Heterogeneity of Antibody Response to Human Platelet Transfusion

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ABSTRACT To study the antibody response to human platelet transfusions, nine thrombocytopenic patients with bone marrow failure were given 6 U (3×10^{11}) of random platelet concentrates twice a week. Before transfusion, none of the patients had preexisting antibodies detectable with lymphocytotoxicity, platelet aggregation, or capillary leukoagglutination techniques. After receiving 18–78 U of platelets, they became refractory to further transfusions of random platelets and alloantibodies were detectable. Two patterns of antibody response could be identified. In three patients, the sera were not lymphocytotoxic with a panel of standard cells in which all the known HLA antigens in the first and second series were represented at least once. Yet, they caused platelet aggregation with 30, 24, and 60%, respectively, of a donor population studied. The aggregating activities were inhibited by antihuman IgG but not by antihuman IgA or antihuman IgM antiserum. The aggregating antibodies could be absorbed out with donor platelets but not lymphocytes or granulocytes. Antibodies from two of these patients aggregated platelets of their respective siblings matched for both HLA haplotypes. Transfusion of platelets from these two siblings did not increase the platelet count while platelets obtained from aggregation-negative donors did. The sera from the remaining six patients were lymphocytotoxic with 15–100% of the panel of standard cells. They also had aggregating antibodies, which could be absorbed out by both platelets and lymphocytes, suggesting that they were HLA antibodies. These data suggest that the development of platelet-specific antibodies may play an important role in the immunological rejection of isologous platelets, and should be considered in the selection of donors for patients who are refractory to platelets from random donors.

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INTRODUCTION

With the increasing demands for the long-term use of platelet concentrates in the supportive therapy of patients with severe thrombocytopenia due to bone marrow hypoplasia, platelet alloimmunization and refractoriness to random platelet transfusions have become an important and challenging problem. Despite the fact that a great deal is recognized with respect to the platelet iso-antigenic systems including A and B antigens (1, 2). HLA antigens (3, 4), and platelet specific antigens (5, 6), little is known about the antigenic specificities of the platelet isoantibodies developed during platelet transfusions in man and consequently very little is known about the immunogenicity of various antigens. Indirect evidence derived from recent in vivo transfusion studies suggests that HLA antigens may play a key role in the platelet alloimmunization. Transfusion of poorly HLA matched platelets resulted in rapid rejection while platelets obtained from HLA matched donors were not rejected (7, 8). Yet, production of HLA lymphocytotoxic antibodies after platelet transfusions has not been systematically studied. In addition, other types of antibodies that can be detected with platelet aggregometry (9–11) and platelet serotonin release techniques (12, 13) have not been simultaneously compared. To characterize the antibody response to human isologous platelet transfusion, we conducted a systmatic transfusion study in nine platelet recipients who had severe bone marrow hypoplasia.

METHODS

Lymphocytotoxicity studies. Lymphocytotoxicity (LCY) [1] tests by a slight modification of the two stage tests of Amos et al. (14) were used in the HLA typing. All assays were performed in micro test-plates and one lambda antiserum was mixed with one lambda lymphocyte concentrate isolated from the peripheral blood. A panel of antisera detecting HLA-A1, 2, 3, 9, W23, W24, 10, W25, W26, 11, 28, W29, W30, W31, W32, W33, W36, and B5, 7, 8, 12, 13, 14, W15, 18, W16, W17, W21, W22, 27, W35, W37, W38, W39, W40, was available for the lymphocyte HLA typing. Each specificity was assayed by at least two and usually three or more sera. Antisera were derived from National Institutes of Health bulk supplies, other investigators, and our own sources. In all, 70 sera or more are employed for typing. Although both parents were commonly available, as many family members (usually 6–10 members) as needed were typed to establish family inheritance for both recipient and donor HLA haplotypes. To detect anti-HLA antibodies developed in these patients, LCY test (14) was performed using antiserum obtained from the patient against a lymphocyte panel obtained from 50 HLA typed unrelated donors on which the HLA antigens were represented more than once. Leukocyte capillary agglutination was described previously (15).

Platelet aggregometry. Platelet aggregation (PA) tests were performed with an aggregometer (Payton Associates, Inc., Buffalo, N. Y.) following the principle of Born (16). All tests were done by adding 0.1 ml patient antiserum to 0.3 ml donor platelet-rich plasma (PRP) which was preincubated at 37°C for 2 min in the aggregometer. The mixture was stirred with a siliconized stirring bar at 1,000 rpm at 37°C for 8 min. In each determination, 0.4 ml of donor PRP was simultaneously run to measure the spontaneous aggregation. Preparation of donor PRP and recipient heated serum was described previously (11). The platelet concentration in each PRP was determined. No adjustment was made when the concentration was within the range of 200,000–400,000/mm^3. The count was adjusted to 300,000/mm^3 using autologous platelet poor plasma if it was higher than 400,000/mm^3. The interval between collection of PRP and performance of the test was usually within 30 min and always within 2 h. The platelet aggregometry was so standardized that the platelet poor plasma would induce 100% light transmission and the PRP from the same subject 0% light transmission. The extent of light transmission was measured by the height (cm) of the aggregation recorded on a chart paper. The result was expressed as the increase in light transmission induced by the test serum minus that of the spontaneous aggregation. The baseline of the aggregometer was extremely stable. Pretransfusion and/or presensitization sera from a group of 50 patient recipients who participated in our transfusion program were studied to serve as controls. The mean increase in light transmission as derived from 104 recipient-donor testings was 0.61±SD 0.99%. In addition, the mean increase in light transmission of 20 testings using 20 normal serums was 0.71±0.89%. To summarize the results, the sera were divided into five classes depending on the degree of increase in light transmission: (*a*) < 5% designated by the sign −; (*b*) 5–10% designated as +; 10–25% designated as ++; (*d*) 25–50% designated as +++; and (*e*) > 50% designated as ++++.

[1] *Abbreviations used in this paper:* LCY, lymphocytotoxicity; PA, platelet aggregation; PRP, platelet-rich plasma.

Donors. Normal donors for platelet transfusions and antibody studies were obtained from the Blood Donor Center of the University of Iowa Hospitals. These were composed of college students. None of the normal donors or family members took aspirin or other drugs known to inhibit platelet aggregation for at least 7 days before the study.

Patients. Nine adult patients, seven women and two men, with bone marrow hypoplasia due to aplastic anemia in two and acute leukemia in seven were studied (Table I). The acute leukemic patients were treated with a regimen of cytosine arabinoside and 6-thioguanine (17) whereby their bone marrow became severely suppressed. All but three women were multiparous. Serum samples were obtained before transfusion and weekly after platelet transfusions had been started. All the recipients were given 6 U of platelet concentrates from multiple unrelated donors twice weekly until they had become refractory to further random transfusions. The refractory state was defined clinically as failure to achieve a corrected increment ($\times M^2/10^{11}$ platelets) in the platelet count of 10,000/mm^3 at 1 h and 8,000/mm^3 at 20 h after platelet transfusions on two occasions in the absence of sepsis, fever (> 38°C), hypersplenism, active hemorrhage, or disseminated intravascular coagulation. HLA typing of the patients and their family members was performed usually at an early phase of the disease. None of these patients had taken aspirin or other drugs that might inhibit platelet aggregation. The period of sequential follow-up of antibody responses in these recipients ranged from 6 to 16 wk.

Platelet concentrates were prepared by either a double plateletpheresis or using a Latham Centrifuge (Latham model 10 Blood Processor, Haemonetics Corp., Natick, Mass.) described previously (18). To assess the response to platelet transfusion, platelet counts were done a few hours before and 1 and 20 h after platelet transfusion. The increment in platelet counts was calculated in a manner similar to that of Yankee et al. (7), except that the result was expressed as an increase in platelet count $\times M^2/10^{11}$ platelets. Platelet counts were determined with a model B Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.) and confirmed with phase microscopy when necessary.

Cell separation and absorption studies. Separation of platelets, granulocytes, and lymphocytes from aggregation-positive and control donors was performed following the method of Severson and Thompson (19). After these cells were separated, they were washed twice with Gelatine-EDTA-KCl buffer (19) and then centrifuged at 2,000 *g* at 22°C for 30 min to obtain cell pellets. Granulocyte and lymphocyte concentrations were determined in the last cell suspension with a hemocytometer and the platelet concentration was determined with a Coulter counter and a hemocytometer under a phase-contrast microscope. For the absorption studies, 0.3 ml of the patient serum was added to the cell pellet which contained 10^7, 10^8, or 10^9 cells. The mixture was stirred gently. After the cell pellet had been evenly resuspended, the mixture was incubated at 37°C for 60 min with frequent mixing followed by centrifugation at 2,000 *g* for 20 min to collect the supernate. The aggregometry was then performed using the absorbed antiserum and freshly prepared PRP of the same donors from whom cells for absorption were obtained. Three aggregation-positive donors were used for the three antisera studies. One donor was used for each antiserum and all the cells were prepared from the same donor. The original unabsorbed antiserum was tested in each study.

TABLE I

Summary of Clinical and Transfusion Data in Nine Patients with Bone Marrow Hypoplasia

Recipients	Age/Sex	Diseases*	Transfusions‡		Pregnancies	LCY§
			Platelet concentrates	Packed erythrocytes		
			U	*U*		%
Group I						
H. P.	30/F	AMML	78	25	0	0
S. D.	43/F	AMML	18	7	0	0
W. J.	31/F	CML & BC	38	6	0	0
Group II						
C. A.	61/F	AML	30	4	2	80
S. D.	42/M	AML	42	4	—	75
C. E.	69/F	AA	24	9	2	75
Y. B.	48/F	AML	24	5	2	100
H. L.	44/F	AMML	24	5	2	100
W. L.	68/M	AA	24	4	—	15

* AML, Acute myelocytic leukemia; CML, Chronic myelocytic leukemia; BC, Blastic crisis; AA, Aplastic anemia; AMML, Acute myelomonocytic leukemia.

‡ Number of transfusions before these patients became refractory to random platelets.

§ Positive lymphocytotoxicity with a panel of lymphocytes of known HLA types in which all the HLA antigens were represented usually more than once.

Antiserum absorbed with cells prepared from aggregation-negative donors was also studied to serve as control.

Antibody characterization. The nature of the aggregating factors detected in the sera of H. P., W. J., and S. D. was characterized by the addition of one part of purified anti-human IgG, IgM, or IgA antiserum to five parts of the patients' serum following the method of Abramson et al. (20). After a 1-h incubation at 37°C, the mixture was centrifuged at 2000 *g* for 20 min to remove any precipitate. One part of the supernate was then added to one part of PRP prepared from aggregation-positive donors. The same donors were used in this study as in the cell-absorption study described above. To serve as positive controls, the serum from these patients was treated in a similar fashion except that the antihuman Ig's were replaced by the same quantity of saline. To serve as negative controls, PRP from aggregometry-negative lymphocytotoxicity-negative donors was used.

TABLE II

Frequency of Positive Platelet Aggregometry by Three Lymphocytotoxicity-Negative Sera

Recipients	Total number of donors*	Positive platelet aggregation
		%
H. P.	47	14 (30)
S. D.	55	13 (24)
W. J.	37	22 (60)

* The same panel of donors was used.

RESULTS

The sera of all nine patients obtained before transfusion did not cause positive LCY, leukocyte capillary agglutinating, or PA reactions. After transfusions of 18–78 U of platelet concentrates (9–39 × 10^{11} platelets), and some packed erythrocytes from multiple unrelated donors, rejection of further platelet transfusion became evident and the lymphocytotoxic antibodies became detectable in some patients (Table I). The antibody response was heterogeneous and could be divided into two groups: group one in which LCY was negative with a panel of 50 lymphocytes and group two in which LCY was positive. Although the LCY was negative in three sera of group one, the PA was positive with 30, 24, and 60%, respectively, of a random donor population studied (Table II). These three sera were not lymphocytotoxic to any of the cells prepared from this random population. They were also PA positive but LCY negative with family members. A poor correlation between the PA, LCY, leukocyte capillary agglutinating and HLA haplotypes was noted (Table III). Please note that two sibling pairs (H. P.-G. C. and W. J.-W. K.) who were matched for both haplotypes and compatible by the lymphocytotoxicity test were PA-positive. The platelet aggregating factors detected in these three patients were inhibited by antihuman IgG but not by antihuman IgM or IgA antisera (Table IV). They were absorbed by 10^9 platelets but not by 10^7 platelets. The PA factors in the serum of W. J. was partially absorbed by 10^8 plate-

TABLE III

Comparison of HLA Haplotypes, PA, LCY, and Capillary Leukoagglutinin LCA Activities in Three Recipients and Their Family Members

Family members	Relationship	HLA haplotype	PA	LCY	LCA
Family of H. P.					
H. P.	Recipient	X, BW40/A3, B18			
G. C.	Father	Al, B12/A3, B18	—	—	—
G. S.	Mother	X, BW40/A3, B7	+ + +	—	—
G. R.	Brother	X, BW40/A1, B12	—	—	—
G. P.	Brother	X, BW40/A1, B18	—	—	—
G. Ca.	Sister	X, BW40/A3, B18	+ +	—	—
Family of W. J.					
W. J.	Recipient	A2, B13/A2, B8			
W. Jo.	Sister	A2, B8/A1, B27	—	—	—
W. K.	Sister	A2, B13/A2, B8	+	—	—
W. M.	Sister	A2, B13/X, Y	—	—	—
Y. M.	Cousin	A2, B7/A3 B7	—	—	—
Family of S. D.					
S. D.	Recipient	A2, BW39/AW36, Y			
S. G.	Father	A2, BW39/A2, BW40	—	—	—
S. Ra.	Mother	X, B5/AW36, Y	—	—	—
S. Ro.	Brother	X, B5/A2, BW40	+ + +	—	—
S. Da.	Nephew	X, B5/AW36, BW40	—	—	—
S. V.	Niece	X, B5/A2, BW40	—	—	—
W. D.	Uncle	A3, B7/X, Y	+ + +	—	+
W. L.	Uncle	A3, B7/AW30, Y	—	—	NT*

* NT = Not tested.

lets (Table V). In contrast to the failure to absorb the platelet aggregating factors in group one sera by 10^6 and 10^8 lymphocytes, PA factors of a group two serum from patient H. L. were readily absorbed out with 10^7 lymphocytes. Granulocytes failed to absorb out the PA factors in sera of either group (Table V). Platelets, lymphocytes, and granulocytes obtained from an aggregation-negative lymphocytotoxicity-negative donor did not have any effect on the aggregating factors. The results of transfusion studies on H. P. and W. J. are shown in Table VI. Despite matches of both haplotypes and compatibility of LCY and leukocyte capillary agglutinating tests, platelets from these two sibling donors failed to induce an expected platelet increment. On the other hand, post-transfusion increment in the platelet counts tended to be better with the transfusion of platelet concentrates obtained from the PA-negative donors.

DISCUSSION

Although it is well recognized that platelet alloantibodies may be present in the serum of patients receiving multiple blood transfusions (21–23), few systematic studies of platelet allosensitization have been carried out. Baldini et al. (24) infused 10 normal subjects with platelet concentrates prepared from 500 ml of blood at weekly intervals and found that the infused platelets were rejected after 2–8 wk as demonstrated by reduced platelet survival. Platelet rejection was presumably due to allosensitization although no alloantibodies were detected by serologic techniques. Dausset et al. (25) immunized 12 normal subjects with intradermal injections of platelet suspension from a single donor and a skin graft from the corresponding donor was applied 15 days later. Their results indicated that the survival of platelets from the donor might be shortened in the re-

TABLE IV

Platelet Aggregating Activity in Group I Sera after Addition of AntiHuman Globulin Antiserum

Serum	Platelet aggregation grade*			
	Saline control	Anti-IgG	Anti-IgA	Anti-IgM
H. P.	+ + +	—	+ + +	+ + +
S. D.	+ + + +	—	+ + + +	+ + + +
W. J.	+ + +	—	+ + +	+ + +
Normal	—	—	—	—

* Source of PRP was from three different aggregation-positive donors.

TABLE V

Platelet Aggregating Activities in Three Group I and 1 Group II Sera after Absorption with Platelets, Lymphocytes, and Granulocytes

Serum	Platelet aggregation grade*								
	Non-Absorbed	Platelets			Lymphocytes			Granulocytes	
		10^9	10^8	10^7	10^9	10^8	10^7	10^8	10^7
Group 1									
H. P.	+ + + +	—	+	+ + + +	‡	+ + + +	+ + + +	+ + + +	+ + + +
S. D.	+ + + +	+	+ + + +	+ + + +	‡	+ + + +	+ + + +	+ + + +	+ + + +
W. J.	+ + + +	—	+ +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
Group 2									
H. L.	+ + + +	—	+ + +	+ + + +	—	—	—	+ + +	+ + + +
Normal	—	—	—	—	—	—	—	—	—

* Source of cells was from aggregometry-positive donors. Four different donors were used. PRP from corresponding donors was used for the final PA testing.

‡ Not done.

cipient immunized with 4–5 × 10^9 platelets while no acceleration of rejection of the skin graft was observed. In the present study, we sensitized nine recipients with 6 U of platelet concentrates prepared from multiple random donors twice weekly and the platelet increment after each transfusion as well as the development of alloimmunization was followed sequentially. No alloantibodies could be detected before allosensitization and platelets transfused into these recipients survived normally. In most recipients, platelet allosensitization and rejection occurred after they had received 18–30 U of platelets, i.e. 1–2 × 10^{12} isologous platelets. Two patients seemed to tolerate the isologous platelets better and did not become refractory until they had received 42–78 U of platelet concentrates respectively. As small quantities of leukocytes and platelets are present in erythrocyte concentrates and even the platelet concentrates are usually contaminated with leukocytes, the platelet antigenic dosage cited above is at best an underestimate.

As several complex antigenic systems are present on human platelets, it is conceivable that the antibodies developed during alloimmunization may be heterogeneous. From this study, we observed two patterns of antibody responses. In one group of antisera, the alloantibodies were lymphocytotoxic, platelet aggregating, and leukocyte agglutinating and were correlated with the matches of HLA antigens. The antibodies could be absorbed out by both lymphocytes and platelets. There is little doubt that they were polyspecific HLA antibodies. In contrast, three sera were not lymphocytotoxic to a panel of cells on which all the known HLA-A and HLA-B antigens were present. Similar results have been reported by Bucher et al. (26) and by Mittal et al. (27). This virtu-

TABLE VI

Comparison of Transfusion Responses with Matches of HLA Types, Platelet Aggregometry and Lymphocytotoxicity

Patients	Donors	Relation	HLA types	PA	LCY	Platelet increment × $M^2/10^{11}$ platelets	
						1 h	*20 h*
W. J.			A2, B13/A2, B8				
	W. K.	Sister	A2, B13/A2, B8	+	—	2,336	584
	Y. M.	Cousin	A2, B7/A3, B7	—	—	11,680	9,424
	P. J.	Unrelated	A1, A10, BW27, BW14	+ + + +	—	−3,504	−4,088
H. P.			X, BW40/A3, B18				
	G. Ca.	Sister	X, BW40/A3, B18	+ +	—	−584	−1,168
	G. S.	Mother	X, BW40/A3, B7	+ + +	—	3,504	3,504
	R. J.	Unrelated	A2, A10, X, Y	—	—	20,148	15,768
	P. J.	Unrelated	A1, A10, BW27, BW14	+ + +	—	584	0
	J. C.	Unrelated	A1, A3, B7, B8	—	—	1,168	7,592

ally ruled out the possibility that they contained antibodies against HLA antigens of the first or the second segregant series. Despite negative lymphocytotoxicity, the sera were positive with platelet aggregometry. Platelet alloantibodies have been shown to induce platelet aggregation and the immunoglobulin class of the alloantibodies was shown to be an IgG (9). In this study, we further provided evidence that the platelet aggregometry detected platelet alloantibodies and the alloantibodies were in the IgG class. These antibodies could be absorbed out by platelets but not by lymphocytes or granulocytes. Although it is possible that these may be induced by third segregant series or other still unidentified antigens of the HLA complex, the absorption studies strongly suggest that they may be developed against platelet-specific antigens. The exact nature of these antigens remains to be elucidated. Several important questions remain to be answered. Are these antibodies monospecific or polyspecific? Are they related to known platelet-specific antigen systems such as Pl^A, Pl^B, Ko or are they directed against new platelet antigenic systems? What are their inheritance patterns? Several projects are currently undertaken in our laboratory to provide answers to these important questions.

Both groups of antibodies appear to play a major role in platelet rejection and detection of these antibodies using a profile of serological techniques may be useful in differentiating true immunological rejection from other causes of resistance to platelet transfusion in these patients. In addition, these techniques may be valuable adjuncts to the HLA serotyping in the selection of suitable platelet donors for patients who have developed HLA antibodies. To select donors for patients who have developed "non-HLA antibodies" is a more complex problem. It is obvious that the selection of donors can not be based on the matches of HLA types alone. As a matter of fact, HLA typing may be of little value as illustrated in two recipients whose platelet counts failed to rise even with the transfusion of platelets from their four-antigen matched siblings. Since the antibodies were not detected with lymphocytotoxicity test, this technique appears of little value also. In contrast, platelet aggregometry seems to be useful as a cross-match test. The limitation of this technique lies in its inability to detect antibodies unless a state of true immunological rejection of platelets has been reached. Whether this method can be used for platelet typing depends on our further knowledge with respect to the immunogenicity and genetic pattern of the platelet specific antigens as well as the importance of newly discovered HLA antigens in platelets transfusions.

In conclusion, the data suggest that development of platelet-specific antibodies may play an important role in the immunological rejection of isologous platelets and should be considered in the selection of donors for patients who are refractory to random platelet transfusion.

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