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THE BERASHIS CELL : A REVIEW IS IT SIMILAR TO THE EMBRYONIC STEM CELL?

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Abbreviations: HLA = Human lymphocyte antigen, SLE = systemic lupus erythematosus.

Introduction

This article is an attempt to define very primitive pleuripotential and probably totipotential primitive cells found in umbilical cord blood and their clinical significance. For lack of a better name and in an effort to separate it from other "stem" cells, we have called it the "Berashis Cell" meaning "*in the beginning*" (Ende, 1995). Currently, we believe this cell exists in limited numbers in human umbilical cord blood, has never impacted on stroma, probably has some functional capacity at 4°C and has few if any recognition antigens. In its functional capacity it may be similar to that predicted for the embryonic stem cell derived from a fetus or embryos (Gearhart, 1998).

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It is the development of the concept of this primitive cell and its possible impact on clinical medicine to which this article is devoted. These primitive cells may provide the possibility of implanting an infantile immune system in an adult human with the creation of a partial or complete chimera. This may have a considerable effect on many chronic diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, diabetes mellitus and others, many of which have limited or no treatment. In addition, it may have reparative assets for various organs similar to embryonic stem cells by directly or indirectly creating new cells in various organ systems.

It probably is not necessary to have the "Berashis Cell" to produce a successful marrow transplant in humans when there is HLA compatibility, but it may be more essential where there is minimal or no HLA compatibility between donor and recipient.

Background

During the 1960's, my brother Milton Ende, M.D., in a period when there was limited treatment for lymphoma or malignant neoplasms, gave a series of cord blood transfusions to patients with malignancies in which conventional therapy had failed (Ende, 1966, Ende and Ende 1972). This was based on the fact that neoplasms were extremely rare in newborns and perhaps the hematopoietic cells existing in the cord blood of newborns would produce some inhibiting effect on the neoplastic growth.

In reviewing the data on these cases at that time, it was noted that the patients had an unusual jump in the hematocrit. This was far greater than could be accounted for from the amount of cord blood given to the patients even if several units (500cc) were given at one time. It was postulated that a temporary hematopoietic graft may have occurred and several case studies were set up to evaluate this concept. In a current retrospective review of this early data, several of the patients probably had temporary chimeras of the hematopoietic system but only one was published. Approximately 30 years later in China, a similar study using human cord blood, unmatched for HLA, produced changes in the recipient consistent with a successful marrow transplant (Shen, Hou, *et al.*, 1991).

Prior to 1970, successful transplantation without HLA matching was reported (Pegg, 1966). Experimentally xenographs had been reported between mice and rats using relatively large doses of cells (Nowell, Cole, *et al.*, 1956; Vos, Crouch, *et al.*, 1956), and to protect irradiated mice with

homogenized spleen from infant mice (Cole and Ellis, 1953). In 1989, we and others (Broxmeyer, Douglas, *et al*, 1989; Ende, Rameshwar, *et al*, 1989) recommended that human cord blood could be banked and used for marrow transplantation. In 1990 with the use of irradiation we were able to obtain a temporary xenograph in relatively normal mice (SJL/J) (Ende, Giuliani, *et al*., 1990). At that time we suggested that HLA close identity between donor and recipient may be unnecessary. Although we were unable to identify human cells in the peripheral blood of the animals, human cord blood mononuclear cells could produce survival of mice that had received lethal levels of irradiation and was dose related (Ende, Lu, *et al*, 1996). Trace amounts of human DNA, however, were found in mice even one year after injection of human cord blood mononuclear cells (Ende, Ponzio, *et al*., 1995). Furthermore, mice survival after lethal irradiation was directly dependent on the number of human cord blood mononuclear cells administered (Ende, Lu, *et al*., 1996).

Clinically, bone marrow-transplantation can modify and improve the course of autoimmune disease (Van Bekkum, 1998). Experimentally, we have found human umbilical cord blood can modify autoimmune disease in mice (Ende, Czarneski *et al*., 1995). MRL Lpr/Lpr mice have an autoimmune disease similar to systemic Lupus that occur in humans. In xenograph experiments we have found that human umbilical cord blood mononuclear cells given to MRL Lpr/Lpr mice could significantly increase the length of life and delay the onset of pathological changes (vasculitis) in these animals (Ende, Czarneski *et al*., 1995). This finding led us to believe that cord blood may be useful in treating amyotrophic lateral sclerosis. Although controversial, amyotrophic lateral sclerosis is considered by some advocates to be an autoimmune disease (Rowland, 1992). The effects of cord blood mononuclear cells on SOD1 mice were startling. In some instances in the initial studies, the life span was increased by 60%. Furthermore, additional studies indicated the length of survival appeared to be dose related. The greater the dose of human cord blood cells, the longer the life span (Chen and Ende, 2000). This was similar to survival of mice exposed to lethal levels of irradiation; the greater the dosage of cells, the greater the number of animals that survived (Ende, Lu, *et al*., 1996). The value of the megadose of human cord blood mononuclear cells became apparent in these studies. These findings were further accentuated when it appeared that pooled cord blood was more effective than single units of cord blood, both in terms of life span of the SOD1 mice

and the presence of human DNA in long term survivors (Chen and Ende, 2000). It appears that the pooling of cord blood, necessary to produce megadose quantities of cells, is not detrimental, but appears to be beneficial.

Use of Cord Blood in Various Disease States

Materials and Methods

Collection, Storage and Separation of Cord Blood Mononuclear Cells

Our procedure for the collection of cord blood cells has been consistent over several years. The cord blood was obtained from the placentas of healthy full-term neonates. The collection of cord blood was made into 50 mL sterile polypropylene test tubes containing 5 mL of citrate phosphate dextrose as an anticoagulant. Samples were sent to the blood bank for storage at 4°C. Initially, we used standard blood bank bags (polyvinyl) which are not gas permeable. After finding that the stem cells were present, but dormant (Fernandez, Lu, *et al.*, 1994) we attempted multiple methods unsuccessfully to aerate the cells while stored at 4°C. Finally, we adopted the polyolefin collection bag (Cryocyte Freezing Container, Baxter Healthcare, Deerfield, IL) which are gas permeable (Lemoli, Tafuri, *et al.*, 1992). This allowed collection of multiple samples into one unit with storage at 4°C and thereby provided us with megadoses of cord blood mononuclear cells. Other gas permeable bags are probably as effective for storage, and survival of the "Berashis Cell".

Mononuclear cells were separated from the whole cord blood by centrifugation in a ficoll hypaque density gradient. The mononuclear cells in our early experiments were given intravenously and intraperitoneal. In our latter experiments, the mononuclear cells were all administered retro-ocularly.

Standard procedures were used for clonal growth, replating efficiency in methylcellulose cultures, immunophenotyping by flow cytometry and mixed lymphocyte cultures. Standard polymerase chain reaction procedure was used for detecting human DNA (Chen and Ende, 2000; Ende, Lu, *et al.*, 1999).

Antisera (anti-killer cell antisera)

Since existing host natural killer cells are not destroyed by 900 cGy of irradiation and it was believed that natural killer cells could play a

central role in bone marrow transplantation; it was reasoned that removal of natural killer cells would facilitate acceptance of a xenograft of cord blood cells. Rabbit anti-Asialo, GM_1 antisera were obtained from Wako Chemicals, Richmond, VA. These sera were given from 6-24 hours prior to irradiation of the animals. Injection of 100 μL of this antibody intravenously depletes natural killer effector cells, as measured by the inability of splenic effector cells to cause lysis of the N.K.-susceptible target cell (Yac-1), for a period of 2 weeks after injection (Ende, Giuliani, *et al.*, 1990). Because our initial studies appeared to be successful, we used anti-killer sera (Aislo GM_1) in conjunction with irradiation and did not drop the use of these antisera and irradiation until we began to use megadose of human cord blood mononuclear cells.

Survival After Lethal Irradiation and Chemoablation

In 1989, we realized that human cord blood mononuclear cells could provide a temporary xenograft in mice following lethal irradiation (Ende, Giuliani, *et al.*, 1990). It was not for several years however, that we realized that animal survival was directly related to the dose of human cord blood mononuclear cells administered (Ende, Lu, *et al.*, 1996). After 7 days of storage, $18.5x10^6$ nucleated cells produced 82% survival in SJL/J mice receiving 900 cGy of irradiation. Syngenec bone marrow produced 78% survival.

In an experiment where SJL/J mice were given 390 mg/kg of cyclophosphamide and 800 cGy of irradiation, at 50 days, 26% of the animals that received human cord blood mononuclear cells survived while those that received syngenic marrow cells were dead ($p<0.05$) (Ende, Ponzio, *et al.*, 1995). Additional studies indicated that human cord mononuclear cells not only could serve as a temporary graft but also aided reconstitution of the animals' own immune system and might serve as an adjunct in the treatment of advanced malignancies (Czarneski, Lin, *et al.*, 1999; Rameshwar, Smith, *et al.*, 1999).

Following a recent nuclear accident in Japan, (late 1999), in which two patients received lethal doses of irradiation, we forwarded information recommending megadose therapy with umbilical cord blood cells. From personal communications, (no official reports are available) one patient received a large dose of cord blood cells and initially responded. Since he was leaning over the radioactive material, he suffered severe radiation burns to his lung and face, but the initial response was very favorable.

Fig I

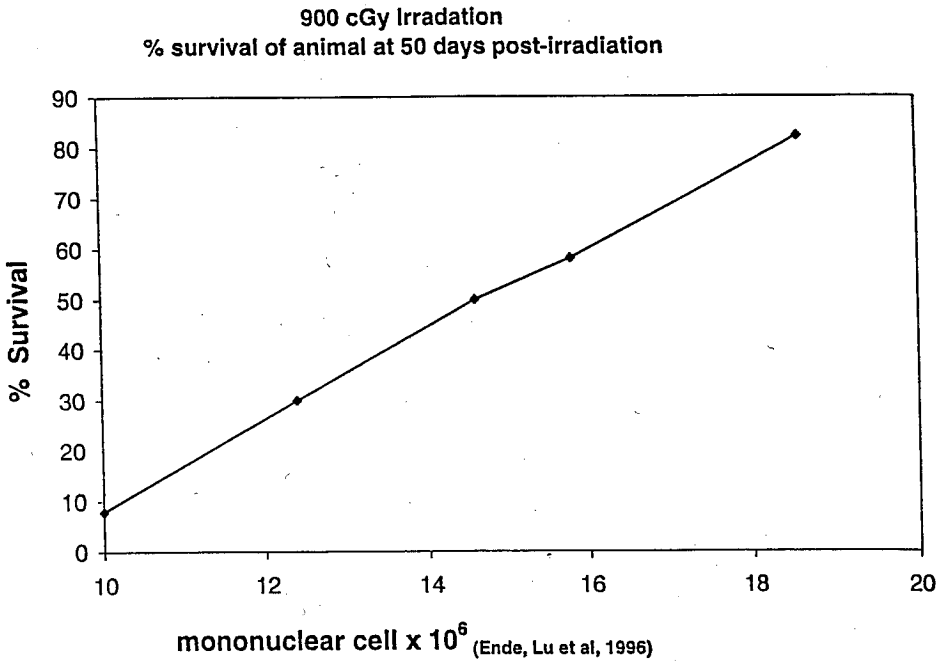


Fig II

Survival of SOD1 Mice After Treatment with or without irradiation and umbilical cord blood mononuclear cells

			Average Days survival	
Control		n+n'+n"=14	126.2±2.5	
anti-killer Sera, Bone Marrow 5 x 10^6 mononuclear cells	Radiation 800 cGy	n'+n=11	132.5±2	BM vs Control: p<0.05
Anti-killer Sera, HUCB 33.2-34.0 x 10^6MNC	800cGy	n'=11	147±7.8	CB vs Control: p<0.01
70.2-73.3 x 10^6 MNC	800 cGy	n'=8	162±10.5	CB vs Control: p<0.001
survival with umbilical cord blood mononuclear cells only				
103-117 x 10^6MNC		n"=7	172.1±9.5	CB vs Control: p<0.01
Survival with umbilical cord blood mononuclear cell only given after onset paralysis				
103-117 x 10^6MNC	800 cGy	n"=6	140±5.5	CB vs Control: p<0.05
103-117 x 10^6MNC		n"=6	148.3± 7.5	

n: 1st experiment
n": 2nd experiment
n": 3rd experiment

Autoimmune Disease

Using MRL Lpr/Lpr mice, as a murine model of autoimmunity, we attempted to modify their disease with the use of human umbilical cord blood cells. These mice served particularly well as a model for human systemic lupus erythematosus (SLE), with certain SLE-specific autoantibodies. With the use of human cord blood cells, we were able to double the life span of MRL Lpr/Lpr mice and delay the onset of vasculitis (Ende, Czarneski, *et al.*, 2000). The effect of human cells on the MRL Lpr/Lpr mice and the effect of human cells on lethally irradiated mice, led us to believe that the most primitive cells ("Berashis cells") in human cord blood may have a direct effect or indirect effect on other mammalian cells. It was the ability of human cord blood mononuclear cells to modify a murine autoimmune disease, which led us to consider treating mice carrying a human transgene associated with amyotrophic lateral sclerosis

SOD1 Mice (Amyotrophic Lateral Sclerosis)

Amyotrophic lateral sclerosis is considered by some to be an autoimmune disease (Rowland, 1992). These SOD1 mice have a mutant transgene related to the genetic form of amyotrophic lateral sclerosis in humans. In our laboratory SOD1 mice, untreated, live an average of 126 days (Figure II). We have found a direct relationship between the life span of SOD1 mice and the dosage of human umbilical cord blood mononuclear cells administered the animals (Fig. II). This extension of life in SOD1 mice occurred in animals receiving irradiation and cord blood mononuclear cells (Chen and Ende, 2000; Ende, Weinstein, *et al.*, 2000) and also those receiving only cord blood mononuclear cells (unpublished, Fig. II). Even after the onset of paralysis, an increase in survival could be accomplished with megadosage of cord blood cells. With the use of megadose human cord blood mononuclear cells the life span of the mice with irradiation averaged 162 days survival without irradiation but with a larger dose of cells (103-117) $x10^6$, the length of life was 172 days. The average length of time the animals survived after the onset of paralysis with the use of human cord blood mononuclear cells (with and without irradiation) increased from an average of 4 days to an average of 19 days (Fig. II). In some instances, the mice lived 30 days after the onset of paralysis.

Aged Mice

To study the effects of aging we used C51BL/6J mice. At the end of 102 weeks the ten control mice in this study were all dead. Of the five mice receiving $8.8x10^6$ nucleated syngenic marrow cells, three of the five lived beyond the death of the controls with two animals living to 118 weeks (Figure III). In the animals receiving cord blood mononuclear cells without any form of immune suppression, there appeared to be a direct correlation with the length of survival and dosage of cells. Four out of seven mice that received ($30x10^6$ mononuclear cells) lived over 125 weeks, and these four long-lived animals received pooled cord blood specimen. Human DNA was found in one of the four mice (the entire animal was not examined, but only portions of the liver, spleen, lung and bone marrow). One of the mice that received $30x10^6$ cells lived 140 weeks while all control animals were, as noted, dead by 105 weeks Fig. III. The autopsies on the 3 mice that lived the longest revealed that two developed lymphoma and one developed amyloidosis. The fourth mouse only showed infection and no evidence of neoplasia.

SJL/J Mice and Lymphoma

Ninety percent of SJL/J mice develop lymphoma by 13 months of age (Erianne, Wajchman, *et al.*, 2000). In our earlier studies in which the animals received human cord blood mononuclear cells and irradiation, none developed lymphoma by 13 months of age (Ende, Ponzio, *et al.*, 1995). In a separate experiment where six SJL/J mice were given only cord blood mononuclear cells in megadose amounts ($105\text{-}112 \times 10^6$), DNA by P.C.R. could not be detected in the peripheral blood in any of the six mice at 30 days. Human DNA could, however, be detected in the animals peripheral blood at 50, 70 and 90 days and in some animals at 110 days after transplantation (Figure IV). Only one of the animals developed lymphoma at 13 months. One animal died at 30 days and one animal was sacrificed at 30 days. In the sacrificed mouse human DNA was identified in the liver, spleen, bone marrow and lungs (Fig. IV).

Megadose Therapy and Storage at 4°C

Since 1993, we have been aware that very primitive stem cells (Berashis Cells) remain alive in cord blood stored at 4°C (blood bank conditions) for varying periods of time (Ende, 1995). With the advent of availability of gas permeable bags (Lemoli, Tafuri, *et al.*, 1992), that can

Fig III

Age mice survival after transplanted Human Cord Blood

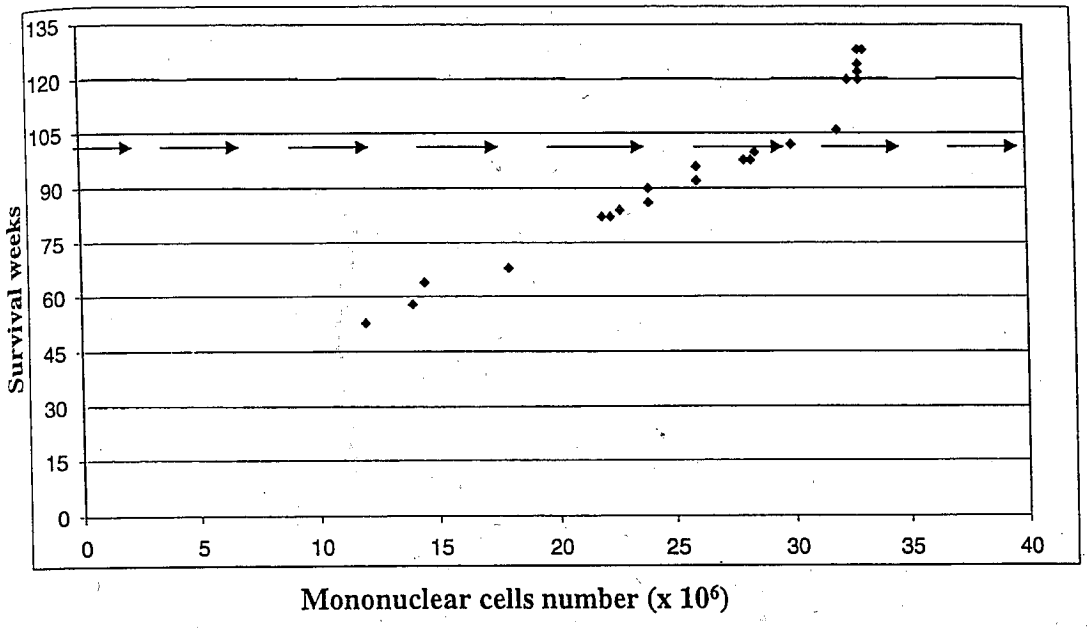


Fig IV

SJL/J mice: PCR results on detection of human DNA in peripheral blood after injection 105-112x10^6 mononuclear cells

Age of mice prior to injection (Months)	6.5	8.5	7.5	6	7	7	6	5.5
After injection date	#1	#2	#3	#4	#5	#6	#7	#8
30 days	-	-	-	-	±	-	-	-
50 days	+	+	-	±	-	-	-	-
70 days	+	+	+	±	-	+	-	±
90 days	+	+	+	-	-	+	+	+
110 days	*	•	+	-	-	+	+	+
130 days			-	±	-	-	-	-
150 days			-	-	-	-	-	-

-: did not find human DNA
±: trace of human DNA
+: human DNA found

*: mouse died
•: mouse sacrificed
: mouse died of Lymphoma

be used for storage at 4°C, we expanded our research in this area. It was found that the buffy coat cells and Ficol Histopaque separated mononuclear cells (Ende, Lu, *et al.*, 1996) after storage in gas permeable bags (Figure I). could both produce significant survival in lethally irradiated mice. There was a drop off, however, in survival of animals that received 21 day stored cord blood which may have indicated that by 21 days of storage some of the most primitive cells either died off or became inactive due to lack of some critical cytokine (Lu and Ende, 1997).

It has become evident by measuring colony formation and flow cytometry, that the more primitive cells, such as CD34, CD117 and GPA cells, not only stay alive when stored at 4°C, but their ratios increase significantly while the other cells (T-cells) either die or become nonfunctional. It has been noted by others (Grunow, Lubert, *et al.*, 1976) that a significant drop off of T cells occurs in banked blood stored at 4°C in 72 hours. In our laboratory it has also been noted that a drop off of T cells occurs, and after 14 days of storage at 4°C, the mixed lymphocyte culture becomes relatively non-reactive (Ende, Lu, *et al.*, 1999). This could indicate there may be little or no graft vs. host reaction if these stored specimens are used for bone marrow transplantation.

The concept of megadose therapy in marrow transplantation is not new (Raveche, Santoro, *et al.*, 1985). In the use of umbilical cord blood, however, the term megadose may be a misnomer. The most primitive or immature cells, the "Berashis Cells", may be so uncommon in the cord blood that pooling is necessary only to obtain the minimal number of cells to accomplish a temporary graft (Ende, Lu, *et al.*, 1999; Lu and Ende, 1997). According to our data, based on the number of replatable blast cell colonies obtained per milliliter of blood, we estimate that fifty milliliters of cord blood may contain an average of 400 "Berashis Cells" (Ende, Lu, *et al.*, 1999; Lu and Ende, 1997). The necessity to pool blood to obtain the larger numbers of the most primitive cells, had no adverse effect but appears to increase the proliferation of some immature cells particularly of the erythroid series (Ende, Lu, *et al.*, submitted). Storage of single cord blood specimens at 4°C for 10-21 days in gas permeable bags produces an apparent increase in the number of immature cells (CD34, CD117, GPA) and the mitotic activity (S+G2/M cells). However, with similar storage of pooled specimens of cord blood, there is a further increase in the ratio of both the number of immature colonies produced when cultured and CD34, CD117, GPA and S+G2M cells. One of the most interesting events with

storage at 4°C in gas permeable bags was the proliferation of the erythroid cells, the normoblast increased markedly in the first 10 days of storage and in some specimens, the increase was exponential (Ende, Lu, *et al.*, submitted).

Cord Blood Mononuclear Cells Effect on Neoplasms

Mice that develop malignant neoplasms of the breast were utilized in this study (MMTV neu Jackson Lab). These mice develop malignant breast neoplasm by 5-6.5 months of age. In our studies once the animals developed breast masses (0.18-0.38 inches in greatest diameter) they were entered into the experimental procedure and were measured at regular intervals. In the initial experiments 900 cGy of irradiation, anti-killer sera (0.1mL Asialo GM_1 antisera) and 16.5-20.0x10^6 human cord blood mononuclear cells from a single specimen were administered to one group of mice. Anti-killer sera (Asialo GM_1) were given to the animals 24 hours prior to irradiation. The controls consisted of mice that developed breast malignancy and did not receive any therapy. Another group of animals received 900 cGy of irradiation and bone marrow cells (5.6x10^6) obtained from congenic mice. Following irradiation both groups demonstrated a decrease in the size and rate of regrowth of the neoplasms. The animals receiving human cord blood mononuclear cells, however, had a greater response and a much slower rate of regrowth of the neoplasms to pretherapy size as compared to mice which received irradiation and congenic bone marrow (Figure V).

This study was repeated again using MMTV neu mice and larger doses of human umbilical cord blood mononuclear cells. When 23.0x10^6 mononuclear cells were used with a lower dose of irradiation (800 cGy) the neoplasms became smaller in size and had a slower rate of re-growth as compared to animals that received bone marrow and irradiation. In animals receiving 45.0-100x10^6 human cord blood mononuclear cells and similar doses of irradiation, one neoplasm disappeared entirely and has not recurred in 6 ½ months and two neoplasms 0.66-0.73 inches in diameter have remained stationary with no evidence of growth for 4 months (Figure VI).

These findings would indicate cord blood mononuclear cells could serve as an adjunct in the treatment of breast tumors, similar to the use of bone marrow. With a megadose of cord blood mononuclear cells, however, it might serve not only as an adjunct to therapy but could provide enough

Fig V

Average Rate of Growth of Breast Tumors in MMTVneu Mice After 800 cGy of Irradiation

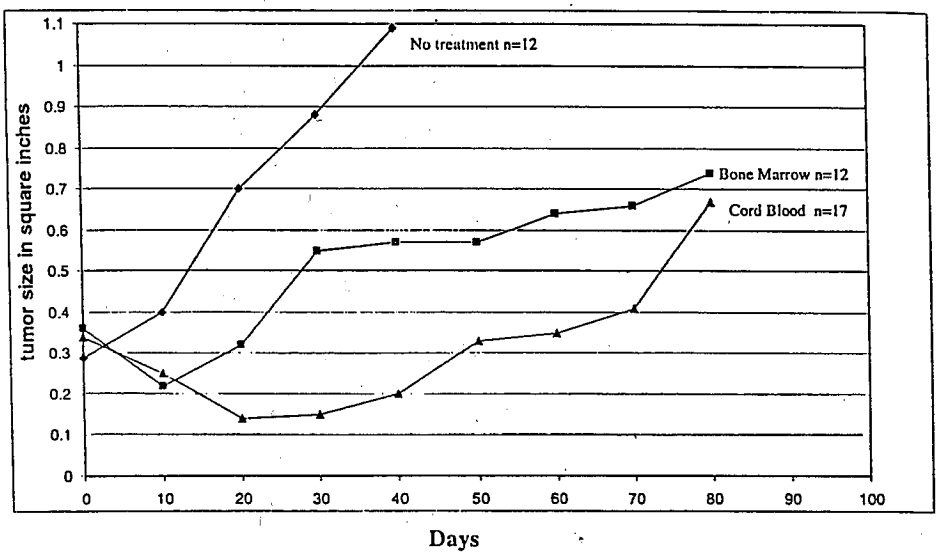
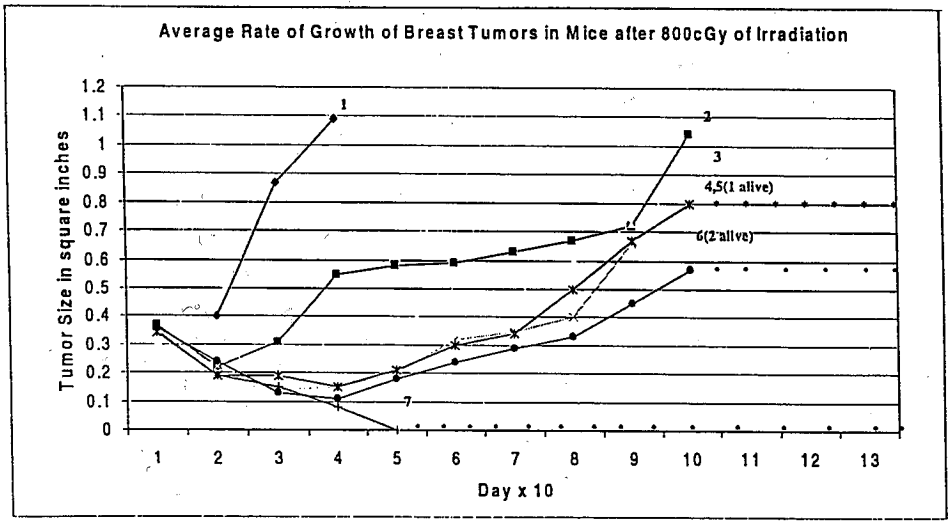


Fig VI



1. No treatment (n=5

2. Bone Marrow 5x 10^6 (n=5)

3. HUCB 20x10^6 (n=2)

4,5. HUCB 35x10^6 (n=4)

6. HUCB 100x 10^6 (n=2)

7. HUCB 45x10^6 (n=1) tumor has not returned in 8 months

of an infantile immune system to significantly suppress the neoplastic growth or regrowth.

Huntington Disease

In initial studies on Huntington Disease mice [B6CBA-TgN(Hdexon 1) 62Gpb] using 800 cGy of irradiation and antikiller sera, the average length of survival using 71-74x10^6 mononuclear cells was 95 days compared to controls of 87 days (Figure VII). When the animals were given 100-105x10^6 human cord blood mononuclear cells the average life span was 104 days ($p<0.05$).

The Huntington transgene is expressed in all the cells of the animals and they usually develop choreaform movements and lose weight early in their illness. The difference in weight loss between the animals treated with cord blood mononuclear cells and untreated controls was striking, $p<0.05$.

Planned experiments: Parkinson's Disease Mice, Type I and type II diabetic mice. Cerebral neoplasm, traumatic cerebral cortex lesions and traumatic spinal cord lesions.

Theory

Since we believe the "Berashis Cell" has few recognition surface markers and are few in number per mL of cord blood, much of the evidence of their existence is presumptive. Based on the number of replatable blast colonies we obtain from multiple individual cord blood samples, we estimate there may be only 8± cells per milliliter. From our studies we have found some cord blood units may contain as many as 18 blast replatable colonies per milliliter of cord blood and some cord blood samples have no replatable blast colonies. The wide variability that we have encountered in tissue culture, flow cytometry and the clinical response of the SOD1 mice, Huntington disease mice and Alzheimer mice; all indicate that these cells are both variable and few in number in a single unit of cord blood. From our studies on the therapeutic effect, there is reasonable certainty that the Berashis Cell is pleuripotential and probably totipotential, similar in function to what has been proposed for the human embryonic stem cells recently described (Soltor and Gearhart, 1999; Thomson, Itskovitz-Eldor *et al.*, 1998). Probably, in the simplest explanation, the "Berashis Cells" may have the ability to produce a temporary infantile immune system in an

adult mammal (mouse) to provide control, repair and surveillance (Ende and Ritter, 1998).

It is my opinion that these most primitive cells may arise at the 21st day of fetal life with the formation of the cardiac cavity. It is also possible that these cells arise at, or prior to the appearance in the embryo of the aorta, mesonephros-gonad region (Medvinski and Dzierzak, 1996). We have found some evidence of hematopoiesis at 4°C which may be related to the primodial nature of the "Berashis Cell".

The effect of cord blood mononuclear cells on an auto immune disease in mice (MRL Lpt/Lpr) is similar to that produced by bone marrow and immunosuppression in humans (Marmont, 1997). The evidence that the "Berashis" cells can effect an autoimmune disease in mice and the presence of trace amounts of human DNA found one year after injection, may not only indicate a lack of recognition antigens on the cell, but the acceptance of a human cell by another mammal.

Our studies on SJL/J mice, where megadose of mononuclear cells have been given without immunosuppression, suggests that the "Berashis cell" appears to be tolerated in the mouse for approximately 60-90 days and either stops its division or the host builds up antibodies to shut the progeny down, but leaves a few cells still in the host which may represent the original "Berashis cell" at rest.

Since recent publications have shown that under experimental conditions, hematopoietic cells can produce neurons and neural stem cells produce hematopoietic cells (Kopen, Prockop, *et al.*, 1999; Marshall, Moore, *et al.*, 1999); there would be little reason to believe that these primitive cord blood hematopoietic cells would not also produce neurogenic cells. Our studies on SOD1, Hdxon1, and Alzheimer mice all have indicated that human cord blood mononuclear cells could have a significant direct or indirect effect on neurogenic cells. Most importantly, these cells although given intravenously, despite the blood-brain barrier, seem to go to or effect the area where needed in the central nervous system.

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