Canine Hemophilia and Hemopoietic Grafting

By Rainer Storb, Thomas L. Marchioro, Theodore C. Graham, Mary Willemin, Cecil Hougie, and E. Donnall Thomas

Two hemophilic beagles were given 1200 R whole-body irradiation followed by a successful marrow graft from a normal sibling. During observation periods of 7 and 24 mo, there was no evidence of factor VIII synthesis.

ORTHOTOPIC TRANSPLANTATION of a normal liver into a hemophilic dog results in complete correction of the deficiency of factor VIII.1-3 However, there are significant extrahepatic sources of factor VIII, since hepatectomized normal dogs bearing a transplanted liver from a hemophilic animal show factor VIII levels equivalent to that seen in the heterozygous state.2,3 Transplantation and perfusion experiments have suggested that the spleen may be a source of factor VIII production in animals.4-8 However, it has now been clearly shown that canine hemophilia is not corrected by orthotopic transplantation of a normal spleen.1-3,9 The consensus seems to be that the spleen may serve as a storage reservoir for factor VIII, thus accounting for the transient elevations of factor VIII following transplantation of a normal spleen and for the factor VIII released from the spleen in perfusion experiments.2,3,9 The kidney does not appear to be a significant source of factor VIII.3 Thus, we are left with the unresolved question of the source of the extrahepatic factor VIII. Among the possibilities are production of factor VIII by myeloid cells or by lymphoid cells. In view of the recent demonstrations of successful human marrow engraftment,10-12 it appeared important to explore the possibility of therapy of hemophilia by marrow transplantation.

Canine marrow grafting following whole-body irradiation provides an experimental method to evaluate whether the marrow or the lymphoid system takes part in factor VIII production, since the hemopoietic and lymphoid systems in the grafted animals are exclusively repopulated by donor cells.13-18 In
the present study, two hemophilic dogs were given marrow grafts from normal siblings, and factor VIII levels were studied for up to 2 yr after grafting.

MATERIALS AND METHODS

The hemophilic beagles and their siblings were provided by Dr. R. G. Buckner of Oklahoma State University. These dogs are mild bleeders and have levels of factor VIII less than 1%. Each pair of siblings was selected on the basis of histocompatibility matching with eight canine lymphocytotoxic isoantisera. Details of the development of the eight sera, the testing technique, and data on the usefulness of the sera in selecting compatible donor-recipient combinations for grafting have been described. Recipients were conditioned to accept the grafted marrow by exposure to 1200 R (midline air exposure) of total-body irradiation at a rate of 9.3 R/min from two opposing 60Co sources. The midpoint body exposure was approximately 900–1000 rads. Donors were killed with intravenous pentobarbital sodium, and marrow was removed under aseptic conditions from long bones and ribs and prepared as a single-cell suspension for intravenous infusion. Donor marrow cells, 10 or 15 × 10^9, were infused within 4 hr after irradiation of the recipient. The recipients were given parenteral fluid and electrolyte support twice daily for the first 8 days following irradiation. Postgrafting immunosuppressive therapy consisted of methotrexate 0.25 mg/kg on days 1, 3 and 6, 0.5 mg/kg on day 11, and once weekly thereafter until day 102. Marrow engraftment was assessed by a prompt rise in white blood cell and platelet counts following the post-irradiation nadir, and by marrow histology. Cytogenetic analysis of cells of the hemopoietic system was carried out at various intervals after grafting, since both recipients were given marrow from donors of opposite sex.

For factor VIII assays, blood was collected in plastic tubes containing 3.8% sodium citrate (volume ratio 9:1). The blood was centrifuged at 3000 rpm, and the plasma was pipetted into a plastic tube. The plasma was kept frozen at -30°C until the time of the test. Clotting tests were performed in glass tubes at 37°C, and all dilutions were made in 0.14 M saline at pH 7.0.

The one-stage, factor VIII assay technique of Langdell et al. was used. The substrate was plasma from an untreated hemophilic dog from the same colony. To 0.1 ml of hemophilic plasma was added 0.1 ml of cephalin, prepared by the method of Bell and Alton, and 0.1 ml of dilutions of test or normal plasma; after 6 sec incubation, 0.1 ml M/40 CaCl2 was added. The test plasmas were diluted 1:5. Pooled normal plasma from three normal beagles was used as the standard and diluted 1:5, 1:40, 1:80, 1:200, and 1:500. In some instances, indicated in the text, human hemophilic plasma was used as the substrate as described above, except that the activated, partial thromboplastin time method using kaolin was employed.

The function of the grafted lymphoid system was measured by antibody formation to chicken and sheep red blood cells; by bacteriophage φX174 clearance, by analysis of the immunoglobulins involved in the primary and secondary immune response to bacteriophage, and by delayed hypersensitivity skin tests with PPD and BCG.

RESULTS

Both dogs showed the usual fall in white blood cell and platelet counts following irradiation and marrow grafting, followed by a rapid rise and return to normal after grafting. Function of the newly formed blood granulocytes was normal as determined by the in vitro radioiodination assay. Cyto-
bacteriophage antibodies on a Sephadex G-200 column showed that the antibodies involved in the first response were mainly gamma M, while those of the secondary response were chiefly gamma G as is seen in normal dogs. There was a normal bacteriophage clearance. The delayed hypersensitivity skin tests were within the normal range. The lymph node histology 213 days and 1 1/2 yr after grafting showed normal nodal architecture.

The two recipients had neither evidence of graft-vs.-host disease nor increased susceptibility to infection. Both had several episodes of subcutaneous and intramuscular hemorrhage following small injuries. Dog E16 had a fatal hemorrhage shortly after a diagnostic marrow aspiration from the iliac crest performed 7 mo following marrow grafting. Autopsy findings were normal except for the signs of bleeding. Dog D7 is in good health 2 yr after grafting.

Factor VIII assays were performed at regular intervals beginning on day 14 following marrow grafting (Table 2). Both dogs failed to show evidence of

| Table 1. Chromosome Analysis of Hemopoietic Cells in Two Female Hemophilic Dogs Given Marrow Grafts From Normal Male Dogs |
|---|---|---|
| Dog No. | Days After Grafting | Tissue Tested | Postgrafting Analysis of Chromosome Counts |
| | | | No. of Cells |
| | | | XX | XY |
| D7 | 234 | Peripheral blood | 0 | 4 |
| | 362 | Peripheral blood | 0 | 22 |
| | 582 | Marrow | 0 | 25 |
| | 582 | Lymph node | 0 | 26 |
| E16 | 211 | Marrow | 0 | 15 |

| Table 2. Factor VIII Values* |
|---|---|---|
| Date of Sampling† | Factor VIII‡ % | Untreated Hemophilic Dog |
| | D7 | E16 | |
| 9/3/69 | 1 | 1 | 1 |
| 9/19/69 | 1 | 1 | 1 |
| 9/25/69 | 4 | 4 | — |
| 10/27/69 | 3 | <1 | — |
| 11/14/69 | <1 (3) | <1 | (4) |
| 2/13/70 | <1 | (4) | <1 (3) |
| 4/17/70 | (4) | (7) | (8) |
| 7/11/70 | (3) | — | (4) |
| 2/27/71 | (7) | — | (7) |
| 4/22/71 | <1 | — | <1 |
| 5/13/71 | <1 | — | <1 |

* Marrow donor for D7 averaged 105% factor VIII, while that for E16 assayed at 100% just prior to transplantation.
† Dates of marrow grafting D7, 10/8/69; E16, 10/15/69.
‡ Figures in parentheses are values using human hemophilic substrate and normal canine plasma as the standard. Other values were obtained using canine hemophilic plasma.
factor VIII synthesis. The factor VIII assays of the transplanted hemophilic dogs did not differ from those of an untreated hemophilic control.

DISCUSSION

Our results unequivocally show that a successful and sustained hemopoietic and lymphoid graft from a normal canine donor into a hemophilic recipient does not result in factor VIII levels detectable by one-stage methods. Furthermore, both dogs continued to show clinical evidence of bleeding. These results are not in accord with the possibility of a production of factor VIII by lymphocytes, as suggested in experiments indicating that lymphocytes may release factor VIII during in vitro culture and by observation of increased factor VIII levels in a hemophilic patient with acute lymphoblastic leukemia. Indirect evidence that the lymphocyte is unimportant in factor VIII synthesis is the finding that the factor VIII level is lower in thoracic duct lymph than in blood plasma.

Despite genetic and functional similarities, the possibility remains that human factor VIII might be produced by myeloid or lymphoid cells while canine factor VIII is not. Our results in the dog are in accord with the general clinical observation that factor VIII level in man is not reduced after splenectomy, in aplastic anemia, or in diseases of impaired RES function. Maurer et al. reported normal factor VIII levels in a patient with combined immunodeficiency disease who showed functional abnormalities of the RES and virtual absence of lymphocytes and plasma cells in marrow, blood, and lymphoid tissues.

We conclude that factor VIII is not produced by myeloid or lymphoid cells. Our studies do not rule out the possibility of factor VIII production by the RES, since we were unable to distinguish donor from host reticuloendothelial cells in our canine chimeras.

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