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SURFACE ANTIGENS OF MURINE HEMOPOIETIC STEM CELLS

II. Rosetting Method Used to Compare the Cross-Reactivity of Antisera against Mouse Brain, Sperm, and Testis Cells with Bone Marrow Stem Cells and Progenitor Cells[1]

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Antisera raised in rabbits against mouse brain, sperm, and testis cells cross-react with hemopoietic stem cells (CFU_s) in mouse bone marrow. The specificity of this cross-reaction for bone marrow CFU_s has been examined by rosetting antibody-treated bone marrow cells with sheep red blood cells coated with antiglobulin and separating the rosetted and nonrosetted cell populations on Isopaque-Ficoll. Nonrosetted cells isolated at the Isopaque-Ficoll interface are then quantitated for CFU_s and for the granulocyte-macrophage progenitor cell CFU_c. This method permits direct comparison of antibody bound to different cell populations in the bone marrow (e.g., CFU_s and CFU_c), since cells are selected on the basis of surface concentration of bound antibody before and independently of the assay method. When bone marrow cells were coated with anti-brain IgG that had been absorbed with erythrocytes and thymocytes, and then rosetted, increased specific activity of CFU_s and CFU_c was observed in the nonrosetted fraction. Thus, anti-brain IgG did not show selectivity for these bone marrow cells. In contrast, antiserum against testis cells selectively depleted bone marrow cells of both CFU_s and CFU_c. No significant antigenic differences were observed between CFU_s and CFU_c with regard to anti-brain IgG and anti-testis serum.

Antigenic identity has become a key element in studying the function and properties of minor cell populations, particularly with regard to subsets of T lymphocytes (1). In addition to providing a means of morphologically and functionally characterizing cells, cell-specific antigens can be used to physically separate subpopulations of cells for *in vitro* study (1–3).

A limiting factor in the study of hemopoietic stem cells and progenitor cells has been a lack of knowledge of their antigenic identity. Recently, several studies have focused on the antigenic nature of the murine pluripotent stem cell, CFU_s,[2] and antigens held in common between CFU_s and brain (4), sperm (5), and mature hemopoietic cells (6, 7) have been described. The question of whether any of these antigens held in common with CFU_s show specificity for CFU_s in the bone marrow has not been unequivocally resolved, although it has been claimed by correlating stem cell purification with immunofluorescence data that the murine pluripotential stem cell can be directly identified by using rabbit anti-mouse brain serum (8). A major problem of comparing the cytotoxic sensitivity of different cell types to a particular antiserum lies in variations in cell sensitivity to complement (C) lysis. For example, using a C-dependent cytotoxic assay and a C-fixation assay, Rodt *et al.* (9) have shown large differences in the sensitivity of bone marrow cells to anti-brain serum and conclude that cytotoxicity may give erroneously low titers. In addition, evidence suggests that C treatment of bone marrow cells coated with heterologous antiserum is not necessary in order to produce maximum sensitivity of CFU_s (assayed *in vivo*) to antisera (6). Thus, factors other than *in vitro* C lysis are implicated in controlling sensitivity of CFU_s to antisera. It follows that antibody binding to CFU_s as measured by a reduction in spleen colony-forming units in the assay of Till and McCulloch (10) cannot be compared directly with antibody binding to bone marrow and progenitor cells in assays involving C lysis.

We have attempted to overcome the problems associated with determining cell sensitivity to antisera in different assays by preselecting bone marrow cells on the basis of surface concentration of bound antibody. This was achieved by rosetting antibody-treated bone marrow cells with sheep red blood cells (SRBC) coated with antiglobulin and separating rosetted and nonrosetted cells on Isopaque-Ficoll. Measurement of cell distribution between the Isopaque-Ficoll pellet and interface and specific activity of functionally defined minor cell populations (e.g., CFU_s and CFU_c) enabled comparison of bound antibody between the different cell populations.

The results indicate that anti-brain IgG does not show selectivity for either CFU_s or CFU_c in the bone marrow. On the other hand, antiserum prepared against mouse testis cells showed selectivity for both CFU_s and CFU_c in mouse bone marrow. Furthermore, no significant antigenic differences were observed between CFU_s and CFU_c with either antiserum.

MATERIALS AND METHODS

Animals. CBA/T6 T6 mice (2 to 3 months) bred at Victoria University were used for the preparation of antisera, for cell assays and absorptions, and as donors in the spleen colony assay. CBA × C57BL mice (2 to 3 months) were used as

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[1] This work was supported by the Malaghan Trust.
[2] Abbreviations used in this paper: CFU_s, colony-forming units in the spleen, stem cells; CFU_c, colony-forming units in culture; NRS, normal rabbit serum; SARGG, sheep anti-rabbit γ-globulin; mSBSS, mouse iso-osmotic-buffered balanced salt solution.

recipients in the spleen colony assay. New Zealand White rabbits were used as a source of normal rabbit serum (NRS) or for raising antisera.

Preparation of antisera. Antiserum against mouse brain was prepared according to Golub (4). Antisera against mouse sperm and testis cells were prepared by injecting rabbits at multiple subcutaneous and intramuscular sites with 2 to 3 × 10^7 nucleated cells homogenized in complete Freund's adjuvant. Four to 5 weeks later the animals were given i.v. booster injections on 3 consecutive days with a similar number of cells. One week later rabbits were bled, and the antisera were pooled, heated at 56°C for 30 min, and stored at −17°C. All antisera were absorbed with an equal volume of CBA erythrocytes at 4°C for 1 hr.

An IgG fraction from anti-brain serum was prepared by passage of a 50% ammonium sulphate fraction through DEAE-cellulose equilibrated with 0.01 M phosphate, pH 8. The yield of IgG was 93 mg from 12 ml antiserum, and resuspension was at 10 mg/ml. Anti-brain IgG was absorbed with 0.2 volumes of CBA erythrocytes and twice with 0.1 volumes of CBA thymocytes.

Sheep anti-rabbit γ-globulin (SARGG) was prepared by injecting sheep at multiple subcutaneous and intramuscular sites with DEAE-purified rabbit IgG (4 mg/sheep) in complete Freund's adjuvant. Booster injections were given 2 and 6 weeks later, and the sheep were bled out after a further 3 weeks. An IgG fraction from sheep anti-rabbit IgG serum was prepared as described above.

Cell preparations. Mouse iso-osmotic-buffered balanced salt solution (mSBSS) (11) was used throughout. Thymocytes, testis cells, and sperm from the caudal epididymus were obtained by teasing tissue through stainless steel gauze into mSBSS, allowing debris to settle for 10 min, then washing the cell preparations three times with mSBSS. Bone marrow cells were flushed from the femur and tibia with mSBSS, and washed with mSBSS.

For cytotoxicity experiments thymocytes and bone marrow cells were purified in Isopaque-Ficoll ($\rho = 1.09$) (12), azide being omitted from the fractionation procedure. The resulting cells were greater than 95% viable as measured by trypan blue exclusion.

Sperm isolated from the caudal epididymus were 80% pure, whereas testis cells were contaminated with 12% sperm or sperm heads.

SRBC in Alsever's solution were obtained commercially and used within 2 weeks of collection.

Peritoneal exudate cells enriched in neutrophils were obtained from CBA mice that had been injected i.p. 18 hr previously with 1.5 ml 10% proteose peptone (Difco) made up in 0.85% saline. Such preparations contained about 78% neutrophils, as determined by staining with orcein in acetic acid.

Spleen colony inhibition assay. The method of Till and McCulloch (10) was used to measure viable pluriopotent stem cells, CFU_s (6). Bone marrow cells (3 × 10^7 nucleated cells/ml) were treated with antisera at 4°C for at least 30 min in mSBSS before dilution and injection into groups of five lethally irradiated (900 R, 300 R/min) mice. Percent inhibition of spleen colony formation was calculated according to the formula:

$$\frac{\text{Number of colonies with NRS-treated cells} - \text{Number of colonies with antibody-treated cells}}{\text{Number of colonies with NRS-treated cells}} \times 100$$

The serum titer is defined as the dilution of antiserum (per unit volume) that gives a 50% inhibition of spleen colony formation. In some experiments antibody-coated bone marrow cells were incubated with C before injection into lethally irradiated mice, but this step has been found to be unnecessary for maximum sensitivity of CFU_s to antisera and was omitted from most experiments (6).

Inhibition assay for colony-forming units in culture (CFU_c). Viable CFU_c were measured according to Metcalf *et al.* (13). Endotoxin-treated mouse serum (0.1 ml per 1 ml culture dish) was used as a source of colony-stimulating factor.

Formation and isolation of rosettes. Washed SRBC were coated with SARGG by the chromium chloride method described by Parish and Hayward (14). Bone marrow cells purified on Isopaque-Ficoll were suspended in mSBSS containing 10% fetal calf serum (FCS) at 3 × 10^7 nucleated cells/ml and were treated with an equal volume of diluted antiserum of 4°C for 30 min. The antibody-coated cells were washed twice with mSBSS containing 10% FCS, suspended at 1 to 3 × 10^7 cells/ml, and rosetted with an equal volume of 10% E anti-Ig as described by Parish *et al.* (15). Rosetted bone marrow cells were counted in 0.1% methyl violet in mSBSS. A rosette was defined as any bone marrow cell that bound five or more erythrocytes. Separation of rosettes from nonrosetted cells was accomplished on Isopaque-Ficoll as described by Parish *et al.* (15). Red cells in the rosette pellet were lysed by osmotic shock (16), and following restoration of isotonicity, the remaining cells were pelleted at 300 × G for 5 min at 4°C and washed in mSBSS containing 10% FCS.

Cytotoxic assays. Antibody binding to thymocytes was determined by C-dependent cytotoxicity by using trypan blue exclusion to measure dead cells.

RESULTS

Anti-stem cell activity in antisera against mouse brain, sperm, and testis cells. Antisera prepared in rabbits against mouse brain, sperm, and testis cells were absorbed with CBA mouse erythrocytes and tested for their ability to deplete bone marrow of cells capable of forming spleen colonies in lethally irradiated mice (i.e., CFU_s). Figure 1 shows the titration curves of these antisera for CFU_s. Titers were calculated from these curves and are summarized in Table I. Each antiserum cross-reacted with CFU_s, their titers being 338 for anti-brain serum, 630 for anti-sperm serum, and 1780 for anti-testis serum.

An IgG fraction prepared from anti-brain serum showed reduced activity against CFU_s (titer 152; cf., 338 for anti-brain serum), and absorption of anti-brain IgG with CBA thymocytes yielded a preparation with an anti-CFU_s titer of 74 (Fig. 1*B*, Table I). Anti-brain IgG absorbed with erythrocytes and thymocytes was subsequently used to characterize the anti-stem cell activity of anti-brain serum.

It has previously been demonstrated in a C-dependent cytotoxic assay that anti-brain serum lacks activity against the granulocyte-macrophage progenitor cell (CFU_c) in mouse bone marrow (17). Thus each antiserum was examined for activity against CFU_c in a C-dependent assay. Table I shows that although anti-brain serum and anti-testis serum showed slight activity against CFU_c resulting in a 50% loss of *in vitro* colonies at a serum dilution of 1:3, no detectable activity against CFU_c was observed with anti-sperm serum.

Comparison of anti-stem cell activity with thymocyte cytotoxicity. The cytotoxicities of anti-brain serum, anti-sperm serum, and anti-testis serum for thymocytes were determined, and their titers are summarized in Table I. Anti-brain IgG retained 28% of the anti-thymocyte activity originally present

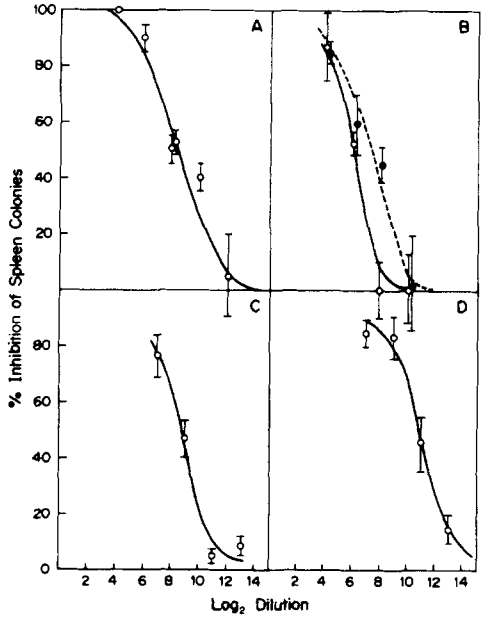

Figure 1. Anti-stem cell activity of antisera against brain, sperm, and testis cells. Bone marrow cells were treated *in vitro* with antisera that had previously been absorbed with erythrocytes, and CFU_s measured in the spleen colony assay (10). *A*, anti-brain serum; *B*, anti-brain IgG (●—●), anti-brain IgG absorbed with 2 × 0.1 volumes thymocytes (○—○); *C*, anti-sperm serum; *D*, anti-testis serum.

TABLE I

Titers of antisera for stem cells and thymocytes

Antisera[a]	Absorption[b]	Titer			Ratio	CFU_s Titer
		CFU_s	CFU_c	Thymocyte		Thymocyte titer
ABrS	RBC	388	3	231		1.7
ABrIgG (10 mg/ml)	RBC	152		64		2.4
	RBC + thymocytes	74		3		24.7
ASpS	RBC	630	0	29		21.7
ATsS	RBC	1780	3	74		24.1
	RBC (X2) + thymocytes + neutrophils	445		0		∞

[a] ABrS, anti-brain serum; ABrIgG, anti-brain IgG; ASpS, anti-sperm serum; ATsS, anti-testis serum.

[b] Sera were absorbed with an equal volume of CBA erythrocytes, 0.5 volumes thymocytes, or 0.5 volumes of neutrophils. Anti-brain IgG was absorbed with 0.2 volumes erythrocytes and/or twice with 0.1 volumes thymocytes.

in anti-brain serum. Absorption of anti-brain IgG with thymocytes, or anti-testis serum with thymocytes and neutrophils largely removed anti-thymocyte activity resulting in greatly increased anti-CFU_s activities relative to thymocytes (Table I).

Cross-reaction of brain, testis, and CFU_s antigens. Both brain and testis cells express antigens that cross-react with antigens on the cell surface of CFU_s and thymocytes. The extent to which brain and testis express common antigens that cross-react with CFU_s and thymocytes was determined by absorption. Table II shows that about 21% of the anti-CFU_s activity in anti-brain serum (averaged results from the single and double absorptions) was removed by absorption with testis cells, whereas 67% of the anti-CFU_s activity in anti-testis serum

TABLE II

Cross reaction of brain and testis antigens with CFU_s and thymocytes

Antisera	Absorption[a]	Thymocyte Titer	Dilution of Antiserum for Treatment of Bone Marrow	Average Colonies[b] per Spleen ± S.E.	% Loss Anti-CFU_s Activity
ABrS	RBC	247	64	5.8 ± 1.4	
	RBC, testis	100	64	11.2 ± 3.1	29
	RBS, 2 × testis	ND[c]	64	8.0 ± 0.6	12
	RBC, brain	0	64	21.6 ± 2.0	84
	RBC, 2 × brain	ND[c]	64	24.6 ± 0.3	100
			16	25.6 ± 1.4	105
ATsS	RBC	112	256	6.8 ± 0.9	
	RBC, brain	0	256	17.0 ± 1.9	57
	RBC, 2 × brain	ND[c]	256	20.4 ± 2.6	76
	RBC, testis	0	256	23.6 ± 2.3	94
	RBC, 2 × testis	ND[c]	256	24.4 ± 0.7	100
			64	24.0 ± 1.6	97
NRS	RBC	0	64	26.5 ± 1.6	
			256	22.5 ± 2.5	

[a] Antisera were absorbed once or twice with an equal volume of packed cells or tissue.

[b] 5×10^4 antibody-treated cells were injected into each of five lethally irradiated mice.

[c] ND, not determined.

was removed by absorption with brain. Quantitative absorption studies in which up to four volumes of each absorbing tissue were used have confirmed these values as being on the plateau with respect to removal of anti-stem cell activity. Thus, brain and testis overlap with respect to antigens held in common with CFU_s. Similarly, Table II shows that brain and testis express common antigens that cross-react with thymocytes. Although all anti-thymocyte activity in anti-testis serum was removed by absorption with brain, about 60% of the anti-thymocyte activity in anti-brain serum was removed by absorption with testis cells. With both anti-brain serum and anti-testis serum, anti-CFU_s and anti-thymocyte activity were completely eliminated by absorption with the homologous tissue, indicating that these activities were not nonspecific effects.

Ability of antisera against mouse brain, sperm, and testis cells to rosette bone marrow cells. Although antisera against mouse brain, sperm, and testis cells cross-reacted with bone marrow CFU_s, the usefulness of this anti-stem cell activity for isolating and characterizing stem cells depends on its specificity of CFU_s in the bone marrow. Thus, an indication of the specificity of each antisera for CFU_s was obtained by measuring the fraction of bone marrow cells with bound antibody. Bone marrow cells were coated with antibody, and that fraction of cells capable of forming rosettes with SRBC coated with antiglobulin was measured. The results of several experiments are summarized in Figure 2. At plateau values, 55% of bone marrow cells rosetted when treated with either anti-brain serum or anti-sperm serum. This plateau was reduced to 30% when anti-brain IgG (thymocyte absorbed) was used instead of anti-brain serum. Antiserum against mouse testis cells, however, rosetted only 18% of bone marrow cells, this plateau being reached at a relatively high dilution of antiserum (1:256, cf., about 1:8 for anti-sperm and anti-brain serum). Because anti-testis serum showed the highest titer for CFU_s and yet rosetted the least number of bone marrow cells, this antiserum appeared to have the greatest potential for selecting stem cells.

Ability of anti-brain IgG to rosette CFU$_s$ in bone marrow. Anti-brain IgG rosetted 30% of bone marrow cells. The question of whether CFU$_s$ reside in this rosetted fraction remains unclear. To resolve this question, bone marrow cells treated with anti-brain IgG (thymocyte absorbed) were rosetted with SRBC coated with antiglobulin, and the rosetted and nonrosetted cells were separated on Isopaque-Ficoll. Red cells in the rosette pellet were lysed by hypotonic shock, and the distribution of CFU$_s$ between the interface and pellet was determined in the spleen colony assay. Table III shows that whereas 90 to 98% of CFU$_s$ were recovered at the Isopaque-Ficoll interface when bone marrow cells were treated with normal rabbit IgG or with anti-brain IgG at high dilution (1:2048), only 45% were recovered in the nonrosetted interface region when treated with a higher concentration of anti-brain IgG (1:128). This loss of CFU$_s$ from the Isopaque-Ficoll interface was not associated with decreased specific activity of CFU$_s$ at the interface. On the contrary, CFU$_s$ were enriched 2-fold in the nonrosetted interface fraction after anti-brain IgG treatment. Anti-brain IgG thus appears to have negative selection value for distinguishing between CFU$_s$ and bone marrow cells as a whole.

A comparison of the loss of bone marrow cells, CFU$_s$, and CFU$_c$ from the nonrosette interface after treatment of bone marrow cells with anti-brain IgG is shown in Figure 3. The concentration of anti-brain IgG required to produce a 50% loss of CFU$_s$, CFU$_c$, or total bone marrow cells from the Isopaque-Ficoll interface varies by little more than one serial dilution between the three cell classes, suggesting that there is little quantitative difference between CFU$_s$, CFU$_c$, and total bone marrow cells with respect to antigenic sites recognized by anti-brain IgG. If anything, total bone marrow cells bind more antibody per cell than do CFU$_c$ or CFU$_s$ (see 1:32 and 1:128 dilution of anti-brain IgG).

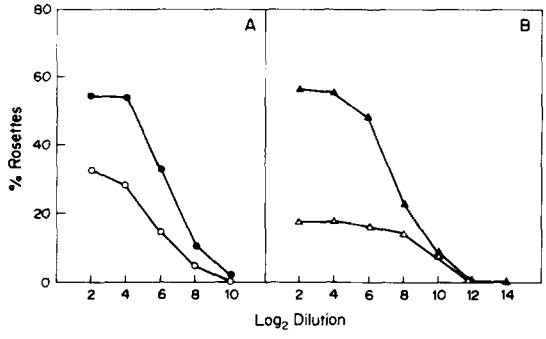

Figure 2. Rosetting of mouse bone marrow cells with antisera against mouse brain, sperm, and testis cells. Bone marrow cells were coated with antisera and rosetted with SRBC coated with antiglobulin. Percent rosettes were determined relative to control bone marrow cells treated with NRS or normal rabbit IgG before rosetting. Each curve is the average of three separate experiments. *A*, Anti-brain serum (●——●), anti-brain IgG absorbed twice with 0.1 volumes thymocytes (○——○); *B*, Anti-sperm serum (▲-----▲), anti-testis serum (△----△).

Overall recovery of CFU$_s$ in the rosette pellet and at the Isopaque-Ficoll interface decreased with increasing antibody concentration. Although 90 to 100% of CFU$_s$ were recovered at anti-brain IgG dilutions of 1:128 or greater, only 45% recovery of CFU$_s$ was observed at a dilution of 1:32. Apparently stem cells in bone marrow treated with high concentrations of antibody are unable to form spleen colonies when recovered from the rosette pellet. At intermediate dilutions of anti-brain IgG (1:128), most (99%) CFU$_s$ were recovered as spleen colonies after Isopaque-Ficoll fractionation even though a 20% loss of spleen colonies was observed by directly injecting the antibody-coated bone marrow cells into lethally irradiated mice (Fig. 1*B*). This high recovery is probably caused by some loss and/or masking of bound antibody on stem cells during the manipulations involved in cell fractionation (i.e., washings and lysing red blood cells). Comparison of the rosetting titration curves obtained by directly counting rosettes (Fig. 2) and the curves obtained by separating rosetted cells on Isopaque-Ficoll (Fig. 3) show the latter technique to be more sensitive with a particular antiserum, probably due to the different criteria used to distinguish rosetted cells. Although rosettes are defined visually by the presence of at least five bound erythrocytes, the criteria in density separation is probably only a single erythrocyte, since

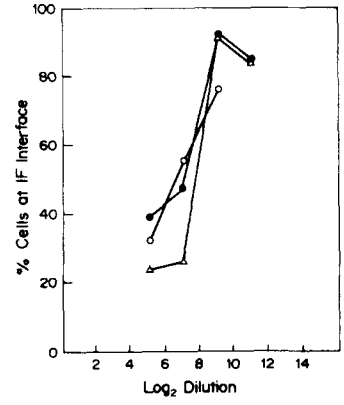

Figure 3. Effect of anti-brain IgG on the recovery of CFU$_s$, and CFU$_c$ from Isopaque-Ficoll interface. Bone marrow cells were treated with anti-brain IgG (erythrocyte and thymocyte absorbed) and rosetted with SRBC coated with antiglobulin. Nonrosetted cells were separated from rosettes and SRBC on Isopaque-Ficoll, washed twice in mSBSS and CFU$_s$, and CFU$_c$ determined as described previously. Cells isolated at the Isopaque-Ficoll interface after treatment of bone marrow with anti-brain IgG are expressed as a percentage of cells at the interface with normal rabbit IgG-treated bone marrow subjected to the same rosetting procedure. CFU$_s$ at interface (●——●), CFU$_c$ at interface (○——○), whole bone marrow cells at interface (△——△).

TABLE III

Rosetting of bone marrow cells and CFU$_s$ with anti-brain IgG

Cell Treatment[a] (3×10^7 Bone Marrow Cells)	IF Interface			IF Pellet		
	Nucleated cells ($\times 10^{-7}$)	Average spleen[b] colonies ± S.E.	Total CFU$_s$ ($\times 10^{-3}$)	Nucleated cells ($\times 10^{-7}$)	Average spleen[b] colonies ± S.E.	Total CFU$_s$ ($\times 10^{-3}$)
NRIgG (1:128)	1.78	34 ± 2.9	12.1	0.90	4 ± 1.7	0.7
ABr IgG (1:128)[c]	0.46	62 ± 3.2[d]	5.7	2.18	16 ± 2.3	7.0
NRIgG (1:2048)	1.86	30 ± 0.8	11.2	0.87	1 ± 0.6	0.2
ABr IgG (1:2048)	1.57	30 ± 2.0	9.4	1.18	4 ± 2.5	0.9

[a] IgG fractions absorbed with 0.2 volumes CBA erythrocytes.
[b] 5×10^4 antibody-treated cells were injected into each of five lethally irradiated mice.
[c] ABrIgG absorbed additionally with 2×0.1 volumes thymocytes.
[d] Estimate only as high density of spleen colonies resulted in some overlap.

erythrocytes were not observed bound to cells at the Isopaque-Ficoll interface.

Antiserum against testis cells but not brain shows selectivity for CFU_s in bone marrow. The fact that antiserum against testis cells contains relatively high anti-stem cell activity (Fig. 1) and yet rosettes fewer bone marrow cells at plateau values than other antisera tested (see Fig. 2) suggests that this antiserum may exhibit selectivity for CFU_s. We therefore examined anti-testis serum for its ability to rosette CFU_s in bone marrow. The results are summarized in Figure 4*B*. Increasing concentrations of anti-testis serum reduced the specific activity of nonrosetted bone marrow cells at the Isopaque-Ficoll interface until at a serum dilution of 1:16, 0.4 CFU_s were recovered per 5×10^4 nucleated cells. This represents a 100-fold depletion of CFU_s from this fraction compared with NRS.

In comparison, analysis of results obtained with anti-brain IgG (Fig. 4*A*) showed that increasing immunoglobulin concentrations caused an increase in the specific activity of CFU_s recovered from the Isopaque-Ficoll interface. Thus, although anti-brain IgG selected against CFU_s in bone marrow, anti-testis serum showed a positive concentration-dependent selection for CFU_s, in that CFU_s were selectively depleted from the bone marrow cell population.

Comparison of sensitivity of CFU_s and CFU_c to anti-brain and anti-testis serum. The results shown in Figure 3 demonstrate that CFU_c in bone marrow treated with anti-brain IgG rosette to about the same titer as CFU_s. Figure 5 compares the activities of anti-brain IgG and anti-testis serum against CFU_s and the granulocyte-macrophage progenitor, CFU_c. Although anti-brain IgG selected against both CFU_s and CFU_c, resulting in increased specific activity of cells at the nonrosette interface, anti-testis serum selected for both CFU_s and CFU_c, resulting in decreased specific activity of nonrosetted cells. Dilution of either anti-brain IgG or anti-testis serum to a concentration that produced background rosetting resulted in the specific activities of CFU_s and CFU_c in the nonrosette fraction, approximating results obtained with NRS.

Thus, we conclude that although anti-brain IgG selects against CFU_s and CFU_c in murine bone marrow, anti-testis serum is selective for CFU_s and CFU_c, resulting in a 5-fold reduction in the specific activity of CFU_s and CFU_c in the nonrosetted cell population at an antiserum dilution of 1:64.

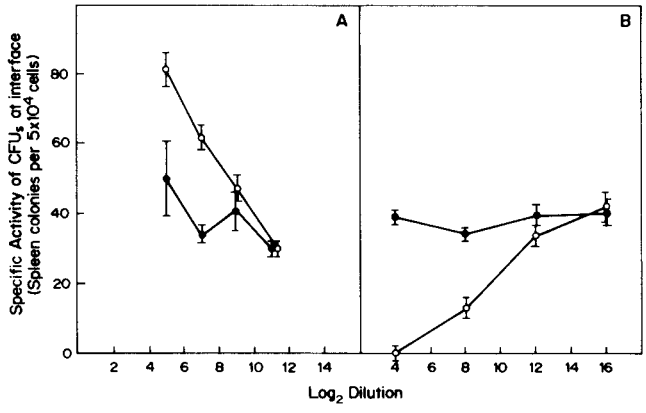

Figure 4. Effect of anti-brain IgG and anti-testis serum on the specific activity of CFU_s recovered from the interface of Isopaque-Ficoll following rosetting and fractionation. Bone marrow cells were coated with antibody, rosetted with SRBC coated with antiglobulin, and the nonrosetted cells separated on Isopaque-Ficoll. Fractionated cells were injected into lethally irradiated mice for determination of spleen colonies. *A*, Bone marrow cells were treated with anti-brain IgG (erythrocyte and thymocyte absorbed) (○——○), or normal rabbit IgG (●——●). Four mice were injected per experimental point; *B*, Bone marrow cells were treated with anti-testis serum ○——○, or NRS (●——●). Five mice were injected per experimental point.

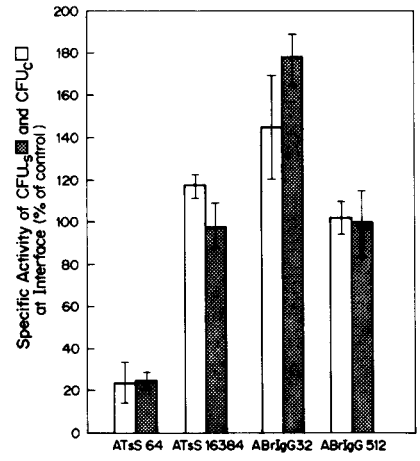

Figure 5. Comparison of the effects of anti-brain IgG and anti-testis serum on the specific activity of CFU_s and CFU_c isolated from Isopaque-Ficoll following rosetting and fractionation. Bone marrow cells were coated with antibody, rosetted with SRBC coated with antiglobulin, and the nonrosetted cells separated on Isopaque-Ficoll. CFU_s and CFU_c were determined as described previously. The results presented are an average between two separate experiments and S.E. were averaged between the two experiments. CFU_c (□); CFU_s (▨).

DISCUSSION

Although the hemopoietic stem cell, CFU_s, is known to be devoid of several of the antigenic markers that characterize differentiated lymphoid cells (18–20), antigens specific for stem cells have not yet been demonstrated. The results presented in this paper suggest that antiserum prepared against mouse testis cells shows selectivity for CFU_s in the bone marrow. In contrast, anti-brain serum, which, is known to cross-react with stem cells in the bone marrow (4), does not selectively bind to stem cells. These conclusions are based on a rosetting procedure that eliminates the need to treat antibody-coated bone marrow cells with C, and thus bypasses the problem of differential sensitivity of cells to C lysis. The procedure involves rosetting antibody-coated bone marrow cells with SRBC coated with antiglobulin, then separating the nonrosetted cells from rosettes and SRBC by centrifugation on Isopaque-Ficoll. Comparison of the titers of anti-brain IgG for CFU_s, either by direct determination of viable CFU_s in the spleen colony assay (Fig. 1*B*) or by rosetting before determination of CFU_s (Fig. 3), showed values to be within one serial dilution, the rosetting procedure being slightly more sensitive. In contrast, by using a C-dependent cytotoxic assay, CFU_c was insensitive to all antisera tested (Table I), results which are in general agreement with those of Van den Engh and Golub (17) and Krogsrud *et al.* (5). Preselection of CFU_c with bound antibody by rosetting, however, gave different results. With anti-brain IgG, CFU_c did not differ markedly from either CFU_s or total bone marrow cells in their sensitivity to anti-brain antibodies (Fig. 3). Thus, CFU_c rosetted to a titer of 100 with anti-brain IgG (cf., titer of 3 by using the cytotoxic assay, Table I). As with CFU_s, the specific activity of CFU_c at the nonrosette interface was greater with bone marrow cells treated with anti-brain IgG than with normal rabbit IgG (Fig. 5), indicating that CFU_c show lower antigenic cross-reactivity with anti-brain IgG than do the majority of bone marrow cells.

In contrast to anti-brain serum, anti-testis serum showed

selectivity for both CFU_s and CFU_c in bone marrow, in that both cell types were preferentially eliminated from the nonrosetted cell fraction (Figs. 4 and 5). Again, although CFU_c were insensitive to anti-testis serum in the cytotoxic assay (Table I), 63% of CFU_c formed rosettes at an antiserum dilution of 1:64 (data not shown). The discrepancy between CFU_c sensitivity to antisera by using cytotoxic and rosetting assays suggest that CFU_c may be relatively insensitive to C lysis or that CFU_c may quickly eliminate bound antibody. Our results regarding the insensitivity of CFU_c with bound antibody to C lysis concur with the observations of Rodt *et al.* (9), who showed that bone marrow cells coated with anti-brain serum showed high titers in a C-fixation test, but were relatively insensitive in a cytotoxic test. Thymocytes, on the other hand, gave similar titers to anti-brain serum in both assays.

Van den Engh and Golub (17), in claiming an antigenic difference between CFU_s and CFU_c, based their interpretation on the results of an *in vivo* transfer experiment in which CFU_s but not CFU_c were eliminated when a known number of stem or progenitor cells coated with antibody were passaged through lethally irradiated mice. In these experiments it is possible that CFU_c, being metabolically more active than CFU_s (21), rapidly turn over membrane-bound antibody and become refractory to *in vivo* elimination processes. We have not observed differences in the antigenic cross-reactivity of CFU_s and CFU_c to anti-brain and anti-testis sera (Fig. 5), and our general conclusion would be one of the close antigenic similarity between CFU_s and CFU_c with respect to anti-brain and anti-testis sera. Furthermore, our results are in direct conflict with those of Filppi *et al.* (8), who contend that an $F(ab)_2$ fraction of rabbit anti-mouse brain serum shows specificity for the murine pluripotential stem cell. The main argument supporting their claim is the close correspondence between the number of fluorescing cells in fractionated bone marrow cell preparations treated with anti-brain $F(ab)_2$ and the number of stem cells calculated from the number of spleen colonies. However, at their best purification only 2.6% of the fractionated cells were stem cells, and correlation of stem cell enrichment with increased numbers of fluorescent cells does not prove identity.

A comparison of the results presented here with those of Krogsrud and Price (22) show some interesting differences. Analysis of mouse bone marrow cells by fluorescence-activated cell sorting showed both rabbit anti-human brain serum and human anti-human sperm serum to enrich for CFU_s 4 to 14.5-fold. Thus, antiserum against human brain appears to be selective for mouse stem cells, whereas anti-mouse brain serum does not. It is possible, however, that the different tissue absorptions used in preparing these antisera may account for the differences observed.

Although we have used rosetting techniques to separate bone marrow cells on the basis of surface concentration of bound antibody, we have routinely experienced difficulty in recovering functional stem cells from rosette pellets, a problem that increases with the concentration of antibody used to coat the bone marrow cells. Several factors may contribute to these problems. The use of hypotonic shock to lyse red cells in the rosette pellet may damage CFU_s, or alternatively, CFU_s with bound antibody coupled to red cell membranes may be functionally incapacitated. In this context, CFU_s consist of cells largely in G_0 phase (21) and may be slow to turn over membrane components, resulting in an inability of "tagged" stem cells to seed on the spleen in the *in vivo* assay. Thus, although Parish (3) was able to recover functionally normal B lymphocytes assayed both *in vivo* and *in vitro* from rosette pellets, the situation with bone marrow stem cells appears to be different. Failure to recover functional CFU_s in the rosette pellet does not appear to be a major problem where antibody is used at concentrations suboptimal for rosette formation (Table III). However, at higher concentrations of antibody where few CFU_s are found in the nonrosetted fraction, total CFU_s recovery declined markedly, due to an inability to demonstrate viable CFU_s in the rosette pellet.

Fractionation of rosetted bone marrow cells on Isopaque-Ficoll results in some carry-over of nonrosetted cells from the interface into the rosette pellet. Even with control serum, between 25 and 40% of bone marrow cells fractionate in the red cell pellet. Although this "carry-over" is not a major problem in demonstrating specificity of antisera for CFU_s, since with control sera 92 to 98% of CFU_s are recovered at the Isopaque-Ficoll interface (Table III), it presents a severe limitation on the use of rosetting procedures to isolate stem cells. The fact that CFU_s comprise less than 0.5% of bone marrow cells (23) suggests that even with considerable improvement the rosetting procedure is unlikely to be an efficient method of isolating stem cells. Once antisera specific for stem cells have been demonstrated, other more efficient methods of selecting cells with bound antibody can be used, e.g., cellular affinity chromatography by using a digestible collagen bridge (24), cell separation by using a fluorescence-activated cell sorter (2), or immunoadsorbant plates (25).

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