Stability of Antigens on Leukocytes in Banked Platelet Concentrates: Decline in HLA-DR Antigen Expression and Mixed Lymphocyte Culture Stimulating Capacity Following Storage

By Mark E. Sherman and Walter H. Dzik

Repeatedly transfused thrombocytopenic patients frequently form antibodies directed against human leukocyte antigens (HLA) and become unresponsive to random donor platelet transfusions. Although exposure to foreign antigens borne on donor leukocytes appears necessary to provoke primary sensitization, the stability of leukocyte antigens during routine platelet storage is largely unknown. Accordingly, we serially measured the expression of surface markers on leukocytes derived from platelet concentrates during storage using immunofluorescence and flow cytometry. Our results indicate that the expression of class I HLA antigens, Leu-4 (T cell), and HLe-i (pan leukocyte) remained stable on lymphocytes under standard platelet storage conditions, but that the percentage of lymphocytes bearing class II HLA antigens declined significantly over time. This decline in lymphocyte HLA class II expression was associated with a significantly diminished ability to stimulate blastogenesis in mixed lymphocyte culture. However, leukocytes retained the ability to respond in mixed lymphocyte culture (MLC) following storage. We also performed studies on lymphocytes cultured in the presence of cyclohexamide, which suggested that the expression of class I HLA antigens and B2 microglobulin are highly sensitive to the inhibition of protein synthesis, whereas the expression of class II HLA antigens, Leu-4, and HLe-i are not. Our results may prove useful in understanding the mechanisms that lead to platelet refractoriness and in designing strategies to prevent HLA alloimmunization.

Material and Methods

Platelet concentrates were prepared by a standard double-spin technique from volunteer whole blood donations collected in CPDA-1 (citrate phosphate dextrose adenine) and stored for five days at 22°C on a rotator. Twenty-one units were randomly selected for study. We sampled and analyzed a 15-mL aliquot of each unit at three different times: 12 hours following phlebotomy, day 3 and day 5 of storage. Before testing, the leukocyte count of each sample was determined on a Coulter model S plus (Coulter Electronics, Hialeah, FL). Chamber leukocyte counts were performed on specimens that contained <500 cells/μL. The total leukocyte counts of the platelet concentrates were estimated by multiplying the leukocyte concentration per unit by the average volume of 50 randomly selected units prepared in our laboratory (48 mL/U).

Isolation of leukocytes. Each 15-mL aliquot was washed three times with RPMI 1640 medium (25 mmol/L HEPES buffer supplemented with L-glutamine, penicillin, and streptomycin; Whittaker MA Bioproducts, Walkersville, MD) and centrifuged at 250 g for ten minutes to reduce platelet contamination. To achieve additional purification, the cell pellet was resuspended in 3.0 mL RPMI containing 50 units of bovine thrombin (Armour Pharmaceutical, Kankakee, IL), rotated for three minutes, and then spun for 20 seconds at 185 g in a Sorvall GLC-2B centrifuge (DuPont, Newton, CT) to remove the resulting platelet aggregates. The supernatant was harvested and spun at 250 g for ten minutes. The resulting platelet-poor suspension was incubated for ten minutes in tris ammonium chloride to lyse contaminating erythrocytes. Following two additional washes with RPMI, the cell concentration was adjusted to a maximum of 10 x 10^6 cells/mL.

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Surface markers. One hundred microliters of each preparation were incubated with mouse monoclonal antibodies directed against the following antigens: HLA-ABC (class I HLA; Cappel Laboratories, Cochranville, PA), HLA-DR (Class II HLA), Leu-4 (pan T cell), HLe-1 (pan leukocyte), Leu-M3 (monocyte; Becton Dickinson, Mountain View, CA), and MY8 (monocyte/macrophage; Coulter Immunology, Hialeah, FL). Antibodies recognizing HLA-ABC, HLA-DR, Leu-M3, and HLe-1 were directly fluorescein conjugated. The cells were incubated with saturating concentrations of monoclonal reagent for 20 minutes at 4°C in a light-protected box (PBS). Following a second incubation with a fluoresceinated goat anti-mouse F(ab′)2 monoclonal reagent (Cappel Laboratories) and two washes with cold PBS, the cells were resuspended in cold 1% paraformaldehyde.

Fluorescence analysis. Each specimen was analyzed for fluorescence intensity on the day of its preparation by counting 10,000 viable cells with a flow cytometer (FACS Analyzer, Becton Dickinson). Lymphoid and nonlymphoid cell populations were distinguished by analysis of volume and right-angle light scatter. Histograms displaying numbers of antigen-positive cells as a function of linear fluorescence were analyzed to yield the highest percentage of fluorescence positive cells. Background immunofluorescence determined by substituting mouse ascites (Bethesda Research Laboratories, Gaithersburg, MD) for monoclonal reagent was subtracted from each result. The number of lymphoid and nonlymphoid cells was determined by multiplying the proportion of cells in each gate by the total leukocyte count. Data obtained on days 3 and 5 of testing were compared with results obtained on day 1 using the paired Student’s t test.

Metabolic inhibition studies. In order to assess the role of protein synthesis in maintaining leukocyte antigen expression, we compared the density of surface markers on lymphocytes cultured in the presence of cyclohexamide with that of untreated cells. Mononuclear cells were collected from heparinized whole blood by Ficoll-Paque (Pharmacia, Piscataway, NJ) density centrifugation and placed into tissue culture flasks containing RPMI, 10% bovine serum albumin, and 100 µg/mL cyclohexamide (Sigma, St Louis), a potent inhibitor of protein synthesis. The experimental preparations consisted of 50 µL cell suspensions containing <1.0 x 10⁶ cells/mL. Control preparations were identical but contained no cyclohexamide. The samples were analyzed by immunofluorescence and flow cytometry following 18 hours of gentle rocking at 37°C in a 7.5% CO₂ incubator. Monoclonal reagents recognizing HLA-ABC, B₂ microglobulin (Cappel Laboratories), HLA-DR, Leu-4, and HLe-1 were tested. We assessed the effect of cyclohexamide on the intensity of surface antigen expression by comparing the mean fluorescence intensity of the treated cells with that of their untreated paired controls.

Mixed lymphocyte cultures. Leukocytes were isolated from ten platelet concentrates at days 1, 3, and 5 of storage using the procedure outlined above (isolation of leukocytes) with the thrombin step omitted. The ability of stored leukocytes to function as stimulators and responders in one-way MLC was examined by culturing aliquots of ten platelet units with cells from a normal male donor. MLCs were established in triplicate in round bottom wells using combinations of 5 x 10⁶ irradiated stimulator cells and 5 x 10⁶ responder cells. Cells were cultured in 5% CO₂ at 37°C for five days and pulsed 18 hours before harvesting with ³H-thymidine. Following automated cell harvesting, the mean radioactivity per well was determined in a liquid scintillation counter (Beckman Minaxi B; Packard, Downers Grove, IL) and a relative stimulation provoked by the cells determined as follows:

Test cells as stimulators in MLC: Relative stimulation = (PC)N - N/N/(Pool), N - N, where (PC), represents irradiated lymphocytes derived from platelet concentrate; N, fresh normal lymphocytes from a single normal individual; N, fresh normal lymphocytes irradiated from a single normal individual; and (Pool), pool of fresh irradiated lymphocytes from three unrelated individuals different from N.

This ratio compares the ability of lymphocytes from platelets to stimulate a normal individual (numerator) compared with the maximal stimulation of that individual (denominator). Cells were tested as responders in MLC using the following combinations: Relative response = (Pool),(PC) - (PC),(Pool), N - N, where (PC) represents lymphocytes derived from platelet concentrates.

This ratio compares the ability of lymphocytes derived from platelet concentrates to respond to maximal stimulation from a pool of unrelated individuals (numerator) with the ability of fresh lymphocytes from a normal individual to respond to the same maximal stimulation.

The mean (±SD) relative stimulation and relative response of lymphocytes from fresh and stored platelet concentrates were compared using the two-tailed paired Student’s t test.

RESULTS

Leukocyte concentrations during platelet storage. The leukocyte counts and percentage of lymphoid vs nonlymphoid cells present in individual platelet concentrates varied greatly between different donor units. The leukocyte concentrations of individual units on day 1 ranged from 200/µL to 2,800/µL with 37% to 91% lymphocytes and on day 5 from 200/µL to 1,000/µL with 77% to 98% lymphocytes. The average leukocyte concentration of the platelet units decreased by approximately 250/µL on each subsequent day of testing (Table 1). As shown in Table 1, mean leukocyte concentrations obtained on day 3 (633/µL) and day 5 (381/µL) were 72% and 43% of that obtained on day 1 (881/µL). The ratio of lymphoid to nonlymphoid cells progressively increased during storage and by day 5 was significantly different from day 1 (89% vs 76%, P < .02). Dot plots displaying volume v light-scatter characteristics of 10,000 cells from each sample demonstrated that granulocytes appeared to rapidly degranulate and disappear with storage and were consistently sparse by day 5 (Fig 1).

Antigen expression on leukocytes during platelet storage. The percentage of lymphocytes staining positive

Table 1. Changes in Leukocyte Content of Platelet Concentrates During Storage

<table>
<thead>
<tr>
<th>WBC/µL (± SEM) (n = 21)</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>881 ± 123</td>
<td>633 ± 117*</td>
<td>381 ± 54*</td>
<td></td>
</tr>
<tr>
<td>Total WBC/platelet concentrate (n = 21)</td>
<td>4.2 x 10⁵</td>
<td>3.0 x 10⁷</td>
<td>1.8 x 10⁷</td>
</tr>
<tr>
<td>Percentage of lymphocytes (± SEM) (n = 11)</td>
<td>76 ± 5</td>
<td>80 ± 3</td>
<td>89 ± 2†</td>
</tr>
<tr>
<td>Total lymphocyte/platelet concentrate (n = 11)</td>
<td>3.0 x 10⁷</td>
<td>2.8 x 10⁷</td>
<td>1.8 x 10⁷</td>
</tr>
</tbody>
</table>

The mean results (± SEM) at intervals of storage are shown. The results at each storage time are compared with day 1.

*P < .02, two-tailed, paired Student’s t test.
†P < .001, two-tailed, paired Student’s t test.
Class I HLA antigens (HLA-ABC) were strongly expressed by over 90% of lymphocytes on each day of testing with little change over time. In contrast, 20 of 21 samples analyzed demonstrated a decline in the percentage of cells reacting with antibodies to class II HLA antigens (HLA-DR). The average percentage of HLA-DR-positive cells progressively declined from 6% on day 1 to 4% on day 3 ($P < .01$) and 1.9% on day five ($P < .001$) of storage. Leu-4 was well expressed throughout storage with little decline in the degree of fluorescence. Although HLe-i expression declined slightly over time, 92.7% of cells remained reactive on day 5.

The relative antigen density as estimated by mean intensity of fluorescence was also stable for HLA class I, Leu-4, and HLe-i-positive lymphocytes (data not shown).

Median background fluorescence was <2.3% for all lymphocyte antigens studied except Leu-4, which demonstrated 5% median background staining on days 1 and 3 and 7% on day 5 of testing. In contrast to lymphocytes, nonlymphoid cells demonstrated strong background staining, which prevented accurate evaluation of antigen expression. The median background fluorescence for the HLA-ABC and HLe-i antigens was 17% and 15% on day 3 and 34% and 24% on day 5, respectively. Nonetheless, these markers remained detectable over background on at least 75% of nonlymphoid cells on day 3 and approximately 50% on day 5 of testing. A higher percentage of specific immunofluorescence due to true antigen positivity cannot be excluded for these markers.

Metabolic inhibition studies. The effect of inhibiting protein synthesis on lymphocyte antigen expression in culture is shown in Fig 2, which compares the mean fluorescence intensity of cyclohexamide-treated cells with their paired controls. The intensity of staining with antibodies directed against HLA-ABC and B2 microglobulin decreased dramatically ($P < .01$) when lymphocytes were cultured in the presence of 100 μg/mL of cyclohexamide for 18 hours. In contrast, inhibition of protein synthesis produced only a modest decline in the mean fluorescence of HLA-DR, which did not reach statistical significance ($P = .06$). The expression of Leu-4 and HLe-i by lymphocytes in culture was also not affected by inhibiting protein synthesis. At least 85% viability was maintained in all samples following culture as demonstrated by trypan blue exclusion. Only viable cells were included within the analytical gates.

Mixed lymphocyte cultures. Lymphocytes demonstrated a marked decline in stimulating function in MLC following storage in platelet concentrates. Normal lympho-

### Table 2. Changes in the Percentage of Antigen-Positive Lymphocytes in Platelet Concentrates During Storage

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-ABC (n = 7)</td>
<td>96.0 ± 0.6</td>
<td>96.7 ± 0.3</td>
<td>95.0 ± 0.9</td>
</tr>
<tr>
<td>HLA-DR (n = 21)</td>
<td>6.0 ± 0.6</td>
<td>4.0 ± 0.4*</td>
<td>1.9 ± 0.3†</td>
</tr>
<tr>
<td>Leu-4 (n = 21)</td>
<td>72.9 ± 1.4</td>
<td>72.7 ± 1.7</td>
<td>75.9 ± 2.0</td>
</tr>
<tr>
<td>HLe-i (n = 21)</td>
<td>95.9 ± 0.6</td>
<td>92.9 ± 1.0†</td>
<td>92.7 ± 1.2*</td>
</tr>
</tbody>
</table>

The mean percentage (± SEM) of cells staining positive at intervals of storage are shown. The results are compared with those of day 1.

* $P < .01$, two-tailed paired Student’s $t$ test.
† $P < .001$, two-tailed paired Student’s $t$ test.
cytes demonstrated a mean (±SD) relative response of 0.55 ± .26 with stimulating lymphocytes derived from day 1 platelet concentrates compared with 0.15 ± .15 using lymphocytes obtained from day 5 platelet concentrates (P < .001, two-tailed paired Student’s t test). In contrast, the ability of lymphocytes to function as responders in MLC remained stable following storage. The relative response of lymphocytes following 1 day of storage (1.04 ± .49) was not significantly different from that obtained on day 5 (0.97 ± .63).

**DISCUSSION**

Alloimmunization to HLA antigens often complicates the medical management of transfusion-dependent thrombocytopenic patients. The immunologic mechanisms that result in HLA sensitization are incompletely understood, but exposure to foreign antigens borne on donor leukocytes appears to play an essential role in provoking antibody formation. Although leukocyte-poor RBCs may be prepared by several different techniques, available methods of producing leukocyte-depleted platelet concentrates are not completely satisfactory. Thus, transfusion of platelet concentrates remains an important stimulus for HLA alloimmunization.

Platelets stored at room temperature with constant agitation remain hemostatically effective for over five days, but the expression of leukocyte surface antigens under these conditions is largely unknown. Few studies even consider the effects of storage on leukocytes in banked platelet concentrates. Gottschall et al. examined platelet units 72 hours following preparation and noted that granulocytes could be identified only occasionally on Wright-stained smears. Margo and Dzik showed that lymphocytes derived from platelet concentrates following storage exhibited a blunted response to mitogenic lectins. Recently, Skinnider et al. found that the ratio of B to T lymphocytes and OKT4 to OKT8 positive cells remained unchanged in banked platelet concentrates. In this study, we examined the changes in leukocyte antigens during routine platelet storage conditions.

In agreement with previous reports, we found that the leukocyte content of individual units prepared from different blood donations varied widely. The leukocyte counts ranged from 200/μL to 2,800/μL on day 1 and from 200/μL to 1,000/μL on day 5. The total leukocyte counts of the 21 platelet concentrates tested fell an average of 57% from a mean of 4.2 x 10^7 per unit on day 1 to 1.8 x 10^7 per unit on day 5.

Wright-stained smears prepared on days 3 and 5 showed rare granulocytes with nuclear pyknosis and cytoplasmic degeneration. Similarly, analysis of dot plots displaying light-scatter characteristics v volume demonstrated that granulocytes rapidly disintegrated during platelet storage and that the relative percentage of lymphocytes increased significantly by day 5 (Fig 1). Since the presence of cell aggregates within the nonlymphoid gate may have contributed to a slight overestimation of the relative percentage of nonlymphoid cells, the true percentage of lymphocytes may have been even higher. Thus, lymphocytes, at least numerically, appear to represent the most important antigen bearing leukocytes in platelet transfusion.

The class I HLA marker was consistently expressed by over 95% of lymphocytes throughout the period of storage. Moreover, the mean fluorescence intensity, which reflects the degree of antigen expression per cell, exhibited little decline during storage. The stable expression of this antigen is consistent with the recognized significance of leukocyte exposure in the development of antibodies to HLA class I antigens. In contrast, expression of the HLA class II marker by lymphocytes declined during storage. The percentage of antigen-positive lymphocytes fell from 6% to <2% by day 5. Since class II HLA antigen expression is felt to be important in the stimulation of the mixed lymphocyte reaction, we tested the ability of lymphocytes isolated from fresh and stored platelet concentrates to stimulate and respond in MLC. As expected, there was a marked decrease in the ability of cells to stimulate in MLC, which developed in parallel with the decrease in class II HLA antigen expression. Previously, similar results were obtained with cells maintained in tissue culture. An alteration in MLC stimulation resulting from changes in HLA-DR or HLA-DQ antigens is also possible. The ability of lymphocytes to respond in MLC did not diminish during the five days of platelet storage.

Studies suggest that concurrent expression of both class I and class II HLA antigens by viable cells is necessary to evoke a primary humoral response to HLA antigens. Studies of dialysis patients receiving pretransplant blood transfusions have shown diminished HLA sensitization following transfusion of stored refrigerated RBCs compared with fresh RBCs. Such results may be due to changes that occur in leukocyte antigens during refrigerated blood storage. In a similar way, the apparent instability of HLA-DR expression and MLC stimulation by lymphocytes during platelet storage may affect the strength of the immune response elicited by transfusion of fresh v stored platelet concentrates. Since platelets are transfused as pools of individual units, and since each unit may be stored for a different period before pooling, the degree of antigen exposure resulting from a given transfusion will reflect the heterogeneous effects of varying periods of storage.

The data obtained with cyclohexamide-treated cells suggest that active lymphocyte metabolism and protein synthesis are required for stable expression of class I HLA antigens during in vitro storage. Using flow cytometry, we found that the expression of both the class I HLA-ABC antigen and B2 microglobulin declined during culture in the presence of this protein inhibitor. Our results with respect to class I HLA antigens confirm earlier studies using complement-mediated lysis and alloantisera. In contrast the expression of HLA class II, CD-3 (Leu-4), and HLe-1 leukocyte antigens appeared relatively stable following inhibition of protein synthesis. These data suggest that the observed decline in HLA class II antigen expression on lymphocytes during storage in platelet concentrates is not due solely to ineffective protein synthesis.

Antigen expression by nonlymphoid cells may be of less
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importance since these cells are far less numerous than lymphocytes in platelet concentrates. HLA class I antigens were detected on approximately 75% of nonlymphoid cells on day 3 and 50% of nonlymphoid cells on day 5 of storage. Flow cytometric analysis of nonlymphoid cells was hampered by high background fluorescence, presumably due, in part, to progressive loss of cell viability. We suspect that the progressive disappearance of nonlymphoid cells during storage is of greater importance than the relative capacity of these cells to maintain antigen expression. The interpretation of our results with respect to in vivo phenomena must be viewed cautiously. Antigens that are lost or altered in vitro, and therefore not detectable with monoclonal reagents, may be re-expressed following transfusion. In addition, the fate of soluble HLA alloantigens present in platelet concentrates was not examined.

The antigenic challenge presented by blood transfusion and the mechanisms leading to alloimmunization are largely unknown. It would seem reasonable that the immunologic response elicited by transfusion of different blood components stored for varying periods of time under different conditions may vary. This study demonstrates that the alterations that occur during routine blood bank storage of platelet concentrates may affect the HLA antigenic exposure, which results from platelet transfusion. Future studies of the clinical effects of platelet transfusion may need to consider the changes that occur in leukocyte antigen expression during storage of platelet concentrates.

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Stability of antigens on leukocytes in banked platelet concentrates: decline in HLA-DR antigen expression and mixed lymphocyte culture stimulating capacity following storage

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