Downregulation of the Anti-HLA Alloimmune Response by Variable Region-Reactive (anti-idiotypic) Antibodies in Leukemic Patients Transfused With Platelet Concentrates

By Ella Atlas, John Freedman, Victor Blanchette, Michel D. Kazatchkine, and John W. Semple

Approximately 30% to 40% of patients with acute leukemia receiving repeated pooled random-donor platelet transfusions develop anti-HLA alloantibodies. Over time, however, serum anti-HLA concentrations decrease in approximately 50% of these patients, despite continued exposure to platelet and/or red blood cell transfusions. Using an enzyme-linked immunosorbent assay to measure serum Igs, the present study demonstrates that the sera of 67% of 82 transfused patients exhibiting a decrease in anti-HLA contain antibodies (anti-idiotypes) that react with the variable (V) region of anti-HLA antibodies. Anti-HLA binding to platelet membranes could be inhibited by these serum antibodies in 36% of the patients, indicating they had paratope-related reactivity. Protein G sepharose absorption showed that the anti-HLA V region-reactive antibodies were IgG. Of the 43 patients who had a decrease in anti-HLA levels, there were 16 whose anti-HLA decreased to undetectable levels; 7 (44%) developed anti-idiotype antibodies that could specifically inhibit their own previously anti-HLA–positive serum. In contrast, antibodies with reactivity to the V region of anti-HLA antibodies (anti-idiotypes) were not demonstrable in patients who developed anti-HLA that did not decrease or disappear. The findings suggest that the development of anti-HLA V region-reactive antibodies (anti-idiotype antibodies) correlates with a decrease in anti-HLA antibody formation in patients multiply transfused with platelet concentrates. The observations indicate that anti-idiotype antibodies may downregulate alloimmune responses in patients undergoing repeated allostimulation during platelet transfusion therapy.

PLATELET USE has increased markedly in recent years with a resulting awareness of problems associated with platelet transfusion therapy. A major problem in patients who receive multiple platelet transfusions has been the development of platelet alloimmunization and a consequent state of clinical refractoriness to random-donor platelet transfusions. Patients develop antiplatelet antibodies that shorten the survival of the transfused platelets; anti-HLA antibodies are most common, but platelet-specific antibodies can also occur. The incidence of alloimmunization to HLA in patients with acute leukemia is approximately 30% to 40%.1-5

Antibody specificities in alloimmunized recipients have been shown to change over time; in some cases, narrow alloantibody specificities may broaden, while in others, antibodies may actually disappear.6-8 It has become evident that in approximately 50% of patients who develop anti-HLA antibodies, the antibodies may disappear, or become less reactive, despite continued exposure to platelet and red blood cell (RBC) transfusions.7,8 On the other hand, some transfusion recipients never become alloimmunized despite long-term antigenic stimulus.

One of the mechanisms by which the peripheral antibody repertoire is regulated is via idiotypic interactions.9-11 One possible explanation of the above phenomenon in transfusion recipients is that anti-HLA and antiplatelet antibody reactivity in alloimmunized patients may be under the regulation of antibodies reactive with V regions, including anti-idiotype antibodies.12-14 This study provides evidence that the disappearance of anti-HLA antibodies in multiply-transfused patients with acute leukemia is related to appearance of antibodies reactive to the V region of anti-HLA alloantibodies.

PATIENTS AND METHODS

Patients. Eighty-two patients (39 females and 43 males) were studied from a population of 134 newly diagnosed patients with acute leukemia receiving multiple platelet transfusions. Anti-HLA (Ab1) antibody reactivity in the patients sera was determined by three different methods (lymphocytotoxicity, flow cytometry, and solid-phase RBC adherence), as described elsewhere.15-16 None of the patients had detectable anti-HLA antibodies before transfusion therapy. Criteria for inclusion in the current study were either that the patients never made anti-HLA antibodies (N = 39) or that they made anti-HLA that decreased over time (N = 43, in 16 of whom antibody decreased to undetectable levels). Females were classified into those who had been previously pregnant (N = 26) and those who were never pregnant (N = 13). Patients who made anti-HLA antibodies received a mean number of platelet units of 59 ± 43. The mean number of days to detectable anti-HLA formation was 49 ± 42. The average length of follow-up was 189 ± 196 days. In addition, 27 patients who developed anti-HLA that persisted were examined.

Twenty-two normal volunteers (7 females with no history of pregnancy and 15 males) were also studied. All patients received essentially the same chemotherapeutic regimen for their leukemia.

Reagents. Intravenous gamma globulin (IVIg; Ivigem; Cutter Biologicals, Etobicoke, Ontario, Canada) was supplied by the Toronto Centre of the Canadian Red Cross Blood Transfusion Service.

Sera. Nine hundred twelve sera were available from 134 patients studied over a 3-year period. All patients were newly diagnosed leukemias and serum samples were obtained before transfusion, every 2 weeks during remission-induction, and then monthly; sera were stored at −70°C until use. Of these, 236 sera from 82 patients were investigated in the current study. Sera from 22 normal volunteers were used for comparison. As a source of reference Ab1 antibodies, 10 sera known to contain strong polyspecific anti-HLA, from patients repeatedly transfused with platelet concentrates, were pooled.
Preparation of Ab1 F(ab')2 fragments. IgG was purified from the pooled anti-HLA reference serum or from normal sera by the method of Hardy.17 The IgG fraction was precipitated by 50% saturated ammonium sulphate, dialyzed against 50 mmol/L Tris-saline, pH 8.0, and albumin removed by adsorption with QAE-Sephadex A-50 (Pharmacia, Mississauga, Ontario, Canada). The IgG fraction (1% to 3% wt/vol) was dialyzed against 0.2 mol/L sodium acetate, pH 4.5, and digested with 2% (wt/wt) pepsin (Sigma Chemical Co, St Louis, MO) for 24 hours at 37°C. The F(ab')2 fragments were purified by Sephacryl S-200 HR (Pharmacia) gel filtration and protein A-Sepharose (Pharmacia) chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions showed that the purity of the F(ab')2 protein was greater than 97%.

Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies reactive with anti-HLA F(ab')2 fragments. Patients' sera not containing anti-HLA were examined for the presence of anti-HLA V region reactive antibodies (Ab2) by ELISA. F(ab')2 fragments of reference anti-HLA or of normal sera were adjusted to 10 μg/mL in carbonate/bicarbonate buffer, pH 9.6, and coated on 96-well ELISA plates (50 μL/well) for 18 hours at 4°C. The plates were washed three times with phosphate-buffered saline (PBS-TWEEN) (washing buffer, 0.05% Tween 20), blocked with 3% bovine serum albumin (BSA) in PBS (300 μL/well) for 2 hours at 20°C, and again washed three times with washing buffer. Patient sera, serially diluted from 1:10 to 1:180 in the blocking buffer, were added to the plates (50 μL/well) and incubated for 2 hours at 37°C. Similarly diluted normal sera were used as negative controls. The plates were then washed three times in washing buffer and 100 μL of alkaline phosphatase-conjugated F(ab')2 goat antihuman IgG (Cedarlane Laboratories, Hornby, Ontario, Canada) was added. After incubation at 20°C for 2 hours, the plates were washed four times and 100 μL of substrate solution (p-Nitrophenyl phosphate; BioRad Labs, Mississauga, Ontario, Canada) was added until color conversion occurred. The reaction was stopped by the addition of 25 μL of 3 mol/L NaOH and the OD405 was read on an ELISA reader (Titertek Multiscan; ICN-Flow Laboratories, Mississauga, Ontario, Canada). Anti-HLA V region-reactive antibodies were not detected in the normal volunteers, nor in the patients (not strictly included in this study) who had anti-HLA antibodies that did not decline.

Preparation of platelet membranes. Pooled group O platelets from normal donors were washed in Tris-buffered saline (TBS), pH 6.5, and platelet membranes prepared by the method of Woods et al.16 Briefly, 2 × 109 platelets/mL were lysed in 0.5% Triton X-100/TBS, pH 7.4, for 30 minutes at 4°C and centrifuged for 18,000g for 1 hour at 4°C. The pelleted membranes were stored at −70°C until use.

ELISA to measure the inhibition of antibody binding and characterization studies. Reference anti-HLA serum (1:4 final concentration) was incubated at 37°C for 2 hours with equal volumes of tiritated (1:2 to 1:16 in PBS) patient sera found positive for V region-dependent antibodies. Similarly diluted normal sera in place of the patient sera were used as negative controls. The mixtures were then tested for residual anti-HLA activity in a solid-phase platelet membrane ELISA. ELISA plates were coated with 50 μL of platelet membranes diluted 1:10 in PBS. After 18 hours at 4°C, the plates were washed three times in PBS-TWEEN 20 (0.05%), blocked with 3% BSA for 1 hour at 37°C, and again washed three times. Each Ab1-Ab2 mixture was then added (50 μL/well) and the plates were incubated for 2 hours at 37°C. The plates were then washed and bound IgG was measured as described above. Percent inhibition was calculated from the formula:

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\frac{1 - OD_{405}(\text{Ab1 sera alone}) - OD_{405}(\text{Ab2 sera:Ab1 sera})}{OD_{405}(\text{Ab1 sera alone})} \times 100
\]

Sera from those patients who showed a disappearance of, or decrease in, their anti-HLA antibody despite continued antigenic stimulus (transfusion) were tested for their ability to "internally" inhibit the anti-HLA reactivity of the patient's own previously positive anti-HLA serum. Isotypic characterization of the inhibitory antiidiotypes was performed by preabsorption of the serum with protein G-Sepharadex beads (Pharmacia), ie, 50 μL of test sera was mixed with an equal volume of beads and gently agitated for 1 hour at 37°C. The mixture was then centrifuged and the supernatants were tested for inhibition of anti-HLA by ELISA as described above.

Statistical methods. Significance between percentages was determined by one-tailed Z-values.19

RESULTS

Screening for antibodies reactive with the V region of anti-HLA antibodies. Using an ELISA technique, patient sera were tested for reactivity with the F(ab')2 fragments of anti-HLA. Overall, as shown in Table 1, the sera of 55 of 82 (67%) patients contained antibodies reactive with the V region of anti-HLA F(ab')2 fragments. We analyzed 263 sera from the 82 patients for these antibodies. Figure 1 shows the quantitative differences between those sera that contained anti-HLA V region-reactive antibodies and those that did not. Of the 263 sera, 133 sera (representing 55 patients) showed increased reactivity against the F(ab')2 fragments. None of the sera tested from 22 healthy volunteers showed reactivity against anti-HLA F(ab')2 fragments. In addition, none of the patients' sera or normal sera showed reactivity against F(ab')2 fragments derived from normal donor sera (not shown).

Of 52 patients not formally included in this study because of high, persistent anti-HLA titers, 27 were tested for serum reactivity against the F(ab')2 fragments and all were found to be negative.
Fig 1. One hundred thirty-three of 263 sera from 55 of 82 patients contained IgG anti-idiotypes specific for reference anti-HLA F(ab')2 fragments. The results presented are mean OD(405) values ± SEM of the titrated sera. (c) Positive anti-idiotypes (N = 133 sera; 55 patients); (d) negative anti-idiotypes (130 sera; 27 patients); (v) normal sera (22).

When IV Ig was tested for its reactivity against either the reference anti-HLA F(ab')2 fragments or against normal serum F(ab')2 fragments, enhanced reactivity was seen at low dilutions (OD(405) of 1/20 to 1/80 serum dilutions ranged from 0.35 ± 0.08 to 0.28 ± 0.04, respectively; for comparison, see Fig 1).

Serum reactivity against anti-HLA F(ab')2 fragments, ie, the presence of anti-HLA V region-reactive antibodies, was examined in relation to whether the patients had a history of previous pregnancy. Table 1 indicates that, although reactivity was seen in all six women who had been previously pregnant but never made anti-HLA, there was, overall, no significant difference in anti-HLA F(ab')2 reactivity between percentages in the patient groups related to pregnancy.

"Binding to platelets by patient anti-HLA sera is inhibited by antibodies reactive with the V region of anti-HLA antibodies." Patients' sera that contained anti-HLA V region-reactive antibodies were tested for their ability to neutralize or inhibit the binding of the reference anti-HLA serum to platelet membranes. Such inhibition would suggest that the anti-HLA V region-reactive antibodies were specific for anti-HLA paratopes. Overall, the sera of 36% (20 of 55) of the patients who made V region-reactive antibodies inhibited anti-HLA binding to platelet membranes (Table 1). The results presented in Fig 2 are the mean percentages (±SEM) of inhibition at various ratios of test sera with reference anti-HLA sera. The percentage of inhibition varied depending on the ratio of V region-reactive serum to anti-HLA reference serum (10% inhibition at a ratio of 1:1 to 50% inhibition at a ratio of 4:1).

Table 1 also shows that approximately twice as many patients who had been pregnant had antibodies that inhibited the anti-HLA binding compared with patients who had never been pregnant (50% v 29%, P = .005). These findings applied whether the patients had failed to make anti-HLA at all or whether they made anti-HLA that subsequently decreased or disappeared.

To confirm the class of the inhibitory anti-HLA V region-reactive antibodies, the sera were preabsorbed on protein G-Sepharose beads. The inhibitory effect of the sera was reversed by protein G-Sepharose absorption in all cases, indicating that the inhibitory antibodies were IgG. Table 2 shows results from three experiments showing the reversal of inhibition by protein G absorption.

"Internal" inhibitions. Sixteen patients had made anti-HLA antibodies whose reactivity disappeared completely despite continued platelet transfusions. To determine whether this disappearance correlated to the appearance of anti-HLA V region-reactive antibodies, "internal" inhibitions were performed with the sera of these patients, ie, the sera lacking any detectable anti-HLA antibody were used to neutralize the patient's own previously detectable anti-HLA (Ab1). Table 1 shows that 50% (6 of 12) of previously pregnant patients and 25% (1 of 4) of those patients who had never been pregnant had developed anti-HLA V region-reactive antibodies over time that could specifically inhibit platelet membrane binding of the anti-HLA they had previously made. Figure 3 shows that the extent of "internal" inhibition for each of the seven patients varied, ranging from 17% to 66%.

**DISCUSSION**

One of the major problems encountered with platelet transfusion therapy is the development of anti-HLA anti-
Anti-idiotypic antibodies in leukemic patients

Anti-idiotypic antibodies may either disappear or become less reactive, despite continued exposure to platelet and RBC membranes. The results presented are the maximum percent inhibition ± SEM. The maximum inhibition was found at Ab2 serum:Ab1 serum ratio of 4:1 for all patients except patient 4 (2:1). C, average percent inhibition ± SEM of control sera mixed with patients Ab1-positive sera.

Fig 3. The sera of 7 of 16 patients (44%) at the time they became Ab1 negative contained IgG anti-idiotypes that were able to inhibit their previously Ab1-positive serum from binding platelet membranes. The results presented are the maximum percent inhibition ± SEM. The maximum inhibition was found at Ab2 serum:Ab1 serum ratio of 4:1 for all patients except patient 4 (2:1). C, average percent inhibition ± SEM of control sera mixed with patients Ab1-positive sera.

bodies that can lead to the refractory state. In approximately 50% of patients who develop anti-HLA antibodies, the antibodies may either disappear or become less reactive, despite continued exposure to platelet and RBC membranes. However, some transfusion recipients never become alloimmunized despite long-term antigenic stimulation. We investigated the possibility that failure to make anti-HLA, or reduction in anti-HLA despite continued transfusion, was due to the appearance of anti-HLA V region-reactive antibodies.

V region-reactive antibodies, which include anti-idiotypic antibodies (Ab2), are known to regulate the antibody repertoire through variable region interactions. It has been suggested that these interactions may have beneficial effects in a variety of immune-mediated processes by downregulating the immune response. For example, anti-idiotypic antibodies have been shown to be present in the sera of patients receiving blood transfusions before renal transplantation, as well as in the sera of women alloimmunized by pregnancies. In most of these studies, the method used to detect anti-idiotypic antibodies was inhibition of complement-dependent cytotoxicity, although some investigators demonstrated the presence of Ab2 by inhibition of binding of Ab1 to HLA antigens.

Sixty-seven percent of patients developed anti-idiotypic antibodies specific for anti-HLA F(ab')2 fragments that did not react with F(ab')2 derived from normal donor sera. Because these V region-reactive antibodies bound only to anti-HLA and not to normal serum F(ab')2 and, further, because they could inhibit anti-HLA interactions, they appear to be anti-idiotypic antibodies. In addition, the demonstration of anti-HLA–specific anti-idiotypic antibodies in patients who did not have detectable anti-HLA at any time during the study supports the possibility that these patients may have been previously stimulated to produce anti-idiotypic antibodies, either by alloimmunization and/or antigenic (anti-HLA) mimicry.

The development of the anti-idiotypic antibodies corresponded to a decrease or absence of Ab1, because anti-idiotypic antibodies could not be detected in the sera of patients with high, persistent anti-HLA reactivities. However, it should be noted that a lack of detectable anti-idiotypic antibodies in these patients may be due to competitive inhibitory influences by the anti-HLA antibodies.

It has been demonstrated that anti-idiotypic antibodies are present in IV Ig preparations. When IV Ig was tested in the ELISA against F(ab')2 fragments derived from either the reference anti-HLA or normal sera, higher reactivities were recorded than seen with normal donor sera. This is consistent with a previous report showing that anti-idiotype antibodies specific for anti-HLA are present in IV Ig. We also observed that γ-globulins from a variety of species sera (rat, human, and mouse) generally have higher reactivities than untreated sera against F(ab')2 fragments in the ELISA (not shown).

Anti-idiotypic antibodies can be classified as either paratope-related (antigen binding site) or paratope-nonrelated (nonsite-associated). Both types are believed to be involved in immune regulation at the B-cell (via surface Ig) and possibly T-cell (via the T-cell receptor) levels. In 36% of the patients whose sera contained anti-idiotypic antibodies, we found inhibition of binding of the reference anti-HLA sera to platelet membranes. This suggests that these antibodies have paratope-related specificities. This anti-idiotype activity was contained in the IgG fraction of the serum, because preabsorption of the serum by protein G-Sepharose, which binds all IgG subclasses, completely reversed the inhibitory effect. There was a consistent trend for a higher proportion of previously pregnant women to have paratope-related anti-idiotypes than observed in the never-pregnant patients. Alloimmunization to paternal HLA can occur as early as 8 weeks of gestation, which may induce an anti-idiotype response. Furthermore, Chaas et al have demonstrated that platelet transfusions in previously alloimmunized mice can induce a strong anamnestic alloimmune response. It may be that prior alloimmunization from pregnancy, together with subsequent re-exposure to HLA via transfusions, results in hyperimmunization of the patient so as to generate cross-reactive anti-HLA idiotypes that preferentially induce inhibitory anti-idiotypes. This possibility is supported, in part, by the observation that anti-HLA specificities in patients' sera often changed over time with repeated transfusions (unpublished observations).

We next determined whether the development of anti-idiotypes correlated with a loss in anti-HLA reactivity. The anti-idiotype antibodies could specifically inhibit the binding of their own previously anti-HLA–positive sera to platelet membranes. Because our assay system did not, however, measure IgM anti-idiotypes, or because anti-idiotype antibodies may be present in amounts too small to be detected in the system used, it cannot be absolutely excluded that all
patients have “internal” anti-idiotypic antibodies. In limited study of the patients, however, examination for anti-idiotypes of the IgM class was negative. Urlacher et al., on the other hand, has reported that sera of multiparous women contain IgM anti-idiotypes specific for IgG anti-HLA antibodies. The present results nonetheless do strongly suggest that in many patients who become alloimmunized by repeated platelet transfusions, the development of anti-idiotypes correlates with a decrease in anti-HLA alloantibodies.

The data support the possibility that anti-idiotypic antibodies can downregulate alloimmunization in multiply-transfused patients and suggests that anti-idiotypes may have potential therapeutic benefits in manipulations of the autoimmune response. However, circulating anti-idiotypes (eg, exogenously administered) may not directly mediate this control, as the process may also be dependent on B-lymphocyte surface interactions, ie, the circulating anti-idiotypes may be solely markers for the process.

In summary, a majority of leukemic patients who had decreased or undetectable levels of alloimmunization (anti-HLA antibodies), despite multiple platelet transfusions, developed anti-HLA-specific anti-idiotypes that could inhibit anti-HLA reactivity. Importantly, the loss of anti-HLA antibodies might be correlated with the appearance of anti-HLA–specific anti-idiotypic antibodies and could potentially result in a better survival of transfused platelets.

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E Atlas, J Freedman, V Blanchette, MD Kazatchkine and JW Semple