

A NEW CLASS OF CELL SURFACE ANTIGENS
Quantitative Absorption Studies Defining
Cell-Lineage-specific Antigens on Hemopoietic Cells*

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The mechanisms of commitment and differentiation of hemopoietic stem cells are at present not known. Some evidence suggests that erythropoietin can act directly at the level of the stem cell (1) resulting in commitment to differentiation but proof that any hormone or stimulatory factor acts specifically and directly on the pluripotent stem cell is lacking. Recently Till (2) proposed a model in which stem cells are characterized by a multiplicity of surface markers and suggested that particular markers may be restricted to a single pathway of differentiation. Furthermore, as a means of reconciling various models for the induction of stem cell differentiation, it was suggested that pluripotent stem cells express low concentrations of cell surface markers which increase in numbers as differentiation proceeds, a process which parallels surface marker restriction to a particular pathway of differentiation.

We have tested this model with respect to cell surface markers held in common between mature hemopoietic cells and the stem cells from which they were derived. Platelets, thymocytes, and macrophages each express antigens in common with the pluripotent stem cell (colony-forming unit), whereas erythrocytes appear to be devoid of such antigens (3). In the present paper we have characterized the anti-stem cell activity in antisera against CBA mouse platelets, thymocytes, and macrophages using quantitative absorption and show that this activity is restricted to a particular hemopoietic cell type (cell-lineage-specific), is tissue specific and species specific but common to both the inbred and randomly bred mouse strains tested.

Materials and Methods

Animals. CBA T6/T6 mice (2-3 mo) were bred at Victoria University and used in the preparation of antisera and for erythrocyte absorptions. C57Bl/6 mice were bred at Victoria University whereas BALB/c and NHI/A mice were bred at the National Health Institute, Wellington, New Zealand. CF₁ mice were from SPF stock bred at Wallaceville Animal Research Centre, Upper Hutt, New Zealand. CBA mice were used as donors and CBA × C57Bl mice (2-3 mo) were used as recipients in the spleen colony assay. New Zealand white rabbits were used for raising antisera and for relevant absorptions. Randomly bred albino rats of Wistar and Sprague Dawley origin were used to prepare cells for absorption, whereas human thymocytes were obtained from a 4-mo-old male child who died of asphyxia 18 h before cell preparation.

Preparation of Antisera. Antisera against CBA mouse platelets, thymocytes, and macrophages were prepared according to Gozzo et al. (4) by injecting rabbits with $2-3 \times 10^7$ nucleated cells

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or $1-2 \times 10^9$ platelets homogenized with complete Freund's adjuvant at multiple subcutaneous and intramuscular sites. 4-5 wk later, the animals were given intravenous booster injections on three consecutive days with a similar number of cells. 1 wk later, the rabbits were bled and the antisera heated at 56°C for 30 min and stored at -17°C . Before the assay or further absorptions, all antisera were absorbed with an equal volume of CBA erythrocytes at 4°C for 1 h. Erythrocyte absorption was routinely used with all antisera because it has previously been shown that antisera against CBA mouse erythrocytes when appropriately absorbed with other hemopoietic cells does not cross-react with the pluripotent stem cell (3).

Cell Preparation and Absorption. Mouse iso-osmotic-buffered balanced salt solution (mSBSS, 5)¹ was used throughout. Hemopoietic cells were prepared as described previously (3). Macrophages used for absorption were obtained from peritoneal washes of G75 Sephadex (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.)-stimulated mice or rats and contained 80-90% monocyte-macrophage cells as determined by staining with methylene blue/acetic acid, latex bead uptake, and carbonyl iron uptake. For the preparation of antisera, macrophages were further purified by adherence to plastic Petri dishes (3).

Absorptions were carried out on neat antisera at 4°C for 1 h with packed volumes of tissue or cells that had been washed three times in mSBSS. Calculations of the cell surface area involved in various absorptions were based on the observation that 1 ml packed cell volume contained 7.3×10^8 macrophages, 3×10^9 thymocytes, 10^{10} erythrocytes, and 10^{11} platelets. Assuming macrophages and thymocytes to have average Diam of $15 \mu\text{m}$ and $8 \mu\text{m}$, respectively (6), erythrocytes to have a surface area of $140 \mu\text{m}^2$ (7) and platelets $14 \mu\text{m}^2$ (8), the total cell surface area of 1 ml packed cell volume of macrophages is $5.2 \times 10^{11} \mu\text{m}^2$, thymocytes $6.0 \times 10^{11} \mu\text{m}^2$, erythrocytes $14 \times 10^{11} \mu\text{m}^2$, and platelets $14 \times 10^{11} \mu\text{m}^2$. Thus, the differences in surface area of the cells used for absorption would have affected the comparative quantitative absorption results in Fig. 3 by little more than one serial dilution. Differences in cell size between mouse, rat, rabbit, and man would also have a small effect on quantitative absorption results but comparative figures for these calculations are not readily available.

Spleen Colony Inhibition Assay. The method of Till and McCulloch (9) was used to measure viable pluripotent stem cells after treatment of bone marrow cells with antiserum. Bone marrow cells ($2.5-3.0 \times 10^7/\text{ml}$, 0.075 ml) purified on Isopaque-Ficoll ($\rho = 1.09$; [3]; Isopaque [sodium metrizamide], Wyegaard & Co., Oslo, Norway; Ficoll, Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) were incubated for at least 30 min at 4°C with 0.075 ml of antiserum diluted in mSBSS. Immediately before injection (within 3 h of mixing cells and antiserum) the antibody-treated cells were diluted 120-fold and 5×10^4 nucleated cells in 0.4 ml mSBSS injected into each of five mice that had been lethally irradiated (900 rad, 300 rad/min) within 6 h of injection. Spleen colonies were counted after 8 d. The serum titer is defined as the dilution of antiserum (per unit volume) that gives a 50% inhibition of spleen colony formation and was obtained from the cytotoxic curve. All serum dilutions represent the final dilution of antiserum in the presence of cells.

Results

Anti-Stem Cell Activity in Antisera against CBA Mouse, Platelets, Thymocytes, and Macrophages. Antisera raised in rabbits against CBA mouse platelets, thymocytes and macrophages and absorbed with erythrocytes cross reacted with hemopoietic stem cells obtained from the bone marrow of CBA mice. Fig. 1 shows the titration curves of each antiserum against stem cells as measured by inhibition of spleen colony formation in lethally irradiated mice. The titers of each antiserum were: anti-platelet serum 7131, anti-thymocyte serum 2574, and anti-macrophage serum 2272. With increasing concentration each antiserum killed between 92 and 98% of stem cells.

Tissue Specificity of Anti-Stem Cell Activity in Antisera. Neat antisera that had been absorbed with an equal volume of CBA erythrocytes were used for further absorptions

¹ Abbreviation used in this paper: mSBSS, mouse iso-osmotic-buffered balanced salt solution.

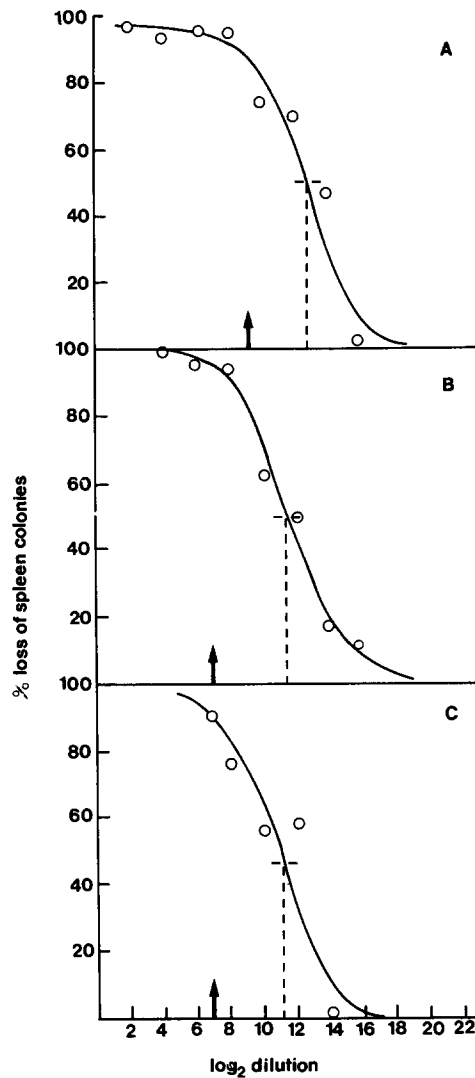


FIG. 1. Anti-stem cell activity in antisera against mouse platelets, thymocytes, and macrophages. Bone marrow cells were treated *in vitro* with antiserum before injection into lethally irradiated mice. A. anti-platelet serum, B. anti-thymocyte serum, C. anti-macrophage serum. The arrow shows the concentration at which each antiserum was used in subsequent experiments. This varied between 12 and 16 times the 50% titer of the antiserum and routinely gave >85% loss of spleen colonies with erythrocyte-absorbed antisera.

and absorbed antisera were tested for anti-stem cell activity at ~16 times their titer for stem cells (see arrow in Fig. 1). Absorption of each antiserum with tissues other than those used to prepare the antiserum, for example, brain, kidney, liver, and testis, had little effect on anti-stem cell activity (Fig. 2). With four volumes of absorbing liver or kidney tissue ~25% of the anti-stem cell activity was removed from anti-platelet serum. Similarly liver absorption removed 18% of the anti-stem cell activity from anti-thymocyte serum and ~9% of the anti-stem cell activity in anti-macrophage serum.

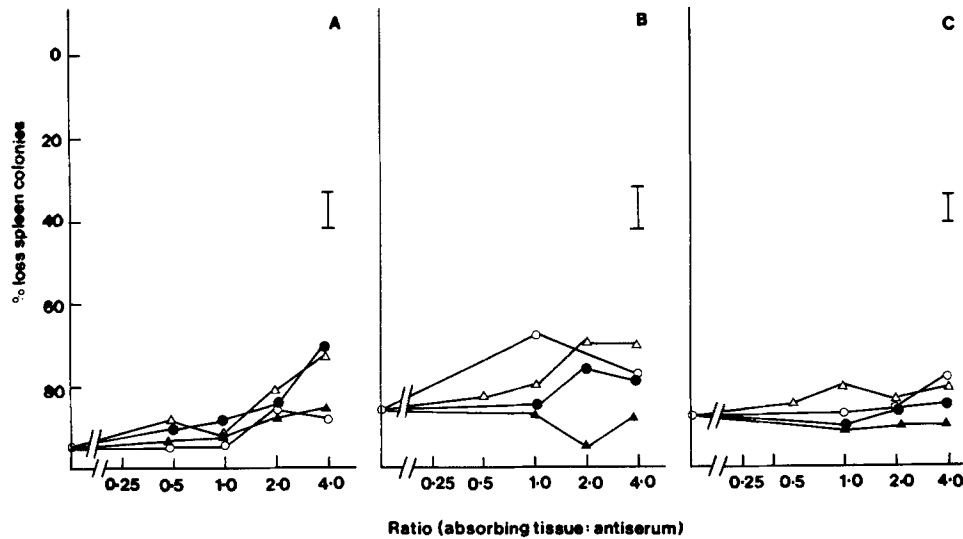


FIG. 2. Anti-stem cell activity in antisera absorbed with CBA mouse tissues. Bone marrow cells were treated with antisera that had been absorbed with brain (○), kidney (●), liver (△), or testis (▲) from CBA mice, before injection into lethally irradiated mice. A. anti-platelet serum, B. anti-thymocyte serum, C. anti-macrophage serum. The average standard error is shown to the right of each graph. In the absence of antiserum the average spleen colonies were 22.2 ± 1.5 .

With each absorbing tissue there was a minimum ninefold difference in absorbing capacity between the particular tissue and the homologous cell type used to prepare the antiserum.

Cell-Lineage-specificity of Anti-Stem Cell Activity in Antisera. Quantitative absorption was used to investigate the degree to which antigens held in common between differentiated hemopoietic cells and stem cells are specific for a particular cell lineage. If antigens that cross react with stem cells are present in significant quantities on more than one differentiated hemopoietic cell type, then this activity would be demonstrated as a loss of anti-stem cell activity after absorption with the cross-reacting cell type. Fig. 3 shows the results of quantitative absorptions of each antiserum with various hemopoietic cell types. Neither thymocytes nor macrophages removed a significant proportion of the anti-stem cell activity in anti-platelet serum whereas absorption with 1 volume of homologous platelets completely removed the anti-stem cell activity (Fig. 3A). Absorption of anti-thymocyte serum with peritoneal macrophages (Fig. 3B) removed 65% of the anti-stem cell activity at a 4-volume absorption. A plateau was not obtained with this absorption. Absorption of anti-thymocyte serum with platelets resulted in a 33% loss of anti-stem cell activity at plateau values, whereas absorption with homologous thymocytes completely removed anti-stem cell activity.

With anti-macrophage serum (Fig. 3C) thymocyte absorption did not reduce anti-stem cell activity whereas platelets removed ~25% of the anti-stem cell activity, a figure which appeared to reach a plateau at 1 volume of absorbing platelets. Again, homologous macrophage absorption removed all of the anti-stem cell activity from anti-macrophage serum.

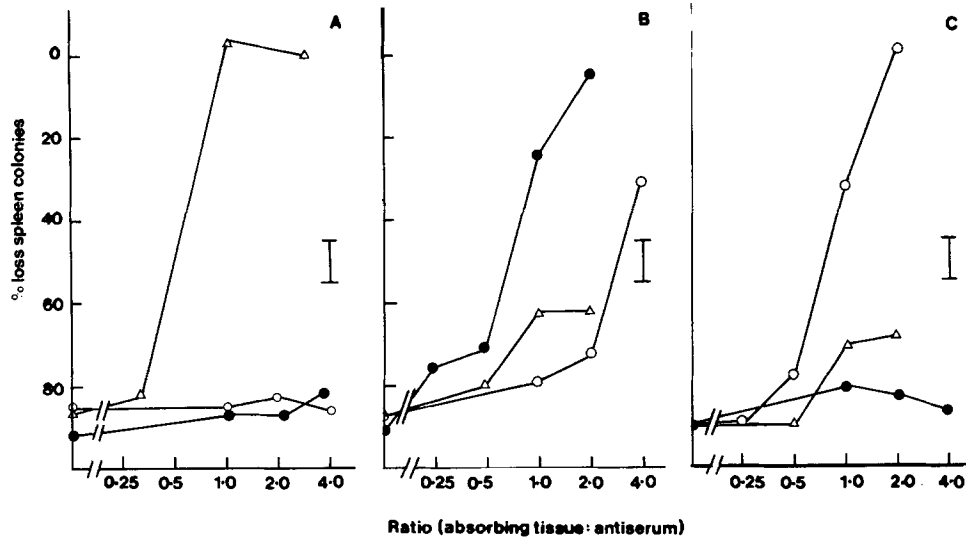


FIG. 3. Anti-stem cell activity in antisera absorbed with CBA mouse platelets, thymocytes, and macrophages. Bone marrow cells were treated with antisera that had been absorbed with platelets (Δ), thymocytes (\bullet), or macrophages (\circ) from CBA mice, before injection into lethally irradiated mice. A. anti-platelet serum, B. anti-thymocyte serum, C. anti-macrophage serum. The average standard error is shown to the right of each graph. In the absence of antiserum the average spleen colonies were 22.8 ± 1.2 .

The small (25–33%) loss of anti-stem cell activity in anti-thymocyte and anti-macrophage serum after absorption with platelets was not associated with a reciprocal loss of anti-stem cell activity after absorption of anti-platelet serum with thymocytes and macrophages.

Mouse Strain Specificity of Anti-Stem Cell Activity in Antisera. The presence of common cell-lineage-specific antigens on the hemopoietic cells of different mouse strains was investigated by quantitative absorption of each antiserum with homologous hemopoietic cells from various mouse strains. Of five mouse strains tested (i.e., CBA, C57Bl, BALB/c, NH1/A, and CF₁) hemopoietic cells from each were able to completely remove the anti-stem cell activity in antisera against CBA mouse platelets or thymocytes (Figs. 4A and B). With anti-macrophage serum, absorption with macrophages from mouse strains other than CBA resulted in 61–72% loss of anti-stem cell activity at plateau values (Fig. 4C).

Although quantitative differences in absorbing capacity were observed between the different mouse strains, these differences did not vary by more than 2.5-fold for a particular antiserum.

The results show cross reacting cell-lineage-specific antigens to be present on both the inbred (H2^k, H2^b, and H2^d) and outbred strains tested.

Species Specificity of Anti-Stem Cell Activity in Antisera. The degree to which cell-lineage-specific antigens on mouse hemopoietic cells cross-react with hemopoietic cells from other species such as rat, rabbit, and human is shown in Fig. 5. No loss of anti-stem cell activity was observed after absorption of antiserum against CBA mouse platelets with platelets from other species. Thus, a minimum 16-fold difference in absorbing capacity is evident between CBA mouse platelets and platelets from the

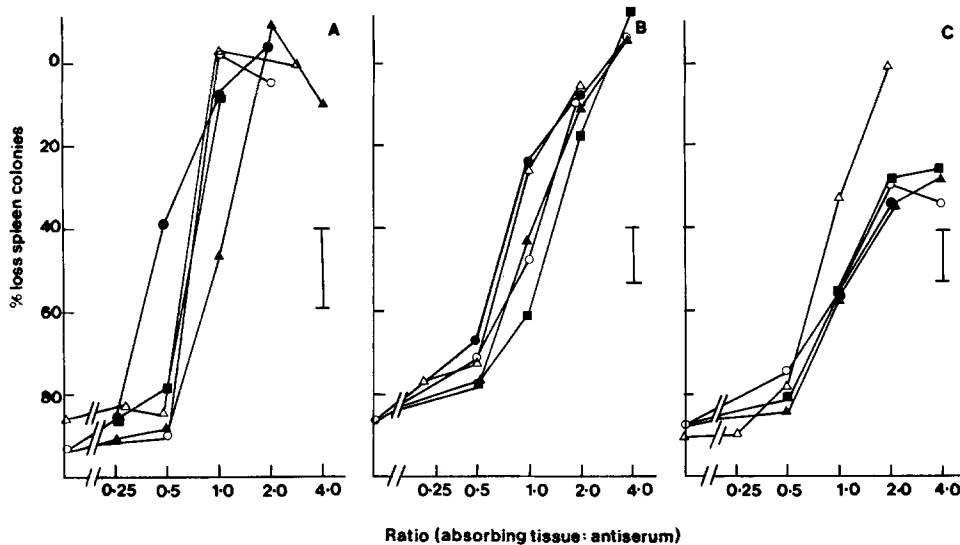


FIG. 4. Anti-stem cell activity in antisera absorbed with homologous hemopoietic cells from various mouse strains. Bone marrow cells were treated with antisera that had been absorbed with the appropriate hemopoietic cells against which each antiserum had been raised, before injection into lethally irradiated mice. Absorbing cells were obtained from CBA/T6T6 (Δ), C57Bl/6 (\circ), BALB/c (\blacksquare), NH1/A (\blacktriangle), or CF₁ (\bullet) mice. A. anti-platelet serum absorbed with platelets, B. anti-thymocyte serum absorbed with thymocytes, C. anti-macrophage serum absorbed with macrophages. The average standard error is shown to the right of each graph. In the absence of antiserum the average spleen colonies were 22.3 ± 1.1 .

other species tested. With anti-thymocyte serum, both rat and rabbit thymocytes produced a small loss of anti-stem cell activity (33 and 21%, respectively) with 4 vol of absorbing cells, whereas no reduction in anti-stem cell activity was observed after absorption with human thymocytes. Rat macrophages resulted in some (39%) loss of anti-stem cell activity in anti-macrophage serum. Difficulties in obtaining sufficient quantities of rabbit and human macrophages have limited our analysis of anti-macrophage serum.

Discussion

The antigenic analysis of hemopoietic cells, in particular of lymphocytes, has centered around investigation of the acquisition of cell surface determinants that are not demonstrably present at earlier stages of differentiation (10, 11). Collectively, these antigens which arise in the mature animal as a result of differentiation processes have been termed differentiation antigens. Although knowledge of these antigens has proven to be of considerable value in functionally defining subpopulations of differentiated cells, the role of most of these antigens has yet to be elucidated. In contrast to differentiation antigens, we present evidence for a group of cell surface antigens, termed cell-lineage-specific antigens, that appear to be selectively conserved during differentiation. That this class of antigens is fundamentally distinct from differentiation antigens is indicated by the absence of antigens such as Ia (12, 13), FcR (14), Thy-1 and Ig (M. V. Berridge, unpublished observations) from hemopoietic stem cells. Furthermore, cell-lineage-specific antigens appear to be distinct from histocom-

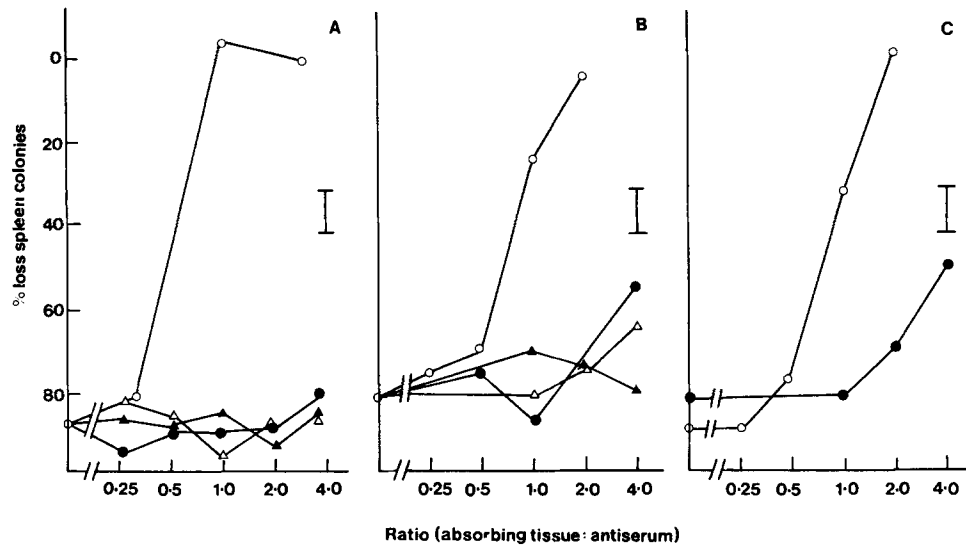


FIG. 5. Anti-stem cell activity in antisera absorbed with homologous hemopoietic cells from various species. Bone marrow cells were treated with antisera that had been absorbed with the appropriate hemopoietic cells against which each antiserum had been raised before injection into lethally irradiated mice. Absorbing cells were obtained from CBA mouse (O), rat (●), rabbit (Δ), and human (▲). A. anti-platelet serum absorbed with platelets, B. anti-thymocyte serum absorbed with thymocytes, C. anti-macrophage serum absorbed with macrophages. The average standard error is shown at the right of each graph. In the absence of antiserum the average spleen colonies were 18.2 ± 1.9 .

patibility antigens which are thought to be present on most cells, in that they are largely restricted to a certain pathway of differentiation (Fig. 3). For example, the anti-stem cell activity in anti-platelet serum was not removed by absorption with either thymocytes or macrophages. The small (25–33%) loss of anti-stem cell activity in anti-thymocyte and anti-macrophage serum after absorption with platelets may be a true cross reaction because the loss of anti-stem cell activity reached a plateau value at 1 volume of absorbing cells, a result that would not have been expected if white cell contamination of absorbing platelets were the responsible factor. In contrast, macrophage absorption of anti-thymocyte serum resulted in increasing losses of anti-stem cell activity. This pattern of absorption is consistent with thymocyte contamination of absorbing macrophages. Quantitation of the difference in absorbing capacity between macrophages and homologous thymocytes for the anti-stem cell activity in anti-thymocyte serum showed a 4.2-fold difference, a result which closely corresponds to the fraction of small lymphocytes observed in cell preparations from G75 stimulated peritoneal washes (Materials and Methods). Conversely, thymocyte absorption of anti-macrophage serum did not result in significant loss of anti-stem cell activity. The fact that homologous absorptions removed all anti-stem cell activity at between 1 and 2 volumes of absorbing cells suggests that the anti-stem cell activities observed in the various antisera were not a result of minor cell populations in the inoculating cell type. Furthermore, the evidence indicates that most (92–97%) of the stem cells in bone marrow express antigens that cross-react with each of the cell-lineage-specific antisera tested (see also Fig. 1).

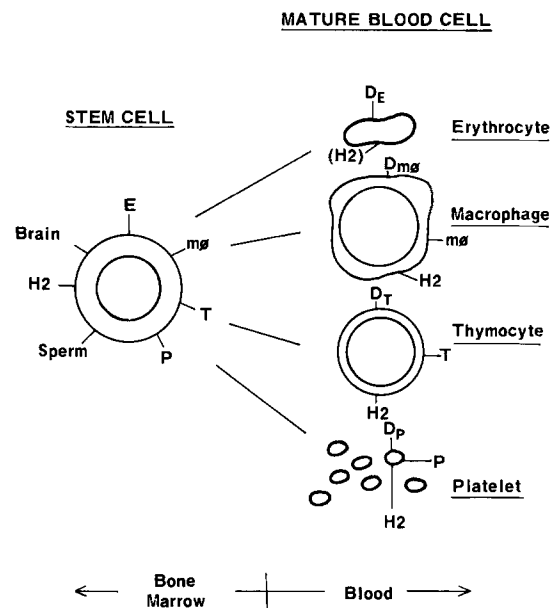


FIG. 6. Model of the major classes of cell surface antigens on hemopoietic cells. H2 represents histocompatibility antigens expressed on all hemopoietic cells; D_E , $D_{m\phi}$, D_T , and D_P represent differentiation antigens expressed on differentiating and mature blood cells; E, $m\phi$, T, and P represent cell-lineage-specific antigens expressed on hemopoietic stem cells and on respective differentiating cell lines. Such antigens are apparently lost after enucleation and terminal differentiation of the erythrocyte. Brain and sperm represent antigens present on these nonhemopoietic tissues that cross-react with the hemopoietic stem cell (15, 16).

A model showing the presence of cell-lineage-specific antigens on hemopoietic cells is shown in Fig. 6. Cell-lineage-specific antigens are shown to be distinct from differentiation antigens (present only on differentiating cells) and from histocompatibility antigens (present on all hemopoietic cells). The stage of differentiation at which antigens held in common between stem cells and differentiated cells become restricted to a particular pathway of differentiation is not known but cell membrane turnover and antigen dilution through membrane synthesis required for cell division may quickly result in the loss of a group of antigens at an early stage of commitment and differentiation. In terms of the model proposed by Till (2) we have not been able to demonstrate antigens on erythrocytes (3) or reticulocytes (M. V. Berridge, unpublished observations) that cross-react with stem cells. In addition, spleen cells from phenylhydrazine-treated mice which contained >95% nucleated erythrocyte precursors exhibited only weak antigenic cross reactivity with stem cells (titer 64). Even so, our results indicate that there is greater anti-stem cell activity in antisera against nucleated erythrocytes precursors than in antisera against the more mature enucleated erythrocytes and reticulocytes, and justify the inclusion of an erythroid cell-lineage antigen on the pluripotent stem cell in the model presented in Fig. 6.

The extent to which cell-lineage-specific antigens are quantitatively conserved during differentiation remains unclear. Because stem cells comprise only a small fraction of bone marrow cells and cannot be morphologically identified, it is not

possible to determine the number of antibody molecules bound to stem cells. Furthermore, there is no obvious way of determining the number of antigens on differentiated cells that cross-react with stem cells. Thus, we have not been able to show whether antigens characteristic of a particular hemopoietic cell lineage increase their cell surface concentration as differentiation proceeds as suggested by Till (2).

In addition to showing cell-lineage-specificity, antisera against CBA mouse platelets, thymocytes and macrophages were also shown to be tissue specific and species specific, although rat thymocytes and macrophages showed some ability to absorb the anti-stem cell activity in their respective antisera against CBA mouse cells (Fig. 5). In contrast, homologous hemopoietic cells from all mouse strains tested readily absorbed all of the anti-stem cell activity in anti-platelet serum and anti-thymocyte serum and most (61–72%) of the anti-stem cell activity and anti-macrophage serum. The reason why macrophages from mouse strains other than CBA absorbed <100% of the anti-stem cell activity in antiserum against CBA macrophages is not known, but the results suggest a small group of cell-lineage-specific antigens on CBA mouse macrophages that are not present in significant amounts in other mouse strains. Therefore, results are in general agreement with previously reported experiments (3) where bone marrow stem cells from C57Bl and BALB/c mice were shown to be sensitive to antisera against CBA mouse platelets, thymocytes and macrophages and confirm that cell-lineage-specific antigens are common to a variety of mouse strains.

The possibility that cell-lineage-specific antigens may be involved in commitment of the pluripotent stem cell to a particular pathway of differentiation and that the presence of these antigens may be required for continued differentiation is an attractive hypothesis that is presently under investigation.

Summary

A new class of cell surface antigens are described which are expressed on cells within a particular differentiation pathway. These antigens, termed cell-lineage-specific antigens, are shown to be distinct from differentiation antigens and from histocompatibility antigens. The presence of these antigens was demonstrated by raising antisera against terminally differentiated hemopoietic cells such as platelets, thymocytes, and macrophages and showing cross-reaction with the pluripotent stem cells from which these cells were derived. Quantitative absorption studies of each antiserum showed the antigens to be largely cell-lineage-specific. For example, the anti-stem cell activity in anti-platelet serum was not absorbed out with thymocytes or macrophages from the same mouse strain but was removed by absorption with platelets. Absorption of each antiserum with nonhemopoietic mouse tissues such as brain, kidney, liver, and testis did not reduce anti-stem cell activity. Thus, each antiserum was shown to be tissue specific and species specific. Hemopoietic cells from mouse strains other than CBA absorbed out most of the anti-stem cell activity indicating cell-lineage-specific antigens to be common to the mouse strains tested.

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