Accurate Rh phenotype determination by reticulocyte mRNA typing shortly after multiple transfusions

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ABSTRACT

Alloimmunization is a common phenomenon after transfusion, with an estimated incidence of 0.5% increasing to 20-60% in chronically transfused patients. In recently transfused patients, serological typing can be hampered by mixed field agglutination. We established RT-PCR methods for *RHD*, *RHC/c* and *RHE/e* typing using mRNA from reticulocytes. Molecular typing was performed soon after 51 separate mismatched transfusion events involving 30 patients. Accurate identification of the transfused patients' phenotype was confirmed in all cases. Reticulocyte maturation studies revealed that temperature is a crucial parameter for transition into mature red blood cells.

Key words: multiple transfusions, reticulocytes, molecular *RH*-typing

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Introduction

Multiply transfused patients are particulary at risk of alloimmunization to antigens on erythrocytes. Transfusion practice requires that the donor's blood group is compatible with the recipient's regarding the ABO system and preferably RhD. Antibodies in chronically transfused patients, such as those with thalassemia and sickle cell disease, are most frequently directed towards other antigens in the Rh blood group system.[1,2] As alloantibodies can cause hemolysis of transfused RBCs, their specificities must be identified for further compatible transfusions. Phenotyping by hemagglutination assay less than three months after transfusion can be difficult and often impossible because of mixed-field agglutination. An alternative method for blood group typing is presented here where we demonstrate how mRNA from reticulocytes can be used to identify a patient's phenotype after multiple transfusions even when the sample is taken within hours after the last transfusion. Correct molecular typing of the patient's own antigens has been demonstrated for *RHD*, *RHE/e* and *RHC/c* using mRNA as template. The approach also facilitates the genotyping of *RHC* which has been difficult in RhD-positive subjects.[3,4] As reticulocytes are known to have a long lifespan at 4°C we studied their *in vitro* maturation time to see if transfused reticulocytes might interfere with our method. No interference was observed as reticulocyte maturation is rapid at body temperature.

Design and Methods

Peripheral blood samples were collected from 30 patients belonging to surgical, hematologic and intensive care units at Ulleval University Hospital. Inclusion criteria were transfusion with at least one mismatched Rh-antigen compared with pretransfusion phenotyping. None of the patients included had been transfused within the last three months. Leukocyte reduced RBC units were used in all transfusion events apart from patient #18 who received 4 units of whole blood in addition to 45 leukocyte reduced RBC units. The study was approved by the Regional Committee for Medical Research Ethics, Health Region East, Norway. RhC, -c, -E, -e and -D antigens in patients were determined using standard serological methods. Validation of the mRNA method was made by typing 50 healthy blood donors and 50 patients using the LightCycler real-time thermocycler (Roche, Mannheim, Germany) and melting curve analysis (Figure 1).[5] The molecular typing methods were performed with one-step RT-PCR using the LightCycler – RNA Amplification Kit Hybridization Probes (Roche) according to the manufacturer's instructions.

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CD71+cells were isolated using Dynabeads Pan Mouse IgG (Dynal Biotech, Oslo, Norway) coated with mouse monoclonal anti-human transferrin receptor/CD71 (Diatec, Oslo, Norway). Coating and cell isolation were performed according to the manufacturer's protocol. Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Absolute red cell number and percent reticulocytes were determined on an EPICS XL flow cytometer (Beckman-Coulter, Florida, USA). For total red cell count, cells were incubated with fluorescein isothiocyanate (FITC)-labeled monoclonal mouse anti-human glycophorin A/CD235a, (Dako-Cytomation, Glostrup, Denmark) or isotype control (FITC-labeled mouse monoclonal IgG1, Diatec). Before analysis, 50 µL Flow-Count Fluorospheres (Beckman-Coulter) were added to samples and 5,000 Flow-Count Fluorospheres were counted from each sample. Percent reticulocytes were determined using thiazole orange fluorescent dye (Retic-Count, Becton Dickinson, San Jose, CA. USA). Up to 50,000 cells from whole blood or 5,000 cells from isolated reticulocytes were analyzed.

Reticulocytes are known to have a long shelf life at 4°C[6] and to investigate if transfused reticulocytes could interfere with our method we studied their *in vitro* maturation time. A healthy volunteer donated blood cells and serum. Reticulocytes were isolated using CELLection Pan Mouse IgG Kit as described above.

Results and Discussion

To verify our methods for cDNA detection of Rh-antigens 50 healthy blood donors and 50 patients were typed. In addition, we investigated the possibility of using mRNA extracted from reticulocytes as a reliable source for identifying the transfused patient's own blood type by studying a panel of 30 patients transfused with mismatched Rh-antigens. For all cases, analysis of reticulocyte mRNA after transfusions was representative of the patient's own phenotype (Table 1). In total, we made 629 comparisons between our cDNA-based method and serological typing and we used an interactive calculator available on the Internet for validation of agreement between molecular typing and phenotyping.[7] The proportion of agreement was 1.000 (complete agreement); 95% confidence interval: 0.992-1.000.

In the transfusion study twelve women and eighteen men from surgical (n=15), hematologic (n=7) and trauma units (n=8) were included. Age varied between 19-86 years. The number of Rh-mismatched units ranged between 1-49, with a total of 245 and an average of 8.2 per patient. Some of the patients were transfused with more than one mismatched Rh-antigen, allowing a total of 51 mismatched transfusion events to be studied. Apart from four units of whole blood, all units were leukocyte filtered, packed red blood cells.

In 88% of cases the first sample for cDNA testing was collected within 24 hrs. after last transfusion and samples were never collected later than 72 hrs. after transfusion. The average time span for testing after last transfusion was 0.8 days. One patient (#18, Table 1) had 44

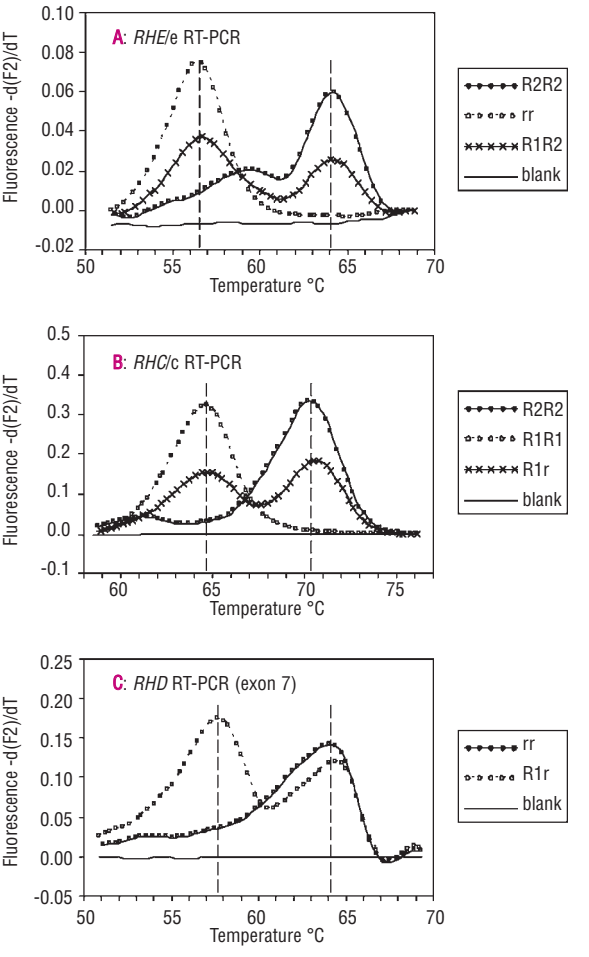

Figure 1. Melting curves for RT-PCR analysis of the different Rh-antigens. Figure 1 shows the melting curves from the molecular typing of mRNA derived from reticulocytes. A shows the simultaneous typing of *RHE/e* in exon 5. Primers and probes were: Sense *RHDCE*-327: 5'-ACT-gTT-CAg-TAT-TCg-gCT-3'; reverse *RHCE*-1074: 5'-CAT-gCC-ATT-gCC-gTT-C-3'; Probes: detection *RHEe*-661: 5'-CCA-AgT-gTC-AAC-TCT-CCT-CTg-C-FITC-3'; Anchor *RHEe*-685: 5'-LC-Red640-AgA-AgT-CCA-ATC-CAA-Agg-AAg-AAT-gCC-P-3'; B shows the simultaneous detection of *RHC* and *RHc* in exon 2. Primers and probes were: Sense *RHDCE*-56: 5'-CAC-Tgg-AAg-CAg-CTC-TCA-TTC-TCC-3'; reverse *RHCE*-526: 5'-CgA-ACA-CgT-AgA-AgT-gCC-TCA-g-3'; Probes: detection *RHDc*-288: 5'-LC-Red640-CTT-CCT-gAg-CCA-gTT-CCC-TCC-Tgg-P-3'; Anchor RHDC-260: 5'-gTg-TgC-AgT-ggg-CAA-TCC-TgC-Tgg-ACg-FITC-3'; C shows detection of *RHD* in exon 7 with a positive control in exon 7 of *RHCE*. Primers and probes were: sense *RHDCE*-843: 5'-ggg-TAC-CTC-gTg-TCA-CCT-g-3'; reverse *RHDCE*-1098: 5'-gCT-gAg-gAg-gAC-CTg-gAA-3'; probes: detection *RHCE*-1043: 5'-LC-Red640-ATC-ACC-TAC-ATT-gTg-CTg-CTg-gT-P-3': anchor *RHDCE*-1019: 5'-CTT-CAg-CTT-gCT-ggg-TCT-gCT-Tgg-AgA-FITC-3'. For detection of *RHD*, primers were chosen to amplify both the *RHD* transcript and the *RHCE* transcript. The detection probe was designed to distinguish between *RHD* and *RHCE*. *RHD* is either present or not, whereas *RHCE* is always present. An *RHD* positive sample (here: phenotype R1r) will depict two melting peaks, one for *RHD* and one for *RHCE*, whereas an *RHD* negative sample only displays one curve representing *RHCE* indicating the presence of template in the PCR reaction. The letters before the sequence indicate which allele is amplified.

mismatched transfusions within 24 hrs. and 4 of the units transfused were whole blood. Only one day after the last transfusion, cDNA analyses represented only the patient's own phenotype. Gel card typings performed at the same time all showed mixed field agglutination.

Serological typing can be problematic for weeks after multiple transfusions. The need for an easy and reliable method of identifying original blood group antigen status has led several groups to use DNA from blood as template.[8-12] These studies show a high degree of concordance between genotype and phenotype indicating that DNA typing is sufficient in most cases. There is, however, an advantage with our protocol that the RHC/c detection can be simultaneously carried out in one single PCR reaction without the need for RFLP and gel electrophoresis.[8,10,13] The epitope difference between RHC and RHc is encoded in exon 2 and what identifies the *RHC* allele is identical to the *RHD* genotype. The difference between Rhc and RhC/D is enclosed in a 4247 basepair stretch of near nucleotide identity between the two genes making specific DNA detection of the *RHC* allele by simple PCR virtually impossible in *RHD* positive individuals.[3,4] Our method may also be useful if there is concern about microchimerism after bone marrow or organ transplant, or after blood transfusion.[14-16]

As preliminary experiments showed that reticulocytes survive for at least 34 days when stored at 4°C, we wanted to investigate the maturation pattern of reticulocytes under different temperatures and oxygen tensions.

Table 1. Mismatched Rh-antigens and number of units transfused.

Mismatched Rh-antigen transfused	*Patient's phenotype (number*)*	*Number of Rh-mismatched units transfused*	*Days between first and last transfusion*	*Days between last transfusion and cDNA testing*
D	rr (6)	1	0	1 and 2
D	rr (10)	4	0	1
D	rr (26)	4	0	2
D	rr (29)	5	1	0
e	R2R2 (18†)	44	1	1
e	R2R2 (18†)	49	4	3
e	R2R2 (21)	5	0	1
e	R2R2 (22‡)	6	2	0
e	R2R2 (28)	16	0	1
c	R1R1 (5)	5	2	2
c	R1R1 (8‡)	5	1	0 and 1
c	R1R1 (11)	27	2	1
c	R1R1 (12)	4	2	0
c	R1R1 (13)	18	3	0
c	R1R1 (15)	3	0	1
c	R1R1 (23)	4	1	0
c	R1R1 (27‡)	2	0	1
C	rr (6)	1	0	1 and 2
C	R2r (7)	2	1	1
C	R2r (9)	16	1	0 and 2
C	rr (10)	3	0	1
C	R2r (16)	14	3	1
C	R2r (17)	3	1	0
C	R2R2 (18†)	36	1	1
C	R2R2 (18†)	41	4	3
C	R2R2 (21)	4	0	1
C	R2R2 (22‡)	3	2	0
C	R2r (25‡)	4	6	0
C	rr (26)	4	0	2
C	R2R2 (28)	11	0	1
C	rr (29)	4	1	0
C	R2r (30)	6	2	0
E	R1r (1)	25	5	0 and 3
E	R1r (2)	1	0	1
E	R1r (3)	1	0	1
E	R1r (4)	1	0	2
E	R1R1 (5)	4	1	0 and 3
E	R1R1 (8‡)	3	1	0 and 1
E	rr (10)	2	0	1
E	R1R1 (11)	14	2	1
E	R1R1 (12)	1	0	1
E	R1R1 (13)	4	3	0
E	R1r (14)	3	0	1
E	R1R1 (15)	1	0	1
E	R1r (19‡)	6	21	0 and 1
E	R1R1 (20‡)	1	0	1
E	R1R1 (23)	3	1	0
E	R1r (24‡)	5	7	1
E	rr (26)	1	0	2
E	R1R1 (27‡)	1	0	1
E	rr (29)	2	0	1

**Some patients were transfused with more than one mismatched antigen and appear in the table more than once. †Patient number 18 had two transfusion events and was tested after each. ‡Hematologic patients.*

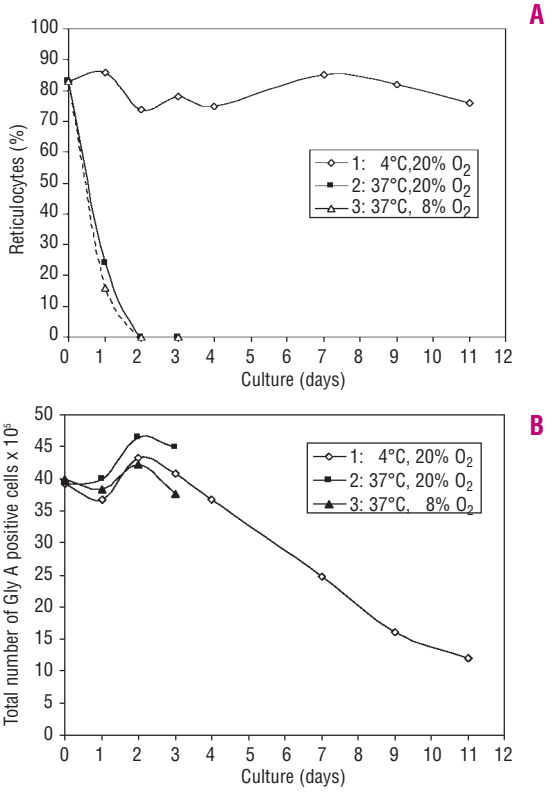

Figure 2. (A) Maturation of reticulocytes under various culture conditions. Percent reticulocytes are calculated as the number of thiazole orange positive cells divided by the number of glycophorin A positive cells. All cultures were in an atmosphere of 5% CO_2. (B) Total number of red blood cells was measured by glycophorin A. There was no loss of cells during the approximately two day maturation period of reticulocytes. Cells cultivated at 37°C were not followed after day 3 as no reticulocytes remained.

Reticulocytes were isolated from one donor and cultivated in 100% autologous serum at three different culture conditions: 1) 4°C, 20% O_2, 5% CO_2 (close to blood storage conditions); 2) 37°C, 20% O_2, 5% CO_2 (standard culture conditions); 3) 37°C, 8% O_2, 5% CO_2 (representing average conditions in blood capillaries). Temperature is the most important factor for reticulocyte maturation in these experiments (Figure 2A). When cultivated at 37°C and 20% O_2, more than 70% of the reticulocytes had transformed into mature red blood cells after 24 hrs. Reticulocytes cultivated at an oxygen tension closer to *in vivo* conditions showed a similar maturation pattern. At 4°C there was no significant maturation of reticulocytes after 11 days of culture. The reticulocytes did not die, as total red cell count measured by glycophorin A was stable during the maturation period (Figure 2B). As the reticulocytes in this study were isolated using CD71, identifying recently released reticulocytes, and counted using thiazole orange, which binds RNA, the whole lifespan should be covered.[17]

In conclusion, this approach identifies a method for correctly typing patients' own Rh-antigens after multiple transfusions. Transfused reticulocytes do not interfere with the method as they mature quickly at 37°C. By using mRNA from reticulocytes, the molecular typing of *RHC/c* in *RHD* positive individuals can be simultaneously carried out in one PCR reaction and the method can be expanded to other relevant antigens.

Authorship and Disclosures

IR is responsible for study concept, research design, data analysis and writing the manuscript; KS contributed to writing the manuscript and performed molecular analysis and cell culture; RH and ABD performed molecular analysis and approved the manuscript; MRM performed flow cytometry and approved the manuscript; KMT and JKK participated in research design and contributed to writing the manuscript.

The authors reported no potential conflicts of interest.

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