Advances in Forensic Haemogenetics 2
(c) Springer-Verlag Berlin Heidelberg

Flow Cytometry: An Alternative Quantitative Method to Determine Antigen Zygosity and Its Application to Paternity Testing

M.E. McCloskey, C.R. Harrison, and R. Heinz

Department of Pathology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284-7751

Single indirect exclusions are a common problem in paternity testing. Approaches to resolve single indirect exclusions include: repeat testing with different reagents, testing for variant alleles, use of additional genetic markers and determining the zygosity by titration scores.[1] This study investigated the use of flow cytometry as an alternative technique for determining antigen zygosity as well as antigen strength.

MATERIALS AND METHODS

Samples of red blood cells were collected in acid citrate dextrose. 100 µl of a 0.25% suspension of red cells was incubated with 100 µl of a 1:2 dilution of commercial anti-c in U bottom microtiter plates and incubated for 30 minutes at 37°C. A blank control containing phosphate buffered saline (PBS pH 7.3) in place of antiserum was included. After washing twice with PBS, 100 µl of PBS was added to the dry cell button, followed by 100 µl of optimal dilution (1:20) of fluorescein conjugated Fab fragment of goat anti-human IgG heavy and light chains specific (FITC). This was incubated for 30 minutes at 22°C in the dark and washed twice with PBS. 100 µl PBS was added and each well content was transferred to FACS tubes, and analyzed on a Becton-Dickinson Fluorescence Activated Cell Sorter (FACS 420). The FACS made an analysis of 10,000 cells and printed a single histogram representing the number of cells versus channels of fluorescence. Each day a control consisting of a mixed population of cells of known phenotypes including a negative, a heterozygous and a homozygous cell was tested and used to set the FACS parameters (Fig 1).

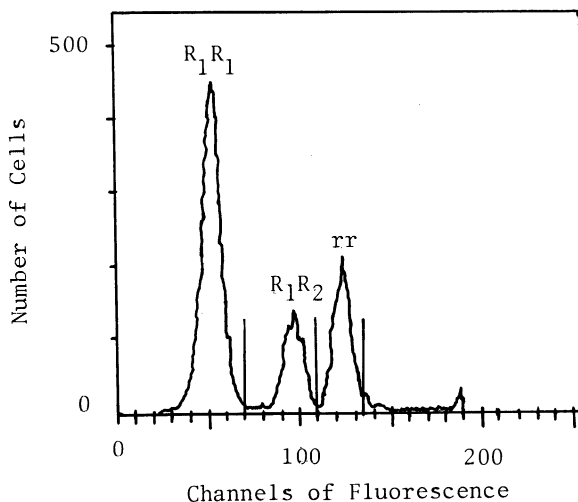

Figure 1 - Mixed cell population of cells negative, heterozygous and homozygous for the c antigen

Advances in Forensic Haemogenetics 2
Edited by W. R. Mayr
© Springer-Verlag Berlin Heidelberg 1988

Advances in Forensic Haemogenetics 2
(c) Springer-Verlag Berlin Heidelberg

The zygosity and antigen strength of each unknown red cell was determined by comparing the mode fluorescence channel with those of the control mixed cell population. Direct typing by conventional agglutination technique was then performed to determine the Rh phenotype.

RESULTS

The results of the phenotypic determination by agglutination and the corresponding fluorescence channels are displayed in Table 1.

Table 1. Mode channel of fluorescence with anti-c

Phenotype	Anti-c	PBS	Difference
dce	120	50	70
DCcEe	92	49	43
DCce	93	56	37
DCe	47	48	0
DC(c)(E)e	55	49	6

For each phenotype the mode channel of fluorescence using anti-c is compared with the mode of a blank control using PBS. The difference between the two values gives a semi-quantitative evaluation of the antigenic strength. It is apparent that results obtained by flow cytometry are consistent with the direct agglutination results. A homozygous c red cell demonstrates a difference of 70, when a heterozygous DCcEe has a difference of 43. Differences in channels among heterozygous phenotypes such as DCcEe (43) and DCce (37) may suggest varying degrees of antigen strength. The very weak c antigen of a red cell with the phenotype DC(c)(E)e, detected only by absorption-elution techniques, showed small changes in mode fluorescence channel (6) consistent with the weakness of the antigen. This phenomenum was consistently repeatable.

CASE STUDY

A paternity trio demonstrated a single indirect exclusion in the Rh system, with a residual paternity index of 178 (AF:DCEe M: DcEe; Ch: DcE) Titration studies with anti-c yielded the following results:

Child 1:2 (score 13); homozygous control 1:8 (score 25); and heterozygous control 1:2 (score 18).

Flow cytometry analysis with anti-c gave the following results.

Advances in Forensic Haemogenetics 2
(c) Springer-Verlag Berlin Heidelberg

Table 2. Mode channel of fluorescence of the child's red cells with anti-c

Phenotype	Anti -c	Blank	Difference
dce	84	43	41
dce	84	41	43
DCce	71	43	28
DCcE	65	39	26
DCe	38	40	0
Child	53	40	13

By comparing channels of fluorescence to the homozygous and heterozygous controls one can conclude that the child is heterozygous for the c antigen. This confirmed the titration studies. The data suggests that the c antigen in the child has a weaker strength than a normal heterozygous control. It is accepted that the C antigen produced by the R^z (*DCE*) gene, is less in quantity than that produced by R^1 (*DCe*). Commercial anti-C typing reagents often contain mostly anti-Ce (anti-rh_i)[2]. Considering these two facts, the child was retested with additional anti-C reagents. The incubation times and the serum to cell ratio were increased and a weak C antigen was demonstrated on the child's red cells by using more sensitive techniques than those recommended by the anti-sera manufacturers.

DISCUSSION

Flow cytometry methods work well in determining antigen zygosity. However thorough knowledge of the instrument's capabilities is necessary to adjust it properly. For example, changes in the instrument's fluorescence gain can markedly enhance resolution between peaks. With the ability to change fluorescence gain, small differences among antigen strengths is more easily distinguished. Absolute differences of antigen strength can only be compared within a run since mode fluorescence channels will vary with the gain. Clear separation of the three cell populations is important in monitoring day to day fluctuation of the instrument's resolution and sensitivity. Careful instrument calibration is essential in order to obtain reproducible quantitative FACS measurement[3]. This is especially important when examining weak antigenic strength.

This method has been successfully applied to the determination of zygosity with anti-D and anti-E. However, at present, reliable results have not been obtained with anti-C and anti-e. The purity of anti-C antisera containing mostly anti-Ce presents problems not only with direct agglutination but also in FACS analysis. Optimal dilutions were never obtained for the C antigen in order to obtain clear separation of mixed cell populations of the C antigen. Thus the strength of the C antigen was not determined by FACS techniques in this case.

This study demonstrates the value of flow cytometry as a method for determining antigen zygosity and its application in the evaluation of the validity in a single indirect exclusion.

Advances in Forensic Haemogenetics 2
(c) Springer-Verlag Berlin Heidelberg

REFERENCES

1. Lee CL, Williams G. How to deal with an indirect exclusion of paternity. Am J Clin Pathol 77:204-206, 1982.

2. Issitt PD. Applied Blood Group Serology. 3rd ed. Miami: Montgomery Scientific, 1985, pp. 239-240.

3. VanBockstaele DR, Bermenan AW, Muylle L, Cole-Dergent J, Peetermuns ME: Flow cytometric anlaysis of erythrocytic and antigen density profile. Vox Sang 51:40-46, 1986.