Technical methods

Determination of Rh(D) genotype: use of human monoclonal antibodies in flow cytometry

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Recent developments in human monoclonal antibodies have led to the production of different varieties of rhesus anti-D.¹ These have in turn allowed new serological techniques to be developed, and in this paper we examine their use in determining Rh D genotype. Knowledge of the father's genotype is helpful when assessing the risk of immunisation of an Rh D negative mother during pregnancy, and Masouredis² and Hughes Jones³ have shown that the number of D antigen sites on the red cell is partly a function of genotype.

In 1975 a semiquantitative method for D genotyping, using an electronic cell counter working by aggregate exclusion, was developed by Lopez *et al*,⁴ but the technique was time consuming and only allowed small numbers of samples to be studied. Monnet and Cabadi⁵ described an alternative rapid automatic method using an aggregometer, and Rouger *et al*⁶ developed another using a continuous flow system. All these techniques were based on cell agglutination.

Using a different approach, Loven *et al*⁷ distinguished homozygosity from heterozygosity using antibody coated cell rosette formation, but we have now developed a simple new technique detecting antibody binding using monoclonal anti-D reagents and a cytofluorograph.

Material and methods

All the red blood cells used were frozen in liquid nitrogen and thawed just before testing. Eighty seven subjects of known genotype were studied.

Two human monoclonal IgG anti-D (Rho) antibodies QA37C3 and H2D5D2 (described by Goossens *et al*¹) were used. The ratio between the number of IgG anti-D molecules and antigen sites in the experimental conditions varied from five to 20 (always with a large excess of antibodies).

FLUOROMETRIC ANALYSIS

The tests were carried out using tubes with 50 µl of a red blood cell suspension containing 2×10^7 cells/ml. The red blood cells had been washed three times in phosphate buffered saline (PBS) (pH 7.20). The 50 µl cell suspensions were incubated with 50 µl of the monoclonal antibody solution at 37°C for two hours.

After three washings in phosphate buffered saline 50 µl of rabbit antihuman IgG conjugated with fluorescein isothiocyanate (Pasteur Diagnostic, France) was added, and this mixture was incubated for 30 minutes at 20°C.

After three additional washings the cells were suspended in phosphate buffered saline at a concentration of 10^6 cells/ml. The amount of bound antibody on each red blood cell was analysed with a cytofluorograph (cytofluorograph system 50 L, Ortho Diagnostic Systems, Westwood, Minnesota), using a 488 nm line with 40 mW light power from an argon ion laser. The exciting light was excluded by the dichroic filters and a narrow band pass (514–540 nm) filter. One thousand cells were analysed each second. Non-aggregated red blood cells were selected by the right and forward angle light scatter. The fluorescence distribution was visualised by histograms (relative fluorescence plotted against number of cells). The histograms are close to Gaussian shaped point spread functions. The mean was assimilated to the mode and directly determined on the histogram. The coefficient of variation (CV) was also determined graphically.

Results

We obtained a direct association between mean fluorescence and genotype. Table 1 shows the mean fluorescence corresponding to the presence of one or two active alleles, R¹ or R². The mean fluorescence of red blood cells from R²r people is 60% of the mean fluorescence of red blood cells from R²R² people. This percentage is 64 between R¹r and R¹R¹ and 51 between R¹R¹ and R²R². The CV was low: with the monoclonal antibody H2D5D2, it was 18, and with the monoclonal antibody QA37C3, it was 19.

We analysed samples from men married to women of phenotype rr (dce/dce; Rh negative) over two years. The genotype of each of the twenty cases deter-

Table 1 Mean (SD) fluorescence obtained by two anti-D monoclonal antibodies with different red blood cells of known genotypes R^1R^1 , R^2R^2 , R^1r , R^2r

Antibody	Genotype			
	R^2R^2 (n 16)	R^2r (n 18)	R^1R^1 (n 14)	R^1r (n 19)
IgG ₁ 1λ anti-D H2D5D2 (25 µg/ml)	230 (50)	140 (20)	120 (30)	80 (10)
IgG ₁ K anti-D QA37C3 (5 µg/ml)	190 (40)	115 (20)	100 (20)	60 (10)

Table 2 Possible genotypes (control cells) compared with those of an RhD positive father

Genotype of control red blood cells	Mean fluorescence
R^0r^1	135
R^1R^0	200
R^1R^1	130
R^1r	85
rr	<5
Case 1	80

This man is a D + C + c + E - e + phenotype. Two genotypes are possible; R^1R^0 ; R^0r^1 . The cyto-fluorometric analysis data are compatible with heterozygous status. The child was rr^1 (D - C + c + E - e +).

mined conventionally was always consistent with the data from flow cytometry. Table 2 shows an example of a patient studied in our laboratory. For each case we used red blood cell controls corresponding to the possible genotypes.

Discussion

The technique outlined seems suitable for studying the zygosity of a given red cell character and makes it possible to determine if an Rh (D) positive subject is genetically D//D or D//d (or D// -). The process is simple and based on antibody binding. The same results were obtained with fresh and thawed red cells. All previous simple methods have depended on aggluti-

nation, where only the kinetics of the reaction are correlated with genotype.

The method described relies on two things. First, the use of monoclonal antibodies of high and homogenous affinity; and secondly, the use of flow cytometry, which allows direct quantitation of the amount of bound antibody.

References

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Simple reagent modification of Technicon H6010C that permits processing of uraemic samples

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The Technicon H6010C performs an automated differential leucocyte count. Leucocytes are classified

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according to their size and reaction with cytochemical dyes. This classification relies on prior lysis of erythrocytes. Blood samples from patients with a raised concentration of urea (> 15 mmol/l (90 mg/100 ml)) cannot be processed on the analyser because the erythrocytes will not lyse using standard Technicon reagents. Where lysis is incomplete, the instrument records a spuriously high leucocyte count and an increased number of cells classified as lymphocytes and "large unstained cells."

In hospitals with large renal units this may result in a considerable number of samples having to be processed on instruments that do not have this limitation. Alternative instruments, however, do not provide a full differential leucocyte count.