic level.

Davila et al. [31] and Bradley et al. [32] collected peritoneal macrophages from young and old rats, after which they stimulated the cells with IFN-γ and LPS or

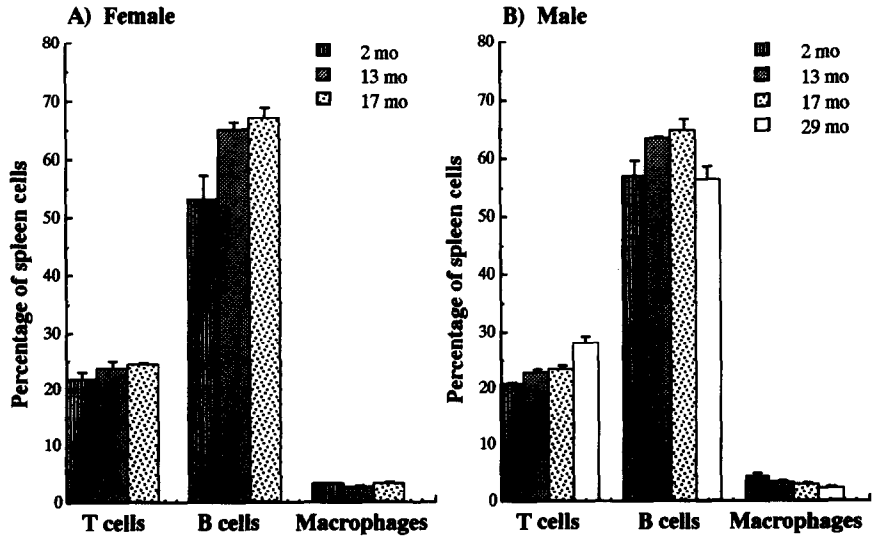

Fig. 6. Age-associated changes in the composition of spleen cells in male and female C3H/He mice. The percentages of T cells, B cells, and macrophages in spleens of female and male C3H/He mice from three and four different age groups respectively, were analyzed by flowcytometry. Columns and bars represent the mean value and standard error for three mice.

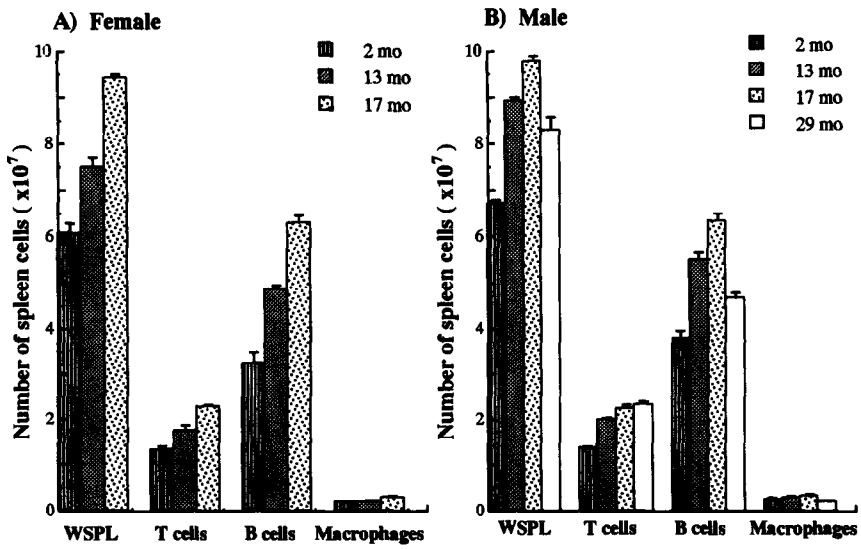

Fig. 7. Effect of aging on the recovery of unfractionated spleen cells (WSPL), T cells, B cells, and macrophages in the spleen of female and male C3H/He mice. The number of WSPL, T cells, B cells, and macrophages in the spleen was counted by flowcytometry in three and four different age groups of female and male C3H/He mice, respectively.

Staphylococcus spp. and found that TNF production by macrophages from old rats was significantly lower. These results do not support the above mechanism (1) and are inconsistent with our findings. With respect to other functions of macrophages, monocytes from aged human donors and macrophages from aged mice were reported recently to show a decrease in the production of reactive oxygen intermediates and IL-1 [33, 34]. However, many other studies indicated that various functions of macrophages do not necessarily deteriorate with aging [35–38]. Furthermore, Foster et al.[39] reported that macrophage TNF production increased with aging, and these results are in agreement with ours. Therefore, the conclusions of the above two reports on the aging in TNF production by macrophages are still open to discussion. On the other hand, our results suggested that TNF production by spleen cells also tended to increase with age. Hobbs et al. [40, 41] compared $CD4^+$ T cells between young and old mice and indicated that TNF production did not decrease with aging and that synthesis of TNF-α and TNF-β mRNA tended to increase in old animals. Aroeira et al. [42] compared responses of young and old mice to an *in vivo* immunization with a super antigen, staphylococcal enterotoxin B and observed that old mice produced more TNF-α than young mice. These results suggest that TNF production by TNF-producing cells, such as macrophages, generally increase with aging.

Since the host becomes more sensitive to infection with aging, it is likely that macrophages are more readily activated in the elderly. In the present study, OK-432 was used to induce TNF production in cells from mice housed in a conventional

environment. Therefore, there is the possibility that the mice acquired more potent immunity against *Streptococcus spp.* with aging, leading to an increase of activated macrophages and OK-432-specific T and B cells. This may help to explain the increase of TNF production by immunocompetent cells, especially macrophages, in the old mice.

Immune reactions involve antibody production and inflammation related to cellular immunity. These reactions are regulated by T helper cells (Th), including Th1 and Th2 cells. It is well known that cytokines secreted by these Th cells enhance various reactions, and at the same time suppress other reactions [43]. For example, IFN-γ suppresses the proliferation of Th2 cells and IL-10 inhibits the secretion of cytokines by Th1 cells [44]. IL-4, IL-10, and IL-13 secreted by Th2 cells all inhibit cytokine secretion by macrophages [45, 46]. The inflammatory response regulated by Th1 cells and the antibody production system regulated by Th2 cells have a suppressive effect on each other. Our present results suggested that antibody production may be predominant in young mice and this system may suppress the production of inflammatory cytokines by macrophages. With increasing age, however, the immune system may gradually shift from the predominance of antibody production to predominance of the inflammatory system.

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