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Vitamin B_{12} and Folic Acid Imbalance Modifies NK Cytotoxicity, Lymphocytes B and Lymphoprolipheration in Aged Rats

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Abstract: Different vitamin B_{12} and folic acid concentrations could exacerbate the immune response. The aim was to evaluate different dietary folic acid and vitamin B_{12} levels on the immune response in aged rats. Male Sprague Dawley aged rats were assigned to three folic acid groups (deficient, control, supplemented) each in absence of vitamin B_{12} for 30 days. Several parameters of innate and acquired immune responses were measured. Serum and hepatic folate levels increased according to folic acid dietary level, while vitamin B_{12} levels decreased. There was a significant decrease in natural killer cell-mediated cytotoxicity in the spleen for the vitamin B_{12} deficient diet and folic acid control diet groups. Significant changes in CD45 lymphocyte subsets were also observed according to dietary imbalance. Lymphoproliferative response to concanavalin A and phytohemagglutinin did not differ significantly between groups. The spleen response to lipopolysaccharide increased significantly, but was unmodified for the other organs. An imbalance between dietary vitamin B_{12} and folic acid concentrations alters some immunological parameters in aged rats. Therefore, the ratio between folate and vitamin B_{12} could be as important as their absolute dietary concentrations.

Keywords: folic acid; vitamin B_{12}; rats; aging; immunology

1. Introduction

From 1998 the United States of America (USA) and Canada implemented a nutrition policy of mandatory fortification with the vitamin folic acid (FA) in flour and grain products. This nutrition policy was established following a proposal by the Food and Drug Administration [1], aimed at preventing neural tube defects (NTD). The fortification program has achieved considerable success in its main goal, since the incidence of NTD in the USA has declined in a range of 19%–27% [2–5]. In Spain where no mandatory fortification policy exists, we have shown that overages are a current practice in FA fortified breakfast cereals and milk products, and total folate values were higher than those declared by manufacturers in most cases [6,7]. The most well known adverse effect of supplementation and for food fortification with FA is the masking of the diagnosis of B_{12} deficiency, because megaloblastic anaemia caused by cobalamin deficiency can be reversed, but not the potential long-term neurological effects [8]. Morris *et al.* [9] observed that low B_{12} status and high serum folate levels were associated with cognitive impairment and anaemia in the elderly. By contrast, adequate B_{12} status and high serum folate levels were associated with protection against cognitive impairment. This has led to a controversy about the effects of supplementation and/or fortification with FA in subjects with vitamin B_{12} deficiency.

Moreover, the two vitamins play a potentially important role in immune function [10]; in fact, folate deficiency could lead to many clinical alterations including reduced immune function [11]. More specifically, folate deficiency in cultured phytohaemaglutinin (PHA)-activated human T lymphocytes, is able to reduce T lymphocyte proliferation, but also to induce apoptosis and increase the $CD4^+$ to $CD8^+$ ratio due to a marked reduction of $CD8^+$ cell proliferation [12], which may lead to a lower resistance to infections [11]. Therefore, another potential effect of supplementation with FA could be the improvement in immune function. Field *et al.* [13], by supplementing rats with additional folate for three weeks, observed that the proliferative response to mitogens, the distribution of T cells in mesenteric lymph node and age-related changes in cytokine production in the spleen were all improved.

It is now proposed that dietary folate requirements may be higher during the aging process, to support and protect immune function, since immunity deteriorates with age [14,15]. Decreased T cell memory and exhaustion of the naive T cell population with involution of the thymus are commonly observed in the elderly [16]. Troen *et al.* [17] found in postmenopausal women a "U-shaped" relation between total folate intake and natural killer cells (NK) cytotoxicity. They also showed that unmetabolized FA in plasma is associated with decreased NK cytotoxicity. Approximately 40% of older adults in the USA have unmetabolized serum folic acid that persists after fasting [18]. Thus, fortification or supplementation with FA may suppress the NK function, which is critical for normal immune function. Tamura *et al.* [19] have suggested that vitamin B_{12} may play an important role in cellular immunity, mainly affecting the $CD8^+$ cells and the NK cell system, which suggests effects on cytotoxic cells.

In consequence, the present study aimed at developing an aged animal model in order to evaluate some critical immunological parameters related to both FA and B_{12} status in the aging process. For this purpose, the influence of FA status is evaluated using different dietary FA levels, from deficiency to a supplemented state under experimental B_{12} induced deficiency.

2. Material and Methods

2.1. Animals and Diets

Twenty-month-old OFA male (Sprague Dawley) rats were obtained from Charles River Laboratories (Barcelona, Spain). Animals were maintained on a 12:12 h dark/light cycle, under temperature and humidity controlled conditions at the Animal Care Unit (Universidad CEU San Pablo, Madrid, Spain). All animal use and handling were performed following the European Union Normative (2003/65/CE) and were reviewed and ethically approved by the Animal Experimentation Committee of the Universidad CEU San Pablo.

2.2. Dietary Treatment

The animals were randomly assigned to be fed either a vitamin B_{12} deficient diet ($n = 27$) or a control diet ($n = 8$). After receiving the assigned diet for eight weeks, they were divided in four different groups on the basis of the experimental diet used, that were adjusted to rat requirements and based on a pure amino-acid diet (Dyets, Bethlehem, PA, USA) [20] modifying the FA and B_{12} content as follows: B_{12} and FA deficient diet (Group $D_{B12}D_{FA}$, 0 μg B_{12}/kg diet and 0 mg FA/kg diet, $n = 9$), B_{12} deficient diet and FA control diet (Group $D_{B12}C_{FA}$, 0 μg B_{12}/kg diet and 2 mg FA/kg diet, $n = 9$), B_{12} deficient diet and FA supplemented diet (Group $D_{B12}S_{FA}$, 0 μg B_{12}/kg diet and 8 mg FA/kg diet, $n = 9$), and control diet (Group $C_{B12}C_{FA}$, 50 μg de B_{12}/kg diet and 2 mg FA/kg diet, $n = 8$). Rats were fed *ad libitum* their respective diets for 30 days.

2.3. Sacrifice and Tissue Handling

Animals were sacrificed by decapitation; liver, spleen, thymus and axillary nodes were quickly removed. Whole blood was collected from all rats and serum and plasma were separated by centrifugation and kept at −80 °C until further analyses.

2.4. Serum Folate

Serum folate levels were analyzed by a microbiological method using *Lactobacillus casei* (ATCC 7469) (American Type Culture Collection), as previously reported by Horne and Tamura [21,22].

2.5. Hepatic Folate

Folate content in the rat liver was assessed by a microbiological method above described for serum folate determination. Previously, the livers were prepared through extraction and enzyme treatments carried according to the described trienzyme extraction method [23].

2.6. Serum Vitamin B_{12}

Serum vitamin B_{12} levels were determinated by commercial kit (Immundiagnostik AG, Bensheim, Germany). The serum was pretreated and diluted in a buffer and then the samples were transferred into a 96 well-plate and then coated with *Lactobacillus delbrueckii* (ATCC 7830). The vitamin B_{12}

quantification depends on the growth of the organism after incubation at 37 °C for 44–48 h. The growth of *Lactobacillus* turbidimetrically is determined at 610–630 nm. The vitamin B_{12} concentration is directly proportional to the turbidity.

2.7. Isolation of Rat Blood Neutrophils and Lymphocytes

Peripheral blood lymphocytes were obtained following a previously described method [24], by gradient sedimentation using a 1.077 density Hystopaque (Sigma-Aldrich, Spain) for lymphocytes. Cells from both interfaces were washed twice in phosphate-buffered saline solution. Pelleted cells were resuspended and diluted to a final concentration of 2×10^5 lymphocytes/mL medium. The lymphocytes were then incubated with monoclonal antibodies at 4 °C for 25 min in the dark. The following lymphocyte subsets were evaluated by flow cytometry: CD3, CD4, CD8, CD45RA and CD161. The control samples were incubated with purified phycoerythrin-labeled mouse immunoglobulin G1, fluorescein isothiocyanate-labeled mouse immunoglobulin G2 and *allophycocyanin*-labeled mouse immunoglobulin M. The fluorescence of the subsets was analyzed with a Facstar Plus dual-laser cytometer (Becton Dickinson, Sunnyvale, CA, USA) [25].

2.8. Lymphoproliferation

A previously described method was used [24,26,27]. Two hundred microliters of lymphocyte suspension, adjusted to 10^6 cells/mL in complete medium (RPMI-1640, PAA; plus 10% fetal bovine serum, Gibco; plus 1% gentamicin, PAA, Pasching, Austria), was cultured in plates with concanavalin A (Sigma-Aldrich, Spain) (Con A, 5 µg/mL in well), lipopolysaccharide (Sigma-Aldrich, Spain) (LPS, *Escherichia coli*, 055:B5, 5 µL/mL in well) and phytohaemagglutinin (Sigma-Aldrich, Spain) (PHA, 50 µg/mL in well) as stimulated samples or in complete medium as non-stimulated samples. After 48 h of incubation, 5 µ Ci [^{3}H]thymidine (Biolink 2000, Spain) was added to each well and cells were harvested. Results were expressed as the "stimulation index", which is the percentage of stimulation in response to Con A, LPS or PHA, with non-stimulated values (counts per minute) being 100%.

2.9. Cytotoxicity Assay

Murine lymphoma YAC-1 cells were used as targets in the NK activity assay [26]. An enzymatic colorimetric assay was used for cytolysis measurements of target cells (Cytotox 96 TM Promega, Madison, WI, USA) based on determination of lactate deshydrogenase using tetrazolim salts. Cells were cultured at an effector/target rate of 10/1 and incubated for 4 h. Then lactate deshydrogenase enzymatic activity was measured in 50 µL/well of supernatants by addition of the enzyme substrate and absorbance spectrophotometrically recorded at 490 nm. Results were expressed as percentage of lysis of target cells.

2.10. Interleukins (IL), Granulate-Macrophage Stimulating Factor Precursor (GM-CSF), Interferon Gamma (IFN-γ), Tumor Necrosis Factor Alpha (TNF-α) Levels

IL-1A, IL-1B, IL-4, IL-12, GM-CSF, IFN-γ, TNF-α levels were determined on culture supernatants of lymphocyte after a 48 h incubation with the mitogen Con A, LPS and PHA, following a method

previously described by Carrasco *et al.* [28] and measured using the multiplex antibody kits for the Luminex® system. All tests were determined by Cytokine 10-Plex rat panel (Invitrogen, Germany) for the simultaneous determination of IL-1a, IL-1b, IL-4, IL-12, TNF-α and IFN-γ in cell culture. The results were expressed as picograms per milliliter.

2.11. Statistical Analysis

Parametric data were statistically analyzed by a one way Analysis of variance (ANOVA). When ANOVA resulted in differences, multiple comparisons between means were studied by Bonferroni tests. Values are expressed as mean (95% confidence interval). Differences were considered significant at $p < 0.05$. Variables were tested for normality using a Kolmogorov-Smirnov test (SPSS 15.0, SPSS Inc.: Chicago, IL, USA).

3. Results

To demonstrate the usefulness of our experimental model, we determined serum and hepatic FA and serum vitamin B_{12} levels after dietary treatment. Serum vitamin B_{12} concentration was significantly decreased in the vitamin B_{12} deficient diet groups ($p < 0.001$) when compared to control animals (Table 1). Serum and hepatic folate levels were increased, as expected, in accordance with the dietary vitamin supplementation level ($p < 0.05$ and $p < 0.001$), as also shown in Table 1.

Table 1. Serum and hepatic folate and serum vitamin B_{12} concentrations.

Group	Serum Folate (ng/mL)	Liver Folate (µg/g liver)	Serum Vitamin B_{12} (ng/L)
$C_{B12}C_{FA}$	38.6	18.3	1203.4
$n = 8$	(34.1 to 43.1)	(14.7 to 22.0)	(1036.6 to 1370.3)
$D_{B12}D_{FA}$	4.6 ***,###	7.6 **,#	744.4 ***
$n = 9$	(4.0 to 5.2)	(4.9 to 10.2)	(663.0 to 825.7)
$D_{B12}C_{FA}$	62.5 *	16.1	906.8 ***
$n = 9$	(54.2 to 70.8)	(11.0 to 21.2)	(845.7 to 967.9)
$D_{B12}S_{FA}$	114.5 ***,###	29.6 **,##	807.6 ***
$n = 9$	(92.8 to 136.3)	(23.6 to 35.7)	(738.1 to 877.0)

Serum and hepatic folate and serum vitamin B_{12} concentrations in male rats fed B_{12} and FA deficient diets (Group $D_{B12}D_{FA}$, 0 µg B_{12}/kg diet and 0 mg FA/kg diet), B_{12} deficient diet and FA control diet (Group $D_{B12}C_{FA}$, 0 µg B_{12}/kg diet and 2 mg FA/kg diet), B_{12} deficient diet and FA supplemented diet (Group $D_{B12}S_{FA}$, 0 µg B_{12}/kg diet and 8 mg FA/kg diet), and control diet (Group $C_{B12}C_{FA}$, 50 µg B_{12}/kg diet and 2 mg FA/kg diet). Values are means (95% confidence interval per group). * $p < 0.05$ *vs.* group $C_{B12}C_{FA}$; ** $p < 0.01$ *vs.* group $C_{B12}C_{FA}$, *** $p < 0.001$ *vs.* group $C_{B12}C_{FA}$; # $p < 0.05$ *vs.* group $D_{B12}C_{FA}$; ## $p < 0.01$ $D_{B12}C_{FA}$; ### $p < 0.001$ $D_{B12}C_{FA}$. (Bonferroni's test).

There was a significant decrease in the NK cytotoxicity (Figure 1) in the spleen, in the DB12CFA group respective to the control group ($p < 0.05$) but neither in the thymus nor the axillary nodes. Furthermore, we observed a significant decrease in the B lymphocyte subsets in groups DB12CFA and DB12SFA ($p < 0.05$) but not for lymphocyte subsets helper T cells, cytotoxic-supressor T cells, mature T cells and natural killer cells (Table 2). Consequently, it seems that B_{12} deficiency decreases both B-cell diversity and the NK activity.

Figure 1. Natural killer activity in spleen, thymus and axillary nodes. Natural killer activity in male rats fed B_{12} and FA deficient diets ($n = 4$, Group $D_{B12}D_{FA}$, 0 µg B_{12}/kg diet and 0 mg FA/kg diet), B_{12} deficient diet and FA control diet ($n = 3$, Group $D_{B12}C_{FA}$, 0 µg B_{12}/kg diet and 2 mg FA/kg diet), B_{12} deficient diet and FA supplemented diet ($n = 4$, Group $D_{B12}S_{FA}$, 0 µg B_{12}/kg diet and 8 mg FA/kg diet), and control diet ($n = 3$, Group $C_{B12}C_{FA}$, 50 µg B_{12}/kg diet and 2 mg FA/kg diet). Values are means (95% confidence interval per group) ***** $p < 0.05$ *vs.* group $C_{B12}C_{FA}$ (Bonferroni's test).
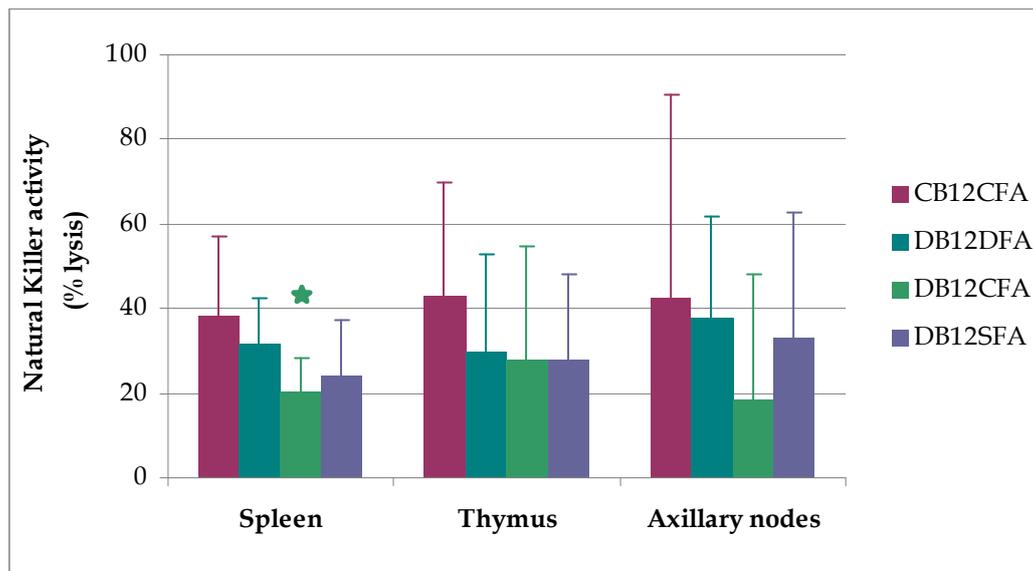
Table 2. Lymphocyte subsets in peripheral blood lymphocytes (%).

Group	Helper Tcells	Cytotoxic-Supressor T cells	CD4/CD8	Mature Tcells	B lymphocytes	Natural Killer Cells
$C_{B12}C_{FA}$	24.6	9.6	2.7	35.5	18.2	15.6
$n = 7$	(18.1 to 31.1)	(6.7 to 12.5)	(2.0 to 3.3)	(26.9 to 44.1)	(12.3 to 24.0)	(9.82 to 21.4)
$D_{B12}D_{FA}$	20.6	10.0	2.2	32.2	12.5	15.7
$n = 8$	(13.7 to 27.4)	(6.6 to 13.3)	(1.5 to 2.8)	(22.8 to 41.6)	(6.2 to 18.9)	(4.8 to 26.5)
$D_{B12}C_{FA}$	26.5	10.4	2.6	38.4	7.3 *	5.0
$n = 7$	(20.1 to 32.8)	(7.5 to 13.4)	(2.1 to 3.1)	(29.2 to 47.5)	(2.1 to 12.5)	(1.7 to 8.4)
$D_{B12}S_{FA}$	21.6	7.5	3.5	30.4	8.9 *	28.0
$n = 8$	(18.3 to 24.9)	(4.5 to 10.6)	(2.0 to 5.0)	(24.3 to 36.4)	(4.0 to 13.7)	(11.9 to 44.1)

Helper T cells (CD4), cytotoxic-supressor T cells (CD8), CD4/CD8 ratio, mature T cells (CD3), B lymphocytes (CD45RA), and natural killer cells (CD161) in male rats fed B_{12} and FA deficient diets (Group $D_{B12}D_{FA}$, 0 µg B_{12}/kg diet and 0 mg FA/kg diet), B_{12} deficient diet and FA control diet (Group $D_{B12}C_{FA}$, 0 µg B_{12}/kg diet and 2 mg FA/kg diet), B_{12} deficient diet and FA supplemented diet (Group $D_{B12}S_{FA}$, 0 µg B_{12}/kg diet and 8 mg FA/kg diet), and control diet (Group $C_{B12}C_{FA}$, 50 µg B_{12}/kg diet and 2 mg FA/kg diet). Values are means (95% confidence interval per group). * $p < 0.05$ *vs.* group $C_{B12}C_{FA}$ (Bonferroni's test).

Lymphoproliferation induced by mitogenic agents (Table 3) showed the response to ConA for the three organs (spleen, thymus and axillary nodes). As it is shown, it did not differ significantly between groups, and the same phenomenon was observed for PHA. However, the response to LPS in the spleen increased significantly in the group $D_{B12}S_{FA}$, but not in the other organs. Therefore, the spleen seems to

be more sensitive to changes in the presence of endotoxins when compared to the thymus or the axillary nodes.

Table 3. Lymphoproliferative response to concanavalin A (Con A), lipopolysaccharide (LPS) and phytohemagglutinin (PHA) in spleen, thymus and axillary nodes (%).

Organ	Mitogen	$C_{B12}C_{FA}$ $n = 8$	$D_{B12}D_{FA}$ $n = 9$	$D_{B12}C_{FA}$ $n = 9$	$D_{B12}S_{FA}$ $n = 9$
Spleen	Con A	138.1 (87.4 to 188.8)	168.0 (111.1 to 224.9)	185.4 (173.6 to 197.2)	146.3 (110.7 to 181.9)
	LPS	140.0 (98.3 to 181.7)	178.1 (129.1 to 227.2)	223.0 (142.5 to 303.5)	207.4 * (133.3 to 281.6)
	PHA	177.1 (90.1 to 264.2)	164.1 (130.9 to 197.4)	270.8 (156.8 to 384.8)	180.6 (97.0 to 264.1)
Organ	**Mitogen**	$C_{B12}C_{FA}$ $n = 8$	$D_{B12}D_{FA}$ $n = 9$	$D_{B12}C_{FA}$ $n = 8$	$D_{B12}S_{FA}$ $n = 9$
Thymus	Con A	111.3 (58.9 to 163.7)	161.1 (126.7 to 195.5)	151.3 (102.5 to 200.1)	125.2 (116.9 to 133.5)
	LPS	152.9 (59.2 to 246.6)	148.6 (108.8 to 188.3)	141.0 (92.7 to 189.3)	157.8 (131.8 to 183.8)
	PHA	161.3 (53.3 to 269.3)	149.9 (124.7 to 175.0)	204.9 (132.9 to 276.8)	154.8 (121.2 to 188.4)
Organ	**Mitogen**	$C_{B12}C_{FA}$ $n = 6$	$D_{B12}D_{FA}$ $n = 8$	$D_{B12}C_{FA}$ $n = 7$	$D_{B12}S_{FA}$ $n = 7$
Axillary nodes	Con A	115.6 (102.2 to 129.0)	152.0 (74.1 to 229.9)	171.0 (42.0 to 300.0)	131.5 (108.9 to 154.1)
	LPS	110.2 (63.8 to 156.6)	191.2 (109.8 to 272.5)	211.7 (27.7 to 395.6)	139.0 (105.9 to 172.1)
	PHA	145.2 (125.0 to 165.4)	190.2 (148.3 to 232.0)	181.0 (43.6 to 318.4)	149.5 (81.0 to 218.0)

Lymphoproliferative response to Con A, LPS and PHA in spleen, thymus and axillary nodes in male rats fed B_{12} and FA deficient dies (Group $D_{B12}D_{FA}$, 0 µg B_{12}/kg diet and 0 mg FA/kg diet), B_{12} deficient diet and FA control diet (Group $D_{B12}C_{FA}$, 0 µg B_{12}/kg diet and 2 mg FA/kg diet), B_{12} deficient diet and FA supplemented diet (Group $D_{B12}S_{FA}$, 0 µg B_{12}/kg diet and 8 mg FA/kg diet), and control diet (Group $C_{B12}C_{FA}$, 50 µg B_{12}/kg diet and 2 mg FA/kg diet). Values are means (95% confidence interval per group). * $p < 0.05$ *vs.* group $C_{B12}C_{FA}$ (Bonferroni's test).

IL-1a, IL-1b, IL-4, IL-12, GM-CSF, IFN-γ, TNF-α, TNF-α/IL-4 concentrations are reflected in Table 4. The concentrations of these interleukins are not modified by the effect of cyanocobalamin deficiency or by the FA level. The proportion of TNF-α to IL-4 is reflected in Table 4. As shown, there is no variation under the four experimental conditions tested.

Table 4. Interleukins, granulate-macrophage stimulating factor precursor (GM-CSF), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) levels in basal conditions and in presence of concanavalin A (ConA), lipopolysaccharide (LPS) and phytohemagglutinin (PHA).

Cytokines	Mitogen	$C_{B12}C_{FA}$ $n = 5$	$D_{B12}D_{FA}$ $n = 5$	$D_{B12}C_{FA}$ $n = 5$	$D_{B12}S_{FA}$ $n = 5$
IL-1a (pg/mL)	**Basal**	54.9 (53.9–55.9)	54.6 (46.7–62.6)	53.8 (49.6–57.9)	53.5 (50.7–56.3)
	Con A	56.0 (43.3–68.7)	54.3 (44.7–63.8)	53.3 (51.9–54.8)	58.0 (46.2–69.8)
	LPS	64.3 (54.3–74.4)	58.0 (55.5–60.5)	62.0 (48.9–75.1)	62.6 (56.8–68.5)
	PHA	58.0 (54.6–61.4)	59.1 (53.8–64.5)	55.8 (50.7–60.9)	54.3 (52.8–55.8)
IL-1b (pg/mL)	**Basal**	13.8 (3.0–24.7)	15.0 (6.9–23.1)	18.3 (8.8–27.7)	13.1 (10.7–15.5)
	Con A	9.7 (2.1–17.3)	9.1 (−6.1–24.3)	14.2 (5.3–23.1)	12.1 (8.5–15.6)
	LPS	20.5 (17.1–23.9)	17.3 (17.3–17.3)	26.6 (9.6–43.6)	18.5 (11.3–25.8)
	PHA	18.0 (3.05–33.0)	18.1 (6.4–29.8)	18.5 (12.0–25.1)	12.1 (8.9–15.4)
IL-4 (pg/mL)	**Basal**	4.39 (4.35–4.42)	4.40 (4.34–4.45)	4.39 (4.35–4.42)	4.38 (4.36–4.41)
	Con A	4.42 (4.18–4.67)	4.39 (4.32–4.45)	4.38 (4.33–4.43)	4.39 (4.35–4.42)
	LPS	4.39 (4.34–4.45)	4.39 (4.34–4.45)	4.39 (4.34–4.45)	4.38 (4.35–4.41)
	PHA	4.43 (4.41–4.45)	4.41 (4.41–4.41)	4.41 (4.41–4.41)	4.31 (4.13–4.49)
IL-12 (pg/mL)	**Basal**	31.5 (−2.8–65.7)	24.8 (9.3–40.3)	27.4 (9.5–45.2)	35.5 (27.3–43.7)
	Con A	18.9 (−67.5–105.3)	28.2 (18.1–38.3)	29.1 (14.6–43.6)	18.7 (27.3–36.7)
	LPS	54.1 (33.0–75.2)	30.8 (18.3–43.4)	61.1 (−4.5–126.7)	54.3 (27.2–81.4)
	PHA	29.4 (−8.2–66.9)	29.1 (14.6–43.6)	41.2 (12.4–69.9)	32.5 (22.8–42.2)
GM-CSF (pg/mL)	**Basal**	12.9 (11.9–14.0)	13.6 (12.6–14.5)	13.3 (13.3–13.3)	12.5 (11.6–13.4)
	Con A	13.9 (6.1–21.7)	13.5 (12.4–14.6)	12.4 (10.5–14.3)	12.9 (11.9–14.0)
	LPS	13.7 (11.9–15.4)	13.7 (11.9–15.4)	12.8 (11.0–14.7)	13.6 (12.6–14.5)
	PHA	13.7 (11.9–15.4)	13.7 (11.9–15.4)	13.3 (13.3–13.3)	13.3 (13.3–13.3)
IFN-γ (pg/mL)	**Basal**	3.0 (1.3–4.7)	2.0 (−0.2–4.1)	2.7 (0.7–4.6)	3.0 (0.7–5.2)
	Con A	2.3 (−6.0–10.8)	1.7 (1.7–1.7)	1.2 (−1.0–3.3)	2.98 (0.6–5.4)
	LPS	3.4 (−0.3–7.1)	2.1 (0.2–4.0)	3.0 (−0.2–6.2)	4.2 (−0.0–8.4)
	PHA	2.8 (1.9–3.7)	2.1 (0.2–4.03)	3.0 (1.4–4.6)	2.7 (2.0–3.5)
TNF-α (pg/mL)	**Basal**	13.6 (−1.0–28.2)	13.9 (0.1–27.7)	11.9 (5.7–18.2)	17.6 (11.6–23.5)
	Con A	13.9 (1.5–29.1)	9.9 (3.5–16.3)	11.0 (0.2–21.9)	15.5 (5.7–25.3)
	LPS	80.9 (66.1–95.7)	56.0 (−23.8–135.9)	67.3 (−11.8–146.3)	64.4 (41.4–87.3)
	PHA	27.0 (−11.8–65.9)	17.03 (0.4–33.6)	44.4 (0.7–88.1)	34.9 (14.2–55.6)
TNF-α/ IL-4	**Basal**	3.1 (−0.2–6.4)	3.2 (−0.0–6.4)	2.7 (1.3–4.2)	4.0 (2.7–5.3)
	Con A	3.1 (−1.2–7.5)	2.1 (1.2–2.9)	2.7 (1.2–4.3)	4.2 (1.8–6.7)
	LPS	19.7 (15.3–24.1)	13.2 (3.7–22.8)	13.2 (1.6–24.7)	14.7 (9.5–19.9)
	PHA	8.4 (−0.2–16.9)	3.9 (0.1–7.6)	10.1 (0.2–20.0)	8.1 (3.3–13.0)

IL-1a, IL-1b, IL-4, IL-12, GM-CSF, IFN-γ, TNF-α and TNF-α/IL-4 in male rats fed B_{12} and FA deficient diets (Group $D_{B12}D_{FA}$, 0 μg B_{12}/kg diet and 0 mg FA/kg diet), B_{12} deficient diet and FA control diet (Group $D_{B12}C_{FA}$, 0 μg B_{12}/kg diet and 2 mg FA/kg diet), B_{12} deficient diet and FA supplemented diet (Group $D_{B12}S_{FA}$, 0 μg B_{12}/kg diet and 8 mg FA/kg diet), and control diet (Group $C_{B12}C_{FA}$, 50 μg B_{12}/kg diet and 2 mg FA/kg diet). Values are means (95% confidence interval per group).

4. Discussion

In the present study, we examined the effects of an imbalance between vitamin B_{12} and FA dietary concentration on NK cytotoxicity, B lymphocytes and lymphoproliperation in aged rats. A marked decrease in the spleen NK activity was observed in the $D_{B12}C_{FA}$ group, although this effect was not statistically significant in the thymus or axillary nodes.

Although just few studies have examined the relationship between immune response and both B_{12} and FA, it has been observed in humans that either a deficiency or an excessive dietary folate appear to be related to an alteration of the immune response, as determined by NK cytotoxicity. In this intervention study, 105 healthy women were therefore exposed to the mandatory fortification with FA in flour and grain products: women whose diets were low in folate and consumed supplements increased the NK cytotoxicity. However, women who consumed a folate-rich diet plus FA supplements had reduced NK cytotoxicity compared with those consuming a low-folate diet and no supplements. In another study by contrast, there was not correlation between plasma total folate and cytotoxicity of NK cells [29].

In experimental animal models, moderate folate deficiency appears not to affect the NK-mediated cytotoxicity, in contrast to that observed in severe folate deficiency [30]. It has been also reported that decreased NK mediated cytotoxicity in aged animals was reverted when feeding them with diets supplemented with antioxidants [31,32].

Following this same pattern, our experiments also revealed that lymphocyte subsets (T-helper cells, cytotoxic or suppressor cells, mature T cells, B cells and Natural Killers) were lower for B cells in $D_{B12}C_{FA}$ and $D_{B12}S_{FA}$ groups, which demonstrates the importance of an equilibrium between folate and B_{12} intake. Gibson *et al.* [33] have recently observed that B-cell diversity may dramatically decrease with age and may have important implications for the immune health of elderly people. More studies are necessary to determine the possibility of a causal link between B_{12} deficiency and loss of B-cells diversity, and whether B-cells could be improved by restoring normal levels of B_{12} and/or folate.

It should be noted that the best-known contribution of B-cells in the immune system is the production of antibodies. These cells are highly effective as antigen-presenting cells and essential for the development of T-cells [34]. There is also evidence to support the role of B-cells as immune regulators, because they are able to secrete IL-10 which could prevent inadequate stimulation of the immune system (e.g., autoimmune diseases), and could also serve to limit the aggressiveness of the immune response [35]. On the other hand, some authors [12,19,36,37] observed reductions of cytotoxic cells and an increase in the CD4/CD8 ratio following folate and vitamin B_{12} deficiency. These observations, however, have not been confirmed in the present study.

The proliferative response in mixed cell cultures is frequently used to estimate "adaptive immunity". When the proliferative capacity against different mitogens in lymphoid organs was evaluated, we did not observe any change due to the dietary treatment, except for an increase in the spleen lymphoproliferative capacity in response to LPS in the B_{12} deficient/FA supplemented group. Therefore, feeding aged rats with additional folate resulted in an improved response of the spleen to mitogens. Otherwise, it is suggested that vitamins B_6 and B_{12} deficiencies may induce a decrease in lymphocyte proliferative response against a mitogen [38] and that a dietary folate deficiency may reduce the stimulation of lymphocytes by PHA [39]. It is necessary to emphasize that there are not

studies in experimental animal models to validate this hypothesis. Further testing would be of interest to determine some guidelines to determine if FA supplementation may reverse the decline in lymphocyte proliferation when it is associated with a vitamin B_{12} deficiency, and if a deficient state in both vitamins actually decreases the proliferative response.

The cytokines can be modulated by nutrients and their participation is essential in establishing certain mechanisms involved in the development of infectious processes [40]. Therefore, it is necessary to establish whether an inadequate or poor state of FA and vitamin B_{12} may affect the balance between Th1 and Th2 and thus impair the immune response. In this sense, cytokine production was measured in our study not only under spleen cell cultures stimulation with different endotoxins, but also after ConA, LPS and PHA stimulation. IL-1a, IL-1b, IL-4, IL-12, GM-CSF, IFN-γ and TNF-α levels were quantified. These cytokines were selected because they belong to the subset 1 and 2. In light of our results, no significant differences in any cytokine production in unstimulated cells or cells stimulated with ConA, LPS and PHA were observed. It therefore appears that both vitamin B_{12} and FA have no effects on cytokine production in the spleen, and FA supplementation does not improve the cellular immune response when there is a vitamin B_{12} deficiency, but also does not show any negative and/or adverse effect.

5. Conclusions

An imbalance in B_{12} and FA alters the NK cytotoxicity and B lymphocyte in aged rats after just a short-term dietary treatment. These effects have not been observed in control or in induced deficiency for both vitamins. The overall balance of folate and B_{12} could be as important as their absolute dietary concentration.

Acknowledgments

Conflicts of Interest

The authors declare no conflict of interest.

References

1. US Food and Drug Administration. Food standards: Amendment of standars of identity for enriched grain products to require addition of folic acid. *Fed. Regist.* **1996**, *61*, 8781–8797.
2. CDC. Trends in Spina Bifida and Anencephalus in the United States, 1991–2006. Available online: http://www.cdc.gov/nchs/products/pubs/pubd/hestats/spine_anen.htm (accessed on 7 May 2013).
3. Honein, M.A.; Paulozzi, L.J.; Mathews, T.J.; Erickson, J.D.; Wong, L.Y. Impact of folic acid fortification of the US food supply on the ocurrence of neural tube defects. *JAMA* **2001**, *285*, 2981–2986.
4. Mathews, T.J.; Honein, M.A.; Erickson, J.D. Spina bifida and anencephaly prevalence: United States, 1991–2001. *MMWR Recomm. Rep.* **2002**, *51*, 9–11.

5. Centers for Disease Control and Prevention (CDC). Spina bifida and anencephaly before and after folic acid mandate—United States, 1995–1996 and 1999–2000. *Morb. Mortal. Wkly. Rep.* **2004**, *53*, 362–365.

6. Samaniego Vaesken, M.L.; Alonso-Aperte, E.; Varela-Moreiras, G. Elaboración de una base de datos del contenido en folatos de los alismentos: Fortificación voluntaria con ácido fólico en España. *Nutr. Hosp.* **2009**, *24*, 459–466.

7. Samaniego-Vaesken, M.L.; Alonso-Aperte, E.; Varela-Moreiras, G. Analysis and evaluation of voluntary folic acid fortification of breakfast cereals in the Spanish market. *J. Food Compos. Anal.* **2010**, *23*, 419–423.

8. Mills, J.L. Fortification of foods with folic acid: How much is enough? *N. Engl. J. Med.* **2000**, *342*, 1442–1445.

9. Morris, M.S.; Jacques, P.F.; Rosenberg, I.H.; Selhub, J. Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. *Am. J. Clin. Nutr.* **2007**, *85*, 193–200.

10. Field, C.J.; Johnson, I.R.; Schley, P.D. Nutrients and their role in host resistance to infection. *J. Leukoc. Biol.* **2002**, *71*, 16–32.

11. Dhur, A.; Galan, P.; Hercberg, S. Folate status and the immune system. *Prog. Food Nutr. Sci.* **1991**, *15*, 43–60.

12. Courtemanche, C.; Elson-Schwab, I.; Mashiyama, S.T.; Kerry, N.; Ames, B.N. Folate deficiency inhibits the proliferation of primary human CD8$^+$ T lymphocites *in vitro*. *J. Immunol.* **2004**, *173*, 3186–3192.

13. Field, C.J.; van Aerde, A.; Drager, K.L.; Goruk, S.; Basu, T. Dietary folate improves age-related decreases in lymphocyte function. *J. Nutr. Biochem.* **2005**, *17*, 37–44.

14. Gomez, C.R.; Nomellini, V.; Faunce, D.E.; Kovacs, E.J. Innate immunity and aging. *Exp. Gerontol.* **2008**, *43*, 718–728.

15. Dorshkind, K.; Montecino-Rodriguez, E.; Signer, R.A. The ageing immune system: Is it ever too old to become young again? *Nat. Rev. Immunol.* **2009**, *9*, 57–62.

16. Weng, N.P. Aging of the immune system: How much can the adaptive immune system adapt? *Immunity* **2006**, *24*, 495–499.

17. Troen, A.M.; Mitchell, B.; Sorensen, B.; Wener, M.H.; Johnston, A.; Wood, B.; Selhub, J.; McTiernan, A.; Yasui, Y.; Oral, E.; *et al.* Unmetabolized folic acid in plasma is associated with reduced natural killer cell cytotoxicity among postmenopausal women. *J. Nutr.* **2006**, *136*, 189–194.

18. Bailey, R.L.; Mills, J.L.; Yetley, E.A.; Gahche, J.J.; Pfeiffer, C.M.; Dwyer, J.T.; Dodd, K.W.; Sempos, C.T.; Betz, J.M.; Picciano, M.F. Unmetabolized serum folic acid and its relation to folic acid intake from diet and supplements in a nationally representative sample of adults aged > or = 60 year in the United States. *Am. J. Clin. Nutr.* **2010**, *92*, 383–389.

19. Tamura, J.; Kubota, K.; Murakami, H.; Sawamura, M.; Matsushima, T.; Tamura, T.; Saitoh, T.; Kurabayshi, H.; Naruse, T. Immunomodulation by vitamin B12: Augmentation of CD8$^+$ T lymphocytes and natural killer (NK) cell activity in vitamin B12-deficient patients by methyl-B12 treatment. *Clin. Exp. Immunol.* **1999**, *116*, 28–32.

20. Walzem, R.L.; Clifford, A.J. Folate deficiency in rats fed diets containing free amino acids or intact proteins. *J. Nutr.* **1988**, *118*, 1089–1096.

21. Tamura, T. Microbiological Assays of Folates. In *Folic Acid Metabolism in Health and Disease*; Wiley-Liss Inc.: Wilmington, DE, USA, 1990; pp. 121–137.

22. Horne, D.W.; Krumdieck, C.L.; Wagner, C. Properties of folic acid gamma-glutamyl hydrolase (conjugase) in rat bile and plasma. *J. Nutr.* **1981**, *111*, 442–449.

23. Hyun, T.H.; Tamura, T. Trienzyme extraction in combination with microbiologic assay in food folate analysis: An updated review. *Exp. Biol. Med.* **2005**, *230*, 444–454.

24. De la Fuente, M.; Victor, V.M. Anti-oxidants as modulators of immune function. *Immunol. Cell Biol.* **2000**, *78*, 49–54.

25. Baker, J.W. An innovate lymphocyte preparation system for flow cytometry. *Am. Clin. Lab.* **1988**, *120*, 320–324.

26. Alvarado, C.; Alvarez, P.; Jiménez, L.; De la Fuente, M. Improvement of leukocyte functions in young prematurely aging mice after a 5-week ingestion of a diet supplemented with biscuits enriched in antioxidants. *Antioxid. Redox Signal.* **2005**, *7*, 1203–1210.

27. De la Fuente, M.; Hernanz, A.; Guayerbas, N.; Victor, V.M.; Arnalich, F. Vitamin E ingestion improves several immune functions in elderly men and women. *Free Radic. Res.* **2008**, *42*, 272–280.

28. Carrasco, M.; Hernanz, A.; De La Fuente, M. Effect of cholecystokinin and gastrin on human peripheral blood lymphocyte functions, implication of cyclic AMP and interleukin 2. *Regul. Pept.* **1997**, *70*, 135–142.

29. Ravaglia, G.; Forti, P.; Maioli, F.; Bastagli, L.; Facchini, A.; Mariani, E.; Savarino, L.; Sassi, S.; Cucinotta, D.; Lenaz, G. Effect of micronutrient status on natural killer cell inmune function in health free-living subjects aged >90. *Am. J. Clin. Nutr.* **2000**, *71*, 590–598.

30. Kim, Y.I.; Hayek, M.; Mason, J.B.; Meydani, S.N. Severe folate deficiency impairs natural killer cell-mediated cytotoxicity in rats. *J. Nutr.* **2002**, *132*, 1361–1367.

31. De la Fuente, M. Effects of antioxidants on immune system ageing. *Eur. J. Clin. Nutr.* **2002**, *56*, S5–S8.

32. De la Fuente, M.; Miquel, J. An update of the oxidation-inflammation theory of aging: The involvement of the immune system in oxi-inflamm-aging. *Curr. Pharm. Des.* **2009**, *15*, 3003–3026.

33. Gibson, K.L.; Wu, Y.C.; Barnett, Y.; Duggan, O.; Vaughan, R.; Kondeatis, E.; Nilsson, B.O.; Wikby, A.; Kipling, D.; Dunn-Walters, D.K. B-cell diversity decreases in old age and is correlated with poor health status. *Aging Cell* **2009**, *8*, 18–25.

34. Crawford, A.; Macleod, M.; Schumacher, T.; Corlett, L.; Gray, D. Primary T cell expansion and differentiation *in vivo* requires antigen presentation by B cells. *J. Immunol.* **2006**, *176*, 3498–3506.

35. Fillatreau, S.; Gray, D.; Anderton, S.M. Not always the bad guys: B cells as regulators of autoimmune pathology. *Nat. Rev. Immunol.* **2008**, *8*, 391–397.

36. Funada, U.; Wada, M.; Kawata, T.; Mori, K.; Tamai, H.; Kawanishi, T.; Kunou, A.; Tanaka, N.; Tadokoro, T.; Maekawa, A. Changes in $CD4^+CD8^-/CD4^-CD8^+$ ratio and humoral immune functions in vitamin B12-deficient rats. *Int. J. Vitam. Nutr. Res.* **2000**, *70*, 167–171.

37. Funada, U.; Wada, M.; Kawata, T.; Mori, K.; Tamai, H.; Isshiki, T.; Onoda, J.; Tanaka, N.; Tadokoro, T.; Maekawa, A. Vitamin B-12-deficiency affects immunoglobulin production and cytokine levels in mice. *Int. J. Vitam. Nutr. Res.* **2001**, *71*, 60–65.

38. Grimble, R.F. Effect of antioxidative vitamins on immune function with clinical applications. *Int. J. Vitam. Nutr. Res.* **1997**, *67*, 312–320.

39. Gebhardt, B.M.; Newberne, P.M. Nutrition and immunological responsiveness. T-cell function in the offspring of lipotrope and protein-deficient rats. *Immunology* **1974**, *26*, 489–495.

40. Grimble, R.F. Malnutrition and the immune response. 2. Impact of nutrients on cytokine biology in infection. *Trans. R. Soc. Trop. Med. Hyg.* **1994**, *88*, 615–619.