Crossreaction of antilymphocyte globulin with human granulocyte colony-forming cells

A. J. BARRETT, P. LONGHURST, N. ROSENGURT, J. R. HOBBS, AND J. G. HUMBLE

From the Departments of Haematology and Chemical Pathology, Westminster Medical School, and the Tissue Typing Department, Westminster Hospital, London SW1P 2AP, UK

SUMMARY Clinical preparations of horse antilymphocyte globulin (ALG) were found to inhibit human bone marrow granulocyte colony growth. This effect was enhanced by complement and was dose dependent, being almost complete at ALG concentrations of 100 μ g/ml. Inhibition was a property of ALG but not of normal horse globulin. However, short incubation of ALG with bone marrow cells occasionally stimulated colony growth and normal horse globulin regularly stimulated it. Three hours' incubation of bone marrow cells with ALG was needed to produce consistent colony inhibition, which was measurable as a reduction in the expected number of colonies and as a fall in the colony:cluster ratio of surviving cell aggregates. Absorption of ALG on acute myeloid leukaemia blast cells removed the inhibiting property of the ALG while preserving its lymphocytotoxic action. Serum from two patients receiving ALG treatment inhibited colony growth for up to 48 hours after ALG administration. The results suggest the presence in ALG of antibodies specifically cytotoxic to myeloid stem cells which may relate to its myelosuppressive properties *in vivo*, and also indicate that it should be possible to remove antimyeloid antibodies from ALG by absorption. The use of such purified ALG would have advantages in clinical bone marrow transplantation.

Antilymphocyte globulin (ALG) and antithymocyte globulin (ATG) inhibit human granulocyte colony growth (Rodt *et al.*, 1974; Barrett *et al.*, 1975). There is evidence from animal experiments that ALG also inactivates bone marrow stem cells *in vivo* and *in vitro* (Field and Gibbs, 1968; DeMeester *et al.*, 1968), and ALG and ATG have occasionally been implicated in the failure of bone marrow transplants to establish a graft in patients with aplastic anaemia (Storb *et al.*, 1974; Barrett *et al.*, 1975). We have investigated (1) the inhibition of human granulocyte colony-forming cells (CFU) by ALG, (2) methods to remove this inhibition, and (3) the clinical relevance of this interaction.

Material and methods

The ALG preparations tested are shown in Table 1. All globulins were purified and preliminarily absorbed by the manufacturer for clinical use.

Table 1 Globulin preparations tested

Designation	Manufacturer	Batch No.
ALG 1	Burroughs Wellcome	TD4
ALG 2	Hoechst	514
ALG 3	Hoechst	509
ALG 4	Hoechst	817027
ALG 5	Hoechst	0288
ALG 6	Hoechst	070170
ALG 7	Hoechst	506
NHG (normal horse globulin)	Hoechst	161097

INCUBATION OF BONE MARROW CELLS WITH ALG

Human bone marrow was obtained from diagnostic aspirates of haematologically normal patients or from bone marrow obtained from the donor during marrow transplantation. Bone marrow was aspirated into preservative-free heparin (20 iU/ml) and the buffy coat obtained after sedimentation for 1½ hours at 37°C. The cells were washed twice and resuspended in McCoy 5A medium. The nucleated cells were

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counted and the volume of the cell suspension was adjusted to obtain 10^6 cells/ml: 0.7 ml volumes of this suspension were pipetted into sterile tubes containing either 0.1 ml normal horse globulin (NHG) or ALG in final concentrations varying from 100 to 0.1 $\mu\text{g/ml}$: 0.2 ml of fresh autologous serum was added to each tube. In some experiments duplicate tubes were prepared with autologous serum heated to 56°C for 30 minutes to inactivate complement. A control tube containing 0.2 ml serum but no horse globulin was also prepared for each experiment. The cells were incubated at 37°C for varying periods of time in different experiments. They were then washed twice in McCoy 5A medium containing 15% fetal calf serum or human AB serum and put into culture.

GRANULOCYTE COLONY CULTURE

Colony culture followed the technique described by Pike and Robinson (1970), but in later experiments 15% human AB serum was used in place of fetal calf serum since it was found to support colony growth better. The washed cell buttons obtained by centrifugation after incubation with globulin were resuspended in 3.5 ml of 0.3% agar and medium and 1-ml volumes were pipetted into triplicate culture dishes on blood leucocyte feeder layers in 0.5% agar prepared at least four days in advance to avoid interference of any residual ALG with colony-stimulating factor production by the feeder layer.

Colonies were counted after 12 to 14 days' incubation at 37°C in 7.5% CO_2 and scored as cell groups of 40 or more. Clusters (groups of 5-39 cells) were counted separately. Results were expressed as mean $\pm$ standard deviation for triplicate samples.

ABSORPTION OF ALG 2

Chronic lymphatic leukaemia (CLL) cells were obtained from an untreated patient by separation of lymphocytes on Lymphoprep (Nygaard Ltd). This preparation contained over 99% lymphocytes.

Chronic granulocytic leukaemia (CGL) cells were obtained from an untreated patient with Philadelphia chromosome positive CGL whose leucocyte count was $200 \times 10^9/\text{l}$, with 1% lymphocytes and 4% myeloblasts.

Acute myeloid leukaemia (AML) blast cells were obtained by kind permission of Dr R. Powles from an untreated patient undergoing leucopheresis. This preparation was preserved at -70°C in dimethyl sulphoxide. It contained over 99% myeloblasts and no lymphocytes.

ALG 2 was absorbed overnight on washed preparations of packed cells in the ratio of 1 mg of globulin to 10^8 cells. The mixture was resuspended and incubated for a further hour before centrifugation at 600 *g*. The clear supernatant was then

removed for testing. Using packed leucocytes the volume of globulin before and after absorption did not alter significantly, and absorbed globulin was diluted identically with the unabsorbed globulin for testing at concentrations of 100, 10, and 1.0 $\mu\text{g/ml}$.

LYMPHOCYTOTOXICITY TESTS

ALG was tested for lymphocytotoxicity by the Terasaki two-step method in microtoxicity plates. Of each globulin dilution 1 μl was added to 2 μl of lymphocytes obtained from normal individuals and incubated for $\frac{1}{2}$ hour before adding rabbit complement. The cells were incubated for a further $1\frac{1}{2}$ hours. Eosin was then added and cytotoxicity assessed using an inverted objective microscope. The globulin was tested absorbed and unabsorbed at final concentrations of 100, 10, and 1 $\mu\text{g/ml}$ on lymphocytes from normal subjects. Results were expressed as the mean lymphocytotoxicity for four experiments using a different lymphocyte donor on each occasion.

TESTS USING SERUM OF PATIENTS TREATED WITH ALG

Two patients were given 40 mg/kg/day of ALG 2 for five days by intravenous infusion during treatment for pure red cell aplasia (case A) and severe aplastic anaemia (case B). Blood was taken before, during, and after ALG administration; serum was separated and stored at -40°C and tested for colony-inhibiting properties on a single bone marrow sample. 10^6 nucleated cells in 0.7 ml were incubated for two hours at 37°C with 0.2 ml volumes of patients' serum and 0.1 ml of fresh AB serum and the cells were then washed and put into culture.

Results

EFFECT OF COMPLEMENT AND COMPARISON OF ALG WITH NORMAL HORSE GLOBULIN

A preliminary experiment showed that ALG 1 inhibited colony growth in a dose-dependent manner and that the effect was enhanced by fresh autologous serum used as a source of complement (Fig. 1).

The results of three similar experiments are shown in Table 2. Inhibition was greater in the presence of a source of complement, but this difference was reproducibly significant only for concentrations of globulin of 100 $\mu\text{g/ml}$.

ALG in the absence of complement showed significant inhibition only when compared with NHG at concentrations down to 10 $\mu\text{g/ml}$, while the addition of complement produced significant inhibition at concentrations as low as 1 $\mu\text{g/ml}$.

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Table 2 Effect of ALG and NHG in three similar experiments after marrow was incubated with serum for two hours. Figures indicate percentage of control colony growth incubated without added globulin

Experiment	Globulin concentration ($\mu\text{g/ml}$)			
	0.1	1	10	100
ALG + complement	60	43	36	8
	54	48	45	35
	100	75	91	37
ALG, no complement	94	92	46	22
	51	112	66	51
	80	77	85	77
NHG + complement	82	82	97	134
	131	131	138	127
	87	87	112	

Significance of differences (P)

NHG ν ALG	NS	NS	0.01	0.005
NHG ν ALG + complement	NS	0.05	0.02	0.0005
ALG + complement ν ALG, no complement	NS	NS	NS	0.05

Fig. 1 Experiment showing dose-dependent inhibition of colonies by ALG. Without added complement ---, with complement —. Cells were incubated with ALG and 10% fresh autologous serum as a source of complement for two hours.

EFFECT OF NHG

NHG tended to stimulate colony growth in a dose-dependent manner and never showed dose-dependent inhibition.

DURATION OF INCUBATION

Repeated experiments where ALG 1 was incubated with bone marrow cells for one to two hours produced considerable variation in the amount of inhibition. Occasionally ALG stimulated colony growth (Fig. 2). Experiments where the incubation

time of ALG with marrow cells was varied were carried out (Table 3). In experiment A exposure of cells for five minutes to NHG and ALG produced an increase in colony numbers compared with the control, which diminished after 1½ hours' incubation with ALG but persisted with NHG. In experiment B ALG and NHG stimulated colony growth initially but after three hours the ALG showed inhibitory properties.

Fig. 2 Experiment showing stimulation of colony growth by ALG after incubation of bone marrow with ALG and complement for one hour. — Complement added, --- no complement (10% fresh autologous serum was used as complement).

Table 3 Two experiments in which bone marrow was incubated with ALG or NHG for varying periods of time before being put into culture

Duration of incubation	Colonies/ 2×10^5 cells (mean $\pm$ SD)		
	ALG 1 (10 $\mu\text{g/ml}$)	NHG (10 $\mu\text{g/ml}$)	Control (no globulin)
Experiment A			
5 min	25 $\pm$ 4.5	23 $\pm$ 5	10.6 $\pm$ 3
100 min	11 $\pm$ 4	27 $\pm$ 3.5	—
Experiment B			
1½ h	162 $\pm$ 9.5	187 $\pm$ 2.5	137 $\pm$ 9
3 h	117 $\pm$ 5.5	138 $\pm$ 6	142 $\pm$ 4
6 h	61 $\pm$ 12	101 $\pm$ 9.5	112 $\pm$ 4
18 h	79 $\pm$ 8	100 $\pm$ 11	92 $\pm$ 8.5

COLONY INHIBITION ASSAY

After these results further studies were carried out in conditions designed to produce optimum ALG-mediated colony inhibition. Bone marrow cells were incubated for three hours at 37°C in the presence of complement before being washed and put into culture. Two identical experiments were carried out to test the colony-inhibiting properties of six ALGs.

The mean results of these two experiments are shown in Figure 3. All preparations showed inhibition; only one (ALG 7) was significantly less inhibitory and NHG did not cause dose-dependent inhibition. Analysis of the colony:cluster ratio in these experiments showed a close correlation between the degree of inhibition observed and the fall in the colony:cluster ratio ($r = 0.96$; $P = 0.001$, Fig. 4).

Fig. 3 Colony inhibition by ALG 2-7 after three hours' incubation in presence of complement. ALG 7 was significantly less inhibitory than the other preparations.

ABSORPTION OF ALG

The results obtained with ALG in single absorption on AML, CLL, or CGL cells are shown in Figure 5. Lymphocytotoxicity and colony inhibition of these preparations are compared. AML absorption did not modify lymphocytotoxicity but almost completely removed colony inhibition. A further single absorption of ALG on AML cells was carried out using 10^7 cells/mg of globulin, which showed that at least 10^8 cells/mg were required effectively to remove anticolony activity but that a single absorption with this number of cells was satisfactory. CLL absorption greatly reduced lymphocytotoxicity without removing anticolony activity, while CGL cells produced reduction in lymphocytotoxicity and colony inhibition.

COLONY INHIBITION IN SERUM OF PATIENTS TREATED WITH ALG

Serum from two patients during ALG administration inhibited colony growth after 48 hours (case B) and after 24 hours (case A). Serum taken before ALG administration did not inhibit colony growth when compared with a control (autologous) serum (Table 4).

Fig. 4 Relationship of colony inhibition to colony:cluster ratio. Each point represents the mean results of values for ALG 2-7 on a single bone marrow sample. $r = 0.96$. $P = 0.001$.

Table 4 Colony inhibition in serum of patients undergoing treatment with ALG 2. Normal bone marrow cells were incubated for two hours in presence of 20% patient's serum and 10% fresh AB serum before being put into culture

Relationship to ALG treatment	Colonies/ 2×10^5 cells (mean $\pm$ SD)
Case A	
Before treatment	11.3 ± 2.8
During first infusion	5.0 ± 0.7
18 hours after infusion	2.5 ± 0.7
48 hours after infusion	2.0 ± 0.7
Case B	
Before treatment	11.3 ± 0.6
21 hours after infusion	4.5 ± 0.7
48 hours after infusion	$8.0 \pm \text{—}$
4 days after infusion	14.6 ± 3.2
Control	
Autologous serum	12.5 ± 3.0

Discussion

The variable results obtained after short exposure of bone marrow cells to ALG may be explained by opposing properties of horse ALG, which showed an immediate stimulating action with NHG but also a slower inhibiting effect on CFU. The mechanism of this stimulating action is not clear. It may be the

Fig. 5 Effect of prior absorption of ALG 2 on CFU inhibition and lymphocytotoxicity. Absorbed and unabsorbed sera were tested in parallel on bone marrow samples. Lymphocytotoxicity represents mean results obtained using lymphocytes from four normal subjects.

same phenomenon as the stimulation of bone marrow cells by exposure for one hour to horse ATG described by Ascensão *et al.* (1976) in a patient with aplastic anaemia. They tentatively attributed this to inactivation of suppressor T lymphocytes by ATG, but the fact that inert horse globulin also stimulated CFU suggests that the effect may not be immunologically mediated.

The increasing inhibition seen by prolonging the incubation times of bone marrow cells with ALG suggests that the 10% autologous serum used was not potent enough to cause rapid complement mediated cell death and that prolonged incubation was necessary before CFU was irreversibly damaged.

More clear-cut results might have been obtained by using a potent rabbit complement. However, the aim of these experiments was to produce a technique for preincubation of clinical marrow graft material. Therefore we decided to restrict the foreign antigen in the system to horse globulin to avoid sensitising patients unnecessarily. ALG appeared to affect the CFU directly rather than colony stimulating-factor production by the feeder layer since (1) the effect depended on exposure of cells to ALG in the liquid phase and (2) all feeder layers were prepared at least four days in advance. Previous experiments have shown that most feeder-layer colony stimulating activity has been produced by that time.

CFU inhibition was measurable in two ways: firstly, reduced colony number compared with control values and, secondly, reduction in the colony: cluster ratio (that is, the fall in colonies with increasing ALG concentration exceeded the fall in clusters). This suggests that partial damage to the growth of some CFU may have occurred, resulting in a cell that was capable of forming only cluster-sized groups. The enhancement by complement and the specific removal of colony inhibition by ALG absorbed on AML cells but not on CLL cells is strong evidence that inhibition was mediated by antibodies, although this has not been formally proved.

The inhibiting effect on CFU found in the serum of patients during and shortly after ALG administration indicates that significant damage to granulocyte CFU may occur *in vivo*, and the inhibition observed *in vitro* occurred at ALG concentrations that would be reached in the serum during treatment. However, the relationship of the *in vitro* phenomenon to possible myelosuppression caused by ALG is not clearly established. In haematologically normal individuals ALG does not cause overt bone marrow depression, although Krantz (1973) demonstrated toxicity of horse ATG to human erythroblasts and suggested that it might be responsible for the anaemia sometimes seen after treatment. On the other hand, ALG given shortly after bone marrow transplantation may prevent successful engraftment. Presumably ALG toxicity to stem cells is clinically significant only when the size of the stem cell pool is very small. Use of ALG as an immunosuppressive agent in treating patients with aplastic anaemia, as Ascensão *et al.* (1976) proposed, might equally cause an initial worsening of the condition.

ALG absorbed to remove stem cell toxicity would have advantages in three situations: (1) in the experimental treatment of aplastic anaemia with ALG alone, (2) in bone marrow transplantation to permit early postgraft treatment (or prophylaxis) of graft versus host disease, and (3) in the preincubation of the bone marrow graft with ALG to eliminate mature lymphocytes from the graft. This last procedure has successfully prevented graft versus host disease in incompatible donor-recipient matches in mice (Trentin and Judd, 1973) and rats (Müller-Rucholz *et al.*, 1976) and might find clinical application in situations where no fully compatible donor was available for a patient with severe aplastic anaemia. These experiments show that it is possible selectively to remove anti-CFU activity in ALG by absorption, and other studies using fetal liver cells as an absorbing antigen have produced similar results (Mosedale *et al.*, 1976). However, there would be practical difficulties in obtaining sufficient fetal liver cells for preparing therapeutic amounts of ALG.

The possibility also remains that the removal of CFU inhibition does not correlate with any reduction in toxicity to the totipotent stem cell responsible for marrow engraftment. Antigens present on spleen CFU and not on agar CFU have been identified in the mouse (Golub, 1972), and until a human stem cell assay is available the efficacy of absorption in removing anti-stem cell activity could be confirmed only by clinical trial.

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