

The effect of fludarabine on interferon- γ production by lymphoid cells from healthy donors and patients with B-cell chronic lymphocytic leukemia

Fludarabine treatment in patients with B-cell chronic lymphocytic leukemia (B-CLL) can trigger or exacerbate the development of autoimmune hemolytic anemia (AHA) through a currently ill-defined mechanism. We here show that exposure of peripheral blood lymphocytes from healthy donors and B-CLL patients to fludarabine increases *in vitro* production of interferon- γ , a key cytokine in the pathogenesis of AHA.

haematologica 2006; 91:574-576

(<http://www.haematologica.org/journal/2006/04/574.html>)

Fludarabine is a strong immunosuppressive nucleoside that is particularly efficient in the treatment of B-cell chronic lymphocytic leukemia (B-CLL).¹ B-CLL is frequently associated with autoimmune hemolytic anemia (AHA) which, in a proportion of patients, is exacerbated after fludarabine therapy through a currently ill-defined mechanism.² Recent studies have demonstrated considerable modifications of normal Th1-Th2 balance in AHA, although the respective contribution of cytokines from these two T-cell subsets to the pathogenesis of AHA is still a matter of debate.^{3,4} There is evidence that T cells from AHA patients predominantly secrete interferon- γ (IFN γ) in response to red blood cells antigen, but little or no interleukin-4.^{3,5} Moreover, IFN γ

production seems to account for the exacerbation of autoantibody-mediated diseases induced by a range of viral infections in mice.⁶ We recently reported that fludarabine stimulates activator protein 1 (AP-1), a transcription factor involved in IFN γ gene regulation.⁷

We here investigated whether fludarabine could enhance the production of IFN γ by activated lymphoid cells, a mechanism that might be implicated in AHA exacerbation. Peripheral blood lymphocytes (PBL) from healthy donors, who were informed about the objectives of the study and gave their consent, were obtained after monocyte depletion by adherence onto plastic. PBL were exposed to graded doses of fludarabine (2-200 μ M) 20 min before polyclonal activation with the addition of PMA (10 ng/mL) and Ionomycin (5 μ g/mL). We found that fludarabine increased the release of IFN γ by activated PBL (Figure 1A) and the percentage of CD2⁺IFN γ ⁺ cells evaluated by flow cytometry at 24 hs (%CD2⁺IFN γ ⁺ cells: 12 \pm 2 vs 26 \pm 6, mean \pm SE, n=20, p <0.05, PMA + ionomycin vs PMA + ionomycin + fludarabine). Representative dot plots are shown in Figure 1B. A similar trend was observed upon PBL activation with PHA (Figure 1C). Differences in IFN γ -producing cells could not be ascribed to a reduced sensitivity of this subset to fludarabine since apoptotic levels were comparably low between activated cells cultured in the presence or absence of the drug (% apoptosis assessed by fluorescence microscopy: 7.5 \pm 0.2 vs 5.3 \pm 0.2, mean \pm SE, PHA vs PHA + fludarabine).

We further confirmed that fludarabine increases the percentage of CD2⁺IFN γ ⁺ cells by using purified CD2⁺ lymphocytes (pCD2⁺) obtained from peripheral blood mononuclear cells which were depleted of B cells and monocytes with specific antibodies and magnetic beads. pCD2⁺ cultures (>98% of CD2⁺ cells), composed of both T cells and NK cells, were cultured with PHA

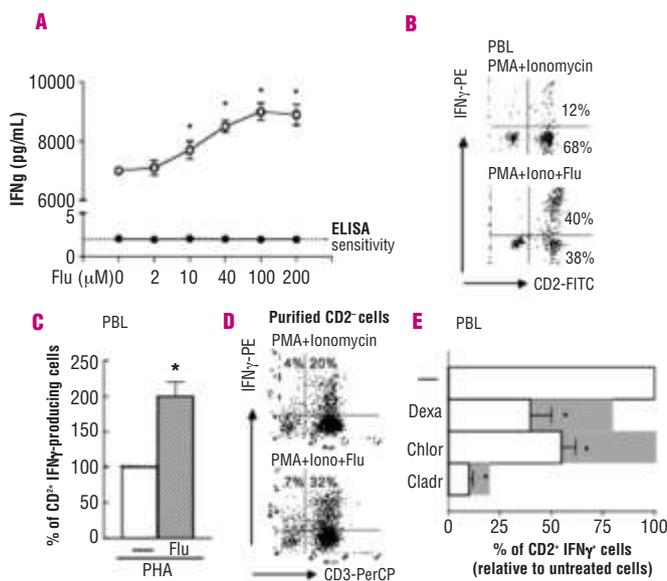


Figure 1. Fludarabine increases IFN γ production by activated lymphocytes from healthy donors. **A.** Peripheral blood lymphocytes (PBL) (2×10^6 cells/mL) were cultured with fludarabine (2-200 μ M) for 20 min before adding PMA (10 ng/mL) and ionomycin (5 μ g/mL) (open circle) or medium (solid circle). IFN γ release was evaluated in culture supernatants at 24 h by ELISA. Values are the mean \pm SEM (n=6). * p <0.05 for fludarabine-treated vs. untreated cells: Student's paired t test. **B.** PBL were cultured with fludarabine (40 μ M) for 20 min before adding PMA (10 ng/mL) and ionomycin (5 μ g/mL). Brefeldin A (10 μ g/mL) was added during the last 6 hs of culture in order to inhibit IFN γ release. IFN γ producing cells were detected by flow cytometry at 24h by immuno-fluorescence staining using antibodies against IFN γ (PE-conjugated) and CD2 (FITC-conjugated). The figure shows the dot plots from a representative experiment (n=20) with the percentages of CD2⁺IFN γ ⁺ and CD2⁺IFN γ ⁻ cells. **C.** PBL were cultured with fludarabine (40 μ M) 20 min before the addition of PHA (2.5 μ g/mL). IFN γ producing cells were detected at 24h as described above. The percentage of CD2⁺IFN γ producing cells relative to untreated cultures are shown as mean \pm SEM (n=10). * p <0.01 for fludarabine-treated cultures vs untreated cells: Student's paired t test. **D.** Purified CD2⁺ lymphocytes were cultured with fludarabine (40 μ M) for 20 min before adding PMA (10 ng/mL) and ionomycin (5 μ g/mL). Brefeldin A (10 μ g/mL) was added during the last 6 h of culture in order to inhibit IFN γ release. IFN γ -producing cells were detected by flow cytometry at 24h using immuno-fluorescence staining and antibodies against IFN γ (PE-conjugated) and CD3 (PerCP-conjugated). The figure shows the dot plots from a representative experiment (n=4) with the percentages of IFN γ producing cells. **E.** PBL were cultured with dexamethasone (Dexa, 10^{-7} M), chlorambucil (Chlor, 16 μ M) or cladribine (Cladr, 35 μ M), for 20 min before adding PMA (10 ng/mL) and ionomycin (5 μ g/mL). IFN γ -producing cells were detected at 24h as described above. The percentage of CD2⁺IFN γ producing cells relative to untreated cultures are shown as mean \pm SEM (n=7). * p <0.01 for drug-treated cultures vs untreated cells: Student's paired t test.

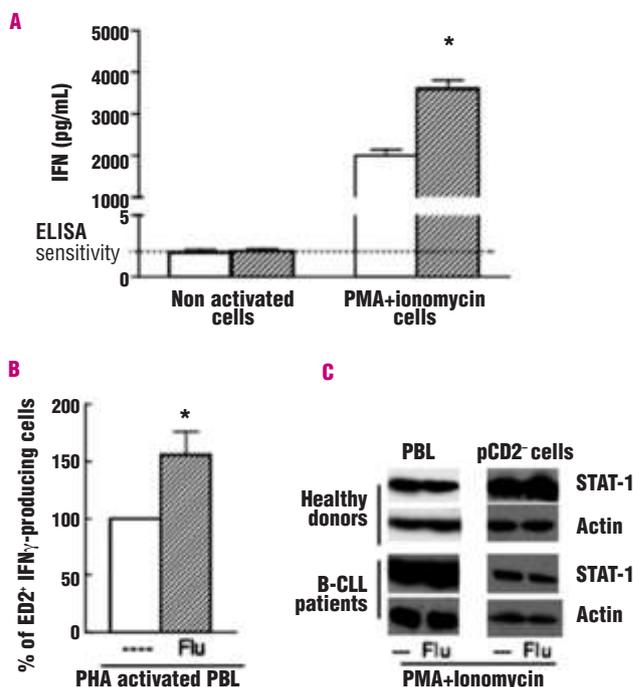


Figure 2. Fludarabine increases IFN γ production by activated lymphocytes from B-CLL patients. **A.** Peripheral blood lymphocytes (PBL) from B-CLL patients (2×10^6 cells/mL) were cultured with (dashed bar) or without (open bar) fludarabine ($40 \mu\text{M}$) for 20 min before adding PMA (10 ng/mL) and ionomycin ($5 \mu\text{g/mL}$). Experiments were performed with B-CLL samples containing at least 15% of CD2⁺ lymphocytes. IFN γ release was evaluated in culture supernatants at 24 h by ELISA. Values are the mean \pm SEM (n=5). * $p < 0.05$ for fludarabine-treated vs. untreated cells: Student's paired t test. **B.** PBL from B-CLL patients were cultured with fludarabine ($40 \mu\text{M}$) for 20 min before the addition of PHA ($2.5 \mu\text{g/mL}$). Brefeldin A ($10 \mu\text{g/mL}$) was added during the last 6 h of culture. IFN γ producing cells were detected as described in Figure 1B. Experiments were performed with B-CLL samples containing at least 15% of CD2⁺ lymphocytes. The percentage of CD2⁺ IFN γ producing cells relative to untreated cultures are shown as mean \pm SEM (n=12). * $p < 0.01$ for fludarabine-treated cultures vs. untreated cells: Student's paired t test. **C.** PBL (2×10^6) and purified CD2⁺ lymphocytes from healthy donors and B-CLL patients were cultured with fludarabine ($40 \mu\text{M}$) for 20 min before adding of PMA (10 ng/mL) and ionomycin ($5 \mu\text{g/mL}$) or PHA ($2.5 \mu\text{g/mL}$). STAT-1 protein detection was evaluated at 24 h by western blot. To this aim, cell pellets were washed with cold saline, immediately resuspended in loading buffer (60 mM Tris pH6.8, 2.3% SDS, 10% glycerol, 0.01% bromophenol blue and 5% β -mercaptoethanol) and boiled at 96°C for 5 minutes. Samples were then separated on a standard 10% SDS-PAGE and transferred to PVDF membranes. Membranes were blotted with antibodies against STAT-1 and actin to compare the total amount of protein in each sample.

and fludarabine as described above. Fludarabine ($40 \mu\text{M}$) increased IFN γ production by CD3⁺ and CD3⁻ lymphocytes (Figure 1D), suggesting that both T cells and NK cells were responsible for IFN γ generation. A similar trend was observed at lower doses of fludarabine (20 or $10 \mu\text{M}$) (not shown).

Additionally, we found that other pro-apoptotic drugs, such as dexamethasone (10^{-7}M) and chlorambucil ($16 \mu\text{M}$) decreased the percentage of CD2⁺IFN γ ⁺ (Figure 1E). Unexpectedly, cladribine ($35 \mu\text{M}$), a purine analog with structural similarity to fludarabine, also impaired IFN γ production without increasing apoptosis. Given that PBL from B-CLL patients include a reduced percentage of non-leukemic lymphocytes with an array of abnormalities and dysfunctions,⁸ it was important to determine whether they could respond in the same way as PBL from healthy donors. Experiments were performed with B-CLL samples containing at least 15% of CD2⁺ lymphocytes. Fludarabine increased the secretion of IFN γ by activated PBL from B-CLL patients (Figure 2A) and the percentage of CD2⁺IFN γ producing lymphocytes (Figure 2B). Leukemic cells from the samples evaluated did not produce IFN γ (not shown). Fludarabine did not affect interleukin-4 production by activated PBL from B-CLL patients (interleukin-4 evaluated by ELISA at 24 h: $36 \pm 6\text{pg}$ vs $28 \pm 2\text{pg}$, mean \pm SE, n=5, PHA vs PHA+fludarabine).

Since Frank *et al.* previously reported that incubation of peripheral blood mononuclear cells with fludarabine impaired IFN γ production due to sustained depletion of signal transducer and activator of transcription-1 (STAT-1),⁹ a key transcription factor required for mitogen activation, we also evaluated STAT-1 protein by western blot. As we expected, fludarabine did not reduce STAT-1 expression in PBL or pCD2⁺ cultures

from normal or B-CLL samples (Figure 2C). Although our observations contrast with those obtained by Frank *et al.*, the effect of fludarabine on STAT-1 is unclear. Other authors recently reported, in line with our data, that fludarabine does not affect the STAT-1 pathway.¹⁰ These observations should be taken into account since it has been suggested that fludarabine-induced depletion of STAT-1 protein may explain some of the immunosuppressive effects of the drug.⁹

In conclusion, our results indicate that fludarabine increases *in vitro* IFN γ production by activated PBL from healthy donors and B-CLL patients. Given that IFN γ has been linked to the pathogenesis of AHA, its enhanced production might be involved in the increased risk of AHA associated with fludarabine treatment in B-CLL patients.

Romina Gamberale,* Paula Fernández-Calotti,*
Julio Sánchez-Ávalos,^o María Fabiana Alberto,[#]
Jorge Geffner,* Mirta Giordano*

*Laboratorio de Inmunología, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Buenos Aires and Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires;
^oHospital de Clínicas José de San Martín, Universidad de Buenos Aires; [#]Departamento de Hemostasia y Trombosis, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Buenos Aires, Argentina

Acknowledgments: We would like to thank Ms Selma Tolosa, Ms. Evelia López and Mr Héctor Sotelo for their excellent technical assistance. This work was supported by grants from CONICET, Agencia Nacional de Promoción Científica y Tecnológica (FONCyT) and Fundación Alberto J. Roemmers.

Key words: fludarabine, IFN γ production, autoimmune hemolytic anemia.

Correspondence: Romina Gamberale, PhD, Laboratory of Immunology, Institute of Haematologic Research, National Academy of Medicine, Pacheco de Melo 3081 (1425), Buenos Aires, Argentina. Phone: international + 0054.11.48053411. Fax: international + 0054.11.48039475. E-mail: rgamberale@hematologia.anm.edu.ar

References

1. Keating MJ, Kantarjian H, Talpaz M, Redman J, Koller C, Barlogie B, et al. Fludarabine: a new agent with major activity against chronic lymphocytic leukemia. *Blood* 1989;74:19-25.
2. Mauro FR, Foa R, Cerretti R, Giannarelli D, Coluzzi S, Mandelli F, et al. Autoimmune hemolytic anemia in chronic lymphocytic leukemia: clinical, therapeutic, and prognostic features. *Blood* 2000;95:2786-92.
3. Elson CJ, Barker RN. Helper T cells in antibody-mediated, organ-specific autoimmunity. *Curr Opin Immunol* 2000;12:664-9.
4. Fagiolo E, Toriani-Terenzi C. Th1 and Th2 cytokine modulation by IL-10/IL-12 imbalance in autoimmune haemolytic anaemia (AIHA). *Autoimmunity* 2002;35:39-44.
5. Hall AM, Ward FJ, Vickers MA, Stott LM, Urbaniak SJ, Barker RN. Interleukin-10-mediated regulatory T-cell responses to epitopes on a human red blood cell autoantigen. *Blood* 2002;100:4529-36.
6. Meite M, Leonard S, El Azami-El Idrissi M, Izui S, Masson PI, Coutelier JP. Exacerbation of autoantibody-mediated hemolytic anemia by viral infection. *J Virol* 2000;74:6045-9.
7. Fernández-Calotti P, Gamberale R, Costas M, Sánchez-Avalos J, Geffner J, et al. Fludarabine induces pro-inflammatory activation of human monocytic cells through a MAPK/ERK pathway. *Int Immunopharmacol* 2006;(in press).
8. Ravandi F, O'Brien S. Immune defects in patients with chronic lymphocytic leukemia. *Cancer Immunol Immunother* 2005;16:1-13.
9. Frank DA, Mahajan S, Ritz J. Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. *Nat Med* 1999;5:444-7.
10. Martinez-Lostao L, Briones J, Forne I, Martinez-Gallo M, Ferrer B, Sierra J, et al. Role of STAT-1 pathway in apoptosis induced by fludarabine and JAK kinase inhibitors in B-CLL. *Leuk Lymphoma* 2005;46:435-42.

Monthly CME program

Haematologica/The Hematology Journal is owned by a non-profit organization, the Ferrata Storti Foundation. The aim of the Foundation is to stimulate and promote the study and research on diseases of the blood and their treatment by supporting the journal and by promoting continuing medical education in clinical hematology.

The monthly Continuing Medical Education (CME) program is designed for physicians who wish to advance their current knowledge of clinical hematology. In order to participate in this program, physicians must be one of the following: a) a member of the European Hematology Association (EHA); b) a member of the Italian Society of Hematology (SIE); c) a member of the Spanish Society of Hematology (AEHH); or d) an individual subscriber to the print journal or the online journal. Detailed information is available at the web site (<http://cme.haematologica.org/>). The online CME program has been approved by the European Hematology CME System (ECAH: EUR/02/ C/F/TH-84902) of the European Hematology Association (EHA). The system has designated participation in the CME program as worth one hour of credit for any exam passed. Four exams will be available each month, and the total number of credits that any participant can earn in a year is 48.

Participants should complete a program evaluation form within December 31st of each year and will then receive by e-mail their certificates of the credits obtained, as PDF documents. Haematologica/The Hematology Journal assumes that each physician personally participates in the online CME program and provides the answers, and therefore that he or she claims only the hours of credit actually spent in this educational activity.