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Dietary folate improves age-related decreases in lymphocyte function☆

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Abstract

Although low folate status is thought to be fairly common in the older population, its implication on immunity has not been adequately investigated. Using 11-month-old and 23-month-old male rats (Fisher 344), the present study was undertaken to examine the modifying effects of feeding a control diet (NIH-07) supplemented with folate (35.7 mg/kg) for 3 weeks on the immune cells of spleen and mesenteric lymph node (MLN) origin. The serum concentrations of folate along with vitamin B_{12} were elevated in response to the folate supplementation ($P<.05$). These results were accompanied by an improved proliferative response (stimulation index) to mitogens in both the spleen and MLNs ($P<.05$). The proportion of T cells in the MLNs, but not in the spleen, was significantly increased in rats fed a diet supplemented with folate. In the spleen, the folate-supplemented diet prevented the age-associated decrease ($P<.05$) in the production of interferon (IFN)α by unstimulated cells and the decrease in T-helper (Th)1/Th2-type response after stimulation with phorbol myristate acetate and ionomycin. In the MLNs, on the other hand, the folate-supplemented diet failed to influence any age-related increase in interleukin (IL)-2, tumor necrosis factor α and IFNγ following stimulation but did result in a significantly increased production of IL-4 ($P<.05$). Overall, this study provides data suggesting that aging is associated with changes in the proportion of T cells, the ability of immune cells to proliferate and the production of cytokines after stimulation. Supplementing a folate-sufficient diet with additional folate improves proliferative response to mitogens, the distribution of T cells in the MLNs and the age-related changes in cytokine production in the spleen. These results suggest that the dietary folate requirement may be higher in the older population than in the younger population to support immune functions.

Keywords: Cytokines; Proliferation; Mitogens; Elderly; Rodents; Vitamin B_{12}; Folate

1. Introduction

A variety of immune changes occur in both animals and humans with increasing age. The aging of the immune system (immunosenescence) is associated with dramatic reductions in immune responsiveness as well as functional dysregulation [1]. This translates into less effective innate and adaptive immune responses, increased reactivity against self-antigens (autoimmunity) and increased incidences of infectious diseases and cancer [1,2]. The most dramatic change with age is reported to occur within the T-cell compartment where there is a declined function of both $CD4^+$ [2,3] and $CD8^+$ [4] cells. This decline in T-cell function is preceded by involution of the thymus gland and dramatic declines in thymic hormone levels [5]. Although changes occurring in the CD4/CD8 ratio are somewhat variable in the literature, a shift toward a memory cell population, as compared with a naive cell population, is a widely observed aging phenomenon in both humans and experimental animals [6]. This shift in the proportion of memory T cells is hypothesized to contribute to inappropriate responses to either new or previously encountered infectious, autoplastic or neoplastic antigens. While it is unequivocally accepted that T-cell activity is modulated due to aging, there are controversies regarding the nature of some of the immunological parameters studied (reviewed in Refs. [2,7]). B-cell function also undergoes considerable changes with age and these may be independent of those in T cells [7].

Innate immunity, the first line of defense that precedes the antigen-specific T-cell and B-cell responses, also changes with advancing age. The age-associated changes in the innate immune system include those of macrophages [8,9], neutrophils [9], dendritic cells [10], natural killer cells

Abbreviations: Con A, concanavalin A; IFN, interferon; IL, interleukin; Iono, ionomycin; MLN, mesenteric lymph node; PMA, phorbol myristate acetate; PHA, phytohemagglutinin; SI, stimulation index; Th, T-helper; TGFβ, transforming growth factor β; TNF, tumor necrosis factor.

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[9] and complement [9]. The mechanisms responsible for the decline of immune potential in senescence have not been fully elucidated but likely involve a complex relationship between cell types and environmental (i.e., suboptimal nutrition) and behavioral factors [1]. Nutrition plays a crucial role in immune function [11]. Most studies on age-associated changes in immunocompetence in healthy adults have not examined the nutritional status of participants. Inadequate nutritional status may confound the relationship of aging and immune response as older adults are at risk for malnutrition [12]. It is therefore quite possible that some of the reported age-related immune changes in the elderly population may be secondary to deficiencies of macronutrients and micronutrients.

One of the micronutrients that the elderly population is at risk for is folate [12]. Although the role of folate in the immune system has not been fully characterized, folate is required for one-carbon transfer reactions and the formation of purines and pyrimidines for DNA and RNA syntheses and would therefore be essential to immune function. Indeed, deficiency of folate can lead to many clinical abnormalities including reduced immune function [13]. More specifically, folate deficiency is associated with reduced T-cell proliferation, increased apoptosis, a marked decrease in $CD8^+$ cells and an increase in the CD4/CD8 ratio [14]. These are similar immune changes reported to occur in the aging population. Providing folate to deficient animals restores the function of CD8 cells [14]. Although, clearly, folate deficiency is associated with immune abnormalities, it is not clear if a milder level of folate insufficiency, more likely to occur in the elderly population, would alter immune function. The purpose of this study was to examine in a rodent model the impact of supplementing a folate-sufficient diet with additional folate on age-related changes in immune function.

2. Materials and methods

2.1. Animals and diet

Male Fisher 344 rats ($N=22$) aged 11 and 23 months were obtained from the National Institute of Aging colony (Harlan Sprague–Dawley, Indianapolis, IN, USA). The animals were housed and treated in accordance to Canadian Council on Animal Care regulations and the protocol was reviewed and approved by the Committee of Animal Policy and Welfare of the Faculty of Agriculture, Forestry and Home Economics. The animals were fed ad libitum for 1 week the nutritionally adequate maintenance NIH-07 diet (Zeigler Bros., Gardeners, PA, USA) for them to acclimatize to the facility. The 11-month-old group of animals ($n=6$) continued on the NIH-07 control diet while the 23-month-old group was randomized to continue consuming the NIH-07 diet ($n=8$) or was fed an NIH-07 diet ($n=8$) supplemented with additional folic acid (35.7 mg/kg, Sigma, St. Louis, MO, USA) for 3 weeks. This level of folate represented 10 times the amount present in the basal diet. Food intake and body weight were recorded on alternate days, all animals were killed over a 3-day period under 3–5% halothane by cardiac puncture and blood and spleen were collected for further analysis.

2.2. Measurement of vitamin B_{12} and folic acid status

Vitamin B_{12} and folate were measured in the plasma by a commercially available competitive binding radioimmunoassay (Solid Phase No Boil Dualcount, Diagnostic Products, Los Angeles, CA, USA) and the concentrations were calculated by measuring the precipitate in a dual-channel gamma counter (Packard 500C AutoGamma Counter, Packard Instrument, Meridine, CT, USA) against standard curves for folate and B_{12}.

2.3. Isolation of immune cells and estimation of proliferation

Immune cells were isolated from the spleen and mesenteric lymph nodes (MLNs) as previously described [15]. Briefly, using aseptic technique and supplies, tissues were pushed through a nylon mesh screen (100 μm) using a KRH buffer supplemented with 5 g/L of BSA (Sigma, Oakville, ON, USA) to separate connective tissue and fat from immune cells and red blood cells. Cell suspensions were centrifuged $250\times g$ (J2-HC floor model, Beckman, Mississauga, ON, USA) and treated with lysis buffer (155 mM NH_4Cl, 0.1 mM EDTA, 10 mM $KHCO_3$, Fisher Scientific, Edmonton, AB, Canada) to remove excess red blood cells. Cells were then washed and resuspended in complete culture media (RPMI 1640 media supplemented with 5% vol/vol heat-inactivated fetal calf serum, 25 mM HEPES, 2.5 mM 2-mercaptoethanol and 1% antibiotic/antimycotic, pH 7.4, Gibco Life Technologies, Burlington, ON, USA). Cells were counted using trypan blue dye (Sigma) exclusion using a hemocytometer and diluted to a final concentration of 1.25×10^6 cells/ml for the proliferation and cytokine assays.

2.4. Phenotype determination

Isolated splenocytes (200,000 cells/well) were used to determine cell phenotype using 2-color indirect immunofluorescence as previously described [15]. The following antibodies were used in combination/singly: W3/13 (pan T cells, Cedarlane, Hornby, ON, USA) with W3/25 [$CD4^+$ T-helper (Th) cells] or OX8 ($CD8^+$ T-cytotoxic/suppressor cells), CD19 ($CD5^+$ T cells) and OX12 (B cells) and OX42 (monocytes) (unless otherwise indicated, these were a gift from Dr. A. Rabinovitch, University of Alberta). Anti-mouse IgG fluorescein isothiocyanate conjugate and phycoerythrocen (Cedarlane, Hornby, ON, USA) were used as secondary antibodies. The percentage of lymphocytes expressing each of the antibody markers was determined by flow cytometry (FacScan, Becton Dickinson, Sunnyvale, CA, USA) as previously described [15].

2.5. Estimation of mitogen proliferation

Cells (1.25×10^6 cells/ml) were incubated in a 96-well microtiter plate, in triplicate, in the presence or absence of mitogens [20 ng/L of phorbol myristate acetate (PMA)+ 0.5 nmol/L of ionomycin (Iono), 5 μg/ml of concanavalin A (Con A), 25 μg/ml of phytohemagglutinin (PHA), all supplied by ICN, Montreal, PQ, Canada] for 48 and 72 h. Eighteen hours prior to harvesting, cells were pulsed with 0.5 μCi of ^{3}H-thymidine (Amersham Life Sciences, Baie D'Urfe, PQ, Canada), harvested on glass-fiber paper filters using a multi-well harvester (Skatron, Lier, Norway) and counted in a beta counter (LS-5801 Beckman Canada, Mississauga, ON, USA). The ability to proliferate was defined for each mitogen as a stimulation index (SI), calculated as: the rate of ^{3}H-thymidine incorporated after incubation with mitogen/the rate of ^{3}H-thymidine incorporated by the cells incubated in the absence of mitogens.

2.6. Cytokine determination

Splenocytes and MLNs (1.25×10^6 cells/ml) were incubated (48 h) in the presence or absence of PMA (20 ng/L)+ Iono (0.5 nmol/L). Supernatants were removed and stored at −70°C until all the samples were collected. The concentrations of interleukin (IL)-4, IL-6 and transforming growth factor β (TGFβ) were determined using OptEIA ELISA kits (BD Bioscience, Mississauga, ON, USA) according to kit specifications using a standard curve (7.8–500 pg/ml). The concentrations of IL-2, tumor necrosis factor (TNF)α and interferon (IFN)γ were determined using matched paired sandwich ELISA analysis (Pharmingen, Mississauga, ON, USA) using biotinylated anti-rat secondary, horseradish peroxidase avidin D enzyme and ABTS substrate (all from Vector Laboratories, Burlington, ON, USA). For these cytokines, when samples fell outside the standard curve (30–2000 pg/ml), they were diluted with 10% vol/vol fetal calf serum and reanalyzed. All the samples were performed in duplicate and only those with a CV value <10% were included in statistical analysis. All the plates were read at 450 nm in a microplate reader (SpectraMax 190, Molecular Device, Sunnyvale, CA, USA).

2.7. Statistical analysis

Statistical analysis was preformed using SAS (version 8.0, SAS Institute, Cary, NC, USA). Differences among groups were tested using one-way or two-way ANOVA on the effect of diet and/or age. For all measures, a probability <.05 was accepted as being statistically significant. Results are presented as means±S.E.M.

3. Results

3.1. Body weight, food intake and plasma folate and vitamin B_{12} levels

Two rats in the 24-month-old control group (final, $n=6$) and one rat in the folate-supplemented group (final, $n=7$) died prior to the completion of the study. The control 12-month-old rats weighed more and consumed more food than did the 24-month-old rats (Table 1). Folate supplementation had no effect on the body weight or food intake of the 24-month-old rats. There was a significant increase in spleen weight with age; however, the total number of cells isolated from the spleen or MLNs did not differ among groups (Table 1). There was no difference in plasma folate or vitamin B_{12} concentrations between the 12-month-old and 24-month-old rats fed the control diet. However, supplementation with folate significantly increased serum folate levels as compared with both groups fed the control diet and significantly increased vitamin B_{12} levels compared with the 12-month-old rats (Table 1).

3.2. Phenotypes of immune cells from the spleen and MLNs

3.2.1. Spleen

In animals fed the control diet, there was a significant decrease in the proportion of $CD5^+$ ($OX19^+$) cells with age and a significant increase in the proportion of macrophages/monocytes ($OX42^+$) (Table 2). Supplementing the 24-month-old rats with folate did not significantly affect any of the measured phenotypes in the spleen.

3.2.2. MLNs

In 24-month-old animals fed the control diet, there was a significantly lower proportion of all T-cell populations ($CD5^+$, $W3/13^+$, $W3/13^+CD4^+$, $W3/13^+CD8^+$) and a significantly higher CD4/CD8 ratio compared with the 12-month-old rats. Twenty-four-month-old rats fed additional folate resulted in a proportion of T cells ($CD5^+$, $W3/13^+CD8^+$)and a CD4/CD8 ratio that did not differ from those of the 12-month-old rats (Table 2). Despite these apparent changes, none of the phenotype proportions measured in the folate-supplemented rats differed significantly from those of the 24-month-old control-fed rats. Additional dietary folate did not prevent the age decrease in the proportion of $CD4^+$ cells (Table 2).

Table 1
Effect of diet and age on body weight, spleen weight and food intake

Group	12-Month-old control group ($n=6$)	24-Month-old control group ($n=6$)	24-Month-old folate group ($n=7$)
Initial body weight (g)	425±12	459±12	457±12
Weight change (g)	11 ± 3^a	-4 ± 3^b	-3 ± 3^b
Food intake (g/day)	21 ± 1^a	18 ± 1^b	18 ± 1^b
Spleen weight (g)	0.87 ± 0.03^a	1.44 ± 0.16^b	1.27 ± 1.05^b
Total spleen cells ($\times10^6$)	189±21	191±31	201±19
Spleen cells/ml ($\times10^6$)/ spleen weight (g)	22 ± 2^a	13 ± 1^b	17 ± 2^b
Total MLNs ($\times10^6$)	88±11	71±7	68±6
Serum folate (nmol/L)	242 ± 20^a	190 ± 20^a	348 ± 20^b
Serum vitamin B_{12} (pmol/L)	861 ± 79^a	1094 ± 79^{ab}	1264 ± 79^b

Values are expressed as mean±S.E.M. Across a row, mean values that do not share a common superscript letter are significantly different ($P<.05$).

Table 2
Effect of diet and age on phenotypes in the spleen and MLNs

Experimental group	OX19 ($CD5^+$)	W3/13$^+$ (pan T cells)	W3/13$^+$W3/25$^+$ (T cells+$CD4^+$)	W3/13$^+$OX8 (T cells+$CD8^+$)	CD4/CD8	OX12 (B cells)	OX42 (macrophages)
	Percentage of total cells (%)						
Spleen							
12-month-old control group ($n=5$)	51±2^a	35±6	27±1	17±1	1.6±0.1	35±3	10±1^a
24-month-old control group ($n=6$)	38±2^b	44±4	32±4	18±3	1.9±0.3	42±1	24±3^b
24-month-old folate group ($n=7$)	41±2^b	44±4	33±3	25±2	1.4±0.2	40±2	24±2^b
MLNs							
12-month-old control group ($n=5$)	54±1^a	49±2^a	42±2^a	16±1^a	2.0±0.1^a	34±2	11±1
24-month-old control group ($n=6$)	47±3^b	41±2^b	35±2^b	11±1^b	2.9±0.2^b	39±2	9±1
24-month-old folate group ($n=7$)	49±3^{ab}	42±3^b	35±1^b	14±1^{ab}	2.5±0.3^{ab}	39±2	8±1

Values are expressed as mean±S.E.M. For the spleen or MLNs, down a column, mean values that do not share a common superscript letter are significantly different ($P<.05$).

3.3. Mitogen responses by splenocytes

3.3.1. Estimation of proliferation (SI)

There was a significantly lower response to PMA+Iono (72 h), Con A [48 h (Table 3) and 72 h (Fig. 1)] and PHA (72 h) by cells from the 24-month-old rats compared with that by cells from the 12-month-old rats fed the control diet. The response by cells from the folate-supplemented rats did not differ from that by cells from the 12-month-old rats for PMA+Iono (72 h), Con A (48 h) and PHA (72 h) and was significantly higher than the response by cells from the 24-month-old control-fed rats for PHA (48 h) (Table 3, Fig. 1). The response by cells from the folate-fed group was significantly different from that by cells from either control group for PMA+Iono (48 h) and Con A (72 h).

3.3.2. Cytokine production (after 48 h of stimulation with PMA+Iono)

There was no difference in the amount of cytokines produced after stimulation between the control-fed groups except for a decrease in the TNFα/IL-4 ratio in the 24-month-old rats compared with the 12-month-old rats (Table 4). There was also a significantly lower production of IFNγ by unstimulated cells from the 24-month-old rats

Table 3
Effect of diet and age on unstimulated and mitogen responses in the spleen and MLNs

	Unstimulated 48 h (dpm)	PMA+Iono 48 h (SI)	Con A 48 h (SI)	PHA 48 h (SI)
Splenocytes				
12-month-old control group ($n=5$)	1906±361	19±1^a	36±7^a	1.5±0.4^a
24-month-old control group ($n=6$)	3199±940	14±3^a	12±3^b	1.7±0.4^a
24-month-old folate group ($n=7$)	2524±826	47±6^b	34±1^a	3.9±1.0^b
MLNs				
12-month-old control group ($n=5$)	965±261^a	95±17^a	49±6^a	19±2
24-month-old control group ($n=6$)	1411±355^{ab}	41±10^b	30±6^b	14±1
24-month-old folate group ($n=7$)	2339±424^b	66±9^{ab}	38±4^{ab}	18±2

Values are expressed as mean±S.E.M. For the spleen or MLNs, down a column, mean values that do not share a common superscript letter are significantly different ($P<.05$).

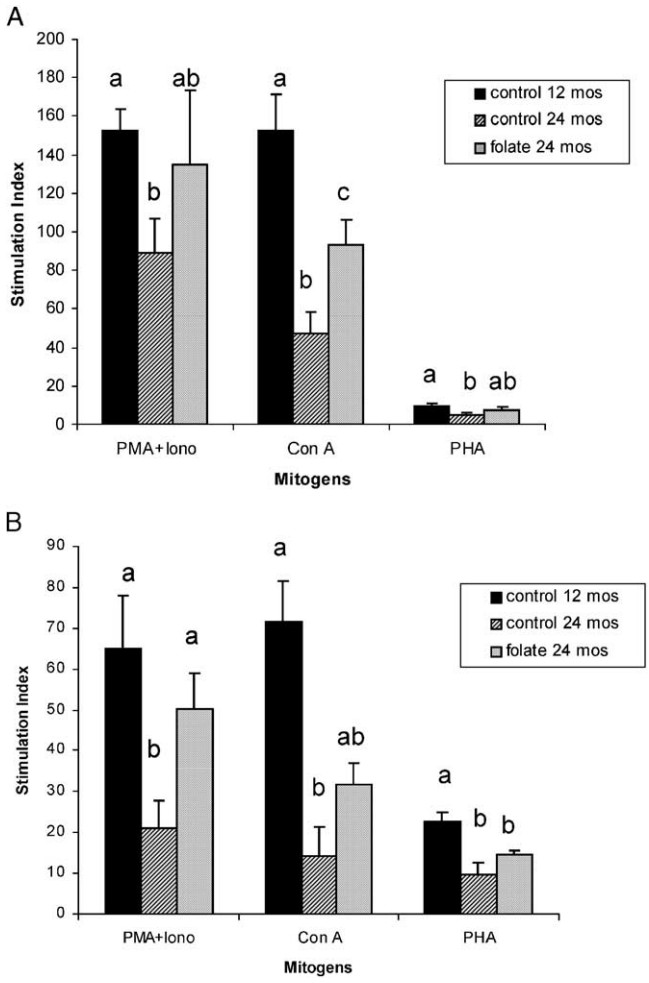

Fig. 1. The effect of age and diet on the response to mitogens in the spleen and MLNs after 72 h. Bars represent the mean±S.E.M. for cells (A) isolated from the spleen and those (B) isolated from the MLNs, for 12-month-old rats fed the control diet ($n=5$), 24-month-old rats fed the control diet ($n=6$) and 24-month-old rats fed the folate diet ($n=7$). For each mitogen, bars that do not share a common letter are significantly different ($P<.05$).

Table 4
Cytokine production unstimulated or after incubation for 48 h with PMA+Iono

	Experimental groups		
	12-month-old control	24-month-old control	24-month-old folate
Spleen PMA+Iono stimulated (pg/ml)			
IL-2	2048±119	2039±161	2219±101
TNFα	1054±41	996±94	1180±61
IFNγ	754±76^{ab}	689±85^{a}	1007±119^{b}
IL-4	72±12	131±43	130±17
IL-6	948±157	715±60	870±86
IL-10	513±76	456±58	687±114
TGFβ	193±13	214±55	208±60
TNFα/IL-4 (Th1/Th2)	16±2.3^{a}	6.6±0.9^{b}	9.5±1.0^{ab}
Unstimulated spleen cells (pg/ml)			
IL-6	1885±125	2064±103	1956±146
TNFα	66±8^{ab}	91±3^{a}	80±8^{b}
IFNγ	327±32^{a}	51±7^{b}	349±48^{a}
MLN PMA+Iono stimulated			
IL-2	763±40^{a}	1614±110^{b}	1637±77^{b}
TNFα	229±19^{a}	527±26^{b}	529±33^{b}
IFNγ	534±51^{a}	1211±170^{b}	1272±142^{b}
IL-4	108±33^{a}	337±100^{a}	498±107^{b}
IL-6	958±176	731±224	852±230
IL-10	199±75	302±58	319±54
TGFβ	243±41	240±99	171±39
TNFα/IL-4 (Th1/Th2)	3.3±1.2^{a}	2.7±0.9^{ab}	1.4±0.3^{b}
Unstimulated MLN cells			
IL-6	1116±28	1112±26	1132±22

Values are expressed as mean±S.E.M. Within a row, mean values that do not share a common superscript letter are significantly different ($P<.05$).

compared with the 12-month-old rats fed the control diet (Table 4). The lower unstimulated production of IFNγ in the 24-month-old rats was not observed in cells from the folate-fed rats and these cells produced a significantly greater amount of TNFα when unstimulated and higher IFNγ when stimulated compared with cells from the 24-month-old control-fed rats (Table 4). None of the cytokines measured in either the stimulated or the unstimulated state differed between the 12-month-old control-fed group and the 24-month-old folate supplement group.

3.4. Mitogen responses by MLNs

3.4.1. Estimation of proliferation (SI)

There was a significantly lower response to PMA+Iono (48 and 72 h), Con A (48 and 72 h) and PHA (72 h) by cells from the 24-month-old rats compared with that by cells from the 12-month-old rats fed the control diet (Table 3; Fig. 1). The response by cells from the folate-supplemented rats did not differ from that by cells from the 12-month-old rats for PMA+Iono (48 and 72 h), Con A (48 h) and PHA (48 h) and the response to PMA+Iono (72 h) was significantly higher than that by cells from the 24-month-old control-fed rats (Table 3; Fig. 1). Supplementation with folate did not appear to increase the low PHA and Con A responses at 72 h that occurred in the 24-month-old rats. The unstimulated response by cells from folate-fed rats at 48 h was significantly higher than that by cells from the 12-month-old control rats.

3.4.2. Cytokine production (after 48 h of stimulation with PMA+Iono)

Despite a lower proliferative response to PMA+Iono by cells from the 24-month-old control-fed rats compared with cells from the 12-month-old rats, there was a higher production of IL-2, TNFα and IFNγ (Table 4). Feeding folate did not change the higher production of these three cytokines observed with age (Table 4). However, cells from the folate-fed group produced significantly more IL-4 than did the cells from either of the control-fed groups (Table 4). The ratio of TNF/IL-4 was lower for cells from the folate-supplemented rats compared with the 12-month-old control rats (Table 4).

4. Discussion

The male Fisher 344 rat is a well-studied model of aging [16–18] but has not been used to study the effects of aging on folate status and immune function. The median life span of Fisher 344 rats [18] fed ad libitum is 28–29 months; thus, at 24 months, the rats are close to the end of their natural life span. Consistent with previous studies, the rats, regardless of diet, lost weight during their last 12 months of life. The age-associated decline in serum folate is in accordance with that shown in other studies [19] and consistent with that reported in elderly humans [20]. Supplementation with folate significantly improved plasma folate levels as compared with both the 12-month-old and 24-month-old rats fed the control diet. This suggests that the requirement for folate increases considerably with age and that the older population may benefit from folate supplementation, without detrimental effects on B_{12} status. Vitamin B_{12} concentrations did not decrease with age and folate-supplemented rats had a higher vitamin B_{12} concentration in serum.

4.1. Reduced mitogen response in the spleen and MLNs

The proliferative response in mixed cell cultures is frequently used to estimate adaptive immunity. Adaptive immunity relies on three types of lymphocytes: B cells, cytotoxic T cells ($CD8^+$) and helper T cells ($CD4^+$). In the spleen and MLNs, there was a lower proliferative response (defined by the SI) to most mitogens at 48 h and to all three mitogens at 72 h (Fig. 1). This age-associated decline in T-cell responsiveness, irrespective of the stimulating agent used, is a well-documented phenomenon [6,7]. Feeding elderly rats additional folate resulted in an improved response to mitogens, both by cells from the spleen and those from the MLNs. Undernutrition is common in the aged population, and a lower serum folate level has been correlated with decreased proliferation of T cells from elderly subjects [21]. A higher response to mitogens in vitro has been demonstrated to be associated with reduced

infections [22] and a better response to vaccine [3], suggesting a physiological effect of this observation.

Differences were observed between groups in the types of cells present in the spleen and MLNs. In the present study, there was a lower proportion of T cells in the spleen and MLNs of the older rats. As to when this specific decline started is not clear as only two time points were measured in the present study; however, it was previously reported in mice that the proliferative decline is only apparent during the last quarter of the median life span (age, >20 months; [2]). A decrease in the number, proportion and function of T cells with age has been postulated to be the result of involution of the thymus, the organ where T cells differentiate and mature [7,23,24]. This results in fewer new T cells being produced and exported to secondary lymphoid organs and has consequences for the maintenance of the naive T repertoire (the cells able to respond to new antigens) with age [7]. Feeding folate did not alter the proportion of T cells in the spleen (a mixture of immune cells that closely represents peripheral blood), which is consistent with a study comparing peripheral blood phenotypes from young with those from old well-nourished adults [25]. Therefore, changes in mitogen responses between the 24-month-old rats were not due to the proportion of T cells and B cells present in the assay. Although not assessed in the current study, multiple steps of the T-cell signal transduction cascade have been reported to be defective in aged animals [6,7] and it is possible that an improvement in folate status may have influenced this. Additionally, there is a growing body of evidence indicating that age-associated defects in non-T cells contribute to the aging of the immune response [1]. Monocytes, macrophages and NK cells are present in spleen isolations (and peripheral blood) and are stimulated by the mitogens used, and their secretory products such as IL-1, IL-6 and PGE2 may have up-regulatory or down-regulatory roles in T-cell activation [6].

In MLNs (primarily T-cell tissues), although aging was associated with a lower proportion of all T-cell populations, the effect was greatest on the $CD8^+$ population, as illustrated by the increase in the CD4/CD8 ratio. A decline in T-cell suppressor function has been reported to be one of the first immunological lesions of aging in rodents [7]. $CD4^+$ and $CD8^+$ T cells are known to contribute differently to the host immune defense and any alteration in their ratio can influence a host's immune response. For example, the inability of $CD8^+$ cells to respond in the elderly has been demonstrated to increase the lethal effects of severe staphylococcal infections and viral infections [7,26]. In this tissue, feeding folate resulted in a proportion of $CD8^+$ T cells and a CD4/CD8 ratio that were not different from those of the 12-month-old rats, and this may have contributed to the improved mitogen response. Consistent with our findings, folate repletion of folate-deficient cells was reported to increase in the proportion of $CD8^+$ cells and the capacity of these cells to proliferate in response to activation [14].

4.2. Altered cytokine production

Cytokines are a class of substances that are produced by cells of the immune system (in addition to other tissues) that affect the immune response. The production and interaction of cytokines produced by immune cells are very complex, but the timing and relative signal strength of these cytokines are crucial to the overall immune response. The cell types present in the spleen and MLNs are very different, resulting in differences in cytokine production, and will therefore be discussed separately. Most researchers have studied peripheral blood and spleen and not T-cell tissues such as MLNs.

4.2.1. Spleen

Unlike most previous studies, cytokine production in the current study was measured after stimulation with PMA+Iono. Age had few effects on cytokine production in the spleen despite a significantly lower mitogen response. Age-related changes in T-cell cytokines are inconsistent in the literature, which may relate not only to differences between species (mouse studies differ markedly from human studies) but also to the nature of the stimulating agent used to assess production. As an example in old adults, surface stimulation by Con A was found to result in a lower production of IFN and IL-4 [27]. However, cytokine production after stimulation with PMA+Iono, the mitogens used in the present study (which bypass surface receptor signaling), did not differ between young and old subjects [27]. Additionally, the nutritional status of the rats in the current study may have contributed to the normal cytokine production as it has been reported that the impact of age on the ability to produce cytokines is much less in a healthy well-nourished population [5].

Most investigators have reported that a shift from a Th1 response (IFNγ, TNFα, IL-2) toward a Th2 response (IL-4, IL-5, IL-6, IL-10 and IL-13) occurs with age [6,24]. Experimentally, the ratio of TNFγ to IL-4 is commonly used to examine this balance. In the present study, we observed a shift toward a Th2 response in the spleen with age and this was significantly improved (albeit only partially correctly) by feeding additional folate. It has been suggested that a shift toward a Th2 response is associated with improved humoral immunity and a better response to vaccines [6].

There were a few unexpected effects of age and folate supplementation on the production of cytokines by splenocytes cultured in the absence of mitogens (unstimulated). The lower production of IFNγ with age was prevented by feeding additional folate, and this suggests an effect on macrophage function. Inhibition of IFN can contribute to a decline in the tumoricidal property of macrophages as well as impair the cytotoxic activity of $CD8^+$ cells directed against virally infected cells [6]. We also observed a higher production of TNFα by the unstimulated cells with folate supplementation compared with the control-fed 24-month-old rats, the implications of this remain to be determined.

4.2.2. MLNs

T-cell division requires the synthesis of IL-2 as well as IL-2 receptor function. A lower production of IL-2 by immune cells from both older individuals and rodents after stimulation with primarily T-cell mitogens (i.e., Con A and PHA) has been reported in the literature [6]. Others using non-T-cell mitogens have not found a decrease with age in IL-2 production [28,29]. In the present study, aging was associated with a twofold increase in IL-2 production after stimulation. PMA+Iono are mitogens that stimulate all cell types via activation of protein kinase C and an increase in intracellular calcium. Despite producing more IL-2, the older rats (at least fed the control diet) did not proliferate as well, suggesting an effect on the function of IL-2 in T cells. Impaired expression of the IL-2R and defective upstream postreceptor signaling (phosphorylation of mitogen-activated protein kinases) have been described in cells from aged animals [5,7].

There exist somewhat inconsistent reports with regard to the effect of age on cytokine production (IL-4, TNFα and IFNγ) after mitogen stimulation (reviewed in Ref. [6]). In the present study, stimulation with PMA+Iono resulted in more TNFα and IFNγ being produced by the cells from older animals. During aging, excessive accumulation of advanced glycosylation end products has been associated with enhanced production of IFNγ [30]. An increase in TNFα production by T cells (most cells in the MLNs) has been suggested to be part of the proinflammatory response in the older population [5]. Feeding folate resulted in higher IL-4 production and a more skewed Th2 response than those produced by either of the control-diet fed groups. IL-4 promotes IL-2 proliferation and differentiation and might have contributed to the improved mitogen response in the folate-fed rats.

4.3. Conclusions

In conclusion, this study confirmed that aging is associated with changes in the proportion of T cells and the ability of immune cells to proliferate and produce cytokines in response to stimulation. We also demonstrated that supplementing a diet with additional folate significantly improved the distribution of T cells (particularly $CD8^+$ cells), increased mitogen responses and corrected most of the aberrant cytokine productions in the spleen but not in the MLNs. Despite insufficient information to define optimum immune function in the elderly, attempts have been made to improve or postpone the decline in immune responsiveness that occurs with aging. The results of the current study suggest that, as the elderly population increases, public health measures aimed at significantly increasing folate intake should be considered.

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