Development of flow cytometry for detection and quantitation of red cell bound immunoglobulin G in autoimmune hemolytic anemia with negative direct Coombs test

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Summary

About 2-10% of patients with warm-antibody autoimmune hemolytic anemia (WAIHA) exhibit a negative direct Coombs test (DAT), requiring more sensitive tests, including detection of RBC-bound immunoglobulins by flow cytometry, for diagnosis. In this study, the optimal conditions for detection and quantitation of RBC-bound IgG by flow cytometry were studied using blood samples from six patients with AIHA and two healthy individuals. Quantitation of RBC-bound IgG was performed using quantum simply cellular (QSC) beads coated with goat anti-mouse IgG antibodies. For detection of RBC bound IgG, a 60-minute incubation of all blood samples with 40 μl of 1:10 dilution of FITC-conjugated mouse anti-human IgG gave mean fluorescent intensity (MFI) values comparable to experiments using larger amounts or higher concentrations of the anti-human IgG. The acquired antibody binding capacity (ABC) values (or IgG molecules) for each QSC bead level, at 40 μl of 1:5 and 1:10 dilution of anti-human IgG for 60 minutes were close to the manufacturer-assigned ABC values. The IgG molecules per RBC in all six patients with positive DAT of 4+, 3+, 2+, 1+, trace and negative DAT were 31,725, 3,823, 1,753, 524, 260 and 88 respectively and in two healthy individuals with negative DAT they were 104 and 78. *(Asian Pac J Allergy Immunol 2011;29:364-7)*

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Submitted date: 10/5/2011
Accepted date: 17/11/2011

Key words: *red blood cell, autoimmune hemolytic anemia, flow cytometry, red cell bound immunoglobulin G, Quantum Simply Cellular (QSC) bead*

Introduction

Autoimmune hemolytic anemia (AIHA) is an acquired hemolytic anemia caused by the production of antibodies against the individual's own red blood cells (RBCs).[1] The direct antiglobulin test (DAT) using the conventional tube technique (CTT) or direct Coombs test is the most commonly used method and is still the gold standard test for the diagnosis of AIHA.[2] However, a negative DAT by CTT does not exclude the diagnosis of this disease, as 2-10% of patients with the clinical characteristics of AIHA, including corticosteroids responsiveness, exhibit a negative DAT by CTT. Probably the most likely cause of this phenomenon is that the DAT by CTT is not visually sensitive enough to detect IgG of less than 500 molecules per red cell.[3] Flow cytometry has been applied to detect RBC-bound Ig due to its high accuracy, reproducibility and comparability to other sensitive assays.[2] In addition, it can also be used for quantitation of Ig molecules expressed on cell surfaces, which will increase its significance for the diagnosis of Coombs-negative AIHA. This can be done by measuring and comparing the fluorescence intensity (expressed as the geometric mean area) of antigen expressed on the cell surface using an external standard,[4] such as quantum simply cellular (QSC) beads. The QSC beads are covalently coated with goat anti-mouse IgG (Fc-specific) antibodies which bind mouse monoclonal IgG with different predefined goat anti-mouse antibody concentrations and can be used to estimate the mouse monoclonal IgG molecules and their corresponding epitopes (such as human Ig molecules on blood cells) by a calibrating comparison of fluorescence intensity on a histogram scale. This study was designed to investigate the optimal

conditions for the detection and quantitation of RBC-bound IgG (IgG molecules) in patients with AIHA by flow cytometry.

Methods

Blood samples

EDTA blood samples were taken from 2 patients with AIHA who were undergoing regular-interval follow-up (samples 1, 2), 2 patients with newly diagnosed AIHA (samples 3, 4), 2 patients with AIHA which was in disease remission (samples 5, 6) and 2 healthy individuals (samples 7, 8). All of the patients had been regularly followed at the Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital. After obtaining informed consent, blood samples from the patients were collected from the remaining (blood) specimens from routine CBC and analyzed on the same day. This study was approved by the Faculty's IRB.

Tube agglutination DAT

The DAT was performed in a standard glass tube according to a standard protocol.[5]

Detection of red blood cell bound IgG by flow cytometry

EDTA blood was centrifuged at 1500 rpm for 10 minutes. The plasma and buffy coat were removed. Five microliter pellets of washed packed red cells[6] were incubated with 20 µl, 40 µl and 60 µl of each dilution at 1:5, 1:10, 1:20 and 1:40 of fluorescein isothiocyanate (FITC)-conjugated mouse anti-human IgG (AbD Serotec, Oxford, UK) at room temperature for 30 minutes and 60 minutes in the dark. In the control tube, RBCs were incubated with FITC-conjugated mouse IgG1. The cells were washed with PBS and suspended in 1% para-formaldehyde solution. Finally, the RBCs were examined with a flow cytometry analyzer (FACS Calibur, Becton and Dickinson, USA).

Determination of antibody binding capacity (ABC) values for quantitation of IgG molecules by flow cytometry

Quantitative analysis of RBC bound IgG was performed using the quantum simply cellular (QSC) beads (Bangs Laboratories Inc., Fisher, USA). Briefly, each combination of the 4-level bead populations in a single tube was incubated with 20 µl, 40 µl and 60 µl of each dilution at 1:5, 1:10, 1:20 and 1:40 of fluorescein isothiocyanate (FITC)-conjugated mouse anti-human IgG at room temperature for 30 minutes and 60 minutes in the dark. Then, the beads were washed with PBS and suspended in 500 µl of PBS. The tube with the blank bead population was run first, followed by all the other tubes with antibody-coated beads. A calibration curve was constructed by plotting the acquired fluorescence intensities from the QSC beads, expressed as the geometric mean area, against the manufacture-assigned antibody-binding capacity (ABC) values using an Excel spreadsheet template-QuickCal software provided by Bangs Laboratories. Using the same instrument setting, the ABC values of unknown samples were then determined by comparing their fluorescence intensities against the calibration curve.

Results

The agglutination strengths of the direct Coombs test for the four groups of samples are shown in Table 1.

For the optimal conditions for detecting red blood cell bound IgG, we found that, with all the 8 tested samples, a 1:5 dilution of (FITC)-conjugated mouse anti-human IgG antibody gave the highest mean fluorescence intensity (MFI) values. With each dilution of the antibody, the volume of 20 µl rendered almost the lowest MFI while the MFI values from 40 µl and 60 µl of the diluted antibody were comparable. As several studies of the detection of RBC bound IgG by flow cytometry used 30 minutes as the incubation time,[5,7] we therefore applied the same time interval as the standard incubation time to investigate for other variables affecting the optimal conditions. However, we found that the MFI values from using 40 µl of 1:10 dilution of the antibody with 60-minute incubation were close to the values with 40 µl of 1:5 dilution of the antibody at 30 minutes as the standard incubation time. Thus for the economical purpose, we chose a condition of 40 µl of 1:10 diluted antibody at 60 minute-incubation as optimal for detection of red blood cell bound IgG. The MFI values of the 8 tested samples are shown in Table 1.

For quantitation of antibody binding capacity (ABC) or IgG molecules, the MFI values of each QSC bead level were used to construct a calibration curve using the Bangs Laboratories' Software (QuickCal), to quantitate the ABC values or IgG molecules (Figure 1). With the 1:5 and 1:10 dilutions of FITC-conjugated mouse anti-human IgG antibody the acquired ABC values of each QSC bead level were close to the manufacturer-assigned ABC values and ABC thresholds (of blank beads) were less than 1,000 after incubation for 60 minutes.

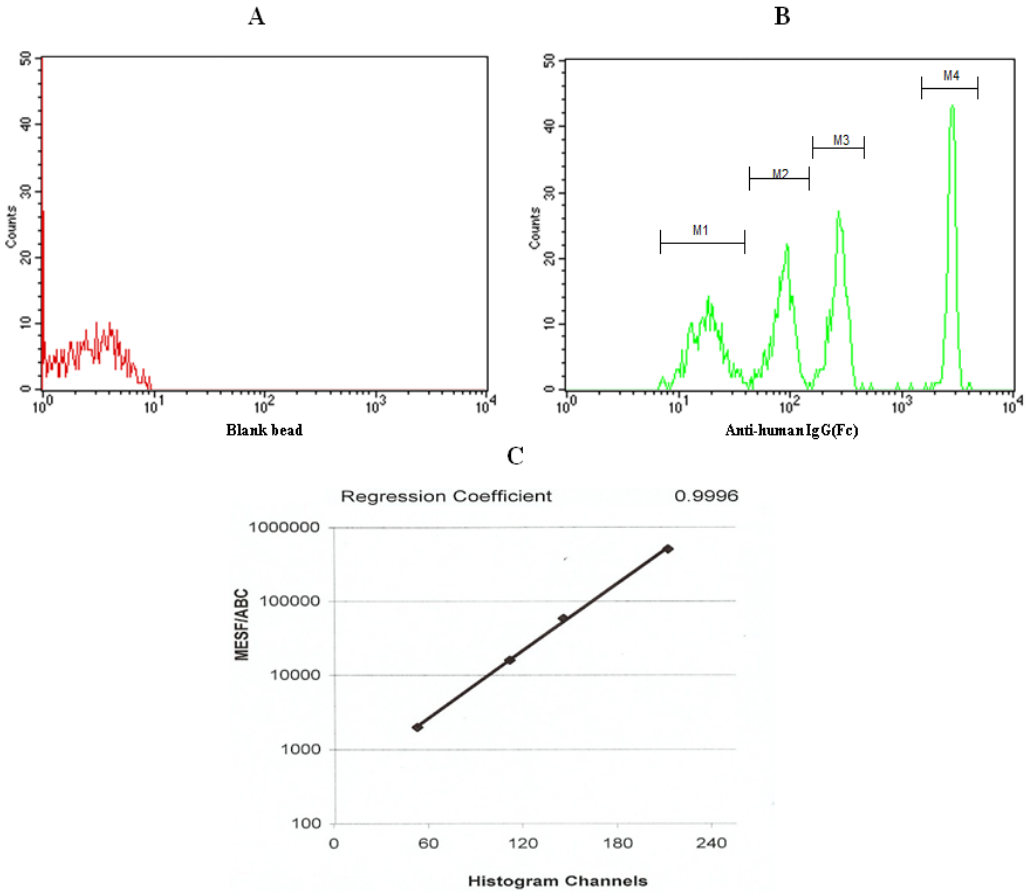

Figure 1. Panel A represents the fluorescence histogram of the blank bead population, panel B represents the fluorescence histograms of the four bead populations which are M1 = bead level 1, M2 = bead level 2, M3 = bead level 3 and M4 = bead level 4 and panel C represents calibration curve for conversion of fluorescence intensity into antibody binding capacity (ABC) values (or IgG molecules). This curve relates the manufacturer-assigned ABC value of FITC-labeled microbead to the mean histogram channel number.

We also found that the acquired ABC values at 40 µl of 1:5 dilution of the antibody for 60 minutes were mostly close to the manufacturer-assigned (ABC) values. Thus, we decided to use the calibration curve from a condition of 40 µl of 1:5 dilution of antibody with incubation for 60 minutes as the optimal condition for determination of ABC values or IgG molecules and the numbers of IgG molecules of all the 8 tested samples are shown in Table 1.

Discussion

As the major objective of our study was to investigate the optimal conditions for detection and quantitation of RBC-bound IgG by flow cytometry, we used only eight blood samples from individuals with different *in vivo* red cell sensitization for this purpose. With all the 8 tested samples, the MFI values were not different between the 40 µl and 60 µl of 1:5 dilution of FITC-conjugated mouse anti-human IgG antibody as well as other dilutions. On the contrary, in some samples the amount of antibody at 60 µl yielded lower MFI values than at 40 µl of the same dilution. This phenomenon may be explained by the loose affinity of the antigen-antibody complex which may easily be dissociated during washing of the red blood cells. Regarding the incubation times, most of previous studies used an incubation time of 30 minutes for detection of RBC bound IgG by flow cytometry.[5,7] In this study we investigated the optimal conditions by comparing 30-minute incubation with 60-minute incubation. The condition of 40 µl of 1:10 diluted antibody at 60 minute-incubation was chosen as the optimal condition because less amount of antibody was used,

Table 1. Results of the direct Coombs test and the MFI values and ABC values (IgG molecules) for the 8 tested samples under the optimal condition of flow cytometry.

Sample (No.)	Tube DAT (Polyspecific IgG+C3d)	Mean fluorescence intensity (MFI)	ABC values (IgG molecules)
1	4+	114.64	31,725
2	2+	5.75	1,753
3	1+	1.65	524
4	3+	12.87	3,823
5	trace	0.80	260
6	negative	0.26	88
7	negative	0.31	104
8	negative	0.23	78

and more samples can therefore be studied at lower cost.

Furthermore, we found that the acquired ABC thresholds (by blank beads) at 1:20 and 1:40 dilution of FITC-conjugated mouse anti-human IgG antibody were close to the acquired ABC values of bead level 1. Therefore, with these dilutions of the antibody, the ABC values for normal controls will not be clearly separated from those for patients, as abnormally high numbers of IgG molecules may be encountered on red cells from normal controls. Thus, these dilutions of the antibody were not chosen as optimal for the test. In addition, at any dilution of the antibody, the incubation time of 60 minutes gave acquired ABC values closer to the manufacturer-assigned ABC values than the 30 minute-incubation,with an ABC threshold of less than 1,000. This phenomenon implies that mouse anti-human antibody optimally binds to the goat anti-mouse IgG on QSC beads with this incubation time. Based on our data, we chose a condition at 40 μl of 1:5 dilution of the antibody for 60-minute incubation as optimal because the acquired ABC values obtained were mostly close to the manufacturer-assigned (ABC) values while the ABC threshold was less than 1,000. Furthermore, we found that the concentrations of antibody required to saturate the QSC beads were more than to those needed to saturate red blood cells (RBCs). Our findings were consistent with those of Serke et al.[8] who reported that the concentrations of fluorochrome-labeled monoclonal antibodies required to saturate QSC beads were not necessary the same as those for cellular epitopes.

In conclusion, we have shown that the optimal conditions for detection and quantitation of RBC-bound IgG by flow cytometry can be determined. These techniques are potentially applicable for the diagnosis of Coombs negative AIHA. Quantitative DAT from flow cytometry can be used to confirm the presence and the significance of red blood cell bound antibody in anemia due to immune hemolysis. This issue is currently being investigated in our laboratory.

Acknowledgement

This study was supported by a developmental fund from Division of Routine to Research (R to R), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

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