RAPID COMMUNICATION

Human Rh D Monoclonal Antibodies (BRAD-3 and BRAD-5) Cause Accelerated Clearance of Rh D+ Red Blood Cells and Suppression of Rh D Immunization in Rh D− Volunteers

By Belinda M. Kumpel, M. Jean Goodrick, Derwood H. Pamphilon, Ian D. Fraser, Geoffrey D. Poole, Colin Morse, Graham R. Standen, George E. Chapman, Duncan P. Thomas, and David J. Anstee

The use of prophylactic anti-D to prevent Rh D immunization in Rh D− women and subsequent hemolytic disease in Rh D+ infants is widespread, but has led to shortages of the anti-D Ig. With the aim of substituting monoclonal anti-D for Rh D prophylaxis, we have compared the abilities of monoclonal and polyclonal anti-D to clear Rh D+ red blood cells (RBCs) infused into Rh D− male volunteers and to suppress Rh D immunization. Two human monoclonal antibodies (MoAbs), BRAD-3 (IgG3) and BRAD-5 (IgG1), produced from stable Epstein-Barr virus–transformed B-lymphoblastoid cell lines, were selected because of their proven in vitro activity in promoting RBC lysis in antibody-dependent cell-mediated cytotoxicity assays. RBC clearance was assessed by intravenous injection of 3 mL of 56chromium-labeled D+ RBCs into 27 volunteers 48 hours after intramuscular injection of monoclonal or polyclonal anti-D. Further 3-mL injections of unlabeled D+ cells were administered at 6 and 9 months to induce immunization. Blood samples were taken throughout the 12-month period of study for the serologic detection of anti-D. The mean half-life (t½) of RBCs in 7 recipients of 300 µg BRAD-5 (5.9 hours) was similar to that in 8 recipients of 500 IU polyclonal anti-D (5.0 hours), whereas D+ cells were cleared more slowly in some of the 8 subjects injected with 300 µg BRAD-3 (mean t½, 12.7 hours) and in 1 individual administered 100 µg BRAD-3 (t½, 41.0 hours). The rate of RBC clearance in both groups administered 300 µg monoclonal anti-D correlated with the amount of antibody bound per cell, determined by flow cytometry. There was no evidence of primary immunization having occurred in any subject after 6 months of follow-up. Five of 24 subjects produced anti-D after one or two further injections of RBCs, confirming that they were responders who had been protected by the monoclonal or polyclonal anti-D administered initially. Four of these responders were recipients of monoclonal anti-D (3 BRAD-3, 1 BRAD-5). One individual who received BRAD-5 produced accelerated clearance of D+ RBCs at the third unprotected RBC challenge but did not seroconvert. This study shows that the human MoAbs BRAD-3 and BRAD-5 can prevent Rh D immunization, and indicates that they may be suitable replacements for the polyclonal anti-D presently used in prophylaxis of Rh D hemolytic disease of the newborn. This report is one of the first to show the therapeutic utility of blood group MoAbs.

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SINCE THE INTRODUCTION of hybridoma technology nearly 2 decades ago,1 many hundreds of monoclonal antibodies (MoAb) have been made. Although some have been developed specifically for therapeutic applications, few have yet shown acceptable clinical utility without undesirable side effects.2 Here we present the results of a clinical trial of two human MoAbs specific for the Rh D antigen on red blood cells (RBCs).

The introduction of immunoprophylaxis to prevent Rh D hemolytic disease of the newborn by postpartum administration of human anti-D Ig to Rh D− women has reduced the incidence of maternal Rh D immunization to 2% or less.3 Antenatal prophylaxis further reduces cases of maternal sensitization and fetal hemolytic disease,4 but this has put additional demands on the supply of anti-D Ig. Human MoAbs derived from immortalized cell lines would enable a potentially unlimited supply of specific standardized anti-D to be used, without the need for repeated immunization and plasmapheresis of volunteer donors.

The mechanism whereby passively administered IgG anti-D mediates suppression of Rh D immunization is as yet unclear. It is known that anti-D–coated RBCs are removed from the circulation by mononuclear phagocytes, principally in the spleen,5 following interaction between RBC-bound IgG and IgG Fc receptors (FcyR) on the effector cells. Immunosuppression may be effected by clearance of the sensitized RBCs within 5 to 8 days6 followed either by the rapid destruction of the D antigen before it is able to elicit an immune response, or by specific antibody-mediated suppression7 of primed B cells,8 or by a combination of these events. The first test of the efficacy of a monoclonal anti-D in vivo is to determine whether it is capable of clearing D+ RBCs from the circulation. Secondly, it must be shown that the monoclonal anti-D can prevent Rh D immunization.

For this study, two human MoAbs, BRAD-3 and BRAD-5, were selected on the basis of their IgG subclass, high affinity for the D antigen, good functional interactions with FcyR-bearing effector cells in vitro, and the stability of the cell lines in culture. One monoclonal anti-D (BRAD-3) has previously been shown to be effective in clearing sensitized autologous RBCs in D+ volunteers.9 In this study, the clearance of D+ RBCs was assessed in Rh D− volunteers 2 days after intramuscular (IM) injection of BRAD-3, BRAD-5, or polyclonal anti-D. Each subject was then monitored

From the International Blood Group Reference Laboratory: the National Blood Service, Bristol; the Bristol Royal Infirmary, Bristol; and the Bio Products Laboratory, Elstree, Herts, UK.

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Address reprint requests to Belinda M. Kumpel, PhD, International Blood Group Reference Laboratory, Southmead Rd, Bristol BS10 5ND, UK.

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for evidence of seroconversion after this and subsequent injections of Rh D\(^+\) RBCs.

**MATERIALS AND METHODS**

The study was approved by the local Medical Research and Ethics committees, and written informed consent was obtained from all volunteers.

**Subjects.** We recruited 27 healthy male Rh D\(^+\) volunteer plasma donors (age range 25 to 52 years, mean 37.9 years) who satisfied the UK criteria for blood donation,\(^{11}\) including a specific enquiry concerning prior transfusion. They were extensively phenotyped for RBC antigens and HLA class I and class II antigens. In addition, monocyte function in each subject was assessed immediately before the clearance study using an in vitro phagocytosis assay as previously described.\(^2\) Briefly, \(O\) Rh D\(^+\) RBCs (phenotype CcDeDe from a single donor) were sensitized with serial dilutions of a standard polyonal anti-D and incubated with adherent monocytes for 2 hours at 37°C: the percentage of monocytes with one or more adherent or phagocytosed RBCs was determined microscopically.

**Anti-D.** The human MoAbs BRAD-3 (IgG3 \(\kappa\), also known as IA3-3) and BRAD-5 (IgG1 \(\kappa\), also known as IA11), with specificity for the Rh D antigen on RBCs, were produced from Epstein-Barr virus (EBV)–transformed B-lymphoblastoid cell lines from two donors by methods previously described.\(^3,4\) The affinity constants of the binding of BRAD-3 and BRAD-5, and the number of sites bound, were determined by radioimmunossay (BRAD-3)\(^5\) and enzyme-linked immunosorbent assay (ELISA) (BRAD-3, BRAD-5) (Kumpel BM, Chapman GE, Gardner B, unpublished, April 1994). For therapeutic use, the cell lines were grown in hollow fiber bioreactors (Acusyst Jr; Cellex Corp, Coon Rapids, MN) in serum-free medium.

**Human polyclonal anti-D.** Human polyclonal anti-D was prepared from pooled immune plasma using cold ethanol fractionation (Bio Products Laboratory, Elstree, UK). This comprised IgG1 (88.0%), IgG2 (1.2%), IgG3 (10.4%), and IgG4 (0.3%) (Kumpel BM, unpublished).

**In vitro assays of anti-D function.** The monocyte-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) assay was performed by incubating peripheral blood mononuclear cells (PBMC) with \(^{51}Cr\)-labeled (sodium chromate; Amersham, Bucks, UK) anti-D coated RBCs at a ratio of 10:1 (PBMC:RBCs) for 4 hours at 37°C.\(^12\) For the lymphocyte-mediated ADCC assay, PBMC were depleted of monocytes by incubation in plastic flasks for 90 minutes at 37°C, and the nonadherent lymphocytes incubated with \(^{51}Cr\)-labeled papain-treated RBCs and anti-D for 16 hours at 37°C at a lymphocyte-to-RBC ratio of 15:1.\(^13,14\) In both assays each test sample included a control without effector cells (spontaneous lysis) and maximum lysis was determined by the addition of 1% (vol/vol) Triton X-100 (Sigma, Poole, UK) to the RBCs. After incubation, radioactivity was determined in aliquots of supernatants, and the percent specific lysis (%SL) was calculated:

\[
%\text{SL} = \frac{\text{cpm(test)} - \text{cpm(control)}}{\text{cpm(max)} - \text{cpm(control)}} \times 100
\]

The number of molecules of IgG anti-D bound to sensitized RBCs was determined by ELISA quantification of IgG released from solubilized RBCs.\(^15\) The IgG concentration of monoclonal anti-D was determined by ELISA.\(^15\) The quantification of anti-D in polyclonal anti-D Ig was performed by AutoAnalyser (Technicon Instruments Corp, Tarrytown, NY); 500 IU (the standard dose) contained approximately 100 \(\mu\)g anti-D.\(^20\)

**In vivo RBC clearance mediated by anti-D.** Rh D\(^+\) RBCs were obtained from volunteer donors and cryopreserved in aliquots in liquid nitrogen until the donors had completed the recommended accreditation schedule.\(^11\) For each subject approximately 3 mL RBCs matched for \(S, s, K, Fy,\) and \(Jk\) antigens were reconstituted and then labeled with \(^{51}Cr\) using standard techniques.\(^21\) Group O cells of the following probable genotypes were used: CDcDee, cDE/cDee, cDe/cDe, CDe/cDe, and cDe/cDe.

Each volunteer received either 100 \(\mu\)g \((n = 1), 200 \(\mu\)g \((n = 2), or 300 \(\mu\)g \((n = 8)\) BRAD-3; 300 \(\mu\)g \((n = 7)\) or 600 \(\mu\)g \((n = 1)\) BRAD-5; or 500 IU (100 \(\mu\)g) polyclonal anti-D \((n = 8)\) by intramuscular injection. Forty-eight hours later a blood sample was taken for quantification of plasma anti-D levels by AutoAnalyser to assess the percentage of the dose which was in the plasma. Labeled RBCs were then injected intravenously (see Fig 1). Blood samples were taken into EDTA at 3 minutes, 1, 3, 5, 24, 48, and 72 hours and the proportion of surviving D positive RBCs calculated from the level of residual radioactivity in lysed whole blood, taking the 3-minute sample as 100%. On each occasion a sample was separated immediately after collection to assess free plasma radioactivity.

The number of molecules of anti-D bound to the D\(^+\) RBCs 3 hours after infusion was assessed using a flow cytometric technique, after incubation of the blood sample for 1 hour at 37°C; after labeling with fluorescein isothiocyanate (FITC) anti-IgG the fluorescence of the test sample was compared with that of standards, and cell-bound IgG in duplicate standards was quantified by ELISA.\(^16\)

**SeroLogic detection of anti-D.** Blood samples taken approximately every 2 weeks were examined for the presence of anti-D by testing with Rh D\(^+\) (phenotype O cDe) and Rh D\(^-\) (phenotype O cde) bromelain-treated RBCs by standard serologic methods\(^3\) and by AutoAnalyser. Antibodies detected in the screening tests were identified using a panel of bromelain-treated RBCs. The presence or absence of anti-D in each sample was further confirmed and quantified by AutoAnalyser using the British Standard /35/515. Determination of the IgG subclass of anti-D was performed by agglutination of sensitized RBCs with monoclonal anti-IgG subclass antibodies.

**Detection of antibody responses to anti-D.** Anti-allotype antibodies were determined by incubation of equal volumes of plasma or serum (serially diluted 1/1 to 1/8) with 1% washed cells (phenotype O cDe, native or presensitized with BRAD-3, BRAD-5, or polyclonal anti-D) at 37°C for 30 minutes followed by centrifugation and detection of agglutination. Anti-idiotypic responses were determined by incubation of equal volumes (50 \(\mu\)L) of plasma or serum (serially diluted 1/1 to 1/8) with anti-D (BRAD-3, BRAD-5, polyclonal IgG anti-D, or MAD-2 [monoclonal IgM anti-D] at twice the minimum hemagglutinating dose, ie, 2.2 ng/mL) for 30 minutes at 22°C, followed by addition of 50 \(\mu\)L of 2% papainized phenotype O cDe cells, centrifugation, and detection of agglutination.\(^22\) A positive control comprised MAD-2 incubated with a monoclonal anti-idiotypic antibody, 9G4, which binds to MAD-2 and blocks the agglutination of RBCs in this assay.\(^23\) Negative controls used anti-D incubated with AB serum (no inhibition of agglutination) or AB serum used alone (no agglutination).

**Unprotected RBC challenges.** Further 3 mL injections of D\(^+\) RBCs were administered to the subjects at approximately 6 and 9 months after the initial RBC clearance study; at this stage no anti-D protection was given (see Fig 1). Blood samples were taken and screened for anti-D formation as described above. If no anti-D was detected after a total of 12 months of study, a third and final unprotected RBC injection was administered using \(^{51}Cr\)-labeled RBCs.
SUPPRESSION OF Rh D IMMUNIZATION BY MONOCLONAL ANTI-D

Fig 1. Schematic plan of study.

Samples were then taken at 3 minutes, 24 hours, 3 or 4 days, then weekly for up to 5 weeks to allow extended measurement of RBC survival. Cell survival was expressed as the tCr. tCr survival and the mean cell life span (MCL) after correction for isotope elution from RBCs (approximately 1% per day) and decay during the period of study.

Calculations. The percentage of the dose of anti-D which was in the plasma 48 hours after injection was calculated using the formula:

$$\frac{100 \times \text{Plasma Volume (mL)} \times \text{Anti-D Level (µg/mL)}}{\text{Injected Dose of Anti-D (µg)}}$$

where the plasma volume was taken as 40 mL/kg.4

The time taken to clear 50% of the injected RBCs (t50%) was determined by plotting percentage of residual radioactivity against time on log-linear plots, logarithmic interpolation of the plots as follows:

$$t_{50\%} = t_1 + \frac{(t_2 - t_1) \times (\log (r_1) - 1.699)}{(\log (r_1) - \log (r_2))}$$

where r1 and t1 were the percent remaining radioactivity and the time for the last sample where the percent residual radioactivity was greater than 50%, and r2 and t2 were the corresponding values for the first sample where the percent residual radioactivity was less than 50%. Clearance rates were defined as 0.693/t50%, assuming that the RBC clearance is a first-order process, which is not strictly true (because of the time taken for the antibody to bind to the RBCs), but is valid for the purposes of comparison.

Statistical comparisons of t50% were made using the Mann-Whitney U test.

RESULTS

In vitro functional activity of anti-D. In the monocyte-mediated ADCC assay, BRAD-3 promoted greater RBC lysis than polyclonal anti-D at equivalent levels of RBC sensitization; BRAD-5 was less active (Fig 2A). In contrast, in the lymphocyte-mediated ADCC, BRAD-5 promoted more lysis than BRAD-3 (Fig 2B), with polyclonal anti-D the most active.

The affinity of both MoAbs for D⁺ (phenotype CcDeEe) RBCs was approximately $3 \times 10^9$ mol/L.

Antibody recovery. All anti-D preparations were well tolerated in all volunteers with no side effects. The mean percentage of the dose of 300 µg monoclonal anti-D which was in the plasma 48 hours after IM injection was less than that of polyclonal anti-D, although there was a wide variation between individuals who received polyclonal anti-D (Table 1). Plasma uptake of anti-D was very low in subject 1, who was administered 100 µg BRAD-3 (Table 1). The subjects had no detectable antibody responses (anti-allotypic or anti-
duced accelerated RBC clearance. The mean tsos of D+ cells (idiotypic) to the administered anti-D 18 to 22 weeks after injection (data not shown).

**RBC clearance.** All three antibody preparations produced accelerated RBC clearance. The mean percentages of D+ cells in subjects administered 300 μg BRAD-3 (12.7 hours) was significantly greater than in recipients of polyclonal anti-D (5.0 hours) \( (P = .014) \) or BRAD-5 (6.0 hours) \( (P = .037) \). There was no significant difference in the half-lives or clearance rates of RBCs coated with BRAD-5 or polyclonal anti-D \( (P = .191) \). In all subjects, plasma levels of radioactivity after 24 hours were less than 1% of that of the original (3-minute) sample of whole blood, and averaged 0.8% at 24 and 48 hours.

There was greatest variation in the half-lives and rates of RBC clearance in the group administered 300 μg BRAD-3 (Table 1), and at 24 hours the percentage of D+ cells remaining ranged between 1.1% and 58.0% \( (P = .014) \) or BRAD-5 (6.0 hours) \( (P = .037) \). There was no significant difference in the half-lives or clearance rates of RBCs coated with BRAD-5 or polyclonal anti-D \( (P = .191) \). In all subjects, plasma levels of radioactivity after 24 hours were less than 1% of that of the original (3-minute) sample of whole blood, and averaged 0.8% at 24 and 48 hours.

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The means ± 1 SD are calculated for the groups administered 300 μg BRAD-3, 300 μg BRAD-5, and 500 IU polyclonal anti-D. Abbreviation: ND, not determined.

* Dose of anti-D injected is given in μg for BRAD-3 and BRAD-5 and IU for polyclonal anti-D.

† Plasma anti-D concentrations are expressed in ng/mL for BRAD-3 and BRAD-5 and IU/mL for polyclonal anti-D.
SUPPRESSION OF Rh D IMMUNIZATION BY MONOCLONAL ANTI-D

A % Radioactivity

B % Radioactivity

C % Radioactivity

Fig 3. In vivo clearance of \(^{51}\) chromium-labeled D\(^+\) RBCs in 24 D\(^+\) subjects after IM injection of anti-D. (A) Subjects 4 (Δ), 5 (Δ), 6 (V), 7 (V), 8 (O), 9 (O), 10 (O), and 11 (O) administered 300 µg BRAD-3. (B) Subjects 12 (Δ), 13 (Δ), 14 (V), 15 (V), 16 (O), 17 (O), and 18 (O) administered 300 µg BRAD-5 and subject 19 (O) administered 600 µg BRAD-5. (C) Subjects 20 (Δ), 21 (Δ), 22 (V), 23 (V), 24 (O), 25 (O), 26 (O), and 27 (O) administered 500 IU polyclonal anti-D.

Fig 4. Relationship between the amount of IgG monoclonal anti-D bound to RBCs and the rate of RBC clearance. Subjects 4 through 19 received 300 µg or 600 µg BRAD-3 (Δ) or BRAD-5 (O).
munized. The administered anti-D was present for up to 3 to 6 weeks (BRAD-3), 8 to 16 weeks (BRAD-5), and 6 to 10 weeks (polyclonal anti-D) after injection, compatible with the half-lives of these antibodies.25 With one exception (subject 6), anti-D was then no longer detected in the volunteers’ serum. Subject 6 exhibited a “collapse curve” of RBC clearance (Fig 3A), indicating rapid removal of D+ RBCs after 2 to 3 days, with anti-D detected in all serum samples both before and after an RBC challenge. Although apparently eligible for the study, on reviewing his past history he admitted to having received an emergency blood transfusion over 20 years earlier, raising the possibility that he had been presensitized although anti-D was not detectable in prestudy serum samples. For this reason he was excluded from further analysis.

None of the remaining 26 subjects produced detectable anti-D by 6 months after the first RBC injection. However, 5 of these volunteers developed anti-D after either one or two subsequent injections of unprotected D+ cells (Table 2). Anti-D levels were highest 8 to 12 weeks after the second challenge, and antibody was of the IgG1 subclass in all cases. Anti-C and/or anti-E was also produced by subjects 1, 4, and 15. In subjects 4 and 15, who produced anti-D 10 to 14 weeks after the first challenge, levels of anti-D increased 20 and 10 times, respectively, after the second challenge. After a third challenge of D+ RBCs, anti-D levels increased to 200 to 850 IU/mL after 2 to 5 weeks, but these levels were not maintained; the anti-D decreased to half these maximum values by 8 to 12 weeks. After a fourth challenge (boost) of RBCs, a similar pattern of antibody response was obtained in the subjects, with the exception of subject 1 whose anti-D remained at about 500 IU/mL for 5 months of study.

Two subjects (24 and 11) withdrew at 16 and 14 weeks after the first and second challenge, respectively. At that time neither subject had seroconverted.

**RBC survival of a third unprotected challenge of D positive RBCs.** The 19 subjects who had no detectable anti-D 12 to 20 weeks after the second challenge were given a final injection of 31Cr-labeled RBCs to determine if there was any evidence for accelerated RBC clearance in the absence of detectable anti-D. In 15 of these subjects, survival of the D+ RBCs was normal (mean tso% 28 days, range 24 to 31 days, MCL 105 days) compared with fresh cells26 (mean tso% 31 days, range 25 to 37 days, MCL 115 days) (an example is shown in Fig 5). In three subjects (10, 14, and 19) there was an initial cell loss of greater than 20% at 24 hours, but the remaining cells survived normally (an example is shown in Fig 5); none of these subjects showed evidence of seroconversion. One subject (18) demonstrated accelerated RBC clearance (tso% 6 days, MCL 10 days) similar to that of subject 7 (tso% 3 days, MCL 4 days) who had seroconverted (with a plasma anti-D level of 2.2 IU/mL at the time of the clearance study) and who had agreed to act as a positive control for this part of the study (Fig 5). Subject 18 had not made anti-D when last tested 4 weeks after the final (third) challenge.

**DISCUSSION**

Both monoclonal anti-Ds caused accelerated clearance of D+ RBCs from the circulation of D- subjects, although there was some variation in the rate of clearance between individual volunteers. The rate of clearance may have been affected by several factors, including the level of IgG bound to the RBCs, the affinity of the monoclonal anti-D for Fc receptors on macrophages in the spleen, the HLA haplotype and

![Image](https://www.bloodjournal.org/content/blood/121/15/1706.full/08a9fd4e-60e3-4f0b-8d7f-4e3835c4b2c3)

**Table 2. Serologic Detection of Anti-D in D- Subjects Responding to Immunization With D+ RBCs**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Anti-D</th>
<th>Dose</th>
<th>Phenotype of Immunizing RBCs</th>
<th>After Initial-Unprotected Challenge</th>
<th>After First-Unprotected Challenge</th>
<th>After Second-Unprotected Challenge</th>
<th>Maximum Anti-D Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BRAD-3</td>
<td>100 μg</td>
<td>cDE (R_R)</td>
<td>2 (0.16)</td>
<td>14 (0.15)</td>
<td>2 (0.30)</td>
<td>37.0 (10)</td>
</tr>
<tr>
<td>4</td>
<td>BRAD-3</td>
<td>300 μg</td>
<td>CoDe (R_R)</td>
<td>10 (0.02)</td>
<td>14 (0.15)</td>
<td>10 (0.02)</td>
<td>56.0 (8)</td>
</tr>
<tr>
<td>7</td>
<td>BRAD-3</td>
<td>300 μg</td>
<td>CDe (R_R)</td>
<td>14 (0.15)</td>
<td>2 (0.30)</td>
<td>2 (0.16)</td>
<td>4.2 (10)</td>
</tr>
<tr>
<td>15</td>
<td>BRAD-5</td>
<td>300 μg</td>
<td>CDEe (R_R)</td>
<td>4 (0.02)</td>
<td>10 (0.02)</td>
<td>10 (0.02)</td>
<td>7.3 (12)</td>
</tr>
<tr>
<td>23</td>
<td>Polyclonal</td>
<td>500 IU</td>
<td>CDe (R_R)</td>
<td>14 (0.15)</td>
<td>2 (0.30)</td>
<td>2 (0.16)</td>
<td>4.2 (10)</td>
</tr>
</tbody>
</table>

![Figure 5](https://www.bloodjournal.org/content/blood/121/15/1706.full/08a9fd4e-60e3-4f0b-8d7f-4e3835c4b2c3)

**Fig 5.** Survival of third unprotected D+ RBC challenge in representative subjects. Dotted lines indicate the upper and lower limits for survival of normal fresh RBCs. Subject 13 (●) showed normal RBC survival, subject 19 (▲) had normal RBC survival after an initial cell loss, subject 18 (●) showed reduced RBC survival without detectable anti-D, and subject 7 (○), a responder who had produced anti-D, had reduced RBC survival.
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splenic function of the recipients, the use of RBCs from different donors, and the effects of freezing and thawing the RBCs on their subsequent survival.

In previous studies it has been shown that the rate of clearance of RBCs sensitized in vitro with polyclonal or monoclonal anti-D is related to the amount of IgG on the RBCs. We found the clearance rate correlated well with the estimated sensitization level of the D+ cells, for both BRAD-3 and BRAD-5. The numbers of molecules of anti-D on the RBCs, as determined by flow cytometry, were close to the theoretical levels calculated from the affinity of the antibodies and the plasma anti-D levels achieved in the subjects. It should be noted that the estimates of RBC bound anti-D were determined after incubation of the blood in vitro, and that these levels may not have been attained in vivo as the cells may have been cleared once a critical number of IgG molecules per cell had been reached.

In a study in chimpanzees, an IgG1 monoclonal anti-D was found to clear human D+ RBCs slightly more rapidly than an IgG3 monoclonal anti-D when sensitization levels of the RBCs were about 6,000 and 4,000 molecules IgG/cell, respectively. RBCs were injected before the anti-D in these two animals.

Anti-D–coated RBCs are removed from the circulation by splenic macrophages, which express three classes of IgG Fc receptor: FcγRI, FcγRII, and FcγRIII. FcγRI, with high affinity for monomeric IgG, is thought to be blocked by splenic macrophage, which express three classes of FcγRIII on splenic macrophages is involved, at least initially, in binding IgG-opsonized cells, after which monomeric IgG may be displaced from FcγRI to enable this receptor to engage cell bound IgG, resulting in activation of effector mechanisms. FcγRIII was shown to be involved in clearance of IgG-coated RBCs in chimpanzees because RBC survival was dramatically increased by prior administration of anti-FcγRIII antibodies. The in vivo clearance of RBCs coated with monoclonal and polyclonal anti-D may depend to a large extent on the ability of the anti-D to interact with both FcγRIII and FcγRI on macrophages in the spleen. Because of the difficulty of obtaining splenic macrophages, no direct assessment of these interactions has been made, but the following indirect evidence supports the involvement of both receptors in the clearance of monoclonal anti-D-coated RBCs.

PB K/natural killer (NK) cells express FcγRIII and have been used as models of FcγRIII-mediated interactions. Anti-D MoAbs vary in their abilities to mediate lysis of RBCs by K cells in ADCC assays and to promote adherence of RBCs to NK cells, which suggests that not all antibodies are equally effective at interacting with FcγRIII and therefore of mediating RBC clearance. We found that whereas BRAD-5 and polyclonal anti-D were efficient at FcγRIII-mediated interactions (Fig 2B, ref 14), BRAD-3 was less active in these assays. In FcγRI-mediated monocyte ADCC, however, BRAD-3 and polyclonal anti-D were more lytic than BRAD-5 (Fig 2A). Assuming that the in vitro functional activity of anti-D reflects the efficiency of interactions with splenic FcγRIII and FcγRI in vivo, then it might be expected that polyclonal anti-D would be slightly better than either BRAD-3 or BRAD-5 alone because it interacts well with both receptors (Fig 2). We found this generally to be the case. One of the criteria for selection of monoclonal anti-D for the current trial was the fact that BRAD-5 and BRAD-3 showed good activity in FcγRIII- and FcγRI-mediated activity, respectively. FOG-1, an IgG1 monoclonal anti-D which has little activity in either FcγRII- or FcγRI-mediated ADCC assays, was not very effective at clearing autologous presensitized RBCs in vivo.

We found that of the six subjects who were of the HLA haplotype B8 DR3, two had both low monocyte phagocytosis in vitro and low RBC clearance in vivo. Up to 50% of individuals with this haplotype have reduced clearance of anti-D–coated RBCs in vivo. Phagocytosis of anti-D–coated RBCs is mediated by FcγRI on monocytes, suggesting that this receptor may be used in RBC clearance in vivo. We used this in vitro assay as a measure of splenic function, instead of the normal procedure that involves in vivo measurement of clearance of anti-D–sensitized Cr-labeled RBCs. The concentration of anti-D in the extravascular space (which is 1.3 times the plasma volume) would be about 50% of that of the plasma at 48 hours after IM injection. At this time, the percentage of the dose of the MoAbs that were in the plasma was less than that of the polyclonal anti-D. This may reflect differences in formulation or glycosylation of the IgG. The anti-D was administered 2 days before the infusion of RBCs to optimize the coating of RBCs in vivo and thus enable estimation of RBC clearance more reliably. A previous study had shown that peak plasma anti-D levels were obtained 2 to 4 days after IM injection of BRAD-3, BRAD-5, or polyclonal anti-D. In 2 of the 5 individuals who responded to the antigenic challenge (Table 2), anti-D first became detectable either 10 or 14 weeks after the first unprotected challenge, a feature compatible with the time course of a primary immune response. Three individuals produced anti-D rapidly after a second unprotected challenge (2 to 4 weeks), indicating a secondary immune response in these individuals who had presumably produced a primary immune response to the first challenge which had not been detected. Thus, in these subjects the evidence shows that administered monoclonal anti-D (3 individuals received BRAD-3 and 1 BRAD-5) or polyclonal anti-D (1 recipient) had prevented primary Rh D immunization to the first injection of Rh D+ RBCs. Overall, the responder rate in this study (21%: 5 of 24 subjects produced a primary anti-D response [excluding subjects 11 and 24 who withdrew, and subject 6 who appeared to be immunized by a previous transfusion]) was lower than would have been expected from previous data. It is possible that the passive injection of anti-D before injection of RBCs in some way induced a certain degree of tolerance. It has earlier been found that the response rate to repeated RBC injections was 36% in those subjects who had previously been administered 15 to 75 μg anti-D compared with 57% in those who had only received RBCs.
that in the responders, anti-D levels increased to over 200 IU/mL after a further challenge but then tended to decrease relatively rapidly, unlike responses in individuals immunized by pregnancy or transfusion when anti-D levels after several booster immunizations are maintained for some months.

Rapid clearance of a second or third injection of D" cells in an individual without detectable anti-D, as in subject 18, has been observed before. In those studies most of the individuals required one or more further D" stimuli before anti-D was detected, and in a few cases anti-D was never detected even though survival of D" cells was reduced. It is likely that a state of sensitization may be attained, when an immune response has been formed without production of sufficient antibody to enable its serologic detection.

In this study all the responders were protected by the initial injection of anti-D, whether administered 100 µg BRAD-3, 300 µg BRAD-3, 300 µg BRAD-5, or 500 IU polyclonal anti-D. The initial rate of RBC clearance did not appear to influence the effectiveness of protection; for instance subject 1 had low plasma uptake of 100 µg BRAD-3 and slow RBC clearance, but was still protected against Rh D sensitization.

Clinical use of monoclonal anti-D in place of the currently available anti-D Ig would eliminate both the possibility of transmissible disease and the need to recruit and immunize volunteer donors in the future. This is the first report of a clinical trial showing the safety and efficacy of human monoclonal anti-D in clearing Rh D" RBCs and in preventing Rh D immunization. If further trials of antenatal and postnatal prophylaxis in Rh D" women are successful, BRAD-3 and BRAD-5 may prove suitable for the prevention of Rh D hemolytic disease of the newborn.

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