No evidences for B-cell clonality by spectratyping analysis in patients with idiopathic thrombocytopenic purpura undergoing rituximab therapy

Recent evidence concerning the cellular pathway of idiopathic thrombocytopenic purpura (ITP) indicate a strict relationship between T and B-cells. T-cell deregulation, probably antigen driven, results in an increase of the CD4 Th1/Th2 ratio with oligoclonal CD4 expansion. According to this model, B-cells appear to have a secondary role and the production of anti-platelet antibodies appears to be mainly an epiphenomenon of T-cell defect.[1,2] However, a central immunomodulating role of B-cells on T-cells was recently proposed.[3] Furthermore, data from clinical experience with rituximab therapy in patients with ITP highlighted the favourable impact of B-cell depletion.[4] These findings demand a reassessment of the pathological implications of B-lymphocytes.

Previous studies in acute and chronic ITP have highlighted the presence of clonal anti-platelet antibodies (mainly against GPIIb/IIIa) and clonal B-cell expansion.[5-7] These are possibly predictive of rituximab response and could, therefore, help optimize the therapeutic choice.

Given this, we performed a prospective study in 18 patients with symptomatic ITP treated with rituximab from November 2005 to February 2007. Aims of the study were to explore B-cell clonal expansion and match results with therapeutic outcome. Subjects were 11 women and 7 men, median age 47 years (range, 16-70). The median platelet count was 32×10^9/L. Patients were treated with rituximab 375 mg/m^2 or 100 mg total dose on days 1, 7, 14, 21 as part of two prospective clinical trials (ML 18542 and LD-RTX). Peripheral blood samples were collected at baseline and subsequently two and six months after the beginning of rituximab therapy. DNA was extracted from all samples using GeHealthcare kit. Specific sequences of immunoglobulin heavy chain gene have been amplified according to the manufacturer's recommendations by means of Identiclone IgH Gene Clonality Assay (InVivoScribe). The multiplex PCR of CDRIII region was conduced according to BIOMED 2 Concerted Action.[8] Five hundred ng of total DNA were amplified. The amplification products (amplicons) were denatured for 3 mins at 94°C, size separated on a high resolution polyacrylamide gel and analyzed using the GeneMapper software on 310 ABIPRISM (Applied Biosystems). Each sample was analysed with its internal standard (ladder). Tests were perfomed in duplicate and repeated in a second run. A perfect reproducibility was verified. In each run, a polyclonal control, a monoclonal control and a no-template control were analyzed. To define a B-cell clonality, two criteria were used according to the manufacturer's instructions and as previously described.[8] The distribution of the amplicons with a size ranging from 100 to 170 nucleotides (nt) was evaluated: a correct fit of a Gaussian curve was considered an indicator of normal polyclonal population, while the appearance of one sharp fluorescence peak was considered an expression of clonal IgH population (Figure 1). The immunoglobulin heavy chain gene rearrangement was judged oligoclonal if a Gaussian distribution was absent and the presence of a reduced number of peaks (<5) documented. B lymphocytes were detected in peripheral blood

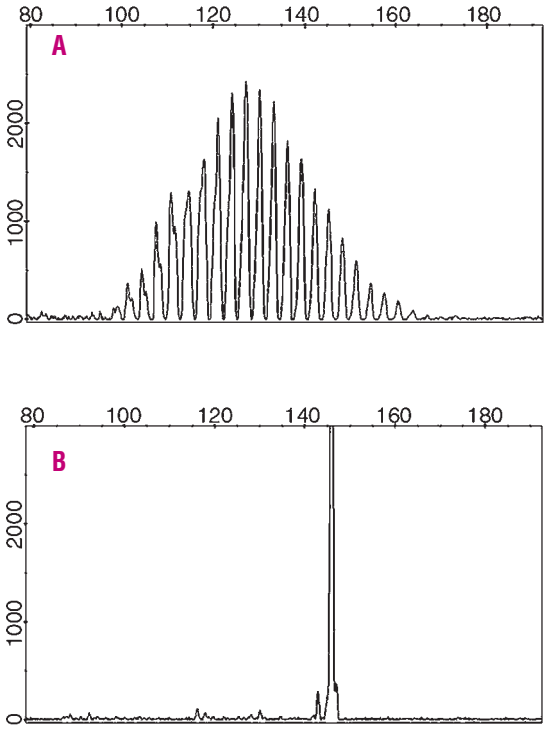

Figure 1. Polyclonal control (A) and monoclonal control (B) for comparison of all obtained electropherograms. Relative fluorescence intensity (y-axis) and PCR fragment size in basepairs (x-axis).

evaluating simultaneously both CD19 and CD20 antigens, using anti-CD19 PE and anti-CD20 FITC (Beckton Dickinson) by flow cytometry (FACScalibur, Becton Dickinson), at baseline and subsequently at two and six months after the start of rituximab treatment.

Median number of B-cells at baseline was 0.142×10^9/L (range $0.052\text{-}0.998\times10^9$/L, 12% of sample's lymphocytes), with a CD20 median Mean Fluorescence Index (MFI) of 164 (range 70-298). Thirteen patients responded to rituximab (8 complete and 5 partial response). No relationship between baseline number of B-cells, CD20 MFI and response to rituximab was found. B-cell depletion was documented in all patients two months after the start of rituximab therapy (median value 0×10^9/L, range $0\text{-}0.07\times10^9$/L) At month +6, a detectable B-cell count was evident in 7 out of 11 valuable patients, with a median B-cell count of 0.00028×10^9/L (range $0\text{-}0.087\times10^9$/L). All runs were of good quality, all no-template controls were uncontaminated and no problems in amplifications in positive controls were seen. At baseline, electropherograms of all samples showed a Gaussian distribution of the CDRIII amplicons, similar to polyclonal control. This showed the presence of polyclonal B-cells in all patients (Figure 2). At month 2, a very low fluorescence peak (almost zero) meant a correct evaluation of amplicon distribution was not feasible. At month +6, the fluorescence intensity was again normal and the amplicons presented a Gaussian distribution with the same characteristics observed at diagnosis. In this study, we used a size fragment analysis on ABIPRISM 310 of PCR products to study the presence of clonal B-cell expansion in a population of ITP patients treated with rituximab. The ABIPRISM detection based assay

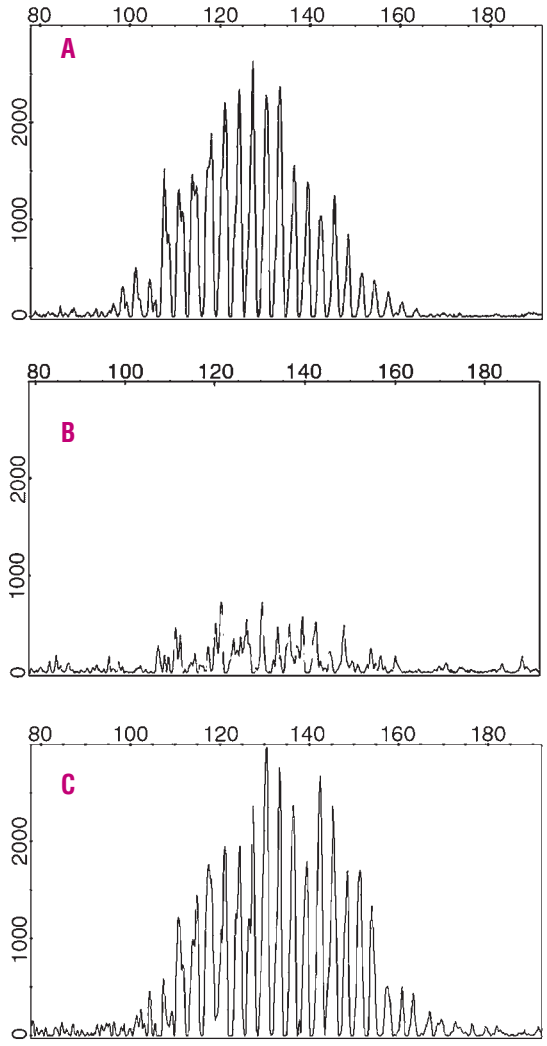

Figure 2. Electropherograms of CDRIII IgH of one patient at baseline (A), two and six months after the start of rituximab therapy (B and C). The baseline electropherogram (A) presents the same characteristics of polyclonal control. The analysis after two months (B) revealed a diffuse decrease of all peaks in agreement with B-cell depletion. The polyclonal pattern reappears after six months (C). This pattern is common to all the study population.

can detect at least 1% B-cell clonal population among the total lymphocyte population. With this limit, none of the patients showed pre-treatment evidence of B-cell clonality. Therefore, no correlation with response to therapy could be performed. This result is in contrast with previous studies[5,6] probably due to differences in methodology. Our analysis was performed with a more sensitive method[8,9] to genetically characterize B-cell populations. By contrast, the determination of B-cell clonality in other studies[5,10] was defined on the basis of light chain restriction in anti-platelet auto-antibodies by flow cytometry. This method, as stated by the authors, may have some limitations, possibly leading to the detection of a false clonal population. Furthermore, studies carried out on human monoclonal antibody obtained applying EBV or combinatorial technology, showed partial or complete light chain restriction despite DNA sequencing evidence of poliClonality.[11,12]

The hypothesis that rituximab eliminates one or more specific B-cell clones is in contrast with the findings of this report. Therefore, the key B-cell role in the cellular pathway of ITP does not appear to be related to a clonal expansion but to a co-stimulating polyclonal B-cell population.

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