



Relative sensitivity of direct antiglobulin test, antibody's elution and flow cytometry in the serologic diagnosis of immune hemolytic transfusion reactions

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ABSTRACT

Background and Objectives. Current immunohematology practice dictates that serologic diagnosis of immune hemolytic transfusion reactions (IHTR) is based on the finding of a positive post-transfusion direct antiglobulin test (DAT). However, since DAT may fail to detect antibody-coated cells when they constitute a minor population amid a large number of non-sensitized ones, we investigated whether antibody detection in eluates or by flow cytometry is more sensitive than DAT in this context.

Design and Methods. Ten samples of red blood cells sensitized with allo- or autoantibodies were diluted in non-sensitized red blood cells to final concentrations ranging from 10% to 0.1%. DAT, antibody detection in eluates, and immunofluorescence by flow cytometry were performed on each mixture.

Results. DAT failed to detect sensitized cells in all but two cases in that only the 10% dilution yielded a positive DAT. Antibody detection in eluates and by flow cytometry was able to detect up to 1% sensitized cells in most cases.

Interpretation and Conclusions. Antibody detection in eluates and by flow cytometry is more sensitive than DAT for detecting minor populations of IgG-coated cells. These techniques should be included in the routine investigation of suspected cases of IHTR.

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Acute and delayed immune hemolytic transfusion reactions (IHTR) are rare, albeit serious hazards of blood transfusion. Diagnosis of IHTR is usually suspected on clinical and analytical grounds, and confirmed by serologic methods. Reference textbooks on immunohematology state that serologic diagnosis of IHTR is based on a positive post-transfusion direct antiglobulin test (DAT). Elution of the antibody attached to red blood cells (RBCs) is recommended only in DAT-positive cases, in order to identify the antibody's specificity.¹⁻³ DAT-

positivity in post-transfusion blood samples has been used as the main criterion of delayed IHTR in several studies,⁴ and the belief that it is sensitive enough to detect most delayed IHTR constituted the basis for the inclusion of autocontrol in compatibility testing.

Recently, we have seen an acute IHTR in which the post-transfusion DAT was negative, despite the corresponding alloantibody being easily detected in the eluate. This finding led us to speculate that hemagglutination-based techniques, such as DAT, may fail to detect antibody-coated cells when they constitute a minor population amid a large number of non-sensitized cells, as may occur in some IHTR. Flow cytometry may have a greater sensitivity than DAT in this context, since it has proven to be useful in detecting minor cell populations in the diagnosis of both fetal maternal hemorrhage and RBC chimerism after bone marrow transplantation.⁵ In addition, eluates of post-transfusion samples demonstrated the corresponding alloantibody in the majority of patients with delayed IHTR in one series, even in those cases in which DAT was negative for IgG.⁶

We undertook the present study to investigate whether these two methods are more sensitive than DAT in detecting minor populations of sensitized RBCs, as may be present in IHTR. For this purpose, mixtures with decreasing proportions of IgG-coated RBCs in non-coated ones were tested by DAT, flow cytometry, and antibody detection in eluates.

Design and Methods

RBCs with their corresponding antigens were sensitized with plasma from patients or blood donors with anti-D (3 cases), anti-E or anti-K (one case each). Plasma was mixed in a 2:1 proportion with washed, concentrated RBCs, and the mixture was incubated at 37°C for one hour. After washing four times, an adequate sensitization was demonstrated by a strong agglutination (+3-+4) in DAT. In addition, we also used IgG-coated RBCs from five patients with immune hemolytic anemia (IHA).

Sensitized and non-sensitized, antigen-negative RBCs were diluted in PBS and adjusted to a hematocrit of 50±4% (measured by a Coulter counter MD2). For each antibody specificity, six different mixtures with decreasing proportions of sensitized RBCs (10%, 5%, 2.5%, 1%, 0.5% and 0.1%) in non-sensitized ones were prepared. The direct antiglobulin test, antibody detection in eluates and indirect

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immunofluorescence by flow cytometry were performed on each mixture.

For the DAT one volume of RBCs was washed four times in 10 volumes of PBS pre-warmed to 37°C. A drop of a 2-5% suspension of washed RBCs in PBS was mixed with two drops of either monospecific anti-IgG antiglobulin (Gamma Biologicals, Houston, TX, USA) or 30% bovine serum albumin. The tubes were immediately centrifuged and macroscopic agglutination was assessed and graded on a scale of +1 to +4. All negative results were validated with commercially available Coombs-positive cells.

Eluates were obtained by the chloroform-trichloroethylene method.⁷ Briefly, 1 mL of thoroughly washed and concentrated RBCs were mixed with 1 mL of normal saline and 2 mL of a 50% mixture of chloroform in trichloroethylene. After shaking the mixture vigorously for one minute, it was incubated at 37°C for ten minutes, shaken again for one minute and centrifuged at 2,200 x g for ten minutes. After filtering the eluate and allowing the chloroform-trichloroethylene to evaporate, it was tested against a panel of three reagent RBCs (Immucor Inc., Norcross, GA, USA) by a LISS-enhanced indirect antiglobulin test. To assure absence of free antibody after washing the sensitized RBCs, the supernatant of the last washing was tested in parallel.

For the flow cytometry analysis, samples were diluted to 0.5% in PBS, and 100 µL of this suspension were incubated with 10 µL of FITC-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark) at 22°C in the dark for 40 minutes. After being washed three times with PBS the cells were resuspended in 1 mL of PBS. Samples were accurately resuspended before analysis to remove macroscopic agglutination. Flow cytometry analysis of the labeled samples was performed on a FACScan (Becton Dickinson, Mountain View, CA, USA) equipped with a 15-mW air cooled 488-nm argon laser and analyzed using Lysis II software. Forward scatter (FSC), side scatter (SCC) and fluorescence channels were set at logarithmic

Table 1. Minimum proportion of antibody-coated RBCs in non-sensitized RBCs that gave a positive test result.

	Direct antiglobulin test	Antibody detection in eluate	Flow cytometry
Autoantibody (1)	> 10%	5%	1%
Autoantibody (2)	> 10%	10%	1%
Autoantibody (3)	> 10%	2.5%	1%
Autoantibody (4)	10%	1%	1%
Autoantibody (5)	> 10%	0.5%	1%
Anti-D (1)	> 10%	1%	5%
Anti-D (2)	> 10%	1%	2.5%
Anti-D (3)	10%	1%	1%
Anti-E	> 10%	2.5%	5%
Anti-K	> 10%	1%	1%

gain. RBCs were selected using FSC and SCC gates and read on a dot plot (FL1 versus FSC). Ten thousand cells were analyzed from each sample. Samples from populations of 100% sensitized and non-sensitized RBCs were used as positive and negative controls, respectively.

Results

Sensitivity of each method in detecting minor populations of antibody-coated RBCs is shown in Table 1. As can be seen, DAT failed in all but two cases to detect even a 10% proportion of sensitized RBCs. Flow cytometry and antibody testing in eluates were able to detect a proportion of IgG-coated RBCs below 5% in near all the cases. However, only antibody elution detected a less than 1% proportion of sensitized cells. Figure 1 shows the flow cytometry results of a representative case.

Discussion

These results show that flow cytometry and antibody detection in eluates are more sensitive techniques than DAT for revealing minor populations of

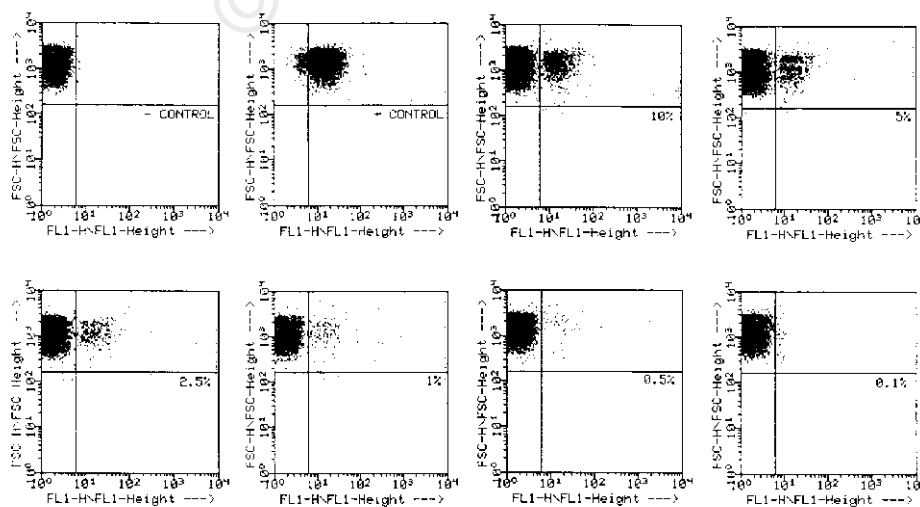


Figure 1. Representative flow-cytometry dot-plots of a typical case of immune fluorescence detection of IgG-coated RBCs. Percentage numbers in the panels represent the proportions of sensitized cells. The negative and positive controls are samples with 0% and 100% sensitized RBCs, respectively. IgG-coated cells appear in the right upper quadrant, whereas non-sensitized ones are in the left upper quadrant.

IgG-coated RBCs. Indeed, both methods could detect up to 1% of sensitized RBCs in non-sensitized RBCs. The relative sensitivity of flow cytometry and antibody detection in eluates varied from case to case, so neither of the two methods was consistently better than the other. Such variability was probably due to differences in antibody concentrations in eluates or in the intrinsic affinity of each antibody. In our study DAT showed a sensitivity below that which is currently believed to be the detection sensitivity of minor populations of antibody-coated RBCs. It has been reported that hemagglutination procedures may detect one antigen-positive RBC per 100 antigen-negative RBCs.^{8,9} In these studies, however, antigen-positive RBCs were detected by direct agglutination with commercially available IgM antibodies, which is a more sensitive method than the antiglobulin test. Thus, while direct agglutination can detect as few as 50 IgM molecules per RBC, the spin-antiglobulin test requires a minimum of 100-150 IgG molecules per cell.^{10,11}

It should be noted that in some patients with IHTR, DAT-positivity can also involve the patient's own RBCs, which would increase the diagnostic yield of DAT over that which is suggested by our results. Thus, in acute and delayed IHTR involving complement-fixing antibodies, complement can also be activated on the patient's own cells.⁶ It has also been suggested that in some delayed IHTR,⁴ as well as in cases of emerging alloimmunization,¹² autoantibodies can be formed in addition to alloantibodies, thus also increasing the proportion of DAT-positive cells.

Despite the fact that the above factors operating *in vivo* can make DAT more useful for detecting IHTR than suggested by our results, the present study shows that flow cytometry and antibody detection in eluates are more sensitive techniques than DAT in revealing minor populations of IgG-coated RBCs. Therefore, they should be included in the routine investigation of suspected cases of IHTR.

Contribution and Acknowledgments

AA wrote the article and participated in the experimental design and the performance and analysis of the immune hematology experiments. SR and SM collaborated in the performance of the experiments and data analysis, and in the critical review of the manuscript. CS was mainly responsible for the flow cytometry experiments, she also participated in the study's design, data analysis, and revision of the manuscript. AP was responsible for the conception of this investigation, data interpretation, and critical review of the manuscript. The list of authors was arranged in increasing order of seniority.

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Disclosures

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Manuscript processing

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Potential implications for clinical practice

- ◆ Flow cytometry and antibody detection in eluates should be included in the routine investigation of suspected cases of immune hemolytic transfusion reactions.

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