Marrow graft rejection and inhibition of growth in culture by serum in aplastic anaemia

A. J. BARRETT¹, A. FAILLE, F. SAAL, N. BALITRAND, AND E. GLUCKMAN

From the Institut de Recherches sur les Leucémies et les Maladies du Sang, Centre G. Hayem, Hôpital Saint-Louis, 75010 Paris, France

SUMMARY The sera of 28 patients with aplastic anaemia were examined for their effect on granulocyte colony growth in soft agar. Normal sera did not affect colony growth, but 13 sera from patients with aplastic anaemia, three from multiparous women, and six from patients polytransfused for various disorders caused colony inhibition. This inhibition was not due to the presence of HLA antibodies in aplasia patients because some sera inhibited HLA compatible bone marrow, and polyspecific HLA antibodies were not found in all inhibitory sera. All patients who failed to show engraftment or who rejected their bone marrow graft within three weeks had serum inhibitory to normal bone marrow cell culture, but inhibition could not be demonstrated against autologous bone marrow cells in these patients with aplastic anaemia.

The results show that patients with serum inhibitors have an increased risk of early graft rejection and suggest that this rejection is mediated by antibodies directed against bone marrow stem cells.

When bone marrow cells are incubated with anti-lymphocyte globulin in the presence of complement subsequent granulocyte colony growth in soft agar is inhibited. The inhibition is dependent on complement and can be specifically removed by absorption, suggesting that the effect is antibody-mediated (Rødt *et al.*, 1974; Mosedale *et al.*, 1976; Barrett *et al.*, 1975, 1978).

Using this preincubation procedure, we examined the effect of sera from patients with aplastic anaemia as compared with sera from HLA sensitised or non-sensitised controls to see whether inhibition of granulocyte colony growth occurred and if it had any specific relevance to the pathophysiology of aplastic anaemia or to bone marrow graft rejection.

Patients and methods

This study was retrospective. The clinical results of marrow transplantations in these patients are reported in full elsewhere (Gluckman *et al.*, 1977).

Of 28 patients with aplastic anaemia studied, 26 fitted the diagnostic criteria for severe aplastic anaemia (neutrophils less than $0.5 \times 10^9/l$, platelets less than $20 \times 10^9/l$, reticulocytes less than 1%,

with hypocellular bone marrow aspirates and biopsy). Two patients (cases 24 and 25) had higher neutrophil counts.

Most patients had been referred for consideration for bone marrow transplantation, and 20 subsequently received a bone marrow graft from a sibling who was HLA identical and compatible by mixed lymphocyte culture. Marrow was transplanted in accordance with the Seattle protocol (Thomas *et al.*, 1975) using either cyclophosphamide (Cy), procarbazine, ALG and cyclophosphamide (PAPA Cy), or cyclophosphamide and total body irradiation preconditioning (Cy TBI) (Table 1). One patient was first treated unsuccessfully with ALG and was subsequently given a bone marrow graft from a compatible sibling. Sera were taken before any specific treatment had been started and at various times after marrow transplantation. No sera were available from patients before they had been transfused.

As controls, sera were collected from normal subjects not immunised by pregnancy or transfusion, and from patients with various haematological disorders who had been given multiple transfusions. Other sera used were from four multiparous women found to have high titres of polyspecific HLA antibodies. All sera were stored at -70°C until they were tested.

¹Present address: Westminster Hospital, London
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Table 1 Relation of colony inhibition in serum taken 10 days pregraft to presence of HLA antibodies and marrow graft rejection

Patient (no, sex, age)	Pregraft conditioning	Bone marrow cells ($\times 10^6$ /kg infused)	Outcome (day of rejection)	Colony inhibition of pregraft serum		HL-A antibodies in serum
				Unmatched marrow	Donor marrow	
2	F 12	Cy	6.0	No take	+	None
3	F 7	PAPA Cy	6.4	No take	+	+++
4	M 16	Cy	0.8	No take	+	+++
5	M 18	Cy	1.2	No take	+	+++
6	F 30	Cy	4.0	Rejection (19)	+	None
7	M 8	PAPA Cy	5.0	Rejection (24)	-	+
8	F 24	Cy	2.5	Rejection (28)	-	+
9	M 29	Cy	1.6	Rejection (29)	-	None
10	F 4	Cy	5.6	Rejection (40)	-	None
11(a)	M 23	Cy	3.3	Rejection (60)	+	+++
11(a)	F 26	Cy	3.0	Rejection (90)	+	+
12	F 15	PAPA Cy	5.0	Rejection (90)	-	None
13	M 4	Cy	8.4	Rejection and autologous reconstitution (60)	+	++
1(b)	M 23	Cy TBI	3.5	(Take)*	-	++
14	M 24	Cy	3.0	Take GVH	-	None
11(b)	F 20	Cy	2.0	Take GVH	-	+
15	M 27	Cy TBI	3.3	Take GVH	+	+++
16	M 10	PAPA Cy	4.4	Take	+	++
17	M 27	Cy	1.9	Take	+	None
18	M 20	PAPA Cy	2.4	Take	-	+
19	M 18	Cy	2.4	Take	-	None
20	M 20	PAPA Cy	2.2	Take	-	None

+ colony growth of less than 70% of control, which differed by more than 2 SD from control colony growth

HL-A antibodies: +++ polyspecific

++ positive on approximately 50% of a lymphocyte panel of 25 donors

+ positive on less than 10% of a lymphocyte panel of 25 donors

*Patient died 19 days after graft with histological evidence of marrow restoration. (a) First graft (b) Second graft

COLONY INHIBITION TECHNIQUE

Fresh bone marrow was aspirated from haematologically normal subjects including bone marrow donors. Marrow was separated on a Ficoll-Triosil density gradient, and the supernatant cells were harvested, resuspended, and washed in medium. Aliquots of 6×10^5 nucleated cells in 0.1 ml of McCoy's 5A medium were pipetted into sterile 8 mm plastic tubes, and 0.2 ml of normal or test serum was added. The cells were incubated with serum for 90 minutes at 37°C, 0.03 ml rabbit complement (10% final concentration) was added, and the cells were reincubated for a further 30 minutes before being washed three times in medium. The whole cellular sediment was resuspended in 0.3% agar and medium containing 15% fetal calf serum and plated in triplicate 1 ml volumes into culture dishes containing a feeder layer of 10^6 buffy coat cells in 0.5% agar as a source of colony-stimulating factor. After 12 days' incubation at 37°C in a humidified atmosphere containing 5% CO₂, colonies (groups of 50 or more cells) were counted and the mean and standard deviation for each triplicate culture were calculated. The colony inhibition index was calculated from the fraction:

$$\frac{\text{colonies}/2 \times 10^5 \text{ cells in test serum}}{\text{colonies}/2 \times 10^5 \text{ cells in control serum}} \%$$

The controls used to represent 100% growth were (i) tubes with no added human serum incubated with 15% fetal calf serum identical with that used in culture (normal sera only), and (ii) tubes in which AB or autologous serum was used. Colony numbers in the incubated controls were comparable to uninoculated values, and experiments on normal bone marrow produced an average of 85 colonies/ 2×10^5 cells with a range of 42-140.

COMPLEMENT

The rabbit complement was a single batch stored at -80°C, which did not alone cause colony inhibition at the 10% concentration used but produced 100% lymphocytotoxicity in the presence of an anti HLA 2 antiserum in a Terasaki microcytotoxicity test at this concentration. Horse ALG (Merieux), 10 µg/ml, was shown to cause complete colony inhibition when incubated together with bone marrow cells and complement in the manner described.

Results

Figure 1 shows the results of 18 experiments in which normal bone marrows were preincubated with complement and serum from normal subjects, multiparae, and polytransfused and aplastic anaemia patients. Sera from normal controls did not inhibit colony growth either when autologous or AB serum incubation was compared with a no-serum control, or when sera of various ABO groups were compared with autologous or AB serum controls. These values were therefore grouped to define a range for normal serum activity of $103 \pm 32\%$ (2 SD).

Fig. 1 Effect on colony growth of preincubation of different sera with normal HLA and ABO unmatched bone marrows and complement. Mean and SD shown for nine patients for whom repeated tests were performed on the same serum.

Three sera from the four multiparae inhibited colony growth, as did all the sera from the six polytransfused patients. Thirteen out of 28 patients with aplastic anaemia showed serum inhibition of colony

growth. Fourteen aplasia sera were tested on two or more occasions either on unmatched bone marrow, or on the donor HLA compatible marrow, or on autologous (aplastic) marrow (Table 2). Repeated

Table 2 Repeated tests on 12 pretreatment sera with unmatched, donor, and autologous marrow cells

Case no	Marrow			Actual colony growth of patients' marrow Colonies/2 × 10 ⁵ cells		
	Unmatched	Donor	Autologous	Control serum	AB serum	Autologous serum
14	68*	10*	—	—	—	—
7	140 107 108	99	—	—	—	—
2	49* 57*	49*	75	4 ± 2	3 ± 2	—
6	52*	100	100	5 ± 2	5 ± 3	—
10	71	156	160	3.3 ± 2	5.3 ± 0.5	—
13	79 115	90	104	4.3 ± 2	4.5 ± 2	—
22	21* 3*	—	150	3.3 ± 1	5 ± 3	—
24	50* 60*	—	100	9 ± 5	9 ± 4	—
25	66*	—	118	8 ± 4	9.5 ± 1	—
28	62*	—	82	4 ± 0	3.3 ± 2	—
3	2* 1*	—	—	—	—	—
23	1* 12*	—	—	—	—	—

Figures represent percentage colony growth against control normal serum for each experiment. Asterisks show sera that produced 70% or less than control growth and differed by more than 2 SD from the mean control colony numbers of that experiment.

tests on different unmatched marrows gave consistent results. Only two out of the seven sera tested against HLA compatible marrow caused colony inhibition. Inhibition never occurred with autologous bone marrow, but these values had less significance because of the low numbers of colonies in these patients.

Changes in serum inhibitory properties after bone marrow transplantation were studied in eight patients (Fig. 2). Ten days after bone marrow transplantation there was a trend towards greater inhibition in all the sera, which later disappeared in most cases irrespective of the outcome of the graft. Patients who rejected their graft showed the greatest inhibition 10 days after transplantation, with the exception of case 16, who had sustained marrow engraftment with an increasing degree of inhibition in the serum. The relationship of inhibition in pre-transplantation sera to the presence of polyspecific HLA antibodies and the fate of the marrow transplant is shown in Table 1. Of 10 patients who had serum colony inhibitors, eight subsequently failed to show engraftment or rejected the bone marrow graft, one with autologous recovery of the bone marrow (case 13). All the patients whose graft failed to take had serum inhibitors but five patients who rejected their graft did not have serum inhibitors. There was some correlation of the presence of polyspecific HLA antibodies in the serum with colony inhibition,

Fig. 2 Serial testing of sera from patients before and after marrow transplantation. Figures indicate case numbers. Cases 1a, 4, 9, and 17 received cyclophosphamide alone as preconditioning.

but the association was not absolute. Three sera without detectable HLA antibodies showed colony inhibition and six sera with HLA antibodies failed to inhibit colony growth of unrelated bone marrow. On six occasions the donor marrow was tested with the patient's serum. The two patients with inhibitors rejected their grafts, but no inhibition was found in a third patient (case 10) who rejected the graft.

Two patients were successfully regrafted after rejection of the first graft. Case 1, who rejected the first graft after 60 days, showed inhibition in the pre-graft serum (1a) but when tested against the second donor and an unmatched marrow with serum taken 10 days before the second graft (1b) inhibition did not occur. This patient died 19 days after the graft with histological evidence of marrow restoration. Case 11 had an inhibitor in the serum before the first graft, which was rejected, but no inhibitor was present 10 days before the second graft (from another sibling), which was not rejected.

Discussion

Colony inhibition was not found to be a property of normal human serum with this technique. This indicates that non-antibody inhibitors of colony

growth previously described in human serum (Chan *et al.*, 1971) are unlikely to have been responsible for the inhibition we found in pathological sera.

There was no effect on colony growth due to ABO incompatibility between serum and test marrow, but inhibition did occur in subjects sensitised by either blood transfusion or pregnancy to HLA antigens, and also in patients with aplastic anaemia. This suggests that colony inhibition was due to antibodies in the serum, independent of the ABO system, which have either HLA specificity or anti-stem cell specificity, or both. The possibility that anti-colony forming cell antibodies are distinct from HLA antibodies is strengthened by the observation that some patients' sera without HLA antibodies demonstrated colony inhibition, and that inhibition was also produced using HLA identical bone marrow.

There was no evidence that colony inhibition represented a specific feature of aplastic anaemia, since all the patients with aplastic anaemia had received blood transfusions before being tested, and the degree of inhibition seen matched that of the polytransfused or sensitised non-aplasia groups. In addition (although it might be argued that the patients' cells were already blocked *in vivo*), no inhibitory effect was demonstrable when the patient's serum was incubated with autologous bone marrow cells.

These results contrast with the findings of Gordon (Royal Marsden Hospital Bone Marrow Transplant Team, 1977), who demonstrated inhibitory activity exclusively in the serum of a patient with aplastic anaemia and not in the sera of control polytransfused patients. Since complement was not used in their studies inhibition may not have been antibody-mediated.

The strong relationship of colony inhibition to early graft rejection or complete lack of marrow restoration suggested that the graft failure in these patients may have been due to circulating antibodies against stem cells. This mechanism of early graft rejection has already been proposed for the dog by Epstein *et al.* (1975). It may be relevant that the colony inhibition of the sera of patients who failed to show engraftment or who rejected their marrow graft increased 10 days after the graft at a time when increasing anti-graft antibodies might be expected. Testing of pre-graft serum with the patient's donor marrow cells could be used as a predictive test for early graft rejection, permitting intensification of the pre-graft immunosuppressive regime, or pre-graft plasmapheresis in the patients found to have colony-inhibiting sera, to reduce the chance of graft rejection.

Storb has described several prognostic factors that

help to predict the outcome of the graft: the sex match of the donor-recipient pair; the cell dose infused; and the relative response index (RRI) of the mixed lymphocyte culture (Storb *et al.*, 1977a, 1977b). In our series of marrow transplantations, there was no significant relationship between the number of cells infused, the RRI, or the sex match with the fate of the graft; nor was there any correlation between the degree of HLA sensitisation and graft rejection (Gluckman *et al.*, 1977). No single predictive criterion is likely to be reliable for an individual patient, and the best indication of the graft outcome would probably be obtained by evaluating all the criteria together.

Further studies are required to confirm that the colony inhibition found in these sera is antibody-mediated. However, the demonstration of colony inhibition in serum appears to have clinical relevance for bone marrow transplantation, which could be confirmed by a larger study using the bone marrow donor cells as test material.

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References

- Barrett, A. J., Humble, J. G., and Hobbs, J. R. (1975). Bone marrow suppression by antilymphocyte globulin. *British Medical Journal*, **3**, 541.
- Barrett, A. J., Longhurst, P., Rosengurt, N., Hobbs, J. R., and Humble, J. G. (1978). Crossreaction of antilymphocyte globulin with human granulocyte colony-forming cells. *Journal of Clinical Pathology*, **31**, 129-135.
- Chan, S. H., Metcalf, D., and Stanley, E. R. (1971). Stimulation and inhibition by normal human serum of colony formation *in vitro* by bone marrow cells. *British Journal of Haematology*, **20**, 329-341.
- Epstein, R. B., Debelak-Fehir, Bennett, B. T., Hess, A., and Zander, A. (1975). Detection of presensitization by granulocyte colony inhibition in canines. *4th Annual Conference of the International Society for Experimental Haematology, Trogir, Yugoslavia*, Abstracts, p. 68.
- Gluckman, E., Devergie, A., Marty, M., Bussel, A., Rottembourg, J., Dausset, J., and Bernard, J. (1977). Allogeneic bone marrow transplantation in aplastic anaemia. Report of 25 cases. *Transplantation Proceedings*, **10**, 141-145.
- Mosedale, B., Smith, M. A., and Courtenay, J. S. (1976). Preparation and characterization of antithymocyte serum and globulin without stem cell activity. *Transplantation*, **22**, 122-131.
- Rodt, H., Netzel, B., Brehm, G., and Thierfelder, S. (1974). Production of antibodies specific for human thymus derived lymphocytes purified from antibodies crossreacting with colony-forming cells. *Blut*, **29**, 416-422.
- Royal Marsden Hospital Bone-Marrow Transplantation Team (1977). Failure of syngeneic bone-marrow graft without pre-conditioning in post-hepatitis marrow aplasia. *Lancet*, **2**, 742-744.
- Storb, R., Prentice, R. L., and Thomas, E. D. (1977a). Treatment of aplastic anemia by marrow transplantation from HLA identical siblings. *Journal of Clinical Investigation*, **59**, 625-632.
- Storb, R., Prentice, R. L., and Thomas, E. D. (1977b). Marrow transplantation for treatment of aplastic anaemia: factors associated with rejection. *New England Journal of Medicine*, **296**, 61-66.
- Thomas, E. D., Storb, R., Clift, R. A., Fefer, A., Johnson, F. L., Neiman, P. E., Lerner, K. G., Glucksberg, H., and Buckner, C. D. (1975). Bone marrow transplantation. *New England Journal of Medicine*, **292**, 832-843 and 895-902.

Requests for reprints to: Dr A. J. Barrett, Westminster Hospital, Horseferry Road, London SW1, UK