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Basic nutritional investigation

Natural killer cell cytotoxicity is not regulated by folic acid in vitro

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ABSTRACT

Objectives: Folate supplementation may be associated with an increased risk of developing several types of cancer and a derangement of immune function. Among the latter, Natural killer (NK) cells are involved in non–MHC-restricted natural immunity against malignant target cells. Abnormalities in NK cell number or function have been associated with a higher cancer risk. The aim of this study was to study in vitro the possible effect of different concentrations of 5-methyltetrahydrofolic acid (5-MTHF) or folic acid on NK cell cytotoxic function, and expression of the stimulatory and inhibitory receptors KIRDL4, KIRDL3, and NKG2D.

Methods: Volunteer-derived peripheral mononuclear cells (PBMC) and highly enriched NK cells (95% CD56+ CD16+) were grown in folic acid free–RPMI 1640, supplemented either with folic acid or 5-MTHF (15–100 nM) during 72 h to 96 h.

Results: No differences in the cytolytic activity of PBMC and enriched NK cells were observed. After 96 h of in vitro culture without folate or supplemented with FA or 5-MTHF (30 or 100 nM), there were no changes in the percentage of HPNK receptor-positive cells.

Conclusions: Our data indicate that a high dose of 5-MTHF or folic acid does not influence NK cell function in vitro.

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Introduction

Folates play a key role in several metabolic processes, acting as coenzymes in one-carbon transfer reactions, which are important for nucleic acids synthesis, methylation reactions, and therefore for the regulation of gene expression [1]. Folate is the generic term used for a group of vitamins with equivalent biological activities. This water-soluble vitamin is naturally present in food, especially in green-leafed vegetables, but the synthetic form has also been incorporated in supplements and fortified food. Dietary folates predominantly exist as polyglutamates, which have to be hydrolyzed to monoglutamates to be transported [2]. On the other hand, the synthetic form, folic acid, is fully oxidized and contains only one conjugated glutamate residue (monoglutamate form), showing higher stability and bioavailability than natural folates and being rapidly absorbed across the intestine. Folic acid has an 85% bioavailability when taken with food and a 100% bioavailability when taken on an empty stomach with water [3]. During its passage through the intestinal mucosa, folate and folic acid are metabolized to N^5-methylenetetrahydrofolate (5-MTHF), which is the principal circulating folate form found in circulation and used for cellular methylation reactions. However, when high amounts of folic acid are consumed (400 mg/d), a percentage of it appears unchanged in the peripheral circulation [4].

Because folate deficiencies during pregnancy have been associated with neural tube defects in the newborn, several countries have started successfully food folic acid fortification programs to cover the requirement (400 μg) of the target population. However, in 20% to 37% of the general population, serum folate levels reached above 40 nmol/L, considered as supraphysiologic levels [5,6].

There is an ongoing debate about the possible unsafe effects of folic acid intake from fortified products, motivated by conflicting reports about increasing the risk of cancer and heart disease and masking vitamin B12 deficiency [7]. There are

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suggestions that circulating unmetabolized folic acid may have adverse effects [8–10].

Epidemiologic studies have reported a temporal relationship between mandatory fortification with folic acid and a greater incidence of colorectal cancer [11,12]. Nevertheless, there are also studies that reported a significant inverse association or no relationship between high intake of food folates, serum folate levels, and cancer incidence [13,14]. Several mechanisms have been proposed to explain the association between folic acid and cancer, including the reduction of effective natural killer (NK) cell activity caused by supraphysiological levels of serum folate [15,16], thus impeding this first antitumoral barrier and increasing the risk of developing cancer [17].

NK cells are effector lymphocytes of the innate immune system that control several types of tumors by limiting their growth and dissemination. Multiple studies in vivo and in vitro have provided evidence that tumor cells are recognized as NK cell targets. NK cells have the ability to mediate cytotoxicity against susceptible target cells to secrete cytokines [18]. These functions are regulated by several stimulatory and inhibitory receptors (KIR2DLs, NKG2D), whose expression in NK cells is epigenetically regulated through DNA methylation [19].

On the basis of these premises, we performed a clinical study to investigate the relationship between folate and NK activity in a group of healthy adults. We did not find any association between folate levels and NK activity [20]. However, none of these subjects had low folate levels, and non-metabolized folic acid was not measured. To overcome the limitations of this clinical study, we decided to assess in vitro the effect of different levels of unmetabolized folic acid and its active molecule, 5-MTHF, levels on NK activity.

To further study the effect of folic acid on NK activity, we extracted NK cells from healthy humans and studied in vitro the effects of different concentration of 5-MTHF or folic acid on NK cell cytotoxic function and expression of receptors KIRDL4, KIRDL3, NKG2D.

Materials and methods

Chemicals

Folic acid (FA) and 5-methyltetrahydrofolate (5-MTHF) from Sigma-Aldrich (St. Louis, USA).

Antibodies

For flow cytometric analysis, the following monoclonal antibodies were used: FITC-conjugated antihuman KIR2DL3, PE-conjugated antihuman KIR2DL4 from R&D Systems (Minneapolis, MN, USA); APC-conjugated antihuman NKG2D from BD Pharmingen (San Diego, CA, USA); antihuman CD3−/CD16+CD56+ from Becton Dickinson (Mississauga, ON, Canada).

Peripheral blood mononuclear cell isolation

Normal peripheral blood mononuclear cells (PBMC) were obtained from EDTA-blood of healthy adult volunteers from two blood banks after obtaining an informed consent. PBMC were isolated by density centrifugation using a lymphocyte separation medium (Cellgro, Mediatech, VA, USA). After monocyte depletion (plastic adherence; 1 h, 37°C and 5% CO2) peripheral blood lymphocytes (PBL) were washed twice with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone, MA, USA), 50 U/mL streptomycin-penicillin (Hyclone, MA, USA), 20 μg/mL gentamicin (Gibco Invitrogen, Grand Island, NY, USA), and 2 mM L-glutamine (Hyclone, MA, USA), named complete medium.

Human natural killer cell enrichment

NK cells were further enriched by negative selection from PBL using the NK Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer instructions. Briefly, PBL were incubated for 10 min with Biotin-Antibody Cocktail (containing antiCD3, antiCD4, antiCD14, antiCD15, antiCD19, antiCD36, antiCD123, and antiCD235a antibodies) at 4°C to 8°C, after which the Anti-Biotin Microbeads were added for an additional 15 min at 4°C to 8°C. Cells were then passed through a magnetic column for collection of purified NK cells. This technique routinely produced highly purified NK cells (HPNK) >90% CD16+/CD56+ cells, as confirmed by flow cytometric analysis. HPNK cells were cultured in the same conditions that PBMC plus 75 U/mL rhIL-2 (BD Pharmingen, San Diego, CA, USA).

Cell culture and folate stimulation

To establish experimental culture conditions under low and high FA as well as 5-MTHF conditions, PBMC and HPNK cells were cultured in a folate-free RPMI 1640, 10% fetal bovine serum complete medium (Invitrogen, Grand Island, NY, USA) supplemented with 15nM FA to 30 nM FA or 5-MTHF (physiological folic acid), 70 nM to 100 nM FA or 5-MTHF (high folic acid), or in the absence of either, FA or MTHF. To assess the effect of folic acid and 5-MTHF, 5×10^5 cells/mL of HPNK and 1×10^6 cells/mL of PBMC were stimulated as described during 96 h. Thereafter, cytotoxicity against K562 target cell line was performed.

Cytotoxicity assay

Cytotoxicity against NK sensitive K562 cells was performed using a standard 4-h ^{51}Chromium release assay. Briefly, 1×10^6 K562 cells were labeled with 100u Ci/mL ^{51}Cr (Perkin Elmer, Boston, MA, USA) during 1 h at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% fetal bovine serum complete medium. Cells were washed twice with PBS and resuspended in RPMI 1640 complete medium. Then, 5000 ^{51}Cr labeled target cells (K562)/well were incubated with either PBMC or HPNK cells (effector cells) during 4 h, using different effector-to-target (E:T) cell ratios. The supernatants were measured for ^{51}Cr release on a γ-counter (Cobra II, Packard Instrument Company). The percent of cytotoxicity was calculated as follows: 100 × ([experimental release – spontaneous release]/[maximum release – spontaneous release]). Maximum release was obtained from target cells lysed with 2% Triton X-100 (Sigma-Aldrich, St. Louis, USA). Spontaneous release was always below 10% of maximum release.

Table 1
In vitro effect of folic acid on the cytotoxicity of HPNK† cells

Subjects N°. sex	Natural killer cell activity									
	E:T* = 0.5:1					E:T = 1:1				
	Folic acid (nM)									
	0	15	30	75	100	0	15	30	75	100
1. M	28‡	44	36	30	32	51	79	63	54	57
2. M	34	34	36	35	33	56	55	57	56	54
3. F	37	34	28	33	32	66	61	50	59	57
4. M	43	37	41	45	42	76	66	74	81	75
Mean ± SD	35.50 ± 6.24	37.25 ± 4.72	35.25 ± 5.38	35.75 ± 6.50	34.75 ± 4.86	62.25 ± 11.09	65.25 ± 10.21	61.00 ± 10.17	62.50 ± 12.50	60.75 ± 9.60

Two way Anova.
* E:T: target cell ratio.
† HPNK cells: highly purified natural Killer cells.
‡ Values expressed as mean of % of cytotoxicity ± SD.

Table 2
In vitro effect of 5-MTHF[α] on the cytotoxicity of HPNK[†] cells

Subjects N°. sex	Natural killer cell activity									
	E:T* = 0,5:1					E:T = 1:1				
	5- MTHF (nM)									
	0	15	30	75	100	0	15	30	75	100
1. M	28[‡]	31	35	33	31	51	55	63	49	46
2. M	35	36	35	25	31	56	54	54	47	49
3. F	37	22	25	22	31	66	58	63	58	50
4. M	43	50	51	50	54	76	75	77	75	82
Mean ± SD	35.75 ± 6.18	34.75 ± 11.70	36.50 ± 10.75	32.50 ± 12.56	36.75 ± 11.50	62.25 ± 11.09	60.50 ± 9.81	64.25 ± 9.50	57.25 ± 12.76	56.75 ± 16.92

[α]5-MTHF, 5-methyltetrahydrofolic acid; α5-MTHF, 5-methyltetrahydrofolic acid.
Two way Anova.
* E:T: target cell ratio.
† HPNK cells: highly purified natural Killer cells.
‡ Values expressed as mean of % of cytotoxicity ± SD.

Flow cytometric analysis

Determination of NK cell surface receptors (KIR2DL3, KIR2DL4 and NKG2D)

Three-color flow cytometric analysis was performed in HPNK cell surface receptors. 1×10^5/mL HPNK cells were resuspended in 100 μL PBS and stained with 10 μL anti-KIR2DL3, 10 μL anti-KIR2DL4, and 15 μL anti-NKG2D during 45 min at 20°C. Finally, stained HPNK cells were washed twice with PBS and resuspended with 400 μL PBS. Samples were analyzed by flow cytometry (BDFacs Canto II, Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis

Data obtained was analyzed with two-way ANOVA test ($P < 0.05$ was considered as significant) using GraphPad Prism 5 Software.

Results

Effect of different FA and 5-MTHF concentrations on NK in HPNK

HPNK from PBMC of four healthy donors were used. All of the samples used for these experiments were enriched at least 95% in CD3− CD16/56+ cells.

There were no significant differences in the cytotoxic activity of HPNK, measured either using a 0.5:1 or 1:1 E/T ratio, after 96 h of incubation in a RPMI 1640 culture medium supplemented with 0 nM, 15 nM, 30 nM, 75 nM, or 100 nM of FA or 5-MTHF (Tables 1 and 2).

It is interesting to see that the HPNK lytic activity was not modified even in the absence of either FA or 5-MTHF (Table 1 and 2).

Effect of FA and 5-MTHF on NK of peripheral blood mononuclear cells

In human peripheral blood, NK cells are a small population comprising only approximately 10% of all lymphocytes. Under this circumstance, cells communicate among them through the action of several secreted proteins. It is possible to assume that the NK cell lytic function could be regulated through the action of either FA or 5MTHF on other peripheral cells in a paracrine fashion. To address this possibility, we incubated human PBMC isolated from eleven healthy adult donors (seven males and six females,) with folate free RPMI 1640 medium or supplemented with 15 nM, 30 nM, 75 nM, or 100 nM of either FA or 5-MTHF during 96 h of culture. NKCA from PBMC showed no significant changes either without folate or with using different FA or 5-MTHF concentrations at the effector to target ratios used (15:1 and 30:1), as shown in Tables 3 and 4.

Table 3
In vitro effect of folic acid on the cytotoxicity of PBMC[†] samples

Subjects N°. sex	PBMC citotoxicity					
	E:T* = 15:1			E:T = 30:1		
	Folic acid (nM)					
	0	30	100	0	30	100
1. M	16[‡]	20	6	37	50	42
2. M	6	7	15	32	34	39
3. M	36	52	59	51	71	76
4. F	33	29	26	57	55	38
5. M	29	30	31	53	53	55
6. F	35	43	28	58	65	53
7. M	39	37	45	70	67	70
8. M	15	12	20	37	36	42
9. F	16	16	18	23	26	29
10. M	25	22	23	47	45	46
11. F	43	40	49	66	64	68
Mean ± SD	26.64 ± 11.89	28.00 ± 14.04	29.09 ± 15.92	48.27 ± 14.65	51.45 ± 14.88	50.73 ± 15.09

Two way Anova.
* E:T: target cell ratio.
† PBMC: human peripheral blood mononuclear cells.
‡ Values expressed as mean of % of cytotoxicity ± SD.

Table 4
In vitro effect of 5MTHF[α] on the cytotoxicity of PBMC[†] samples

Subjects N°. sex	PBMC cytotoxicity					
	E:T[*] = 15:1			E:T = 30:1		
	5MTHF (nM)					
	0	30	100	0	30	100
1. M	16	14	11	37	35	28
2. M	NA	NA	NA	NA	NA	NA
3. M	36	58	51	51	71	72
4. F	33	34	15	57	61	33
5. M	29	31	41	53	53	57
6. F	35	50	29	58	67	55
7. M	39	42	51	70	71	76
8. M	15	25	22	37	52	40
9. F	16	13	20	23	23	35
10. M	25	19	19	47	42	38
11. F	43	50	51	66	72	73
Total[‡]	28,70 ± 10,25	33,60 ± 16,01	31,00 ± 16,01	49,90 ± 14,36	54,70 ± 16,95	50,70 ± 18,26

[α] 5-MTHF, 5-methyltetrahydrofolic acid; NA, non analyzed.
Two-way Anova.
[*] E:T: target cell ratio.
[†] PBMC: human peripheral blood mononuclear cells.
[‡] Values expressed as mean of % of cytotoxicity ± SD.

Effect of FA and 5-MTHF on the induction of NKG2D, KIR2DL3, and KIR2DL4 receptors surface in HPNK

We analyzed the expression of the NK activating NKG2D and KIR2DL4 receptors as well as the inhibitory receptors KIR2DL3 in HPNK of four healthy donors. After 96 h of in vitro culture without folate or supplemented with FA or 5-MTHF (30 nM or 100 nM), there were no statistically significant changes in the percentage of HPNK receptor-positive cells of the four donors, as shown in Table 5.

Discussion

Our results demonstrate that in vitro, NK cell activity is not modifiable by the concentration of FA or 5-MTHF in culture medium, confirming our in vivo results, showing no association between serum folate levels and NK cell activity [20]. Moreover, even in a folate-free culture medium, NK cell activity does not change. These results refuse the hypothesis that folate can modulate NK cytotoxicity in a U shaped fashion. This assumption was based on animal and human studies. Kim et al. showed that folate deficiency can diminish NK cytotoxicity in rats [21]. Troen et al. [16] found an inverse relation between the presence of unmetabolized FA in plasma and NK cytotoxicity activity among women aged 60 y to 75 y.

On the contrary, our results are in concordance with a study in an Italian population of healthy older people (90–106 y old), which did not find an association between plasma total folate concentration and NK cytotoxicity [22].

The function of NK cells is specifically regulated by an equilibrium between numerous inhibitory and activating cellular receptors. Killer immunoglobulin-like receptors (KIR) are the main family of human leukocyte antigen (HLA) class I specific receptors present on NK cells. The interaction between inhibitory KIR on NK cells and their HLA ligands leads to the generation of a negative signal, which inhibits their cytolytic activity, preventing target cell lysis. KIR2DL3 or KIR2DL4 activators may serve as inhibitory receptors, which play a relevant role in tumor cell lysis [23]. The clonal distribution pattern is entirely maintained by CpG DNA methylation. Maintenance of DNA methylation depends on DNA methyltransferase 1 (Dnmt1) and intracellular S-adenosylmethionine levels, and is inhibited by S-adenosylhomocysteine. Inhibition of the DNA methyltransferase (DNMT) by 5-aza-2′-deoxycytidine (5-Aza-dC) leads to a global expression of KIRs on all NK cells [24]. Thus, folate as an important cofactor in DNA methylation process could regulate the expression of these receptors and subsequently modify NK cell activity. Because we did not demonstrate changes in NK activity, the lack of modifications in KIR2DL3, KIR2DL4, and NKGD2 expression in HPNK cells cultured with different FA or 5-MTHF concentrations, was foreseeable. Li Y et al. showed that restricting folate increased KIR2DL2 mRNA levels in older people but not in young people. Moreover, they observed that KIR2DL2 levels were higher in CD4+ T cells from older compared with younger donors, cultured in media containing 40 nM folate or 10 nM folate [19]. To avoid the effect of age in NK cell activity and KIR expression, we cultured NK cells from young donors. Moreover, in this study we did not observe any effect with lower or higher concentration of FA or 5-MTHF in the culture medium.

In conclusion, our in vitro studies show that the cytolytic activity of PBMC and purified NK cells, as well as the expression of KIR2DL3, KIR2DL4, NKG2D, remains unchanged in the presence of supraphysiological concentrations of FA or 5-MTHF.

Table 5
Effect of folic acid and 5-MTHF[α] on the expression of KIR2DL3, KIR2DL4 and NKGD2 in HPNKC

	Positive cells (%)		
	KIR2DL3-FITC	KIR2DL4-PE	NKG2D-APC
0 nM FA[β] or 5-MTHF	5.2 ± 2.9[*]	44.2 ± 8.7	28.0 ± 9.4
30 nM FA	6.8 ± 5.2	44.7 ± 5.4	24.7 ± 7.1
100 nM FA	8.5 ± 8.7	48.9 ± 3.7	21.9 ± 8.4
30 nM 5-MTHF	8.7 ± 6.7	47.9 ± 2.1	25.6 ± 9.4
100 nM 5-MTHF	4.6 ± 3.5	39.6 ± 4.5	26.5 ± 11.7

[β]FA, folic acid; [α]5-MTHF, 5-methyltetrahydrofolic acid.
Two-way Anova.
HPNK, highly purified natural Killer cell HPNK were obtained from three individuals and cultured during 96 h in the presence or absence of FA or 5-MTHF.
[*] Results represent the mean of three independent experiments ± Std error.

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