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PRODUCTION OF HUMAN TO MOUSE XENOGRAFTS BY UMBILICAL CORD BLOOD

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Summary

Utilizing human umbilical cord blood, it has been possible to create in irradiated animals a human to mouse xenograft. To facilitate hematopoietic reconstitution, SJL/J mice, which are functionally low in natural killer (NK) cells, were treated with anti-Asialo GM_1 antibodies (anti-NK) and irradiation prior to injection of cord blood mononuclear cells. In contrast, SJL/J mice with the "beige" (bg/bg) mutation, which confers a functional NK cell deficiency, required only irradiation for successful transplantation. Human cells, detected by means of DNA probes, were demonstrated in the lungs and lymph nodes of irradiated animals up to 6 months after injection of the human cord blood cells.

In 1971 we reported evidence of a human bone marrow transplantation in an acute myelogenous leukemia patient following the transfusion of human umbilical cord blood (1). Since then, other authors have reported the presence of progenitor cells in human umbilical cord blood (2,3,4) in possibly even greater numbers than that found in normal human adult bone marrow (3).

We recently reported that human umbilical cord blood produced 3-5 times more hematopoietic colonies *in vitro* than did normal human bone marrow (5). The results of these studies suggest that cord blood may hold even greater potential than bone marrow as a source for human marrow transplantation. A similar study concerning the potential of umbilical cord blood for marrow reconstitution has been recently published by other investigators (6).

In this preliminary communication, we report the production of human hematopoietic xenografts in lethally irradiated mice. This was accomplished by adoptive transfer of human umbilical cord blood mononuclear cells into recipient mice that are either genetically deficient in natural killer (NK) cells or that have been treated with antibodies to deplete the NK effector cells.

Toward resolution of the current shortage of human bone marrow and the clinical problems related to bone marrow donors, human umbilical cord blood offers a potentially unlimited source of human stem cells for marrow transplantation for both clinical and experimental purposes.

MATERIALS & METHODS

Mice: SJL/J (8-10 weeks old) mice were obtained from the Jackson Laboratory

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(Bar Harbor, ME). SJL/J mice bearing the "beige" mutation [SJL (bg/bg)] were produced from a breeding colony maintained in the Research Animal Facility at UMD-NJMS. The breeding stock of SJL (bg/bg) mice was obtained from the Jackson Laboratory.

Antisera: Rabbit anti-Asialo GM_1 antisera was purchased from Wako Chemicals (Dallas, TX). Injection of 100 μl of this antibody i.v. depletes NK effector cells, as measured by the inability of splenic effector cells to cause lysis of the NK-susceptible target cell, YAC-1, for a period of 2 weeks after injection (N.M. Ponzio, unpublished observations). Mice were given anti-Asialo GM_1 antibody 24 hours before irradiation, and adoptive transfer of hematopoietic cells was done within 2 hrs of irradiation. Mice in control groups received 100 μl normal rabbit serum (NRS) in lieu of anti-Asialo GM_1 antibody.

Irradiation: Irradiation was delivered from the 8 MeV X-ray beam of a linear accelerator (Philips SL 75-20) using a 25 x 25 cm field with a rate of about 400 Gy/min. Animals were restrained during irradiation in a lucite box measuring 19.8 x 15.9 x 6.5 cm which holds 10 mice in individual compartments. Polystyrene blocks of 5.22 and 5.46 cm thickness respectively were placed above and below the cage to provide dose buildup and back scatter. The source surface distance of the irradiation assembly was 89 cm with mice a mean distance of 100 cm from the X-ray source. Irradiated animals were housed in a laminar flow compartment with sterilized cages, food, water and bedding for the duration of the experiment.

Bone Marrow: Syngeneic bone marrow cells were obtained by direct, repeated flushing of cells from the tibias and femurs of normal donor mice using 2 ml of normal saline delivered from a 3 ml syringe through a 25 ga needle.

Umbilical Cord Blood: Forty ml of human umbilical cord blood was collected directly into 10 ml of Iscoves modification of Dulbecco's medium (Gibco Laboratories, Grand Island, NY) containing 50 units/ml of preservative free heparin. Ficoll hypaque density gradient centrifugation was used to isolate mononuclear cells from the whole cord blood.

Bone marrow and cord blood cells were washed 3 times with normal saline, and assessed for viability by trypan blue dye exclusion. Cells were given in a single intraperitoneal injection within 2 hrs of irradiation.

DNA Probes: Organs selected for DNA analysis were spleen, liver, lungs and lymph nodes. Southern blot analyses were performed by Dr. K. Mehta's Laboratory of the Molecular Probes Services for Lymphoma and Leukemia at UMD-NJMS. DNA was extracted from the murine tissues by reagents obtained from Oncor Laboratories (Gaithersberg, MD), digested with restriction enzymes BamHI, HindIII, and EcoRI, and then subjected to electrophoresis on 0.8% agarose slab gels. After denaturation and neutralization, the DNA was transferred to nitrocellulose membrane by the technique of Southern (7). Hybridization with ^{32}P labeled Alu DNA probe was carried out at 45°C in a solution of hybridization reagents obtained from Oncor Laboratories. Following hybridization, filters were washed (0.1x SSC and .01% SDS) at 52°C for one hour. Autoradiography of the washed filters was carried out for 3 days at -70°C with two intensifying screens (fig I).

For slot blot analysis, DNA was applied to filters using BioRad slot blot apparatus, and hybridization was performed using standard techniques (8). For slot blot analyses human repetitive DNA sequence Alu was used as a probe (performed by Dr. Ragbir Athwal's laboratory in the Department of Microbiology and Molecular Genetics at UMD-NJMS).

RESULTS

Initial studies using irradiated (850cGy) CF_1 recipients of human umbilical cord blood showed a 50% survival rate similar to that of the controls, but 2 out of the 10 experimental group mice survived for 22 and 33 days respectively. A small number of irradiated (800cGy) SJL (bg/bg) mice also showed some long term survivors (2 out of 4) when transplanted with human cord blood. In these initial experiments, however, survival studies were inconclusive, possibly due to the low number of cord blood cells injected or to the temporal delays before injection of cells (5). During these initial studies, some of the long term surviving animals that received cord blood were noted to have hematopoietic colonies in the spleen around the 18th day. Repeated analysis of cells from these colonies with DNA probes failed to reveal the presence of human DNA sequences. These regenerating colonies appeared in the spleen around the 10th day in animals receiving syngeneic marrow.

In an experiment in which SJL/J mice were given anti-Asialo GM_1 antibody and 900 cGy prior to injection of cord blood (Table I, Group E), 4 out of 10 mice survived over 40 days (5 Mice were sacrificed and 1 mouse died prior to day 18). In two of these long term surviving animals, human DNA was detected in lung tissue. The third mouse showed no evidence of human DNA in the lung by Southern blot analysis, and the fourth animal died without benefit of post-mortem examination on day 43.

In this experiment, there were 2 long term survivors in each of the control groups (n=10) that received syngeneic marrow. Mice in these groups received $4X10^6$ nucleated cells and 900 cGy of radiation, one with anti-Asialo GM_1 antibody (Group D) and one without the antibody (Group B). The 10 control animals that received normal rabbit serum and cord blood cells (Group C) had no long term survivors. A dose of 900 cGy is lethal for SJL mice, and all five mice in this experiment that received only irradiation (Group A) succumbed by the 14th day after irradiation.

Table II summarizes the cumulative results of DNA analysis of tissues from long-term survivors. Mice #1 and #2 are from Group E of the experiment presented in Table I. Mouse #3 is from an earlier experiment utilizing SJL/J (bg/bg) mice treated with 800cGy of radiation. Human DNA was detected in the lung of this mouse 6 months after injection of cord blood. Mice #4 and #5 are from another experiment in which 24 SJL/J mice were treated with anti-Asialo GM_1 antibody and received 900 cGy prior to injection of 1.8×10^6 cord blood mononuclear cells. Although there were 18 deaths in this group, two of the three mice that were sacrificed on day 32 showed human DNA in the cervical lymph nodes by slot blot analysis. The remaining 3 mice survived beyond 60 days, but no DNA analysis was performed.

DISCUSSION

There has recently been a series of articles on the creation of human to mouse chimeras using peripheral blood [$10\text{-}90 \times 10^6$ leukocytes] (9), human fetal tissue (10) and human bone marrow [1×10^7 marrow cells] (11). The mice utilized in these studies had various genetic immune deficiencies and in some cases were also given supplemental radiation (11). As far as we can determine, no successful human to mouse radiation chimera has been reported (10). In this study we have produced such radiation chimeras by using anti-Asialo GM_1 antibody (12) to deplete NK cells in a low NK strain of mouse, the SJL/J strain. Similar results were also obtained without the need for treatment with anti-Asialo GM_1 antiserum in NK-deficient SJL/J(bg/bg) mice (Table II).

TABLE 1

INFLUENCE OF HUMAN CORD BLOOD ON SURVIVAL OF IRRADIATED SJL/J MICE[1]

Group	Serum Injected[2]	Cells Injected ($x10^6$) Mouse Nuc. B.M.	Human Mono. C.B.	No. of Mice Total	Died	Sac.	Survival Mice alive on Day 18[3]	30[4]	40[4]
A	None	0	0	5	5	0	0	0	0
B	NRS	4	0	10	4	4	3	2	2
C	NRS	0	4	10	8	2	0	0	0
D	Asialo GM_1	4	0	10	3	5	4	2	2
E	Asiaol GM_1	0	4	10	1	5	4	4	4

[1] SJL/J mice were irradiated (900 cGy) and reconstituted with either mouse bone marrow cells or human cord blood mononuclear cells at the indicated number within 2 hrs of irradiation.

[2] Mice were injected with normal rabbit serum (NRS) or rabbit anti-Asialo GM_1 serum 24 hrs prior to irradiation.

[3] The mice shown here were alive 18 days after irradiation. The other animals had either died or were sacrificed for experimental analysis. Three animals died between day 18 and day 30, 1 in group B and 2 in group D.

[4] The mice shown here survived beyond 30 days. None of the mice died between day 30 and day 40. Thus, the numbers shown are the same mice. The number of mice that survived to day 30 plus the number of mice that died or were sacrificed account for the total number of mice per group.

Sac - sacrificed; Nuc. - Nucleated; B.M. - Bone Marrow; Mono. - Mononuclear; C.B. - Cord Blood

TABLE II

DETECTION OF HUMAN DNA IN MICE INJECTED WITH CORD BLOOD MONONUCLEAR CELLS

MOUSE NO.	TYPE OF MOUSE	ANTI-ASIALO GM_1 ANTISERA	DAYS POST RAD.	# OF MONO. CELLS INJECTED	RAD. DOSE	LOCATION OF HUMAN TISSUE WITH DNA PROBE
1	SJL/J	YES	41	$4X10^6$	900cGy	LUNG
2	SJL/J	YES	87	$4X10^6$	900cGy	LUNG
3	SJL/bg	NO	178	$1.2X10^6$	800cGy	LUNG
4	SJL/J	YES	32	$1.8X10^6$	900cGy	L.N.
5	SJL/J	YES	32	$1.8X10^6$	900cGy	L.N.

L.N. - Lymph Node; RAD. - Radiation

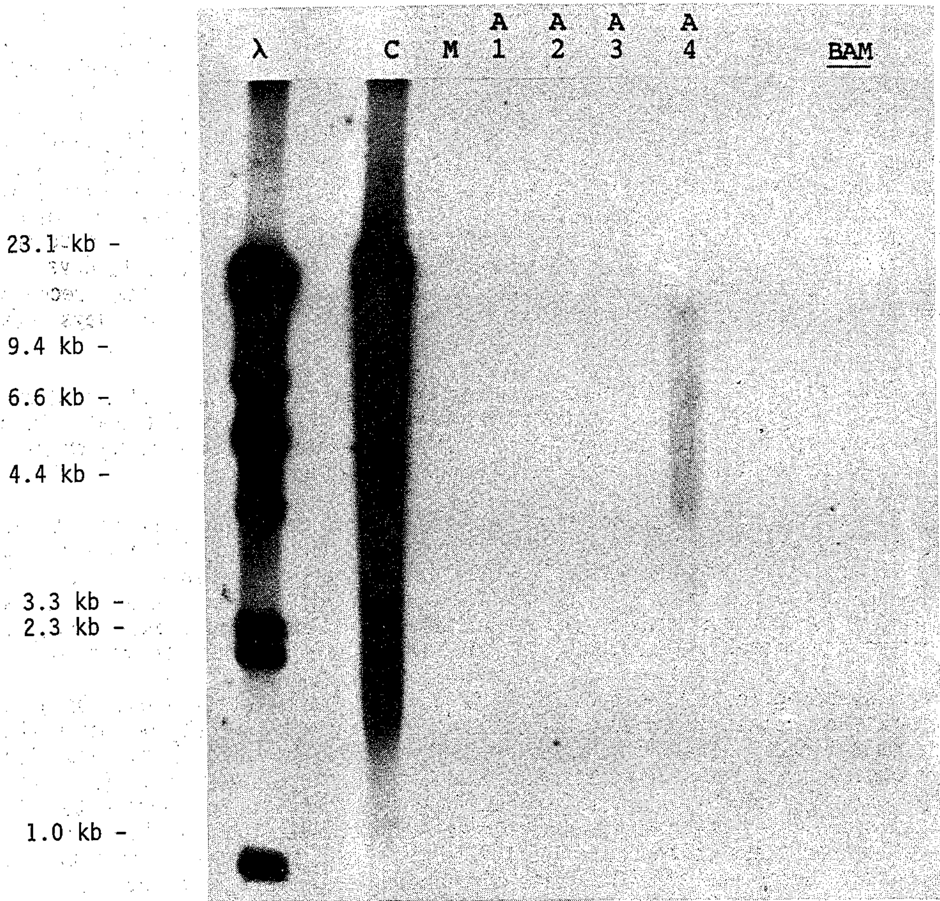

Fig. 1

SOUTHERN BLOT ANALYSIS OF DNA FROM TISSUES OF MOUSE #1 (TABLE II)

λ	LAMBDA MARKER	
M	NORMAL MOUSE CONTROL	
C	PLACENTA (HUMAN CONTROL)	
A-1	SJL/J cord blood-antibody	SPLEEN
A-2	SJL/J cord blood-antibody	KIDNEY
A-3	SJL/J cord blood-antibody	LIVER
A-4	SJL/J cord blood-antibody	LUNG

Except for a diminished number of NK cells, the SJL/J mice are immunocompetent. Mice in this strain do develop lymphadenopathy and paraproteinemia prior to development of B cell lymphomas (13,14). However, these characteristics typically develop in mice that are at least 6-8 months of age, and are irrelevant to the results presented here, since the SJL recipients of human cord blood were only 8-10 weeks old. Moreover, while the SJL/J(bg/bg) mice exhibit deficiencies in NK cell activity and granulocyte production, they have an otherwise intact immune system. Existing host NK cells apparently are not destroyed by 900cGy, and are thought to be the cell type responsible for bone marrow transplant rejection in both allogeneic and xenogeneic models (15). Since these cells play a central role in bone marrow transplantation, we reasoned that removal of NK cells would facilitate acceptance of the cord blood xenograft. The results presented in Table I demonstrate the influence of NK cells on graft survival. None of the mice that were irradiated and received NRS and cord blood cells survived to 18 days. In contrast, out of 10 mice (5 were sacrificed) 4 of the 5 mice that received anti-Asialo GM_1 antiserum and cord blood cells survived 40 days post irradiation.

As shown in Table II, human DNA was detected in the lungs of irradiated mice at 41 days and up to 6 months after receiving fetal cord blood with no evidence of graft versus host disease. This suggests that a lasting chimeric state had been established in some of these animals.

The ability of cord blood to produce a human to mouse chimera in SJL mice with the use of anti-NK antiserum and in NK-deficient SJL(bg/bg) mice without anti-NK antiserum after irradiation clearly indicates the central role of NK cells in marrow transplant rejection (15) and provides a useful and economical model for further research on the human immune system. Unlike SCID mice, SJL mice are considerably less costly, readily available and easy to maintain.

Misleading results were obtained in our earlier pilot studies. On gross examination, spleen colonies were noted in 3 irradiated SJL(bg/bg) mice that were sacrificed on the 18th day after tail vein injection of human umbilical cord blood, and there were no spontaneous deaths in this small group (n=3) that received only 800cGy. These spleen colonies apparently represented the animal's own regenerating hematopoietic cells. Repeated Southern blot analysis of cells from these spleen colonies failed to reveal the presence of human DNA sequences. Unfortunately, the lungs of the animals were not examined for human DNA.

The spleen is one of the first organs in the mouse to regenerate hematopoietic colonies following irradiation. The spleens from all of the animals showing human DNA in the lungs or lymph nodes were either regenerated to normal size or had large hematopoietic colonies. Analysis of these spleens however, failed to show evidence of human DNA. Examination of blood and plasma from these mice did not reveal the presence of human platelet factors, human hemoglobin by electrophoresis or cells that express human surface markers by indirect immunofluorescence. The survival of these mice through the period in which they usually succumb following lethal irradiation, and the finding of human DNA in unexpected locations leads us to believe that the human fetal cord blood cells may have carried the mice through this post-lethal irradiation period. The presence of human hematopoietic cells may have provided a protective effect, allowing endogenous hematopoiesis. A similar phenomenon has been postulated for some of the Chernobyl survivors, where the allogeneic bone marrow transplants provided transient grafts that allowed re-establishment of endogenous hematopoiesis (16).

Our initial studies (1) in 1970 where 70 ml of cord blood effected a temporary bone marrow graft in a leukemic patient, as well as the results of our current studies, provide support for our belief that cord blood may hold considerably more capability to establish a marrow graft than does adult bone marrow. *In vitro* studies in our laboratories suggest that the erythroid colony forming ability of cord blood is 3 to 5 times greater than that of human bone marrow (5); and studies done in other laboratories indicate that the number of progenitor cells may be as much as 20 times greater in umbilical cord blood (3). The ability to produce a bone marrow xenograft in mice by means of umbilical cord blood using a relatively low number of mononuclear cells provides a basis for using cord blood for human transplantation. Potentially, the problem of insufficient numbers of bone marrow donors and the clinical problems related to the marrow donors themselves could be eliminated (17). In a limited number of human cases we did not encounter any adverse reactions when umbilical cord blood was transfused (1,18). Furthermore, we have found no evidence of bacterial contamination in cultures of cord blood maintained for 20 days (5).

With existing methods for efficient storage of bone marrow cells (19,20,21) and the nearly limitless availability of umbilical cord blood, now considered only a waste product, the possibility of establishing human umbilical cord blood banks is entirely feasible. Utilizing cord blood as a source of marrow cells for human transplants could be, both clinically and experimentally, far reaching. These banks could make available 6-antigen HLA matched and blood type specific marrow cells for use in various clinical situations, such as malignancy, organ transplantation (22), immunodeficiency, specific gene replacement therapy, and even auto-transplantation.

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