

Measuring Humanized Antibodies in Plasma of Patients Treated With Antibody-Based Therapy Using Bead-Based Flow Cytometry

The Story of Alemtuzumab

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Summary

Alemtuzumab (Campath), the humanized rat monoclonal antibody that targets the CD52 surface antigen, is currently used for treatment of patients with resistant chronic lymphocytic leukemia. Monitoring levels of the antibody in plasma/serum could provide insight into the optimal dosing and scheduling of therapy. Current methods of detecting alemtuzumab in serum or plasma are complicated and difficult to adapt to high-throughput testing. We describe a novel bead-based assay that measures circulating alemtuzumab by taking advantage of remnant rat sequence in the antibody. Levels of total alemtuzumab complexed with CD52, and free alemtuzumab are quantitated in the serum or plasma by flow cytometry. This approach is applicable to the measurement of other humanized antibodies that contain an appropriate remnant animal sequence.

Key Words: Alemtuzumab; CAMPATH-1H; CD52; chronic lymphocytic leukemia; plasma.

1. Introduction

Alemtuzumab (Campath[®]; Berlex, Montville, NJ) is a humanized rat immunoglobulin (IgG1) monoclonal antibody directed against CD52, which is expressed on the surface of mature T- and B-cells, monocytes, and myeloid cells (1,2) but not in hematopoietic cells (3). Alemtuzumab causes cell lysis through complement fixation in the host and antibody-dependent cellular cytotoxicity (4). It has been clinically successful in previously treated patients with chronic lymphocytic leukemia, with response rates of up to 33% (5).

From: *Methods in Molecular Biology*, vol. 378: *Monoclonal Antibodies: Methods and Protocols*
Edited by: M. Albitar © Humana Press Inc., Totowa, NJ

Alemtuzumab has also been studied for treatment of various T-cell malignancies such as graft-vs-host disease and autoimmune disease (6–9).

The original method of detecting and quantifying alemtuzumab in serum or plasma is based on capturing the alemtuzumab using cell line-expressing CD52 and quantifying the binding antibodies (10). This procedure is complicated and difficult to adapt for high-throughput analysis in clinical laboratories. More importantly, we have reported that free circulating CD52 can be detected in circulation, especially in patients with chronic lymphocytic leukemia and this free CD52 binds to the alemtuzumab in circulation forming immune complexes (11,12). It is important to measure total alemtuzumab, complexed alemtuzumab/CD52, and free alemtuzumab. Alemtuzumab is a fully humanized rat antibody in which most of the Ig framework is of human origin. However, sufficient rat sequence remains and we have reported the development of enzyme-linked immunosorbent assay based on capturing the rat portion of the alemtuzumab and detecting the human portion (13). Here, we report a simple bead-based assay that uses the same approach as that of the enzyme-linked immunosorbent assay with anti-rat Ig antibodies capturing the remnant rat sequence of alemtuzumab on beads instead of the surface of the plate then detecting the humanized portion using anti-human Ig antibodies. Furthermore, we report a method for the detection of the alemtuzumab/CD52 complexes as well as the free alemtuzumab. To detect the alemtuzumab/CD52 complexes, we first capture total alemtuzumab on the surface of beads using the anti-rat Ig antibodies, then we use anti-CD52 antibodies for detecting the complexes. As for detecting the free and not complexed alemtuzumab, we first immunoprecipitate the alemtuzumab/CD52 complexes from the plasma/serum sample using beads coated with anti-CD52, then we use this plasma/serum for detecting the alemtuzumab as previously described. These quantitative assays can be adapted for use in clinical laboratories.

2. Materials

2.1. Reagents and Equipment

1. 6.9 μ M Carboxylated polystyrene beads (Bangs Laboratories, Fishers, IN).
2. Sodium bicarbonate (Sigma Aldrich, St. Louis, MO).
3. Carbodiimide (Sigma Aldrich).
4. Rabbit anti-rat IgG antibody (Sigma Aldrich).
5. Rat anti-human CD52 (Serotec, Raleigh, NC).
6. Phosphate-buffered saline (PBS) (Ca+/Mg+-free) (Sigma Aldrich).
7. Phycoerythrin (PE)-labeled rabbit anti-human IgG (BD Bioscience, San Jose, CA).
8. PE-labeled anti-CD52 (BD Biosciences).
9. Seven-color setup beads (BD Bioscience).
10. Sorvall Cellwasher 2 (Thermo Electron Corporation, Asheville, NC).

2.2. Buffer Preparation

1. Flow PBS: 5.6 g sodium phosphate dibasic, 35.48 g sodium chloride, 2.8 g bovine serum albumin (BSA), and 4.0 g sodium azide. Add deionized water to 4 L with and adjust pH to 7.4.
2. Blocking/dilution buffer (1 $\times$ PBS/5% BSA): 10 $\times$ PBS (Ca+/Mg+-free), 0.5 g BSA. Add deionized water to 1 L with and adjust pH to 7.4.
3. Activation buffer (0.05 M sodium bicarbonate): 4.2 g sodium bicarbonate. Add deionized water to 1 L with and adjust pH to 8.0.
4. Quenching solution (40 mM glycine): 3.02 g glycine. Add deionized water to 1 L and adjust pH to 7.4.

2.3. Flow Cytometric Analysis

1. FACSCanto™ flow cytometer (BD Biosciences).
2. FACSDiva™ software (BD Biosciences).
3. QuantiBRITE™ PE beads (BD Bioscience).
4. FlowJO analysis software (Treestar, Ashland, OR).

3. Methods

The methodology described here is based on immunoprecipitation and detection on beads. Beads are either coated with antibodies directed against rabbit anti-rat IgG or anti-CD52. Upon exposure to plasma, the rat IgG-coated beads immunoprecipitate both total alemtuzumab and alemtuzumab–CD52 complexes; both of which can be separately quantitated with PE-labeled antibodies directed against the human component of the IgG or CD52, respectively. The CD52-coated beads immunoprecipitate alemtuzumab–CD52 complexes leaving free alemtuzumab in the supernatant. Free alemtuzumab is detected by immunoprecipitation with the IgG-coated beads and then detected with a PE-labeled human antibody.

3.1. Instrument Setup

1. Calibration should be performed prior to every acquisition using seven-color setup beads (BD Biosciences). Beads are prepackaged to be reconstituted with the provided buffer.
2. The FACSCanto is prepared by running “Fluidics Startup” and selecting the appropriate acquisition template and instrument settings on FACSDiva software.
3. Standardization is performed before acquisition of samples using QuantiBRITE PE beads, which are reconstituted with 1 mL flow PBS. Five thousand events are acquired on the FACSCanto.

3.2. Bead Conjugation and Preparation

1. All washes are performed with centrifugation at 17,949g or 2 min at room temperature unless otherwise stated. Beads are coated with antibodies directed against

rabbit anti-rat IgG or rat anti-human CD52 according to the manufacturer's protocol. The following is a brief description.

- On day 1, 100 μ L of 6.9 μ M carboxylated polystyrene beads are resuspended in 1 mL of activation buffer (sodium bicarbonate) and washed twice.
- After washing and resuspending in 1 mL of activation buffer, the beads are incubated in the presence of 10 mg carbodiimide for 25 min at room temperature with mixing.
- The beads are then washed and resuspended in 1 mL activation buffer.
- 100 μ L of the beads are then incubated with either 100 μ L rabbit anti-rat IgG or 50 μ L rat anti-human CD52 overnight at 4°C with mixing.
- On day 2, the beads are washed and blocked with 1 mL blocking buffer for 90 min at 37°C with mixing.
- The beads are washed with 1 $\times$ PBS/5% BSA and incubated with quenching buffer for 30 min at room temperature with mixing.
- The beads are then washed and resuspended with 1 $\times$ PBS/5% BSA (*see Note 1*).

3.3. Sample Preparation

- Plasmas are collected from the peripheral blood of a chronic myeloid leukemia (CML) patient and normal individuals. PBS is also used as a negative control.
- The plasmas are diluted 1:10,000 in PBS/2% BSA and denatured in the presence of 2% SDS at 96°C for 5 min, followed by cooling to room temperature and centrifugation at 17,949g for 2 min (*see Note 2*).
- 500 μ L of denatured plasma is then aliquoted into two separate 1.5-mL microcentrifuge tubes.
- Plasma is incubated with 30 μ L of either IgG-conjugated beads or anti-CD52 conjugated beads for 3 h at room temperature with mixing.

3.4. Detection of Total and Complexed Alemtuzumab

- The IgG-conjugated beads are centrifuged at 13,000g for 2 min.
- After removal of supernatant, beads are washed three times with 1 $\times$ PBS/5% BSA.
- The beads are then split into two tubes and incubated with either 3 μ L of anti-human PE (to detect total alemtuzumab) or 5 μ L of CD52-PE (to detect complex alemtuzumab/CD52) for 1 h at room temperature with mixing.
- Beads are washed in the cell washer and resuspended in 500 μ L flow PBS for analysis. An example of the results is shown in **Fig. 1A,B**.

3.5. Detection of Free Alemtuzumab

- The CD52-conjugated beads are centrifuged at 17,949g for 2 min.
- The supernatant (which contains free CD52) is transferred to a fresh 1.5-mL microcentrifuge tube and incubated with 30 μ L IgG-conjugated beads for 3 h at room temperature with mixing.
- After centrifugation at 13,000g for 2 min, the beads are washed and resuspended in 1 $\times$ PBS/5% BSA and then incubated with 3 μ L anti-human PE for 1 h at room temperature in the dark with mixing.

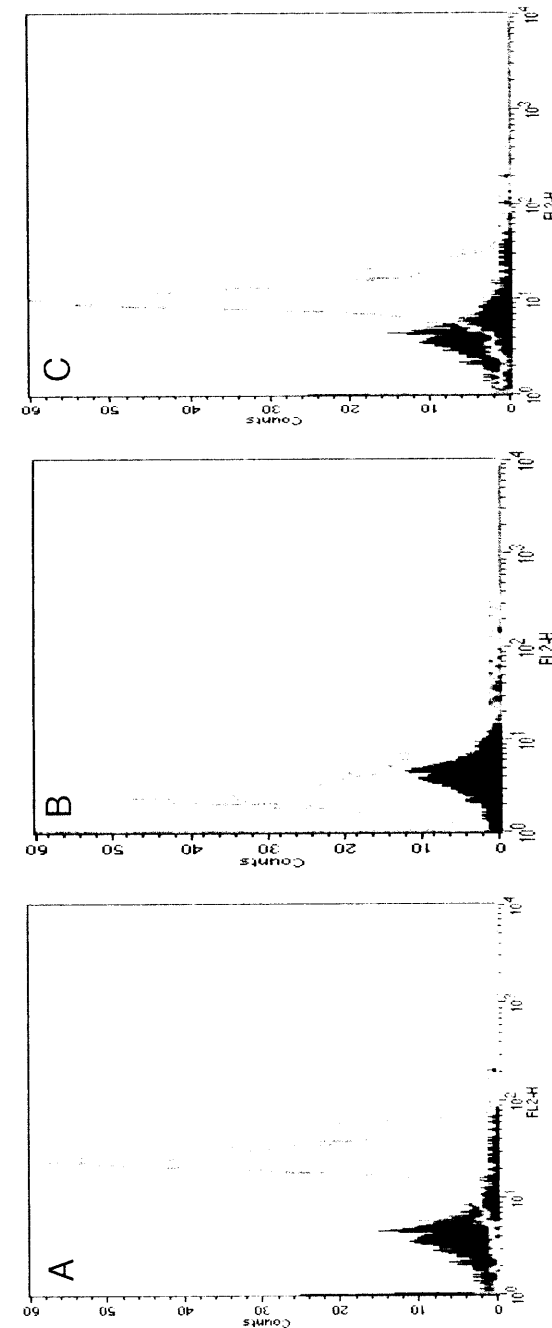


Fig. 1. Histogram overlays representing either unstained control beads (black) or stained beads (grey line). Beads are stained with either rabbit anti-human PE (to detect total Alemtuzumab) (A), anti-CD52 PE (to detect Alemtuzumab-CD52 complex) (B), or rabbit anti-human PE (to detect free Alemtuzumab) (C).

- The beads are then washed in the cell washer and resuspended in 500 μ L flow PBS for analysis. An example of the results is shown in **Fig. 1C**.

3.6. Acquisition and Analysis

- The samples are acquired on the FACSCanto using FACSDiva software. Flow cytometry output is in the form of FCS files.
- Quantitation is based on a fluorescence standard given by QuantiBRITE PE beads.
- Data are analyzed using FlowJo software. Normal samples are used as the baseline for setting regional markers. Samples are analyzed based on gating according to the normal plasma samples.

3.7. Calculations

- Index = % positive protein $\times$ antibody binding capacity (ABC) (*see Note 3*).
- Ratio phosphorylated: total protein = index phosphorylated protein/index total protein.

4. Discussion

Proper dosing of therapeutic agents such as alemtuzumab may vary according to the tumor load and immune system in each patient. Thus, accurate measurement of alemtuzumab in the serum or plasma could provide the information necessary for tailoring therapy for individual patients. The assays described here take advantage of the minimal residual rat sequence in the alemtuzumab antibody, using antibodies against the rat Ig sequence. This approach can be used to monitor levels of alemtuzumab in patients during treatment and to correlate the results with clinical responses and adverse effects. Similar approach can be used for other humanized antibodies.

5. Notes

- It is preferable that the conjugation be confirmed by performing a detection step with anti-mouse PE. The conjugation is detected by incubation of diluted conjugated beads (1:10 with blocking buffer) with 5 μ L anti-mouse PE for 15 min at room temperature in the dark. The beads are washed and resuspended in 500 μ L flow PBS for flow analysis.
- The dilution factor of plasma should be determined empirically.
- Fluctuations of intracellular proteins manifests as changes in percent of cellular positivity and fluorescence intensity. The index is a derived value that accounts for both. Index = (% positivity) $\times$ (ABC).

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