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ORIGINAL ARTICLE

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Natural killer cell activity in chronic lymphocytic leukemia patients treated with fludarabine

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Abstract Fludarabine, the 5′-monophosphate of 9-β-D-arabinofuranosyl-2-fluoroadenine (FaraAMP), is effective in the treatment of chronic lymphocytic leukemia (CLL) and has been demonstrated to increase natural killer (NK) cell lytic activity (NK_a) in humans and mice. To determine the effect of FaraAMP on NK cells in CLL, we analyzed NK_a toward K562 targets after in vitro incubation with FaraAMP and after in vivo exposure to fludarabine. Pretreatment analysis of peripheral blood from 12 CLL patients (9 untreated) revealed: median number of NK cells 500/μl (range 290–1160); median NK_a lytic $unit_{30}/10^6$ cells (range 5–80). These results were similar to those from healthy adult donors. After exposure to 3, 30, or 300 μ*M* FaraAMP, the median maximum stimulation index (NK_a FaraAMP/NK_a) was 1.2 (range 0.9–1.5), within the range observed in normal adults. FaraA also stimulated NK_a in vitro toward autologous CLL cells in two of five patients as measured by a dye-exclusion assay. In three patients following three or more treatment courses of fludarabine (30 mg/m^2 per day for 5 days) the NK cell number and NK_a were maintained near pretreatment values. Phenotypic analysis of the peripheral mononuclear cells in 34 consecutive CLL patients revealed a marked reduction in CD5/CD20 and CD4 cell numbers after three courses of fludarabine with less effect on CD8 and CD56 cells. These results indicate that fludarabine spares NK cells and may stimulate NK_a in some CLL patients.

Key words Natural killer · Chronic lymphocytic leukemia · Fludarabine

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Introduction

Fludarabine monophosphate, 9-(β-D-arabinofuranosyl)-2-fluoroadenine 5′-monophosphate (FaraAMP), is a prodrug for arabinosyl-2-fluoroadenine (FaraA) that has pronounced clinical activity toward chronic lymphocytic leukemia (CLL) and other low-grade lymphocytic malignancies [15, 16, 29, 30]. The triphosphate of this drug acts to inhibit proliferation of cells due to various effects on DNA polymerases, ribonucleotide reductase, DNA primase and DNA ligase I [4, 10, 21, 35]. Certainly, fludarabine is extremely toxic toward proliferating cells, and it prevents lymphocytic immune responses when added to cultures during the proliferative phase [26]. On the other hand, FaraAMP has the interesting property of stimulating certain immune responses when it is present during the "effector" phase such as that observed with activated T cells [24, 26]. Natural killer (NK) cells do not require proliferation for their activity and FaraAMP enhances NK cell activity (NK_a) in mouse and human lymphocytes in vitro [24, 26], and in mice in vivo [24]. Clearly, this property of FaraAMP is distinct from its cytotoxic properties toward proliferating cells, and this action may be mediated via the inhibitory P-site of adenylyl cyclase [25]. Although the stimulation of NK_a is relatively small (approximately 50% increase at the most

effective doses for the biological in vitro assays), it has pharmacological consequences since the inhibitory effects of the drug on antibody production are attenuated in mice lacking NK cells, i.e. beige mutation [27]. Another toxic nucleoside, tubercidin (Tub), produces exactly opposite effects to that of fludarabine; that is, it stimulates antibody formation at doses that inhibit NK_a [24, 26]. The enhancement of antibody formation by Tub is completely abrogated in mice lacking NK_a [27], pharmacological evidence that NK cells provide a modulatory effect on antibody production by B cells [17]. For the murine system studied, the effects of Tub and FaraAMP occur at doses that are chemotherapeutically active. It appears likely, therefore, that FaraAMP has pharmacologic properties that are distinct from mechanisms responsible for its cytotoxic action.

CLL is characterized by the clonal proliferation and accumulation of long-lived, functionally inactive, mature-appearing B lymphocytes. The disease is often accompanied by immunological abnormalities involving B lymphocytes, T lymphocytes and NK cells. Both phenotypic alterations and intrinsic functional defects in NK cells in CLL have been reported [6, 7, 14, 34, 36]. Although the clinical implications of an altered NK population in CLL are not fully understood, association with disease progression [1], increased incidence of second neoplasms [12], infection [1] and hypogammaglobulinemia [13] have been reported. The animal experiments described above in which NK cells were demonstrated to indirectly mediate the effects of FaraAMP on a B-cell function led us to investigate the possible role of NK cells in the action of this drug in CLL. The results indicate that both the absolute number and functional activity of NK cells are normal in CLL, the NK_a is increased by FaraAMP in vitro, and NK cells are spared relative to other lymphocyte subpopulations during FaraAMP therapy. A preliminary report of this work has been presented [31].

Material and methods

Patients and control donors

The peripheral blood of healthy donors and of CLL patients was used in this investigation. All CLL patients fulfilled the National Cancer Institute Working Group (NCI-WG) criteria [5] for the diagnosis of CLL and had been off therapy for at least 6 weeks. Immunophenotyping by dual-parameter flow cytometry revealed coexpression of CD5 with B-cell antigens and isotypic light-chain expression in all but one patient, who was shown to have T-cell CLL. Clinical staging was based on the system described by Rai et al. (28), and similar response criteria to the NCI-WG were used [30].

Freshly obtained blood was fractionated by Ficoll-Hypaque (Winthrop Pharmaceuticals, New York, N.Y.) density sedimentation. Nonadherent mononuclear cells were washed and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 m*M* L-glutamine, penicillin and streptomycin. T-cell depletion was achieved by incubation with anti-CD3 antibody conjugated magnetic beads (Advanced Magnetics, Cambridge, Mass.) for 40 min followed by magnet depletion. NK-cell depletion was achieved by two successive incubations of the lymphocytes with anti-CD16 antibody (Becton-Dickinson, San Jose, Calif.) for 30 min followed by lysis with baby rabbit complement (Calbiochem, La Jolla, Calif.).

NK assay against K562 cells.

The K562 cell line derived from a patient with chronic myelogenous leukemia in erythroid blast crisis was used as a target for human NK_a. K562 cells were maintained in continuous culture in RPMI-1640 medium supplemented as above. For use in the ^{51}Cr-release assay, 5×10^6 cells were incubated with 100–150 μCi of sodium [^{51}Cr]chromate (Amersham Corporation, Arlington Heights, Ill.) for 1 h at 37° C. The release of ^{51}Cr was determined after a 4-h incubation in RPMI-1640 medium supplemented as above. After the 4-h incubation, the released ^{51}Cr was measured using a gamma scintillation counter. In some experiments, various concentrations of FaraAMP (Berlex, Alameda, Calif.) were added directly to the test wells for the 4-h incubation. This agent did not enhance or abrogate the spontaneous release of ^{51}Cr from the target cells in the absence of effector cells during the 4-h incubation period (data not shown). The effector population consisted of T-cell-depleted peripheral blood lymphocytes as described above. The number of effector cells was determined by quantitation of NK cells using dual-parameter flow cytometry. To calculate NK_a, at least four effector:target ratios were used to determine lytic units. The number of NK cells required to release 30% of the ^{51}Cr label was defined as one lytic unit (LU_{30}).

NK assay against CLL cells

CLL cells were purified, as described above, and exposed to various concentrations of FaraAMP with or without prior elimination of NK cells by selective lysis using antiCD16 antibody as described above. After a 4-h drug exposure, viable cells were assessed by trypan blue dye exclusion using a hemacytometer.

Immunophenotyping

Immunophenotyping was performed on peripheral blood collected in EDTA by flow cytometry using a simultaneous dual-color staining technique. This was performed to further document the diagnosis of CLL and determine the absolute NK cell number. Samples were obtained before treatment, 28 days after three treatment courses (immediately prior to the fourth course), and 28 days after six courses of single-agent FaraAMP. The following combinations of phycoerythrin (PE)-conjugated and fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) were used: IgG2-PE/IgG1-FITC (control), CD14-PE/CD45-FITC, HLA-DR-PE/CD2-FITC, CD4-PE/CD8-FITC, CD20-PE/CD5-FITC, CD5-PE/IgM + D-FITC, CD5-PE/k-FITC, CD5-PE/λFITC, CD3-FITC/CD16-PE, and CD3-FITC/CD56-PE. The red blood cells in the sample were lysed by incubation in isotonic ammonium chloride for 10 min, after which the cells were collected and washed. The mAbs were added to the cells (10 μl of each mAb to 1×10^6 cells) and incubated at 2–8° C for 15 min in the dark. The cells were resuspended and fixed with 1% paraformaldehyde. The analysis was performed by FACScan (Becton-Dickinson, San Jose, Calif.) using a Consort 30 program.

Results

Effect of fludarabine on lymphocyte subsets

In 34 consecutive CLL patients, immunophenotyping was performed on peripheral blood by flow cytometry before treatment and after three and six courses of fludarabine. As shown in Fig. 1, after three and six courses of fludarabine, immunophenotyping revealed marked decreases in various lymphocyte subsets. CD20 +cells and CD4 +lymphocytes decreased dramatically. In contrast, the populations expressing CD56 or CD8 were less affected.

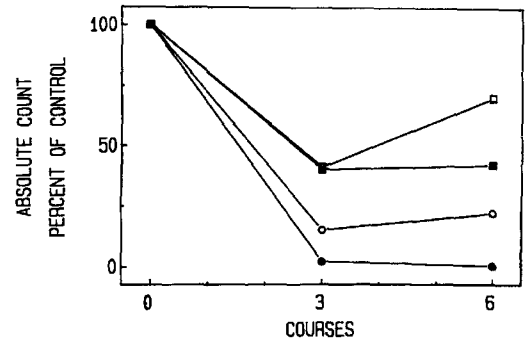

Fig. 1 Effect of fludarabine treatment on lymphocyte subsets. Peripheral blood was obtained before and 28 days after three and six courses of fludarabine (30 mg/m^2 daily for 5 days every 4 weeks) in 34 consecutive CLL patients. Immunophenotyping was performed using flow cytometry and mAb immunofluorescence as described in Materials and methods. Subset values are expressed as the percentage of the absolute count prior to therapy. Median values are shown and the following subsets were analyzed: CD56(□), CD8 (■), CD4 (○), CD20 (●)

NK number and activity in CLL

The absolute number of NK cells and NK_a against K562 targets were determined in 12 patients as described in Materials and methods (Table 1). The number of NK cells was similar to normal controls; however, in three patients NK numbers were increased ($>1000/\mu l$). NK_a was in the normal range in the majority of patients, although, high-level basal cytotoxicity (≥ 50 LU_{30}) was noted in four patients. Neither the NK number nor the NK_a appeared to correlate with clinical stage or prior treatment status in this small group of patients. Treatment of effector cells from CLL patients with 3 to 300 μM FaraAMP during a 4-h incubation was compared with the basal spontaneous NK_a to determine whether FaraAMP exposure enhanced the lysis of K562 targets. The median maximum FaraAMP stimulation index (NK_a in the presence of FaraAMP versus NK_a in the absence of FaraAMP) was 1.2 (range 0.9–1.5). This index did not appear to correspond with the clinical response or disease stage in the seven patients who received therapy with FaraAMP (data not shown). For example, of the three patients having the lowest stimulation index (i.e. 0.90, 0.97, 1.05), two achieved a complete response and the other achieved a partial remission. The stimulation of NK_a in the patients and controls as a function of FaraAMP concentration is illustrated in Fig. 2.

In three CLL patients, NK_a was reassessed 4 weeks after the third or sixth treatment with FaraAMP (Table 2). Although the absolute number of lymphocytes decreased markedly in all three patients, the NK number was relatively unchanged, in accordance with the result from the larger sample given in Fig. 1. Indeed, the NK cells become a significant proportion of the lymphocytes in some FaraAMP-treated patients. This finding suggests that the large numbers of leukemia cells prior to therapy did not interfere with the flow cytometric measurement of NK cells. Likewise, assessment of the NK_a after treatment revealed no marked change from that observed prior to treatment in two patients, whereas the NK_a was reduced to a normal value in the patient having a relatively-high NK_a of 80 LU_{30} prior to therapy.

Peripheral mononuclear cells from five patients were divided into two aliquots. One aliquot was rendered NK cell-deficient by incubation with anti-CD16 antibody and complement (Table 3), and the other aliquot served as control. Untreated and antibody-treated cells were then exposed to three concentrations of FaraAMP to determine whether NK cells were required in vitro for short-term lysis of autologous CLL cells (Table 3). Results obtained with 0 and 3 μM FaraAMP were similar as were the results with 30 and 300 μM (see Fig. 2). Consequently, the results obtained at these concentrations were combined to generate the data given in Table 3. In two of the five patients (patients BT-7 and BT-9), FaraAMP enhanced the lysis of CLL cells. This effect was not observed in the absence of NK cells suggesting that, at least for these two patients, the FaraAMP effect was due to stimulation of NK_a.

Discussion

FaraA and 2-chlorodeoxyadenosine (CldAdo) are analogs of deoxyadenosine (dAdo) that are useful agents in indolent leukemias. Interest in dAdo analogs as potential antileukemic agents was stimulated by the observation that adenosine deaminase (ADA) deficiency is associated with severe combined immunodeficiency disease in a proportion of patients [8]. In the absence of ADA or during its inhibition, dAdo and adenosine (Ado) exhibit toxicity toward a number of lymphoid cells [33]. Thus, it is generally assumed that accumulation of these endogenous substrates mediates immunodeficiency; however, the exact biochemical mechanism is not known. While attempting to mimic in

Table 1 NK cell number and function in CLL patients. NK cell number was determined by flow cytometry and NK cell activity was measured by ^{51}Cr-release from K562 targets as described in Materials and methods (*n.d.* not determined)

Rai stage	Prior therapy	Peripheral blood Lymphocytes ($\times 10^3/\mu l$)	NK cells[a] ($/\mu l$)	NK cell activity[b] ($LU_{30}/10^6$ cells)	Fludarabine stimulation Index[c]
0	−	36.0	349	5	1.21
0	−	11.9	354	5	1.24
0	−	17.4	301	7	1.46
0	−	31.6	294	10	1.26
0	−	31.4	1160	6	1.14
0	−	23.4	290	75	0.97
I	+	10.0	390	50	1.40
I	−	67.3	760	80	1.05
I/II	+	101	1120	5	1.20
II	−	69.7	1050	< 5	n.d.
IV	+	19.5	610	50	1.20
III[d]	−	7.2	680	21	0.90

[a] Normal adult value approximately 100–500/μl
[b] Normal adult median value 5–8, range < 5 to 70, $n = 10$
[c] Maximum increase in cytolytic activity produced by fludarabine, 3, 30 or 300 μ*M* (see Fig. 2)
[d] T-cell CLL patient

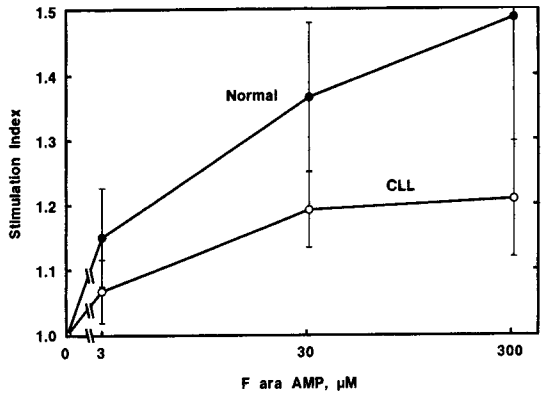

Fig. 2 Stimulation of human NK_a by FaraAMP in vitro. Peripheral blood lymphocytes were harvested from normal adults and from adult CLL patients as described in Materials and methods. The ability of these cells to lyse ^{51}Cr-labelled K562 cells served as a measure of NK_a. Effector to target ratios were selected such that control cells lysed 20–40% of the targets in the absence of FaraAMP. The results shown are mean values ± SE for five (normal) and ten (CLL) subjects

Table 2 NK cell number and activity in CLL patients treated with fludarabine. NK cell number was determined by flow cytometry (see Materials and methods). NK cell activity was measured against K562 targets as described in Materials and methods (*PR* partial response, *CR* complete remission)

	Patient number 1	2	3
Clinical status			
Rai stage	IV	0	I
Numer of course	6	3	3
Clinical response	PR	CR	CR
Pretreatment values			
Lymphocytes/μl	16,400	48,600	67,300
NK cells/μl	290	166	760
NK_a ($LU_{30}/10^6$)	5	5	80
Posttreatment values			
Lymphocytes/μl	400	1900	1200
NK cells/μl	200	160	689
NK_a ($LU_{30}/10^6$)	4	15	3

mice what might occur in humans due to the absence of ADA, we have previously studied the immunomodulatory effects of Ado and dAdo analogs that are poor substrates for ADA [20, 24]. The Ado analog, Tub, and the two dAdo analogs studied, FaraAMP and CldAdo, have opposite effects on NK cells. Specifically, the dAdo analogs stimulate NK and CTL activity while inhibiting primary antibody response to either a T-cell-dependent (sheep red blood cells) or T-cell-independent (TNP-LPS) antigen. Exactly the opposite is observed when Tub is administered to mice, i.e. NK and CTL activity is inhibited while primary antibody response is enhanced [24]. Since NK cells have been reported to negatively modulate primary antibody response, we hypothesized that the diverse effects of these agents on primary antibody response occur indirectly due to their stimulation or inhibition of NK cell activity. This hypothesis has been tested in NK cell-deficient mice, C57/Bl beige mutants [32], and in mice rendered NK cell-deficient by administration of antiasialoGM1 antibody [27]. The stimulation (Tub) or inhibition (FaraAMP) of primary antibody response in the NK cell-deficient mice was abrogated or reduced compared to NK cell-competent controls, i.e. the antibody response occurs secondarily to the effects of these agents on NK cells [27]. These experiments indicate that the

Table 3 Lysis of CLL targets in the presence of FaraAMP. The incubations used 10^5 cells per well. Values shown are the mean number ±S.E. of cells ($\times 10^4$) excluding trypan blue after a 4-h incubation (number of viable cells). Results obtained with 0 and 3 μ*M* FaraAMP or with 30 and 300 μ*M* were combined, $n = 6$. Patients had no prior therapy or had been off therapy ≥ 6 weeks before testing. Samples were rendered NK-deficient by incubation with anti-CD16 antibody and rabbit complement as described in Materials and methods. This antibody treatment reduced the lysis of K562 targets from 15.1 ±1.4% (control) to 1.0 ±0.1% (antibody treated), mean value ±SE, $P < 0.05$, $n = 5$ (these patients)

Patient	+ NK cells		− NK cells		Rai stage
	FaraAMP (μM)		FaraAMP (μM)		
	0 and 3	30 and 300	0 and 3	3 and 300	
BT-7	5.6* ± 0.6	3.3* ±0.3	7.4 ±0.3	7.8 ±0.6	0
BT-11	4.3 ±0.4	5.3 ±0.4	6.0 ±0.6	6.0 ±0.3	0
BT-9	8.1 ±0.4	5.3* ±0.3	7.7 ±0.5	6.9 ±0.4	I
BT-12	4.2 ±0.2	5.2 ±0.5	7.0 ±0.4	6.0 ±0.7	II
BT-8	5.6 ±0.7	4.4 ±0.3	4.8 ±0.7	5.0 ±0.2	IV

*$P < 0.05$ (unpaired *t*-test) versus all other values in this patient

in vivo effects of FaraAMP and Tub on NK cells are real and have physiological consequences. Further, although these agents are extremely cytotoxic, the action on NK cells must occur through a mechanism other than the known actions of these drugs on dividing cells because proliferation is not a requisite for NK_a. The effects of Tub, CldAdo and FaraAMP were observed at chemotherapeutic doses in mice; thus, we were prompted to investigate possible modulation of NK_a by fludarabine in humans.

Fludarabine has become the drug of choice for patients with CLL resistant to treatment with alkylating agents. In spite of the dramatic increase in lymphocytes in the peripheral blood of these patients during the course of their untreated disease, due to their circulating leukemic cells, the number of NK cells and NK_a were relatively normal (Table 1). This is in contrast to other reports in which NK_a has been shown to be decreased in CLL patients [23, 34]. However, several investigators have noted normal activity in some CLL patients [1, 23, 34], and one investigator was able to correlate low cytotoxic activity with advanced clinical stage [34]. Thus, the relatively normal NK_a observed in this study may reflect the early stage and untreated status of our patient population. The B-cell regulatory influences of large granular lymphocytes from CLL patients have previously been reported to be retained [22].

Compared with B cell and helper T cell populations, the NK cells and suppressor/cytotoxic T cells were spared after treatment of CLL patients with fludarabine (Fig. 1). This finding is similar to that previously reported in a group of solid-tumor patients [3]. NK_a in a significant proportion of patients was stimulated by FaraAMP in vitro (Table 1; Fig. 2). The degree of stimulation of the NK cells from healthy donors shown in Fig. 2 is in accordance with the results of our earlier study [26] and with the results observed when mouse splenocytes are cocultured with the drug [25]. The K562-lysis assay is an in vitro bioassay, so it is not possible to translate the quantitative aspects of this assay to the whole organism. For this and other reasons, the results obtained with FaraAMP in beige mutant mice argues in favor of the pharmacological relevance of the assay. Qualitatively similar results have been reported for the related analog, CldAdo, in humans with hairy cell leukemia [18] and for the ADA inhibitor, deoxycoformycin, in mice [19]. It may be significant that the stimulation by FaraAMP is reduced or does not occur in some CLL patients (Table 1). Although nascent NK_a from normal donors was stimulated by FaraAMP, exposure to the drug during the effector phase failed to stimulate further the killing produced by the same cells following interleukin-2 treatment, i.e. production of LAK cell activity [26]. Whether or not this cytokine plays a role in the reduced response seen in some CLL patients' cells (Table 1) was not investigated. Finally, the NK cell number and NK_a appear to be maintained at relatively normal levels following fludarabine treatment (Table 2).

In two of five patients studied, FaraAMP at high concentrations appeared to enhance the action of NK cells toward CLL targets (Table 3). If this effect occurs in vivo, one would anticipate that a proportion of patients would experience a more rapid and/or dramatic decline in their CLL cells due to this action of the drug compared with a cytotoxic mechanism which may require cell division or the induction of apoptosis. The complete reduction in CLL cells has been observed to occur during the first week of therapy in isolated cases (data not shown). The two patients who responded to FaraAMP in this regard (Table 3) were not subsequently treated with the drug; so we were unable to determine whether this sensitivity in vitro translated into an in vivo response.

In conclusion, NK cell number and NK_a were relatively normal in this cohort of CLL patients. The NK_a was increased when effectors were exposed to FaraAMP. The apparent ability of FaraAMP, in some patients, to enhance the NK_a toward autologous CLL cells (Table 3) suggests that this effect of the drug could

be important in a subpopulation of CLL patients. Intuitively, stimulation of NK_a by this or other cytotoxic agents would be a desirable effect because of the established antimetastatic activity of NK cells [2, 9, 11].

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