

Biological and molecular characterization of PNH-like lymphocytes emerging after Campath-1H therapy

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Summary. Campath-1H, an anti-CD52 monoclonal antibody, is therapeutically active in lymphoproliferative and autoimmune diseases. After Campath-1H therapy, lymphocytes with a paroxysmal nocturnal haemoglobinuria (PNH) phenotype have been reported to emerge. We characterized a PNH-like lymphocyte population emerging after Campath-1H therapy, in a patient with fludarabine refractory B-cell chronic lymphocytic leukaemia (B-CLL). We demonstrated a reduction in PIG-A mRNA levels compared with controls,

and of all cytokines tested [interleukin (IL)-4, IL-13, IL-2, interferon(IFN)- γ , IL-6, IL-10, and tumour necrosis factor (TNF)- α], except transforming growth factor (TGF)- β . Given the inhibitory activity of TGF- β , its elevated levels may contribute to the selective pressure of Campath-1H, leading to the emergence of PNH-like lymphocytes.

Keywords: Campath-1H, CLL, cytokines, PIG-A, PNH-like lymphocytes.

Campath-1H, a humanized monoclonal antibody against CD52, is increasingly used in the treatment of lymphoid malignancies, autoimmune disease and bone marrow transplantation. After Campath-1H treatment, the emergence of lymphocytes deficient in glycosylphosphatidylinositol (GPI)-anchored proteins has been described (Hertenstein *et al.*, 1995; Brett *et al.*, 1996; Taylor *et al.*, 1997). The phenotype of these CD52-negative B and T cells closely resembles that of lymphocytes from paroxysmal nocturnal haemoglobinuria (PNH) patients, in which the first step of the GPI-biosynthetic pathway is blocked (Rowan *et al.*, 1998). The molecular involvement by mutation of the PIG-A gene in these PNH-like lymphocytes is controversial (Hertenstein *et al.*, 1995; Taylor *et al.*, 1997) and has been described in one B cell chronic lymphocytic leukaemia (B-CLL) patient who developed PNH-like T lymphocytes after treatment with Campath-1H (Rawstron *et al.*, 1999).

We characterized the PNH-like lymphocyte population that emerged in a patient affected by fludarabine refractory B-CLL after treatment with Campath-1H, using molecular analysis and determination of cytokine production.

The patient, a 57-year-old man, was diagnosed with B-CLL (SmIgM, G λ) Binet stage C in 1991. He was initially treated with alkylating agents, followed by α -interferon (IFN) maintenance, obtaining a partial remission. A progression of disease occurred in 1997 and fludarabine

treatment was started with no response. In October 1998, Campath-1H treatment (30 mg every other day for 12 weeks) was started for disease progression. Post-treatment evaluation revealed a complete remission in the peripheral blood and bone marrow (CD5/CD19 double-positive cells < 3% using flow cytometry). After Campath-1H treatment, the great majority of the patient's lymphoid cells were represented by PNH-like T-lymphocytes, with loss of CD52 (Fig 1A,B), 55, and 59 expression (data not shown). Notwithstanding the lymphocyte PNH phenotype, the study of the entire coding region and flanking intronic sequences of the PIG-A gene, using single-stranded conformation polymorphism (SSCP) analysis, showed no abnormal electrophoretic patterns suggestive of gene mutation. To test the hypothesis that the loss of GPI-anchored proteins may be the result of reduced levels of PIG-A mRNA, we assessed the PIG-A mRNA levels using quantitative-competitive reverse transcription-polymerase chain reaction (RT-PCR). Interestingly, the expression of PIG-A mRNA was reduced compared with normal controls (5×10^3 – 10^4 molecules in four consecutive samples during Campath-1H treatment, versus 10^5 molecules in three normal controls (see Fig 1C). These findings may suggest a possible role for defective PIG-A mRNA production in the genesis of PNH-like lymphocytes, even in the absence of detectable PIG-A gene mutations (Rawstron *et al.*, 1999). Regarding cytokine production (Table I), we found that all cytokines, except transforming growth factor (TGF)- β , were reduced compared with CLL control-patients and to a greater extent than

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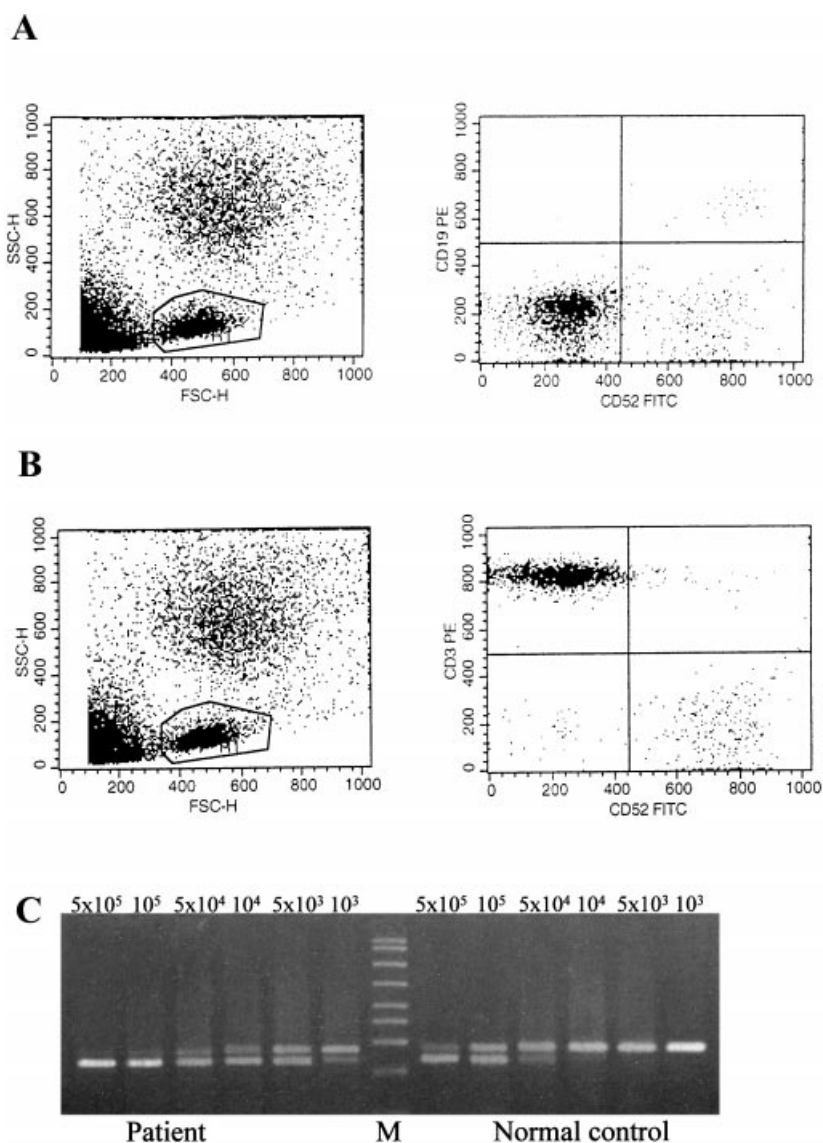


Fig 1. (A and B) Flow cytometric analysis of the patient's lymphocytes after Campath-1H treatment. Mononuclear cells were separated from peripheral blood using a Ficoll-Hypaque density gradient. The cells were analysed by two-colour immunofluorescence staining with the indicated fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labelled monoclonal antibodies. The analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). A portion of each sample was incubated with the appropriate isotype control-antibodies to establish the background level of non-specific staining, and positivity was defined as staining greater than non-specific background staining. (A) left, gated event; right, 1.84% of B lymphocytes co-expressed CD52 antigen. (B) left, gated events; right, 2.47% of T-lymphocytes co-expressed CD52 antigen. Before Campath-1H treatment, >90% of the patient's T-lymphocytes and >90% of the B-lymphocytes co-expressed CD52 antigen (data not shown). (C) Evaluation of the expression of PIG-A gene in the patient and in normal controls by quantitative-competitive PCR. The competitor was obtained using a 36-bp PCR internal deletion of the PIG-A cDNA fragment to be quantified. In the first PCR round forward 1 (5'-CTGTACTAAGAGCAGCACTG-3') and reverse (5'-CTGTCATGCAGCTGGTATCT-3') primers were used, generating a fragment of 274 bp; in the second PCR round, the 274 bp fragment was amplified in the presence of reverse primer and forward 2 primer (5'-CTGTACTAAGAGCAGCACTGGATCCTACTGACTTCACTCC-3'), a hybrid represented by forward 1 sequence fused to a cDNA PIG-A sequence 36 bp downstream. Therefore, a PCR fragment of 238 bp derived from PIG-A cDNA, delimited by forward 1 and reverse sequences was generated. Total RNA (1 µg) was reverse transcribed to cDNA and for quantitative-competitive PCR, 100 ng of product were amplified in the presence of scalar amounts of competitor molecules using forward 1 and reverse primers. PCR conditions were as follows: hot start, 5 min at 95°C; 35 cycles at 95°C for 50 s, 55°C for 50 s and 72°C for 60 s, the same used to generate the competitor. Equivalence (indicated by an arrow) was 5×10^3 molecules/100 ng of total RNA in the patient, and 10^5 molecules/100 ng of total RNA in controls. M, molecular markers (Amplisize Molecular Ruler 50–2000 bp, Biorad, Hercules, California, USA).

Table 1. Cytokine production by mitogen-stimulated blood cultures.

	Patient	Normal controls	CLL
IL-2	35 ± 11	237 ± 17	117 ± 28
IFN- γ	926 ± 265	5147 ± 304	3234 ± 519
IL-4	1.9 ± 0.5	21.5 ± 1.9	9.7 ± 2.9
IL-6	540 ± 235	2245 ± 120	939 ± 161
IL-10	115 ± 37	435 ± 44	218 ± 43
IL-13	1.2 ± 0.5	133 ± 14	129 ± 24
TNF- α	53 ± 11	147 ± 18	96 ± 22
TGF- β	1649 ± 317	1394 ± 78	630 ± 134

Blood cultures were stimulated with phytohaemagglutinin (PHA, 1 μ g/ml) and supernatants collected after 48 h. Cytokine measurement was performed using the commercially available enzyme-linked immunosorbent assay (ELISA) kits. Values are expressed as pg $\times 10^{-9}$ /cell, and were obtained by dividing the cytokine concentration in supernatants by the number of peripheral blood mononuclear cells present in culture. Values for normal controls and CLL patients are the mean \pm SD of 46 and 47 subjects respectively. Values for the patient under study are the mean \pm SD of four consecutive determinations performed between March and December 1999.

normal controls. Interleukin (IL)-4 and IL-13 production was very low and IL-2, IFN- γ , IL-6, IL-10 and tumour necrosis factor (TNF)- α were, on average, 20–30% of normal controls and 50% of CLL patients. Interestingly, TGF- β production was disproportionately high compared with the other cytokines; furthermore, it was comparable to normal controls and threefold greater than the production occurring in CLL patients.

Several abnormalities of cytokine production have been described in CLL regarding both the B and T cell compartment, including reduced IL-4, IFN- γ (Hill *et al.*, 1999) and IL-6 (Hulkkonen *et al.*, 1998), which have been related to the cellular and humoral immune defects of the disease. However, little is known about cytokine production by PNH lymphocytes, except for reduced IL-2 release in patients with aplastic anaemia who progressed to clinical PNH (Nissen *et al.*, 1999). Compared with the CLL group, we found increased production of TGF- β , a well-known inhibitory cytokine. Excessive production of TGF- β by bone marrow stromal cells has been hypothesized to be responsible for the inhibitory activity on haematopoietic precursors in CLL (Lagneaux *et al.*, 1993). Given the inhibitory activity of TGF- β , our results may suggest that elevated levels of this cytokine could contribute to alterations of the micro-environment and synergize with the selective pressure of Campath-1H, leading to expansion of pre-existing PNH-like lymphocytes (Rawstron *et al.*, 1999).

The study of larger series, and in particular of classical

PNH lymphocytes, will be necessary to demonstrate if this cytokine production pattern is characteristic of the PNH-like lymphocytes emerging after Campath-1H treatment.

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