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Folate Deficiency Inhibits the Proliferation of Primary Human CD8$^+$ T Lymphocytes In Vitro[1]

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Folate is required for one-carbon transfer reactions and the formation of purines and pyrimidines for DNA and RNA synthesis. Deficiency of folate can lead to many clinical abnormalities, including macrocytic anemia, cardiovascular diseases, birth defects, and carcinogenesis. The nucleotide imbalance due to folate deficiency causes cell cycle arrest in the S phase and uracil misincorporation into DNA, which may result in DNA double-strand breaks during repair. The role of folate in the immune system has not been fully characterized. We cultured PHA-activated human T lymphocytes in varying concentrations of folate, and measured proliferation, cell cycle, apoptosis, uracil misincorporation, and proportions of Th cells (CD4$^+$) and cytotoxic T (CD8$^+$) cells. Folate deficiency reduced proliferation of T lymphocytes, induced cell cycle arrest in the S phase, induced apoptosis, and increased the level of uracil in DNA. Folate deficiency also increased the CD4$^+$ to CD8$^+$ ratio due to a marked reduction of CD8$^+$ cell proliferation. Folate or nucleoside repletion of folate-deficient cells rapidly restored T lymphocyte proliferation and normal cell cycle, reduced the DNA uracil content, and lowered the CD4$^+$ to CD8$^+$ ratio. These data suggest that folate status may affect the immune system by reducing the capacity of CD8$^+$ cells to proliferate in response to activation. ***The Journal of Immunology*****, 2004, 173: 3186–3192.**

Folate is an essential vitamin that is involved in various biochemical reactions. Folate is required for one-carbon transfer reactions, and the formation of purines and pyrimidines for DNA and RNA synthesis (1). Before the recent folate fortification of grains, the percentage of the U.S. population that had a low folate intake (<50% of the recommended daily allowance) was 50% for women and 25% for men (2, 3). Although food fortification increased folate intake in the U.S. population (4), folate deficiency is common in many other developed and developing countries (5). Deficiency of folate can lead to many clinical abnormalities, including macrocytic anemia, cardiovascular diseases, birth defects, and carcinogenesis (6). The role of folate in carcinogenesis has been best studied in colorectal cancer (7), although epidemiologic studies have also associated folate deficiency with cancers of the lung, esophagus, stomach, brain, cervix, pancreas, and breast, and with leukemia (1, 8). A proposed mechanism by which folate deficiency leads to carcinogenesis is the misincorporation of uracil during DNA synthesis, as folate is required for the synthesis of deoxythymidylate (dTMP)[5] from deoxyuridylate (dUMP), and hence for maintaining a proper ratio of dTMP to dUMP (9). Enzymatic removal of uracil during DNA repair causes transient single-strand breaks; the repair of two nearby opposing lesions can cause a double-strand break, which is the primary cause of chromosomal aberrations and a cause of cancer (10–12). It has also been proposed that alterations in DNA methylation due to folate deficiency contribute to carcinogenesis by modulating gene expression (13). Impaired immune function is also involved in the development of many cancers (14, 15), although the impairment of immune functions is generally not considered to be a mechanism by which folate deficiency may modulate carcinogenesis. Folate plays a crucial role in nucleotide synthesis, and thus may affect immune cell proliferation and responsiveness.

Folate deficiency has been shown to reduce proliferation of various cell types (16–18). Cells lacking folate accumulate in the S phase due to nucleotide imbalance and slow DNA synthesis; such cells also have increased uracil misincorporation and DNA damage (18, 19). When folate is added back to folate-deficient cells, there is a reversal of the S phase accumulation, and proliferation is restored (18, 19). Folate deficiency is more likely to affect proliferative cells, which have a higher nucleotide requirement. It is not known, however, whether the proliferation rate is the only factor determining which cell types are sensitive to the lack of folate.

In humans, folate deficiency has been shown to reduce the proportion of circulating T lymphocytes and their proliferation in response to mitogen activation, which in turn, decreases resistance to infections (reviewed in Ref. 20). The folate antagonist methotrexate, which is used in the treatment of autoimmune and chronic inflammatory diseases, inhibits thymidine synthesis and induces apoptosis of activated T lymphocytes (21, 22). Folate deficiency has also been associated with faster disease progression after infection of T lymphocytes by HIV type 1 (HIV-1) (23, 24). Risk of acute lymphocytic leukemia is increased by low folate status (25, 26). However, NK cell-mediated cytotoxicity is not impaired by folate deficiency (27).

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[5] Abbreviations used in this paper: dTMP, deoxythymidylate; dUMP, deoxyuridylate; GC-MS, gas chromotography-mass spectrometry.

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Although it has been shown that folate deficiency affects immune cell proliferation and function, it is not known whether some subpopulations of immune cells are more sensitive than others to the lack of folate. To better understand how folate deficiency increases cancer risks and decreases resistance to infection, we investigated the effects of folate deficiency on mitogen-activated human T lymphocytes. We measured proliferation of subpopulations of lymphocytes, cell cycle distribution, apoptosis, and uracil incorporation. We show that the ratio of $CD4^+$ (Th) cells to $CD8^+$ (cytotoxic T) cells was increased in folate-deficient conditions, and this was due to a marked decrease in $CD8^+$ cell proliferation. Folate supplementation rapidly restored the rate of $CD8^+$ cell proliferation, and thus restored the $CD4^+$ to $CD8^+$ cell ratio. These results may advance our understanding of how folate deficiency affects the immune function.

Materials and Methods

Cell culture and proliferation

Fresh human mononuclear cells were obtained from AllCells (Berkeley, CA) and cultured in folate-free RPMI 1640 medium (Invitrogen, Carlsbad, CA), supplemented with 10% dialyzed FBS (Invitrogen), and antibiotics. With the addition of dialyzed FBS, the folate content was 0.6 nM as determined by microbiological assay with *Lactobacillus casei* (data not shown). Folate (pteroyl-L-glutamic acid; Sigma-Aldrich, St. Louis, MO) was added to the folate-free medium to a final concentration of 0, 6, 12, 30, or 3000 nM folate. PHA (22.5 μg/ml, Sigma-Aldrich) and IL-2 (10,000 U/ml; Sigma-Aldrich) were added on day 0 to ensure activation and proliferation of T lymphocytes (28). Every three days, the cells were spun down and resuspended at 0.5×10^6 cells/ml in fresh medium (retaining 10% of spent medium), folate, and IL-2 (10,000 U/ml) for a total of 10 days of culture. For the repletion experiments, cells were first cultured for 7 days in folate-deficient medium, and then repleted with either 3000 nM folic acid, or 20 μM thymidine, or a nucleoside mix (20 μM thymidine, 20 μM deoxycytidine, and 60 μM adenosine). Proliferation kinetics were measured at 24-h intervals from day 6 to day 10, using a Coulter Counter (Beckman Coulter, Fullerton, CA) to count cell density.

Flow cytometry

Cells (0.5×10^6) were harvested, washed with PBS containing 1% BSA and 1% FBS, and incubated for 30 min at room temperature with 20 μl of FITC-conjugated anti-CD4 (or anti-CD8) Ab and 20 μl of PE-conjugated anti-CD3 Ab. After a wash in PBS, cells were analyzed using a FACSort flow cytometer (BD Biosciences, San Jose, CA), and the percentages of T cells ($CD3^+$) that were Th cells ($CD4^+$) or cytotoxic T cells ($CD8^+$) were calculated. As a control, cells (0.5×10^6) were incubated with 20 μl of FITC-conjugated anti-CD4 Ab and 20 μl of PE-conjugated anti-CD8 Ab, to ensure that only $CD4^+CD8^-$ or $CD4^-CD8^+$ cells were present, and that there were no $CD4^+CD8^+$ cells (immature T lymphocytes) (data not shown).

Cell cycle analysis

Cells (0.5×10^6) were harvested, washed with PBS, permeabilized with 0.3% saponin, treated with 100 μg/ml RNase A, and stained with 10 μg/ml propidium iodide. After 20 min incubation at room temperature, the distribution of DNA content was measured with a FACSort flow cytometer. The percentages of cells in the G_1, S, or G_2/M phases of the cell cycle were determined using the ModFit software (BD Biosciences). To measure cell cycle distribution for subpopulations of T lymphocytes, cells (0.5×10^6) were first incubated for 30 min with 20 μl of FITC-conjugated anti-CD4 (or anti-CD8) Ab in PBS containing 1% BSA and 1% FBS, and then permeabilized in 0.3% saponin, containing 100 μg/ml RNase A and 10 μg/ml propidium iodide. The distribution of DNA content for $CD4^+$ (or $CD8^+$) cells was measured using a FACSort flow cytometer and the ModFit software.

Apoptosis measurement

Cells (0.5×10^6) were harvested, washed with PBS containing 1% BSA and 1% FBS, and incubated for 30 min at room temperature with 20 μl of PE-conjugated anti-CD4 (or anti-CD8) Ab. Cells were washed with PBS and incubated for 15 min at room temperature in a binding buffer containing Annexin V-Fluorescein (Roche Molecular Biochemicals, Indianapolis, IN). The percentage of $CD4^+$ (or $CD8^+$) that were labeled with annexin V was measured using a FACSort flow cytometer.

Uracil measurement

Uracil analysis was conducted using gas chromotography-mass spectrometry (GC-MS), in a modification of the method by Blount and Ames (29). Cells (5×10^6 cells) were harvested after 10 days of culture, and the DNA was purified using the Qiagen DNeasy kit (Qiagen, Valencia, CA). Three micrograms of DNA were incubated for 1 h at 37°C with 0.2 U of uracil DNA glycosylase (Epicentre Technologies, Madison, WI). After digestion, 50 pg of heavy uracil (m.w. = 343; Cambridge Isotope Laboratories, Andover, MA) was added to each tube, samples were dried and were resuspended in 61 μl of a 50:10:1 mixture of acetonitrile, triethylamine, *N*1,*N*3-(3,5-bis(trifluoromethyl)benzyl bromide. Water (50 μl) and isooctane (100 μl) were added, and the organic layer was analyzed by GC-MS in negative chemical ionization mode using a Hewlett-Packard 5890-Series II GC-MS machine.

Statistics

Results are expressed as mean ± SEM of four independent experiments, each using cells obtained from a different individual. One-way ANOVA with Dunnett's posthoc test was performed to determine significant differences between treatment groups. A value of $p < 0.05$ was considered statistically significant.

Results

Folate deficiency reduces lymphocyte proliferation and induces arrest in S phase

Normal human T lymphocytes can proliferate in culture for a few weeks when activated on day 0 with PHA and subsequently cultured with IL-2. Lymphocytes do not proliferate without PHA activation; with PHA activation and no IL-2, cells proliferate at ~60% of the rate of cells that have been both PHA-activated and cultured with IL-2 (data not shown). About 4 days of culture are necessary to deplete intracellular folate levels of fresh lymphocytes (30, 31). The cultures were started with mononuclear cells containing T lymphocytes, B lymphocytes, monocytes, and NK cells. On day 0, ~50% of the mononuclear cells were positive for CD3, a specific marker of T lymphocytes, and after 6 days of culture >95% of the cells were $CD3^+$ (data not shown). Accordingly, T lymphocyte proliferation measurements were made from day 6 to day 10 of culture. Control cells were cultured in 3000 nM folate, the concentration found in regular RPMI 1640 medium. A folate concentration of 30 nM in the cell culture medium is similar to physiological concentrations in human plasma, and 12 nM folate in plasma is generally considered to be deficiency in humans (28, 32). Cells cultured in 30 nM folate had a growth curve similar to control cells cultured in 3000 nM folate (Fig. 1*A*). The addition of 12 nM, 6 nM, or 0 nM folate to the cell culture medium resulted in a dose-dependent decrease in proliferation relative to that of cells cultured in 3000 nM folate ($p < 0.001$). Lymphocytes cultured in 12 nM folate had a proliferation rate of ~50% of that of lymphocytes cultured in 3000 nM folate; complete folate deficiency (0 nM) almost completely abolished lymphocyte proliferation. Folate deficiency did not inhibit PHA activation, as cells that had been PHA activated on day 0, then blocked in S phase by culturing for 7 days in 0 nM folate, could begin to proliferate when folate was then added back (see Fig. 4). However, T lymphocytes cultured without PHA activation on day 0 did not proliferate and were stopped in the G_1 phase of the cell cycle (data not shown).

When lymphocytes were cultured for 10 days in various folate concentrations, there was an inverse relationship between S phase arrest and folate concentration (Fig. 1*B*). Cells cultured in 30 nM folate showed a cell cycle distribution similar to that of control cells, whereas cells cultured in 12 nM, 6 nM, and 0 nM folate showed a significant increase in the proportion of cells in the S phase ($p < 0.001$), suggesting that folate-deficient lymphocytes have an impaired capacity to synthesize DNA and to complete the cell cycle.

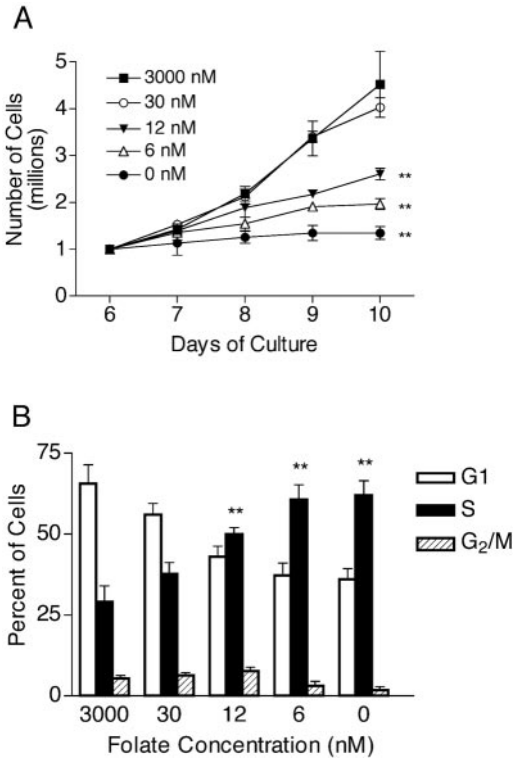

FIGURE 1. Folate deficiency reduced lymphocyte proliferation and induced arrest in S phase. Primary human lymphocytes were cultured for 10 days in varying folate concentrations. *A*, Proliferation was measured by counting cells from day 6 to day 10 of culture. **, $p < 0.001$ lower number of cells on day 10 compared with 3000 nM. *B*, Cell cycle distribution was measured on day 10 of culture by propidium iodine staining. **, $p < 0.001$ increased S phase compared with 3000 nM. Data are expressed as the mean ± SEM of four independent experiments, each using cells obtained from a different donor.

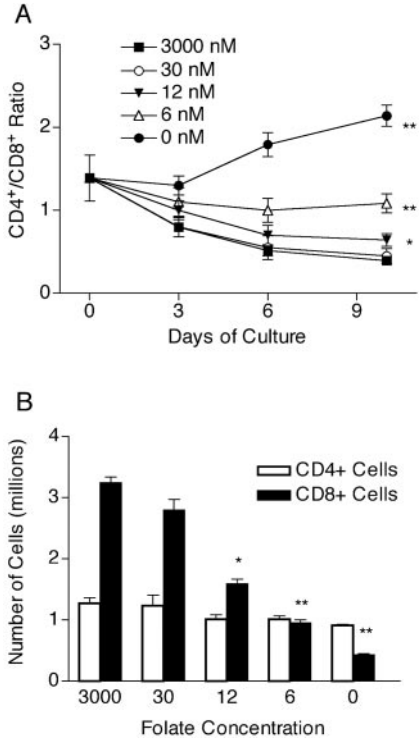

FIGURE 2. $CD4^+$:$CD8^+$ ratio increased and $CD8^+$ cells proliferation decreased in folate-deficient conditions. *A*, Ratio of $CD4^+$ to $CD8^+$ cells from day 0 to day 10 of culture was determined using anti-CD3-PE and anti-CD4-FITC or anti-CD8-FITC Abs. *, $p < 0.05$, **, $p < 0.001$ increased $CD4^+$:$CD8^+$ ratio compared with 3000 nM. *B*, Number of $CD4^+$ and $CD8^+$ cells after 10 days of culture in varying folate concentrations was determined using anti-CD3-PE and anti-CD4-FITC or anti-CD8-FITC Abs. (The cultures were started with 0.3×10^6 $CD4^+$ cells and 0.2×10^6 $CD8^+$ cells) *, $p < 0.05$, **, $p < 0.001$ lower number of CD8 cells on day 10 compared with 3000 nM. Data are expressed as the mean ± SEM of four independent experiments, each using cells obtained from a different donor.

$CD4^+$:$CD8^+$ ratio increases and $CD8^+$ cells proliferation decreases under folate-deficient conditions

Activation of T lymphocytes by PHA and IL-2 resulted in proliferation of both $CD4^+$ (Th) cells and $CD8^+$ (cytotoxic T) cells. Mononuclear cells from different donors had initial $CD4^+$:$CD8^+$ ratios of 1.1:2.2, but after 6 days of culture in different folate concentrations, variation of $CD4^+$:$CD8^+$ ratios between donors was very low (Fig. 2). Within treatment groups, $CD4^+$:$CD8^+$ ratio remained stable from day 6 to day 10 of the culture (Fig. 2*A*). To determine whether one subpopulation of T lymphocytes was more sensitive to folate deficiency than the other, cells were cultured in different folate concentrations, and the $CD4^+$ and $CD8^+$ cell populations were measured. After 10 days of culture in 3000 nM folate, 28% of control cells were $CD4^+$ and 72% were $CD8^+$, giving a $CD4^+$:$CD8^+$ ratio of 0.39 (Fig. 2*A*). Folate deficiency caused a dose-dependent increase in the $CD4^+$:$CD8^+$ ratio. The $CD4^+$:$CD8^+$ ratio was similar to control for cells cultured in 30 nM folate (0.44), but the ratio was 0.64 for cells cultured in 12 nM folate ($p < 0.05$), 1.08 for 6 nM ($p < 0.001$), and 2.12 for 0 nM folate ($p < 0.001$). The increased $CD4^+$:$CD8^+$ ratio under conditions of folate deficiency is likely due to a lower proliferation rate of $CD8^+$ cells, compared with $CD4^+$ cells. The cultures were started with 0.3×10^6 $CD4^+$ cells and 0.2×10^6 $CD8^+$ cells and after 10 days of culture in varying folate concentrations, $CD8^+$ cell number was up to 3.2×10^6 for 3000 nM, but was only 1.6×10^6 for 12 nM ($p < 0.05$), 0.9×10^6 for 6 nM ($p < 0.001$), and 0.4×10^6 for 0 nM ($p < 0.001$) (Fig. 2*B*). $CD4^+$ cell proliferation was not significantly affected by folate deficiency: after 10 days of culture, number of $CD4^+$ cells was from 1.3×10^6 cells for 3000 nM to 0.9×10^6 cells for 0 nM.

S phase arrest is similar for $CD8^+$ and $CD4^+$ cells, but apoptosis is higher for $CD4^+$ cells at a moderate level of folate deficiency

To understand why folate deficiency reduced the proliferation of $CD8^+$ cells more than $CD4^+$ cells, cell cycle distribution and apoptosis were measured for both cell types. Fig. 3*A* shows the proportion of cells in the S phase for both $CD4^+$ and $CD8^+$ cells. A slightly higher proportion of $CD8^+$ cells were arrested in S phase than $CD4^+$ cells. The difference between S phases was 1.3% for cells cultured in 12 nM folate, 4.2% for 6 nM folate, and 10.8% for 0 nM folate. Although the difference between $CD4^+$ and $CD8^+$ cells arrested in S phase was not statistically significant, it was observed for each folate concentration, and in four independent experiments made with lymphocytes from different donors.

Folate deficiency has been shown to induce apoptosis of T lymphocytes in culture (33). In addition to the S phase arrest, the reduced proliferation of $CD8^+$ cells could also be due to an increase in apoptosis. Cells were harvested after 10 days of culture, and apoptosis was determined for $CD4^+$ and $CD8^+$ cells by staining with annexin V. Fig. 3*B* shows that apoptosis was induced in both $CD4^+$ and $CD8^+$ cells, but surprisingly in a lower proportion

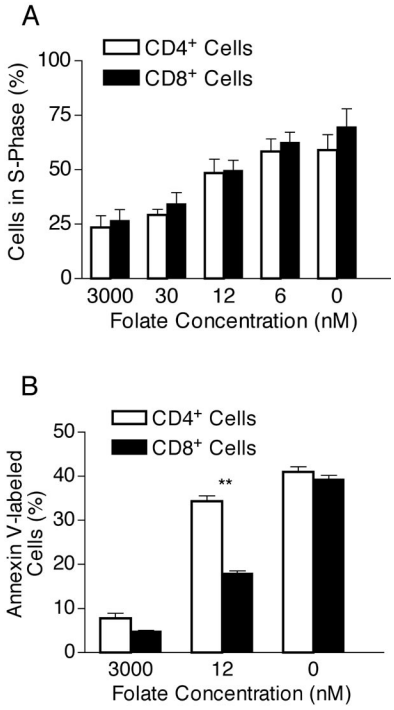

FIGURE 3. S phase arrest and apoptosis for $CD8^+$ and $CD4^+$ cells. *A*, Percentage of $CD4^+$ and $CD8^+$ cells in the S phase of the cell cycle after 10 days of culture in varying folate concentrations. Cells were stained with propidium iodine and labeled with anti-CD4-FITC or anti-CD8-FITC Ab. *B*, Percentage of apoptotic $CD4^+$ and $CD8^+$ cells after 10 days of culture in varying folate concentrations. Cells were labeled with annexin V and with anti-CD4-PE or anti-CD8-PE Ab. **, $p < 0.001$ increased apoptosis for $CD4^+$ cells compared with $CD8^+$ cells. Data are expressed as the mean ± SEM of four independent experiments, each using cells obtained from a different donor.

of $CD8^+$ cells. Cells cultured in 3000 nM folate had <10% of apoptotic cells for both $CD4^+$ and $CD8^+$ cells. At the moderate level of folate deficiency (12 nM), 18% of $CD8^+$ cells were stained with annexin V, whereas 34% of $CD4^+$ were ($p < 0.001$). In complete folate deficiency, both $CD4^+$ and $CD8^+$ cells had ~40% of their cells stained with annexin V.

Arrest of $CD8^+$ cell proliferation is reversed by the addition of either folate or a mix of nucleosides

Knowing that folate deficiency stops $CD8^+$ cell proliferation by inducing an S phase arrest of the cell cycle, we determined whether this block was reversed by folate repletion. Lymphocytes were cultured for 7 days in 0 nM folate, replenished with 3000 nM folate (or different nucleosides), and proliferation was measured for 3 days (Fig. 4*A*). Folate repletion restored the proliferation of folate-deficient cells; the growth curve after repletion was similar to the curve of control cells cultured for 10 days in 3000 nM folate. Adding only thymidine to folate-deficient cells did not restore proliferation. Adding the nucleoside mix (thymidine, deoxycytidine, and adenosine) did restore proliferation, although the growth curve was slightly lower than the curve of control cells.

Folate repletion, by allowing thymidine and purine synthesis, is likely to restore proliferation by reversing the S phase arrest. Cell cycle distribution was measured 24 h after repletion (Fig. 4*B*). There was a significant decrease in the proportion of cells in the S phase after folate repletion, and the cell cycle distribution was similar to control cells that had been cultured in 3000 nM folate. The nucleoside mix, but not thymidine alone, reversed the S phase arrest of folate-deficient cells. Similar results were observed when lymphocytes were cultured in 6 or 12 nM folate for 7 days before repletion with folate, thymidine, or the nucleoside mix (data not shown).

Because folate repletion restored proliferation and reversed the S phase arrest of folate-deficient cells, we examined whether the proliferation of $CD8^+$ cells and the $CD4^+$:$CD8^+$ ratio were affected by folate repletion. Subpopulations of T lymphocytes were measured 24 h after repletion. Fig. 4*C* shows that folate repletion restored the proliferation of arrested $CD8^+$ cells, so that the $CD4^+$:$CD8^+$ ratio was significantly decreased from 2.0 for folate deficient cells to 0.64 for folate-replenished cells. Two days after folate repletion, the $CD4^+$:$CD8^+$ ratio of replenished cells (0.41) was similar to control cells cultured in 3000 nM folate (0.39) (data not shown). Consistent with the cell cycle data, the nucleoside mix, but not thymidine alone, decreased the $CD4^+$:$CD8^+$ ratio of folate-deficient cells to 0.69 (Fig. 4*C*). Similar results were found when lymphocytes were cultured in 6 or 12 nM folate for 7 days before repletion with folate, thymidine, or the nucleoside mix (data not shown).

In addition to cell cycle arrest, the reduced proliferation of T lymphocytes could be due to increased uracil misincorporation, leading to DNA breaks. Uracil content of nuclear DNA was measured after 10 days of culture in different folate concentrations, and 24 h after folate (or nucleoside) repletion of folate-deficient cells. Cells cultured in 0 nM folate, which were not proliferating, had only 1.9 pg uracil/μg DNA, a level slightly higher than control cells (1.0 pg uracil/μg DNA) (Fig. 4*D*). Cells cultured in low concentrations of folate (6 and 12 nM) had a high level of uracil incorporation (4.7 and 5.9 pg uracil/μg DNA, $p < 0.001$ and $p < 0.05$), compared with control. When cells were cultured for 9 days in 12 nM folate and were then replenished for 24 h with folate, thymidine, or the nucleoside mix, the level of uracil incorporation decreased to a level similar to control cells.

Discussion

It has long been known that folate has a role in maintaining the immune system, but this role has not been fully characterized. Folate status is known to modulate immunocompetence and resistance to infections, and to affect cell-mediated immunity by reducing circulating T lymphocytes and decreasing response to mitogen activation (20). Although folate status is known to affect T lymphocyte function, it was not known whether any specific subpopulations of T lymphocytes are more affected by the lack of folate. We show in this study that the proliferation of activated $CD8^+$ cells is more sensitive to the lack of folate than $CD4^+$ cells.

Immature T lymphocytes ($CD4^+CD8^+$) are processed in the thymus and released into peripheral blood in two different subpopulations: Th cells ($CD4^+$ cells), whose function is to activate other immune cells, and CTLs ($CD8^+$ cells), which recognize and destroy infected cells, foreign tissue, and tumor cells. The ratio between the two subpopulations of T lymphocytes is important for proper immune function, and a change in the $CD4^+$:$CD8^+$ ratio has been observed in various diseases (34–36). In the present study, the $CD4^+$:$CD8^+$ ratio changed markedly with the varying folate concentrations. In folate-sufficient conditions, both $CD4^+$ and $CD8^+$ cells proliferated, although $CD8^+$ cells proliferated faster than $CD4^+$ cells after mitogen activation. When T lymphocytes were cultured in lower folate concentrations, the proliferation of $CD8^+$ cells decreased more than the proliferation of $CD4^+$ cells; thus the $CD4^+$:$CD8^+$ ratio was increased. The $CD4^+$:$CD8^+$ ratio was doubled in moderate folate deficiency (12 nM), and was increased 5-fold in complete folate deficiency. Folate deficiency is known to influence the number of circulating T lymphocytes, but

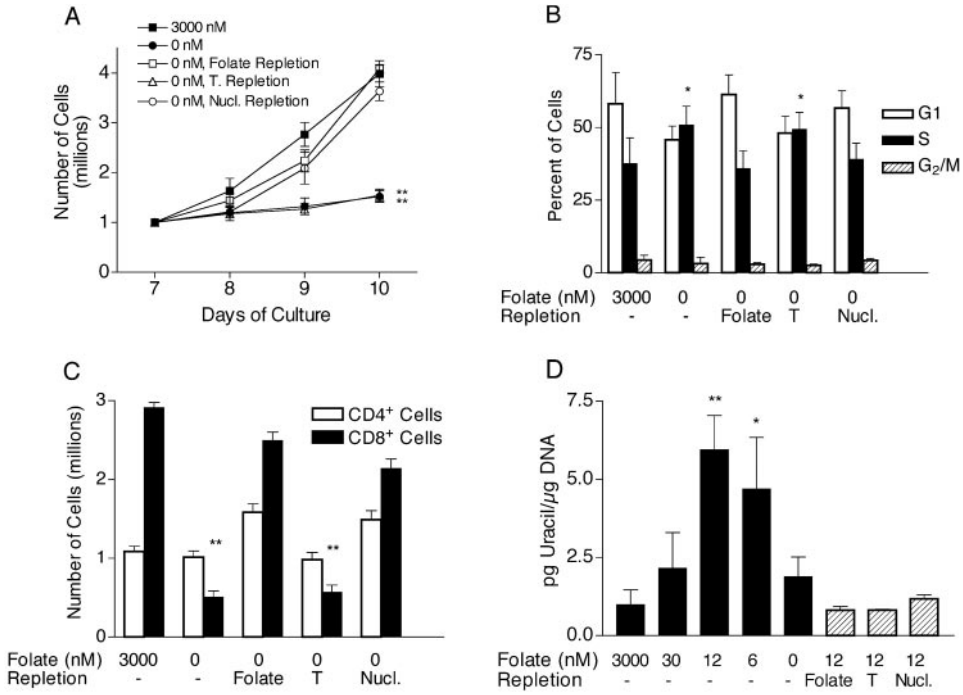

FIGURE 4. Arrest of $CD8^+$ cell proliferation is reversed by the addition of either folate or a mix of nucleosides. Primary human lymphocytes were cultured for 10 days in varying folate concentrations On day 7, folate-deficient cells were replenished with 3000 nM folate (Folate), or 20 μM thymidine (T), or a nucleoside mix of 20 μM thymidine, 20 μM deoxycytidine, and 60 μM adenosine (Nucl.). *A*, Proliferation was measured by counting cells from day 7 to day 10 of culture. **, $p < 0.001$ lower number of cells on day 10 compared with 3000 nM. *B*, Cell cycle distribution was measured 24 h after repletion by propidium iodine staining. *, $p < 0.05$ increased S phase compared with 3000 nM. *C*, Number of $CD4^+$ and $CD8^+$ cells was determined 24 h after repletion using anti-CD3-PE and anti-CD4-FITC or anti-CD8-FITC Abs. **, $p < 0.001$ lower number of CD8 cells on compared with 3000 nM. *D*, DNA uracil content of primary human lymphocyte was measured by GC-MS after 10 days of culture in varying folate concentration, or 24 h after repletion of folate-deficient cells. *, $p < 0.05$, **, $p < 0.001$ increased uracil content compared with 3000 nM. Data are expressed as the mean ± SEM of four independent experiments, each using cells obtained from a different donor.

to our knowledge, it has not been shown to specifically reduce $CD8^+$ cell proliferation after activation. Other micronutrients are known to affect immune function, but most studies have shown effects on $CD4^+$ cells (34, 37). For example, zinc is required for intrathymic maturation of $CD4^+$ cells, and zinc deficiency can lower the $CD4^+$:$CD8^+$ ratio (38). Vitamin A deficiency has also been shown to reduce the $CD4^+$ cell proportion in humans (39). A few in vivo studies showed a decrease in $CD8^+$ cell population with deficiencies in vitamin D (40), vitamin E (41), and vitamin B12 (42–44). Interestingly, vitamin B12 plays an important role in DNA metabolism and B12 deficiency can cause uracil misincorporation into DNA by the same mechanism as folate deficiency

To better understand how folate deficiency lowers $CD8^+$ cell proliferation, we measured the cell cycle distribution of cultured T lymphocytes. At the end of the culture period, there were a greater percentage of cells in S phase at folate-deficient conditions (0, 6, and 12 nM folate) vs folate sufficiency. However, there was no significant difference between the proportions of $CD8^+$ cells that were arrested in the S phase vs $CD4^+$ cells at any of the experimental folate concentrations. Although lower $CD8^+$ cell proliferation in folate deficiency could be explained by $CD8^+$ cells having a higher requirement for nucleotides to proliferate, it remains unclear why $CD4^+$ cells are able to proliferate (calculated from Figs. 1 and 2) in folate deficiency while the proliferation of $CD8^+$ cells seems to have completely stopped. $CD4^+$ cells may have a more efficient folate intake than $CD8^+$ cells in low folate conditions. Interestingly, this phenomenon was completely reversible. When folate was added to folate-deficient T lymphocytes, proliferation and normal cell cycle were rapidly restored. Only 6 h after folate repletion, a substantial proportion of cells were in the G_2/M phase, indicating that these cells were able to complete their DNA synthesis and were undergoing mitosis (C. Courtemanche and B. N. Ames, manuscript in preparation). After 24 h, the cell cycle and the proliferation rates of folate-replenished cells was similar to that of cells cultured in folate-sufficient conditions. This suggests that the decreased proportion of $CD8^+$ cells was due to a cytostatic effect. The decreased proportion of $CD8^+$ cells in folate deficiency does not seem to be due to an increase in apoptosis, as a smaller percentage of $CD8^+$ cells were apoptotic as compared with $CD4^+$ cells.

These findings contrast with the suggested mechanism of operation of the folate antagonists, methotrexate and tomudex, which are believed to induce apoptosis of activated T lymphocytes (21, 22). Moreover, thymidine completely abolishes the apoptosis-inducing effects of methotrexate and tomudex (21), which was not the case in our study. Folate is involved in many biochemical pathways, and it is likely that folate antagonists, which inhibit specific targets, produce different cellular effects than folate deficiency. In our study, the cell cycle arrest due to folate deficiency could be reversed using a mix of nucleosides, containing thymidine, adenosine as a source of purines, and deoxycytidine to overcome the feedback inhibition by dTTP on the reduction of cytidine to deoxycytidine (45). This nucleoside mix, but not thymidine alone, can also reverse the accumulation in the S phase of folate-deficient erythroblasts (16).

In addition to impaired thymidine and purine synthesis, folate deficiency can lead to elevated homocysteine, which can increase oxidative stress (46, 47), and hypomethylation of DNA, which can affect gene expression (8, 48). However, our results suggest that

the growth arrest of $CD8^+$ cells was rather due to nucleotide imbalance, because normal proliferation and cell cycle of folate-deficient cells were restored with a combination of nucleosides. It is possible that $CD8^+$ cell proliferation could also have been reduced because of increased DNA damage. Folate is required for methylation of dUMP to dTMP (1). Under folate deficiency, the excess of dUMP leads to uracil misincorporation into DNA; repair of two nearby opposing lesions can cause a double-strand break (10, 11). Highly proliferative cells such as mitogen-activated T lymphocytes are likely to have more uracil misincorporation, and thus more DNA double-strand breaks, when cultured in folate-deficient conditions. In our study, T lymphocytes had low DNA-uracil content when grown in low or high levels of folate, whereas cells grown under mild folate deficiency had high levels of DNA-uracil. A proposed explanation is that proliferation and the dUTP:dTTP ratio have opposite effects on DNA-uracil content. Maximum DNA-uracil content might then be expected at mild deficiency in which there exists both a relatively high dUTP:dTTP ratio, and enough folate present that cells are still actively trying to proliferate (49). Unlike $CD8^+$ cells, that appear to simply stop proliferating under low folate conditions, $CD4^+$ cells divide despite folate deficiency. This cessation of proliferation may protect $CD8^+$ cells from DNA damage, and therefore apoptosis, and may explain the higher apoptosis rates in $CD4^+$ cells in comparison with $CD8^+$ cells. It could be that $CD8^+$ cells respond to DNA damage by putting forth a cell division block at low levels of damage, whereas $CD4^+$ cells have a stronger impetus to divide despite DNA damage and accumulate damage until apoptosis is triggered.

PHA activation of T lymphocyte proliferation has been shown in some studies to reduce sensitivity to DNA damage-induced apoptosis (50, 51), but these studies did not distinguish between $CD4^+$ and $CD8^+$ cells. However, other studies have shown that subtypes of PHA-activated T cells can differ in their growth or apoptosis rates. The difference in response of the different T cell subtypes may help illuminate why different stresses can cause different immune responses. Onodera et al. (52) found that naive $CD8^+$ T cells exposed to a strong magnetic field were reduced to 27% of the number of unexposed $CD8^+$ cells, whereas $CD8^+$ memory cells were reduced to 78% of the number of unexposed cells (a nonsignificant decrease); in contrast, $CD4^+$ naive and memory cells had similar decreases after exposure (to 38% and 46%, respectively, of the number of unexposed cells). At 0 nM folate levels, DNA-uracil content is low, indicating that DNA damage is also likely to be low, yet apoptosis rates are highest in both $CD4^+$ and $CD8^+$ cells. It may be that other effects due to folate deficiency, such as oxidative stress and cell cycle blockage, may contribute to this high apoptosis rate. In complete folate deficiency, there is also no difference in apoptosis between $CD4^+$ and $CD8^+$ cells, indicating that these other effects due to folate deficiency become dominant factors in determining apoptosis rates. $CD4^+$ and $CD8^+$ cells may also have a different potential for recognizing and repairing DNA damage. Interestingly, repletion with thymidine lowered uracil levels of folate-deficient cells, although it did not restore proliferation, suggesting that DNA repair was active in arrested cells. In support of this hypothesis, when [^{3}H]thymidine was added to folate-deficient cells, the thymidine was rapidly incorporated into the cells (data not shown). Measurements of uracil levels and DNA repair genes for each subpopulation of T lymphocytes may help to clarify whether $CD8^+$ proliferation could also have been reduced because of increased DNA damage.

In addition to causing an S phase arrest and increased DNA damage, folate deficiency could also interfere with the PHA activation of $CD8^+$ cells, or with potential interactions between $CD4^+$ and $CD8^+$ cells. In mice, treatment with high levels of folate (50 mg/kg) enhances the IFN-β-induced production of extrathymic $CD4^+$ and $CD8^+$ cells, as well as the antimetastatic activity of $CD8^+$ cells (53). Folate deficiency could have the reciprocal effect on PHA-activated $CD8^+$ cells, although it does not likely involve an irreversible inhibition of PHA activation. The repletion experiment showed that $CD8^+$ cell proliferation may follow the addition of folate to cells cultured in 0 nM folate, 1 wk after the PHA treatment.

Further research is needed to fully characterize how folate deficiency affects different subpopulations of T lymphocytes, in vitro and in vivo. If folate deficiency reduces in vivo $CD8^+$ cell proliferation after activation, it could impair the capacity of $CD8^+$ cells to eliminate infected or tumor cells; this would provide a third mechanism (in addition to increased DNA damage and altered DNA methylation) by which folate deficiency enhances carcinogenesis. This could also explain why folate deficiency is associated with decreased resistance to infection and faster HIV-1 disease progression (23, 24). HIV-1 is known to reduce $CD4^+$ cells number and function, but $CD8^+$ cells are also affected by the virus (36), and folate deficiency could accelerate this process. Although it may be useful to reduce $CD8^+$ proliferation in the treatment of autoimmune and inflammatory diseases (54, 55), such a reduction of $CD8^+$ cells may be detrimental for normal effective immune function. In summary, we have shown that moderate and high levels of folate deficiency reduce in vitro proliferation of activated $CD8^+$ cells and induce a cell cycle arrest in the S phase. Folate repletion reverses the cell cycle arrest and restores $CD8^+$ cell proliferation.

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