CLINICAL TRIALS AND OBSERVATIONS

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

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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, G1∆nab, devoid of in vitro cytotoxic activity. RhD-positive red blood cells (RBCs), labeled with chromium-51 or technetium-99m, were separately coated to equal levels with wild-type IgG1 or G1∆nab anti-D antibody (Fog-1). After reinjection, there was complete, irreversible clearance of IgG1-coated RBCs by 200 minutes, 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 G1∆nab-coated cells was slower, incomplete, and transient, with whole blood counts falling to 7% to 38% injected dose by about 200 minutes before increasing to 12% to 67% thereafter. There was no appearance of plasma radiolabel and no hepatic accumulation. These findings suggest that G1∆nab-coated RBCs were not hemolysed but temporarily sequestered in the spleen and that our approach merits investigation in larger studies. (Blood. 2006; 107:2619-2626)

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Introduction

Maternal alloimmunization to paternally inherited alloantigens on red cells or platelets can cause pregnancy complications that 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 hemolysis was inhibited by antibodies against FcγR.[1] Although the timing of appearance of FcγR on fixed effector cells in fetal liver and spleen is uncertain, FcγRI-III are present on circulating leucocytes by 18 weeks of pregnancy,[2] when alloimmunity can cause life-threatening fetal platelet destruction.[3]

In contrast to IgG1 and IgG3, IgG2 and IgG4 antibodies rarely cause significant hemolysis,[4] explained by their low binding to FcγR and limited ability to activate effector cells or complement. We have exploited this difference to generate nondestructive 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 2 regions of the immunoglobulin C_H2 domain, which are critical for FcγRI-III and complement C1q binding,[5] 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 minimize the potential to create new immunogenic epitopes. When recombined with model human variable region genes (Fog-1 anti-D[6]), 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 G1∆ab-coated red cells did not trigger either CD16-positive natural killer cell–mediated cytotoxicity (ADCC)[8] or monocyte chemiluminescence (CL),[7] an assay reported to predict clinical outcome in hemolytic disease of the newborn (HDN).[9] Importantly, Fog-1 G1∆ab inhibited ADCC and CL triggered either by Fog-1 IgG1 or clinical anti-D sera.[7,8] Fog-1 G1∆ab has been further modified by removal of 3 allotypic residues[10] and redesignated Fog-1 G1∆nab.

We now report a "proof of principle" investigation in human volunteers to establish whether the nondestructive nature of G1∆nab in vitro is reflected in improved intravascular survival of cells coated with modified antibody. Two aliquots of autologous red cells, labeled with chromium-51 (^{51}Cr) or technetium-99m (^{99m}Tc),[11] were coated with either active Fog-1 G1 or mutated Fog-1 G1∆nab. Since radiolabeling and antibody sensitization do not affect each other,[12,13] this dual labeling strategy provides comparative red cell

survival data for the 2 antibodies without the added complexity of interdonor variation. It permits assessment of both intravascular survival beyond a few hours (^{51}Cr) and sites of red cell destruction by gamma camera imaging (^{99m}Tc).[13]

Subjects, 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 older than 30 and women older than 50 years of age (or sterilized) 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 serologic 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 were performed 4 to 6 weeks after the study.

Production and prerelease testing of Fog-1 IgG1 and G1Δnab antibodies

Fog-1 is a human anti-D, IgG1,κ molecule that recognizes up to 26 000 D sites/cell with a binding affinity of 1 to 2×10^9 M^{-1} and stimulates monocyte phagocytosis.[6,14] The properties of recombinant IgG1 and mutant G1Δab forms of Fog-1 have been described.[7,8,15,16] Fog-1 G1Δnab is a null allotype variant[10] of Fog-1 G1Δab in which the additional mutations Lys214Thr, Asp356Glu, and Leu358Met have removed residues specifying the allotype G1m(1,17). Fog-1 G1Δab and G1Δnab have equivalent activity in binding to FcγRI, FcγRIIa of 131R 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[17] in the National Blood Service Clinical Biotechnology Centre and quality control tested in accordance with European Union guidelines.[18] Fog-1 G1 and G1Δnab cell lines each were cloned 3 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, United Kingdom) in the cell compartment. After 1 week, the basal medium M925 (Hyclone) was changed 3 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, United Kingdom) affinity chromatography and cation exchange chromatography. The eluate was viral filtered then dialyzed and concentrated using a crossflow membrane into phosphate-buffered saline (PBS). Purity was demonstrated by a single band on acid native gel electrophoresis and 2 bands on reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The product was resuspended in 0.1% human albumin (Bioproducts Laboratory, Elstree, United Kingdom), bottled in nitrogen-filled borosilicate glass bottles (Amersham BioSciences), and stored at −20°C. The final concentrations of antibodies, assessed by anti-κ chain enzyme-linked immunosorbent assay (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 R$_2$R$_2$ and R$_1$r cells, with end point titers of wild-type 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,[7] with Fog-1 G1Δnab giving 10^4-fold less binding than Fog-1 G1 to FcγRI transfectants, and no CL stimulation at 1 μg/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, solubilized, coated RBCs were assayed by ELISA (SOL-ELISA) to establish coating conditions for the in vivo studies that gave 75% saturation with each antibody.

SOL-ELISA for measurement of antibody coating

Known volumes of antibody-coated red cells were solubilized[19] and antibody standard prepared in the same buffer. Dilutions were assayed in quadruplicate in a sandwich ELISA using unlabeled and peroxidase-conjugated goat anti–human κ chain antibodies (Sigma, Poole, United Kingdom). 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.

Radiolabeling/antibody coating for subjects 1 to 5

On the day of study, a 17-mL citrated blood sample was taken and split into 2 aliquots. Packed red cells from each aliquot were separately radiolabeled 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 G1Δnab (8 mL of antibody at 50 μg/mL) for 30 minutes at 37°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 to 1.0 MBq ^{51}Cr and 17 to 41 MBq ^{99m}Tc.

Measurement of red cell survival (subjects 1 to 5)

The ^{51}Cr-labeled antibody-coated cells were injected intravenously over 1 minute, followed by the ^{99m}Tc sample. Blood samples were taken via an indwelling catheter from the opposite arm at time points from 5 to 240 minutes. Additional samples were taken on the following day in 4 subjects and after 5 or 6 days in 3 subjects. Because only 80% of injected dose was detected at 5 minutes for subject 1, samples were taken at earlier time points in subjects 2 to 5. Whole blood and plasma samples were counted against standards made from aliquots of labeled, 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 less than 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/liter and to percentage injected dose, using the blood volume predicted from the subject's height and weight.[20] 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.[21] To calculate clearance rates of labeled cells from the blood, whole blood activity was corrected for plasma activity, using the sample hematocrit, to give cell-bound activity. The data were transformed logarithmically, and the curves from 5 to 60 minutes were fitted with a standard least squares routine (Excel, Microsoft, Seattle, WA). Data from the 0- to 300-minute time points of the Fog-1 G1Δnab clearance curves also were fitted to the function C(t) = A + B exp(−bt), using a nonlinear least squares routine (IDL Research Systems, Boulder, CO).

Table 1. Volunteer data

	Subjects					
	1	**2**	**3**	**4**	**5**	**6**
Age/sex	47/F	53/M	45/M	41/M	48/M	38/M
Predicted Rh genotype	R_1R_2	R_2R_2	R_1R_2	R_1r	R_1r	R_1r
G1 antibody molecules/cell*	11 700 (± 2700)	19 900 (± 2300)	6800 (± 500)	17 400 (± 3500)	13 300 (± 1300)	–
G1Δnab antibody molecules/cell*	11 200 (± 2100)	19 900 (± 2500)	6500 (± 600)	15 600 (± 2600)	13 700 (± 2100)	13 500 (± 3000)

– indicates not applicable (subject 6 did not receive G1-coated cells).

*Figures in parentheses refer to estimated error limits for SOL-ELISA determination ("Subjects, materials, and methods").

Gamma camera imaging (subjects 1 to 5)

Gamma camera imaging was performed with the subject lying supine under a dual-headed gamma camera (Prism 2000XP, Picker, OH) positioned over the chest and upper abdomen. Dynamic imaging (40 × 1 minute frames) was performed immediately after injection followed by static imaging at 40 minutes. 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 normalized 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 RBCs were accumulating on microvascular endothelium, a revised protocol was developed for subject 6, using a modification of a method previously described for neutrophils.[22] Fifteen MBq of uncoated ^{99m}Tc-RBCs were injected and 6 blood samples taken over a 20-minute equilibration time. Then 49 MBq of ^{99m}Tc-Fog-1 G1Δnab-coated RBCs 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 RBCs, and the net data were used to assess clearance of coated cells, as above. Dynamic imaging (60 × 1 minute 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 normalized 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–to–soft tissue activity ratio following injection of antibody-coated cells.

Results

### Clinical effects and red cell antibody coating

Subjects 1 to 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 to 4. However, at follow-up, subject 4 reported having felt shivery 1 hour after injection and achy the following day, returning to normal by 48 hours. Subject 5 experienced obvious rigors 1 hour after 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 blood pressure unchanged at 110 over 67. The next day he still felt achy 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 G1Δnab ranged from 6500 to 19 900 molecules/cell in the different subjects (Table 1). The maximum amount of total antibody received was 130 to 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 2 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 wild-type and modified antibodies was considered valid.

Recovery and survival of antibody-coated red cells in subjects 1 through 5

For the majority of injections, there was less than 100% recovery of radiolabeled cells coated with either antibody as determined from whole blood counts of samples taken 1.5 to 5 minutes after injection (Figure 1, Table 2). The exception was the Fog-1 G1-coated red cells in subject 5, where 101% injected dose was measured at 1.5 minutes. In the other subjects, the maximum
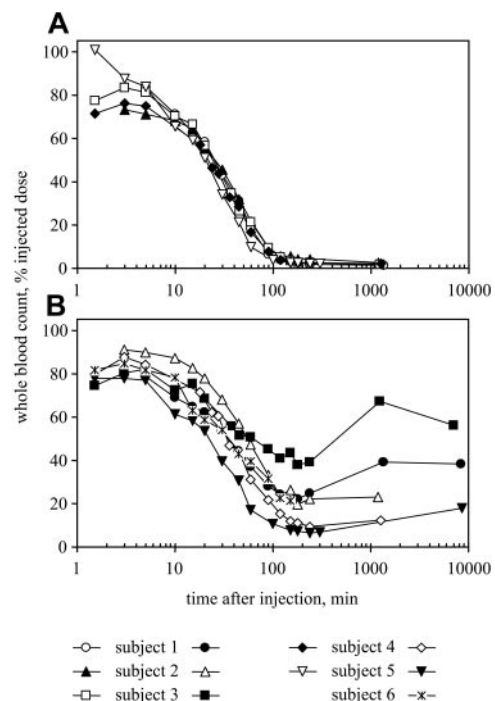
Figure 1. Variation of whole blood count up to 6 days after injection in all subjects. The corrected blood sample counts for each radionuclide are expressed as a percentage of the injected activity. Panel A corresponds to cells coated with Fog-1 G1, and panel B to cells coated with Fog-1 G1Δnab. In both parts, ^{99m}Tc-labeling is indicated by open symbols and ^{51}Cr-labeling by closed symbols.

Table 2. Red cell survival data

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

ND indicates not determined; NA, not applicable (subject 6 did not receive G1-coated cells); and —, plasma values given for ^{51}Cr only due to the high rate of elution of ^{99m}Tc from cells.

*Rate constant, b, obtained by fitting cell clearance data for the 5- to 60-minute period to the function C(t) = B exp(−bt).

†Rate constant, b, and plateau, A, obtained by fitting cell clearance data for the 0- to 300-minute period to the function C(t) = A + B exp(−bt).

‡For subject 5, no samples were taken one day after injection.

recovery of G1-coated cells ranged from 73% to 84%, while 78% to 91% G1Δnab-coated cells were recovered in the 5 subjects.

Cells coated with Fog-1 G1 were removed rapidly from the circulation in all 5 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- to 60-minute data for cell-associated activity (Figure 2A), ranged from 0.026 to 0.046 minutes^{-1} (Table 2) and showed no correlation with either Rh phenotype or level of coating. Cells coated with Fog-1 G1Δnab showed slower clearance in all subjects, demonstrated by comparison of survival at 60 minutes (Table 2). Notably, clearance also was incomplete, with nadir values ranging from 7% to 38% injected dose (Figures 1B,2B), followed by reappearance of these cells in the circulation at late time points. Whole blood counts reached 12% to 67% injected dose in 4 subjects at 20 to 23 hours after injection (Table 2; subject 5 was unavailable for sampling). The short half-life of ^{99m}Tc (6 hours) meant that survival of G1Δnab-coated cells could not be followed further in 2 subjects but, for those having the ^{51}Cr/G1Δnab combination, 38%, 56%, and 18% injected dose remained in the circulation 5 to 6 days after 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 G1Δnab rate constants ranged from 0.009 to 0.026 minutes^{-1} (Table 2) and were significantly lower than the G1 values of 0.026 to 0.046 (Student t tests: unpaired, $P = .008$; paired, $P < .001$). However, since the cell-associated activity curves approached plateau toward the end of the 0- to 300-minute 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 to 0.030 minutes^{-1} and the plateau value from 6% to 40%, neither value correlating with either Rh phenotype or level of coating.

Since ^{51}Cr is a stable red cell label,[11] non–cell-bound activity is a marker of cell destruction. For G1-coated cells, the plasma ^{51}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}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}Tc is considerably less stable in
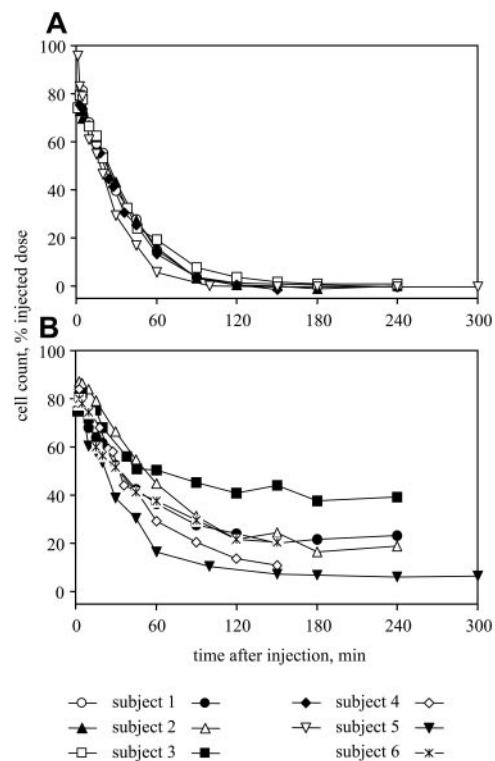
Figure 2. Variation of cell-associated activity over first 5 hours in all subjects. The whole blood counts are adjusted for radiolabel in the plasma and the resulting cell counts expressed as a percentage of the injected activity. Panel A corresponds to cells coated with Fog-1 G1 and panel B to cells coated with Fog-1 G1Δnab. In both parts, ^{99m}Tc-labeling is indicated by open symbols and ^{51}Cr-labeling by closed symbols.
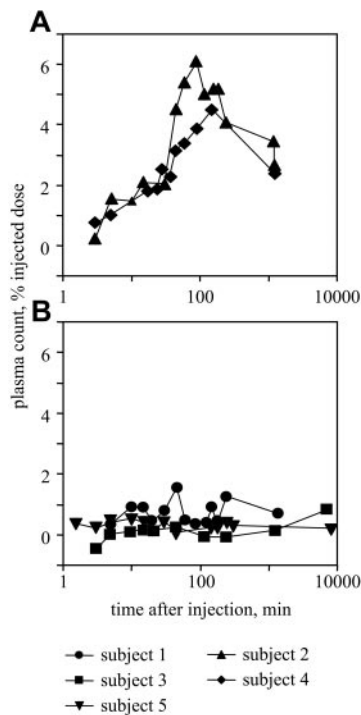
red cells, showing a rate of spontaneous elution of approximately 3% per hour,[23,24] plasma data for this label are not informative of cell destruction. Plasma ^{99m}Tc curves drawn for the 2 coating antibodies were not distinctly different, with levels starting at 3% to 5% injected dose, presumably due to elution prior to injection, and tailing off thereafter due to the clearance of free ^{99m}Tc from the circulation (data not shown). The ^{99m}Tc elution will have negligible effect on the clearance curves of the rapidly destroyed G1-coated cells but impacts 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 hours for subjects 2 and 4 would increase to approximately 39% and 20%, respectively.
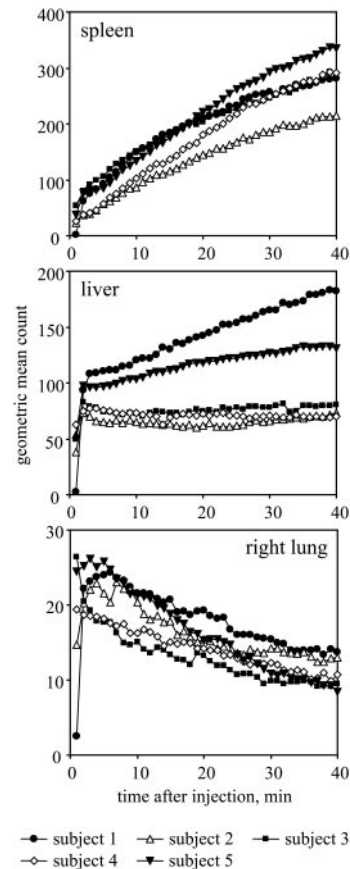
Organ scanning

Uptake of ^{99m}Tc-labeled, antibody-coated cells by the spleen and liver over the first 40 minutes after injection was monitored by gamma camera imaging for subjects 1 to 5. In all subjects, the cells accumulated in the spleen, although uptake tended to be higher in the 3 subjects with the ^{99m}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}Tc-labeled red cells without antibody coating were injected and allowed to equilibrate over 20 minutes. Labeled cells coated with G1Δnab antibody were
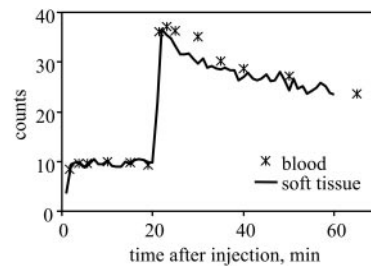
Figure 4. ^{99m}Tc activity in the spleen, liver, and lung. Count rates for the whole spleen, whole liver, and a portion of right lung were obtained from gamma camera imaging data, normalized to a standard injected activity, and plotted against time after injection. Each panel shows activity over an 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-labeled cells were coated with Fog-1 G1Δnab (open symbols).

then injected, and sampling continued for 150 minutes. 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 midrange (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),

Figure 3. ^{51}Cr activity associated with the plasma. The ^{51}Cr plasma counts, expressed as a percentage of the injected dose, are plotted as a function of time after injection. Panel A corresponds to cells coated with Fog-1 G1 (subjects 2 and 4) and panel B to cells coated with Fog-1 G1Δnab (subjects 1, 3, and 5). ^{99m}Tc plasma counts are not informative (see "Recovery and survival of antibody-coated red cells in subjects 1 through 5").

Figure 5. Comparison of whole blood and soft tissue counts following injection of uncoated and Fog-1 G1Δnab-coated RBCs. Subject 6 was injected with 15 MBq uncoated ^{99m}Tc-RBCs followed, after 20 minutes, by 49 MBq Fog-1 G1Δnab-coated ^{99m}Tc-RBCs and was monitored by whole blood counting and gamma camera imaging of the soft tissue of the thighs. Blood sample counts were normalized by the ratio of mean image counts to the mean blood counts for the 0- to 20-minute period and overlaid on the time-activity curve of the soft tissue region.

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 minimize FcγR binding and complement activation would have reduced capacity to trigger antibody-mediated blood cell destruction in vivo. Since antibodies for antenatal therapy must have an Fc portion to maintain the normal half-life and placental transport,[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. Interspecies differences in IgG subclass binding by FcγR[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.[27] Unexpectedly, 2 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,[27] but the volumes of coated cells were nearly 20 times lower than injected here. Since the 2 febrile subjects had both wild-type 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 wild-type 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 minutes, 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 suggests total cell clearance and destruction. The degree of clearance seen was greater than in the previous study of Fog-1,[27] despite comparable levels of coating in some subjects and similar levels of elution from the D antigen (31%/h in vitro, data not shown). The ability of our Fog-G1 to promote faster and more complete hemolysis 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.[28,29] The different killing efficiencies also may be related to cell culture or purification conditions.

In comparison to wild-type antibody, clearance of cells coated with G1Δnab antibodies was significantly slower, incomplete, and transient, with blood cell counts rising again after 3 to 4 hours (Figure 1). Moreover, the level of ^{51}Cr in the plasma remained low in the 3 subjects receiving the ^{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. These plasma data contrast that seen for the G1-coated cells in subject 4, although the 2 populations had similar initial clearance rates. The organ scanning data also support a different pattern of behavior for G1Δnab-coated cells. While cells coated with either antibody accumulated in the spleen, the liver showed considerable uptake of cells carrying the wild-type 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 (6800 molecules/cell), since hepatic uptake has not been detected with fewer than 9000 IgG anti-D molecules per cell.[12,13] These differences are not due to G1Δnab being lost from the cells faster than wild-type 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 FcγR but not triggering signaling and phagocytosis. Binding to FcγRII may be the explanation since, although the mutations have abrogated binding to FcγRI and III,[7,8] some interaction with FcγRIIa and the inhibitory receptor FcγRIIb remains.[16] The clearance curves from 5 to 300 minutes could be described in terms of a rate constant for the clearance of a proportion of cells and a plateau value that 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.[13] Our observations also are consistent with the biphasic model of sequestration followed by phagocytosis proposed for red blood cells (RBCs) coated with other anti-D antibodies (George Chapman, Bio Products Laboratory, e-mail communication, 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 also may be a factor, since vacant receptors are not being regenerated following phagocytosis. The net increase in circulating labeled 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 3 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 underrepresented 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 4 subjects, for cells coated with either antibody, fewer 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-minute sample.[27] 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,[20] 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 2 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 wild-type and mutated antibodies. Although the degree of coating achieved varied considerably between subjects, the variation in coating between the 2 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 R_1R_2, and is thus heterozygous for D.[30]

The lack of correlation between survival of Fog-1 G1-sensitized 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.[31,32] 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.[33] 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 normalization of erythrocyte or platelet survival may not be required. CL studies have shown that HDN sera giving less than 30% of maximum activation are rarely associated with clinical disease,[9] so reduction of the degree of hemolysis 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 bialleleic Human Platelet Antigen-1 system, which accounts for most cases of feto-maternal allo-immune thrombocytopenia, are the result of a single amino acid substitution[34] and therefore may be easier to block by a single monoclonal antibody. We have generated an Fc-modified antibody to HPA-1a,[34] 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.[35] Human studies are planned to examine platelet survival and thus its therapeutic potential.

Acknowledgments

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