Mouse models of IgG- and IgM-mediated hemolysis

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**Left running head:** SCHIRMER et al

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**Abbreviations:**
HTR: hemolytic transfusion reaction; IgM/G-HTR: IgM/G-mediated HTR; RBC: red blood cell(s); AIHA: autoimmune hemolytic anemia; Tg: transgenic or transgene; KO: knockout; mRBC: mouse RBC; hGPA: human glycophorin A; MAb: monoclonal antibody; CWB: column
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ABSTRACT

Well-characterized mouse models of alloimmune antibody-mediated hemolysis would provide a valuable approach for gaining greater insight into the pathophysiology of hemolytic transfusion reactions. To this end, mouse red blood cells (mRBC) from human glycophorin A transgenic (hGPA-Tg) donor mice were transfused into non-Tg recipients that had been passively immunized with IgG or IgM hGPA-specific monoclonal antibodies (MAb). In this novel murine “blood group system,” mRBC from hGPA-Tg mice are “antigen positive” and mRBC from non-Tg mice are “antigen negative.” Passive immunization of non-Tg mice with the IgG1 10F7 and IgG3 NaM10-2H12 anti-hGPA Mab each induced rapid clearance of incompatible transfused hGPA-Tg-mRBC in a dose-response manner. Using various knockout (KO) mice as transfusion recipients, both the complement system and activating Fcγ receptors were found to be important in the clearance of incompatible mRBC by each of these IgG Mab. In addition, the IgM E4 anti-hGPA Mab induced complement-dependent intravascular hemolysis of transfused incompatible hGPA-Tg-mRBC accompanied by gross hemoglobinuria. These initial studies validate the relevance of these new mouse models for addressing important questions in the field of transfusion medicine.
INTRODUCTION

Hemolytic transfusion reactions (HTRs) are dangerous complications of blood transfusion. IgM-mediated HTRs (IgM-HTRs) occur acutely, are usually due to ABO incompatibility, typically cause intravascular hemolysis, and can lead to shock, renal failure, coagulopathy, and death\(^1\). Although infrequent, IgM-HTRs have a high mortality rate. IgG-mediated HTRs (IgG-HTRs), are more common, but usually less severe, than IgM-HTRs; they can occur acutely or be delayed, typically cause extravascular hemolysis, and occasionally result in renal failure, coagulopathy, and death\(^2\). Symptomatic immune-mediated red blood cell (RBC) destruction also occurs in autoimmune hemolytic anemia (AIHA)\(^3\), blood-group incompatible transplantation\(^4\), and immune thrombocytopenic purpura treated with Rh-immune globulin\(^5\). Although various different modalities are used to treat HTRs, there is no definitive evidence regarding efficacy. Given the sporadic nature of HTRs, designing human trials to evaluate treatment options is difficult. Therefore, to study the mechanisms underlying HTRs, to evaluate the efficacy of existing treatments, and to develop new treatments, a relevant, inexpensive, and tractable animal model is required\(^6\). A mouse model would be ideal for this purpose\(^7\), given the abundance of reagents and relevant knockout (KO) and transgenic (Tg) animals. Although mouse blood group polymorphisms exist\(^8\), and transfused incompatible mouse RBC (mRBC) are rapidly cleared\(^9,10\), no one has exploited these findings to develop a model of HTRs. The availability of a Tg mouse expressing human glycophorin A (hGPA) on mRBC\(^11\) offers an approach using a well-described glycoprotein target and well-characterized reagents.

We describe initial studies validating mouse models of IgG- and IgM-mediated alloimmune hemolysis. Thus, mRBC from hGPA-Tg donors were transfused into non-Tg recipients that were passively immunized with IgG or IgM hGPA-specific monoclonal antibodies (MAb). The clearance of the transfused mRBC was quantified and the roles of complement and Fc\(\gamma\) receptors were evaluated.
MATERIALS AND METHODS

Antibodies

Purified MAb NaM26-2H12 (IgG3), NaM26-3F4 (IgG1), and NaM10-6G4 (IgG2a), which recognize peptide epitopes on the M and N forms of hGPA12, were purchased from EFS (Nantes, France). Hybridomas producing MAb 6A7, an IgG1 specific for a sialic acid-dependent epitope on M-type hGPA13-15, 10F7, an IgG1 recognizing a non-polymorphic epitope on hGPA13,14, and J11d, a rat IgM recognizing CD24 on mRBC16,17, were purchased from the ATCC (Manassas, VA). The hybridoma producing MAb N92, an IgG2a anti-N Mab18, was provided by Elwira Lisowska (Ludwik Hirszfeld Institute, Wroclaw, Poland). The hybridoma producing MAb E4, an IgM that recognizes a non-polymorphic epitope present on hGPA19, was provided by Marilyn Telen (Duke University, Durham, NC).

Throughout culture and MAb purification, endotoxin contamination was minimized. IgG MAbs were purified by immunoaffinity chromatography. Thus, 5 ml of goat anti-mouse IgG agarose (Sigma, St. Louis, MO) in a disposable 10 ml chromatography column (Pierce, Rockford, IL) were equilibrated with 50 ml of column wash buffer (CWB; 50 mM Tris, pH 8.0, prepared using endotoxin-free water and 1.0 M Tris-HCl, pH 8.0 (Mediatech, Herndon, VA)). A peristaltic pump and pyrogen-free tubing (VWR, West Chester, PA) were used to perfuse the column with 100 ml of conditioned medium or ascites at 1 ml/min overnight at 4°C. The column was then washed with 50 ml of CWB and eluted with Immunopure IgG elution buffer (Pierce, Rockford, IL) in 4 ml fractions into tubes containing 1 ml of 1.0 M Tris-HCl in endotoxin-free water, pH 8.0. Peak fractions (i.e. A280nm > 0.050) were pooled, changed into endotoxin-free Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl (Teknova, Hollister, CA)), and concentrated to 1 mg/ml (determined by the BCA Assay (Pierce)) using Centriprep YM-10 centrifugal filters (Millipore, Billerica, MA). Similarly, the IgM J11d and E4 MAbs were purified using a protein L column (Pierce) and the mannose binding protein-based Immunopure IgM Purification Kit (Pierce), respectively.

All MAbs used in vivo were purified to electrophoretic homogeneity. Endotoxin levels were quantified using the LAL QCL-1000 kit (Cambrex, East Rutherford, NJ). The purified MAbs were clear solutions with no visible precipitates; however, they were not further evaluated to exclude the presence of higher oligomers or aggregates. To measure Mab activity,
hemagglutination titers were determined (see below), using commercially-available reagent-quality human RBC (Ortho-Clinical Diagnostics, Raritan, NJ). To evaluate IgM Mab function in vitro, hemolytic complement assays were performed. Thus, RBC were incubated for 1h at room temperature (RT) in MAb E4 or J11d diluted in normal saline (NS; 0.154 M NaCl, pH 7.4). Ten microliters of washed RBC (see below) in NS (2 x 10^8 cells/ml) were added to 190 µl of an 18.75% (v/v) mixture of guinea pig serum (Sigma) in gelatin veronal buffer +2 (GVB+2) (Sigma) in a microtiter plate and incubated at 37°C for 30 min. Following centrifugation for 5 min at 4°C, SN absorbance at 414 nm was determined. Non-opsonized human RBC and mRBC were negative controls. As positive controls, RBC were completely hemolyzed using distilled water instead of GVB+2 buffer; after hemolysis, 10 µl of guinea pig serum were added.

**Mice**

Wild type C57BL/6 and BALB/C mice, and FcγRIIb knockout (KO) mice on the BALB/C background, were from Taconic Labs (Hudson, NY). FVB/NJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). FcγRI KO, complement C3 KO, and FcγR/C3 double-KO mice (all on the C57BL/6 background) were maintained by Dr. Clynes; C3 KO breeding pairs were provided by Michael Carroll (Harvard University, Boston, MA). Animal protocols were approved by the Institutional Animal Care and Use Committee at the Columbia University. Narla Mohandas (New York Blood Center) provided breeding pairs of hGPA-Tg mice, which were constructed on the FVB/NJ background using human BAC clone 159F4 (Catalog #982132; Invitrogen), which contains hGPA. FVB/NJ mice do not express functional complement C5 protein.

A multiplex polymerase chain reaction (mPCR) assay identified hGPA-Tg mice, using genomic DNA extracted from tail biopsies to amplify a 291-bp hGPA sequence using forward (5’-TATTAGCTCAGAGCCTCACACATT-3’) and reverse (5’-GAATTTCTGAAACTTCATGAGCTC-3’) primers; as a positive control, an 88 bp segment of the mouse glycophorin gene Gypa was amplified (forward: 5’-CTTTGTCATTCGGCTGATA-3’; reverse: 5’-TGATGATACTCATAATTTTGGG-3’). Fluorescence in situ hybridization (FISH) was performed by standard methods using the 159F4 BAC clone to probe bone marrow cells from femurs of wild type and hGPA-Tg mice.
In humans, hGPA encodes the MN blood group antigens. The MN phenotype of hGPA-Tg-mRBC was determined by standard microtiter plate hemagglutination assays using the 6A7 anti-M\textsuperscript{13,14} and N92 anti-N\textsuperscript{18} MAbs. Briefly, 25 µl aliquots of serial two-fold dilutions of the MAbs were prepared in NS. Washed mRBC, obtained from retro-orbital plexus blood, were re-suspended to a 3% hematocrit in NS and 25 µl aliquots were added to each well. Following a 1h incubation at 37°C, agglutinates were identified macroscopically. Controls included reagent-quality M+N-, M+N+, and M-N+ human RBC and wild type FVB/NJ mRBC.

Passive immunization
Prior to injection, MAbs were dialyzed against endotoxin-free NS (Phoenix Scientific, St. Joseph, MO). Recipients received IgG MAbs by intraperitoneal injection 16h before transfusion; alternatively, the IgM MAb was infused into the right jugular vein 2 min before transfusion.

Preparation of 51-Cr labeled mRBC
Donors were anesthetized, exsanguinated by cardiac puncture using 25-gauge needles and heparin-coated syringes, and euthanized. For each transfusion, a 1 ml aliquot of a 3% suspension of washed mRBC in a pyrogen-free 1.5 ml microfuge tube was centrifuged at 10,000 x g for 3s, SN was removed, and packed RBC (pRBC) were re-suspended in 100 µl of endotoxin-free NS and 20 µl of 51-Cr (1 mCi/ml; GE Healthcare, Piscataway, NJ). Following 30 min at RT, the tube was centrifuged, SN removed, and the pRBC re-suspended in 100 µl of NS.

Transfusion of 51-Cr labeled mRBC
The recipients were anesthetized and, using sterile technique, a 1 cm vertical midline incision was made and the left jugular vein exposed by blunt dissection. The 51-Cr labeled mRBC (~30 µl of pRBC) in a 25-gauge butterfly was transfused over 5s into the jugular vein. Following transfusion, hemostasis was achieved, and the wound was closed. This procedure was completed in less than 5 min. A zero timepoint (T=0) sample of ~25 µl of blood was then obtained from the retro-orbital plexus.
Survival of transfused RBC

At designated times post-transfusion, mice were anesthetized and an endpoint sample of ~25 µl of retro-orbital plexus blood was obtained. Microhematocrit tubes containing the T=0 and terminal blood samples were centrifuged for 1 min; the height of the pRBC column was measured and the tubes analyzed for counts per minute (CPM) in a gamma counter (Perkin-Elmer, Wellesley, MA). The percentage of radiolabeled mRBC remaining at time “n” following transfusion (T=n) was calculated as:

\[
\frac{\text{CPM/mm of pRBC in the T=n sample}}{\text{CPM/mm of pRBC in the T=0 sample}} \times 100
\]

Following the terminal time point sample, recipients were exsanguinated and euthanized.

Determination of hemoglobinuria

In some experiments, recipients were maintained in metabolic cages. Urine was collected from metabolic cages while mice were alive; bladder urine was also collected at autopsy. Urine absorbance was measured by wavelength scanning spectrophotometry at 300-700 nm using a spectrophotometer (Nanodrop Technologies, Wilmington, DE).
RESULTS

Further characterization of hGPA-Tg mice

Animal husbandry
There were no behavioral differences between hGPA-Tg and wild type FVB/NJ mice. hGPA-Tg mice were healthy, bred well, bore litters of expected size (averaging ~8 neonates/litter), and nurtured their offspring attentively.

Serological characterization of hGPA-Tg-mRBC
In the initial study of hGPA-Tg mice, hGPA expression on mRBC was characterized using only two anti-hGPA MAbs, which had no blood group MN specificity. We extended these studies using well-characterized anti-hGPA MAbs. Hemagglutination with anti-M and -N MAbs (6A7 and N92, respectively) revealed that hGPA-Tg-mRBC expressed the M allele (Table 1). These results are particularly interesting because 6A7 recognizes a sialic acid-dependent epitope, and mouse and human RBC sialic acids differ. In addition, the NaM16-1B10, NaM10-2H12, NaM26-3F4, and 10F7 MAbs, which recognize non-allelic hGPA peptide epitopes, agglutinated human RBC and hGPA-Tg-mRBC, but not wild type mRBC (Table 1). Similar results were obtained with the hGPA peptide-specific NaM10-6G4 and E4 MAbs (not shown). The variation in agglutination titer seen with 10F7 and NaM26-3F4 with different RBC isolates is intriguing, but is not yet understood. Taken together, these results demonstrate that a broad array of anti-hGPA MAbs recognize a glycosylated recombinant form of hGPA on hGPA-Tg-mRBC.

Genotyping and phenotyping hGPA-Tg mice
We developed mPCR and hemagglutination assays to provide a definitive means of identifying hGPA-Tg mice. Thus, mPCR of genomic DNA yielded a 218 bp band when the hGPA Tg was present, and an 88 bp band corresponding to mouse glycophorin, as an internal standard (Figure 1). In contrast, mPCR results from mice lacking the hGPA Tg yielded only the 88 bp mouse glycophorin band (Figure 1). For older mice, hemagglutination assays using the 6A7 anti-hGPA MAb allowed phenotypic confirmation of the presence or absence of the Tg. Thus, MAb 6A7
agglutinated both M+N- human RBC (positive control) and hGPA-Tg-mRBC, but not wild type mRBC or M-N+ human RBC (negative controls; not shown). These assays always produced concordant results.

**Identification of the hGPA Tg integration site**

Initially, it was not clear whether the hGPA Tg integrated at one or multiple loci. FISH provides a definitive means of examining chromosomal integration sites. As a positive control, hGPA is a single copy gene in human leukocytes and maps to chromosome 4q28-q31 (Figure 2A). Similarly, in hGPA-Tg mouse bone marrow cells, the hGPA Tg integrated at a single locus (Figure 2C-D). No hybridization was observed with wild type FVB/NJ mouse bone marrow cells (Figure 2B).

**RBC survival studies**

**IgG-mediated hemolysis in vivo of transfused incompatible mRBC**

When transfused into wild type FVB/NJ and C57BL/6 mice that had been passively immunized with varying amounts of the 10F7, NaM10-2H12, and NaM10-6G4 IgG MAbs, incompatible hGPA-Tg-mRBC were promptly cleared from the circulation in amounts proportional to the amount of infused anti-hGPA MAb. For example, when immunized with increasing amounts of MAb 10F7, 24-hour RBC survival progressively decreased (Figure 3). Because of these results, 500 µg of MAb 10F7 were used subsequently. Similar dose-response experiments with NaM10-2H12 and NaM10-6G4 (not shown), revealed that 50 µg of each of these MAbs would suffice for inducing dramatic RBC clearance (Table 2). As negative controls, no destruction of transfused, compatible, wild type mRBC was observed in mice immunized with any IgG anti-hGPA MAbs (not shown). In addition, when an isotype-matched irrelevant IgG1 MAb was used as a control for 10F7, the survival of hGPA-Tg-mRBC was normal at 24 hr (D.A.S, S.L.S., and James C. Zimring, unpublished data). Similarly, no destruction of transfused hGPA-Tg-mRBC was detected in unimmunized recipients (not shown and see Table 3, below). Therefore, despite the H-2 difference between donors (FVB/NJ background) and recipients (i.e. C57BL/6 and BALB/C), no “naturally-occurring” anti-H-2 antibodies caused accelerated mRBC destruction.
Transfused incompatible hGPA-Tg-mRBC were rapidly cleared from passively immunized mice. For example, in mice immunized with MAb 10F7 or NaM10-2H12, incompatible hGPA-Tg-mRBC clearance was complete by 4 hours post-transfusion (Figure 4).

To evaluate the roles of activating Fcγ receptors and the complement system in IgG-mediated clearance of incompatible mRBC, FcRγ KO, C3 KO, and FcγR/C3 double-KO mice, all on the C57BL/6 background, were passively immunized with IgG anti-hGPA MAbs and transfused with incompatible mRBC. When coated by IgG MAbs of different subclasses, incompatible mRBC were cleared by activating different pathways. For example, as compared with wild type C57BL/6 mice, IgG1-mediated clearance of hGPA-Tg-mRBC by MAb 10F7 was markedly inhibited in FcRγ KO mice and only moderately inhibited in C3 KO mice (Table 3B). Thus, although complement participates in IgG1-mediated clearance of hGPA-Tg-mRBC, activating Fcγ receptors play a dominant role. In contrast, when immunized with the IgG3 NaM10-2H12 anti-hGPA MAb, hGPA-Tg-mRBC destruction was markedly inhibited in C3 KO mice and only moderately inhibited in FcRγ KO mice (Table 3A); thus, complement is dominant, with some contribution by activating Fcγ receptors. With both of these MAbs, clearance of hGPA-Tg-mRBC was markedly, but not completely, inhibited in FcγR/C3 double-KO mice, suggesting that other mechanisms may also be involved in mRBC clearance.

Inhibitory Fcγ receptors were also investigated using FcγRIIb KO mice (BALB/C background). No difference in IgG3-mediated clearance of incompatible mRBC by Mab NaM10-2H12 was observed when comparing FcγRIIb KO and wild type BALB/C mice (not shown). This was not unexpected given the low affinity of FcγRII for IgG3; in addition, Kupffer cells, which appear to play a major role in mRBC destruction (D.A.S. and S.L.S., unpublished data; and Ref. 26-29), either lack FcγRIIb or have defective regulation of phagocytosis by FcγRIIb.

To examine the role of terminal complement components in IgG-mediated mRBC clearance, we compared wild type FVB/NJ mice, which lack complement component C5, with wild type C57BL/6 mice. Clearance of hGPA-Tg-mRBC in MAb 10F7-immunized FVB/NJ mice was markedly reduced compared with C5-replete C57BL/6 mice (Tables 2-3). This suggests that complement C5 plays a significant role, perhaps because C5a enhances activating Fcγ receptor function. In contrast, there was little difference between FVB/NJ and C57BL/6 mice in the clearance of incompatible mRBC by the IgG3 NaM10-2H12 MAb (Tables 2-3).
These differences may relate to the individual epitope specificities, affinities, or IgG subclasses of these two MAbs. In addition, it will be interesting to determine whether C5 is completely responsible for the findings with 10F7 by using congenic mouse strains that differ only at the C5 locus\textsuperscript{32}.

*Complement mediated hemolysis in vitro of IgM-coated hGPA-Tg-mRBC*

hGPA-Tg-mRBC are susceptible to complement-mediated hemolysis in vitro in the presence of guinea pig serum. Thus, hGPA-Tg-mRBC coated in vitro with either the IgM E4 anti-GPA MAb or the IgM J11d anti-CD24 MAb were hemolyzed following incubation in complement-containing serum, as were wild type mRBC coated with J11d (Table 4). As negative controls, wild type mRBC incubated with the E4 MAb, and uncoated hGPA-Tg-mRBC, were not hemolyzed. Thus, the hGPA epitope density on hGPA-Tg-mRBC and the avidity of MAb E4 were sufficient to activate complement, up to and including formation of functional C5b-9 membrane attack complexes.

*IgM mediated hemolysis in vivo of transfused incompatible hGPA-Tg-mRBC*

Dose-response and time course studies demonstrated that passive immunization intravenously of wild type C57BL/6 mice with 100 $\mu$g of the IgM anti-hGPA E4 MAb caused dramatic and very rapid clearance of transfused incompatible hGPA-Tg-mRBC (Table 5). For example, by comparing the CPM/mm of pRBC at T=0 of MAb E4-immunized recipients transfused with either wild type or hGPA-Tg-mRBC, approximately 50% of the hGPA-Tg-mRBC were cleared in the short time between transfusion and drawing the T=0 sample (i.e. 2-5 min). In addition, by 2h post-transfusion, hGPA-Tg-mRBC clearance in E4-immunized mice was virtually complete, with little change at the 24hr time point (not shown). Finally, no significant RBC clearance occurred in mice immunized with MAb E4 and transfused with wild type mRBC (Table 5).

By 2h post-transfusion, abundant, soluble, red-brown pigment was seen in the urine of all MAb E4-immunized wild type C57BL/6 mice transfused with hGPA-Tg-mRBC (Figure 5A). This pigment was not pelleted by centrifugation (not shown); thus, this color change was not due to gross hematuria. However, spectrophotometry confirmed that the pigment was free hemoglobin with a peak absorbance at 414 nm (Figure 5B); thus, the rapid clearance of transfused hGPA-Tg-mRBC produced gross hemoglobinuria (Figure 5; Table 5). In contrast, no
abnormal pigment was seen with MAb E4-immunized mice transfused with wild type mRBC. These results strongly suggest that the IgM E4 anti-hGPA MAb rapidly produced dramatic, intravascular, complement-mediated hemolysis of transfused incompatible hGPA-Tg-mRBC.

To confirm the role of the membrane attack complex, FVB/NJ mice were used, which are C5 deficient\textsuperscript{22}. Interestingly, the survival of transfused hGPA-Tg-mRBC in MAb E4-immunized FVB/NJ mice was only slightly less than that observed in complement-replete wild type C57BL/6 mice (not shown). However, no hemoglobinuria was observed in FVB/NJ recipients. Thus, although complement-mediated intravascular hemolysis was strikingly evident in C57BL/6 mice, the predominant clearance mechanism was probably CR3-mediated phagocytosis of IgM/C3b-coated mRBC. This will be evaluated further in future studies using C3 KO and/or CVF-treated, complement deficient mice. The specific role of CR3, as opposed to CRIg\textsuperscript{33}, can also be evaluated using CR3 KO mice\textsuperscript{34} and the M1/70 CR3-blocking MAb\textsuperscript{35,36}. Finally, the role of C5 can be explored further using congenic strains that only differ at this locus\textsuperscript{32}. 
DISCUSSION

The need for mouse models to study immune-mediated hemolysis

Various animal models were used to study immune-mediated RBC destruction and its sequelae. However, these are not optimal for studying human HTRs due to their expense and the limited flexibility. Although mRBC blood group systems were described, and although antibody-mediated clearance of transfused, incompatible mRBC occurs, these polymorphisms were not exploited to study HTRs. However, murine AIHA models, which clear mRBC following infusion of monoclonal mRBC-specific autoantibodies, allowed evaluation of the roles of antibody isotype, IgG subclass, complement, and Fcγ receptors. Nonetheless, incomplete knowledge of target epitopes, the inability to evaluate early kinetics of mRBC destruction, and in vivo agglutination by IgM, make these less than ideal models for human HTRs. For example, autoantibody-induced agglutination in vivo almost never occurs in humans. Finally, transfusion of hen egg lysozyme (HEL) Tg mRBC into wild-type mice actively immunized to HEL surprisingly caused removal of HEL from the mRBC, with no mRBC destruction. Therefore, the current study describes novel mouse models of alloantibody-mediated intravascular and extravascular hemolysis, potentially suitable for studying HTR pathogenesis.

The relevance of Tg hGPA for immune-mediated hemolysis

Using hGPA-Tg-mRBC to study antibody-induced mRBC clearance is highly relevant for the human situation. Thus, hGPA is the most abundant human RBC glycoprotein. Its primary importance results from carrying the MN antigens, glycopeptide antigens encoded by amino acid polymorphisms at positions 1 and 5, that also depend on O-glycosylation at positions 2-4. In addition, hGPA carries other types of blood group antigens. Antibodies to hGPA antigens cause HTRs, hemolytic disease of the newborn, and AIHA.

The hGPA-Tg mouse provides a genetically inheritable, murine “blood group system.” Thus, wild-type mRBC are “antigen-negative” and hGPA-Tg-mRBC are “antigen positive.” Wild-type mice can be actively immunized with hGPA and many mouse MAbs are available. This type of blood group polymorphism (i.e. the presence or absence of an entire
protein) also occurs in humans: En(a-) RBC lack hGPA, S-s-U- RBC lack human glycophorin B, and “Rh-negative” RBC lack the Rh(D) protein.

**IgG-mediated hemolysis in passively immunized mice transfused with hGPA-Tg-mRBC**

This study provides useful insights into the pathogenesis of antibody-mediated hemolysis. For example, the kinetics of IgG1- and IgG3-mediated clearance of hGPA-Tg-mRBC (Figure 4; Table 2) correlated well with that seen in human IgG-HTRs. In addition, although complement participates in IgG1-mediated clearance of hGPA-Tg-mRBC, Fcγ receptors play a dominant role (Table 3). Interestingly, in a recent study, the IgG1 subclass caused minimal platelet destruction due to its strong binding to the inhibitory FcγRII; the efficacy of the IgG1 10F7 MAb in hGPA-Tg-mRBC clearance is consistent with these findings due to the liver being the likely locus of RBC clearance in mice (Ref. 26-29; D.A.S. and S.L.S., unpublished data) and the absence of, or defective regulation by, FcγRII on mouse Kupffer cells. Indeed, when using IgG class-switch variants of a monoclonal mRBC-specific autoantibody to induce AIHA, the IgG1 variant was moderately pathogenic; furthermore, this variant’s pathogenicity resulted from its interaction with activating Fcγ receptors. Therefore, the efficacy 10F7 (Table 2; Figures 3-4) correlates well with what is known of IgG1’s in murine immune-mediated hematological disorders.

The difference between FVB/NJ and C57BL/6 mice in IgG1-mediated mRBC clearance (Tables 2-3) was particularly intriguing because FVB/NJ mice are deficient in C5. Therefore, C5a and/or the C5b-C9 membrane attack complex may be important in mRBC clearance by MAb 10F7. In contrast, in a mouse AIHA model (i.e. comparing mRBC clearance in DBA/2 (C5 deficient) and BALB/C mice (C5 replete)), C5 was not involved in the anemia induced by several mRBC-specific MAbs, including an IgG1. Thus, our alloimmune IgG-HTR model provides novel information as compared to existing AIHA mouse models. Additional studies using congenic mice that only differ at the C5 locus will further clarify these findings.

Both complement and activating Fcγ receptors are important in IgG3-mediated clearance by MAb NaM10-2H12, with complement being dominant (Table 3). Although in the initial description murine IgG3 did not activate complement, later studies clearly demonstrated complement activation. Our results that activating Fcγ receptors contribute to IgG3-mediated clearance are particularly interesting in light of recent papers concerning murine IgG3 antibodies. Thus, it was originally proposed that a unique IgG3-specific Fcγ receptor facilitates
phagocytosis of IgG3-coated mRBC by murine macrophages. In addition, recent results suggest that no known murine Fcγ receptors function as IgG3 receptors because they do not bind IgG3 in a quantitative binding assay. Our results in vivo with FcRγ KO mice (Table 3) are consistent with this hypothesis; that is, there may be an additional, as yet undescribed, activating FcγR requiring the FcRγ chain. Finally, when human RBC or hGPA-Tg-mRBC are coated in vitro with NaM10-2H12 in serum-free medium, they are phagocytosed by RAW 264.7 mouse macrophages (D.A.S., S.L.S., and Sunny Seo, unpublished data), also suggesting the presence of an activating Fcγ receptor.

In contrast, others suggested that FcγRI is the only IgG3 receptor, in that IgG3 binds to transfected cells expressing recombinant FcγRI and macrophages from FcγRI KO mice do not phagocytose IgG3-coated RBC. Interestingly, although FcRγ KO mice express ~20% of normal levels of FcγRI, they cannot phagocytose IgG3-coated RBC because phagocytosis presumably requires the ITAM function of FcRγ.

Finally, in mouse ITP and AIHA models, IgG3 antibodies are either non-pathogenic or slightly pathogenic, respectively. In addition, in the latter, only complement was involved for this mild effect. However, in our in vivo studies NaM10-2H12 led to rapid and pronounced clearance of incompatible mRBC involving both complement and Fcγ receptors. These results are similar to those found with an IgG3 monoclonal anti-mRBC autoantibody, although the roles of complement and Fcγ receptors were not assessed. Thus, the mechanisms of mRBC clearance by IgG3 Mabs may depend on additional factors, such as antigen specificity and antigen affinity. Studies directly comparing these systems (e.g. infusing anti-hGPA Mabs into hGPA-Tg mice as an AIHA model, and constructing Tg mice expressing hGPA on platelets as an ITP model) would provide additional insights.

Thus, this new model has characteristics that will deepen and extend the mechanistic understanding of IgG-mediated RBC destruction beyond that provided by existing models.

IgM-mediated hemolysis in passively immunized mice transfused with hGPA-Tg-mRBC

The IgM E4 anti-hGPA MAb produced dramatic, intravascular, complement-mediated hemolysis of transfused hGPA-Tg-mRBC (Table 5; Figure 5). To our knowledge, this represents the first time that an IgM has been effectively used to cause intravascular hemolysis in mice. Although
previous studies used monoclonal IgM anti-RBC MAbs to induce AIHA, the anemia was due to intravascular agglutination\textsuperscript{41}. In contrast, given the small quantities of incompatible mRBC transfused in the current study, their clearance could only be caused by complement-mediated hemolysis and/or phagocytosis. Furthermore, the rapid kinetics of this IgM-mediated clearance agrees well with that in human IgM-HTRs\textsuperscript{64}. Interestingly, despite the presence of the CD59 complement regulatory protein on hGPA-Tg-mRBC, and despite the impression that mouse complement is “weak”\textsuperscript{73}, E4 caused complement-mediated intravascular hemolysis. Thus, this model will prove useful in unraveling and clarifying the pathogenic mechanisms of IgM-HTRs. IgM-mediated intravascular hemolysis could also be enhanced by constructing hGPA-Tg x CD59 KO mice\textsuperscript{74} and using these mRBC for transfusion.

When IgM-opsonized RBC were previously transfused into guinea pigs\textsuperscript{37,75}, they were rapidly cleared by the liver, and then released back into the circulation within 24h. The mechanism involved complement activation and adherence of IgM/C3b-coated RBC to complement receptors (presumably CR3), but not phagocytosis. In contrast, passively infused IgM E4 MAb not only rapidly cleared hGPA-Tg-mRBC, but the cleared mRBC were also not released back into the circulation (Table 5). One explanation is that the CR3 integrins in our mice were already activated (even though the mice are maintained in a barrier facility and less than 0.5 µg of endotoxin contaminated the infused E4 MAb). Alternatively, the newly described CRIg complement receptor\textsuperscript{33}, which does not require prior activation, may be responsible. Finally, unique aspects of the specificity and/or affinity of MAb E4 may be relevant.

These mouse studies provided a surprising result that may be relevant to the pathogenesis of human IgM-HTRs. Thus, complement-mediated intravascular hemolysis has long been thought to be primarily responsible for destroying transfused incompatible RBC, as well as for the associated morbidity and mortality of human IgM-HTRs. Indeed, the signs of human IgM-HTRs, including hemoglobinemia and hemoglobinuria, provide solid evidence of intravascular hemolysis. However, without studying IgM-HTRs in a controlled setting, it has not been possible to determine the extent to which intravascular hemolysis clears transfused, incompatible RBC. For example, the dramatic hemoglobinuria in MAb E4-immunized C57BL/6 recipients transfused with hGPA-Tg-mRBC provides clear evidence of intravascular hemolysis (Figure 5). Coupled with its rapid kinetics, it initially seemed that most, if not all, of the incompatible mRBC were destroyed intravascularly. Thus, the results of MAb E4-mediated mRBC clearance
in C5-deficient FVB/NJ recipients were particularly interesting. As expected, no hemoglobinuria occurred in any FVB/NJ mice; however, these recipients cleared incompatible mRBC with the same kinetics and almost the same efficiency as the complement-replete C57BL/6 recipients. This suggests that, in contrast to what is believed with human IgM-HTRs, although complement does cause intravascular hemolysis of transfused, IgM-coated RBC, the IgM-mediated clearance of the incompatible RBC may be predominantly due to complement-mediated extravascular hemolysis resulting from phagocytosis by hepatic Kupffer cells and/or splenic macrophages. Studies evaluating complement receptors (e.g. CR3 KO mice), early components in the complement cascade (e.g. C3 KO mice), and macrophage phagocytosis (e.g. using liposomal chlodronate) will provide more insight.

**Future directions**

Future studies will evaluate the relevance of this mouse model for human HTRs. In the current study, small amounts of radiolabeled incompatible mRBC were transfused into anesthetized mice. Despite rapid clearance of transfused mRBC, recipients did not display the clinical sequelae seen in human HTRs. Therefore, future studies will transfuse large quantities of incompatible mRBC and will monitor various physiological parameters.

**Acknowledgements:** This work was initially inspired by conversations with Dr. Louis Fink. The authors wish to thank Drs. Elwira Lisowska, Marilyn Telen, and Narla Mohandas for providing valuable reagents, Dr. Murty Vundavalli for performing the FISH analysis, Drs. Raymond Baggs and Carl Pinkert for advice regarding the design of mouse experiments, Mary Bolognino and Sunny Seo for technical assistance during the initial studies, Drs. Robert Pierce and James Zimring for helpful discussions, and Dr. Michael Shelanski for support and encouragement.

All authors participated in designing and performing the research; D.A.S and S.L.S controlled and analyzed the data; D.A.S and S.L.S. wrote the paper; and all authors critiqued various drafts and checked the final version of the manuscript.
FIGURE LEGENDS

**Figure 1. Identification of hGPA-Tg mice by mPCR.** The presence of the hGPA Tg was detected using mPCR of genomic DNA extracted from biopsied tail tissue with the primers described in Materials and Methods. This yielded a 219 bp band if the hGPA Tg was present. Amplification of the endogenous mouse glycophorin gene was used as an internal standard and yielded an 88 bp band in each case.

**Figure 2. Localizing the hGPA gene by FISH.** FISH was performed using the 159F4 BAC probe to examine the chromosomal location of the hGPA gene, which encodes the hGPA protein. As a positive control, the probe hybridized to human chromosome 4 (A), and, as a negative control, the probe did not hybridize to any wild type FVB/NJ mouse chromosome (B). However, the probe did hybridize to one chromosome from hemizygous hGPA-Tg mice (C-D). The locations of hybridization are indicated by white arrowheads.

**Figure 3. IgG1-mediated clearance of incompatible mRBC.** Wild type FVB/NJ mice were passively immunized intraperitoneally with the indicated amounts of purified 10F7, an anti-hGPA IgG1 MAb; 16h later they were transfused with 51Cr-labeled wild type (i.e. (-) control) or hGPA-Tg-mRBC, and 24h RBC survivals were quantified. Results are recorded as percentage of transfused RBC remaining in the vasculature after 24 hours for groups of 2-5 mice (mean ± 1 SD). These results show that incompatible hGPA-Tg-mRBC were cleared from the circulation of mice in proportion to the dose of purified 10F7 MAb that they received.

**Figure 4. IgG-mediated clearance of incompatible hGPA-Tg-mRBC: Time course studies.** Wild type FVB/NJ mice were passively immunized intraperitoneally with 500 µg of the purified 10F7 anti-hGPA IgG1 MAb (A) or with 50 µg of the purified NaM10-2H12 anti-hGPA IgG3 MAb (B). Sixteen hours later they were transfused with 51Cr-labeled hGPA-Tg-mRBC, and then RBC survival was quantified at the indicated time points. The number of mice evaluated at each time point is indicated in parentheses; the mean ± 1 SD is provided for each time point.
Figure 5. Gross hemoglobinuria in wild type C57BL/6 mice following passive immunization with the IgM E4 anti-hGPA MAb and transfusion with incompatible hGPA-Tg-mRBC.

Mice were passively immunized intravenously with 100 µg of the purified IgM E4 MAb. (A) Representative post-transfusion urine samples were obtained at autopsy from mice receiving incompatible hGPA-Tg-mRBC (left sample) or receiving compatible wild type mRBC (right sample). (B) Urine samples from mice transfused with incompatible hGPA-Tg-mRBC displayed absorbance peaks at 414 nm (for example, curve A); no absorbance peak at 414 nm was detectable in any urine sample obtained from mice transfused with compatible wild type mRBC (for example, curve B).
Table 1. Serological characterization of hGPA expressed on the surface of hGPA-Tg-mRBC.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Specificity</th>
<th>Source of RBC</th>
<th>RBC type</th>
<th>Isotype</th>
<th>6A7</th>
<th>N92</th>
<th>NaM16-1B10</th>
<th>10F7</th>
<th>NaM10-2H12</th>
<th>NaM26-3F4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Human #1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6A7</td>
<td>M</td>
<td>M+N-</td>
<td>&gt;128</td>
<td>0</td>
<td>64</td>
<td>16</td>
<td>1600</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N92</td>
<td>N</td>
<td>M+N-</td>
<td>128</td>
<td>0</td>
<td>64</td>
<td>64</td>
<td>1600</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaM16-1B10</td>
<td>hGPA</td>
<td>Mouse #1</td>
<td>hGPA-Tg</td>
<td>128</td>
<td>0</td>
<td>64</td>
<td>1600</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10F7</td>
<td>hGPA</td>
<td>Mouse #2</td>
<td>hGPA-Tg</td>
<td>128</td>
<td>0</td>
<td>64</td>
<td>1600</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaM10-2H12</td>
<td>hGPA</td>
<td>Mouse #3</td>
<td>hGPA-Tg</td>
<td>&gt;128</td>
<td>0</td>
<td>64</td>
<td>128</td>
<td>1600</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>NaM26-3F4</td>
<td>hGPA</td>
<td>Mouse #4</td>
<td>wild type</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

A panel of mouse MAbS recognizing either a sialic acid-dependent M epitope on hGPA (i.e. 6A7), a sialic-independent N epitope on hGPA (i.e. N92), or peptide epitopes on both M- and N-type hGPA (i.e. NaM16-1B10, 10F7, NaM10-2H12, and NaM26-3F4) were used to evaluate hGPA expression on hGPA-Tg-mRBC. Human RBC of defined MN type were positive controls. RBC from a wild type FVB/NJ mouse were used as a negative control. The inverse agglutination titers are provided in each case, except for the results of the N92 MAb with the RBC obtained from Human #2, in which strong agglutination was seen, but titers were not performed. As additional controls, wild type and hGPA-Tg-mRBC were agglutinated by a rabbit polyclonal anti-mRBC IgG antibody (not shown).

Table 2. IgG anti-hGPA MAbS rapid cleared hGPA-Tg-mRBC in vivo.

<table>
<thead>
<tr>
<th>Mab</th>
<th>IgG subclass</th>
<th># of mice</th>
<th>% 4h RBC survival (mean ± 1 SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10F7</td>
<td>IgG1</td>
<td>5</td>
<td>59.9 ± 3.4</td>
</tr>
<tr>
<td>NaM10-6G4</td>
<td>IgG2a</td>
<td>4</td>
<td>29.6 ± 9.2</td>
</tr>
<tr>
<td>NaM10-2H12</td>
<td>IgG3</td>
<td>10</td>
<td>17.0 ± 7.2</td>
</tr>
</tbody>
</table>

Wild type FVB/NJ recipient mice that had been passively immunized IP with MAb 10F7 (500 µg), NaM10-6G4 (50 µg), or NaM10-2H12 (50 µg), were then transfused 16 hours later with 51Cr-labeled hGPA-Tg-mRBC and the 4 hour RBC survivals were determined as described in Materials and Methods.
Table 3. Evaluation of the role of activating Fcγ receptors and complement component C3 in IgG-mediated clearance of incompatible hGPA-Tg-mRBC.

A

<table>
<thead>
<tr>
<th>Mice</th>
<th>Passive Immunization</th>
<th># of mice</th>
<th>% 4h RBC survival (mean ± 1 SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>None</td>
<td>6</td>
<td>98.2 ± 1.6</td>
</tr>
<tr>
<td>Wild type</td>
<td>NaM10-2H12</td>
<td>6</td>
<td>26.8 ± 8.1</td>
</tr>
<tr>
<td>FcRγ KO</td>
<td>NaM10-2H12</td>
<td>2</td>
<td>56.5 ± 0.4</td>
</tr>
<tr>
<td>C3 KO</td>
<td>NaM10-2H12</td>
<td>6</td>
<td>81.7 ± 7.2</td>
</tr>
<tr>
<td>FcγR/C3 double-KO</td>
<td>NaM10-2H12</td>
<td>6</td>
<td>80.6 ± 9.1</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Mice</th>
<th>Passive Immunization</th>
<th># of mice</th>
<th>% 4h RBC survival (mean ± 1 SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>None</td>
<td>6</td>
<td>98.2 ± 1.6</td>
</tr>
<tr>
<td>Wild type</td>
<td>10F7</td>
<td>6</td>
<td>27.2 ± 14.5</td>
</tr>
<tr>
<td>FcRγ KO</td>
<td>10F7</td>
<td>6</td>
<td>85.7 ± 19.9</td>
</tr>
<tr>
<td>C3 KO</td>
<td>10F7</td>
<td>6</td>
<td>58.6 ± 4.5</td>
</tr>
<tr>
<td>FcγR/C3 double-KO</td>
<td>10F7</td>
<td>4</td>
<td>80.2 ± 9.2</td>
</tr>
</tbody>
</table>

All mice were on the C57BL/6 background. Mice were passively immunized IP with either 50 µg of the IgG3 NaM10-2H12 anti-hGPA MAb (Panel A) or 500 µg of the IgG1 10F7 anti-hGPA MAb (Panel B) 16h prior to transfusion. All mice were then transfused with 51Cr-labeled hGPA-Tg-mRBC. In mice immunized with 10F7, clearance of incompatible mRBC was highly dependent on the presence of activating Fcγ receptors, and moderately dependent on C3. In contrast, clearance of incompatible RBC in mice immunized with NaM10-2H12 was moderately dependent on activating Fcγ receptors and highly dependent on C3.

Table 4. Complement mediated lysis in vitro of IgM-opsonized mRBC.

<table>
<thead>
<tr>
<th>MAb</th>
<th>J11d (2 µg/ml)</th>
<th>E4 (0.25 µg/ml)</th>
<th>E4 (0.1 µg/ml)</th>
<th>E4 (0.05 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type mRBC</td>
<td>91.8</td>
<td>0.0</td>
<td>0.0</td>
<td>1.9</td>
</tr>
<tr>
<td>hGPA-Tg-mRBC</td>
<td>118.0</td>
<td>67.9</td>
<td>38.9</td>
<td>31.9</td>
</tr>
</tbody>
</table>

Wild type and hGPA-Tg-mRBC were incubated with distilled water as a positive control (defined as 100% hemolysis) or with guinea pig serum alone as a negative control (defined as background hemolysis). Test samples contained guinea pig serum as a source of complement and defined concentrations of purified MAbs. Hemolysis was determined by spectrophotometric evaluation of absorbance at 414 nm of the supernatant of each reaction and the results are presented as percent hemolysis compared to the distilled water control. All samples were tested in duplicate and the average results are presented. Binding of the mRBC-specific J11d anti-CD24 IgM MAb led to hemolysis of both types of mRBC; the E4 anti-hGPA IgM MAb only hemolyzed hGPA-Tg-mRBC.
Table 5. IgM-mediated clearance of incompatible hGPA-Tg-mRBC.

<table>
<thead>
<tr>
<th>Experiment #</th>
<th>MAb</th>
<th>mRBC transfused</th>
<th># of mice</th>
<th>% 2h RBC survival (mean ± 1 SD)</th>
<th>% 24h RBC survival (mean ± 1 SD)</th>
<th>Urine Absorbance at 414 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E4</td>
<td>wild type</td>
<td>4</td>
<td>91.3 ± 6.3</td>
<td>ND</td>
<td>0.04, 0.05, 0.10, 0.06</td>
</tr>
<tr>
<td>1</td>
<td>E4</td>
<td>hGPA-Tg</td>
<td>4</td>
<td>19.1 ± 2.0</td>
<td>ND</td>
<td>0.28, 1.63, 0.74, 0.77</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>hGPA-Tg</td>
<td>3</td>
<td>99.6 ± 1.1</td>
<td>98.9 ± 3.9</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>E4</td>
<td>hGPA-Tg</td>
<td>5</td>
<td>29.4 ± 5.1</td>
<td>23.2 ± 3.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

Wild type C57BL/6 mice were passively immunized IV with 100 μg of the purified E4 IgM anti-hGPA MAb, or with the same volume of NS, 2 min prior to transfusion with 51Cr-labeled wild type FVB/NJ mRBC or hGPA-Tg-mRBC. The RBC survival at 2 and 24 hours was then determined as described in Materials and Methods. In addition, scanning wavelength spectrophotometry was used to identify hemoglobin (which has a characteristic absorption peak at 414 nm) in urine collected from the bladder at autopsy. Experiments 1 and 2 were performed on different days with different lots of MAb E4; the mice were euthanized at 2 hours post-transfusion in Experiment 1 and at 24 hours post-transfusion in Experiment 2. Bladder urine obtained at autopsy in all mice in Experiment 2 was clear yellow; therefore, the absorbance was not determined (ND).
Figure 1

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>100</th>
<th>101</th>
<th>102</th>
<th>103</th>
<th>104</th>
<th>105</th>
<th>106</th>
<th>107</th>
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<tbody>
<tr>
<td>219 bp</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>88 bp</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Agglutination

Figure 2

A  
Human 4q28-q31

B  
Wild-type FVB mouse

C  
hGPA-Tg mouse

D  
hGPA-Tg mouse
Figure 3

![Graph showing % 24 hr survival of hGPA-Tg mRBC against Passive Immunization (10F7: ug per mouse (# mice)).](image)

Figure 4

![Graphs showing % survival of hGPA-Tg mRBC over time.](image)
Figure 5
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Mouse models of IgG- and IgM-mediated hemolysis

David Austin Schirmer, Shuh-Chyung Song, Jeffrey P Baliff, Stephanie O Harbers, Raphael A Clynes, Anna Krop-Watorek, Gregory R Halverson, Marcin Czerwinski and Steven L Spitalnik

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