Intravascular survival of red cells coated with a mutated human anti-D antibody engineered to lack destructive activity.

Short title: Intravascular survival of modified IgG1-coated RBC

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* Three of the authors (KLA, MRC, LMW) have filed patent applications (WO 99/58572), owned by the University of Cambridge and covering use of the mutant IgG constant region studied in this work.

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ABSTRACT

Alloimmune feto-maternal destruction of blood cells is thought to be mediated by binding of alloantibodies to Fc receptors on effector cells. Blocking the antigen using inert antibodies might prolong cell survival. We have performed a ‘proof of principle’ study in volunteers to measure the intravascular survival of autologous red cells coated with human recombinant IgG antibody containing a novel constant region, G1Δnab, devoid of in vitro cytotoxic activity. RhD-positive RBC, labelled with chromium-51 or technetium-99m, were separately coated to equal levels with wildtype IgG1 or G1Δnab anti-D antibody (Fog-1). After reinjection, there was complete, irreversible clearance of IgG1-coated RBC by 200 min, concomitant with appearance of radiolabel in plasma. Gamma camera imaging revealed accumulation in spleen and, at higher coating levels, in liver. In contrast, clearance of G1Δnab-coated cells was slower, incomplete and transient, with whole blood counts falling to 7 – 38% injected dose by about 200 min before increasing to 12 - 67% thereafter. There was no appearance of plasma radiolabel and no hepatic accumulation. These findings suggest that G1Δnab-coated RBC were not haemolysed but temporarily sequestered in the spleen and that our approach merits investigation in larger studies.
INTRODUCTION

Maternal alloimmunisation to paternally-inherited alloantigens on red cells or platelets can cause pregnancy complications which threaten the life of the fetus. Involvement of Fc receptors for IgG (FcγR) in destruction of alloantibody-coated blood cells is suggested by studies in which antibody-mediated haemolysis was inhibited by antibodies against FcγR.1 Although the timing of appearance of FcγRI-III on fixed effector cells in fetal liver and spleen is uncertain, FcγRI-III are present on circulating leukocytes by 18 weeks of pregnancy2, when alloimmunity can cause life-threatening fetal platelet destruction.3

In contrast to IgG1 and IgG3, IgG2 and IgG4 antibodies rarely cause significant haemolysis4, explained by their low binding to FcγR and limited ability to activate effector cells or complement. We have exploited this difference to generate non-destructive antibody constant regions for potential therapeutic use. In antibody-mediated diseases, for instance fetal alloimmune disorders, such antibodies, specific for the target epitope, would compete with pathogenic antibody for antigen binding without causing blood cell destruction. Since two regions of the immunoglobulin C1 domain which are critical for FcγRI-III and complement C1q binding5 have unique sequences in IgG2 and IgG4, we produced versions of IgG1 substituted with combinations of the IgG2 (Δb or Δc) and IgG4 (Δa) motifs. This approach, substituting up to 7 residues from very highly homologous molecules, was used to minimise the potential to create new immunogenic epitopes. When recombined with model human variable region genes (Fog-1 anti-D6), some mutated constant regions resulted in antibodies with minimal binding to FcγRI and III and less capacity for cell lysis than IgG2 or IgG4.7,8 In particular, Fog-1 G1Δab-coated red cells did not trigger either CD16-positive NK cell-mediated cytotoxicity (ADCC)8 or monocyte chemiluminescence (CL)7, an assay reported to predict clinical outcome in haemolytic disease of the newborn (HDN)9. Importantly, Fog-1 G1Δab inhibited ADCC and CL triggered either by Fog-1 IgG1 or clinical anti-D sera.7,8 Fog-1 G1Δab has been further modified by removal of three allotypic residues10 and redesignated Fog-1 G1Δnab.
We now report a ‘proof of principle’ investigation in human volunteers to establish whether the non-destructive nature of G1Δnab in vitro is reflected in improved intravascular survival of cells coated with modified antibody. Two aliquots of autologous red cells, labelled with chromium-51 (51Cr) or technetium-99m (99mTc)\textsuperscript{11}, were coated with either active Fog-1 G1 or mutated Fog-1 G1Δnab. Since radiolabelling and antibody sensitisation do not affect each other\textsuperscript{12,13}, this dual labelling strategy provides comparative red cell survival data for the two antibodies without the added complexity of inter-donor variation. It permits assessment of both intravascular survival beyond a few hours (51Cr) and sites of red cell destruction by gamma camera imaging (99mTc)\textsuperscript{13}.
MATERIALS AND METHODS

Selection and assessment of subjects
Six healthy D-positive subjects were recruited from the donor panel at the National Blood Service Cambridge Centre, with written informed consent and permission from the Local Research Ethics and Administration of Radioactive Substances Advisory Committees. Only men over 30 and women over 50 years of age (or sterilised) were recruited and assessed by a doctor. Exclusions were:- splenectomy, transfusion in the previous 6 months, red cell alloantibodies, severe allergy or reactions to blood products, clinical trial participation in the previous 30 days, or administration of radioisotopes within the previous 12 months.

A full medical assessment was performed in the 28 days preceding the study. Blood samples were taken for full blood count, urea and electrolytes, liver function and mandatory infection markers. Full Rh phenotype was performed by conventional serological techniques using monoclonal antibodies to C, c, D, E and e (Reagents Department, National Blood Service). All injection and sampling procedures were carried out for one subject at a time in the Department of Nuclear Medicine, Addenbrooke’s Hospital. Subjects remained under medical supervision for 4 hours after administration of the antibody-coated red cells. An independent Data and Safety Monitoring Committee was informed of any adverse events. A follow-up medical check and repeat blood sampling was performed at 4 - 6 weeks post-study.

Production and pre-release testing of Fog-1 IgG1 and G1∆nab antibodies
Fog-1 is a human anti-D, IgG1,κ molecule which recognises up to 26,000 D sites/cell with a binding affinity of 1-2 x 10⁹ M⁻¹ and stimulates monocyte phagocytosis. The properties of recombinant IgG1 and mutant G1∆ab forms of Fog-1 have been described. Fog-1 G1∆nab is a null allotype variant of Fog-1 G1∆ab in which the additional mutations Lys214Thr, Asp356Glu and Leu358Met have removed residues specifying the allotype G1m (1,17). Fog-1 G1∆ab and G1∆nab have equivalent activity in binding to FcγRI, FcγRIIa of 131I.
and 131H allotypes, FcγRIIb and FcγRIIIb of allotypes NA1 and NA2 and in CL and ADCC (data not shown).

Clinical-grade antibodies were produced under European Union good manufacturing practice conditions in the NBS Clinical Biotechnology Centre and quality control tested in accordance with European Union guidelines. Fog-1 G1 and G1Δnab cell lines were each cloned three times and adapted to serum-free medium. For production, cells were grown in Integra CL350 flasks (Integra Biosciences, Chur, Switzerland), seeded at 10^7 cells in CCM1 medium (Hyclone, Cramlington, UK) in the cell compartment. After one week, the basal medium M925 (Hyclone) was changed three times per week. Culture was maintained for up to 70 days with IgG concentrations of up to 1 mg/ml. Antibodies were purified by a combination of rProtein A Sepharose FF (Amersham BioSciences, Buckinghamshire, UK) affinity chromatography and cation exchange chromatography. The eluate was viral filtered then dialysed and concentrated using a crossflow membrane into PBS. Purity was demonstrated by a single band on acid native gel electrophoresis and two bands on reducing SDS-PAGE. The product was resuspended in 0.1% human albumin (Bioproducts Laboratory, Elstree, UK), bottled in nitrogen-filled borosilicate glass bottles (Amersham BioSciences) and stored at –20°C. The final concentrations of antibodies, assessed by anti-κ chain ELISA, were 200 µg/ml for Fog-1 G1 and 50 µg/ml for Fog-1 G1Δnab following precipitation losses.

Anti-D specificity was confirmed in the indirect antiglobulin test against R2R2 and R1r cells, with endpoint titres of wildtype and mutated antibodies occurring at equal concentrations and no reactivity with rr cells (data not shown). Clinical-grade antibodies showed the same characteristics as previously, with Fog-1 G1Δnab giving 10^4-fold less binding than Fog-1 G1 to FcγRII transfectants, and no CL stimulation at 1µg/ml, compared with a detectable CL response to Fog-1 G1 at 20 ng/ml (data not shown). Since the low concentration of Fog-1 G1Δnab did not permit saturation of D sites on red cells, the SOL-ELISA was used to establish coating conditions for the in vivo studies which gave 75% saturation with each antibody.
**SOL-ELISA for measurement of antibody coating**

Known volumes of antibody-coated red cells were solubilised and antibody standard prepared in the same buffer. Dilutions were assayed in quadruplicate in a sandwich ELISA using unlabelled and peroxidase-conjugated goat anti-human \( \kappa \) chain antibodies (Sigma, Poole, UK). Mean absorbance was plotted against amount of antibody/volume of lysate (log scale). The standard deviations of the means were used to obtain equations for the minimum and maximum versions of the linear portions of the curves. This allowed calculation of the minimum and maximum amounts of standard antibody and lysate corresponding to the midpoint absorbance value. These were equated and converted to antibody molecules per cell. The result was given as the midpoint, with the range expressed as an estimated error.

**Radiolabelling/antibody coating for subjects 1 - 5**

On the day of study, a 17 ml citrated blood sample taken and split into 2 aliquots. Packed red cells from each aliquot were separately radiolabelled with either 1.2 MBq \( ^{51} \)Cr sodium chromate or stannous medronate solution followed by 60 MBq \( ^{99m} \)Tc sodium pertechnetate by incubation for 15 minutes at room temperature. After washing, the aliquots were incubated with Fog-1 G1 or G1\( \Delta \)nab (8 ml of antibody at 50\( \mu \)g/ml) for 30 minutes at 37\(^{\circ} \)C, consecutive subjects having opposite radiolabel/antibody combinations. After washing, each aliquot was resuspended to 9.5 ml in saline, providing 8 ml for re-injection, plus samples for a counting standard and SOL-ELISA. The injected activities were 0.5 – 1.0 MBq \( ^{51} \)Cr and 17 – 41 MBq \( ^{99m} \)Tc.

**Measurement of red cell survival (subjects 1 - 5)**

The \( ^{51} \)Cr-labelled antibody-coated cells were injected intravenously over 1 min followed by the \( ^{99m} \)Tc sample. Blood samples were taken via an in-dwelling catheter from the opposite arm at time points from 5 to 240 min. Additional samples were taken on the following day in four subjects and after 5/6 days in three subjects. Because only 80% of injected dose was detected at 5 min for subject 1, samples were taken at earlier time points in subjects 2 - 5. Whole
blood and plasma samples were counted against standards made from aliquots of labelled, antibody-coated cells in an automatic well scintillation counter (Compugamma 1282, LKB/Wallac, Turku, Finland). Dual windows were set for $^{99m}$Tc and $^{51}$Cr on the morning after injection to allow $^{99m}$Tc activity to decay to give counter deadtime of <10%. Cross-talk factors were calculated from counts of the standards. Measured counts of whole blood and plasma samples were corrected for deadtime, background, cross-talk, radionuclide decay and volume. The known sensitivities of the counter were used to determine the fraction of each injected dose represented by the standards. The sample to standard corrected count ratio was thus converted to percentage injected dose/litre and to percentage injected dose, using the blood volume predicted from the subject’s height and weight. Only $^{51}$Cr counts were considered for samples taken on the second day or later, and were corrected for elution of $^{51}$Cr from the cells. To calculate clearance rates of labelled cells from the blood, whole blood activity was corrected for plasma activity, using the sample haematocrit, to give cell-bound activity. The data were transformed logarithmically, and the curves from 5 - 60 min were fitted with a standard least squares routine (Excel, Microsoft, Seattle USA). Data from the 0 – 300 min time points of the Fog-1 G1Δnab clearance curves were also fitted to the function: $C(t) = A + B \exp(-bt)$ using a non-linear least squares routine (IDL Research Systems, Boulder, USA).

**Gamma camera imaging (subjects 1 - 5)**

Gamma camera imaging was performed with the subject lying supine under a dual-headed gamma camera (Prism 2000XP, Picker, Ohio, USA) positioned over the chest and upper abdomen. Dynamic imaging (40 x 1 min frames) was performed immediately after injection followed by static imaging at 40 min. Count rates for regions drawn around the whole liver, whole spleen and a portion of right lung were corrected for background and geometric means of anterior and posterior views of each organ were calculated. These means were normalised to a standard injected activity and time-activity curves plotted.
Survival and gamma camera imaging of Fog-1 G1Δnab-coated cells (subject 6)
To assess whether Fog-1 G1Δnab-coated RBC were accumulating on microvascular endothelium, a revised protocol was developed for subject 6, using a modification of a method previously described for neutrophils. 15 MBq of uncoated $^{99m}$Tc-RBC were injected and six blood samples taken over a 20 min equilibration time. Then 49 MBq of $^{99m}$Tc-Fog-1 G1Δnab-coated RBC were injected and venous sampling performed as previously until 180 minutes. The mean $^{99m}$Tc-RBC count at equilibrium was subtracted from the whole blood counts after injection of antibody-coated RBC and the net data were used to assess clearance of coated cells, as above. Dynamic imaging (60 x 1 min frames) was performed from the time of the first injection. A region of interest was drawn over soft tissue (representing the capillary bed) adjacent to the major blood vessels in each thigh. The blood sample counts were normalised by the ratio of mean image counts to the mean blood counts during the flat $^{99m}$Tc-RBC phase and overlaid on the time-activity curve of the soft tissue region to look for change in the whole blood:soft tissue activity ratio following injection of antibody-coated cells.
RESULTS

Clinical effects and red cell antibody coating
Subjects 1 - 5 were studied within a 6-week time frame. The injections were generally well tolerated, with no symptoms reported on the day of study and no changes in pulse, temperature or blood pressure in subjects 1 - 4. However, at follow-up, subject 4 reported having felt shivery 1 hour post-injection and 'achey' the following day, returning to normal by 48 hours. Subject 5 experienced obvious rigors 1 hour post-injection and felt cold and shivery. There was no immediate change in pulse, temperature or blood pressure, and he was treated with paracetamol and oral fluids. By 5 hours he felt well enough to go home but was beginning to feel 'fluish', with aches and flushing. His temperature was 38.2°C, pulse 98, with BP unchanged at 110/67. The next day he still felt achey but became entirely well over the next 48 hours, and was well at review visits on days 7 and 28. The Data and Safety Monitoring Committee considered that the study should continue, with later subjects being warned of a possible reaction and treated with paracetamol if required. However, subject 6, who received modified antibody only, had no reactions or changes to vital signs.

SOL-ELISA results showed that the degree of red cell coating by Fog-1 G1 and G1.\(\Delta\)nab ranged from 6,500 - 19,900 molecules/cell in the different subjects (Table 1). The maximum amount of total antibody received was 130 – 430 µg. The degree of coating showed only limited correlation with Rh phenotype, with considerable overlap between D homozygous and heterozygous individuals. Importantly, coating levels for the two antibodies were within 4% of each other for subjects 1, 2, 3 and 5 and within 10% for subject 4. Thus, direct comparison of intravascular survival of red cells coated with wildtype and modified antibodies was considered valid.

Recovery and survival of antibody coated red cells in subjects 1 - 5
For the majority of injections, there was less than 100% recovery of radiolabelled cells coated with either antibody as determined from whole blood counts of samples taken 1.5 – 5 min after injection (Figure 1, Table 2). The exception was
Cells coated with Fog-1 G1 were removed rapidly from the circulation in all five subjects, with complete clearance by 200 minutes and no reappearance at later time points (Figures 1a, 2a). The rate constant of clearance of cells coated with Fog-1 G1, calculated from the 5 – 60 min data for cell-associated activity (Figure 2a), ranged from 0.026 – 0.046 min\(^{-1}\) (Table 2) and showed no correlation with either Rh phenotype or level of coating. Cells coated with Fog-1 G1\(\Delta\)nab showed slower clearance in all subjects, demonstrated by comparison of survival at 60 min (Table 2). Notably, clearance was also incomplete, with nadir values ranging from 7 - 38% injected dose (Figures 1b, 2b), followed by reappearance of these cells in the circulation at late time points. Whole blood counts reached 12 - 67% injected dose in four subjects at 20 – 23 h post-injection (Table 2; subject 5 was unavailable for sampling). The short half-life of \(^{99m}\)Tc (6 h) meant that survival of G1\(\Delta\)nab-coated cells could not be followed further in two subjects but, for those having the \(^{51}\)Cr/G1\(\Delta\)nab combination, 38, 56 and 18% injected dose remained in the circulation 5 - 6 days post-injection. Approximations of the initial clearance rates could be made using a simple exponential function as for the G1-coated cells, with all \(R^2\) values between 0.94 and 1.00. The G1\(\Delta\)nab rate constants ranged from 0.009 – 0.026 min\(^{-1}\) (Table 2) and were significantly lower than the G1 values of 0.026 – 0.046 (Student’s \(t\)-tests: unpaired \(p=0.008\), paired \(p=0.0002\)). However, since the cell-associated activity curves approached plateaux towards the end of the 0 – 300 min period (Figure 2b), these data were better described by the function \(C(t) = A + B \exp(-bt)\) , allowing estimation of a rate constant (\(b\)) for the disappearance of a portion of cells and a plateau value (\(A\)) representing the level remaining in circulation (Table 2). This second rate constant, for which there is no corresponding value for G1-coated cells, ranged from 0.020 – 0.030 min\(^{-1}\) and the plateau value from 6 – 40%, neither value correlating with either Rh phenotype or level of coating.
Since $^{51}\text{Cr}$ is a stable red cell label\textsuperscript{11}, non-cell-bound activity is a marker of cell destruction. For G1-coated cells, the plasma $^{51}\text{Cr}$ curves were consistent with complete cell destruction, as suggested by the rapid disappearance of the whole blood signal, and peaked at 4.5 and 6.1% injected dose (Figure 3, Table 2). In contrast, the plasma $^{51}\text{Cr}$ plots for G1Δnab-coated cells were flat, with maximum values of 0.5, 0.8 and 1.6% injected dose, suggesting very little cell destruction. Since $^{99m}\text{Tc}$ is considerably less stable in red cells, showing a rate of spontaneous elution of approximately 3%/h\textsuperscript{23,24}, plasma data for this label are not informative of cell destruction. Plasma $^{99m}\text{Tc}$ curves drawn for the two coating antibodies were not distinctly different, with levels starting at 3 – 5% injected dose, presumably due to elution prior to injection, and tailing off thereafter due to the clearance of free $^{99m}\text{Tc}$ from the circulation (data not shown). The $^{99m}\text{Tc}$ elution will have negligible effect on the clearance curves of the rapidly destroyed G1-coated cells but impacts on the G1Δnab cell data. If adjusted for Tc elution, nadir blood values rise by about 10% and, more significantly, cell counts at 20 or 21 h for subjects 2 and 4 would increase to approximately 39% and 20% respectively.

**Organ scanning**

Uptake of $^{99m}\text{Tc}$-labelled, antibody-coated cells by the spleen and liver over the first 40 min post-injection was monitored by gamma camera imaging for subjects 1 – 5. In all subjects, the cells accumulated in the spleen, although uptake tended to be higher in the three subjects with the $^{99m}\text{Tc}$/Fog-1 G1 combination (Figure 4). Two of these subjects (1 and 5) also showed increased activity over the liver which was not apparent for subject 3 (Fog-1 G1) or for subjects 2 and 4 (G1Δnab). In all subjects, a low count rate was recorded over the lungs, excluding the possibility of adhesion of cells to pulmonary vascular endothelium.

**Investigation of early loss of injected cells**

The protocol used for subject 6 was designed to investigate whether the apparent loss of cells immediately upon injection was due to adhesion to microvascular endothelium. $^{99m}\text{Tc}$-labelled red cells without antibody coating were injected and allowed to equilibrate over 20 min. Labelled cells coated with G1Δnab antibody were then injected and sampling continued for 150 min. The whole blood and cell
counts, corrected to correspond to antibody-coated cells only, fell in line with G1Δnab cells in the other subjects (Figures 1b, 2b) with rate constant and plateau values in the mid-range (Table 2). A gamma camera was used to obtain image counts for areas of soft tissue in the thighs. The ratio of soft tissue to whole blood counts remained constant throughout (Figure 5), making cell accumulation on vascular endothelium unlikely. The proportions of injected activity in plasma were similar following injection of uncoated and G1Δnab-coated cells (data not shown), confirming that appearance of $^{99m}$Tc label in plasma does not indicate cell destruction.
DISCUSSION

This study tested the hypothesis that antibodies with constant regions modified to minimise FcγR binding and complement activation would have reduced capacity to trigger antibody-mediated blood cell destruction \textit{in vivo}. Since antibodies for antenatal therapy must have an Fc portion to maintain the normal half-life and placental transport\textsuperscript{25}, we attempted to abrogate destructive functions by specifically modifying the Fc, rather than using either Fab or single-chain variable region fragments of antibodies. Inter-species differences in IgG subclass binding by FcγR\textsuperscript{26} preclude use of animal studies, so we have chosen human anti-D antibodies as our model system. There is extensive accumulated evidence over several decades of administering anti-D antibodies to humans and the original Fog-1 was assessed in volunteers for its suitability in HDN prophylaxis.\textsuperscript{27}

Unexpectedly, two of our subjects had mild febrile reactions coincidental with the appearance of radiolabel in the plasma, suggesting a response to the destructive process. The previous study of active Fog-1 reported no reactions\textsuperscript{27}, but the volumes of coated cells were nearly 20 times lower than injected here. Since the two febrile subjects had both wildtype and modified antibody, it is not possible to attribute the reactions to either one.

The key finding of the study is that there was a clear difference between the survival curves for cells coated with wildtype IgG1 and mutant G1Δnab antibodies. The clearance of cells coated with Fog-1 G1 antibody was rapid, with a fall of at least 70\% between 5 and 60 min, and was essentially complete by 3 hours (Figure 2). The concurrent appearance of $^{51}$Cr in plasma (Figure 3) with no re-emergence of IgG1-coated cells suggest total cell clearance and destruction. The degree of clearance seen was greater than in the previous study of Fog-1\textsuperscript{27}, despite comparable levels of coating in some subjects and similar levels of elution from the D antigen (31\%/hour \textit{in vitro}, data not shown). The ability of our Fog-G1 to promote faster and more complete haemolysis than in the earlier study may be due to the different expression system used here. The original Fog-1 was obtained from human-mouse heterohybridoma cells whereas we used rat myeloma cells for the production of recombinant Fog-1 G1 and G1Δnab.
Different cell lines are known to produce antibodies with alternative glycosylation profiles which, in turn, affect potency in ADCC.\textsuperscript{28,29} The different killing efficiencies may also be related to cell culture or purification conditions.

In comparison to wildtype antibody, clearance of cells coated with G1Δnab antibodies was significantly slower, incomplete and transient, with blood cell counts rising again after 3 - 4 hours (Figure 1). Moreover, the level of \textsuperscript{51}Cr in the plasma remained low in the three subjects receiving the \textsuperscript{51}Cr/G1Δnab combination (Figure 3). This is particularly remarkable for subject 5 whose plasma count did not rise above 0.5\% injected dose despite the whole blood count falling to 7\% injected dose. This plasma data contrasts that seen for the G1-coated cells in subject 4 although the two populations had similar initial clearance rates. The organ scanning data also support a different pattern of behavior for G1Δnab-coated cells. Whilst cells coated with either antibody accumulated in the spleen, the liver showed considerable uptake of cells carrying the wildtype antibody (subjects 1 and 5), but no accumulation of cells coated with higher amounts of the mutant antibody (subjects 2 and 4; Table1, Figure 4). Subject 3 was unusual in showing no hepatic uptake with IgG1 which was probably due to the low coating level (6,800 molecules/cell), since hepatic uptake has not been detected with less than 9000 IgG anti-D molecules per cell.\textsuperscript{12,13} These differences are not due to G1Δnab being lost from the cells faster than wildtype antibody since, as expected for antibodies containing the same variable regions, elution rates and titration curves against D homozygous and heterozygous cells were identical.

These results suggest that G1Δnab-coated cells are not destroyed but show exaggerated pooling within the spleen, adhering to splenic Fc\textsubscript{γ}R but not triggering signalling and phagocytosis. Binding to Fc\textsubscript{γ}RII may be the explanation, since, although the mutations have abrogated binding to Fc\textsubscript{γ}RI and III\textsuperscript{7,8}, some interaction with Fc\textsubscript{γ}RIIa and the inhibitory receptor Fc\textsubscript{γ}RIIb remains.\textsuperscript{16} The clearance curves from 5 - 300 minutes could be described in terms of a rate constant for the clearance of a proportion of cells and a plateau value which limits the clearance in this period. This has some similarities with the biexponential clearance kinetics observed for heat-damaged red cells, which are either
temporarily sequestered in the spleen or permanently trapped and destroyed. Our observations are also consistent with the biphasic model of sequestration followed by phagocytosis proposed for RBC coated with other anti-D antibodies (George Chapman, Bio Products Laboratory, email, November 30, 2004). Individual variation in numbers of FcγR-bearing cells and the avidity of their interaction with G1Δnab-coated cells will affect the rate of uptake. The level at which receptor binding reaches saturation may also be a factor, since vacant receptors are not being regenerated following phagocytosis. The net increase in circulating labelled cells at later time points may occur due to elution of G1Δnab from the D antigen, decreasing the likelihood of red cell capture or retention by splenic FcγR. Unfortunately, because reappearance of the cells was unexpected, we did not collect sufficient late samples for the three subjects receiving the $^{51}$Cr/G1Δnab combination to clarify the extent of cellular reappearance and, thus, how few cells were actually destroyed. For the $^{99m}$Tc/G1Δnab combination, the cell counts are likely to have significantly under-represented reappearance of cells due to elution of the radiolabel. Despite these limitations, the lack of hemolysis seen with the modified antibody confirms that Fc-mediated cytotoxicity is an important mechanism of alloimmune blood cell destruction.

In four subjects, for cells coated with either antibody, less than 100% injected dose of radiolabel was detected in the blood at the earliest time points. It is unclear whether this was seen previously with Fog-1, since survival results were expressed as a percentage of the 3 min sample. The discrepancy could be partly explained by an underestimation of blood volume, since there is up to 25% error in blood volumes calculated from height and weight, but it seems unlikely that the actual volume would be higher than predicted in every subject tested. Rapid cell clearance by an individual organ was considered, but none has blood flow high enough to account for the degree and speed of loss. Using Fog-1 G1Δnab in subject 6, we examined whether adhesion to capillary endothelium throughout the vasculature could be rendering cells unavailable for sampling. However, comparison of the blood counts relative to the soft tissue signal between uncoated and Fog-1 G1Δnab-coated cells did not support this hypothesis (Figure 5), and early losses of cells remain unexplained. These
disparities do not affect the overall conclusions of the study, since we have compared survival of the two cell populations simultaneously in each donor.

We wished to achieve 100% saturation of D sites on the red cells, to mimic the worst case scenario in HDN. The concentration of antibody this required could not be attained for Fog-1 G1Δnab due to purification limitations, so we compared clearance at a calculated 75% saturation for both wildtype and mutated antibodies. Although the degree of coating achieved varied considerably between subjects, the variation in coating between the two antibodies in each individual was small enough to permit a valid paired comparison of red cell survival (Table 1). We saw no clear correlation between the degree of coating and Rh phenotype. Subject 3, in particular, showed surprisingly low coating for an apparently homozygous individual (Table 1). Since we performed Rh phenotyping and not genotyping, it is possible that this subject has the rare genotype Rzr, rather than the common R1R2, and is thus heterozygous for D.

The lack of correlation between survival of Fog-1 G1-sensitised cells and degree of coating may be due to FcγR polymorphisms. FcγRIIa-131H and FcγRIIIa-158V molecules show higher IgG binding than receptors of the alternative allotypes, influencing susceptibility to disease and efficacy of therapeutic antibodies. A previous clinical trial of an IgG1 anti-D showed that clearance rates of D-positive cells were independent of antibody dosage but were faster in FcγRIIa-131H or FcγRIIIa-158V homozygotes. However, we did not have permission to genotype our subjects and a larger study would be needed to show significance. If FcγRII binding is responsible for the sequestration of G1Δnab-coated red cells, then the FcγRIIa polymorphism may be affecting the rate of uptake. Although red cells coated with mutant anti-D did not show normal survival, this does not preclude a therapeutic benefit in alloimmune fetal disorders, since complete normalisation of erythrocyte or platelet survival may not be required. CL studies have shown that HDN sera giving <30% of maximum activation are rarely associated with clinical disease, so reduction of the degree of haemolysis might permit the fetal bone marrow to compensate.
It was not the intention of this study to show that a single modified anti-D would be able to prevent red cell destruction caused by maternal polyclonal anti-D. Because of the complex nature of the RhD antigen, the Fog-1 variable region may not be optimal for this purpose. In contrast, the antigens of the biallelic Human Platelet Antigen System-1 system, which accounts for most cases of feto-maternal allo-immune thrombocytopenia, are the result of a single amino acid substitution, and therefore may be easier to block by a single monoclonal. We have generated an Fc-modified antibody to HPA-1a, which has been shown to inhibit binding of maternal sera to HPA-1a1b platelets and subsequent activation of effector cells (Cedric Ghevaert, Craig Turner, Kathryn Armour, et al, manuscript in preparation), while having no significant effect on their activation or function. Human studies are planned to examine platelet survival and thus its therapeutic potential.
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## Table 1. Volunteer data.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age / Sex</td>
<td>47 / F</td>
<td>53 / M</td>
<td>45 / M</td>
<td>41 / M</td>
<td>48 / M</td>
<td>38 / M</td>
</tr>
<tr>
<td>Predicted Rh genotype</td>
<td>R₁R₂</td>
<td>R₂R₂</td>
<td>R₁R₂</td>
<td>R₁r</td>
<td>R₁r</td>
<td>R₁r</td>
</tr>
<tr>
<td>G₁ antibody molecules/cell $^{1,2}$</td>
<td>11700 (± 2700)</td>
<td>19900 (± 2300)</td>
<td>6800 (± 500)</td>
<td>17400 (± 3500)</td>
<td>13300 (± 1300)</td>
<td></td>
</tr>
<tr>
<td>G₁Δnab antibody molecules/cell $^1$</td>
<td>11200 (± 2100)</td>
<td>19900 (± 2500)</td>
<td>6500 (± 600)</td>
<td>15600 (± 2600)</td>
<td>13700 (± 2100)</td>
<td>13500 (± 3000)</td>
</tr>
</tbody>
</table>

$^1$ Figures in parentheses refer to estimated error limits for SOL-ELISA determination (see Materials and Methods).

$^2$ Subject 6 did not receive G₁-coated cells.
Table 2. Red cell survival data.

<table>
<thead>
<tr>
<th>Subject</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>G1 antibody-coated cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Label</td>
<td>$^{99m}$Tc</td>
<td>$^{51}$Cr</td>
<td>$^{99m}$Tc</td>
<td>$^{51}$Cr</td>
<td>$^{99m}$Tc</td>
<td>$^{51}$Cr</td>
</tr>
<tr>
<td>Whole blood count at 5 min time point (% injected dose)</td>
<td>84</td>
<td>71</td>
<td>81</td>
<td>75</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Whole blood count at 60 min time point (% 5 min sample)</td>
<td>22</td>
<td>30</td>
<td>26</td>
<td>22</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Maximum plasma value (% injected dose)</td>
<td>6.1</td>
<td>4.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rate constant for clearance of all cells (min$^{-1}$)</td>
<td>0.030</td>
<td>0.027</td>
<td>0.026</td>
<td>0.031</td>
<td>0.046</td>
<td></td>
</tr>
<tr>
<td><strong>G1Δnab antibody-coated cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Label</td>
<td>$^{51}$Cr</td>
<td>$^{99m}$Tc</td>
<td>$^{51}$Cr</td>
<td>$^{99m}$Tc</td>
<td>$^{51}$Cr</td>
<td>$^{99m}$Tc</td>
</tr>
<tr>
<td>Whole blood count at 5 min time point (% injected dose)</td>
<td>79</td>
<td>90</td>
<td>82</td>
<td>84</td>
<td>77</td>
<td>82</td>
</tr>
<tr>
<td>Whole blood count at 60 min time point (% 5 min sample)</td>
<td>47</td>
<td>52</td>
<td>61</td>
<td>37</td>
<td>22</td>
<td>48</td>
</tr>
<tr>
<td>Maximum plasma value (% injected dose)</td>
<td>1.6</td>
<td>0.8</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rate constant for clearance of all cells (min$^{-1}$)</td>
<td>0.014</td>
<td>0.012</td>
<td>0.009</td>
<td>0.019</td>
<td>0.026</td>
<td>0.013</td>
</tr>
<tr>
<td>Rate constant for clearance to asymptote only, (min$^{-1}$)</td>
<td>0.024</td>
<td>0.020</td>
<td>0.027</td>
<td>0.020</td>
<td>0.030</td>
<td>0.022</td>
</tr>
<tr>
<td>Plateau/asymptote (% injected dose)</td>
<td>21</td>
<td>19</td>
<td>40</td>
<td>6</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>Rebound: Time of highest measured rebound (hours)</td>
<td>23</td>
<td>20</td>
<td>21</td>
<td>21</td>
<td>144</td>
<td>nd</td>
</tr>
<tr>
<td>Whole blood count at rebound (% injected dose)</td>
<td>39</td>
<td>23</td>
<td>67</td>
<td>12</td>
<td>18</td>
<td>nd</td>
</tr>
</tbody>
</table>

1 Subject 6 did not receive G1-coated cells.

2 Plasma values given for $^{51}$Cr only due to the high rate of elution of $^{99m}$Tc from cells.

3 Rate constant, b, obtained by fitting cell clearance data for the 5 – 60 min period to the function $C(t) = B \exp(-bt)$.

4 Rate constant, b, and plateau, A, obtained by fitting cell clearance data for the 0 – 300 min period to the function $C(t) = A + B \exp(-bt)$.

5 For subject 5, no samples were taken one day post-injection.

nd, not determined
Figure Legends

Figure 1

Variation of whole blood count up to six days post-injection in all subjects. The corrected blood sample counts for each radionuclide are expressed as a percentage of the injected activity. Part a corresponds to cells coated with Fog-1 G1 and part b to cells coated with Fog-1 G1\(\triangle\)nab. In both parts, \(^{99m}\text{Tc}\)-labelling is indicated by open symbols and \(^{51}\text{Cr}\)-labelling by closed symbols.

Figure 2

Variation of cell-associated activity over first five hours in all subjects. The whole blood counts are adjusted for radiolabel in the plasma and the resulting cell counts expressed as a percentage of the injected activity. Part a corresponds to cells coated with Fog-1 G1 and part b to cells coated with Fog-1 G1\(\triangle\)nab. In both parts, \(^{99m}\text{Tc}\)-labelling is indicated by open symbols and \(^{51}\text{Cr}\)-labelling by closed symbols.

Figure 3

\(^{51}\text{Cr}\) activity associated with the plasma. The \(^{51}\text{Cr}\) plasma counts, expressed as a percentage of the injected dose, are plotted as a function of time post-injection. Part a corresponds to cells coated with Fog-1 G1 (subjects 2 and 4) and part b to cells coated with Fog-1 G1\(\triangle\)nab (subjects 1, 3 and 5). \(^{99m}\text{Tc}\) plasma counts are not informative (see text).
Figure 4

\(^{99m}\)Tc activity in the spleen, liver and lung. Count rates for the whole spleen, whole liver and a portion of right lung were obtained from gamma camera imaging data, normalised to a standard injected activity and plotted against time post-injection. Each panel shows activity over an organ for subjects 1, 3 and 5 where \(^{99m}\)Tc coated cells were coated with Fog-1 G1 (closed symbols) and for subject 2 and 4 where \(^{99m}\)Tc labelled cells were coated with Fog-1 G1Δnab (open symbols).

Figure 5

Comparison of whole blood and soft tissue counts following injection of uncoated and Fog-1 G1Δnab-coated RBC. Subject 6 was injected with 15 MBq uncoated \(^{99m}\)Tc-RBC followed, after 20 min, by 49 MBq Fog-1 G1Δnab-coated \(^{99m}\)Tc-RBC and was monitored by whole blood counting and gamma camera imaging of the soft tissue of the thighs. Blood samples counts were normalized by the ratio of mean image counts to the mean blood counts for the 0 – 20 min period and overlaid on the time-activity curve of the soft tissue region.
Figure 1
Figure 2

(a) and (b) show the cell count as a percentage of the injected dose over time after injection in minutes. Each graph contains lines representing different subjects, distinguished by their markers.

- **subject 1**: ○
- **subject 2**: ▲
- **subject 3**: □
- **subject 4**: ●
- **subject 5**: △
- **subject 6**: ■

The graphs illustrate the decay rate of cell counts over time for different subjects.
Figure 3
Figure 4
Figure 5
Intravascular survival of red cells coated with a mutated human anti-D antibody engineered to lack destructive activity

Kathryn L Armour, David R Parry-Jones, Nigel Beharry, James R Ballinger, Rosey Mushens, R K Williams, Cynthia Beatty, Simon Stanworth, Paul Lloyd-Evans, Marion Scott, Michael R Clark, A M Peters and Lorna M Williamson