In Vitro Tests for Distinguishing Possible Immune-Mediated Aplastic Anemia From Transfusion-Induced Sensitization

By Beverly J. Torok-Storb, Colin Sieff, Rainer Storb, John Adamson, and E. Donnall Thomas

Forty-two patients with aplastic anemia (AA) were studied to determine whether or not transfusion-induced sensitization is responsible for the in vitro inhibition by patient lymphocytes of HLA-identical erythroid burst-forming units (BFU-E). The results indicate that lymphocytes from 12 of 34 transfused patients inhibited normal colony growth. In contrast, lymphocytes from none of the 8 untransfused patients demonstrated inhibition. These data were interpreted to mean that coculture studies would not be useful for identifying immune-mediated AA in transfused patients. Therefore, in order to identify possible immune-related AA, we assayed BFU-E from patient blood before and after T-cell depletion. In all 32 patients studied, BFU-E failed to grow from peripheral blood cells before T-cell depletion, but in 8 cases, normal-appearing BFU-E grew after T cells had been removed. Growth of patient BFU-E colonies was inhibited in 6 cases when patient T cells were added back to the culture, indicating that in these 6 patients, an "autoimmune" mechanism may have been present.

RECENT REPORTS that lymphocytes from patients with aplastic anemia (AA) suppress in vitro growth of hematopoietic colonies from healthy HLA-nonidentical unrelated individuals have suggested that some cases of AA have an immune etiology.[1-4] However, subsequent studies have shown that in vitro inhibition of HLA-nonidentical granulocytic colony growth (CFU-C) by patient cells may be due to transfusion-induced sensitization of patient lymphocytes.[5] Also, studies in dogs have shown that even a single transfusion from a DLA-identical littermate can induce sensitization as detected in vitro by reduced growth of erythroid colonies when cells from the transfusion donor are cocultured with recipient lymphocytes.[6] Furthermore, reduced colony growth in these dogs was predictive of rejection of subsequent transplants of marrow from the transfusion donors.[7] These findings suggested that minor antigens outside of the major histocompatibility complex were responsible not only for transfusion-induced sensitization but also for marrow graft rejection in dogs. Since most patients with AA have a history of blood transfusions, we hypothesized that in vitro inhibition of normal erythroid colony growth by AA patient cells, even colonies from an HLA-identical sibling, is most likely related to transfusion-induced sensitization. In the case of transfused patients, therefore, an endpoint other than inhibition of normal erythroid colonies by patient lymphocytes would be needed to identify potential cases of autoimmunity. In this study, we tested this hypothesis by two approaches. First, we compared the effect of lymphocytes from transfused and untransfused patients with AA on the growth of erythroid burst-forming units (BFU-E) by cells from their HLA-identical siblings. Second, we demonstrated erythroid colony growth from the peripheral blood of some patients with AA after removal of E-rosette-forming cells and that BFU-E growth was suppressed when these cells were added back to the culture.

MATERIALS AND METHODS

Patients

Included in this study were 42 patients who met the criteria for severe AA.[8] Thirty-four patients were treated with high doses of cyclophosphamide, followed by marrow transplantation from an HLA-identical sibling (with the exception of one HLA-D mismatch).[9,10] Eight patients did not have HLA-identical donors. Patients were divided into 3 groups according to their transfusion history: group 1 included 8 patients who had not received any transfusions of blood products before the study; group 2 included 10 patients who had transfusions within 48 hr of the study; and group 3 included 24 patients who had had multiple transfusions 48 hr or more before the study.

Preparation of Cells

Twenty milliliters of heparinized whole blood were collected from both the patient and an HLA-identical sibling. Blood was diluted 1:1 in Hanks balanced salt solution and layered over Ficoll-Hypaque (Lymphoprep, Nyegard Co., Oslo, Norway). After centrifugation for 30 min at 400 *g*, peripheral blood mononuclear cells (PBMC) retained at the interface were collected, washed 3 times, and suspended in Eagle's minimum essential medium with 20% fetal calf serum. An aliquot of these cells was retained and the remaining cells separated into rosetting cells, designated T cells, and nonrosetting cells, designated null', by rosetting with neuraminidase-treated sheep red blood cells (SRBC).[11] T cells were retained for culture after treatment with hemolytic buffer (NH_4Cl tris lysing buffer).

From the Divisions of Hematology and Oncology, Department of Medicine, University of Washington School of Medicine, Veterans Administration Hospital, and the Fred Hutchinson Cancer Research Center, Seattle, Wash.

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Address reprint requests to Beverly J. Torok-Storb, Ph.D., Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Wash. 98104.

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BFU-E Assays

The unfractionated mononuclear cells, null$^+$ cells, and T cells were washed two additional times and then suspended in supplemented alpha-medium at a concentration of 10^6/ml. The three cell populations from both the patient and donor were cultured alone and in various combinations at a total concentation of 4×10^4 cells/0.1 ml plasma clot. T cells and null$^+$ cells were cultured in a 1:1 ratio (i.e., 2×10^4 T cells with 2×10^4 null$^+$ cells). The plasma clots were formed as described by McLeod and coworkers[12] and had a final concentration of 3 U of erythropoietin (sheep plasma, step III, Connaught Lab, Willowdale, Ontario) per milliliter culture. Cells were incubated 14 days at 37°C in 5% CO_2, clots were harvested, fixed on glass slides, and stained with benzidine for enumeration of erythroid bursts. At least 8 replicates were done for every combination of cells.

Table 1. Effect of Patient PBMC on the Growth of HLA-Identical Donor BFU-E* as Indicated by Ratios

Unique Patient Number	Mean ± SE Number of BFU-E/10^5 Donor PBMC: With Patient PBMC	Mean ± SE Number of BFU-E/10^5 Donor PBMC: Without Patient PBMC	Erythroid Colony Ratio	
Group 1. Patients receiving no blood products before testing				
841	13.7 ± 2.0	11.7 ± 1.1	1.2	
849	11.0 ± 2.4	8.0 ± 0.9	1.4	Not transplanted
838	4.4 ± 0.6	1.9 ± 0.2	2.3	
879	14.4 ± 3.1	5.0 ± 0.7	2.9	
896	17.0 ± 0.7	11.0 ± 0.3	1.6	
872	24.0 ± 1.1	5.3 ± 0.1	4.5	
1003	10.8 ± 0.9	5.3 ± 0.5	2.0	
1025	45.2 ± 2.1	15.0 ± 1.3	3.0	
Group 2. Patients receiving blood products within 48 hr of testing				
851	10.5 ± 1.1	10.5 ± 0.9	1.0	
877	1.3 ± 1.0	5.0 ± 1.1	0.3	
939	0	8.0 ± 0.7	0.0	Rejected
842	19.7 ± 1.7	15.0 ± 1.3	1.3	
919	12.6 ± 0.8	7.5 ± 0.3	1.7	
843	5.7 ± 2.1	5.7 ± 2.0	1.0	
796	29.2 ± 2.7	17.8 ± 4.3	1.6	
795	5.2 ± 1.9	16.7 ± 4.0	0.3	
1005	0	5.0 ± 0.2	0.0	Rejected
1015	19.0 ± 1.4	15.0 ± 0.8	1.3	
Group 3. Patients receiving blood products greater than 48 hr before testing				
855	4.2 ± 0.5	4.2 ± 0.3	1.0	
861	26.7 ± 2.0	17.1 ± 2.1	1.6	
863	0.7 ± 0.1	4.7 ± 0.2	0.2	
850†	46.0 ± 0.5	26.0 ± 0.6	1.8	
866	16.9 ± 2.1	15.6 ± 1.7	1.1	
882	2.5 ± 1.0	1.2 ± 0.1	2.1	
923†	7.0 ± 0.2	7.0 ± 0.5	1.0	Rejected
927	8.1 ± 1.1	6.3 ± 1.0	1.3	
949	2.0 ± 0.1	4.0 ± 0.3	0.5	
955†	6.9 ± 1.0	6.3 ± 0.7	1.1	Failure to engraft
954	18.8 ± 1.3	6.9 ± 0.7	2.7	
976	7.5 ± 0.1	3.2 ± 0.1	2.0	
979	19.0 ± 0.4	33.0 ± 0.9	0.6	
983	7.2 ± 0.9	4.6 ± 0.4	1.6	
984	30.0 ± 2.6	15.0 ± 1.7	2.0	
996†	9.4 ± 0.7	9.5 ± 0.6	1.0	HLA-D mismatched, rejected
1010	9.1 ± 0.3	8.7 ± 0.9	1.04	
854	3.5 ± 0.4	4.4 ± 0.8	0.8	Not transplanted
859	5.3 ± 0.3	2.4 ± 0.7	2.2	Not transplanted
869	1.25 ± 1.0	3.8 ± 1.4	0.3	Not transplanted
874†	0.8 ± 0.8	3.7 ± 1.1	0.2	Not transplanted
876†	14.0 ± 1.2	35.0 ± 1.7	0.4	Not transplanted
884	5.6 ± 0.6	1.8 ± 1.0	3.1	Not transplanted
902	25.0 ± 1.1	38.1 ± 1.7	0.7	Not transplanted

*Patients identified as "not transplanted" were tested in culture with PBMC from unrelated normal controls.

†Possible immune-mediated AA.

Table 2. Mean ± SE Number of Erythroid Bursts/10^5 PBMC From 22 Normal Individuals

Cells Studied			
Unfractionated	Null$^+$	T	T + Null$^+$
5.7 ± 0.84	13.5 ± 2.31	0.4 ± 0.11	24.6 ± 3.98

RESULTS

Effect of Unfractionated PBMC From Transfused and Untransfused AA Patients on BFU-E Growth From HLA-Identical Siblings

To compare the effect of lymphocytes from transfused and nontransfused patients on the growth of normal BFU-E, unfractionated PBMC from an HLA-identical sibling were grown alone and with an equal number of PBMC from the patient. Results were expressed as ratios derived by dividing the number of erythroid bursts obtained in the presence of patient cells by the number obtained without patient cells. In this way, ratios >1 indicate that patient cells increased growth of normal colonies, whereas ratios <1 indicate that patient lymphocytes inhibited normal colony growth.

Of the 42 patients studied, 8 had not received blood transfusions before testing (Table 1, group 1). Mononuclear cells from all 8 nontransfused patients increased donor BFU-E growth as indicated by ratios >1. In contrast, 4 of the 10 patients receiving blood products 48 hr before testing (group 2) and 8 of the 24 patients receiving blood products greater than 48 hr before testing (group 3) inhibited donor BFU-E growth.

BFU-E Growth After SRBC Rosette Separation of PBMC

Healthy individuals. Results with cells from 22 healthy individuals are shown in Table 2. In most experiments, 10×10^6 unfractionated PBMC were separated by SRBC rosetting into approximately 1.5–3.0 $\times$ 10^6 null$^+$ cells and 3–6 $\times$ 10^6 T cells, with cell recovery ranging from 60% to 90%. The null$^+$ cell fraction showed a significantly increased concentration of BFU-E/10^5 cells when compared to unfractionated PBMC. T cells alone formed significantly less BFU-E than unfractionated PBMC. The addition of T cells to the null$^+$ fraction in a 1:1 ratio significantly increased the number of erythroid bursts obtained from 10^5 cells over that with null$^+$ cells alone.

Patients with AA. Results in 32 patients are shown in Table 3. Unfractionated PBMC from patient blood failed to grow erythroid bursts in all 32 instances. In 8 cases, normal-appearing BFU-Es grew from the null$^+$ fraction of patient cells, while in 24 cases, no BFU-E growth was seen. Addition of the patient's own T cells to the null$^+$ fraction totally inhibited BFU-E growth in 6 of the 8 cases. In contrast, addition of T cells from healthy individuals did not suppress BFU-E growth in the 6 cases so tested. This suggests that in 6 of 8 patients, colony growth after T-cell depletion was not due merely to an increased concentration of precursors but rather due to the removal of an inhibiting population of lymphocytes, presumably T cells.

DISCUSSION

Lymphocytes from 12 of 34 transfused AA patients inhibited growth of normal BFU-E. This was true not only for patients who had received transfusions over a long period of time, but also for those who had received blood products within 48 hr of testing. In striking contrast, lymphocytes from all 8 untransfused patients stimulated erythroid colony growth in a manner similar to that seen with cells from normal

Table 3. Mean ± SE Number of BFU-E/10^5 PBMC From Patients With AA

		Patient Null$^+$ Cells			
Unique Patient Number	Patient Unfractionated PBMC	Alone	With Patient T Cells	With Control T Cells	
850*	0	62.5 ± 3.7	0	ND	Sustained graft
923*	0	1.0 ± 1.0	0	ND	Rejected
955*	0	2.0 ± 0.9	0	4.5 ± 0.8	Failure to engraft
996*	0	3.4 ± 0.4	0	6.0 ± 1.0	HLA-D mismatched, rejected
876*	0	3.1 ± 0.2	0	8.4 ± 0.9	Not transplanted
874*	0	20.2 ± 1.6	0	22.3 ± 4.3	Not transplanted
1015	0	4.0 ± 0.3	4.0 ± 0.8	4.0 ± 0.7	Successful graft, day 57
1025	0	4.2 ± 1.0	3.9 ± 1.1	5.0 ± 0.2	Successful graft, day 18
All others (n = 24)	0	0	0	0	

*Possible immune-mediated aplasia.

individuals. Our present data in man, as well as our previous data in dogs,[6] therefore suggest that inhibition of in vitro erythroid colonies seen with lymphocytes from patients with AA is generally the result of transfusion-induced sensitization to histocompatibility antigens rather than the result of an autoimmune disease as suggested by previous publications.[1-4] Data in agreement with this were reported by Singer et al. showing that inhibition of normal granulocytic colony formation occurred most frequently when transfused patient cells were cocultured with normal marrow.[5] Our studies indicate that the true incidence of autoimmune AA assessed by coculture studies can only be determined for patients who have never been transfused. In the current study, none of the eight untransfused patients showed inhibition of colony growth. In a similar study involving granulocytic colony growth, inhibition was seen in 3 of 16 untransfused patients.[13] However, in this latter study, "untransfused patients" included those who had received transfusions within 48 hr of study. It has been common practice to group patients in this manner,[14] but the current study suggests this may no longer be appropriate.

Previous studies in transfused dogs indicated that in vitro tests positive for inhibition were found to predict subsequent marrow graft rejection with a good degree of accuracy (13 of 14).[7] The data presented here suggest that inhibition of donor colony growth by patient lymphocytes correlates with transfusion history but not rejection. This is most likely due to the fact that specific conditioning regimens used for AA patients are more successful in overcoming transfusion-induced "sensitization" than the 1200-R whole body irradiation used in dogs.[15]

Since colony inhibition observed in patient–donor coculture studies correlates with transfusion history, we believe this technique is of limited value for identifying an immune mechanism in transfused AA patients. For this reason, we attempted to develop a method by which autoaggressive T cells might be identified.[16] We hypothesized that at least three criteria were necessary: (1) that patient-derived colonies would not grow from unfractionated cell pools, (2) that after removing T cells normal-appearing colonies would grow, and (3) that adding back patient T cells would abrogate that growth. The third criterion seemed important to rule out the possibility that T-cell depletion served merely to increase the proportion of BFU-E/10^5 cells up to detectable levels. If growth of patient colonies after T-cell depletion were due merely to increased concentrations and not to removal of a suppressing T cell, then adding back patient T cells should either not affect or increase colony numbers as demonstrated with normal cells. Several authors have shown increased growth of colonies from patients with AA after removal of T cells by red cell rosetting, treatment with antithymocyte globulin plus complement, or velocity sedimentation.[2,17-19] None, however, have demonstrated that patient T cells can reduce this growth if added back.

Thirty-two patients were tested for BFU-E growth after T-cell depletion, and of these eight showed normal growth. Only six of these had T-cell populations that inhibited autologous BFU-E and thereby satisfied all three criteria for immune-mediated disease. Three of these patients were transplanted with HLA-compatible sibling marrow, but only one of these, no. 850, has a successful sustained engraftment. None of the four transplanted patients identified as having a possible "immune-mediated aplasia" demonstrated inhibition of HLA-identical donor BFU-E in coculture studies. This observation can be interpreted in at least two ways. First, an infectious process or chemically induced lesion could cause the expression of "neoantigens" on patient cells making them targets for immune reactions. These neoantigens would not be present on HLA-identical donor cells tested in vitro, so donor cells would not be subject to attack. Alternatively, T cells that prevent BFU-E growth in vitro could occur normally, their inhibiting potential usually suppressed by other cells. However, patients with AA could lack this suppressor population. In coculture studies with donor peripheral blood cells, suppressor cells that prevent T cells from inhibiting BFU-E growth could be provided by the donor cell population. Obviously, more extensive studies of lymphocyte subpopulations are needed to elucidate mechanisms responsible for immune-mediated AA. Regardless of the interpretation, the observation that immune-mediated aplasia need not be associated with inhibition of donor BFU-E in vitro is important because it reemphasizes the limited value of using coculture studies alone for either elucidating the mechanisms of aplasia or predicting the outcome of marrow transplantation.

It is interesting to note that only 5 patients of the 34 transplanted had a failure of marrow engraftment or graft rejection and that all 5 were associated with either 100% inhibition of donor BFU-E or possible immune-mediated aplasia. Further study will determine whether or not the two tests described in this report, used in combination, will be useful for identifying patients at risk of graft rejection.

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