Non-Hemolytic Antibody-Induced Loss of Erythrocyte Surface Antigen

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Nonstandard Abbreviations: Hen Egg Lysozyme (HEL), Direct Antiglobulin Test (DAT), chloromethylbenzamido 1,1'-dioctadecyl-3,3,3',3'- tetramethylindocarbocyanine perchlorate (CM-DiI), 3,3'-dihexadecyloxycarbocyanine perchlorate (DiO), Ovalbumin (OVA), allophycocyanin (APC), horseradish peroxidase (HRP), Reticuloendothelial system (RES).
Abstract:

Transfusion of red blood cells (RBC) into patients with anti-donor RBC antibodies (crossmatch incompatible transfusion) can result in lethal antibody-mediated hemolysis. Less well appreciated is the ability of anti-RBC antibodies to specifically remove their target antigen from donor RBC without compromising cell survival or adversely affecting the transfusion recipient. In an effort to elucidate the mechanistic details of this process, we describe the first animal model of non-hemolytic antibody-induced RBC antigen loss. RBC from transgenic mHEL mice express surface hen egg lysozyme (HEL) as a transmembrane protein. Transfusion of mHEL RBC into mice immunized with HEL results in selective loss of HEL antigen from donor RBC without affecting other blood group antigens or reducing the circulatory lifespan of the transfused RBC. While this process does not require the presence of a spleen, it requires both anti-RBC IgG antibodies and the FcgammaIII receptor. These studies provide mechanistic insight into the phenomenon of antigen loss during incompatible transfusion in humans.
Introduction:

It has long been appreciated that antibodies against red blood cell (RBC) antigens are a major cause of both hemolytic transfusion reactions in the case of alloantibodies and autoimmune hemolytic anemia (AIHA) in the case of autoantibodies. Most acquired anti-RBC antibodies promote phagocytosis of RBC by cells of the reticular endothelial system (RES) leading to a delayed extravascular hemolysis that occurs over the course of days to weeks. Patients with AIHA exhibit significant and potentially life-threatening hemolysis that can involved the destruction of the majority of their own RBC. However, some RBC can escape antibody-mediated hemolysis through selective loss of the antigen recognized by alloantibodies or autoantibodies. "Antigen suppression" of this type has most often been observed with Kell blood group antigens, but the phenomenon has also been reported with Rh (including D and e), Kidd, Duffy, Lutheran and other blood group antigens such as LW, Co, Ge, En and AnWj.

When suppression of antigen occurs, patient specimens test negative by the direct antiglobulin test (DAT), which measures the presence of antibody on the surface of RBC by incubating RBC with anti-Human Ig. As many as 10% of AIHA patients exhibit a negative DAT. In some cases, DAT negative AIHA is due to levels of RBC binding antibody below the threshold of detection by DAT, low-affinity IgG autoantibodies that elute during the DAT procedure, or autoantibody isotypes not detected by standard Coombs reagent (i.e. IgA or IgM). However, in some cases of DAT negative AIHA, antigen suppression has been formally demonstrated by showing a loss of detectable antigen by western blot analysis of RBC. In this setting, masking of the antigen by an immunoglobulin not recognized by DAT is not feasible, since the western blots are
performed under denaturing conditions that would dissociate antigen-antibody complexes. While loss of antigen in the presence of binding antibody has been clearly demonstrated for each of the main clinically significant RBC antigens, no mechanistic elucidation of this process has been carried out.

In the current report, we present an animal model of antibody-induced RBC antigen suppression. Using a transmembrane form of hen egg lysozyme (mHEL) on the surface of murine erythrocytes as a model blood group antigen, we report that transfusion of mHEL RBC into mice that were pre-immunized with HEL resulted in selective loss of mHEL from the erythrocyte surface without a decrease in the circulatory lifespan of the RBC. This antigen loss was highly specific, as other RBC surface antigens not recognized by antibodies in the transfusion recipient were not decreased. Loss of mHEL antigen in the presence of anti-HEL antibodies did not occur in vitro, indicating that both anti-HEL and other in vivo factors were required. Additionally, while splenectomy did not decrease the extent of antigen loss, antigen loss was prevented by targeted deletion of Fcγ receptors, suggesting the requirement for an interaction between antibody-coated RBC and FcγR expressing host cells. Together, these studies demonstrate the first animal model of antigen suppression on transfused RBC and provide unique mechanistic insight into this phenomenon that is known to occur in the setting of human transfusion and AIHA.
Materials and Methods

Mice

C57BL/6 mice, RAG knockout -/- and FcgrIII KO mice were obtained from Jackson Laboratories (Bar Harbor, ME). Fcgr2B KO mice were obtained from Taconic Labs (Germantown, NY). mHEL mice (available from Jackson Labs) were bred by the Emory Division of Animal Resources Animal Husbandry service. All knockout mice and the mHEL mice are on a C57BL/6 background. All mice were male, aged 8-12 weeks.

Leukoreduction of Blood

Peripheral blood was obtained from donor mice by retro-orbital bleed and was passed over a sterile pediatric leukoreduction filter (Purecell Neo Neonatal High Efficiency Leukocyte Reduction Filter, PALL Biomedical Products, NY) pre-equilibrated with PBS, adjusted to 340 Osm with NaCl (MPBS).

Fluorescent Labeling of RBC and Transfusion

Leukoreduced RBC were labelled with CM-DiI or DiO according to manufacturer's instructions (Molecular Probes, Eugene OR). All transfusions were carried out by left lateral tail vein injection.

Immunization with HEL or OVA

HEL and chicken ovalbumin (OVA) (Sigma, St. Louis MO) were emulsified in complete Freund's adjuvant (CFA) at a concentration of 2 µg/µL. 100 µg were injected into the left hind footpad and an additional 100 µg were injected into the left flank. All animals were used 2-3 weeks post immunization. After confirmation of adequate seroconversion
following HEL/CFA immunization, surgical splenectomy was performed on some animals, which were then subsequently transfused with mHEL RBC.

**HEL-Specific ELISA**

96 well plates were coated with HEL or OVA and then blocked with ELISA buffer (10 µg/µL BSA in PBS) for one hour. Test serum was diluted in ELISA buffer and was incubated with the wells for one hour. Wells were washed and incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (HRP). HRP linked anti-IgG, anti-IgA, anti-IgE and anti-IgM (Jackson immunologicals, West Grove, PA) were used at a dilution of 1:5000. HRP linked anti-IgG1 and anti-IgG2a were used at a dilution of 1:5000, while anti-IgG2b, anti-IgG2c and anti-IgG3 were used at 1:10,000 (Bethyl Labs, Montgomery TX). ABTS HRP substrate (Roche, Indianapolis IN) was added to the wells to detect HRP and absorbance was read at 415 nM. To properly subtract background signal, each sample was incubated with wells coated with either HEL or OVA. The value for each OVA coated well was subtracted from the values from the corresponding HEL coated wells. A separate background value was generated for every sample at every dilution to control for animal-to-animal variation.

**Flow Cytometry**

Peripheral blood was obtained by retro-orbital bleeding and was collected in an RBC preservative (adenine/citrate/dextrose). Since DiI and DiO fluorescence was detected on FL-1 and FL-2, respectively, all antibody staining was performed with labels that emit on FL-3 or FL-4. RBC were stained with a 1:50 dilution of serum from mice immunized
with HEL/CFA (anti-HEL) or OVA/CFA (anti-OVA) followed by a 1:100 dilution of goat anti-mouse immunoglobulin conjugated to APC (Pharmingen, San Diego CA). DAT were performed with only goat anti-mouse APC. For analysis of IgG subtypes bound to mHEL RBC, subtype-specific antisera linked to HRP (Bethyl labs) was used at a dilution of 1:100 followed by staining with Cy5-labeled anti-HRP (Jackson Immunologicals). All samples were analyzed using a 4-color FacsCaliber flow cytometer.
Results:

Erythrocytes from mHEL mice express surface-bound HEL. The mHEL mouse expresses a transgene consisting of a fusion between HEL and a single-pass transmembrane domain\textsuperscript{15}. To assess expression of mHEL on RBC, peripheral blood was analyzed by flow cytometry. Since mHEL mice are on a C57BL/6 background, we used wild type C57BL/6 mice as a negative control. RBC from mHEL mice showed significantly greater immunoreactivity with anti-HEL antisera compared to RBC from wild type C57BL/6 mice (Figure 1A). This interaction was specific to the anti-HEL antisera, since mHEL cells were negative using antisera to a separate antigen, ovalbumin (OVA). Preincubation of anti-HEL antisera with soluble HEL, but not OVA, inhibited anti-HEL binding (Figure 1B and 1C), confirming that anti-HEL was recognizing HEL epitopes on the surface of mHEL RBC. The mHEL-RBC+anti-HEL specimen in panels A-C (solid line) represents the same sample displayed in three panels for clarity.

To determine if the presence of HEL on the surface of mHEL RBC altered any biological properties of RBC, we utilized two lipophillic tracking dies that have long half-lives and typically do not alter membrane characteristics of the labeled cells. RBC from C57BL/6 mice were labeled with DiO and RBC from mHEL mice were labeled with CM-DiI. DiO and CM-DiI fluoresce at different wavelengths, which allows simultaneous tracking of both populations by flow cytometry. Initial flow cytometry studies established appropriate fluorescence compensation parameters such that no crossover in fluorescence was observed with DiO- or CM-DiI-labeled cells (data not shown).
To prevent contaminating leukocytes from interfering with interpretation, blood was stringently leukoreduced prior to labeling with DiO or CM-DiI by using commercially available RBC leukoreduction filters. Leukoreduction resulted in an approximate 4-log_{10} reduction in contaminating leukocytes (data not shown). Leukoreduced C57BL/6 and mHEL RBC labeled with DiO or CM-DiI were mixed and transfused into RAG knockout recipients on a C57BL/6 background. Using RAG knockout mice ensured that interference by potential anti-HEL humoral immune responses to transfused mHEL RBC would not occur. Blood from untransfused mice was used to establish flow cytometry gates such that there were no cells in the DiO or CM-DiI gates (data not shown).

Blood was collected at periodic points over a 5-week time course and the rate of clearance of C57BL/6 or mHEL RBC was calculated by determining the percentage of remaining cells by flow cytometry, an example of which is shown (Figure 1D). RBC from C57BL/6 and mHEL RBC had a similar half-life of approximately 11 days (Figure 1E). Relative rates of clearance were analyzed by dividing the percentage of remaining mHEL RBC by the percentage of remaining C57BL/6 RBC at each time point. This calculated percentage was constant over 5 weeks indicating that the circulatory half-life of mHEL RBC was not altered compared to wild type RBC (Figure 1F).

To determine the persistence of the mHEL antigen over the lifespan of transfused mHEL RBC, blood obtained at each time point was stained with anti-HEL followed by a secondary antibody conjugated to APC. The fluorescence of APC at a wavelength distinct from either DiO or CM-DiI allowed analysis of HEL antigen on the transfused cells. Transfused RBC from mHEL mice (CM-DiI +) stained positive with anti-HEL
compared to transfused RBC from C57BL/6 mice (DiO⁺) at each of the tested time points over a 5-week period (Figure 1G). Since no significant decrease in mean fluorescence of anti-HEL staining was observed, we conclude that the mHEL antigen is stable on transfused mHEL RBC. The staining with anti-HEL was specific, since no shift was seen in either CM-DiI or DiO populations stained with anti-OVA (data not shown). Together, the above data demonstrate that mHEL RBC have a normal circulatory half-life and that the mHEL antigen is stable in vivo on the RBC surface.

**Immunization of recipient mice with HEL/CFA results in high titer IgG1 anti-HEL that binds efficiently to mHEL RBC.** To generate transfusion recipients that are crossmatch incompatible with mHEL RBC, wild type C57BL/6 mice were immunized with HEL in complete Freund's adjuvant (HEL/CFA). Two weeks after immunization, serum was isolated from peripheral blood and the presence of anti-HEL antibodies was assayed using a HEL specific ELISA.

Serum from HEL/CFA immunized mice contained high levels of anti-HEL IgG antibodies compared to serum from unimmunized mice (Figure 2A). No significant anti-HEL of the IgA, IgE or IgM type was detected. Each of the secondary antibodies was reactive with wells coated with whole mouse serum (data not shown), indicating that the lack of IgA, IgE or IgM was not an artifact of nonreactive secondary antibodies. To determine which subtypes of IgG were involved in the anti-HEL response, additional ELISA assays were carried out using IgG subtype specific antibodies (Figure 2B). IgG1 was the most abundant anti-HEL antibody, detectable at a titer of at least 1:20,000. Considerable amounts of IgG2b and lesser but significant amounts of IgG2c and IgG3
were also observed. No anti-HEL IgG2a was detectable in any specimens from HEL/CFA immunized mice. Since IgG2a is not expressed by C57BL/6 mice, the absence of detectable IgG2a is a predicted finding that serves as a negative control for the specificity of the anti-IgG reagents.

To test the capacity of the different subtypes of IgG to bind to mHEL RBC, serum from HEL/CFA immunized mice was incubated with RBC from either mHEL or C57BL/6 mice, followed by staining with secondary antibodies specific for IgG subtypes (Figure 2C). Based upon this flow cytometric crossmatch procedure, IgG1 was the predominant isotype that bound to mHEL RBC. Lesser amounts of IgG2b and IgG2c were detected, whereas no significant IgG3 or IgG2a were observed binding to mHEL RBC. Thus, HEL/CFA-immunized mice were crossmatch-incompatible with mHEL RBC and IgG1 was the predominant subtype of antibody that bound mHEL RBC.

**Transfusion of mHEL RBC into HEL immune mice.** We hypothesized that transfusion of mHEL RBC into HEL/CFA immunized mice would result in rapid removal of mHEL RBC. To test this hypothesis, leukoreduced mHEL RBC were transfused into HEL/CFA immunized or unimmunized mice (Figure 3). At 6 hours post transfusion, the amount of antibody bound to mHEL RBC was visualized by staining with anti-mouse Ig. This assay is the functional equivalent of a DAT, which is the methodology by which crossmatch incompatible transfused RBC are monitored in human transfusion recipients. A small population of antibody-coated RBC (DAT+) was detected in HEL-immunized mice transfused with mHEL RBC (Figure 3A). This population, which constituted 2.3% of RBC, was absent from either HEL-immunized mice transfused with wild type
C57BL/6 RBC, or from unimmunized mice transfused with mHEL RBC (Figure 3B and 3C). Staining of blood specimens with anti-HEL antibodies demonstrated a population of mHEL+ RBC in the unimmunized mice that were transfused with mHEL RBC, which constituted approximately 5% of RBC (Figure 3F). Thus, transfusion of mHEL RBC into HEL immunized mice results in antibody coating of the transfused cells and rapid removal of HEL+ RBC with an approximately 50% removal by 6 hours.

**Antibody-dependent removal of HEL+ RBC is due to antigen loss.**

To test the extent of hemolysis of mHEL RBC, HEL/CFA-immunized mice and control OVA/CFA-immunized mice were transfused with a mixture of C57BL/6 RBC labeled with DiO and mHEL RBC labeled with CM-DiI. Recipient mice were bled at the indicated time points, and flow cytometry was used to track the survival of transfused cells. At 6 hours post-transfusion, there were fewer mHEL RBC than C57BL/6 RBC in both the HEL/CFA-immunized and control OVA/CFA-immunized mice (Figure 4A). However, the ratio of mHEL:B6 RBC was lower (0.67) in the HEL/CFA-immunized mice than in the control OVA/CFA-immunized mice (0.87). This pattern suggests a degree of selective removal of mHEL RBC in HEL/CFA-immunized mice and is consistent with what is seen in the early stages of a delayed hemolytic transfusion reaction in humans. However, the ratios of mHEL:B6 RBC then stabilized and remained constant until at least 72 hours (Figure 4A).

To assess the extent to which the transfused RBC were being coated with antibodies, DAT testing was performed by staining blood with anti-mouse Ig. Antibody coating of RBC was assessed by gating on transfused mHEL RBC (CM-DiI+) and
comparing their anti-mouse Ig staining with transfused C57BL/6 RBC (DiO⁺) (Figure 4B). At 6 hours post-transfusion, many of the transfused mHEL RBC were positive compared to transfused C57BL/6 RBC. At 17 hours post-transfusion, the transfused mHEL RBC remained positive compared to transfused C57BL/6 RBC, but the binding of anti-mouse Ig was decreased from the 6-hour determination. By 72 hours, no detectable IgG was on the surface of transfused mHEL RBC compared to transfused C57BL/6 RBC. No IgG was detected on mHEL RBC that were transfused into control OVA/CFA immunized mice at any timepoint. The progressive decline in DAT⁺ cells was not an artifact of exhausting the anti-HEL, as anti-HEL titers remained high in HEL/CFA post transfusion (data no shown).

While the above data suggest limited hemolysis of transfused mHEL RBC at early time points, a second population containing the majority of transfused mHEL RBC continue to circulate. Based upon these data, we rejected the hypothesis that the disappearance of DAT⁺ cells from this second population was due to hemolysis. Since the transfused mHEL cells progressed from a DAT⁺ state to a DAT⁻ state from 6 to 72 hours without a concomitant decrease in the number of circulating RBC, we hypothesized that the mHEL cells were progressively losing mHEL antigen. To test this possibility, blood from the 72-hour time point was stained with anti-HEL (Figure 4C). No HEL antigen was detected on mHEL RBC that were transfused into HEL/CFA immunized mice, but high levels of mHEL antigen were present on mHEL RBC that had been transfused into control OVA/CFA-immunized mice. The lack of detectable HEL on transfused mHEL RBC from HEL/CFA-immunized mice was not an artifact of existing antibodies masking the HEL epitope, since the DAT (Figure 4B), which used a pan anti-
mouse globulin reagent that reacts with IgG, IgA and IgM, was negative. Loss of HEL from the RBC surface was an antigen specific event, as no decrease in staining with an antibody against a separate blood group antigen (TER-119) was observed (data not shown).

**Transfused mHEL RBC that have lost their antigen have a normal circulatory lifespan.** In the setting of human transfusions, RBCs undergoing an antibody-mediated hemolysis can have a portion of their membrane removed by cells of the RES. In this scenario, the remaining cell portion usually consists of a spherocyte or schistocyte with an altered size, shape, and a diminished circulatory lifespan. To test the possibility of this occurring in the mHEL model, HEL/CFA-immunized mice were transfused with a mixture of C57BL/6 RBC (DiO labeled) or mHEL RBC (CM-DiI labeled) and were followed over the course of a month. Percentages of surviving cells were determined by flow cytometry at weekly time points. Despite complete antigen loss by day 3 (data not shown), the half-life of transfused mHEL RBC was not significantly altered from that of transfused C57BL/6 (Figure 5A). To directly visualize potential alterations in RBC morphology as a result of antigen loss, CM-DiI-labeled mHEL RBC that had lost their antigen subsequent to transfusion into HEL/CFA-immunized mice were isolated by flow activated cell sorting (FACS). As a control, co-transfused DiO labelled C57BL/6 RBC were also sorted, and both populations were subjected to a peripheral blood smear. Although deformed RBC were visible in both populations, likely as a result of damage during sorting, RBC with normal biconcave morphology were clearly visible in the mHEL RBC that had lost antigen (Figure 5B) with a similar frequency to biconcave cells.
in the control C57BL/6 RBC. No schistocytes were observed in either population. High power magnification confirmed that mHEL RBC have a normal morphology despite antigen loss, and demonstrated that they are normochromic RBC. These data argue against a model in which antigen is lost due to removal of a significant portion of the RBC membrane. Too few RBC were recovered to allow confirmation of antigen loss by western blot analysis.

**Passive transfer of anti-HEL serum results in antigen loss in vivo but not in vitro.**

Cell-free serum was isolated from HEL/CFA- or OVA/CFA-immunized mice and was passively transferred to unimmunized recipients. The level of anti-OVA in serum from OVA/CFA-immunized mice was equivalent to anti-HEL in serum from HEL/CFA-immunized mice (data not shown). Several hours after transfer of serum, recipient mice were transfused with a mixture of mHEL RBC (CM-DiI) and B6 RBC (DiO). At the same time that mice were transfused, a portion of the transfusion mixture was placed in tissue culture with serum from HEL/CFA- or OVA/CFA-immunized mice. Since HEL antigen is generally undetectable on mHEL RBC after 3 days of circulation in HEL/CFA-immunized mice, blood was harvested 2 days post-transfusion to allow analysis of samples at a time point when antigen loss should be ongoing. All specimens were stained with anti-mouse Ig alone (DAT) or with anti-HEL.

Staining of a pretransfusion specimen demonstrated that the mHEL RBC were strongly immunoreactive with anti-HEL compared to C57BL/6 RBC (Figure 6A). After two days, mHEL RBC transfused into mice that received anti-HEL serum showed a low level of antibody coating as measured by DAT using anti-mouse Ig (Figure 6B), while no
DAT⁺ cells were detected in transfusion recipients that received either anti-OVA serum or no serum (Figure 6C and 6D). Staining with anti-HEL revealed high levels of HEL antigen on mHEL RBC transfused into control mice (Figure 6H and 6I) that were comparable to the pretransfusion sample. However, the level of anti-HEL staining of mHEL RBC transfused into mice given anti-HEL serum was significantly reduced and at a level equivalent to the DAT⁺ (Figure 6G). These findings demonstrate that similar to HEL/CFA immunized mice, mHEL RBC become DAT⁻ with loss of detectable HEL antigen when transfused into unimmunized animals that received a passive transfer of anti-HEL serum. In contrast to these *in vivo* findings, cells incubated for the same period of time in the presence of anti-HEL serum *in vitro* remained DAT⁺ and displayed the same level of HEL antigen as the pretransfusion specimen (Figure 6E and 6J). *In vitro* incubation of control anti-OVA serum had no positive signal on DAT and did not alter levels of HEL antigen (Figure 6F and 6K).

Together, these data indicate that passive transfer of cell free immune serum was sufficient to induce loss of mHEL from RBC. This was not an effect of cytokines or other non-specific inflammatory molecules associated with immunization, as serum from OVA/CFA-immunized mice did not induce antigen loss. These findings suggest that anti-HEL antibodies induced by HEL/CFA immunization are responsible for HEL antigen loss and that cellular immunity is not required. In addition, the process of antigen loss does not appear to be an intrinsic property of the antibody-RBC interaction, as no antigen loss was observed with RBC that were incubated with immune serum *in vitro*. Thus, the process of antigen loss appears to require interactions between antibodies and other *in vivo* host factors.
Role of Fcγ receptors in antibody induced antigen loss from transfused RBC. Since mHEL RBC must be both exposed to antibody and circulating in vivo for antigen loss to occur, and anti-HEL in HEL/CFA immunized mice is almost exclusively of the IgG isotype, we hypothesized that Fcgamma receptors (FcγR) would be involved in antibody induced antigen loss from mHEL RBC. Three FcγRs have been described in mice. FcγRI is a high affinity receptor and has the ability to bind uncomplexed monomeric IgG. FcγRII and FcγRIII have a lower affinity than FcγRI. Due to decreased affinity, FcγRII and FcγRIII only bind to IgG that is involved in either an immune complex or bound to a cell surface, such as antibody coated RBC. FcγRIII is a stimulatory receptor, the ligation of which leads to increased phagocytosis and cellular activation, while FcγRII is inhibitory to these processes. It has been reported that FcγRIII is required for phagocytosis of RBC coated with IgG1 in mice and that FcγRIII plays a central role in antibody induced AIHA. While FcγRI can also be involved in phagocytosis of RBC coated in antibody, it preferentially binds to RBC coated with IgG2a antibodies. Since FcγRIII is required for phagocytosis of IgG1 coated RBC by macrophages, and the main anti-HEL antibody coating mHEL RBC transfused into HEL/CFA immunized mice is IgG1 (see figure 2), we hypothesized that FcγRIII would be required for antibody induced antigen loss.

To test this hypothesis, anti-HEL serum was passively transferred into mice with a targeted deletion of the FcγRIII (FcγRIII KO) or the FcγRII (FcγRII KO) genes. As a positive control, antiserum was also transfused into wild-type C57BL/6 mice. Six hours
after transfer of antibody, mice were transfused with a mixture of mHEL RBC and
C57BL/6 RBC labeled with CM-DiI or DiO, respectively. As a negative control, animals
from each group that had received no antisera were also transfused. Peripheral blood was
collected 4 days post transfusion and analyzed by flow cytometry.

Transfusion into C57BL/6 mice that received anti-HEL resulted in a loss of HEL
antigen (Figure 7A and 7B), while HEL antigen persisted on RBC transfused into
C57BL/6 recipients that did not receive anti-HEL antiserum (Figure 7C and 7D). In
contrast, mHEL RBC transfused into FcγRIII KO mice were coated with
immunoglobulin, but did not undergo antigen loss (Figure 7E and 7F) compared to
FcγRIII KO mice that did not receive anti-HEL antiserum (Figure 7G and 7H). This
observation was not an artifact of the anti-HEL antiserum not functioning in the FcγRIII
KO mice, as the mHEL RBC were coated with Ig (Figure 7E). This demonstrates that
antigen loss does not occur in FcγRIII KO mice and suggests that FcγRIII is required for
antibody induced antigen loss from mHEL RBC. HEL antigen was lost from mHEL
transfused into FcγRII KO to the same extent as wild type C57BL/6 mice (Figure 7I and
7J). No antigen loss was observed in FcγRII KO mice that didn't receive anti-HEL
antisera (Figure 7K and 7L). Since the FcγRIII and FcγRII KO mice are on a C57BL/6
background, these findings are not likely the result of genetic differences other than the
knocked out gene.

We hypothesized that splenic macrophages were involved in this process. To test
this hypothesis, after confirming adequate seroconversion of HEL/CFA immunized
C57BL/6 mice, surgical splenectomies were performed followed by transfusion of CM-
DiI labeled mHEL RBC and DiO labeled B6 RBC as above. Neither the kinetics nor the extent of antigen loss was diminished in splenectomized animals (data not shown).

Together, these data indicate that antigen loss of HEL from transfused mHEL RBC depends upon recognition of antibody-coated RBC by FcγRIII bearing cells. Precisely which cell type is unclear, but splenic macrophages are not required.
Discussion:

The above data formally demonstrate the phenomenon of alloantibody induced non-hemolytic antigen loss in a murine model of RBC transfusion. Although the frequency of non-hemolytic antigen loss in the clinical setting is unknown, it is clear that this phenomenon does occur in humans. Suppression of blood group antigens has most often been documented in the setting of AIHA. The precise frequency of antigen suppression in AIHA is uncertain, but approximately 10% of AIHA patients have a negative DAT. Although the percentage of DAT-negative AIHA patients who have antigen suppression is unknown, the frequency of antigen suppression in AIHA may be significant.

Speculation as to the frequency of antigen suppression due to alloantibodies after transfusion of crossmatch-incompatible blood is less straightforward. The main existing paradigm in the field of transfusion-based immunohematology dictates that a hemolytic transfusion reaction is the likely outcome of transfusing crossmatch incompatible blood into a patient with an alloantibody against a clinically significant antigen, such as Rh, Kell, Kidd or Duffy. However, antigens in each of these blood group systems have also been shown to be capable of undergoing a process of antigen suppression in the setting of autoantibodies and antigen suppression on transfused RBC has been confirmed in the case of several alloantibodies. In the rare cases that crossmatch incompatible transfusions are given, either as a "least incompatible" unit or due to a clerical error, a progressive decrease in DAT is interpreted as antibody-mediated destruction of the transfused RBC. Given the clinical signs and symptoms of hemolysis that can occur, some degree of antibody-mediated hemolysis is almost a certainty in at least some cases.
However, since a negative DAT is equally consistent with RBC destruction and antigen loss, and because transfused RBC are not routinely detected by means other than DAT, the extent to which conversion to a DAT-negative status reflects destruction of transfused RBC as opposed to non-hemolytic antigen loss is unclear.

To date, several hypotheses have been proposed to explain the mechanism of antibody-induced antigen suppression. One explanation suggests that the autoantibodies, or other host factors, create an environment in which synthesis of the recognized antigen is inhibited. This hypothesis was ruled out in two cases of AIHA-induced suppression of Kell antigens by demonstrating loss of presynthesized antigens on transfused RBC. Consistent with the notion of loss of presynthesized antigen, several groups have proposed that antigens are degraded on mature erythrocytes by proteases, deglycosidases, or reductases from microbial pathogens infecting the patient during the AIHA illness. This possibility cannot be excluded, as some patients with antigen suppression have documented microbial infections. However, re-appearance of the antigen in question on the RBC surface is routinely seen after resolution of the autoantibody and relapse of the antibody can re-suppress antigen. Thus, antigen suppression is more closely correlated to the presence of antibody than to documented microbial infections.

In our model of non-hemolytic antibody-mediated loss of alloantigen, suppression of antigen synthesis is not a possibility since unlike AIHA, mature antigen positive RBC are transfused into alloimmunized recipients. Thus, the conversion of mature HEL⁺ RBC to HEL⁻ RBC confirms a mechanism of antigen loss rather than suppression of synthesis. In this setting, destruction of antigen by microbial infection is highly unlikely, since mice were kept in pathogen-free environments. In addition, antigen loss occurred in 100% of
animals with anti-HEL antibodies and 0% of animals without anti-HEL antibodies in numerous experiments conducted over the course of 8 months, during which time multiple animals were used from different litters. Based on these factors, we reject the hypothesis of that antigen is destroyed by microbial pathogens.

The ability to induce antigen loss can be transferred with cell-free serum from HEL/CFA-immunized mice, but not serum from OVA/CFA-immunized animals. In addition, antigen loss does not occur in mice with a targeted deletion of FcγRIII, which specifically binds to immunoglobulins. Together, these data support a model in which antigen loss is induced through the interaction of RBC, anti-RBC antibodies, and FcγRIII-bearing cells. The exact mechanism of antigen loss remains to be determined, but may include either shedding of the antigen by the RBC or enzymatic destruction of the antigen through release of proteases from FcγRIII-bearing macrophages that degranulate on antibody coated RBC.

It is unclear why some crossmatch-compatible transfusions in humans clearly lead to hemolysis while others lead to non-hemolytic antigen loss. Differences in RBC antigens are an unlikely explanation, since multiple transfusions of Kell positive RBC units into a patient with anti-Kell AIHA gave consistent antigen loss of Kell from a variety of different RBC donors. It thus seems more likely that a particular characteristic of a given anti-Kell antibody response is responsible for antigen loss in some settings and hemolysis in others. Ongoing investigations into the nature of antibodies capable of inducing non-hemolytic antigen loss may lead to the generation of antibody reagents that can be used to purposefully induce antigen loss. Such reagents...
could have considerable therapeutic potential by allowing transfusion of crossmatch-
incompatible units into multiply alloimmunized patients.
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References:


Figure Legends:

Figure 1. RBC from mHEL mice express HEL as a surface antigen. Blood was harvested from mHEL or C57BL/6 mice and stained with anti-HEL or anti-OVA antisera followed by a fluorescent secondary antibody (A). Blood from mHEL mice was stained with anti-HEL that was pre-incubated with HEL or OVA protein followed by secondary antibody (B and C). The mHEL-RBC+ anti-HEL specimen in panels A-C (solid line) represents the same sample displayed in multiple panels for clarity. Leukoreduced RBC from mHEL and C57BL/6 mice were labeled with CM-DiI and DiO, respectively. The cells were then mixed and transfused into RAG-/- KO mice. Peripheral blood was harvested and the percentages of remaining transfused mHEL or C57BL/6 RBC were determined by flow cytometry (D). Lifespan of transfused mHEL and C57BL/6 RBC was determined by enumerating remaining mHEL or C57BL/6 RBC by flow cytometry at the indicated time points (E and F). At each indicated time point, the level of mHEL on mHEL RBC was determined by staining with anti-HEL and gating on labeled transfused mHEL or C57BL/6 cells (G). The experiments shown in this figure have been reproduced in at least three separate experiments. The data presented in this figure are representative results. Survival studies past two weeks have been performed twice with identical results.

Figure 2. IgG1 is the predominant antibody that binds to mHEL RBC in HEL/CFA Immunized mice. C57BL/6 mice were immunized with HEL/CFA. Two weeks after immunization, serum was harvested. The titer, isotype and subtype of anti-HEL antibodies were determined by HEL-specific ELISA (A and B). The capacity of each
subtype to bind to mHEL RBC was determined by incubating serum from HEL/CFA-immunized mice, at a dilution of 1:50, with RBC from either mHEL or C57BL/6 mice followed by IgG subtype specific antibodies. Binding was measured by flow cytometry \((C)\). Analysis of this type was performed on all groups of immunized mice in all subsequent experiments to confirm similar patterns of immunization. Essentially identical results were obtained in all animals. The experiments shown in this figure have been reproduced in at least three separate experiments. The data presented in this figure are representative results.

**Figure 3. Transfusion of mHEL RBC into HEL/CFA immunized mice results in rapid removal of HEL\(^+\) RBC.** C57BL/6 mice were immunized with HEL/CFA. Two weeks post immunization, immunized mice and control unimmunized mice were transfused with leukoreduced mHEL RBC. Peripheral blood was harvested 6 hours later and stained with anti-mouse Ig to detect antibody-coated cells, which were visualized by flow cytometry \((A-C)\). HEL\(^+\) cells were detected in peripheral blood from each group of animals by staining with anti-HEL followed by anti-mouse Ig \((D-F)\). The experiments shown in this figure have been reproduced in at least three separate experiments. The data presented in this figure are representative results.

**Figure 4. Transfusion of mHEL RBC into HEL/CFA immunized mice results in loss of HEL antigen without clearance of mHEL RBC.** C57BL/6 mice were immunized with HEL/CFA or OVA/CFA. Immunized mice were then transfused with a mixture of leukoreduced CM-DiI labeled mHEL RBC and DiO labeled C57BL/6 RBC. Peripheral
blood was obtained at the indicated time points. The remaining percentages of transfused mHEL or C57BL/6 RBC were determined by detecting residual labeled RBC by flow cytometry (A). At the indicated time points, peripheral blood was stained with anti-mouse Ig and the amount of immunoglobulin coating RBC was determined by gating on transfused mHEL or C57BL/6 RBC and measuring bound anti-mouse Ig by flow cytometry (B). Levels of HEL antigen were measured by staining with anti-HEL followed by anti-mouse Ig and comparing staining on transfused mHEL or C57BL/6 RBC (C). The experiments shown in this figure have been reproduced in at least three separate experiments. The data presented in this figure are representative results.

Figure 5. mHEL RBC that have been transfused into HEL/CFA immunized mice have a normal circulatory lifespan and morphology despite loss of antigen. A mixture of leukoreduced CM-DiI labeled mHEL RBC and DiO labeled C57BL/6 RBC were transfused into HEL/CFA immunized mice. Antigen loss was confirmed by staining with anti-mouse Ig or anti-HEL followed by anti-mouse Ig (data not shown). At the indicated time points, the percentage of remaining transfused mHEL or C57BL/6 RBC was determined by flow cytometry (A). After confirmation of mHEL antigen loss, mHEL RBC (CM-DiI) and C57BL/6 RBC (DiO) were re-isolated by FACS and analyzed by peripheral blood smear (B). The experiments shown in this figure have been reproduced in at least three separate experiments. The data presented in this figure are representative results. Flow sorting for morphological analysis was performed twice.
Figure 6. Passive transfer of cell-free serum from HEL/CFA-immunized mice is sufficient to induce HEL antigen loss from transfused mHEL RBC, but antigen loss does not occur in vitro. A mixture of leukoreduced CM-DiI labeled mHEL RBC and DiO labeled C57BL/6 RBC was transfused into mice that had previously received an intravenous injection of HEL/CFA immune serum (B and G), OVA/CFA immune serum (C and H) or no injection (D and I). The mixture of RBC was also incubated with HEL/CFA or OVA/CFA immune serum in vitro (E-K). Two days after transfusion, peripheral blood was harvested. Antibody coating of RBC was measured by gating on transfused mHEL (CM-DiI+) or C57BL/6 (DiO+) cells and comparing staining with anti-mouse Ig. Remaining HEL antigen was measured by staining with anti-HEL. The experiments shown in this figure have been reproduced in at least three separate experiments. The data presented in this figure are representative results.

Figure 7. Antibody induced antigen loss requires the FcγIII receptor. C57BL/6 mice (A-D) or mice with targeted deletions of either the FcγIIIR (E-H) or FcγIIR (I-L) received an intravenous injection of HEL/CFA immune serum. Six hours later, each group was transfused with a mixture of leukoreduced CM-DiI labeled mHEL RBC and DiO labeled C57BL/6 RBC. Four days after transfusion, peripheral blood was harvested and antibody coating of RBC was measured by gating on transfused mHEL (CM-DiI+) or C57BL/6 (DiO+) cells and staining with anti-mouse Ig. Remaining HEL antigen was measured by staining with anti-HEL. The experiments shown in this figure have been reproduced in at least three separate experiments. The data presented in this figure are representative results.
Figure 1

A. mHEL RBC+ anti-HEL
B. mHEL RBC+ anti-HEL
C. mHEL RBC+ anti-HEL
D. mHEL RBC+ anti-OVA
E. mHEL RBC+ anti-HEL
F. mHEL RBC+ anti-HEL
G. mHEL RBC+ anti-HEL

Gated on mHEL RBC
Gated on B6 RBC

1 day
1 week
2 weeks
3 weeks
5 weeks
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Passive Transfer of anti-HEL

No Transferred antibody

Figure 7
Non-Hemolytic Antibody-Induced Loss of Erythrocyte Surface Antigen

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