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Rituximab associated neutropenia: Description of three cases and an insight into the underlying pathogenesis

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A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: To describe Rituximab associated neutropenia (RAN), and to explore its underlying mechanism.

Case Report: We describe three patients with RAN. The effect of patient's plasma on colony forming unit, Granulocyte-Monocyte (CFU-GM) was measured by the addition of plasma to the culture of a healthy bone-marrow. Repeated tests were performed after recovery of white count.

In the leukopenic period the patient's plasma inhibited CFU growth completely. Control plasma did not have such an effect. Addition of patient's cell supernatant to bone marrow cells did not change the number of CFU. The same effect was demonstrated in normal control. After recovery the patient's plasma did not inhibit colony formation, similar to control.

Conclusions: RAN is a clinically significant side effect. It may take place during treatment or several months afterwards. Circulating antibodies in the plasma may be responsible for this unique BM toxicity.

key words: **rituximab • neutropenia • bone marrow • pathogenesis**

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BACKGROUND

Rituximab is a genetically engineered monoclonal antibody directed against the CD20 antigen found on the surface of both normal and malignant B lymphocytes. Its possible mechanisms of cell lysis include complement dependent cytotoxicity and antibody-dependent cell mediated toxicity. It has been wildly used in the treatment of B-lymphocytic malignancies that express CD20. The toxicity of rituximab is generally mild and transient; the most common side effects are fever, hypotension and hypersensitivity reactions [1].

Recently, awareness has grown regarding an unusual complication of rituximab therapy, rituximab associated neutropenia (RAN). Reports of the incidence of this phenomenon were mostly derived from series describing delayed RAN, ie RAN-occuring after completion of treatment with this agent. Most studies reported an incidence ranging from 0.02% to 6% [2–4]. A recent report suggested this event to be more frequent, with an incidence as high as 25% [5]. The pathogenesis of RAN is unknown, although some suggested that antibody-associated mechanisms play a role in this condition [6,7].

We present three cases of neutropenia related to rituximab therapy: One case occurred during rituximab treatment, and two patients presented with delayed onsent neutropenia. We analyzed the serum of one of these patients, both during the neutropenia and afterwards, in an attempt to shed light on the underlying mechanism of RAN.

CASE REPORT

Case 1

A 33 year old woman suffering from diffuse large B-cell mediastinal non-Hodgkin's lymphoma (NHL) performed a complete blood count (CBC) on a routine follow up 78 days after completing treatment of chemotherapy and Rituximab, and was in complete clinical remission. Results revealed leukopenia of 800/mm^3 and neutropenia of 100/mm^3. Hemoglobin and thrombocytes levels were normal (12.4 gr/dL and 332,000/ml, respectively). Glucose, electrolytes, kidney and liver function tests were all within normal range. Serology for Parvovirus B19 was negative. A subsequent CBC two days later was similar (Figure 1). A few hours later she developed fever of 40°C and was admitted.

The patient had previously received weekly chemotherapy according to the VACOB-P protocol, composed of intravenous cyclophosphamide, adriamycin, vincristine, bleomycin, etoposide, prednisone, and rituximab.

Neutropenia developed after each treatment cycle and was consistent with the expected side effects of chemotherapy. The patient was treated with granulocyte colony stimulating factor (G-CSF) after each cycle, and had normal CBC following termination of treatment (Figure 1). The last treatment with chemotherapy and rituximab was given 78 days before the present neutropenia.

Upon evaluation, BM biopsy demonstrated all three hematopoietic lines with a left shift of the white line, no maturation of granulocytes, normal eosinophils, with average cellularity of 50% and no involvement by lymphoma. Chest X-ray was normal. She received antibiotics and 300 µg/d G-CSF for 7 days and the neutrophil count increased to 7,900/mm^3. Follow up neutrophil level revealed persistent neutropenia. She received 300 µg/d G-CSF each time the neutrophil count decreased below 1500/mm^3. Neutrophil levels at different time points are shown in Figure 1.

The protracted neutropenia lasted five months and recovered spontaneously. During this period she was hospitalized once more due to neutropenic fever and development of severe facial abscesses infected by *Pseudomonas aeruginosa*. She was treated with antibiotics and G-CSF, with complete resolution of all signs and symptoms. This patient is now well, in complete clinical remission for over two years.

Blood was drawn from this patient for analysis when she was first diagnosed with RAN, and two months after recovery. The results of this analysis are described separately (see "Plasma analysis from case 1").

Case 2

A 37 year-old woman with mediastinal diffuse large B-cell NHL was admitted because of fever and neutropenia. She completed six courses of chemotherapy and rituximab according to the R-CHOP protocol (rituximab 600 mg, cyclophosphamide, doxorubicin, vincristine and prednisone) and radiation to the mediastinum (a total dose of 30 Gy, in fractions of 1.5 Gy), and was in complete clinical remission for the previous 3 months.

Upon admission, laboratory results revealed leukopenia of 1,680/mm^3 with a neutrophil count of 50/mm^3. The hemoglobin level and platelet count were unremarkable (11.7 g/dL and 229,000/mm^3, respectively). Blood chemistry was unremarkable and chest X-ray was normal. BM aspiration revealed myeloid cells in all stages of maturation, relatively few stab cells and abundant promyelocytes and myelocytes. Erythropoiesis and megakaryogenesis appeared normal. BM biopsy showed no evidence of lymphoma. The patient was treated with 300 µg/d G-CSF with a recovery of the leukocyte count and neutrophil count to 8,240/mm^3 and 4,040/mm^3, respectively, within four days. The patient's follow up was uneventful and she remains in complete remission with normal CBC for more than 12 months.

Case 3

A 57 year old woman suffering from diffuse large B-cell NHL with BM involvement was admitted with neutropenic fever. She completed three out of six courses of chemotherapy and rituximab according to the R-CHOP protocol. The previous courses were uneventful. Laboratory results revealed leukopenia of 770/mm^3 with neutrophil count of 0/mm^3. The hemoglobin level and platelet count were 10.8 g/dL and 154,000/mm^3, respectively, and blood chemistry was normal too. BM biopsy revealed all three hematopoietic lines, absence of mature granulocytes, and no evidence of lymphoma.

The patient was treated with 300 µg/d G-CSF with recovery of the white count within three days. She continued to receive the same regimen of chemotherapy but without rituximab, without any subsequent episodes of neutropenia.

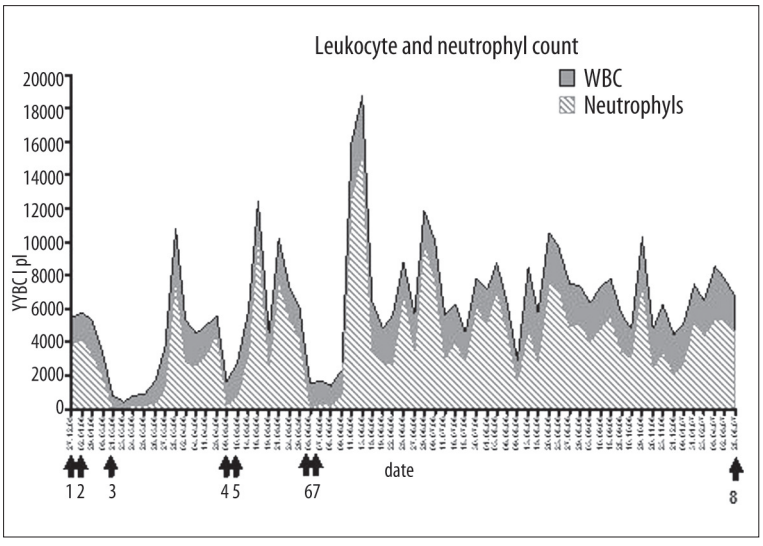

Figure 1. Total leukocytes and the number of neutrophils. The patient no' 1 blood count (CBC) showed variable number of leukocytes and neutrophils in different periods of treatment. The number of these cells at the dates of blood count is shown. The arrows show: 1: The last chemotherapy. 2: The last Rituximab. 3, 4,6: Patient's plasma collection before Neopogen (G-CSF) administration. 4,5, 6,7: Neopogen (G-CSF) administration. 8: Patient's plasma collection after recovery.

Her treatment resulted in complete clinical remission and her follow-up was unremarkable, for more than 4 months.

Analysis of plasma from case 1

Patient's plasma and leukocytes

As described, the patient's plasma and leukocytes were collected after diagnosis but before treatment for leukopenia, and in the recovery period, when she was no longer treated with G-CSF. BM and plasma from a healthy donor were used as a negative control.

Colony formation test

Heparinized blood from the patient (P) and a healthy donor (D) were centrifuged for 10 min, in 1500 RPM (round per minute) at 4°C. Plasma was separated from the cells and saved at –70°C. Cells were hemolysed by the addition of RBC lysing buffer (8.33 g NH_4Cl; 1g $KHCO_3$; 0.2ml 0.5M EDTA pure water added up to 1L; pH 7.4), and centrifuged for 10 min, at 2000 RPM, 4°C. Cell pellets (leukocytes) (P) and (D) were washed twice with Dulbecco's Phosphate Buffered Saline (PBS) and were resuspended (5×10^6 cells/ml) in RPMI medium-1640 (**R**oswell **P**ark **M**emorial **I**nstitute) with 5% Foetal Bovine Serum (FBS), (Beit Haemek cat.# 04-121-1A), supplemented with 2.5% Phytohemagglutinin (PHA), (PHA-M Beit Haemek cat.# 12-006-1H) and incubated at 37°C in a humidified 5% CO_2 incubator for 72 hrs. Supernatant was collected and stored at –70°C. Mononuclear cells from the bone morrow of a healthy donor were separated over Ficoll-Hypaque (Sigma cat. # 1077-1) and washed twice with RPMI medium. Washed cells were plated in triplicate at a concentration of 2×10^5 cells in 0.5 ml methylcellulose (Stem Cell Technologies MethoCult H4531 cat.# 04531) in 17 mm diameter wells (Nunc culture plates, cat.# 134673). The patient's plasma was added to 50 µl/well. The plasma of a healthy donor was used as a control.

In order to investigate the effect of cell secretion on the colony formation, the patient's and healthy donor's supernatants with and without PHA (see above), aliquots of 10 µl/well, were added to triplicate wells containing methylcellulose with a final BM cell concentration of 10^5 per well. The cultures were incubated for 14 days in a humidified incubator with 5% CO_2, 37°C. Aggregates of >20 cells (colony forming unit – CFU) were scored as colonies, using an inverted microscope (Olympus, Tokyo, Japan). The tests were repeated three times.

The same process was performed after the patient's recovery, when her WBC counts were normal.

Laboratory results

The effect of the patient's plasma on CFU growth was measured by the addition of 50 µl plasma to the culture of a healthy BM immersed in methylcellulose. In the leukopenic period (WBC 790/mm^3), the patient's plasma inhibited CFU growth completely (0 colonies), whereas the addition of healthy plasma did not change the number of colonies grown spontaneously from a healthy BM (125±4.3) (Figure 2). In order to investigate the role of cell secretion in CFU inhibition, the patient's cells and donor's cells were incubated for 72 hours in the presence of 2.5% PHA. As a baseline (control), PHA supplemented medium was also incubated with the cells. The addition of 10 µl/well of the supernatants to the methylcellulose culture of 10^5 per well BM cells, did not change the number of CFU, which resembled their quantity in all cases: patient's plasma, donor's plasma and PHA (116±17, 114±12 and 115±5.2 CFU, respectively). This observation suggests that the secretion of cells do not have any function in the inhibition of colony formation (Figure 3).

Two months after the spontaneous recovery of the patient's white count, the test (see above) was repeated. Her serum did not inhibit colony formation. The test results of the healthy donor and the patient were similar (Figure 2), (128±13 and 133±12.6 colonies, respectively). The patient's PHA supernatant (152±10.2) had the same effect as the donor's (143±12.9), similar to the PHA colonies (160±16.4) (Figure 3).

Discussion

In this article we presented three cases of grade 4 neutropenia associated with rituximab treatment for diffuse large

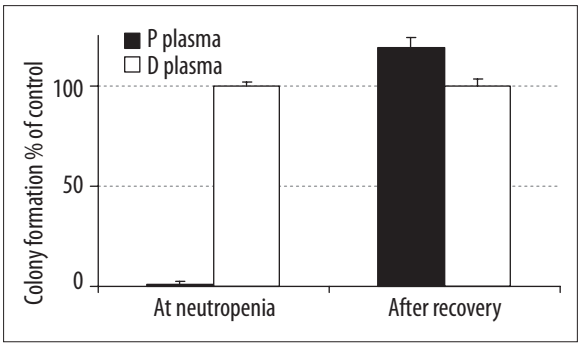

Figure 2. The effect of patient's plasma (P plasma) on colony formation (CFU-GM). The patient's plasma (P plasma) during neutropenia and after recovery was tested on a healthy BM for GM colony formation. The plasma of a healthy donor was used as control. The results are expressed as percent of control and mean of three independent experiments, each of them performed in triplicate. D plasma: Donor's plasma, P plasma: Patient's plasma.

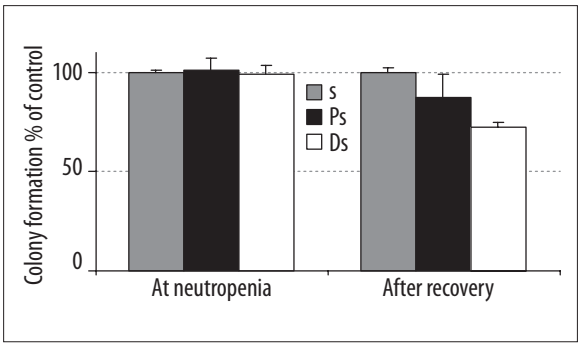

Figure 3. The effect of patient's cell supernatant (s) on colony formation. The effects of patient's mononuclear cells supernatant, induced by PHA at neutropenia and after recovery, were tested on a healthy BM for *(GM)* colony formation. The cell supernatant of a healthy donor was used as control. The results are expressed as percent of control and mean ±SD of three independent experiments, each of them performed in triplicate. Ps: Patients's cells supernatant, Ds: Donor's cells supernatant, s: PHA supernatant (without cells).

B-cell NHL. One patient developed this complication during treatment, while the two others developed it approximately three months after its completion; in one of these the neutropenia lasted five months. We demonstrated that the plasma of one of our patients inhibited cellular growth of the white line of a healthy bone marrow, while supernatant of cells from the patient did not have such effect. We therefore propose a humoral (immunoglobulin-mediated) mechanism as the pathophysiology of this phenomenon.

Previous cases of RAN have been reported. Whereas some authors described the appearance of RAN during treatment [8,9], this phenomenon was usually reported after the completion of treatment, generally within 28 to 204 days afterwards [3,4,10,11]. The duration of RAN varies between four days and one year [3,10–12]. In our patients, RAN started during treatment or three months after its completion. It had both an acute clinical course, lasting several days (two patients), and a protracted course, lasting 5 months (one patient).

The largest study of RAN was reported by Nitta at al. [5] They analyzed 24 out of 107 patients who received rituximab and developed RAN, being the highest incidence ever reported. Contrary to our patients who developed neutropenic fever, none of their patients had serious infections during the neutropenia. A multivariate analysis done suggested several risk factors for RAN: intensive chemotherapy, including ablative therapy followed by hematopietic stem cell transplantation and high dose methotrexate-containing regimens [5]. None of our patients underwent stem cell transplantation, and the patient that did receive high dose methotrexate developed neutropenia before that treatment. Similarly, other reports described RAN following standard regimens for lymphomas [3,11,12].

The etiology of RAN is still obscure. A direct toxic effect of rituximab on neutrophils is highly unlikely since the mean serum half-life of rituximab ranges from 31 to 407 hours, and therefore a direct toxic phenomenon cannot explain RAN appearing three months after treatment [13,14]. Furtheremore, granulocytes and uncommitted hematopoietic precursors do not express CD20, and therefore should not be directly affected by rituximab.

We tried to evaluate the mechanism by which neutrophil precursors of one of our patients were inhibited and demonstrated that her plasma completely inhibited CFU growth of a healthy BM. We also demonstrated that the supernatant of her leukocytes did not have such effect, similarly to supernatant of leukocytes from a healthy donor. This suggests that the secretion of cells do not have a role in the inhibition of colony formation, implying a mechanism of antibody-mediated toxicity.

A similar mechanism was suggested by Voog et al. [6], in their study on eight patients with severe RAN. They demonstrated IgG-type antibodies bound to the surface of neutrophils by immunofluorescence but no antibodies were found in the serum by flow cytometry or granulocyte agglutination test. They hypothesized that rituximab treatment depleted the normal B-lymphocyte populationt, which recovered within 3 to 9 months. During recovery, transient production of autoantibodies may occur, some of which may be directed against neutrophils or hematopoietic precursors[6]. This hypothesis may explain delayed RAN, but fails to explain RAN occuring during or shortly after the sessation of treatment.

Another etiology was hypothesized by Otroc et al. [7], who described two patients with rituximab induced acute thrombocytopenia. They suggested that the presence of CD20 antigen on platelets themselves or a soluble CD20 antigen in the circulation may cause an antigen-antibody reaction and immune-mediated cell lysis [7]. On the other hand Fukuno et al. [4], analyzed plasma and BM of three patients with RAN and did not find circulating immune complexes nor anti-neutrophil antibodies [4].

A different mechanism was suggested by Terrier et al. [15], who assessed serum, peripheral blood and BM of a patient with neutropenia associated with rituximab for Waldenstrom macroglobulinemia. They found lack of granulopoiesis in

the BM with a high level of BAFF, a strong stimulator of B-cell recovery. They hypothesized that the RAN is caused by competition by promotion of B-cell lymphopoiesis over granulopoiesis in the BM [15].

Conclusions

In summary, RAN may be more frequent and more clinically significant than previously thought. It may take place during treatment or several months afterwards. Circulating antibodies in the plasma may be responsible for this unique BM toxicity. Once confirmed, such antibodies may shed light on the exact mechanism by which rituximab induces its toxicities and more importantly, its therapeutic effects.

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