

Late-onset neutropenia following rituximab results from a hematopoietic lineage competition due to an excessive BAFF-induced B-cell recovery

Rituximab is used in the treatment of lymphoma and autoimmune diseases, for which late-onset neutropenia (LON) were reported. LON-related mechanisms remain unclear. To obtain insights into the mechanisms, we assessed serum, peripheral blood and bone marrow (BM) samples of a patient with LON. Factors classically associated with neutropenia such as anti-neutrophil antibodies, T-LGL, soluble Fas Ligand were not detectable. We then evaluated the kinetics of various cytokines involved in B-cell and granulocyte homeostasis. We found that LON is related to a lack of granulopoiesis in the BM that coincides with a very high level of BAFF, a strong stimulator of B-cell recovery, and hypothesized a hematopoietic lineage competition due to an excessive B-cell recovery in the BM by promotion of B-cell lymphopoiesis over granulopoiesis within common developmental niches. Assessment of serum BAFF levels following rituximab could detect patients at risk of developing LON.

Haematologica 2007; 92:(2):e20-e23

Introduction

Rituximab, an anti-CD20 monoclonal antibody, is increasingly used in the treatment of lymphoma and autoimmune diseases. CD20 is expressed on malignant B-cells, normal differentiated B-cells and pre-B-cells, but not on stem cells and granulocyte precursors. Late-onset neutropenia following rituximab (LON) have been reported,^{1,7} occurring 1 to 6 months after last rituximab infusion, as being severe ($<0.5 \times 10^9/L$), spontaneously reversible and without life-threatening infections.

Several studies suggest that LON could be related to an excess of T-Large Granular Lymphocyte (LGL) in the bone marrow (BM) and peripheral blood (PB) which express and secrete large amounts of Fas and Fas Ligand (FasL) leading to apoptosis of mature neutrophils,⁴ or to a production of autoantibodies binding to the neutrophil surface during recovery of a new immune repertoire.^{2,6} On the other hand, a recent study suggests that LON is not related to circulating factors but to perturbations of Stromal-derived Factor 1 (SDF-1) and granulopoiesis homeostasis during B-cell recovery.⁷ This is reinforced by previous studies showing the hypocellularity of the marrow at the time of LON^{1-3,6} and the absence of anti-neutrophil antibodies in the serum or T-LGL in the PB.^{2,6,7}

We report here that LON is related to a lack of granulopoiesis in the BM that coincides with the time of maximum B-cell depletion in PB, and proposed the hypothesis that LON is due to a hematopoietic lineage competition in the BM by promotion of B-cell lymphopoiesis over granulopoiesis within common developmental niches.

Study design

Case report. In August 2005, a 55-year-old woman was referred for severe neutropenia of fortuitous discovery. Her past medical history was remarkable for Waldenström macroglobulinemia (WM) associated with retroperitoneal and renal infiltration diagnosed in March 2005. The patient was treated with rituximab (375 mg/m² day 1), fludarabine (30 mg/m² day 1 to 3) and cyclophosphamide (300 mg/m² day 1 to 3) from March

to June 2005 (last treatment being defined as day 0), for 4 courses monthly, leading to complete remission of WM. After the end of treatment, blood tests performed on day 37 were unremarkable except for a mild decrease in neutrophil count ($1.6 \times 10^9/L$). At admission on day 84, blood tests revealed a severe neutropenia ($0.2 \times 10^9/L$), with normal hemoglobin level and platelet count, but the exact time from last treatment to the development of neutropenia could not be precisely determined with the available data. The serum monoclonal component was still not detectable. There was no recent or concomitant medication. Bone marrow aspiration showed granulocytic hypoplasia (22%) without maturation blockade, and no excess of T-LGL or hemophagocytosis. In the PB, smear analysis was normal without T-LGL, lymphocyte immunophenotyping showed the absence of B cells (CD19/CD20: 0%) and T-LGL, and TCR rearrangement analysis did not reveal any circulating T-cell clone. Viral PCR in PB and BM were negative for parvovirus B19, CMV, EBV, and HHV6. Circulating anti-neutrophil antibodies were not detected by immunofluorescence. Thus, we put forward the diagnosis of rituximab-induced neutropenia since regular causes of neutropenia were excluded. Neutropenia was spontaneously reversible within 10 days ($5400/mm^3$), without the use of G-CSF. A second episode of neutropenia ($340/mm^3$) occurred few days later, spontaneously reversible within 6 days. After 15 months, the patient did not relapse of either neutropenia or WM. Peripheral blood stem cells were collected after stimulation by G-CSF 6 months after the recovery from neutropenia, suggesting that the patient's bone marrow has returned to normal.

Serum samples and cytokines levels. Patient serum samples were either available or have been collected specifically, at -198 (before rituximab), 37, 84 (first episode of LON), 138 and 222 (after resolution of LON)-day time points; day 0 being defined as the last rituximab infusion. Cytokines levels at the five selected time points have been assessed by Enzyme Linked ImmunoSorbent Assay (ELISA) for tumor necrosis factor (TNF)-alpha, interleukin (IL)-6 (BioSource Europe, Belgium), Thymic Stromal Lymphopoietin (TSLP), SDF-1 and B-cell activating factor (BAFF) (R&D systems, MN, USA); by immunoblot for soluble FasL.

Bone marrow samples, CFU-GM culture and analysis of the serum inhibitory effect. Patient's BM sample was collected during the LON episode, at 84-day time point, but not after the neutrophils or B-cells recovery. On this sample, the Colony-forming units granulocyte-macrophage (CFU-GM) growth have been assessed by the quantification of the number of CFU-GM after 10 days of culture. The inhibitory effect of the patient's sera collected at the 5 time-points on CFU-GM growth have been assessed by incubation of CFU-GM from an healthy subject BM with sera and quantification of the number of CFU-GM after 10 days of culture.

Results and discussion

To obtain insights into the LON-related mechanisms, we assessed serum, PB and BM samples of a patient with LON following rituximab.

BM assessment revealed granulocytic hypoplasia and the absence of T-LGL on BM smear. No anti-neutrophil antibodies in the serum or T-LGL in the PB were detected and at the selected time points, serum assessment revealed the absence of soluble FasL (Figure 1A). The patient's sera did not inhibit the growth of healthy donor CFU-GM, and showed a possible stimulation at the 84-day time point (Figure 1B). Finally, the patient's CFU-GM culture from BM at day 84 showed a disturbance of gran-

ulocyte differentiation, with a decrease number of CFU-GM after 10 days of culture (12 vs. 60 to 300 in healthy subjects) (Figure 1C). Our findings suggest: first, a lack of early granulocyte precursors at this time point, since this patient did not have any argument for an intrinsic defect of granulocyte differentiation until the episode of LON; second, the commitment of progenitors to the lymphoid lineage since CFU-GM growth quantifies the progenitors already committed to the myeloid lineage.

We then assessed variations in the serum levels of cytokines involved in B-cell and granulocyte homeostasis: SDF-1 involved in human B-cell homeostasis and granulopoiesis,⁸ TSLP involved in murine B-cell homeostasis and granulopoiesis,⁹ TNF-alpha which inhibits granulopoiesis¹⁰ and IL-6 which exhibits stimulatory effect on granulocyte production.^{11,12} No fluctuations of TNF-alpha, TSLP and SDF-1 in serum were detected (Figure 2A, 2B, 2C, 2D). Regarding SDF-1, perturbations of this cytokine were recently underlined in the pathophysiology of LON, because of its central role in both the regulation of neutrophil egress from the BM and early B-cell lymphopoiesis, raising the notion that B-cell recovery following rituximab involves early stages of B-cell

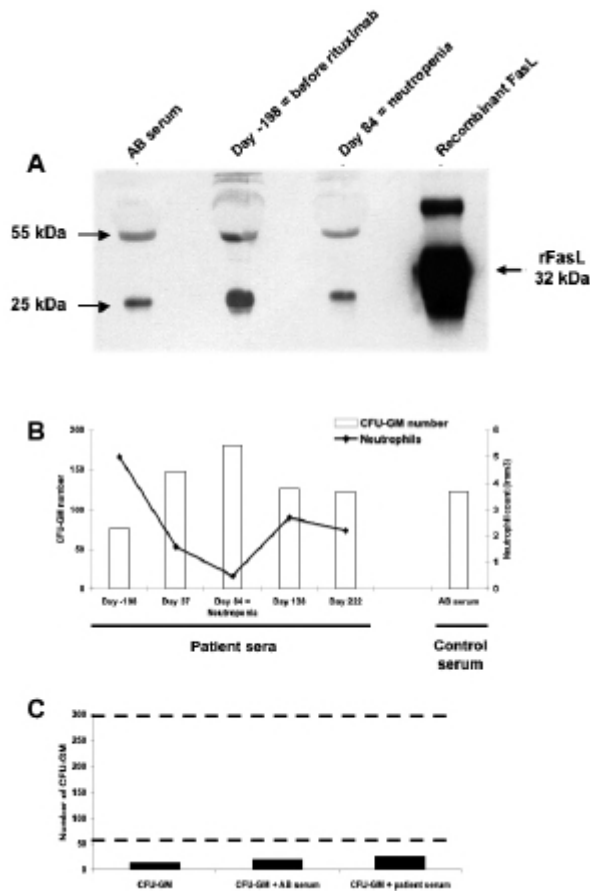


Figure 1. LON is not related to circulating factors but rather to a lack of granulopoiesis in BM. (A) Detection of soluble FasL in the patient's sera by immunoblot. (B) Analysis of the inhibitory effect of the patient's sera at the 5 time-points on the growth of healthy donor CFU-GM. (C) Assessment of patient CFU-GM growth by quantification of the number of CFU-GM after 10 days of culture (normal values are within the dotted lines) at time of LON. Day 0 is defined as the end of treatment (last rituximab infusion).

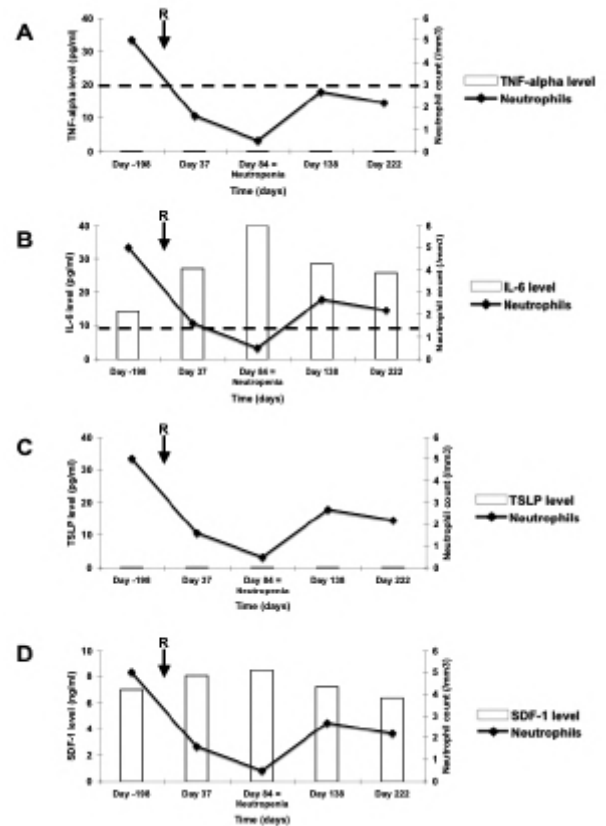


Figure 2. Assessment of the level of (A) TNF-alpha, (B) IL-6, (C) TSLP and (D) SDF-1 in the patient's sera at the selected time points (the upper limits for TNF-alpha and IL-6 are represented by the dotted lines). Day 0 is defined as the end of treatment (last rituximab infusion)

lymphopoiesis.⁷ We did not find significant fluctuations in circulating levels of SDF-1, but SDF-1 is not directly accessible to quantitative analysis. IL-6 paralleled the evolution of the number of CFU-GM after incubation with patient's sera, with an increase following rituximab, a maximum at the time of LON, and a decrease with the neutrophils recovery. IL-6 variation was not linked to an inflammatory process since C-reactive protein level and temperature curve were normal at each time point. The evolution of IL-6 serum level could be explained by its synergistic action with G-CSF on myeloid progenitor cells, as a positive feedback to normalize granulopoiesis.

LON occurred during the time of peripheral B-cell depletion (Figure 3A, 3B, 3C). Interestingly, the neutropenia was also concomitant to the maximum of BAFF serum level, a cytokine involved in human B-cell survival, expansion and development.^{13,14} BAFF, almost undetectable prior to therapy, increased following rituximab until a maximum coinciding with the episode of LON, and then decreased with the neutrophils and B-cells recovery in the PB (Figure 3D). The increase in BAFF levels following rituximab has been already reported and is in part, linked to the physical loss of BAFF-binding B-cells,^{16,17} but also the result of a transcriptional up-regulation of BAFF mRNA after B-cell depletion by a yet unknown mechanism. Thereby, BAFF serum level is increased during the time of peripheral B-cell depletion and the decline in BAFF serum levels is associated with

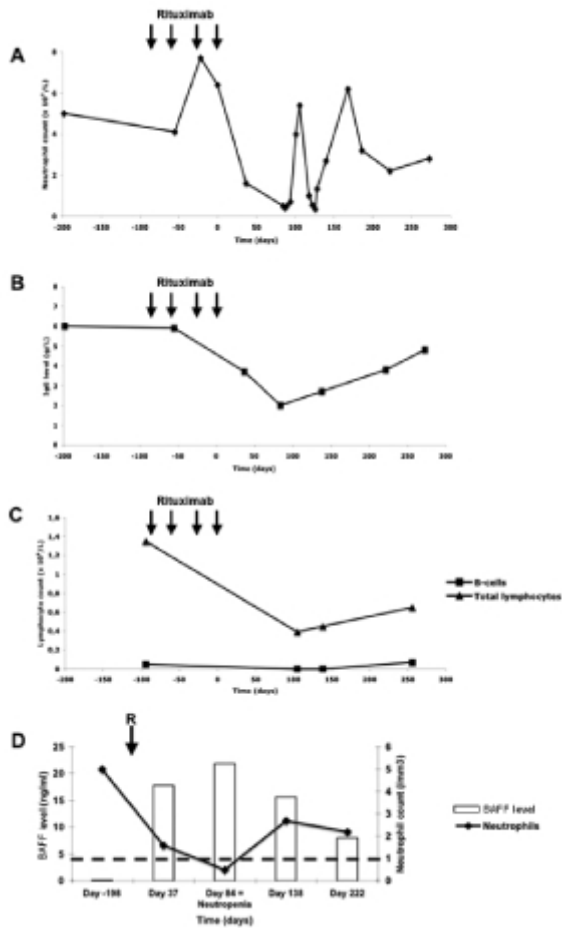


Figure 3. Kinetics of (A) LON, (B) serum immunoglobulins level and (C) B-cell count in PB. (D) Assessment of BAFF level in the patient's sera at the selected time points (the mean value of BAFF serum level after rituximab of 5 patients treated with rituximab but who did not develop LON is represented by the dotted line). Day 0 is defined as the end of treatment (last rituximab infusion).

the reemergence of B cells in the PB. Our results are consistent with the fact that LON could occur just before B-cell recovery as suggested by Dunleavy *et al.*,⁷ and are concordant with the usual timing of occurrence of LON after rituximab therapy. In our patient, BAFF serum level after rituximab was much higher (22 ng/ml) than in patients treated with rituximab for autoimmune systemic disorders but who did not develop LON (n=5, mean 5.0±2.3 ng/mL with the same assay).¹⁷ Such high BAFF serum level is very unusual and represents a very strong activating factor of B-cell recovery. Thus, we hypothesize that post-rituximab LON could be the consequence of a promotion of B-cell lymphopoiesis over granulopoiesis, determined by B-cell depletion-induced variations of growth and retention factors, including SDF-1 and BAFF, to support the reemergence of peripheral B-cells. Ueda *et al.* demonstrated that the reciprocal dynamics of the BM lymphocyte and neutrophil populations were consistent with a cellular competition within the developmental niche defined by a common location and shared growth resources, and that there was a balance between granulopoiesis and lymphopoiesis in the BM in normal condition, balance which could be dis-

turbed following rituximab.¹⁸ BAFF does not seem to be directly involved in LON-related mechanism but could represent a surrogate biomarker of the risk of developing LON following rituximab. The high increase of BAFF following rituximab could be linked to the profundity of tissuer B-cell depletion or to other unknown genetic factors. Another hypothesis could be that a cytokine, which was not measured in our study, exhibit an inhibitory effect on granulopoiesis. However, this hypothesis does not seem relevant since our patient's sera did not inhibit the growth of healthy donor CFU-GM. Studies on a larger cohort of patients are needed to confirm our hypothesis.

In conclusion, LON following rituximab would occur in patients with an excessive B-cell depletion and recovery, in relation to a hematopoietic lineage competition by promotion of B-cell lymphopoiesis over granulopoiesis within common developmental niches in the BM. Assessment of serum BAFF levels following rituximab could detect patients at risk of developing LON.

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Keywords: Neutropenia, rituximab, BAFF, niches, granulopoiesis, lymphopoiesis, lineage competition

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