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Development of anti-Bw6 reactivity in patients receiving r-GCSF: a preliminary report

M. F. LEACH AND J. P. AUBUCHON

Recombinant granulocyte colony-stimulating factor (r-GCSF) is used in autologous bone marrow/peripheral blood progenitor cell transplantation (ABMT/PBPC) to shorten the period of neutropenia. As these patients require platelet transfusions, their sera may be monitored for the presence of platelet/human leukocyte antigen (HLA) antibodies. Sera of some patients on r-GCSF (16 µg/kg/day) became difficult to evaluate in vitro for the presence of HLA and platelet antibodies because of apparently anomalous reactions in solid phase red cell adherence (SPRCA) assays. SPRCA tests were positive only when platelets were adhered to the polystyrene plates in the presence of glucose; when other simple sugars were used, the patients' sera failed to react. HLA Bw6-positive platelets were more likely than HLA Bw6-negative platelets to be reactive (p < .001). The transfusion of HLA Bw6-positive platelets to patients displaying this in vitro reactivity (positive patients) resulted in a 50 percent lower corrected count increment (CCI) than those given to patients without it (negative patients; p = <.001). When all transfusions were considered, the CCI for those of the positive patients was decreased 73 percent when compared to the control patients (p = .0005). The presence of the antibody also was associated with a twofold increase in the number of platelet transfusions given (p = .0005). ABMT/PBPC patients receiving r-GCSF may develop unexpected reactions on SPRCA antibody screens and poor responses to transfusion of Bw6-positive platelets. *Immunohematology* 2001;17:63–69.

Key Words: r-GCSF, marrow/progenitor cell transplant, anti-Bw6

Potent hematopoietic stimulants offer important clinical benefits in chemotherapy and marrow transplant patients. Analogs of naturally occurring hormones and cytokines have reduced morbidity and these patients have shortened hospital stays, but they may elicit undesirable immunologic side effects. This study describes the discovery, investigation, and presumptive resolution of an unexpected serologic finding associated with use of a common hematopoietic stimulant.

Because of the frequency and clinical significance of refractoriness to platelet transfusion associated with the development of human leukocyte antigen (HLA) and/or platelet-specific alloantibodies, some institutions regularly screen patients receiving platelet transfusions to detect the formation of these antibodies and redirect hemotherapy. We encountered a group of patients undergoing autologous bone marrow/peripheral blood progenitor cell transplantation (ABMT/PBPC) who appeared to develop blunted responses to platelet transfusion while receiving recombinant granulocyte colony-stimulating factor (r-GCSF); all demonstrated anomalous serologic activity in HLA/platelet antibody screening associated with development of a novel glucose-dependent antibody. The investigation of this phenomenon and its resolution is the subject of this report.

All hematology-oncology patients (including those undergoing ABMT/PBPC) treated at this institution are routinely tested by a solid phase red cell adherence (SPRCA) assay for the development of HLA- and/or platelet-specific antibodies. Platelet crossmatching is performed when positive HLA/platelet antibody screens are found or when the patient has an unsatisfactory corrected count increment (CCI) following two consecutive platelet transfusions. During this routine evaluation, anomalous positive reactions were encountered in the HLA/platelet antibody screen of some patients. These tests were positive only for a short period of time, beginning after r-GCSF therapy was initiated and continuing until approximately 7 days after r-GCSF therapy was discontinued. As these reactions disappeared from the SPRCA assay following chloroquine diphosphate treatment, the presence of platelet-specific antibodies was ruled out. Concomitantly, an increase in ABO antibody titers was noted in group O patients, unexpectedly poor responses were seen to some platelet transfusions, and there was an apparent requirement for more platelet transfusions. Following these observations, a prospective study was initiated.
Materials and Methods

Patient selection and data collection
A prospective study involving 92 consecutive patients undergoing ABMT/PBPC from 7/3/91 to 7/13/94 was designed. All patients received r-GCSF (Amgen, Thousand Oaks, CA; 16 µg/kg/day) from 2 to 3 days before marrow reinfusion until their absolute neutrophil count rose above 5000/µL or until they had received r-GCSF for 21 days. Sixteen patients were excluded due to the presence of factors that might influence their response to platelet transfusions, such as the presence of drug-associated platelet antibodies (2), the receipt of intravenous gammaglobulin (5), multispecific HLA antibodies for which crossmatch-compatible platelets were not available for transfusion (2), platelet-specific antibodies requiring transfusion of platelets collected from family members (4), renal dialysis (1), or transfusion of plasma-depleted platelets due to repeated severe allergic transfusion reactions (2).

Data collected included timing of r-GCSF administration for PBPC collections and pregnancy history. A platelet transfusion history was also obtained, which included the following information: number and dates of transfusions, the donor ABO group, donor HLA type (including Bw4 and Bw6 type), and the resultant CCI. In addition, aliquots of all available patient sera were frozen for future testing. The day on which the patient was reinfused with previously harvested bone marrow and/or PBPCs was designated as Day 0.

The presence of anomalous reactivity (that is, reactivity not attributable to reactivity with one or more HLA/platelet antigens) in HLA/platelet antibody screens was used to assign patients to either the study (n = 25) or control (n = 48) group. All patients evaluated had at least two PBPC collections prior to transplant using standard ablative regimens of radiation and/or chemotherapy. r-GCSF therapy was initiated 4 days prior to the first PBPC collection and continued until the day before the last PBPC collection. Generally, 1 to 3 weeks elapsed between the last PBPC collection and transplantation. The negative patients included 7 male and 41 female patients with the following diagnoses: breast cancer (n = 36), Hodgkin’s disease (n = 7), non-Hodgkin’s lymphoma (n = 4), and lung cancer (n = 1). These control patients received a total of 308 platelet transfusions, with a CCI being calculated for 246 (80%) of the transfusions. The positive patients included 3 male and 22 female patients with the following diagnoses: breast cancer (n = 18), non-Hodgkin’s lymphoma (n = 2), osteosarcoma (n = 1), ovarian carcinoma (n = 1), Hodgkin’s disease (n = 1), rhabdospheroma (n = 1), and germ cell cancer (n = 1). The positive patients received a total of 341 platelet transfusions, with a CCI being calculated for 269 (79%) of the transfusions.

Hemotherapy
All patients received one daily prophylactic platelet transfusion using single donor platelet units when their platelet counts were less than 20,000/µL. Platelet counts were kept above 50,000/µL in the event of hemorrhage. A 10-minute posttransfusion platelet count was obtained whenever possible, and the CCI was calculated. All platelet and red cell units released for transfusion were gamma irradiated with 25Gy (2500 rad) using a 137Cs Irradiator (CIS-US, Bedford, MA) and leukocyte reduced by bedside adsorption filtration (PL100K and RC50K filters; Pall Biomedical Corporation, Glen Cove, NY). As we had previously demonstrated that there was no effect of storage time on platelet count increment in bone marrow transplant patients,4 units were selected from inventory according to outdate, with the oldest units being transfused first. ABO compatibility was considered only when the recipient demonstrated in vitro or in vivo incompatibility with out-of-group platelet units. (ABO incompatibility was defined as the presence on platelets of A or B substance lacking on the patient’s red cells; plasma incompatibility was not considered.)

SPRCA HLA/platelet antibody screens
SPRCA HLA/platelet antibody screens were performed at least weekly (Capture-P® Ready Screen, Immucor, Inc., Norcross, GA); manufacturer’s directions were followed for all testing. In chloroquine diphosphate treatment to remove HLA antigens from reagent platelets (CDP; Gamma Biologicals, Houston TX), two drops of CDP were added to the test strip, and manufacturer’s directions were followed to complete the testing. The incubation time, after CDP addition, was increased to 1 hour to allow for sufficient time for the CDP to react with the reagent platelets. The CDP pretreatment was performed twice on each reagent test strip to maximize the effect on the HLA antigens. If a transfusion in a control patient did not result in a CCI < 7500/µL without demonstrable clinical cause, HLA and platelet antibodies were assumed to be absent.
Anti-A and Anti-B titers

Anti-A and anti-B titers were performed on all available serum/plasma samples (stored frozen) collected from group O patients ($n = 35$). The results were reported as the reciprocal of the highest dilution that produced at least a 1+ reaction.

Platelets immobilized with various sugars

Platelets in the Capture-P® Ready Screen kits are immobilized by the manufacturer to a polystyrene microtiter plate in the presence of glucose. To evaluate the effect of various sugars on the positive patients sera, plates on which platelets had been immobilized with various simple sugars were supplied by the manufacturer. These sugars included arabinose, fructose, galactose, glucose, lactose, maltose, mannose, melibiose, trehalose, and sucrose (Sigma Chemical Company, St. Louis, MO). Platelets from the same donor were immobilized with different sugars so comparisons of the results could be made. Antibody screening using the platelets that had been immobilized with the various sugars was performed as for routine SPRCA-HLA screening.

Antibody neutralization

When positive HLA/platelet antibody screens were identified in routine SPRCA techniques, the testing was repeated using serum or plasma treated with various substances (Immucor, Inc., Norcross, GA), to determine if the observed reactivity could be neutralized. Solutions (0.1M) of glucose, sucrose, fucose, mannose, fructose, galactose, mannose, or galactosamine (all obtained from Sigma Chemical Company) were prepared in deionized water. Undiluted r-GCSF (300 µg/mL) was also used as a neutralizing substance. One hundred µL of each solution were added to 500 µL of serum/plasma. The mixture was allowed to incubate at room temperature (22 to 24°C) for 5 minutes. These treated samples were used to repeat SPRCA HLA/platelet antibody screens; results were compared to those obtained with untreated samples.

Ion exchange chromatography procedure

Ion exchange chromatography, using diethylaminoethyl Affi-Gel Blue agarose gels (Biorad Labs, Hercules, CA) was performed on 18 individual sera collected from positive patients; samples from three control patients were also included. The manufacturer’s instructions were followed for use of small volumes of sera to allow separation in a conical centrifuge tube rather than a large column.

The effluent was collected in 1 to 5 mL fractions. The fraction size was determined by the amount of buffer added to the gel during the various steps in the chromatography procedure. The estimated total protein concentration of each fraction was calculated by multiplying the optical density (obtained by spectrophotometric analysis) by the volume and the dilution required to obtain an optical density reading that was < 2. Following total protein determinations, the protein composition of each fraction was evaluated by SDS-PAGE gel electrophoresis following manufacturer’s directions (Biorad Labs). Fraction #1 was found to contain the majority of IgG proteins (data not shown). This fraction had a total volume of 1 to 5 mL and was expected to contain the unbound (IgG) protein from the sample. All fractions were placed in a -70°C freezer for use in future testing.

Additional testing

An enzyme-linked immunosorbent assay (ELISA) HLA antibody screening kit (GTI, Brookfield, WI) was used to perform HLA antibody screens on selected fractions obtained from ion exchange chromatography procedures according to the manufacturer’s directions (Table 1).

<table>
<thead>
<tr>
<th>Patient*</th>
<th>Days from Transplant</th>
<th>Fraction contains†</th>
<th>SPRCA screen with Bw6+ Platelets</th>
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<td>-51</td>
<td>IgG</td>
<td>neg</td>
</tr>
<tr>
<td>1</td>
<td>-51</td>
<td>Other</td>
<td>neg</td>
</tr>
<tr>
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<td>-15</td>
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</tr>
<tr>
<td>1</td>
<td>-12</td>
<td>IgG</td>
<td>neg</td>
</tr>
<tr>
<td>1</td>
<td>-12</td>
<td>Other</td>
<td>neg</td>
</tr>
<tr>
<td>1</td>
<td>-2</td>
<td>IgG</td>
<td>neg</td>
</tr>
<tr>
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<td>Other</td>
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</tr>
<tr>
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<td>0</td>
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<td>neg</td>
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<td>pos</td>
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<tr>
<td>1</td>
<td>+8</td>
<td>Other</td>
<td>neg</td>
</tr>
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<td>+9</td>
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<td>+18</td>
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<td>pos</td>
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<td>2</td>
<td>+18</td>
<td>IgG</td>
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</tr>
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<td>neg</td>
</tr>
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<tr>
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* = all samples except those from Patients 3 and 4 are from chromatography fractions from positive patients. Patients 3 and 4 are from negative patients (serum pooled to aid in processing).

† = content of fractions determined by SDS-PAGE electrophoresis
Statistics

The results of the study were analyzed by logistic regression to identify predictors for the presence of the antibody. Gender, blood type (A vs. B vs. O), diagnosis (breast cancer vs. other), HLA Bw type (Bw4 vs. Bw6 vs. other), radiation, days on r-GCSF, days on total parental nutrition (TPN), and increase in anti-A and anti-B titer (group O patients only) were included in the model. An analysis of variance (ANOVA), using natural logarithmic transformation, was performed on the total number of transfusions. Factors in this model included presence of the antibody, patients’ HLA Bw6 types, antibody by Bw6 interaction (Does the patient’s Bw6 type influence the development of the antibody?), diagnosis, and blood type. A $t$-test, using log scale to meet normality assumptions, was performed to compare average CCI per transfusion between the control and the positive patients. Chi-square analysis was used to determine if the observed frequency of HLA Bw6-positive platelets reacting with the sera under study were significantly different than what would be expected based on chance. The observed frequency of patient ABO group and Rh types was analyzed by Yates Chi-square analysis to determine if the incidence observed was significantly different than what would be expected by chance.

Results

Anomalous results in SPRCA HLA/platelet antibody screening

All HLA/platelet antibody screens were evaluated for the presence of unexplained (anomalous) reactivity. A total of 204 antibody screen procedures were performed on the 48 patients in the negative (control) group ($M = 4.25$); 130 procedures were completed on the 25 patients in the positive group ($M = 5.20$). Specific HLA antibodies were identified in eight patients in the control group and in six patients in the positive group ($p > .05$). Eighty-five percent of all the screens done on control patients were negative compared with 58 percent in the positive patients ($p \leq .001$). Thirty-two percent (42/130) of the screens completed on patients in the positive group were found to have anomalous reactivity. All samples in which specific HLA antibodies and/or unexplained reactivity were identified were further tested to determine the cause of the anomalous reactivity. This reactivity was first observed 5 to 16 days ($M = 9.14$) after receiving the first r-GCSF after admission for transplant; this reactivity was no longer detected 1 to 5 days ($M = 2.17$) after r-GCSF had been discontinued.

Relationship of anomalous reactivity to glucose immobilization of platelets

All samples displaying the presence of unexpected reactivity, as well as randomly selected samples from five patients with a negative HLA antibody screen, were tested with platelets immobilized with arabinose, fructose, galactose, glucose, lactose, mannose, melibiose, trehalose, or sucrose. Samples known to contain only HLA-specific antibodies were also tested. In all cases (i.e., samples from the “positive” study group) in which the original HLA/platelet antibody screen had exhibited the anomalous reactivity, negative results were also obtained when the sample was tested with platelets immobilized with any sugar other than glucose. Samples that had not exhibited this reactivity continued to show a negative antibody screen when the other sugars were utilized for platelet immobilization. However, the samples from patients whose samples had not exhibited the anomalous reactivity but had defined HLA specificities continued to react in the presence of the other sugars as they had when the reagent platelets had been immobilized with glucose. Similarly, samples from patients demonstrating the anomalous reactivity as well as defined anti-HLA reactivity continued to show the specific anti-HLA reactivity when the reagent platelets were immobilized with other sugars. No reactivity was observed when sera from the positive study group were reacted with glucose in plates that contained no platelets.

The reagent platelets that showed reactivity on routine antibody screening with positive patients’ sera but were negative following neutralization with glucose were evaluated further to determine if there was any specific HLA antigen that appeared more frequently than would be expected. A total of 152 reagent platelets were evaluated. Reactivity was seen with 140 (92%) of HLA-Bw6+ reagent platelets but only 6 (4%) of Bw6- platelets ($p < .001$). When the patients in the study were phenotyped for HLA-Bw6, 10/48 (21%) of the negative patients were found to be Bw6- compared with 4/25 (16%) of patients in the positive group. This compares with a population distribution of 10 percent HLA-Bw6 negativity (difference = $p > .230$).

Changes in anti-A titer with r-GCSF administration

All patients in the study were evaluated to determine the number of ABO-incompatible platelet transfusions
Anti-A and anti-B titers were performed on 182 samples from patients in the negative group and on 167 samples from patients in the positive group. The median number of titers performed and the mean for the lowest anti-A and anti-B titers were similar in both groups. However, the positive patients showed the median highest anti-A titer of 512 compared to 64 for the negative patients. The median for the highest anti-B titer was slightly higher in the positive patients ($Mdn = 32$) than in the negative patients ($Mdn = 16$). The median change in anti-A titer for the negative patients was 44 (range 0 to 480) compared with 464 (range 14 to 2016) for the positive patients. The median change in the anti-B titer in the negative patients was 12 (range 0 to 120) compared with 22 (range 2 to 480) in the positive patients. The changes in titer were evaluated by the $z$ test for rejection of the null hypothesis of no difference. The $z$ value for changes in anti-A titer in the positive group was $>7$ standard deviations from the mean for the negative patients, a significant difference between the two groups. When the changes in anti-B titer were evaluated, the $z$ value was found to be $<1$ standard deviation from the control mean; the change in anti-B titer between the two groups was not significantly different.

**Antibodies in eluted fractions**

After separation using ion exchange chromatography, a total of 144 fractions (each fraction contained 1 to 5 mL) collected from 18 samples from patients with the anomalous reactivity were evaluated. Each fraction was tested with glucose-immobilized donor platelets to determine which fraction contained the anomalous reactivity. Twenty-one of these fractions, including reactive and nonreactive fractions, underwent additional testing; these fractions were the first collected and contained the IgG component of immunoglobulins as determined by SDS-PAGE electrophoresis. These fractions were selected for evaluation as the reactivity undergoing investigation appeared to be IgG (IgG-sensitive indicator red cells in SPRCA). In addition, six study serum samples, showing negative results with glucose-immobilized platelets, were also included in the additional testing for comparative purposes.

IgG-containing chromatography fractions from samples taken from positive patients about the time when they exhibited the anomalous reactivity clinically showed the same characteristic SPRCA anomalous reactivity. Two of these samples also contained HLA-antigen specific reactivity as previously documented; these specificities were confirmed in ELISA testing. Chromatography fractions containing IgG from negative patient samples shown to contain HLA antibodies displayed antigen-specific reactivity in both SPRCA and ELISA test systems.

When all SPRCA testing was completed, all remaining positive and negative fractions were pooled to allow sufficient volume for additional testing. First, the presence of ABO antibodies was sought by standard techniques. When testing fractions derived from patients who had exhibited the anomalous reactivity, ABO antibodies were not present in the pooled fractions that exhibited the anomalous reactivity (that is, the fractions presumed to contain IgG) but were identified in the pooled fractions that did not show this reactivity (that is, fractions presumed to be of other immunoglobulin classes). Second, glucose neutralization was performed. The anomalous reactivity showed the same neutralization characteristics in these pooled fractions as in the original samples.

**Clinical effect of “anomalous” antibody**

A logistic regression was fitted to identify predictors of the presence of the antibody from among the following factors: gender, blood type (A vs. B vs. O), diagnosis (breast cancer vs. other), HLA-Bw type (Bw4 vs. Bw6 vs. other) of the patient, radiation (yes or no), days on r-GCSF, days on TPN, increase in anti-A or anti-B titer (group O only).

An ANOVA was performed on the total number of platelet transfusions required by patients. Increased likelihood of the antibody’s presence was associated only with the number of platelet transfusions received ($p = .0005$). The geometric mean number of transfusions (with 95% confidence interval) for control patients was 5.01 (4.08, 6.16), whereas that for study patients was 10.49 (7.67, 14.36). No significant differences between the control and positive patients were identified.

The significance of changes in anti-A titer between the control and positive patients was evaluated by ANOVA. Increases in titer in the positive patients was shown to have borderline significance ($p = 0.068$), with an increase in titer of $>100$ being associated with an increase in the probability of developing the unexpected reactivity. The type of ablative therapy used
had no association with the development of the anomalous reactivity. A test was performed to compare average CCI per transfusion (on the log scale, to meet normality assumptions) for the positive vs. the negative patients. The difference in CCI between the two groups was statistically significant ($p = .0005$). The geometric mean CCI (with 95% confidence) for the negative patients was 10,822 (9,888;11,844) compared to 7,951 (6,956;9,090) for the positive patients, a 73 percent difference.

Chi-square analyses were used to determine if CCIs following receipt of Bw–6 or Bw–4 positive platelets were significantly different between groups. No statistically significant differences were found when Bw4+6–, ABO-compatible platelets were transfused. However, when Bw4+6+ or Bw4–6+ ABO-compatible units were transfused (168/216 [77%] transfusions in the negative group vs. 205/244 [84%] transfusions in the study group), the CCIs in the study group were significantly lower than those in the control group ($p = <.001$). No significant differences were seen when ABO-incompatible platelet units ($n = 33$ [negative group] compared to $n = 30$ [positive group]) were received.

Yates corrected Chi-square analyses were used to determine if the incidence of ABO groups in both study and negative patients was significantly different between groups and from the expected incidence in the general population. Half (24/48) of the patients in the control group were group O, whereas 64 percent (16/25) of patients with the anomalous reactivity were group O ($p > .1$). The negative patients included 17 group A patients compared to 8 in the positive group ($p > .1$). There were too few patients of other groups to evaluate.

Discussion

This study documented the presence of an IgG antibody giving anomalous reactions in SPRCA. This was seen in all blood groups, was temporarily associated with r-GCSF administration, could be blocked in vitro with addition of glucose to the sera, and reduced platelet transfusion response. This reactivity was also associated with the presence of the HLA Bw6 antigen on the reagent platelets and an increase in ABO titer (in group O patients only).

The unusual serologic findings in the preliminary observations prompted a closer, prospective investigation of the apparent phenomenon reported here. The source of the unexpected reactivity was ultimately traced to an IgG antibody (reactivity observed with IgG-coated indicator cells) that reacted to platelets bound to a polystyrene solid phase in the presence of glucose. The strong association of the reactivity with the presence of the Bw6 antigen on the reagent platelets clearly suggests a role for this epitope in the antibody’s specificity. The very specific effect of the use of just one sugar might suggest the former as the more plausible mechanism, but ascribing immunogenicity to such a small and ubiquitous molecule, even through haptenic combination, seems unlikely.

The identification of this reactivity in the SPRCA system but its failure to react in ELISA and lymphocytotoxicity testing systems might initially suggest that this is solely a phenomenon restricted to one particular test system. In itself, such an occurrence would still be important to note because anomalous results in SPRCA antibody screening can be confusing and could delay selection of platelet units for transfusion. However, our finding that the presence of this reactivity was associated with 73 percent lower CCIs demonstrates that the in vitro reactivity may have an in vivo counterpart that is clinically significant. The effect may be associated with the need to administer twice as many platelet transfusions during ABMT/PBPC, thus increasing recipient risk and the procedure’s cost. This effect appeared independent of ABO compatibility; however, HLA Bw6+ platelet units were more likely to yield a poor transfusion response in patients with the unexpected reactivity, suggesting a clinical correlation of the increased in vitro reactivity of the positive sera with the presence of HLA Bw6 on platelets.

Red cell antibodies that react only in the presence of certain sugars have been reported. The presence of this unexpected reactivity with red cells suspended in a variety of sugars, including glucose, galactose, mannose, fructose, lactose, or dextran has been shown to occur in 2 to 22 percent of sera tested. However, a new finding that the presence of this reactivity in the SPRCA technique and thus may not have serologic findings in patients such as these that correlate with poor platelet transfusion responses in affected patients. If a patient is currently receiving TPN and r-GCSF and has CCIs < 7500, the patient history should be carefully reviewed and evaluated. As group O patients were 11.4 times more likely to develop the antibody if there was a greater than 100-fold increase in their anti-A titer, this
simple titration test may be important in inferring the possible presence of the antibody. As pretransplant titers are required for this evaluation, it is recommended that a pretransplant sample be frozen and used for isoagglutinin titration when the presence of the anomalous antibody is suspected. If responses to platelet transfusions and patient history indicate that this glucose-dependent reactivity might be present, HLA antibody screen results and determination of the HLA-Bw6 type of transfused platelet units and their CCIs may provide useful information. If the CCIs for HLA-Bw6 negative units are significantly higher than those for HLA-Bw6 positive units without serologic evidence of alloimmunization, the presence of this anomalous antibody should be considered.

It is important to note that all patients included in this study received r-GCSF at a dose of 16µg/kg/day. Shortly after the completion of this study, the r-GCSF dose for ABMT/PBPC patients at our hospital was decreased to 5µg/kg/day; only two additional examples of this unexpected reactivity have been identified since the r-GCSF dose was decreased. This suggests that the dose of r-GCSF received may contribute to the development of this unexpected reactivity.

Similar anomalous results in SPRCA testing on patients who received r-GCSF have recently been reported. These researchers identified the anomalous reactivity described in this report; however, as the reactivity was observed only in SPRCA, they reported its presence as “false-positivity” and attributed the poor posttransfusion platelet response to the presence of infections and other clinical factors. Our research indicates that r-GCSF recipients should be monitored for the development of a glucose-dependent HLA-Bw6 antibody that may have clinical implications. SPRCA techniques are easy to perform and currently are the only techniques that will detect these significant antibodies. For patients displaying this unexpected reactivity, all platelet units should either be ABO identical or group O, and should be negative for HLA-Bw6 as well, of course, as any HLA antigens to which the patient has been sensitized. This protocol should be followed until at least 7 days after r-GCSF discontinuation.


References

Miriam Fogg Leach, MS,MT(ASCP)SBB, (corresponding author), Special Projects Coordinator and Information System Specialist, Transfusion Service Laboratory, Department of Pathology, Dartmouth-Hitchcock Medical Center, Lebanon, NH 03756; and James P.AuBuchon, MD, Department of Pathology, Dartmouth-Hitchcock Medical Center, Lebanon, NH.
Detection of granulocyte antibodies by flow cytometry without the use of pure granulocyte isolates

K. M. Kiekhaefer, K. M. Cipolone, J. L. Procter, K. Matsuo, and D. F. Stroncek

Established methods used to detect serum antibodies to granulocytes require the isolation of granulocytes. Flow cytometric analysis of granulocytes with monoclonal antibodies eliminates the need for granulocyte isolation. The purpose of this study was to develop a method to evaluate reactions of antibodies to granulocytes without separating granulocytes from other leukocytes. Three screening cell samples for granulocyte antibody detection were prepared from whole-blood samples in which the red blood cells (RBCs) were lysed and remaining leukocytes tested against sera at 4°C. Binding of human alloantibodies to the screening cells was determined by flow cytometric analysis using phycoerythrin-conjugated antibody to human immunoglobulin. Forward and side scatter were used to analyze granulocytes separately from other leukocytes. The assay was validated by testing granulocytes with reference alloantibodies directed to NA1, NA2, 5b, and Mart antigens. Samples from 32 patients were tested, and the results of the assays were compared with the results of testing the samples in a granulocyte immunofluorescence (GIF) assay performed by a reference laboratory. In the whole-blood flow cytometric (WBFC) assay the mean fluorescence intensities of reference antisera with antigen-positive cells, expressed in arbitrary units, were anti-NA1 = 48 to 221, anti-NA2 = 24 to 69, anti-5b = 15 to 57, and anti-Mart = 42 to 72. In contrast, the mean fluorescence intensity of type AB-negative control sera ranged from 3 to 11. Of the 32 patient sera tested, 23 were positive (range = 12 to 56) and 9 were negative (range = 3 to 10). When compared with the results obtained by the reference laboratory, 27 sera were concordant between the WBFC and the GIF assays. Four of the samples were positive in WBFC (range = 11 to 31) and negative in GIF and one sample was negative in WBFC (range = 5 to 6) and positive in GIF. Leukocytes prepared from whole blood after lysis of RBCs can be used in flow cytometric analysis to detect granulocyte alloantibodies. The results of testing for granulocyte antibodies with this assay were similar to results of testing sera in GIF. Further comparative studies are indicated to confirm findings and explain the discordant results.

Key Words: granulocytes, flow cytometry, granulocyte agglutination assay, granulocyte immunofluorescence assay, monoclonal antibody immobilization of granulocyte antigens

Granulocyte antibodies are important in the diagnosis of autoimmune neutropenia, neonatal alloimmune neutropenia, and transfusion reactions. Transfusion recipients who have antibodies to donor leukocytes may experience febrile nonhemolytic reactions if they are transfused with blood components containing leukocytes. Transfusion of blood components containing granulocyte antibodies can cause transfusion-related acute lung injury (TRALI). In addition, patients who are transfused with granulocyte concentrates often develop antibodies to granulocyte antigens that render future transfusions ineffective. To diagnose and treat these conditions, it is imperative that an easy-to-perform, rapid, accurate, and sensitive method is available to detect and identify granulocyte antibodies.

Methods for the detection of granulocyte antibodies include the granulocyte agglutination (GA) assay, granulocyte immunofluorescence (GIF) assay, and the monoclonal antibody immobilization of granulocyte antigens (MAIGA) assay. These methods use granulocytes that have been isolated from whole blood by removing red blood cells (RBCs) by sedimentation and lysis and then separating granulocytes from other leukocytes by density gradient solutions. The isolation procedure is time-consuming and technique-dependent.

The purpose of this study was to develop and validate a method to detect granulocyte antibodies using whole blood without removing RBCs by sedimentation and without separating white blood cells (WBCs) using density gradient solutions.

Materials and Methods

Study design

To avoid isolating granulocytes from the samples to be used as screening cells, whole blood was collected and the RBCs were lysed. The granulocytes were tested and analyzed for fluorescence using flow cytometric methods. This procedure was initially tested in a study.
that optimized the variables of temperature of incubation, volume of reactants, and the use of formaldehyde as a fixative. The initial study also assessed the efficacy of the method using high-titer antibodies specific to well-characterized antigens. Next, to validate the whole-blood flow cytometry (WBFC) antibody detection method, granulocytes of known phenotype were tested with reference antisera. Then a blinded study compared the test results of samples that had been previously tested for granulocyte antibodies by traditional methods with test results on the same samples using the WBFC assay.

**Antisera and patient samples**

The reference antisera for neutrophil antigens NA1, NA2, Mart, and 5b were provided by the American Red Cross (North Central Blood Services, St Paul, MN). The group AB sera were obtained from healthy, nontransfused, nonpregnant donors. The patient samples were from individuals who were suspected of having autoimmune neutropenia or who were transfused with granulocyte concentrates and seen at the Warren G. Magnuson Clinical Center (National Institutes of Health, Bethesda, MD).

**Testing serum for granulocyte antibodies**

Initially, three variables were optimized: the temperature of incubation of antibodies with granulocytes, the volumes of cell suspensions and sera, and the step to best fix granulocytes with formaldehyde. For these initial studies, sera containing well-characterized anti-NA1 and anti-NA2 were used as positive controls and AB sera were used as a negative control. The sera were tested against granulocytes from donors whose NA1 and NA2 phenotypes were previously determined using monoclonal antibodies and whose genotypes were confirmed by polymerase chain reaction and restriction enzymes. Varying quantities of granulocytes and sera were tested at either 24°C or 4°C. Incubation at 4°C was better than incubation at 24°C in that nonspecific fluorescence and decreased viability of granulocytes occurred at 24°C (data not shown). In addition, it was found that 10 µL of sera should be incubated with 200 µL of cells to decrease the time needed for the flow cytometer to acquire 10,000 events.

The treatment of granulocytes with fixative broadened the window of time in which testing could be completed. In some methods, granulocytes were fixed after they had been incubated with antibodies. We found that formaldehyde fixation did not affect the test result when used at the end of the procedure, which allowed data acquisition by flow cytometry up to 24 hours after the testing procedure was completed.

On the basis of these results, the following procedure was used to test granulocyte antibodies. After obtaining informed consent, whole blood from each granulocyte donor was collected into two 7 mL vials containing EDTA and stored at room temperature for no more than 24 hours before testing. Approximately 10 mL of whole blood was combined with 40 mL of ACK Lysing Buffer (Quality Biologic, Inc., Gaithersburg, MD) in a 50 mL polypropylene conical tube (Becton Dickinson, Mountain View, CA) and placed on a mechanical rocker for 6 minutes or until lysis of RBCs was complete. The tube was then centrifuged at 1900 x g for 5 minutes. The hemolysate was decanted and the remaining WBC sediment was resuspended and washed x 2 in cold (4°C) Hanks buffered saline solution (HBSS) without calcium (BioWhittaker, Walkersville, MD). After the last wash, each WBC pellet was resuspended in 4 mL of HBSS and 200 µL aliquots of this cell suspension were placed in 5 mL polypropylene test tubes (Becton Dickinson). Ten microliters of test or control sera were added to the 200 µL aliquot of the donor cell suspension and incubated for 30 minutes at 4°C. Following incubation, the WBCs were washed once with cold HBSS. Five microliters of secondary antibody, a phycoerythrin (PE)-conjugated goat anti-human IgG [F(ab')2-PE] (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), were added to each tube and incubated at 4°C for 30 minutes, protected from light. After 30 minutes, the suspension was washed once with cold HBSS and resuspended to a final volume of between 200 and 400 µL with HBSS or an HBSS 1% formaldehyde solution (Polysciences, Warrington, PA). The samples were then analyzed by flow cytometric methods.

**Flow cytometry analysis**

When WBCs were analyzed by flow cytometry (Facsort, Becton Dickinson), forward and side scatter were used to differentiate the granulocyte population from the lymphocyte and monocyte populations. Software (Becton Dickinson CellQuest) was used to further analyze the fluorescence of the granulocyte population. The results of the analysis of granulocytes from each donor were expressed as the peak cell
fluorescence intensity (the fluorescence intensity that corresponded to the greatest number of granulocytes from each donor).

Establishing cutoffs for positive results

To differentiate between positive and negative test results, a cutoff value was established by calculating the mean plus 2 standard deviations using the values of the mean peak channel fluorescence of known negative samples. Both group AB sera and reference sera that were tested against cells that did not possess the corresponding antigen were used to determine the cutoff values. This was done for each of the three WBC cell populations tested. When patient samples were tested, any value greater than the cutoff was considered positive.

Testing for granulocyte antibodies by a reference laboratory

All patient samples were tested by GA and GIF by a neutrophil serology reference laboratory (American Red Cross, North Central Region Blood Services, St. Paul, MN). The interpretations of the GA and GIF assays were based on phase contrast microscopy or fluorescent microscopy readings of reactions of the sera with granulocytes from five different donors. Samples with discordant results in the WBFC and GIF assays were tested by the reference laboratory in the MAIGA assay against CD16 (Fc-gamma receptor IIIb), CD18 ($\beta_2$ integrin), and CD177 (NB1) monoclonal antibodies.

Results

Testing reference antisera and sera without antibodies

Sera known to contain well-characterized alloantibodies to granulocyte antigens were used to demonstrate that antibodies to well-described alloantigens could be detected by the assay. The sera were tested against granulocytes from three different donors (Table 1). Results of testing the control group AB sera against granulocytes from each of the three donors as well as anti-NA1 against antigen-negative granulocytes (Donor C) and anti-NA2 against antigen-negative cells (Donor A) are shown in Table 2. There was no difference in the reactions of group AB sera and anti-NA1 or anti-NA2 with antigen-negative cells. On the basis of these reactions, the cutoff for reactions of patient sera with granulocytes from Donor A was determined to be 10.53 arbitrary units. For granulocytes from Donor B, the cutoff was 9.12. For granulocytes from Donor C, the cutoff was 10.32. The results of testing reference alloantibodies against granulocytes from the three donors are shown in Table 3. Reactions of anti-NA1 and anti-NA2 with antigen-positive cells were much greater than with antigen-negative cells. Reactions of anti-Mart and anti-5b with granulocytes from all three donors were significantly greater than reactions of anti-NA2 and anti-NA1 with antigen-negative granulocytes.

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<tr>
<td>Donor C</td>
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* Determined by polymerase chain reaction and restriction enzymes
† Determined by flow cytometry using NA1- and NA2-specific monoclonal antibodies
‡ Determined in-house using isolated granulocytes, alloantisera, and flow cytometry

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 Asterisk: Peak fluorescence intensity is expressed in arbitrary units
Testing patient samples

Sera from 32 patients were tested and 23 were reactive with granulocytes. However, the peak cell fluorescence intensities of the reactions of the 23 positive patient sera were less than the peak fluorescence intensities of reference antibodies.

The results of testing the 32 patient samples in the WBFC assay were compared with those obtained using the GIF and GA assays. Twenty of the 32 patient samples were positive in the GIF assay and five were positive in the GA assay. The results of the WBFC assay differed from the GIF assay for five of the 32 assays. Four of the five samples with discordant results were positive in the WBFC assay and negative in the GIF and GA assays (Table 4) and one was negative in the WBFC assay, but positive in the GIF assay (Table 5). Of the four samples that were reactive in the WBFC assay but negative in the GIF and GA assays, three were reactive in the WBFC assay with granulocytes from all three donors tested and one was reactive with granulocytes from one donor. All four of these samples were tested in another assay, MAIGA, and were negative. The sample that was negative in the WBFC assay and positive in the GIF assay was negative in the GA and MAIGA assays. All five samples that were positive in the GA assay were also positive in both the WBFC and GIF assays.

Discussion

A new assay to test for granulocyte antibodies was developed and tested. This method has the advantage over other assays, including GIF, in that it avoids the laborious density gradient separation step. Another advantage is that flow cytometry rather than fluorescence microscopy is used to detect the binding of antibodies to granulocytes. Flow cytometry permits more precise scoring of reactions of antibodies to granulocytes and produces a permanent record of test results.

Table 3. Representative positive results of testing reference antisera against granulocytes from three donors in the whole-blood flow cytometry assay

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<td>Anti-5b</td>
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*Bold = Peak fluorescence intensity greater than the negative cutoff

Table 4. Comparison of test results of samples positive in the whole-blood flow cytometry assay (WBFC) with the granulocyte agglutination (GA) and granulocyte immunofluorescence (GIF) test results

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*Not available
The results of testing patients’ samples in the WBFC assay and in the GIF assay were similar, but some discordant results occurred. The reason for these discordant results is not certain, nor is it clear which assay was correct. The differences could be due to variable test results of weak antibodies. Alternatively, slight differences in the test methods could account for the discordant results. In the WBFC assay, granulocytes and serum were incubated at 4°C and granulocytes were treated with formaldehyde after incubation with the serum and secondary antibody. In the GIF assay, granulocytes were fixed with formaldehyde prior to 37°C incubation and subsequent incubation with secondary antibody.

To detect all granulocyte antibodies, a combination of assays must be used. However, the results of this study showed that the WBFC method is most similar to the GIF in its ability to detect granulocyte antibodies and if a laboratory can use only a single assay, the WBFC or GIF assay is preferable to the GA assay to screen for granulocyte antibodies. The GA assay was the most specific assay, but it lacked sensitivity.

The WBFC assay is a valid method to screen patients suspected of having granulocyte antibodies. If the number and phenotype of granulocyte donors permit, this method could also be used to determine specificity of granulocyte antibodies. With the use of well-characterized granulocyte antibodies, this method can also be used to phenotype granulocyte antigens.

The WBFC assay described is a relatively quick and sensitive method that could aid in the diagnosis and treatment of clinical conditions in which granulocyte antibodies are implicated. It may be feasible to use this assay to screen whole blood donors for granulocyte antibodies and discard antibody-containing components in order to prevent the transfusion of granulocyte antibodies that could result in TRALI.

**References**

Flow cytometry for granulocyte antibodies

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Low-incidence MNS antigens associated with single amino acid changes and their susceptibility to enzyme treatment

M. E. Reid and J. R. Storry

MNS antigens are carried on glycophorin A (GPA), glycophorin B (GPB), or their variants. Antigens at the N-terminus of GPA are sensitive to cleavage by ficin, papain, and trypsin but are resistant to α-chymotrypsin. Antigens at the N-terminus of GPB are sensitive to cleavage by ficin, papain, and α-chymotrypsin but are resistant to trypsin treatment. These characteristics have been used to aid in the identification of blood group alloantibodies. Recent molecular analyses have identified changes in amino acids that are associated with several low-incidence antigens in the MNS blood group system. This review relates the molecular studies with the susceptibility or resistance of these antigens to treatment of intact red blood cells by proteolytic enzymes. Immunohematology 2001;17:76–81.

Key Words: MNS blood group system, low-incidence antigens, molecular basis, enzymes, papain, trypsin, α-chymotrypsin, ficin

The many low-incidence antigens of the MNS blood group system are carried on variant forms of glycophorin A (GPA) and glycophorin B (GPB). GPA and GPB are single pass, type I integral membrane glyco-proteins of the red blood cell (RBC) membrane. The proteins are encoded by adjacent genes (GYPA and GYPB, respectively) on chromosome 4. Because these genes share 95 percent identity, hybrid genes caused by simple crossing over and gene rearrangements lead to numerous hybrid proteins.1,2

M, N, S, and s are the polymorphic antigens after which the MNS blood group system was named. The M or N antigen is located at the N-terminus of GPA. GPA carries the ‘N’ antigen at the N-terminus and the S or s antigen at residue 29, where methionine is specific for the S antigen and threonine is specific for the s antigen.3,4 Other antigens in the MNS blood group system arise from single amino acid substitutions, novel amino acid sequences at the junction of the hybrid proteins, or within encoded regions of the GPB pseudogene.2

The purpose of this review is to summarize the molecular basis of low-incidence MNS antigens that are carried on GPA or GPB and are associated with a single point mutation. The molecular structure is consistent with laboratory evidence of known resistance or sensitivity of these antigens to enzyme treatment of intact antigen-positive RBCs. Low-incidence antigens in the MNS system are well-expressed on cord RBCs and many of the corresponding antibodies have been implicated in hemolytic disease of the newborn. The significance of these antibodies in blood transfusion is largely unknown because crossmatch procedures that include an antihuman globulin test will detect incompatibility in the absence of a positive antibody screen and finding compatible RBCs is not difficult.

Low-incidence antigens on GPA

Table 1 lists the low-incidence antigens that have been shown to be associated with a single amino acid substitution in GPA.3 Table 1 also includes the effect of papain, ficin, trypsin, or α-chymotrypsin treatment of antigen-positive RBCs. GPA on intact RBCs does not have an α-chymotrypsin cleavage site. M and N antigens at the N-terminus of GPA are sensitive to treatment of antigen-positive RBCs with trypsin, ficin, or papain. Similarly, Vw, Hut, and Ny antigens, which are located on the N-terminal side of the trypsin cleavage site on GPA,6,8 have the same characteristics (Fig. 1).

The Or antigen has tryptophan, instead of arginine, at amino acid residue 31,9,10 which ablates one of the trypsin cleavage sites. Thus, depending on the extent of trypsin treatment of antigen-positive RBCs, the Or antigen may be sensitive or weakened. The extent of weakened reactivity of anti-Or with trypsin-treated
Or-positive RBCs may also be dependent on the precise specificity of the antibody. The change of Arg31Trp on Or-positive RBCs provides an explanation for the observation that the M antigen on Or-positive RBCs is more resistant to trypsin treatment when compared with the M antigen on Or-negative (normal) RBCs.11

Vr and Os\(^\text{a}\) antigens reside on GPA between the sites for trypsin and ficin/papain cleavage, and both are resistant to trypsin and sensitive to ficin or papain treatment of antigen-positive RBCs (Fig. 1).7,12 The amino acid substitution associated with the Vr antigen (tyrosine at residue 47) introduces a novel \(\alpha\)-chymotrypsin cleavage site. Thus, the Vr antigen is on GPA but sensitive to \(\alpha\)-chymotrypsin treatment.

The Mt\(^\text{a}\) antigen is associated with a point mutation on GPA at residue 5812 and the Ri\(^\text{a}\) antigen is associated with a point mutation on GPA at residue 57,13 These amino acid positions are close to the cleavage sites for ficin and papain and provide an explanation for variation in reactivity with different examples of anti-Mt\(^\text{a}\)14 and anti-Ri\(^\text{a}\).15 The amino acid substitution associated with the Ri\(^\text{a}\) antigen introduces a novel trypsin cleavage site, which explains the trypsin sensitivity of this antigen.15

The amino acid change associated with ERIK (Gly59Arg) is predicted to ablate a ficin cleavage site and introduce a possible cleavage site for papain and trypsin.16,17 The ERIK antigen is reported to be sensitive to papain and partially sensitive to trypsin.18 However, it is possible, due to the position (close to the RBC membrane) and nature of the substitution (Gly59Arg) associated with the ERIK antigen, that other examples of anti-ERIK may react differently with ficin- or papain-treated RBCs as seen with some examples of anti-Mt\(^\text{a}\) and anti-Ri\(^\text{a}\) with different enzyme susceptibilities.14,15 The single amino acid changes associated with MARS and HAG antigens reside on GPA between the ficin/papain cleavage site and the RBC lipid bilayer.19,20 Both antigens are, as expected, resistant to trypsin, \(\alpha\)-chymotrypsin, ficin, or papain treatment (Fig. 1). The amino acid substitution associated with the MARS antigen might be expected to introduce a novel enzyme site. However, because the antigen is resistant to treatment of RBCs with the proteolytic enzymes, it is presumed that the site (close to the lipid bilayer) is not accessible to the enzyme. The presence of either HAG or MARS antigens alters the expression of Wr\(^\text{b}\), a high-incidence antigen located on Band 3, but requiring an

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**Table 1. MNS antigens associated with single amino acid changes in Glycophorin A**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antigen ISBT #</th>
<th>Amino acids involved</th>
<th>Ficin/α-chymotrypsin</th>
<th>Papain</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vw</td>
<td>002009</td>
<td>Thr28Met(^*)</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
</tr>
<tr>
<td>Vr</td>
<td>002012</td>
<td>Ser47Tyr</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Sensitive</td>
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<tr>
<td>Mt(^\text{a})</td>
<td>002014</td>
<td>Thr58Ile</td>
<td>Partially sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ri(^\text{a})</td>
<td>002016</td>
<td>Glu57lys</td>
<td>Partially sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
</tr>
<tr>
<td>Ny(^\text{a})</td>
<td>002018</td>
<td>Asp27Glu</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
</tr>
<tr>
<td>Hut</td>
<td>002020</td>
<td>Thr28Ile</td>
<td>Sensitive</td>
<td>Partially sensitive</td>
<td>Resistant</td>
</tr>
<tr>
<td>Or</td>
<td>002031</td>
<td>Arg31Trp</td>
<td>Sensitive</td>
<td>Partially sensitive</td>
<td>Resistant</td>
</tr>
<tr>
<td>ERIK</td>
<td>002037</td>
<td>Gly59Arg</td>
<td>Papain sensitive</td>
<td>Partially sensitive</td>
<td>Resistant</td>
</tr>
<tr>
<td>Os(^\text{a})</td>
<td>002038</td>
<td>Pro54Ser</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>HAG</td>
<td>002041</td>
<td>Ala65Pro</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>MARS</td>
<td>002043</td>
<td>Gln63Lys</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

\(^*\) Mechanism could be a single nucleotide substitution, or a GPA(A-B-A) hybrid2
interaction with amino acids 59 to 76 of GPA for full expression.

**Low-incidence antigens on GPB**

The low-incidence antigens, M’, Mit, and s^D, are each associated with a single amino acid change on GPB (Table 2). The effect of papain, ficin, trypsin, or \( \alpha \)-chymotrypsin treatment on antigen-positive RBCs is also given in Table 2. The N-terminal 26 amino acids of GPB usually are identical to the amino acid sequence of GPA carrying the N antigen, but occasionally GPB carries the He antigen. The N antigen on GPB is often called ‘N’ (N quotes) in order to differentiate it from the N antigen on GPA. Diagnostic anti-N reagents are formulated to detect the N antigen on GPA, but not on GPB. Wild-type GPB does not have a trypsin cleavage site on intact RBCs. The N or He antigens on GPB are sensitive to treatment of antigen-positive RBCs with ficin, papain, or \( \alpha \)-chymotrypsin, but resistant to trypsin treatment. The M’ antigen, which is also located at the N-terminus of GPB is, as expected, sensitive to papain, ficin, or \( \alpha \)-chymotrypsin. The presence of M’ ablates the ‘N’ antigen.

### Table 2. MN\( ^8 \) antigens associated with single amino acid changes in Glycophorin B

<table>
<thead>
<tr>
<th>Antigen</th>
<th>ISBT #</th>
<th>Amino acids involved</th>
<th>Ficin/Papain</th>
<th>Trypsin</th>
<th>( \alpha )-chymotrypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>002003</td>
<td>Met29</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td>s</td>
<td>002004</td>
<td>Thr29</td>
<td>Partially</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td>M’</td>
<td>002021</td>
<td>Thr3Ser</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td>s^D</td>
<td>002023</td>
<td>Pro39Arg</td>
<td>Partially</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>Mit</td>
<td>002024</td>
<td>Arg35His</td>
<td>Resistant</td>
<td>Resistant</td>
<td></td>
</tr>
</tbody>
</table>

S and s antigens are carried on GPB at amino acid residue 29 (methionine and threonine, respectively) and are sensitive to treatment of antigen-positive RBCs with \( \alpha \)-chymotrypsin, resistant to trypsin treatment, and behave variably after ficin or papain treatment. The S antigen is usually sensitive to ficin or papain treatment of antigen-positive RBCs, but the s antigen is only partially sensitive as determined by most anti-s. Because neither methionine nor threonine are enzyme-cleavage sites, one can only presume that the cleavage sites are more accessible on GPB carrying methionine (S antigen) than on GPB carrying threonine (s antigen) at position 29. The Mit and s^D antigens are located between the \( \alpha \)-chymotrypsin site and the lipid bilayer, which explains the resistance of Mit and of s^D to this enzyme (Fig. 2). The Mit antigen is partially sensitive to papain and resistant to ficin or trypsin treatment (Carole Green, personal communication). The resistance of the Mit antigen to ficin treatment is unexpected. There are only limited data regarding the protease sensitivity of s^D. The single report of anti-s^D notes that the antibody reacts “weaker with enzyme-treated cells,” but the enzyme is not named. The amino acid substitution of Pro39Arg associated with the s^D antigen is C-terminal of the \( \alpha \)-chymotrypsin, papain, and ficin cleavage sites (and, thus, would be expected to be resistant to treatment of antigen-positive RBCs with these enzymes) but creates a novel papain and trypsin cleavage site. This may explain the weakened reactivity of anti-s^D with enzyme treated s^D-positive RBCs, although its close proximity to the lipid bilayer may make the site relatively inaccessible to the enzyme and thereby allow only partial cleavage of the altered GPB.
Weakened expression of S and s antigens

The presence of M′, Mit, or s0 on GPB weakens the expression of S and s antigens.24-29,31 Dahr et al.3 showed that, for expression of S and s antigens, the amino acid at position 29 is critical and that 25Thr (and/or the oligosaccharide attached to this residue), 28Glu, 34His, and 35Arg are also important (at least as defined by some anti-S and anti-s).32 Thus, the change of Arg35 to His associated with the Mit antigen would be expected to alter the expression of the S or s antigen. The replacement of proline with arginine at residue 39 in GPB carrying the s0 antigen would be expected to have a profound effect on the local conformation of GPB. In addition, such a change could alter the expression of the S or s antigen due to the proximity of the amino acid substitution to the S or s antigenic determinant. Based on analysis of the GPB amino acid sequence, the hydrophobic sequence from residues 36 to 66 forms the α-helical membrane-spanning domain.32-34 Proline is most commonly found in the N-terminal sequence flanking the transmembrane region of type I membrane proteins and it is thought to be a helix initiator.35 Arginine is a highly positively charged amino acid, and studies have shown that introduction of a positively charged amino acid at the extracellular boundary will force the transmembrane helix out of the membrane.36 Therefore, it is likely that the amino acid substitution of proline by arginine at position 39 (s0+) will alter the conformation of the glycoporphin, thereby weakening the s antigen and, potentially, the insertion of GPB in the membrane. The substitution with arginine could introduce a trypsin and papain cleavage site, but it is possible that residues close to the lipid bilayer may not be accessible to enzymes.

RBCs that are s positive, M′ positive, have a weakened expression of s,24,30 but there is no apparent effect on S antigen expression when it is carried in cis to M′.24 Because the amino acid substitution associated with the M′ antigen is a considerable distance from that associated with the S antigen, it is hard to understand how M′ exerts its effect. Immunochemical analysis of s-positive, M′-positive RBCs by periodic acid-Schiff’s (PAS) staining of polyacrylamide gels showed that GPB associated with the M′ haplotype is present at approximately 25 percent of normal levels of the s form of GPB.23,25 In contrast, PAS staining of S-positive, s-positive, M′-positive RBC membranes had grossly normal amounts of GPB.22 It is possible that any decrease in the amount of s-positive GPB was masked by the S-positive GPB, as GPB carrying the S antigen is present at 1.5 times the amount of GPB carrying the s antigen.37 Thus, the weakening of s in M′-positive RBCs is most likely due to the reduced GPB copy number in these cells rather than to the amino acid substitution having a direct effect on the s antigen.

Suppression of S or s antigen was more readily demonstrated in early studies with RBCs carrying the M′, s0, or Mit antigens than is likely today. The more potent contemporary reagents are less likely to show weakening of S and s antigens. As genotyping for blood group alleles becomes more widespread, it is important to be aware of variants that may affect oligonucleotide primer design and amplification of alleles.

Acknowledgments

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References


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Use of polyethylene glycol (PEG) to promote adsorption of autoantibodies is reported to give good recovery of concomitant alloantibodies. In initial experiments, PEG and ZZAP (Ficin and DTT) adsorption procedures were compared for removal of autoantibody and recovery of alloantibody. Postadsorption studies \((n = 11)\) were performed and hemagglutination scores compared. In subsequent studies, equal volumes of alloantibody containing sera, PEG, and antigen-negative red blood cells (RBCs) were used in twofold adsorption experiments. Saline was substituted for PEG for control purposes. Postadsorption titers and immunoglobulin levels were determined. Autoantibodies were completely removed by both methods \((n = 5)\); better by PEG \((n = 3)\); better by ZZAP \((n = 1)\); and not adsorbed \((n = 1)\). Alloantibody recovery was comparable in three cases (E, K, Jk\(a\)) but weaker by at least one reaction grade in four (K, E, Jk\(a\), and antibody to low-frequency antigen). The latter anti-Jk\(a\) reacted 1+ with Jk(a+b+) RBCs after ZZAP adsorption but was nonreactive with the same RBCs following PEG adsorption. Titers of six alloantibodies adsorbed with antigen-negative RBCs in PEG were markedly weaker (range 2 to 8) compared to saline controls (range 4 to 32). IgG levels for PEG adsorbed (range 128 to 243 mg/dL) were 50% lower than controls (range 265 to 505 mg/dL). Although PEG adsorption is effective in removing autoantibody, the precipitation of immunoglobulin by PEG may result in failure to detect underlying alloantibody.

Key Words: polyethylene glycol (PEG); PEG adsorption; autoantibody removal; detection of concomitant alloantibody; immunoglobulin precipitation by PEG

The promoting effect of polyethylene glycol (PEG) on red cell antigen-antibody interactions was first described in 1987 by Nance and Garratty. Since then, many transfusion service laboratories have incorporated a PEG method into their antibody detection and/or identification testing protocols. In a 1995 letter to the editor of Transfusion, Liew and Duncan suggested that PEG be used in autoadsorption procedures to decrease adsorption incubation times. They further suggested that the presence of PEG in the adsorbed serum would promote detection of concomitant alloantibodies, if present.

Champagne and Moulds questioned the use of PEG for autoadsorption. They reported that, after PEG adsorption, a weak alloanti-K was not detected and the reactivity of an alloanti-E was markedly weaker. In 1997, an evaluation of the PEG adsorption procedure was published in Immunohematology. The authors reported no problems with the detection of concomitant alloantibodies. Cheng and colleagues recently reported similar results.

Leger and colleagues have compared the efficacy of a variety of methods available for autoantibody removal and the detection of underlying alloantibodies. They found PEG adsorption to give similar results to those obtained by ZZAP adsorption, but PEG had the advantages of eliminating the prior treatment of the adsorbing cells with ZZAP reagent (a mixture of dithiothreitol and cysteine-activated papain), and a 50 percent or more reduction in required adsorption time. They did mention the possibility of losing alloantibody reactivity due to immunoglobulin precipitation. In a letter to Transfusion, they cautioned technologists to test PEG-adsorbed serum on the day of preparation because immunoglobulins precipitated upon storage. They also stated that if the adsorbed serum must be stored, it should be remixed and not centrifuged prior to testing.

We have used a modified ZZAP procedure since the original method was described by Branch and Petz in 1982. We simply substitute ficin for the cysteine-activated papain. Given the favorable reports of the PEG adsorption method, we performed crossover validation studies, with a view to switching to the PEG adsorption procedure should our data support such a change. Our findings are presented in this report.

Materials and Methods

Unless otherwise noted, we used commercially available PEG (PeG; Gamma Biologicals, Houston, TX). Twofold adsorptions were performed using 1 mL each...
of PEG (added only to first adsorption), serum, and adsorbing red blood cells (RBCs). Each adsorption was for 15 minutes at 37°C. Following the second adsorption, the sera were tested by the indirect antiglobulin test (4 volumes of adsorbed serum, 1 volume of 3 to 4% RBCs, 30 minutes incubation at 37°C, anti-IgG). Reactions were graded and scored as described by Marsh.10 Saline was substituted for PEG in control adsorption experiments.

ZZAP adsorptions were performed as described elsewhere.9 Adsorption at 37°C was for 30 minutes. After the second adsorption, the sera were tested by a saline indirect antiglobulin technique (3 volumes of adsorbed serum, 1 volume of 3 to 4% RBCs, 60 minutes at 37°C, polyspecific antihuman globulin reagent).9

Throughout this investigation, samples containing alloantibodies were adsorbed with RBCs that lacked the corresponding antigen(s). Serum samples contained either autoantibodies, autoantibodies spiked with known alloantibodies, or only alloantibodies.

All other serologic procedures were performed as described elsewhere.9 Immunoglobulin (IgG) levels were measured by a standard nephelometric procedure (Beckman Array; Beckman Coulter, Inc., Fullerton, CA).

Results

Figure 1 summarizes the results of adsorption studies to remove warm reactive autoantibodies. In all of the 11 cases, the unadsorbed sera reacted equal to or greater than 2+ by our routine antibody detection method (Löw and Messeter low-ionic-strength saline).11 The assigned score values are the total of the reaction scores observed with all RBCs tested, divided by the number of RBC samples. PEG and ZZAP autoadsorptions were not effective in removing autoantibodies from one sample. Both methods completely removed autoantibody from three samples and reduced reactivity to about 1+ in two samples; trace reactivity with some cells was seen in five samples, in three PEG-adsorbed samples, and in four ZZAP-adsorbed samples. These data confirm the observations of Leger and Garratty6 that PEG and ZZAP methods are essentially comparable for autoantibody removal.

We looked at a total of 12 alloantibodies and studied the effect of both PEG and ZZAP adsorption with antigen-negative RBCs. Figure 2 shows the results from seven cases in which the reactivity of the PEG-adsorbed serum against antigen-positive RBCs was weaker than the reactivity of the ZZAP-adsorbed serum by one reaction grade or more. Except for the antibody to an undefined low-frequency antigen, at least three antigen-positive RBCs were used to test the adsorbed sera; the assigned value represents the average score.

It is of note that an anti-Fy that gave a strong 1+ (score 6) reaction with the ZZAP-adsorbed serum was not detected in the PEG-adsorbed sample. Also, an anti-Fy+ strong after ZZAP adsorption gave only trace reactions (score 2) with PEG-adsorbed serum.
An anti-Jk\(^a\) reacting 2+ with ZZAP-adsorbed serum was equally difficult to detect with PEG-adsorbed serum. Table 1 illustrates the stronger reactivity of this anti-Jk\(^a\) after ZZAP adsorption compared to the PEG-adsorbed sample. To exclude the possibility that the enhanced anti-Jk\(^a\) reactions with the ZZAP-adsorbed serum were due to bound complement (C3), as polyspecific antihuman globulin (containing anti-C3) was used for testing, we repeated tests on the adsorbed serum with anti-IgG and obtained similar results.

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Additional twofold PEG adsorptions of alloantibodies with antigen-negative RBCs were performed. For parallel control adsorptions, PEG was substituted with normal saline. Titration studies were performed on the adsorbed sera using saline as a diluent, 60 minute incubations at 37\(^\circ\)C, and anti-IgG. The results are summarized in Figure 3. With five of seven alloantibodies, the titers after PEG adsorption with antigen-negative RBCs were twofold lower than the control titers. With the other two antibodies, the titers after PEG adsorption were onefold lower than the control titers. Similar findings were obtained using a 20% solution of 3350 MW PEG in isotonic saline (data not shown).

To demonstrate that loss of antibody activity in the PEG-adsorption procedure is not solely an effect of storage of the postadsorbed samples, we incubated equal volumes of serum and PEG in the absence of RBCs for 15 minutes at 37\(^\circ\)C, centrifuged the sample, and immediately measured the IgG levels in the supernatants. Saline was substituted for PEG in control experiments. (Of note: As soon as PEG was added to the sera, a visible, cloudy precipitate formed, and a gelatinous deposit was observed after centrifugation.) We looked at six serum samples and measured the IgG content of the PEG-treated and control samples. Again, the IgG levels in the PEG-treated sera were on average 50 percent lower than saline controls (Fig. 5).

**Discussion**

Based on our data, PEG adsorption appears to be less effective than ZZAP adsorption for detecting concomitant alloantibodies in patients with warm reactive autoantibodies. This is evident from our finding of one anti-Fy\(^a\) that was nonreactive after PEG adsorption.
adsorption with Fy(a+) RBCs, six antibodies that had reduced reactivity by at least one reaction grade after PEG adsorption, and a further seven alloantibodies that had a one- to twofold decrease in titer after PEG adsorption. This loss of alloantibody reactivity is the result of immunoglobulin precipitation by PEG. Autoantibody removal is also likely facilitated by this PEG precipitation of immunoglobulin.

Our data are in conflict with other reports on the use of PEG-adsorbed sera for detecting concomitant alloantibodies in sera containing autoantibodies. In light of these observations, we have chosen not to implement PEG adsorption.

References


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Selecting an acceptable and safe antibody detection test can present a dilemma

M. R. Combs and S. J. Bredehoeft

The Transfusion Service at Duke University Hospital has changed antibody detection methods from the use of albumin in indirect antiglobulin tests to low-ionic-strength solution (LISS), and from LISS to polyethylene glycol (PEG) in an effort to enhance the rapid detection of clinically significant antibodies. In 1996, staffing issues required the consideration of automation. Although previous studies indicated that the gel test was not as sensitive as PEG for detection of clinically significant antibodies, we chose to implement the gel test to be used with the Tecan MegaFlex-ID. We performed a retrospective analysis of identified antibodies and transfusion reactions to compare the outcomes of one year’s experience with gel and PEG. We found comparable detection of potentially clinically significant antibodies by both methods and significantly fewer unwanted or clinically insignificant antibodies detected with the use of gel. Fewer delayed serologic transfusion reactions and no transfusion-associated hemolytic events occurred in the year that gel was used. Although we initially found the selection of the gel test to be a dilemma, our ultimate decision appears to have successfully protected patient safety and balanced sensitivity with specificity. Immunohematology 2001;17:86–89

Key Words: antibody detection, gel, PEG

Since 1981, the Transfusion Service at Duke University Hospital has eliminated many nonessential tests and streamlined protocols while maintaining patient safety. Our course of change has been very similar to that of the University of Michigan described by Judd. Examples of changes include elimination of a routine direct antiglobulin test on all patients, implementation of immediate spin compatibility test for patients with no unexpected antibodies, and elimination of the test for weak D on patients. In addition to elimination of specific tests, we have changed our antibody detection method from use of albumin in the indirect antiglobulin test to low-ionic-strength solution (LISS) in 1981 and from LISS to polyethylene glycol (PEG) 10 years later. Each change in antibody detection method was made to enhance rapid detection of clinically significant antibodies.

In 1996, we faced new challenges. Increasing scarcity of well-trained or adequately experienced technologists began to create recurring difficulty in filling staff vacancies. Also, increasing clinical activity began to threaten our capacity to maintain appropriate laboratory support. Although satisfied with the performance of PEG for antibody detection, we felt compelled to evaluate new technologies that would eventually allow for automation. Our previously reported evaluation of the gel test (Ortho-Clinical Diagnostics, Raritan, NJ), the solid phase test (Immucor, Norcross GA), and the reports of other investigators resulted in our continued use of PEG. When compared with gel, we found that PEG detected more potentially clinically significant antibodies than gel but without loss of specificity. The solid phase test, although exquisitely sensitive, yielded too many unwanted positive results to be suitable for routine antibody detection in our laboratory.

Throughout 1998, staffing and workload issues continued to worsen, prompting reconsideration of transition to automated methods. We chose to implement gel testing paired with the Tecan MegaFlex-ID instrument (Micro Typing Systems Inc., Pompano Beach, FL), which automates pipetting the gel test. We found the gel test to be standardized and simple, with fewer steps and manipulations, and it provided consistent and reproducible test results. As we considered transitioning from PEG to gel, we questioned whether we might be sacrificing clinically significant sensitivity. These preliminary concerns were lessened considerably due to the absence of reports of increased hemolytic reactions by gel users since the test had been in use in the United States. We also reasoned that perhaps some antibodies detected only in PEG might not be clinically significant. For example, in a previous report on the lack of significance of “enzyme-only antibodies,” we found that most of these enzyme-only antibodies that lacked clinical significance were also detected by PEG.
We performed a retrospective analysis comparing the outcome of one year’s experience with gel and PEG. The specificities of identified antibodies as well as transfusion reactions were evaluated.

Materials and Methods

In 1998, PEG (3350 mw; Sigma, St. Louis, MO) was used in antibody detection and identification tests and antiglobulin compatibility tests. Our method was the same as described by Nance and Garratty except that 15% PEG was used instead of 20%. With 20% PEG, we found that there was inadequate removal of PEG-precipitated protein with the use of automated cell washers and, as a result, IgG-coated control cells often were not agglutinated. Reducing the concentration of PEG alleviated much of this problem while still maintaining sensitivity. Two drops of patient's serum, 4 drops of 15% PEG, and 1 drop of 3% reagent or donor red blood cells (RBCs) were incubated at 37°C for 15 minutes, followed by the indirect antiglobulin test with anti-IgG.

During 1999, we implemented gel testing (Ortho-Clinical Diagnostics) following parallel testing with PEG that confirmed comparability in the test sample. In 2000, we were using the gel test for antibody detection, identification, and antiglobulin compatibility tests. Twenty-five µL of patient's serum and 50µL of 0.8% LISS-suspended reagent or donor RBCs were incubated for 15 minutes at 37°C in gel cards containing anti-IgG (Micro Typing Systems). Following incubation, cards were centrifuged for 10 minutes according to manufacturer's directions. Pipetting of reagents and samples for antibody detection tests was performed either manually or on the Tecan MegaFlex-ID.

To evaluate our experience for calendar years 1998 and 2000, we conducted retrospective review of records of antibody specificities and transfusion reactions.

Results

In 1998, while using PEG for antibody detection and identification, 3085 antibodies were detected in 37,832 samples (8%) submitted for pretransfusion testing. In 2000, while using the gel test, 2715 antibodies were detected in 43,405 samples (6%). Table 1 compares the percentages of potentially clinically significant or wanted specificities identified. Table 2 compares insignificant, or unwanted, antibodies identified. Overall, comparison of potentially clinically significant antibodies and clinically insignificant antibodies identified showed that while using PEG, 71 percent of antibodies identified were potentially clinically significant and 29 percent were considered unwanted or insignificant. While using gel, 80 percent of antibodies identified were potentially clinically significant and 20 percent were insignificant.

In 1998, we transfused 40,083 red cell units. Transfusion-related hemolytic events were reported in four patients. One reaction was due to mechanical or thermal effects, one was due to a delayed reaction because of anti-S in a sickle cell patient, and in two cases, there were no immunological causes of hemolysis detected. Nineteen delayed reactions were discovered as part of routine pretransfusion testing following recent transfusion and not as a result of reported clinical hemolysis, thus fitting the definition of delayed serological transfusion reaction (DSTR) as described by Ness et al. (Table 3). This was compared to the use of

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>Rh</td>
<td>44.4%</td>
<td>51.9%</td>
</tr>
<tr>
<td>Duffy</td>
<td>5.4%</td>
<td>6.2%</td>
</tr>
<tr>
<td>Kidd</td>
<td>6.6%</td>
<td>4.0%</td>
</tr>
<tr>
<td>Kell</td>
<td>11.5%</td>
<td>15.0%</td>
</tr>
<tr>
<td>S/s</td>
<td>2.4%</td>
<td>2.7%</td>
</tr>
<tr>
<td>Total</td>
<td>3085</td>
<td>2715</td>
</tr>
</tbody>
</table>

<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>M/N</td>
<td>2.8%</td>
<td>2.6%</td>
</tr>
<tr>
<td>Lewis</td>
<td>6.3%</td>
<td>1.7%</td>
</tr>
<tr>
<td>P_1</td>
<td>1.3%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Inconclusive</td>
<td>7.7%</td>
<td>8.6%</td>
</tr>
<tr>
<td>Cold auto</td>
<td>2.2%</td>
<td>0.9%</td>
</tr>
<tr>
<td>Warm auto</td>
<td>5.8%</td>
<td>4.0%</td>
</tr>
<tr>
<td>HTLA*</td>
<td>2.4%</td>
<td>1.0%</td>
</tr>
<tr>
<td>Total</td>
<td>3085</td>
<td>2715</td>
</tr>
</tbody>
</table>

*High-titer-low-avidity

Table 3. Comparison of DSTR

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>4 anti-E</td>
<td>5 anti-K</td>
</tr>
<tr>
<td>3 anti-c</td>
<td>2 anti-c</td>
</tr>
<tr>
<td>3 anti-Fy*</td>
<td>2 anti-E, -Fy*</td>
</tr>
<tr>
<td>3 anti-Jk*</td>
<td>1 anti-D</td>
</tr>
<tr>
<td>2 anti-Jk*</td>
<td>1 anti-Jk*</td>
</tr>
<tr>
<td>2 anti-D</td>
<td></td>
</tr>
<tr>
<td>1 anti-c,-E</td>
<td></td>
</tr>
<tr>
<td>1 anti-E,-Jk*</td>
<td></td>
</tr>
<tr>
<td>Total: 19 DSTR</td>
<td>Total: 9 DSTR</td>
</tr>
</tbody>
</table>

*Delayed serological transfusion reactions
the gel test in 2000 when 42,204 RBC units were transfused; no hemolytic events were reported and nine DSTRs were detected. During both years, in all cases, new antibody/ies that were detected in the eluate were also detectable in the plasma.

Discussion

Our previous observations suggested the possibility that gel might miss significant numbers of potentially significant antibodies. However, analysis of antibody testing and transfusion reaction experience using gel failed to confirm this possibility. Gel detected slightly higher numbers of Rh and Kell antibodies and modestly lower numbers of anti-Jkα (Table 1). During the year in which gel was being used, only one example of a DSTR was detected due to anti-Jkα, whereas three examples of DSTRs due to anti-Jkα were detected when PEG was being used (Table 3). However, total clinically significant antibodies detected by the two methods are comparable.

Comparison of unwanted antibodies agreed with our perceptions as well as our previous reports; fewer unwanted antibodies are detected with the use of gel. This level of specificity is valuable, as additional testing such as cold antibody screens, prewarm testing, warm and cold adsorptions, and titrations are required to investigate unwanted antibodies, such as warm and cold autoantibodies and insignificant antibodies with high-titer-low-avidity characteristics. In addition to labor savings, unnecessary delays in providing blood to the patient are avoided when fewer unwanted positive tests are detected.

The comparison of transfusion reactions occurring while PEG and gel were used shows that no hemolytic reactions were reported and significantly fewer DSTRs were detected when gel was used. This agrees with the commonly held belief that many of the antibodies detected only in PEG may not be clinically significant.

This report describes our experience of knowingly changing to a method that appeared to have less sensitivity than our current method. In our desire to detect as many clinically significant antibodies as possible, our reluctance to change to the gel test in 1996 suggests that perhaps we too were guilty of overkill as described by Issitt. As Issitt discussed, selection of an antibody detection method in the new millenium must be based on prevailing circumstances. Our prevailing conditions of inadequate staffing required that we consider an alternative antibody detection method that could be automated. Although we initially found this to be a dilemma, our ultimate decision appears to have successfully protected patient safety and balanced sensitivity with specificity. The successful, safe transfusion of nearly 90,000 units of red cells since implementing gel technology reinforces this judgement.

References


Martha Rae Combs, MT (ASCP), SBB, Analytical Specialist, Duke University Hospital, Transfusion Service, Box 2928, Durham, NC 27710; Steven J. Bredehoeft, MD, Medical Director, Duke University, Transfusion Service, Durham, NC.

Attention: Presidents of State Blood Bank Associations—In order to increase the number of subscribers to Immunohematology, we are soliciting membership lists of your organizations. Upon receipt of such a list, each person will receive a complimentary copy of Immunohematology, and, if desired, a personal letter from the association president. For further information, contact: Mary H. McGinniss, Managing Editor, by phone or fax at (301) 299–7443.

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1st week in January for the March issue
1st week in April for the June issue
1st week in July for the September issue
1st week in October for the December issue

Fax these items to Mary H. McGinniss, Managing Editor, (301) 299–7443.
A hemolytic transfusion reaction due to anti-K undetected by a LISS antibody screen

Significant hemolytic transfusion reactions caused by anti-K have been reported, including a case in which the antibody failed to react using various low-ionic-strength solution (LISS) reagents. We identified an anti-K that was initially undetectable by LISS technique and that resulted in an acute hemolytic transfusion reaction.

An 84-year-old, group O– female with a history of atrial fibrillation, permanent pacemaker insertion, osteoarthritis, and Alzheimer’s dementia, underwent open reduction-internal fixation for a right hip fracture. Her transfusion history was unknown although prior transfusion was likely due to hip surgery many years ago. The preoperative antibody screen was found to be negative using LISS antiglobulin technique (N-Hance; Gamma Biologicals, Inc., Houston, TX) with a 15-minute incubation time at 37°C. Two group O– red blood cell (RBC) units were transfused intraoperatively based on compatible immediate-spin saline crossmatch results. As per our blood bank protocol, antiglobulin crossmatches were completed for the two RBC units, although a 35-minute delay was noted due to heavy work flow at the time. One of the two units was found to be 1+ incompatible by the antiglobulin crossmatch, but the unit had been transfused prior to completion of the test.

Clinically, 6 hours after surgery and intraoperative transfusion of the two RBC units, the patient suddenly became hypoxic and unresponsive after receiving narcotic pain medication. She was given oxygen by mask and treated with narcan and sodium bicarbonate, after which she became responsive although agitated. Shortly after, scanty, dark urine was noted along with a drop in hemoglobin to 8 g/dL from 11 g/dL preoperatively, and a hemolytic transfusion reaction was suspected owing to transfusion of the incompatible RBC unit. One hour later, she became hypoxic and hypotensive (blood pressure: 70/50 mmHg), requiring intubation as well as IV fluid and renaldose dopamine supportive therapy. Two additional units of RBCs were also given. Four units of fresh frozen plasma were transfused due to disseminated intravascular coagulation, but the patient later developed ecchymoses and a left groin hematoma. The next morning, she went into cardiac arrest in the surgical intensive care unit and was pronounced dead. An autopsy was not performed.

On follow-up testing using LISS antiglobulin technique, anti-K was identified in the preoperative sample (titer of 8) with a 30-minute incubation at 37°C but was not detectable with a 15-minute incubation, confirming our suspicion that prolonged incubation time was required for detection. Testing of the hemolyzed posttransfusion sample with either 15- or 30-minute incubation time was negative, whereas the direct antiglobulin test was 1+ positive for C3d and negative for IgG: the eluate was also negative. The incompatible unit was confirmed to be K+. Increasing the serum-to-reagent RBC drop ratio from 2:1 to 3:1 and repeating the tests on the pretransfusion sample with 15- to 30-minute incubations once again yielded negative and positive results, respectively.

Although it is likely that a number of factors were involved in this patient’s demise, including her underlying medical condition as well as the possible adverse effect of narcotic medication, we believe the hemolytic reaction contributed significantly. We subsequently eliminated the antiglobulin crossmatch procedure for patients with no antibody history and a negative antibody detection test. We conclude that this was indeed an example of an anti-K that was nonreactive in LISS using our routine testing procedure; however, it is possible that the initial inability to detect the antibody was due to factors unrelated to LISS, such as weakened antigen expression on the reagent screening cells after storage. Because anti-K as well as other RBC antibodies had been detected in many other cases using the same screening cell reagents maintained under the same conditions, we feel that the latter is much less likely. This case, and others like it, should serve to humble us even as we take confidence in our technologic breakthroughs and abbreviated testing procedures.

Mark T. Friedman, DO
Alan P. Carioti, MT(ASCP)SBB
Lutheran Medical Center
Blood Bank
150 55th Street
Brooklyn, NY 11220
Ortho Dedication

This is the 11th year that Ortho-Clinical Diagnostics has sponsored the September issue of *Immuno-hematology*. The editors and readers would like to thank the management of Ortho-Clinical Diagnostics for supporting the publication and distribution of the Journal. In addition to their donation to support the publication of each September issue, they distribute many free copies of each issue to their customers.

Please tell your Ortho sales representative how much you appreciate their generosity and concern for education.

Delores Mallory
Editor-in-Chief

Mary McGinniss
Managing Editor

References


ERRATA

*Vol. 17, No. 2, 2001 Page 58*

The first paragraph of the response letter from Martha Combs, et al. is missing several words. The last two sentences should read:

“Three patients received one CPDA-1 unit each and no other antibody-containing unit. One patient received one CPDA-1 unit and one additive unit containing alloantibodies.”
IN MEMORIUM

Dr. William Sherwood, medical editor of Immunohematology for 5 years and a nationally respected leader and authority on transfusion medicine and blood center operations, died at his home at age 67.

Early in his career, Dr. Sherwood was a captain in the U.S. Army and served as chief of hematology at Second General Hospital, Landstahl, Germany. He was chief of Jefferson Hematology Service at Philadelphia General Hospital.

In 1971 he became the medical director and then director of the Penn-Jersey region of the American Red Cross. He coordinated the efforts of the Penn-Jersey region to create a single system of blood supply in the area.

Dr. Sherwood served as senior vice president of the American Red Cross Biomedical Services. Among his many successes in the blood transfusion field, his interest in and skills with computers directed the establishment of the first American Red Cross blood center computer program in the Penn-Jersey region in 1975. While at National Headquarters as senior vice president, he helped begin the work of creating a single computer system to serve all American Red Cross blood centers.

Dr. Sherwood was known locally and nationally as an innovative expert in transfusion medicine and was honored in 1992 with the American Red Cross Charles R. Drew Award and by the Pennsylvania Association of Blood Banks with the Lyndall Moltman, MD, Memorial Lecture Award.

Dr. Sherwood was on the editorial board as medical editor for Immunohematology from 1996 until his retirement in 2000. He was an advisor to Immunohematology on the construction and content of the Immunohematology Web page, one of the first to appear on the American Red Cross Web site.

Dr. Sherwood was an inspired and inspiring physician and a person who worked tirelessly on behalf of the patients and donors.

Delores Mallory
Editor-in-Chief

Mary McGinniss
Managing Editor
IN MEMORIUM

John Case, FIBMS, FIMLS 1926 – 2001

John Case was born December 29, 1926 in Poole, Dorset, England. He began his career in medical laboratory technology serving in the Royal Army Medical Corps from 1945 to 1948. He worked at Wembley and Whipscross Hospitals in London and the South London Blood Transfusion Center in Sutton, Surrey. He and his family immigrated to New Zealand in 1959, where he was in a charge position at the blood bank at Dunedin Hospital. Moving to Australia in 1971, he was at the Commonwealth Serum Laboratories in Melbourne. In 1976 he came to Houston, Texas to become vice president of Regulatory Affairs at Gamma Biologicals, Inc., where he remained until his retirement in January 1999.

John was an expert in blood group serology well before he became involved with writing of product applications, package inserts, and FDA responses. His early work was with low-frequency antigens. He was an expert on the blood group systems and his own red cells were discovered to be the rare Rg-. He was a faithful panel donor and his red cells have been used in serologic problem solving around the world.

He was known worldwide for his influence on keeping English “proper” in publications.

He was fearless in his commitment to his views and honest to his ideals and his friends. John was a member of committees in the World Health Organization and the American Association of Blood Banks (AABB). He participated in many technical lectures for yearly tutorials at Gamma Biologicals, Inc. and for the AABB. For his contributions to the field, he was greatly honored. From the Australian Society of Blood Transfusion, he received the Ruth Sanger Oratorical Award. The Kay Beattie Award was given from the Michigan Association of Blood Banks. The L. Jean Stubbins Award was presented from the South Central Association of Blood Banks. The AABB presented him with both the Sally Frank Award and the Ivor Dunsford Memorial Award. John was a great communicator both in writing and orally.

John loved his roses and was a talented grower. He grew champion roses and was a long-time member of and an author for an Australian rose association. He will be greatly missed by his many friends and admirers.

Delores Mallory
Editor-in-Chief

Mary McGinniss
Managing Editor
IN MEMORIUM

Fred Stratton, BSc, MD
1913 – 2001

Dr. Fred Stratton was born October 18, 1913 and spent his childhood in a Quaker home in Levenshulme, Manchester, England.

He received a BSc in 1934, an MB ChB in 1937, a diploma in public health in 1939, an MD with commendation in 1944, and a DSc in 1957. He was a founding Fellow of the Royal College of Pathologists and was granted a Fellowship of the Royal College of Physicians for his outstanding work in transfusion medicine.

Dr. Stratton began his career in blood transfusion medicine in 1940 when he joined the “Blood Depot” in Manchester as part of the plans for blood collection in WWII. He continued with various positions until he became regional transfusion director in 1949 and held this post until his retirement March 31, 1980.

In addition to his excellent work in blood transfusion medicine at the Manchester Center, he became interested in blood group serology, particularly complement and blood group antibodies, and was the first to recognize the weaker form of the Rh antigen, which became known as D u. His knowledge of laboratory tests was profound, and in 1958, with his associate Dr. Peter Renton, he published the important text Practical Blood Groupings.

His tremendous accomplishments were honored in 1963 with the Oliver Memorial Fund Award for outstanding contributions to science and practice of blood transfusion. In 1977, he was awarded a Personal Chair in Human Serology at the University of Manchester. In 1978, he was awarded the Karl Landsteiner Award, the highest award of the American Association of Blood Banks. He was a founder of, and in 1981, the inaugural president for the British Blood Transfusion Society. In 1987, the BBTS awarded him its highest award, the James Blundell Award.

Dr. Stratton was truly one of the pioneers of blood transfusion medicine and blood group serology.

Delores Mallory
Editor-in-Chief

Mary McGinniss
Managing Editor
ANNOUNCEMENTS

International Society of Blood Transfusion. The 11th Regional Western Pacific Congress of the International Society of Blood Transfusion will be held in Shanghai, China, from November 10–13, 2001 at the Exhibition and Conference Hall located in the Shanghai Rainbow Hotel. The program will offer plenary lectures, scientific symposiums, and poster sessions on the latest advances in blood banking and transfusion medicine. There also will be planned social activities to promote understanding and camaraderie among the different nationalities and cultural backgrounds.

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For further information, Contact: Congress Secretary, Prof. ZHU Youg Ming, Shanghai Blood Center, #1191, Hong Qiao Road, Shanghai 200051, China; phone: (81–62) 62780789; fax: (86–21) 62958414; e-mail: isbt2001@sbc.org.cn

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   C. Running title of ≤ 40 characters, including spaces
   D. 3 to 10 key words
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      Clinical and/or hematologic data and background serology.
   C. Materials and Methods
      Selection and number of subjects, samples, items, etc. studied and description of appropriate controls, procedures, methods, equipment, reagents, etc. Equipment and reagents should be identified in parentheses by model or lot and manufacturer’s name, city, and state. Do not use patients’ names or hospital numbers.
   D. Results
      Presentation of concise and sequential results, referring to pertinent tables and/or figures, if applicable.
   E. Discussion
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   C. Use inclusive pages of cited references, e.g., 1431–7.
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   A. Number consecutively, head each with a brief title, capitalize first letter of first word (e.g., Table 1. Results of ...), and use no punctuation at the end.
   B. Use short headings for each column, and capitalize first letter of first word.
   C. Place explanations in footnotes (sequence: *, †, ‡, §, ‡‡, ‡‡‡).
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   A. Figures can be submitted either drawn or photographed (5" X 7" glossy).
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5. References—limited to ten.
6. One table and/or figure allowed.

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