RESEARCH ARTICLE

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Evaluation of CD20-Positive B Cells in Libyan Lymphoma Patients Following Rituximab Treatment

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Abstract

Objective: To monitor the depletion of CD20-positive B cells in Libyan lymphoma patients treated with Rituximab using flow cytometry. **Methods:** Peripheral blood samples were collected from 25 newly diagnosed Libyan patients with histologically confirmed CD20+ B-cell non-Hodgkin's lymphoma, receiving Rituximab at the Oncology Departments of Tripoli Medical Center and the National Cancer Institute, Sabratha. Patients received intravenous Rituximab (375 mg/m²) combined with CHOP chemotherapy monthly for up to seven months, except one patient treated weekly for six weeks. Flow cytometry using CD19 and CD20 markers was performed on blood samples collected monthly or weekly post-infusion, employing BD TruCOUNT™ tubes and the Lyse No Wash method for accurate quantification of B cells. **Results:** Of the 25 patients, 24 (96%) showed complete depletion of CD19+ and CD20+ B cells by the seventh month of Rituximab treatment. Three out of five patients assessed at two months had complete depletion, while two showed a gradual decline. One patient (4%) remained resistant, with B cell counts exceeding 100 cells/μL up to the seventh month. Weekly monitoring in a single patient showed full B cell depletion by week four. Rituximab had no effect on CD3+ T cells, confirming its specificity. **Conclusion:** This study presents flow cytometry-based assessment of Rituximab efficacy in Libyan B cell lymphoma patients, providing novel population-specific data.

Keywords: Rituximab- CD20- B-cell Lymphoma- flow cytometry

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Introduction

In 1997, the Food and Drug Administration (FDA) approved the clinical use of a monoclonal antibody (mAb), namely Rituximab (Rituximab, Rituxan; Genentech, Inc, South San Francisco, CA and IDEC Pharmaceutical Corporation, San Diego). Rituximab remains one of the most prominent therapeutic monoclonal antibodies in clinical oncology [1]. It consists of human IgG1 heavy and light chain constant regions fused to variable regions derived from a murine monoclonal antibody [2]. Rituximab specifically targets CD20 antigen expressed on the surface of B lymphocytes. Since its approval, Rituximab has become a standard component in the treatment of follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL), and chronic lymphocytic leukemia (CLL) [3,4]. It is also used in autoimmune diseases where B-lymphocytes are believed to play a pathological role [5].

CD20 is a non-glycosylated phosphoprotein with a molecular weight of 33 to 35 KDa. It is expressed on the surface of B cells from the pre-B cell stage through maturity, but not on terminally differentiated plasma cells. The CD20 antigen is thought to play a role in B cell activation, proliferation, and differentiation [6]. CD20 is expressed in over 95% of B cell lymphomas [7].

Rituximab depletes CD20+ B cells via three primary mechanisms: complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and induction of direct cell death [8, 9]. Evidence supporting these mechanisms primarily stems from in vitro studies using CD20+ cell lines, and their precise contribution to clinical responses; either as monotherapy or in combination with chemotherapy, has yet to be fully determined. .

The aim of this study was to evaluate the efficacy of the anti-CD20 monoclonal antibody Rituximab in the treatment of Libyan NHL patients using flow cytometry as immunomonitoring tool.

Materials and Methods

Patients

Samples were collected from twenty five Libyan patients diagnosed with B-cell NHL, all undergoing Rituximab treatment at the Oncology Department of Tripoli Medical Center and the National Cancer Institute

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Sabratha. They were newly diagnosed patients with histologically documented CD20+ B cell NHL. Treatment involved a monthly intravenous infusion of Rituximab at a dose of 375mg/m2, administered in saline solution alongside CHOP chemotherapy for seven months, except for one patient who received Rituximab weekly for six consecutive weeks.

As a control, peripheral blood samples were also collected from healthy volunteers. Participant ranged in age from 18 to 80 years old, comprising seventeen males and eight females. The study was conducted in accordance with the Declaration of Helsinki, national laws and institutional guidelines. Written informed consent was obtained from all participants prior to their inclusion in the study,

Preparation of samples

Peripheral Blood samples were collected in tubes containing anticoagulant agent and analyzed within 24 hours. Samples were obtained from patients either monthly or weekly, following each Rituximab infusion. To determine cell counts, the Lyse No Wash (LNW) method (BD Bioscience) was employed. This method involves lysing red blood cells with a lysing solution (BD biosciences) and staining cells without wash. For absolute quantification, Becton Dickinson TruCOUNT™ tubes, preloaded with a known number of brightly fluorescent polystyrene beads, was used. During flow cytometry analysis, the bead population was gated, and the exact number of cells was calculated based on the bead to cell ratio [10]. Samples were kept in the dark at room temperature until analysis using FACSCalibur flow cytometry (Becton Dickinson).

Monoclonal antibodies

A gating strategy using CD45 versus side scatter (SSC) was applied to identify B cells in peripheral blood. This approach allows for the accurate identification of lymphocytes, which typically exhibit high 45 expression and low SSC. It also ensures the clear distinction between different white blood cells populations and the bead fluorescence population. Cells of interest were labeled with matched combinations of anti-human murine monoclonal antibodies directly conjugated to fluorescein isothiocyanate (FITC), Phycoerythrin (PE), and Peridinin chlorophyll A protein (PerCP). To analyze B cells, combinations of anti-CD45 (PerCP), anti-CD19 (FITC), and anti-CD20 (PE) were used. All antibodies were purchased from BD Biosciences.

Flow cytometry analysis

Flow cytometry was performed using FACSCalibur system, which is capable of detecting up to four fluorescent parameters. This system was used to monitor the depletion of CD19+ and CD20+ cells following Rituximab treatment.

Briefly, 50µl of anti-coagulated whole blood and 20µL of the relevant monoclonal antibody were added to the TruCOUNT tubes and mixed gently. After a 15-minute incubation in the dark at room temperature, a 450µl of FACS lysing solution (BD Biosciences) was added. Samples were then analyzed using CellQuest software (BD Biosciences). Instrument settings and fluorescence compensation were calibrated using BD CaliBRITE Beads with FACSComp software.

Results

Depletion of peripheral blood B cells by Rituximab

One month after Rituximab treatment, only one patient was available for analysis. This patient exhibited no depletion of CD20-positive B cells. The total lymphocytes count, as well as CD19+ and CD20+ cells, remained elevated compared to healthy control samples (Figure 1). At the two-month mark, an additional four patients were analyzed. Figure 2 shows the expression of CD19+ and CD20+ cells in all five patients following two months of Rituximab treatment. Two patients showed gradual

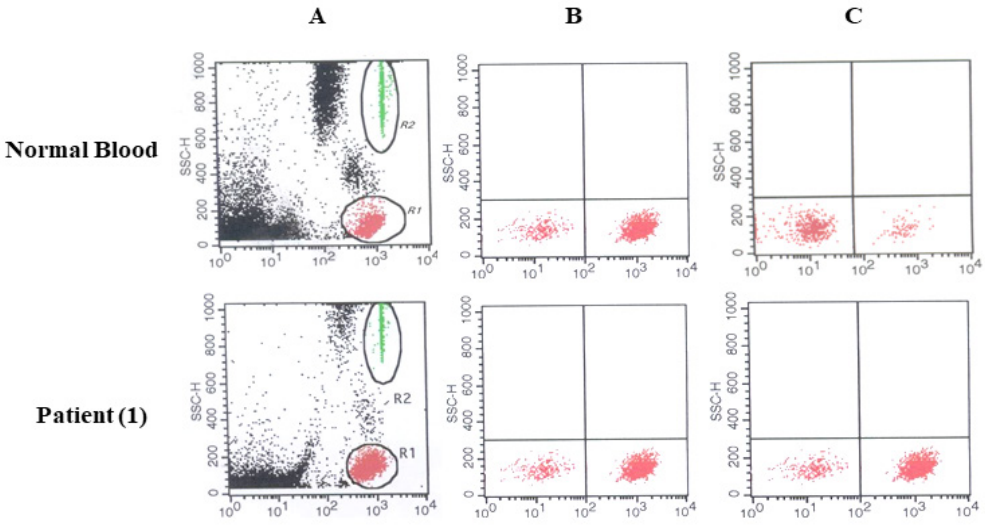

Figure 1. Gating Lymphocytes (R1) and beads (R2) on CD45 vs SSC dot plot column (A), column (B) represents CD19-expressed cells; and column (C) represents CD20-expressed cells. Positive cells are shown in the right side whereas negative cells are in the left side of the dot plots. The results represented for one patient following one month after Rituximab treatment.

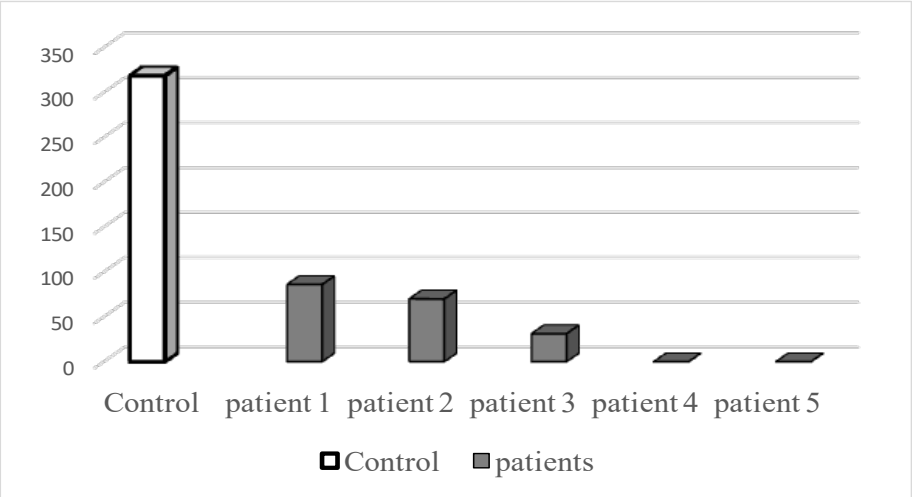

Figure 2. represents the Exact Number of CD20-Positive cells (B cells) in Five Patients Following Two Months of Rituximab Treatment. The results show gradual decrease of CD20-expressed cells in patients one and two (less than 100 cells/µl), and complete depletion of those cells in patients three, four and five (0 cells/µl).

decrease of CD19+ and CD20+ cell numbers, while the remaining three patients showed complete depletion of all B cell subpopulations.

In the subsequent cycles, up to seven months, all patients exhibited a complete depletion of CD19+ and CD20+ B cells (Data not shown). However, one patient showed persistent resistance to treatment. CD19+ and CD20+ cells remained detectable at levels exceeding 100 cells/µl even after five and seven months of Rituximab therapy (Figure 3).

B cell depletion with weekly Rituximab administration

One patient in this study received Rituximab treatment on a weekly basis for six consecutive weeks. Flow cytometry analysis was performed after each weekly infusion. Results showed a progressive decline in peripheral B-cells (CD19+ and CD20+ cells), with a complete depletion observed by the fourth week of treatment (Figure 4).

Specificity of Rituximab (No effect on CD20-negative cells)

As Rituximab is specific to CD20, it should not affect CD20-negative cell population. Figure 5 illustrates a representative flow cytometry plot showing that T lymphocytes (CD3+ cells) were unaffected by Rituximab. This finding was consistent across all patients.

Discussion

Rituximab is a chimeric mouse/human monoclonal antibody, engineered by grafting murine variable regions

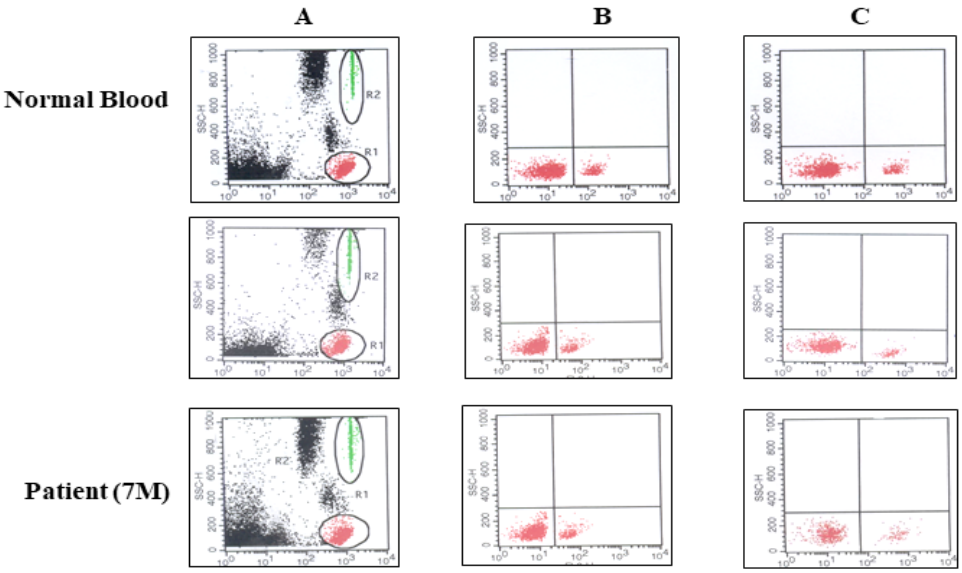

Figure 3. CD19+ and CD20+ cells are detected, showing resistance to Rituximab treatment. Gating lymphocytes (R1) and beads (R2) on CD45 vs SSC dot plot column (A). Column (B) represents CD19-expressed cells; and column (C) shows CD20-expressed cells. Positive cells are in the right side of the plots, whereas negative cells are in the left side. The results represented here are for patient following five and seven months of Rituximab treatment.

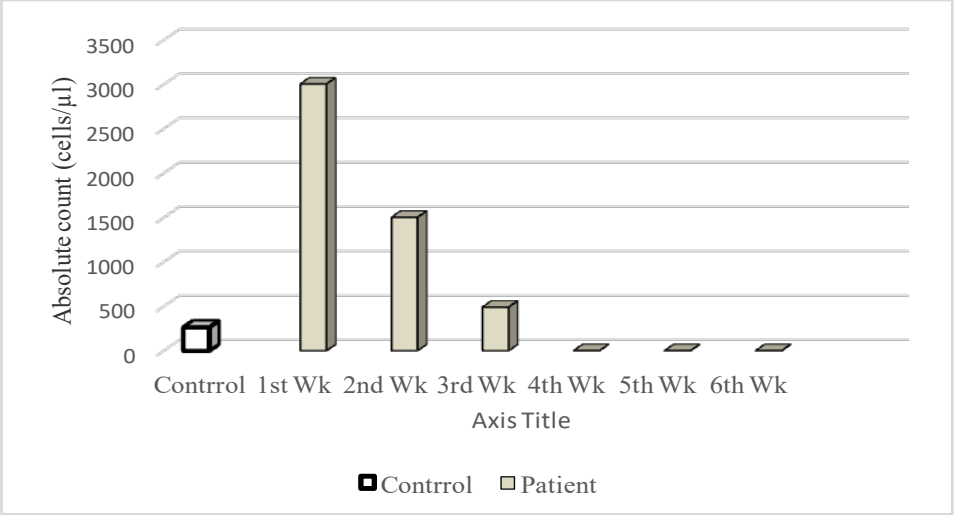

Figure 4. Represents the Exact Number of CD20-Positive Cells in One Patient Following Six Weeks of Rituximab Treatment in Comparison with Normal Blood. The results show gradual decline of CD20-positive cells with complete depletion observed at four weeks.

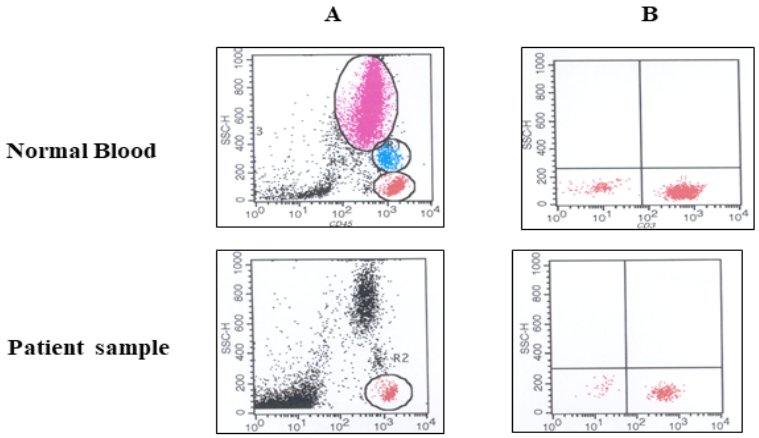

Figure 5. Gating Lymphocytes on CD45 vs SSC Dot Plot Column (A). Column (B) represents CD3-expressed cells (T cells). Positive cells are in the right side of the plots, whereas negative cells are in the left side. The results represented here are for normal blood as well as patient treated for seven months with Rituximab.

targeting the CD20 antigen onto human constant regions. CD20 is an ideal target for antibody-based therapies because it is expressed on the surface of mature B cells, and in approximately 95% of patients with B-cell lymphoma [7], but is absent on precursors B cells and plasma cells [11]. Although Rituximab induces depletion of B cells, normal B-cell populations can be eventually regenerated from CD20-negative stem cells [12].

The mechanisms by which Rituximab depletes B-cell lymphoma include complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity, and induction of apoptosis [8, 9].

This study aimed to assess the depletion of CD20+ B cells in the peripheral blood of Libyan patients with B-cell lymphoma undergoing Rituximab therapy. Flow cytometric analysis was performed using a CD45 vs SSC gate strategy to identify the lymphocyte population, which appears as a bright, low SSC cluster. Although CD45 is also expressed on other leukocytes such as monocytes, granulocytes, eosinophils, and basophils, subsequent staining with CD19 and CD20 antibodies allowed for specific identification of B cells.

Twenty-five patients were included in the study. After the first Rituximab infusion, only one patient was available for analysis, showing no evidence of B-cell depletion. The elevated counts of total lymphocytes, CD19+ and CD20+ cells in this patient likely reflect circulating tumor cells. Notably, complete depletion was observed in this patient by the fourth treatment cycle.

In the second treatment cycle, five patients were analyzed. Two of these showed a gradual decrease in B-cell numbers, while the remaining showed a complete depletion of all B-cell subpopulations. These inter-patient differences are consistent with previous reports highlighting variability in individual responses to Rituximab [13]. By the third cycle (approximately three months into treatment) a complete depletion of B-cells was observed in all patients.

Interestingly, one patient remained refractory to Rituximab throughout the study. Despite seven cycles of treatment, CD20+ B-cells were still detectable, and the patient was subsequently switched to an alternative

therapeutic regimen. Resistance to Rituximab may arise through various mechanisms, including internalization of the CD20 antigen, impairment of ADCC, reduction in CDC due to complement consumption, or intrinsic resistance to apoptosis [11, 14]. Additionally, selective pressure from antigen-specific therapies can lead to tumor cell mutation or phenotypic changes, resulting in the loss of CD20 expression and resistance to further Rituximab treatment. In fact, some studies report that, following a second course of Rituximab, tumor cells may no longer express CD20 on the membrane or within the cytoplasm [15]. Loss of CD20 expression has also been associated with progression from low-grade to high-grade lymphoma [16].

A limitation of this study is the relatively small sample size, which reflects the total number of patients available during the study period. Additionally, some patients were lost to follow-up after certain treatment cycles due to transfers to other hospitals or seeking treatment abroad. Despite these factors, our results provide valuable preliminary insights into Rituximab response and resistance in a patient population that remains underrepresented in lymphoma research.

In conclusion, this study offers novel insight into the dynamics of peripheral CD20+ B-cell depletion in Libyan patients with B-cell lymphoma undergoing Rituximab therapy, a population underrepresented in current research. Our findings demonstrate that Rituximab effectively depletes peripheral CD20+ B cells by the third treatment cycle in most patients. Notably, we document variability in depletion kinetics and highlight a rare but clinically significant case of persistent Rituximab resistance. This resistance, potentially driven by mechanisms such as CD20 antigen modulation or impaired immune effector functions, underscores the complexity of treatment responses and the need for personalized monitoring. By characterizing these responses in a distinct patient cohort, our study contributes valuable data on Rituximab's clinical efficacy and resistance patterns, supporting the development of tailored therapeutic strategies for improved management of B-cell lymphoma across diverse populations.

Author Contribution Statement

AMA: Design of the study, analysis of data, interpretation of results, and drafting and revision of the manuscript. EE: Design of the study, acquisition and analysis of data. SA, ME, ASE: Acquisition of data and interpretation of results. YL: Reviewing the manuscript and editing.

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Ethical approval

This study was approved by the Bioethics Committee at Biotechnology Research Center, (BEC-BTRC). All patient data were collected as part of routine diagnostic procedures and standard clinical follow-up for B-cell lymphoma patients, and were anonymized prior to analysis. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was carried out in the best interest of the patients.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Study registration

This study was not registered in a clinical trial registry, as it involved analysis of routine clinical data and did not constitute a clinical trial or interventional study.

Conflict of interest

The authors declare no conflict of interest.

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