A single recombinant anti-RhD IgG prevents Rhesus D immunization: association of RhD positive red blood cell clearance rate with polymorphisms in the FcγRIIA and IIIA genes.

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Abstract

A single recombinant IgG1 anti-RhD antibody (MonoRho®) was compared with a currently used polyclonal anti-RhD product (Rhophylac®) in a Phase I study for safety, efficacy of Rhesus D positive red blood cell (RBC) clearance and prevention of RhD immunization in RhD negative men challenged with 15mL of RhD positive RBC. Both the polyclonal product and recombinant anti-RhD effectively cleared RhD positive RBC after intravenous and intramuscular injection. The recombinant anti-RhD demonstrated a slower clearance rate compared to the polyclonal anti-RhD. There was no dose response and considerable variation amongst subjects who received the same dose of recombinant anti-RhD. Interestingly, RhD positive RBC clearance rates were strongly associated with FcγRIIA, IIIA but not IIIB polymorphisms. Subjects homozygous for FcγRIIA-131H or FcγRIIIA-158V allotypes showed a faster clearance rate compared to both the heterozygote and the corresponding alternative homozygote allotypes. A similar but less marked trend was seen for the polyclonal anti-RhD. Despite the variation in clearance rates there was no evidence of anti-RhD alloantibodies in any of the subjects at +6 months after the RBC challenge.

Introduction

Rhesus prophylaxis to prevent haemolytic disease of the foetus and newborn has been successfully assured for many years by polyclonal anti-RhD products. However, new viral epidemics e.g. SARS, West Nile virus in transfusion products and concerns about the transmission of variant Creutzfeld-Jacob disease illustrate the potential vulnerability of the hyperimmune plasma donor programmes that are required for production of current RhD immune globulin products. To avoid the necessity for hyperimmune plasma a recombinant anti-RhD antibody has been developed (MonoRho®) and produced in a stable Chinese hamster ovary (CHO) cell line, MDJ8s. Comparison of MonoRho® with a polyclonal anti-RhD product (Rhophylac®) for specificity and Fc function gave similar results in vitro. The next major question concerns the clinical efficacy and safety of a single recombinant antibody compared to a polyclonal antibody preparation.

A Phase I clinical trial was designed to assess MonoRho® and Rhophylac® for their comparative safety, efficacy of RhD positive RBC clearance and prevention of RhD immunization in RhD negative men challenged with 15mL of RhD positive (R1r) RBC followed 24h later by anti-RhD immune globulin. A large 15mL challenge of RhD positive RBC was chosen because it represents a worst case scenario and because a single standard dose of polyclonal anti-RhD (approximately 300 µg) contains sufficient anti-RhD to suppress the immune response to 15mL of RhD positive RBC. A hemorrhage of 5mL or greater occurs in only 0.6% of pregnancies. The parameters measured included concentration of RhD positive RBC in volunteers over time, elimination rate, saturation of RhD binding sites with anti-RhD, serum concentration of anti-RhD, genotyping for the FcγRIIa, FcγRIIIa, and FcγRIIIb polymorphisms, presence of anti-RhD alloantibodies and anti-MonoRho® at 3 and 6 months post challenge and routine clinical laboratory assessments.

As previously reported there is a fundamental difference between the methods for determination of anti-RhD content in human plasma derived products and recombinant or other monoclonal anti-RhD antibodies like MonoRho®. The former are based on the European Pharmacopoeia “AutoAnalyzer” assay which measures agglutinating activity while the latter are based on determination of purified protein by biochemical means. Our unpublished data indicate that MonoRho® is underestimated by a factor of 4 to 5 in the “AutoAnalyzer” assay. Thus an essential part of this study was evaluation of an escalating
dose range of MonoRho® in vivo in order to estimate which dose of MonoRho® would be comparable to the standard dose of plasma derived anti-RhD.

This Phase I study showed that a single recombinant human IgG1 anti-RhD antibody prevented primary immunization by RhD positive RBC.

Materials and methods

Volunteers

Healthy RhD negative male volunteers (18 - 45 yrs, n=46) were enrolled after giving voluntary written informed consent. Subjects were excluded if they had blood group alloantibodies, a history of anaphylactic or other severe systemic reaction to immune globulins, were IgA deficient, had been administered anti-RhD previously, were previously transfused with RhD positive blood or any blood-borne products six months prior to enrolment.

Anti RhD

MonoRho® is a recombinant human IgG1/kappa antibody produced in the CHO cell line MDI8s. Its development was based on RhD specific phage isolated from phage display libraries and subsequent construction of the full length human IgG1 \(^2,9\). MonoRho® recognises a discontinuous epitope on loops 3, 4 and 6 of the RhD protein \(^2\) (and unpublished data). Clinical material was produced in a 200 L batch fermentation process, purified and supplied in ready-to-use syringes containing 300 µg of antibody in 1 mL of solution. No CHO host cell proteins were detectable and more than 95% of the antibody was monomeric IgG with less than 1% aggregates and less than 4% fragments. The production process in CHO cells contained validated virus inactivation and nanofiltration steps and complies with current regulatory requirements.

Fab and Fc functions of MonoRho® tested in vitro were all comparable with the plasma derived anti-RhD product \(^2\). Polyclonal anti-RhD (Rhophylac® ZLB Bioplasma AG, Bern, Switzerland) \(^10\) was supplied in ready-to-use syringes containing 1500 International Units (IU) (= 300 µg) anti-RhD determined by AutoAnalyzer.

RhD positive RBC

RhD positive (R1r) Kell negative, group O RBC were obtained from accredited regular blood donors from Transfusion Medicine, University Clinics Charité, Berlin, Germany. Donor selection and testing of RBC concentrates was performed according to German guidelines \(^11,12\). RBC were cryopreserved, thawed and washed according to Good Manufacturing Practice guidelines \(^13\).

Study design and treatment

The study was an open label Phase I trial performed in accordance with the Declaration of Helsinki (revised version of Edinburgh, Scotland, 2000) and approved by the local Ethics Committee. Subjects were allocated to receive either a single standard dose of Rhophylac® or one of various doses of MonoRho® in consecutive cohorts. The study was performed at the Institute of Clinical Pharmacology, Berlin, Germany.
Each volunteer received 15 mL of RhD positive RBC by intravenous administration and 24 h later a single injection of anti-RhD. 25 subjects received one of various doses (300 µg - 1800 µg) of MonoRho® by intravenous administration and 6 subjects received 1200 µg of MonoRho® by intramuscular administration. Other subjects received 1500 IU of Rhophylac® by intravenous (n = 9) or intramuscular (n = 6) administration (Table 1).

In view of this large challenge prevention of accidental immunization of the volunteers receiving MonoRho® was safeguarded by incorporating a rescue dose of Rhophylac® if the RBC clearance rate had not reached pre-defined limits based on previous experience with polyclonal products. Due to results accumulating during the course of the study, the criterion for satisfactory clearance was changed twice. The first 12 subjects treated with MonoRho® were administered Rhophylac® on day 7 only if less than 92.5% of the RhD positive RBC were cleared from the circulation on day 3. The 100% level was set as the RhD positive RBC concentration measured at 23.5 h, ie 30 min prior to anti-RhD injection. For the following 6 subjects, the Rhophylac® administration was moved to day 11 if the desired clearance level was not reached by day 7. For the remaining 11 subjects, Rhophylac® was to be given on day 11 if less than 50% of RhD positive RBC were cleared by day 7.

**In vivo clearance of RhD positive RBC**

Peripheral blood samples were obtained from all subjects up to at least 72 hours post administration of RhD positive RBC and in some subjects up to a maximum of 17 days. The concentration of RhD positive RBC was measured by FACS analysis after first removing leukocytes and platelets by dextran sedimentation. RBC (5 x 10⁷) were incubated with 200 µL of saturating amounts of anti-RhD (MonoRho® or Rhophylac®, respectively) for 30 min. at 37°C to engage all RhD antigen sites on RhD positive RBC. The samples were washed twice followed by addition of 100 µL of PE goat anti-human IgG F(ab’)2 (Jackson ImmunoResearch, West Grove, PA) and incubated for 30 min at 4°C. After washing the samples were taken up in 1 mL PBS. 4 x 100 µL of each sample were added to 4 staining tubes and incubated with Thiazolorange (Retic-COUNT kit, Becton Dickinson, Basel, Switzerland) for 30 min at room temperature. A total of 250,000 events were counted in each tube (i.e. 1 million events per sample). RhD positive RBC were defined by gating PE positive and Thiazolorange negative events. The percentage of RhD positive RBC in relation to the total RBC number was calculated. Using this method less than 0.005% RhD positive RBC could be reliably detected.

**Elimination half-life of RhD positive RBC**

The elimination half-life of RhD positive RBC following intramuscular or intravenous administration of anti-RhD was calculated using results of RhD positive RBC concentrations in RhD negative blood. The disposition rate constant (λ₂) was calculated by unweighted log-linear regression of the RhD positive RBC concentration-time curve. The half-life (t₁/₂) was calculated as t₁/₂ = ln(2)/λ₂.

**Saturation of RhD positive RBC with anti-RhD IgG**

In a subset of subjects the percentage of RhD positive antigen sites occupied by anti-RhD IgG was determined. The same method was used as described above, but in addition the same samples were also analysed without anti-RhD treatment during the staining procedure. The percentage of saturation was calculated as 100 times the ratio of the median PE fluorescence obtained of samples without and with anti-RhD treatment during staining. Only samples with RhD positive RBC counts of more than 200/ million total RBC were used for calculations.
Concentration of anti-RhD IgG in serum

The serum anti-RhD IgG concentration was measured up to 48 h post anti-RhD injection. A sensitive assay was developed using a modification of the European Pharmacopoeia FACS assay. Briefly, 1.25 x 10^5 RhD positive RBC (R2R2) were incubated with test or standard serum samples containing known concentrations of MonoRho® and Rhophylac® in human AB serum. After washing, samples were incubated in saturating amounts of FITC goat anti-human IgG Fab (Jackson Immunoresearch, West Grove, PA). Controls included samples with RhD negative RBC and spike samples with low, intermediate and high anti-RhD content, which were assessed in every experiment and had to be within 25% of the theoretical anti-RhD concentration. The quantitation limit was 0.39 ng anti-RhD/mL.

Serologic detection of anti-RhD

Serum samples obtained at the screening visit and 3 and 6 months after the challenge with RhD positive RBC were tested for blood cell alloantibodies by the indirect haemagglutination test (ID gel agglutination test, DiaMed, Cressier, Switzerland). Serum was tested with a panel of 11 test RBC. The above sensitive FACS method could not be used due to the presence of recombinant antibody still circulating at low levels.

Detection of antibody responses to MonoRho®

The pre-dose, the 3 and the 6 months samples were checked for the presence of antibodies to MonoRho® using an adaptation of the Particle Gel Immuno-Assay system (ID-PaGIA, DiaMed AG, Cressier, Switzerland). In brief, 10 µL of the serum sample was incubated with 50 µL of MonoRho® coated red polystyrene beads on top of the ID gel-card for 5 minutes at room temperature then centrifuged and read macroscopically. Positive reactions were recognized by agglutination of beads. The assay was validated with rabbit anti-MonoRho® antiserum as no human antiserum against MonoRho® is available. The limit of detection for anti-MonoRho® antibody was between 25 and 50 ng/mL.

Safety evaluations

Blood pressure, sublingual body temperature, and heart rate were measured before and at frequent intervals during the 72 h period following administration of RhD positive RBC and after 7 and 30 days. A physical examination was performed at baseline and after 72 h, 7 days and 30 days; an ECG was performed at baseline and after 72 h. Hematological parameters (hemoglobin, hematocrit, red blood cell counts, white blood cell counts and differential counts, platelet counts), serum chemistry (glucose, creatinine, total bilirubin, urea, total protein, AST and ALT), and dipstick urinalysis (protein, glucose, blood, pH) were measured at baseline, after 23.5 h, 72 h, 7 days and 30 days. Adverse events were recorded throughout the study.

Fc gamma receptor analysis

Genotyping for the FcRIIA, FcRIIIB, and FcRIIIB polymorphisms was performed as previously described with polymerase chain reaction (PCR)-based allele-specific primer amplification.

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### Table 1. Overview of Subjects, FcγR-Polymorphism, Clinical Data and Results

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*Aggl = Blood was tested with a panel of 11 test rbc types. Some of them showed a very weak agglutination, but a specific antibody could not be identified. Agglutination was scored by visual assessment from ++++ (strong agglutination) descending to - (no agglutination) as defined by the DiaMed ID gel scale. The positive agglutination was rated as a 1+ reaction on the ID gel scale. ND (not done).

### Statistical Analysis

The half-lives of the RBC were log-transformed to achieve normal distribution of the data and equality of variance across groups. To evaluate the effect of the FcγR polymorphisms on the elimination of RhD positive RBC after intravenous administration of MonoRho®, an analysis...
of variance (ANOVA) with the factors FcγRIIA, FcγRIIIA, and FcγRIIIB (without interactions) was performed

**Results**

**Anti-RhD IgG serum concentrations**

The serum concentrations of anti-RhD IgG measured after intravenous administration of MonoRho® were dependent on the dose given (Figure 1). One hour after administration, the mean anti-RhD IgG concentrations ranged between 23 ng/mL (300 µg dose) and 300 ng/mL (1800 µg dose). In comparison, one hour after intravenous administration of Rhophylac® 1500 IU, the mean concentration was 47 ng/mL.

The anti-RhD IgG concentrations decreased slightly faster after MonoRho® than after Rhophylac® administration. After 2 days, compared to the concentrations measured at 1 hour, serum anti-RhD had decreased by 80% for the 300 µg dose of MonoRho® and by 60% to 65% for the higher doses of MonoRho®. In the case of Rhophylac® (1500 IU), serum anti-RhD had decreased by 40%. After intramuscular administration the anti-RhD IgG serum concentrations gradually increased reaching 10.2 ± 5.9 ng/mL (Rhophylac® 1500 IU dose) and 0.9 ± 1.3 ng/mL (MonoRho® 1200 µg dose), respectively. The variability of anti-RhD IgG serum concentrations between subjects receiving the same dose of anti-RhD immune globulin by the intramuscular route was much greater than after intravenous administration.

![Figure 1](image_url)

**Figure 1**

**Anti-RhD IgG serum concentration (mean +/- SD) after administration of anti-RhD immune globulin.** Rhophylac® was administered both intravenous and intramuscular at the standard dose of 1500 IU (=300 mcg) anti-RhD as determined by the AutoAnalyser.
MonoRho® was administered both intravenous and intramuscular at different doses defined by measurement of purified anti-RhD antibody (OD280nm). Anti-RhD IgG levels in serum were measured using a sensitive FACS assay as described in materials and methods.

**Clearance of RhD positive RBCs**

The clearance rate of 15 mL RhD positive RBC following administration of Rhophylac® and MonoRho® is shown in Figures 2 and 3 and Table 1. In all subjects, the RhD positive RBC concentration in blood remained relatively stable (~ 0.35%) for the 24 h period until anti-RhD was injected. Following intravenous administration of Rhophylac®, RhD positive RBC disappeared with a mean half-life of 1.46 ± 0.89 hours. On average more than 95% of RhD positive RBC were eliminated from the circulation within 8 hours. After intramuscular administration of Rhophylac®, an average of 12 h was required before approximately 10% of RhD positive RBC were cleared and 4 days before 95% of RhD positive RBC were cleared.

**Figure 2**

*Kinetics of concentration of RhD positive RBC (mean ± SD) measured in blood following intravenous (IV) or intramuscular (IM) administration of 1500 IU (300mcg) of Rhophylac®. The percentage RhD positive RBC remaining in the blood at different times was calculated according to the 100% value determined at +23.5 h after RBC administration by FACS assay.*

The mean half-life of RhD positive RBC elimination was almost 4 times longer after intramuscular administration of 1200 µg of MonoRho® than after intramuscular administration of 1500 IU of Rhophylac® (45.9 ± 44.1 h versus 12.3 ± 9.5 h). The clearance
rate differed significantly between subjects receiving MonoRho® intramuscular with half-lives ranging from 8.6 days to 131.4 days (Table 1). The mean remaining RhD positive RBC at 120 h and 168 h after anti-RhD administration was 32% (range 0.02-59.04) and 6.4% (range 0.00-15.84) respectively.

The RhD positive RBC clearance rate showed no correlation to the dose of MonoRho® administered intravenously and also varied considerably between subjects who received the same dose (Figure 3). Overall the half-life of RhD positive RBC disappearance after MonoRho® intravenous ranged from 2 to 203 hours, irrespective of the dose, (Table 1).

There were 6 subjects who failed to meet the pre-defined “satisfactory” RhD positive RBC clearance criteria (see Material and Methods) and who received 1500 IU of Rhophylac® intravenous either on day 7 or on day 11 (Table 1). In 2 of these subjects the concentrations of RhD positive RBC were measured shortly prior to the Rhophylac® administration and 4 days (subject no. 503) or 6 days (subject no. 1101) thereafter. Surprisingly, in both subjects the Rhophylac® administration did not cause an accelerated clearance of RhD positive RBC, rather the concentration of RhD positive RBC continued to decrease at approximately the same rate as before. For eg in subject 503, 69.2% of RhD positive RBC were cleared on day 7 and 86.3% on day 11. In subject 1101, 51.8% of RhD positive RBC were cleared on day 11.
and 69.4% on day 17. In subjects 501 and 502, who had cleared about 90% of RhD positive RBC at 72 h, more than 99% were already cleared prior to Rhophylac® administration on day 7. The percentages of eliminated RhD positive RBC at 72 h and on day 13, respectively, for the other 3 subjects who all received the Rhophylac® administration on day 7 were: subject 202 (87.7%, 99.8%), subject 301 (51.1%, 97.5%), subject 302 (64.4%, 100%).

In summary both MonoRho® and Rhophylac® after intravenous or intramuscular administration cleared RhD positive RBC from the circulation but at different rates. Overall MonoRho® was slower than Rhophylac® and showed no dose response with respect to the clearance rate.

**Saturation of antibody binding sites on RhD positive RBC**

The kinetics of antibody binding to RhD positive RBC was measured in all subjects who received anti-RhD by intramuscular administration, the 1800 µg MonoRho® dose by intravenous administration and also in some subjects from the 300 µg, 600 µg, and 900 µg MonoRho® intravenous treatment groups (Figure 4). Within 1 hour after intravenous injection of MonoRho® and before the elimination of RhD positive RBC, the 300 µg, 600 µg, 900 µg and 1800 µg doses were sufficient to saturate a mean of 64%, 80%, 94%, and 100%, respectively, of the RhD positive binding sites. Within 3 hours and 12 hours, the saturation levels also increased up to more than 80% in the 600 µg and 300 µg doses, respectively.

![Figure 4](image-url)

**Figure 4**

Kinetics of saturation of RhD positive RBC with anti-RhD IgG according to the dose of MonoRho® and Rhophylac® at 1500 IU (300mcg). 15mL RhD positive RBC were administered 24 h prior to the anti-RhD injection. Saturation of RBC at the indicated time points was measured using a FACS assay on samples treated without and with additional anti-RhD treatment (to allow for saturation of binding) during staining.
Administration of anti-RhD by the intramuscular route, either MonoRho® or Rhophylac® resulted in a slower saturation of binding sites reaching levels of 20-67% after 48 hours (Figure 4). However, this did not jeopardize removal of RhD positive RBC as clearance began when approx. 20% of the binding sites displayed bound anti-RhD, data not shown.

**Fcγ receptor analysis**

The majority of subjects were typed for the FcγRIIA-131H/R, FcγIIA-158V/F, and FcγRIIB NA1/NA2 polymorphisms as follows; 23 subjects receiving MonoRho® intravenous; 6 receiving MonoRho® intramuscular; 7 out of 9 receiving Rhophylac® intravenous and 6 receiving Rhophylac® intramuscular (Table 1). The MonoRho® intravenous data revealed an association of FcγRIIA (p=0.05) and IIIA (p=0.05) allotypes on the RBC clearance rate while no dependence on FcγRIIB polymorphisms was seen (p=0.87) (Figure 5a,b,c). The borderline degree of significance obtained for FcγRIIA and FcγIIIA using this rigorous analysis is related to the small sample size but a clear trend is shown (Figure 5). Subjects homozygous for FcγRIIA-131H or FcγIIIA-158V allotypes showed a faster clearance rate compared to both the heterozygote FcγRIIA-H/R, FcγIIIA-V/F and the alternative homozygote FcγRIIA-RR and FcγIIIA-FF. The RBC clearance rates for subjects receiving Rhophylac® intravenous were all fast and no statistical analysis could be performed (Table 1).

Administration of anti-RhD by the intramuscular route resulted in slower RBC clearance rates compared to the intravenous route. Despite the smaller number of subjects in the intramuscular groups, a similar but not so marked trend of correlation with FcγR polymorphisms was seen (Table 1). In both the Rhophylac® and MonoRho® intramuscular treatment groups the presence of FcγRIIA-131R and FcγIIIA-158F was correlated with a slower removal of antibody coated cells; for example, the 2 subjects (no’s. 1005 and 1006) homozygous for both allotypes IIA-RR and IIIA-FF had the longest RhD positive RBC elimination half-lives (50.3 and 131.4 h) (Table 1).

In summary, these results showed that for both polyclonal and recombinant antibodies there were faster RBC clearance rates after intravenous administration than intramuscular. In particular, for MonoRho® administered intravenous the RBC clearance rate was not dependent on the dose of anti-RhD used but instead showed an interesting association with the FcγRIIA and IIIA polymorphisms.
Figure 5A
Influence of FcγRIIa-131 H/R polymorphisms on the clearance rate of RhD positive RBC after MonoRho® intravenous administration. The polymorphisms were analysed on DNA extracted from peripheral blood samples. 15mL RhD positive RBC were administered 24 h prior to the anti-RhD injection.
Figure 5B
Influence of FcγRIIIa-158 V/F polymorphisms on the clearance rate of RhD positive RBC after MonoRho® intravenous administration. 15mL RhD positive RBC were administered 24 h prior to the anti-RhD injection.
Influence of FcγRIIIb-NA1/NA2 polymorphisms on the clearance rate of RhD positive RBC after MonoRho® intravenous administration. 15mL RhD positive RBC were administered 24 h prior to the anti-RhD injection.

Prevention of RhD sensitization
The serological follow up assessments revealed that after 3 months, weak anti-RhD activity was found in serum samples of 3 of the 9 subjects who were treated with Rhophylac® intravenous and in 3 of the 6 subjects who were treated with Rhophylac® intramuscular. In the MonoRho® treatment group, 9 out of 31 subjects including 6 of the 7 subjects who received Rhophylac® “rescue” medication also had detectable anti-RhD. The other 3 subjects had received MonoRho® only, either 1200 µg by intramuscular administration or 1800 µg by intravenous administration. After 6 months, serum samples from all 46 subjects were negative for anti-RhD (Table 1).

Safety
The administration of RhD positive RBC and anti-RhD immune globulins were well tolerated and had no effect on routine laboratory parameters. There were no adverse events in any of the volunteers. The 3 and 6 months serum samples of all 31 subjects who were administered MonoRho® contained no detectable antibodies to MonoRho®.
Discussion

Recent years have seen an increasing application of therapeutic recombinant monoclonal antibodies in many different clinical situations. In order to maximize their effects, much has still to be learnt concerning their mechanisms of action and interactions with other effector pathways of the immune system. This is also true for candidate recombinant antibodies projected to be used for Rhesus prophylaxis. In this case there is an added threshold to overcome as any potential recombinant antibody must be at least as good as the current polyclonal anti-RhD products. A key question is whether a single IgG1 antibody can replace a polyclonal product containing primarily anti-RhD of IgG1 and IgG3 isotypes and recognizing multiple epitopes on the RhD antigen. Here we report on a successful Phase I clinical study of a single recombinant IgG1 anti-RhD antibody (MonoRho®) designed to assess the safety and efficacy of RhD positive RBC clearance and prevention of RhD immunization in RhD negative male volunteers.

Other clinical studies using a mix of IgG1 and IgG3 monoclonal anti-RhD antibodies have been reported but using challenge RBC volumes of no more than 5 mL. Our study aimed to simulate a worst case scenario in a first time pregnancy such that the anti-RhD was given 24h after a 15 mL RBC challenge. In 24 subjects treated with MonoRho® only and another 7 subjects who received MonoRho® and a rescue dose of Rhophylac® there was no evidence of immunization tested at 6 months out from the original challenge. This is the first time a single IgG1 recombinant anti-RhD has shown prevention of RhD primary immunization after such a large (15 mL) RBC challenge. A control arm of non treatment was not feasible due to ethical considerations but well documented data from historical controls indicates that in an unprotected challenge 50% of subjects exposed to 12.6 to 14.6 mL RBC developed anti-RhD antibodies.

After intravenous administration of MonoRho® there was a clear correlation with the dose given and a rapid decline of serum concentration probably due to rapid distribution of antibody into the extravascular space and/or binding to RhD positive RBC which occurs very quickly. After intramuscular administration of both anti-RhD immune globulin preparations, peak concentrations were not reached within the 48 h post dose observation period. This finding is consistent with results of pharmacokinetic studies performed in healthy RhD negative male volunteers with MonoRho® (unpublished results) and with Rhophylac® in RhD negative pregnant women where maximum anti-RhD IgG serum levels were reached after a mean of 3.4 days and 5.5 days, respectively.

There are many unresolved questions concerning Rhesus prophylaxis including the mechanism of action and whether a single antibody or a mix of antibodies of different epitope specificities and isotypes is an absolute requirement. There is an extensive literature on in vitro functional analysis of Fc mediated effector functions of monoclonal anti-RhD antibodies via FcγR interactions, their utility in predicting prophylactic efficacy and the relative merits of IgG1 versus IgG3 isotypes with respect to phagocytosis and cytolysis.

Rh prophylaxis is thought to be successful due to the efficient clearance of RhD positive RBC from the circulation and phagocytosis of anti-RhD coated RBC by macrophages in the spleen. Accelerated clearance of coated RBC and a relation between the rate of clearance and the degree of coating was observed many years ago. Results from a decreasing dose response trial indicated that a dose of 100 µg polyclonal anti-RhD was an adequate lower limit. Recommendations today vary in different countries and include the addition of antenatal prophylaxis but remain mostly based on these early studies. We considered an escalating dose range of MonoRho® intravenous compared with the standard dose of polyclonal anti-RhD as essential due to the previously mentioned discrepancies, which are seen when monoclonal
antibodies are quantitated using the European Pharmacopoeia AutoAnalyser assay. The RBC clearance rate as an early indicator of efficacy showed no correlation with the intravenous doses of MonoRho®. Clearance was detected starting at approx. 20% saturation of RBC but in some subjects the RBC clearance rate was initially slow compared to the polyclonal product and for safety reasons they received a rescue administration of polyclonal anti-RhD. However, this did not speed up the rate of RhD positive RBC clearance, probably because the RBC were already saturated to more than 90% with anti-RhD even at the lowest dose of 300µg and thus the polyclonal anti-RhD could not bind. In support of this hypothesis it has been shown in vitro that MonoRho® can competitively inhibit the binding of Rhophylac® to the RhD antigen on RBC. Our results confirm other clinical data demonstrating that monoclonal antibodies generated a slower RBC clearance rate than polyclonal anti-RhD and that the speed of RBC clearance was not correlated with the ability to prevent RhD immunization.

Clearance of RhD positive RBC from the circulation implies interactions with FcγR’s on effector cells of the immune system. It is known that polymorphisms of the leukocyte receptors FcγRIIA, FcγRIIIB, and FcγRIIIA influence the IgG binding capacity of the receptor. The NA1 isoform has been reported to induce a higher rate of phagocytosis of IgG sensitized particles, presumably because of its high affinity for both IgG1 and IgG3. We found no correlation with the FcγRIIB NA1/NA2 polymorphism. However, subjects homozygous for the FcγRIIIA-158V isoform had the fastest RBC clearance rates particularly in the MonoRho® intravenous group. It may be relevant in this context that the 158V isoform shows higher binding capacity for IgG1, IgG3 and IgG4 than the 158F variant. Also, recent studies on the therapeutic activity of the chimeric IgG1 anti-CD20 antibody, Rituximab have shown a greater probability of response linked with the homozygous FcγRIIIA-158V patients which is thought to be due to the increased antibody dependent cell cytotoxicity activity on B lymphoma cells.

Interestingly, the RBC half-life was also shorter in subjects with the homozygous FcγRIIA-131H genotype. Previous in vitro functional assays had shown no effect of FcγRIIA polymorphisms on IgG1-RBC immune complexes but instead only an effect on IgG3 mediated immune reactions with the FcγRIIA -131H showing some higher affinity for IgG3.

The variability in clearance rate in subjects protected with the polyclonal product which contains anti-RhD of the IgG1 and IgG3 subclasses was less pronounced than in the MonoRho® treated subjects. Nevertheless, those subjects in the Rhophylac® intramuscular treatment group with the FcγRIIA-131H and FcγRIIIA-158V alleles also tended to have faster RBC clearance rates. This finding agrees with results from a previous study in 13 patients suffering from lupus nephritis, where the half-life of RBC coated with polyclonal anti-RhD was significantly prolonged in subjects homozygous for FcγRIIA-131R genotype, but does not agree with respect to FcγRIIIA where no difference was observed. Another study reported that the FcγRIIA-131R genotype may contribute to impaired removal of circulating immune complexes in patients with lupus nephritis in analogy with the slow RBC clearance seen in this study. In contrast, the FcγRIIA and IIA polymorphisms of subjects from a clinical trial of monoclonal anti-RhD antibodies has reported seemingly opposite results as the RBC clearance rate was more rapid in subjects homozygous for FcγRIIIA-158F than in those expressing the FcγRIIIA-158V allele and no association with FcγRIIA genotypes. However these results apply to an IgG3 anti-RhD antibody, whose functional profile in vitro is strikingly different from comparable IgG1 anti-RhD antibodies.
While there is an accumulating literature on the clinical impact of the FcγR polymorphisms, this study shows they had no effect on the clinical end point of prevention of immunization. Interestingly, a recent paper \(^{51}\) seems to indicate that phagocytosis of anti-RhD coated RBC is initially stimulated and then down regulated in a time period where RBC would still be circulating. The saturating levels of MonoRho on RBC after intravenous administration may indicate that other mechanisms eg. antigen masking may play an important role. However, antigen masking is still controversial because studies with polyclonal and monoclonal antibodies including our MonoRho intramuscular and Rhophylac administration showed non-saturating levels of RhD immune globulin on RBC. \(^{19,52}\). Recent studies have shown that more than 90% of the antibody response in transgenic mice lacking the known receptors for IgG was suppressed \(^{53}\) and that F(ab)\(_2\) fragments as well as IgE are efficient suppressors of antibody responses \(^{53,54}\). These findings strongly suggest that IgG is able to efficiently suppress antibody responses independently of the Fc part and favour an important role for antigen masking \(^{55}\). Additionally, it has been claimed that there is an Fc dependence for suppression of primary antibody responses based on lack of suppression by F(ab)\(_2\) fragments \(^{56-58}\) and non-epitope specificity of suppression \(^{59}\). The above mechanisms would also not exclude a role for inhibition of specific B cells by FcγRIIB signalling once the RBC are cleared from the circulation \(^{60}\). The precise mechanism of action of Rh prophylaxis remains unclear and may depend on multiple additional pathways, reviewed in \(^{20,52,61}\).

Our study has demonstrated that a single, human, recombinant IgG1 antibody expressed in CHO cells effectively prevented RhD immunization in male volunteers after a large RBC challenge volume not previously tested in other clinical trials of monoclonal anti-RhD antibodies. The encouraging results of this study suggest that MonoRho® warrants further development as a safe and efficacious alternative to plasma derived anti-RhD immune globulin products for Rhesus prophylaxis.

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References

24. Hadley AG, Kumpel BM. Phagocytosis by human monocytes of red cells sensitized with monoclonal IgG1 and IgG3 anti-D. Vox Sang. 1989;57:150-151
31. Bredius RG, Fijen CA, De Haas M, et al. Role of neutrophil Fc gamma RIa (CD32) and Fc gamma RIIib (CD16) polymorphic forms in phagocytosis of human IgG1- and IgG3-opsonized bacteria and erythrocytes. Immunology. 1994;83:624-630
32. Wiener E, Jolliffe VM, Scott HC, et al. Differences between the activities of human monoclonal IgG1 and IgG3 anti-D antibodies of the Rh blood group system in their abilities to mediate effector functions of monocytes. Immunology. 1988;65:159-163
33. Hadley AG, Kumpel BM. Synergistic effect of blending IgG1 and IgG3 monoclonal anti-D in promoting the metabolic response of monocytes to sensitized red cells. Immunology. 1989;67:550-552
39. de Haas M. IgG-Fc receptors and the clinical relevance of their polymorphisms. Wien Klin Wochenschr. 2001;113:825-831
42. Koene HR, Kleijer M, Algra J, et al. Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. Blood. 1997;90:1109-1114
43. Shields RL, Namenuk AK, Hong K, et al. High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. J Biol Chem. 2001;276:6591-6604
49. Hadley AG, Zupanska B, Kumpel BM, Leader KA. The functional activity of Fc gamma RII and Fc gamma RIII on subsets of human lymphocytes. Immunology. 1992;76:446-451
A single recombinant anti-RhD IgG prevents Rhesus D immunization: association of RhD positive red blood cell clearance rate with polymorphisms in the FcγRIIA and IIIA genes

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