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A simple method for accurate quantification of complement receptor 1 on erythrocytes preserved by fixing or freezing

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Abstract

The mean number of complement receptor 1 (CR1) molecules on erythrocytes differs between normal individuals within the range of 100–1000 molecules per cell. In some disease states such as systemic lupus erythematosus (SLE), acquired immune deficiency syndrome (AIDS), insulin-dependent diabetes mellitus and malaria, erythrocyte CR1 levels are reduced and CR1 function may be impaired. Current methods for determining erythrocyte CR1 levels by flow cytometry require the use of freshly drawn blood samples because CR1 is lost from erythrocytes during storage. In order to facilitate field studies of associations between erythrocyte CR1 levels and disease, we have developed and validated an assay to quantify CR1 on both healthy and diseased erythrocytes that have been fixed in 5% formaldehyde or frozen in glycerol. These methods enable blood samples to be collected in areas lacking the facilities for flow cytometry and stored for later accurate quantification of CR1. Such procedures will be of particular benefit for future investigations of erythrocyte CR1 expression level and malaria susceptibility.

Keywords: Complement receptor 1; Erythrocyte; Flow cytometry; Formaldehyde; Glycerol; Malaria

1. Introduction

The level of complement receptor 1 (CR1) on erythrocytes is important in a variety of disease processes. Reduced levels of erythrocyte CR1 correlate with disease severity in acquired immune deficiency syndrome (AIDS), systemic lupus erythematosus (SLE) (Kazatchkine et al., 1987) and insulin-dependent diabetes mellitus (Ruuska et al., 1992). In the case of SLE, low CR1 levels may cause pathology because the normal function of CR1 in regulating complement activation and removing immune complexes from the circulation is impaired (Gibson and Waxman, 1994). In malaria infection, reduced levels of erythrocyte CR1

Abbreviations: ABS, AB Serum; AIDS, acquired immune deficiency syndrome; BSA, bovine serum albumin; CR1, complement receptor 1; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; HEPES, *N*-2-hydroxy-ethylpiperazine-*N*-2-ethanesulphonic acid; PBS, phosphate-buffered saline; SLE, systemic lupus erythematosus; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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have been implicated in the pathogenesis of severe malarial anaemia (Waitumbi et al., 2000). Conversely, we have suggested (Rowe et al., 1997) that low erythrocyte CR1 may protect against severe malaria by reducing the virulence-associated rosetting phenomenon whereby *Plasmodium falciparum*-infected erythrocytes bind to uninfected erythrocytes to form clumps of cells that may block microvascular blood flow and contribute to severe disease (Kaul et al., 1990). Further elucidation of the role of erythrocyte CR1 in malaria and other diseases requires the investigation of CR1 expression levels and disease associations in a variety of populations and patient sets.

Several methods currently exist to quantify the mean number of CR1 molecules per erythrocyte, including an ELISA technique and a flow cytometry method (Cohen et al., 1999). Both methods give equivalent results, although flow cytometry is preferable in many circumstances because it can be performed on very small quantities of blood. This is a particular advantage when studying young children or when multiple investigations are to be carried out on a single blood sample. One drawback, however, is that the flow cytometry method can only be used to determine CR1 numbers on fresh blood samples, because CR1 is lost from erythrocytes during storage (Pascual et al., 1993). This is a particular problem when working on malaria and AIDS in some field study areas in developing countries where facilities for flow cytometry may not always be available. We therefore set out to test a variety of methods of erythrocyte preservation in order to develop an assay to determine accurately by flow cytometry the CR1 expression level on erythrocytes after storage.

2. Methods and study design

2.1. Determination of CR1 expression by flow cytometry

The mean erythrocyte CR1 expression level on freshly drawn blood samples was determined using a modification of a previously published method (Cohen et al., 1987). Whole blood (50 μl) was washed three times in PBS (all reagents from Sigma, Poole, Dorset, UK unless otherwise stated) supplemented with 4% RPMI 1640 (Gibco Life Technologies, Rockville, MD) and 1% AB serum (Blood Transfusion Service, Edinburgh, UK) (PBS-RPMI-ABS). The cells were resuspended in 1 ml of PBS-RPMI-ABS and 100 μl of this suspension (i.e. approximately 2.5 μl of cells) was placed in a 96-well plate, spun at 1500 rpm for 1 min and the supernatant removed. The cells were then incubated at 4 °C with 40 μl of 0.5 μg/ml of the CR1 monoclonal antibody J3D3 (Immunotech, Marseille, France) in PBS-RPMI for 1 h with occasional agitation. The cells were washed three times in PBS-RPMI-ABS and resuspended in 10 μg/ml of Alexa Fluor™ 488-conjugated goat anti-mouse IgG (Molecular Probes, Leiden, the Netherlands) followed by incubation at 4 °C for 1 h with occasional agitation. Alexa Fluor™ 488 is equivalent to fluorescein isothiocyanate but is more stable and sensitive and less prone to photo bleaching. Negative control samples were treated as above but without the primary antibody. After the secondary incubation, cells were washed three times in PBS-RPMI-ABS and resuspended in PBS-RPMI-ABS supplemented with 0.37% formaldehyde (BDH, Poole), and analysed on a FacSCAN flow cytometer (Becton Dickinson, San Jose, CA). The mean fluorescence intensity of each sample, minus the mean fluorescence intensity of the negative control was determined. A standard curve was obtained by plotting the fluorescence intensities of blood samples from donors of previously determined CR1 numbers (standards, initially determined by Scatchard analysis using ^{125}I labelled mAb (Cohen et al., 1987)) ranging from 200 to 1000 molecules per cell. The standard curve was used to read off the CR1 expression level of unknown samples.

2.2. Preservation of blood samples

After obtaining informed consent, 5 ml of blood was drawn from 11 donors (five standards and six unknown samples) into Vacutainers containing 0.12 ml of 15% EDTA (BD Vacutainer systems, Plymouth, UK). We then preserved or fixed aliquots of all 11 blood samples in four different ways. The first was simply left in the EDTA Vacutainer into which the blood had been drawn. The second aliquot was fixed in 0.25% glutaraldehyde according to the method of Pattanapanyasat et al. (1993), in which washed cells are resuspended in 0.25% (v/v) glutaraldehyde in PBS at 2% haematocrit for 20 min before being washed in

PBS and resuspended at 50% haematocrit in PBS supplemented with 1% BSA. The third aliquot was fixed in formaldehyde as outlined by Bianco et al. (1986). These cells were first washed three times in incomplete RPMI (RPMI 1640 medium with sodium bicarbonate (Gibco Life Technologies), supplemented with 2 mM L-glutamine, 25 mM HEPES, 20 mM D-glucose and 25 μg/ml gentamicin). Whole blood (50 μl) was resuspended at 4% haematocrit in complete RPMI (as for incomplete RPMI but with the addition of 10% ABS) and an equal volume of fixative solution (10% w/v formaldehyde, 4% w/v glucose in Tris–saline (10 mM Tris, 150 mM NaCl, 10 mM sodium azide adjusted to pH 7.3) was added. The fixed samples and the EDTA-preserved samples were stored at 4 °C for the remainder of the experiment. The final aliquot of blood was frozen in glycerol by adding drop-wise five volumes of freezing solution (42.25% w/v glycerol, 0.1 M sodium lactate, 4 mM potassium chloride, 0.1 M sodium dihydrogen phosphate adjusted to pH 6.8) to three volumes of erythrocytes and then freezing overnight at −70 °C before cryopreservation in liquid nitrogen. At the appropriate time (see below), cryopreserved cells were thawed at 37 °C and 200 μl of 12% NaCl solution was added slowly and drop-wise to each sample, followed by a further slow drop-wise addition of 10 ml of 1.8% NaCl followed by 10 ml of 0.9% NaCl, 0.2% glucose solution. The cells were washed in incomplete RPMI 1640 and were then ready for flow cytometry.

2.3. Study design

To determine whether the flow cytometry could be used to quantify CR1 expression level using preserved erythrocytes from healthy donors we performed the following experiment. We drew blood from five individuals with known CR1 expression who were then used as standard donors for CR1 quantification as described above. We also drew blood from six test subjects who had unknown erythrocyte CR1 levels. We determined the mean number of CR1 molecules per cell from the fresh blood samples of the six unknowns according to the flow cytometry protocol. We then preserved or fixed aliquots of all 11 blood samples according to the methods outlined above. After 1, 2, 4, 6 and 8 weeks, the EDTA preserved, fixed and frozen samples underwent flow cytometry to assess erythrocyte CR1 level. In order to determine the optimum method of measuring CR1 levels on the stored cells, we assessed them in two ways. Firstly, we used a standard curve derived from freshly drawn standards at each time point (absolute standards) and, secondly, we derived a standard curve from the standards that had been drawn and preserved in an identical way to the test samples at the start of the experiment (relative standards).

Having determined the most promising techniques for the preservation of healthy erythrocytes (fixing in 5% formaldehyde and freezing—see Results and discussion) we then proceeded to determine whether these techniques were reliable with diseased erythrocytes which may have reduced CR1 levels. Accordingly, we repeated the experiment using the same CR1 standard donors and unknown samples from seven individuals with SLE whose symptoms met the American Rheumatism Association criteria for the disease. The erythrocytes were preserved by fixing in 5% formaldehyde and freezing in glycerol as described above, and the CR1 expression levels assessed with relative and absolute standards at 4 and 8 weeks.

3. Results and discussion

Mean erythrocyte CR1 levels were measured on six fresh blood samples from healthy donors (unknown samples) by flow cytometry using a standard curve derived from five donors with known CR1 levels (standards) as shown in Fig. 1. Erythrocytes from the six healthy test samples and the five standard donors were then preserved by fixing or freezing as described under Methods and study design. For each of the six test samples, the CR1 levels were then measured on the preserved cells at various time points (1, 2, 4, 6 and 8 weeks after the blood was drawn) using both absolute (freshly drawn) and relative (preserved) standards as described in Methods and study design. At each time point, the CR1 levels of the preserved cells were compared to the CR1 levels of the samples when freshly drawn, and the data were analysed by linear regression. The best results were obtained with erythrocytes preserved by fixation in 5% formaldehyde. Comparison with the relative standards gave accurate results throughout the 8 weeks of the experiment (Table 1 and Fig. 2A, open squares), whereas

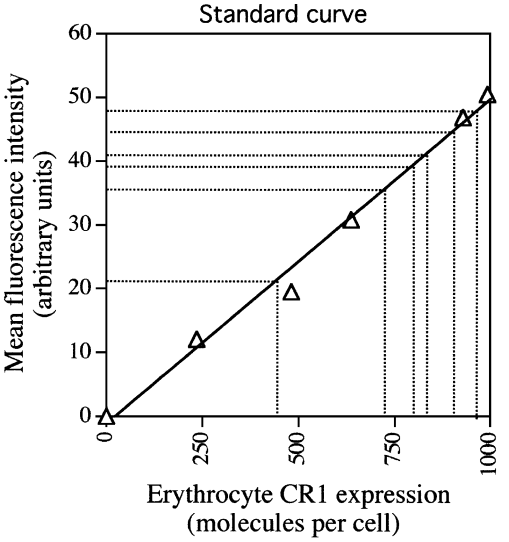

Fig. 1. A standard curve used to determine the mean erythrocyte CR1 expression level of unknown samples. The standard curve was plotted with data from five standards with previously determined CR1 expression levels (△), with the point at the origin representing the negative control sample with no primary antibody. The mean fluorescence intensity of six unknown samples was read off the standard curve to determine the number of CR1 molecules per cell (dotted lines). The mean CR1 expression levels of the six unknown samples were determined as 448, 727, 796, 814, 894 and 967 molecules per cell.

comparison to absolute standards was accurate up to 4 weeks after the blood was drawn, but thereafter gave an underestimate of true CR1 numbers (Table 1 and Fig. 2A, filled squares). Preservation in EDTA also gave good results providing the test samples were compared to relative standards (Fig. 2B, open squares). This was true up to 6 weeks from the date of blood

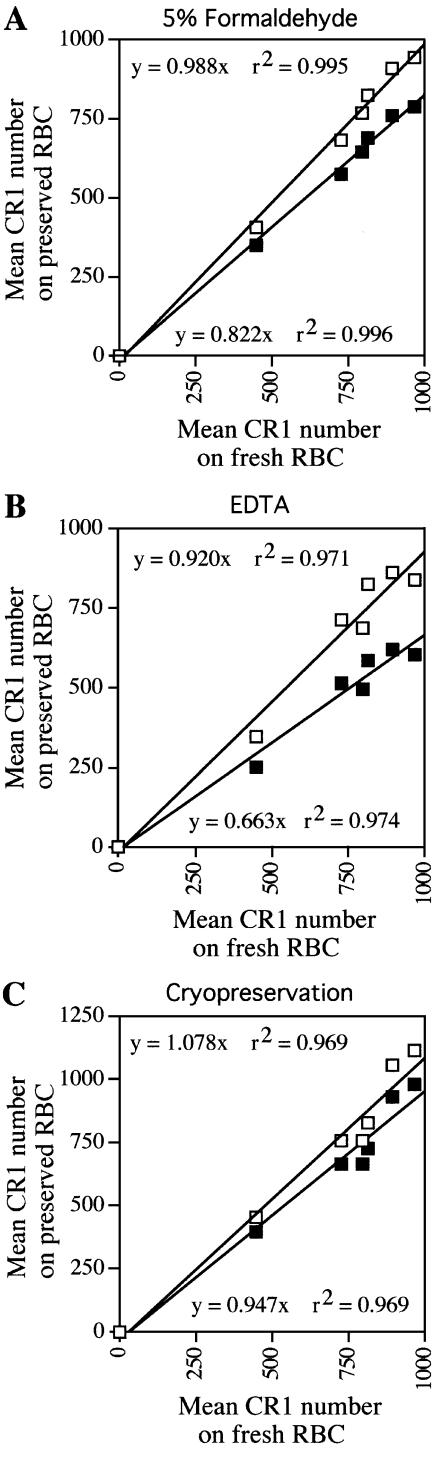

Fig. 2. CR1 expression levels determined after the preservation of healthy erythrocytes compared to CR1 levels determined on fresh erythrocytes. (A) Erythrocytes stored in 5% formaldehyde at 4 °C for 8 weeks. (B) Erythrocytes stored in EDTA at 4 °C for 6 weeks. (C) Erythrocytes cryopreserved in liquid nitrogen for 8 weeks. The CR1 expression levels of the six test samples determined by comparison to freshly drawn (absolute) standards are shown as filled squares (■), whereas the CR1 expression levels of the six test samples determined by comparison to preserved (relative) standards are shown as open squares (□). The relationship between the CR1 levels measured on preserved cells compared to fresh cells was analysed by linear regression. The regression equation for the data determined using relative standards is shown at the top of each graph, and the regression equation for the data determined using absolute standards is shown at the bottom of each graph. RBC, red blood cell.

Table 1
Assessment of CR1 expression level over the course of 8 weeks on erythrocytes fixed with 5% formaldehyde

	No. of CR1 molecules per cell when fresh					
	448	727	796	814	894	967
CR1 measured using absolute standards						
Week 1	425	719	816	826	929	1072
Week 2	421	860	886	1064	1308	1173
Week 4	436	740	804	833	889	1014
Week 6	294	529	585	606	632	746
Week 8	350	575	645	690	760	788
CR1 measured using relative standards						
Week 1	427	757	865	877	993	1152
Week 2	355	732	754	907	1117	1001
Week 4	442	760	826	856	915	1045
Week 6	408	753	835	866	904	1071
Week 8	406	682	769	824	909	944

withdrawal (Fig. 2B), after which time, the extent of haemolysis of the samples became too great for further use. When absolute standards were used to assess the CR1 level of EDTA-preserved cells, this was accurate up to 2 weeks after the blood was drawn, and thereafter resulted in an underestimation of the number of CR1 molecules per cell (Fig. 2B, filled squares). Fixation of erythrocytes in 0.25% glutaraldehyde resulted in high background fluorescence of all samples and it impossible to generate a standard curve (data not shown). Therefore, it was concluded that this method was not suitable for CR1 quantification. Preservation of erythrocytes by freezing in glycerol gave accurate results at all time points when relative standards were used (Fig. 2C, open squares, data from week 8). The use of absolute standards gave good results at some time points

Table 2
Summary of methods for the preservation of erythrocytes for CR1 level determination

Preservation method[a]	Maximum time of storage for accurate CR1 determination
Using absolute standards	
EDTA	Up to 2 weeks after blood drawn
5% formaldehyde	Up to 4 weeks after blood drawn
Freezing in glycerol	Inconsistent results – not recommended
Using relative standards	
EDTA	Up to 6 weeks after blood drawn
5% formaldehyde	Up to 8 weeks after blood drawn[b]
Freezing in glycerol	Up to 8 weeks after blood drawn[b]

[a] Fixation in 0.25% glutaraldehyde was also tested but was not acceptable at any time point due to high background fluorescence.
[b] Longer periods of storage may be acceptable, but, this would require validation. The samples were only tested up to 8 weeks in these experiments.

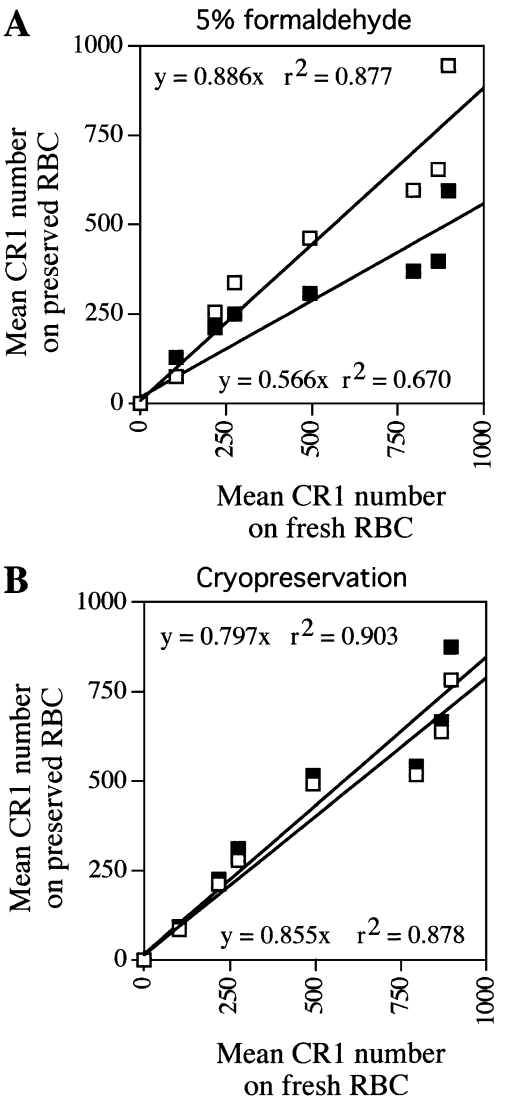

Fig. 3. CR1 expression levels determined after preservation of erythrocytes from seven donors with SLE compared to CR1 levels determined on fresh SLE erythrocytes. The mean CR1 levels when fresh were 104, 218, 275, 493, 794, 867 and 896 molecules per cell. (A) Erythrocytes stored in 5% formaldehyde at 4 °C for 8 weeks. (B) Erythrocytes cryopreserved in liquid nitrogen for 8 weeks. Symbols and analysis as in Fig. 2.

(e.g. week 8, Fig. 2C, filled squares), although, at two earlier time points the use of absolute standards gave widely differing estimates of CR1 numbers (data not shown), and we would not recommend the use of absolute standards with frozen cells. A summary of the results from each method of preservation is given in Table 2.

Our results indicate that CR1 levels can be accurately measured on erythrocytes from healthy donors preserved by formaldehyde fixation or freezing, as long as similarly preserved erythrocytes are used to generate the standard curve. To determine whether these methods are also reliable using erythrocytes from donors with disease states that may reduce CR1 numbers, we repeated the experiment using samples from seven donors with SLE. Once again we found that fixing in 5% formaldehyde and freezing in glycerol both gave good results over 8 weeks when relative standards were used (Fig. 3A and B, open squares). As previously, an underestimation of CR1 expression was observed when fixed samples were used with absolute standards (Fig. 3A, filled squares), while frozen cells with absolute standards gave good results in some cases (Fig. 3B, filled squares; data from week 8) but was not reliable at all time points. Crucially, both the fixing and freezing techniques worked well with samples showing low CR1 expression (<250 molecules per cell).

We conclude that accurate CR1 quantification may be performed after preservation of either diseased or healthy erythrocytes using either a freezing technique or fixation in 5% formaldehyde, providing that the CR1 expression level standards are collected and fixed in an identical fashion. Cryopreservation of erythrocytes in glycerol is reliable and permits potentially long-term storage of samples, but does require liquid nitrogen for freezing and transportation of cells, which may not always be available during field studies. Fixation of erythrocytes in 5% formaldehyde is quick and economical and gives an accurate determination of CR1 level for at least 2 months after sample collection. These methods will facilitate further studies of CR1 expression level and disease susceptibility in areas lacking facilities for flow cytometry.

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References

Bianco, A.E., Battye, F.L., Brown, G.V., 1986. *Plasmodium falciparum*: rapid quantification of parasitemia in fixed malaria cultures by flow cytometry. Exp. Parasitol. 62, 275.

Cohen, J.H.M., Aubry, J.P., Jouvain, M.H., Wijdenes, J., Bancherau, J., Kazatchkine, M., Revillard, J.P., 1987. Enumeration of CR1 complement receptors on erythrocytes using a new method for detecting low density cell surface antigens by flow cytometry. J. Immunol. Methods 99, 53.

Cohen, J.H.M., Atkinson, J.P., Klickstein, L.B., Oudin, S., Subramanian, V.B., Moulds, J.M., 1999. The C3b/C4b receptor (CR1,CD35) on erythrocytes: methods for study of polymorphisms. Mol. Immunol. 36, 819.

Gibson, N.C., Waxman, F.J., 1994. Relationship between immune complex binding and release and the quantitative expression of the complement receptor type 1 (CR1, CD35) on human erythrocytes. Clin. Immunol. Immunopathol. 70, 104.

Kaul, D.K., Roth Jr., E.F., Nagel, R.L., Howard, R.J., Handunnetti, S.M. 1990. Rosetting of *Plasmodium falciparum*-infected red blood cells with uninfected red blood cells enhances microvascular obstruction under flow conditions. Blood 78, 812.

Kazatchkine, M.D., Jouvin, M.H., Wilson, J.G., Fischer, E., Fischer, A., 1987. Human diseases associated with C3 receptor deficiencies. Immunol. Lett. 14, 191.

Pascual, M., Lutz, H.U., Steiger, G., Stammler, P., Schifferli, J.A., 1993. Release of vesicles enriched in complement receptor 1 from human erythrocytes. J. Immunol. 151, 397.

Pattanapanyasat, K., Udomsangpetch, R., Webster, H.K., 1993. Two-color flow cytometric analysis of intraerythrocytic malaria parasite DNA and surface membrane-associated antigen in erythrocytes infected with *Plasmodium falciparum*. Cytometry 14, 449.

Rowe, J.A., Moulds, J.M., Newbold, C.I., Miller, L.H., 1997. *P. falciparum* rosetting mediated by a parasite-variant erythrocyte membrane protein and complement receptor 1. Nature 388, 292.

Ruuska, P.E., Ikaheimo, I., Silvennoinen-Kassinen, S., Kaar, M.L., Tiilikainen, A., 1992. Normal C3b receptor (CR1) genomic polymorphism in patients with insulin-dependent diabetes mellitus (IDDM): is the low erythrocyte CR1 expression an acquired phenomenon? Clin. Exp. Immunol. 89, 18.

Waitumbi, J.N., Opollo, M.O., Muga, R.O., Misore, A.O., Stoute, J.A., 2000. Red cell surface changes and erythrophagocytosis in children with severe *Plasmodium falciparum* anemia. Blood 95, 1481.