CONCISE REPORT

Erythroid Colony Stimulating and Inhibiting Cells in Peripheral Blood of Transfused Dogs: Separation of Function by Velocity Sedimentation

By Beverly Torok-Storb, H. Joachim Deeg, Kerry Atkinson, Paul L. Weiden, John W. Adamson, and Rainer Storb

We have previously shown that the addition of normal dog peripheral blood lymphocytes (PBL) to cultures of allogeneic marrow increases the number of marrow-derived erythroid colonies (EC), but that PBL from transfused dogs usually inhibit EC growth from marrow of the transfusion donor. In this study, the cells in normal dog PBL responsible for stimulating EC growth were shown to sediment in a narrow peak at 4.30 mm/hr. A similar population of stimulating cells exists in transfused dogs and can be separated, on the basis of size, from cells that inhibit EC growth. EC-stimulating cells from transfused dog PBL sediment at 3.3–5.0 mm/hr, while cells responsible for inhibition are larger and sediment more rapidly at 5.4–8.1 mm/hr. These data demonstrate that cells capable of stimulating allogeneic EC are present in transfused dogs, but their stimulating ability is masked by the presence of EC-inhibiting cells. Thus, coculture experiments designed to test lymphocyte/marrow cell interactions may miss significant but opposing effects if unfractionated cells are used.

PERIPHERAL BLOOD LYMPHOCYTES (PBL) or thoracic duct lymphocytes (TDL) from normal dogs added to cultures of either autologous or allogeneic marrow significantly increase the number of erythroid colonies (EC) over that obtained from marrow alone. A possible in vivo correlate for these in vitro studies is provided by previous work showing that allogeneic marrow engraftment in lethally irradiated dogs was enhanced by the addition of either PBL or TDL to the marrow inoculum.3,4

In contrast to the stimulating ability of normal dog PBL, we found that PBL from transfusion-sensitized dogs either failed to stimulate or actually inhibited erythroid colony growth from marrow of the DLA-identical transfusion donor.1 This was also in keeping with earlier in vivo observations that showed that dogs given blood transfusions frequently rejected subsequent marrow grafts from their transfusion donor.5,6 This in vitro inhibition of donor EC by sensitized recipient lymphocytes was shown to predict marrow graft rejection in vivo.7

Transfusion-sensitized PBL specifically inhibited EC from the allogeneic transfusion donor while maintaining their ability to stimulate autologous EC. From this
observation, we hypothesize that a single lymphoid cell population stimulates autologous or allogeneic EC growth, and that this population is present in both normal and transfused dogs. In transfused dogs, however, alloimmunization generates a second lymphoid population that inhibits allogeneic EC growth and masks the coexistence of the EC-stimulating fraction. In this study we confirm the validity of this hypothesis and demonstrate that both functional subsets of cells do exist and can be separated on the basis of size.

**MATERIALS AND METHODS**

To potentiate antigenic differences, donor and recipient dogs were unrelated and DLA-nonidentical. Recipients were sensitized by intravenous transfusions of 50 ml of heparinized whole blood given 24, 17, and 10 days before testing. For repeated testing of the same recipient, an additional transfusion was given 10 days before each subsequent test.

Bone marrow cells (BMC) were aspirated from the humerus head into a 10-ml syringe containing 4 ml TC-199 tissue culture medium and 400 U of preservative-free heparin. Buffy-coat cells were separated by centrifugation at 400 g for 10 min, washed 3 times, and suspended in supplemented alpha medium (Flow Labs, Rockville, Md.). PBL were obtained by diluting heparinized whole blood 1:1 in Hanks balanced salt solution and layering over Ficoll-Hyphaque (Lymphoprep, Nyegard Co., Oslo, Norway). After centrifugation at 400 g for 20 min, the cells retained at the interface were collected and washed 3 times. PBL were then separated on the basis of size by unit gravity velocity sedimentation through a linear gradient of fetal calf serum.

BMC were cultured at a concentration of 2 x 10^6 cells/0.1 ml plasma clot using the technique described by McLeod et al. as modified by Brown and Adamson. PBL were added to cultures at a concentration of 5 x 10^4/clot. Cells were incubated for 72 hr in the presence of 1.0 U of erythropoietin (Step III, sheep plasma, Connaught Lab., Willowdale, Canada) per milliliter culture. Clots were harvested, fixed on glass slides, and stained with benzidine. Aggregates of 8 or more hemoglobinized cells were counted as EC.

**RESULTS**

Dog peripheral blood cells, separated on Ficoll-Hyphaque (density 1.077), are contaminated by polymorphonuclear cells (PMN) that can be removed by velocity sedimentation. As shown in Fig. 1, greater than 90% of the PMN sediment faster than 8.08 mm/hr and can be easily separated from the majority of mononuclear cells. Likewise, contaminating red blood cells can be easily eliminated. Mononuclear cells sediment in a broad band from 2.46 to 9.16 mm/hr. In 4 experiments, stained cytocentrifuge preparations of various fractions of cells have shown that cells sedimenting between 2.46 and 6.0 mm/hr were greater than 80% lympho-
cytes, whereas cells sedimenting between 6.46 and 8.08 mm/hr were greater than 80% monocytes.

Various fractions of PBL from two normal dogs were cultured with bone marrow cells from their prospective transfusion donor. Each dog was tested on two separate occasions prior to transfusion. As shown in Fig. 2, in all four experiments, unfractionated PBL significantly increased EC growth. Normal dog cells capable of stimulating allogeneic EC growth sedimented in a narrow peak between 3.22 and 4.30 mm/hr. The faster sedimenting PBL had no significant effect on EC growth.

After the recipient dogs were transfused, their unfractionated PBL significantly inhibited allogeneic EC growth. However, after separating PBL on the basis of size, cells responsible for inhibiting EC were found only in more rapidly sedimenting fractions. Cells capable of stimulating donor EC growth were detected sedimenting more slowly.

DISCUSSION

It appears that lymphocytes, presumably T cells, play a role in stimulating erythropoiesis. Normal lymphocytes with T-cell characteristics can stimulate growth of human erythroid colonies in vitro and promote erythropoiesis in vivo in mice. Likewise normal dog PBL and TDL have been shown to increase EC growth and enhance marrow engraftment in transplanted dogs. However, after transfusion–induced sensitization, the ability of dog PBL to stimulate allogeneic EC is lost, resulting in most cases in an inhibition of EC growth. In this report we demonstrate that stimulating cells are still present in sensitized dog PBL, but their stimulating effect is masked by the presence of an inhibiting population. Lymphocytes that inhibit allogeneic EC growth are larger and can be separated by unit gravity sedimentation, on the basis of size, from cells that stimulate erythropoiesis.

These data suggest that coculture studies between marrow cells and unfractionated PBL may fail to detect significant but opposing functions. However, it is possible to at least partially separate cells that stimulate erythropoiesis from those that inhibit it.
ACKNOWLEDGMENT

The authors would like to thank Cathy Wise, Nancy Lin, and Ted Graham for their help with these studies and Joni Whitefield for preparing the manuscript.

REFERENCES

Erythroid colony stimulating and inhibiting cells in peripheral blood of transfused dogs: separation of function by velocity sedimentation

B Torok-Storb, HJ Deeg, K Atkinson, PL Weiden, JW Adamson and R Storb