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PROPERTIES OF THE HEMOPOIETIC STEM CELL:
CAN ITS BONE MARROW REPOPULATING ABILITY BE DISSOCIATED
FROM IMMUNOLOGICAL COMPETENCE?*

by

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Introduction:

Death following whole body radiation in the lethal dose range is correlated directly with marrow aplasia and its sequelae. With the exception of the value of antibiotics and transfusions in lower lethal range, the only effective post-radiation therapy is bone marrow transplantation. It has become quite clear from many lines of study that marrow regeneration, whether spontaneous or from transplanted marrow, commences at the stem cell level. The differentiated hemopoietic cells clearly are not capable of continuous self-replication. Accordingly, an understanding of the properties of the stem cell is vital to an understanding of spontaneous marrow recovery and how recovery is induced by marrow transplantation. To date, the properties of this cell have only been characterized indirectly, since it has not as yet been identified and described in proper morphological terms.

The major effect of irradiation in the lethal dose range is the dose-dependent depletion of the stem cell compartment. Many investigators have studied this problem in detail. The consequences of a depletion of the stem cell compartment have been analysed by Bond et al. This results in a progressive depletion of the most immature differentiated compartment, followed by each more mature compartment. As the bone marrow becomes depleted of the proliferating compartments and contributes the mature cells to the blood, a diminution then commences in these items within the blood.

The probability of spontaneous recovery is purely a function of the numbers of surviving stem cells and the time it takes for these cells to re-establish the minimum size of the stem cell pool that permits cells to differentiate. This problem has been discussed in detail by many workers (Lajtha, Cronkite, Bond et al.). Clearly the depleted stem cell pool must expand faster than cells are removed from it by differentiation or the stem cell pool, the differentiating proliferating pool of cells in the marrow, and the peripheral elements in the blood upon which survival depends cannot be replenished. Time is of the essence. In the presence of severe granulocytic and thrombopenia death may take place from infection and/or severe hemorrhage. Accordingly, the doubling time of the stem cell compartment, the time for proliferation and maturation in the differentiated cell lines until cells are ripe enough to be extruded into the blood is critical. Accordingly, the first objective of this presentation will be the presentation of the time parameters of marrow proliferation in the human being, since the time parameters of the lower animals are well-known.

It is now uniformly accepted that regeneration can be substantially accelerated by marrow transplantation, marrow shielding or the introduction of stem cells from any source (peritoneal washings, peripheral blood and bone marrow but not lymph nodes, thymus, thoracic duct or spleen of animals without myeloid metaplasia). Despite all of this knowledge about where stem cells exist, there is no clear-cut acceptable description of the stem cell.

The second objective of this paper will be the summarization of knowledge about the property of the stem cells.

In dealing with syngenic strains of animals, one can almost double the LD₅₀ with marrow transplantation. Man is not syngenic. The allogenic transplantation of stem cells from marrow results in protection of degree but the development of secondary disease (allogenic disease), which is presumably produced by either the transplantation of immunologically competent cells or their development from a totipotential stem cell, which results in a graft-versus-host disease being launched by the expanding population of donor immunologically competent cells. The third objective of this presentation will be a theoretical discussion of whether it is possible to selectively eliminate immunologically competent cells from bone marrow or to prevent a theoretical totipotential cell from producing immunologically competent cells.

Time parameters of human erythropoiesis and granulopoiesis:

The principles of studying the flow of cells from cell compartments in which the DNA is labeled by tritiated thymidine (³HTdr) to more mature compartments utilizing autoradiography and cytology have been presented (Killman et al., Patt et al., Cronkite et al.).

The principle involved will be presented schematically and observations made on human erythropoiesis and granulopoiesis will be presented.

Properties of the Stem Cell:

The Concept:

The stem cell concept is many decades of age, and has been described in detail by Bloom. It is difficult to trace the entire history with satisfaction. Cowdry and Bloom recognized the need for a self-perpetuating cell in hemopoiesis and other tissues. Cell renewal has been discussed by Leblond and Walker. In embryonic development, blood formation is commenced within the primitive mesenchyme in the yolk sac. A vast literature, primarily of polemics, exists as to whether there is a single stem cell for hemopoiesis or multiple cells in adult life. The number and types of stem cells in adult life for hemopoiesis remain still unknown. The existence of pure aplasias of one type or another in human disease can be stated as an argument for the loss of the stem cell for that line or the loss of the specific inductor for that cell line, which acts upon the common stem cell. Recent genetic studies by the Canadian group clearly demonstrate that in mice there is an inherited disorder in which the stem cell has a faulty mechanism for the development of erythropoiesis, and another inherited disorder in which a host factor is absent that permits proper development of erythropoiesis.

The stem cell(s) may be defined as cells with the capability of unlimited self-replication upon appropriate stimulation, the capability of responding to specific differentiating stimuli, and a mechanism of regulating the size of the stem cell pool. The theoretical totipotent hemopoietic stem cell can be further defined as having a broad capability of differentiation into erythrocytic, granulocytic, megakaryocytic, general lymphocytic, specific immunologically competent lymphocytic cells and perhaps other cell lines. This definition corresponds roughly to Maximow's concept of heteroplastic and homoplastic hemopoiesis.

Morphology:

Although there is no definitive description of the stem cell, a considerable amount of evidence has been developed in recent years, showing that stem cell activity is associated with small bone marrow lymphocyte-like cells. Fliedner et al. have

approached the problem in the following manner: dogs were treated with nitrogen mustard to eliminate the differentiated cellular elements in the marrow. Serial bone marrow studies were performed to determine the time when regeneration commenced. Just before any clear-cut cytological differentiation was present, the marrow was taken from these animals to protect fatally irradiated dogs. It was clearly demonstrated that marrow which had no clearly defined erythropoiesis or granulopoiesis would accelerate the regeneration of marrow in fatally irradiated dogs. The donor animals were then carefully studied in respect to DNA synthesis and morphological changes. From these studies it was concluded that there is a small round basophilic cell in the marrow that undergoes proliferation and differentiation into a larger undifferentiated blast-like cell that resembles the hemocytoblast. It was further concluded that the stem cell responsible for the repopulation of the marrow must be amongst mononuclear cells of the marrow which are in a quiescent state or have a long generation time, so that these are rarely found in DNA synthesis and thus cannot be ordinarily labeled under normal steady state conditions. Cudkowiec et al., in studies on separation of cells from the mouse marrow by glass-wool filtration, strongly supports the idea that the mouse stem cell is a small marrow lymphocyte which protects against radiation injury, whereas lymph node lymphocytes with the same appearance do not protect. Mel and Schooley have separated mouse bone marrow by the "Statiflo" technique. This technique involves very small forces and gives very clear separations of cell types. They find the protective activity of the bone marrow to reside in a fraction that contains a mixture of large and small lymphocytes with the latter predominating. The evidence points clearly to the stem cell being within what is commonly called the lymphocyte family of the bone marrow. The evidence seems to be stronger for a small mononuclear cell rather than a large one. The use of the term "lymphocyte" is unfortunate and confusing, but what else is one to call a cell that resembles a lymphocyte?

Another approach to the stem cell has been a revival of embryological studies by Stohlman and associates. They have carefully studied the development of hemopoiesis in the embryonic liver. Within the liver prior to the development of erythropoiesis there are large hemocytoblastic-appearing cells. These have a high proliferative rate as indicated by labeling with tritiated thymidine and mitosis. They find no small round cells similar to the ones that the studies of Fliedner, Cudkowiec and Mel

might implicate in the normal steady state of the adult animal. These observations may not be incompatible. Perhaps the quiescent form of the stem cell in steady state hemopoiesis of the adult is small, round and dense. When it becomes activated it may then transform into a cell that appears like the classical hemocytoblast and resembles the cells in the embryonic liver.

Migration of stem cells:

Perhaps the earliest observation that suggested the possibility of radiation protection within the marrow and migration is that of Fabricius-Müller, who protected guinea-pigs from radiation injury in 1921 by shielding one leg and exposing the rest of the body to radiation. Many later studies have conclusively demonstrated that stem cells can migrate from one part of the body to another. For example, Jacobson shielded the spleen of a mouse which normally has myelopoiesis and found regeneration of the marrow. Subsequent studies clearly demonstrated that this was due to migration of cells from the shielded spleen to the bone marrow with subsequent proliferation. Woenckhaus, and later Brecher and Cronkite, made parabiotic rats and irradiated one while the other was shielded. This resulted in significant protection of the irradiated member of the pair. The only logical explanation is the transfer of stem cells from the non-irradiated animal. Storer et al. found an increased survival in rats from shielding the tail in which red bone marrow production had been induced, but not when non-hemopoietic normal tails were shielded. Swift et al. increased the survival in mice by allowing a 90-minute interval to elapse between exposures of the upper and lower half of the body. They also found an increased survival in rats when one leg was lead shielded during irradiation and then later irradiated. Hanks has measured the rate of migration of repopulating cells from the shielded femur. Hellman also studied the capability of a shielded leg to produce spleen nodules in the mouse by the Till and McCulloch technique of colony forming units. Robinson et al. have quantitatively measured the rate of migration of stem cells from the shielded tail of the mouse. In their studies the degree of colonization in the spleen was found to be a function of the length of time which elapsed between the original whole body irradiation with the tail shielded and a subsequent, suppressive irradiation of the tail. The preceding studies, and the fact that one finds stem cell activity in the peripheral blood of mice (Trobough and Lewis), and the presence of stem cells in the peritoneal washings of mice and rats clearly prove that stem cells migrate from one portion of the body to another.

The colony forming unit:

The mouse behaves somewhat differently from other mammals during spontaneous regeneration from radiation injury. Nodules of hemopoietic proliferation that are grossly visible appear in the spleen roughly 8 to 15 days after exposure, depending somewhat on the dose of radiation and the strain of mouse. Presumably many individuals have wondered why these nodules appear in the spleen of the mouse and not other species of mammals. Why this should occur is still not clear, but presumably is related to the normal myeloid metaplasia of the mouse spleen. This natural phenomenon has been brilliantly exploited by McCulloch, Till and associates in a series of papers since 1960. In the bone marrow, spleen, blood and peritoneal washings of the mouse there are cells which, on being transplanted intravenously into irradiated mice, can proliferate and form the macroscopic colonies. These colonies are discreet and can be easily counted macroscopically. These colonies are similar to the ones that appear in the spleen of mice spontaneously regenerating from non-fatal radiation injury. The colonies appear as a cluster of hematopoietic cells with frequent mitotic figures. Depending upon the time that the colonies are examined, they may be predominantly one cell type or another, or maybe differentiating into the three lines of erythrocytic, granulocytic and the megakaryocytic series respectively. These colonies have been shown in all probability to be derived from single cells. They probably represent the stem cells for hemopoiesis, but since this cell has not been identified by direct cytological means, the Canadian group have chosen to give "the operational name of colony forming cells" to these entities. The colony forming cells are present in hemopoietic tissue in the ratio of approximately one colony forming cell per 10^4 nucleated cells. Clearly these colony forming cells are self-replicating with a great capacity for proliferation. Within 10 days they can give colonies containing in excess of 10^6 cells. In addition, they are capable of differentiation, since, as mentioned earlier, the colonies contain large numbers of histologically recognizable differentiated cells in the erythropoietic, granulopoietic and megakaryocytic series. The proof that the cells are capable of self-renewal lies within the fact that one can extirpate growing colonies and retransplant these cell suspensions into other irradiated mice and see that they have the capacity to produce colonies again. The preceding facts seem almost conclusive evidence that the colony forming cells are probably stem cells. In extending their studies Bruce and McCulloch measured the effect of hypoxia upon the concentration of the colony forming cells in the spleen and bone marrow of hypoxic mice. From their studies they

concluded that "the primitive erythroid precursor that is sensitive to erythropoietic stimulation is not the colony forming cell. We suggest, instead, that the late decrease in colony forming cells in the spleen is a response to a depleted pool of cells sensitive to erythropoietin and that colony forming cells are differentiated to produce more of these cells". This observation is in apparent conflict with the concepts of La jtha, Stohlman and Alpen. The preceding three believe that erythropoietin induces primitive stem cells to differentiate into the erythroid system. However, different species are used and the techniques are substantially different and may account for the differences in interpretation.

McCulloch and Till (1964) have shown that for one day after transplantation the colony forming cells do not proliferate in the irradiated recipients. After this initial lag phase, they increase in an exponential manner for 7 days, with a doubling time of 20 to 25 hours. Siminovitch et al. have clearly demonstrated that about 0.17 of the colony forming cells lodge in the spleen following an intravenous injection. An indeterminate number of these cells lodge in the bone marrow, lymph nodes and thymus.

Two other phenomena have been observed in respect to the colony forming cells that are of considerable importance. First, the phenomenon of repression has been described as a reduction in the number of colony forming unit per cells injected when parenteral marrow is used in F_1 hybrid mice. The colonies formed are significantly reduced, but the survival of treated animals is just as good. There is no clear explanation as to why the parenteral to F_1 hybrid combination should repress the capability of forming macroscopic colonies. It may be related to a graft-versus-host reaction, but this also is not clear. The phenomenon of decline is similar to that observed by Cudkowicz. When colony forming cells are derived successively from colonies in the spleen and repeatedly transferred to successive recipient, the yield of colonies per 10^5 cells injected progressively decreases.

Becker et al. (1965) have treated suspensions of colony forming cells with high concentrations of tritiated thymidine in the hope of being able to kill colony forming units by a tritiated thymidine suicide technique. When embryonic cell suspensions reutilized or colony forming cells from rapidly regenerating colonies they demonstrated a death of about 40 % to 65 % of the cells, indicating that this fraction was in DNA

synthesis. However, when similar amounts of tritiated thymidine were added to cell suspensions of bone marrow or spleen of animals in a normal steady state proliferation, there was an imperceptible effect of the tritiated thymidine upon the number of colony forming cells. From this they conclude that there is an insignificant number of cells of the colony forming type in DNA synthesis in the normal steady state. This is a very nice direct confirmation of the deductions of Flie dner et al.: "stem cells responsible for the repopulation of the marrow must be mononuclear cells which are in a quiescent state or have a long generation time under normal steady state conditions, so that under these conditions they are rarely found in DNA synthesis and thus are not labeled". La jtha et al., in their analysis of probable stem cell models, conclude that a small fraction of the stem cells are cycling during normal steady state hemopoiesis. Tonna and Cronkite have described a comparable situation in the adult femora of the mouse. Less than one per cent of the osteoblasts are in the DNA synthesis phase of the cell cycle. Within 6 to 12 hours after a fracture the osteoblasts burst into proliferative activity and nearly 80 % are found in synthesis by 24 to 36 hours after the fracture. The proliferative activity subsides at the epiphyseal ends of the bone first and finally at the fracture site after healing is complete. The periosteum then slumbers until once again intense proliferation is needed for repair.

During rapid regeneration of colony forming cells in the spleen following transplantation into the heavily irradiated recipient in vitro ³HTdr suicide technique has demonstrated that 40 % to 60 % of the colony forming cells are in DNA synthesis.

Radiation responses of colony forming cells:

The Canadian group has carried out extensive radiobiological studies on these cells. The D₀ and extrapolation numbers of the colony forming cells vary somewhat depending upon the anatomic source:

	D ₀	Extrapolation number
Adult bone marrow	95 ± 9 rads	1.50 ± 0.54
Adult spleen	90 ± 16 rads	.80 ± 0.46
Fetal liver	176 ± 26 rads	1.08 ± 0.50

Fourteen days after transplantation into heavily irradiated mice the colony forming cells derived from marrow, spleen or fetal liver and present in the spleen have become substantially more sensitive to radiation.

In their original work the usual 30 day survival of marrow treated irradiated mice was studied as a function of the number of marrow cells administered and the dose of radiation to these cells in vivo and in vitro. The D_0 was 105 ± 24 rads for the mouse marrow cells. Since only the stem cells within the marrow cell suspension are responsible for regeneration of the marrow and thus prevention of death from marrow aplasia, this dose probably represents the LD_0 for the marrow stem cell. The similarity of D_0 in this study, the colony forming cell study and the fact that colonies in the spleen may include erythropoiesis, granulopoiesis and megakaryocytopoiesis strongly indicate that the colony forming cell(s) are identical to the hemopoietic stem cell responsible for marrow repopulation and survival after otherwise fatal irradiation injury.

The differentiation of colony forming cells has been studied. Liron and Feldman have shown that transfusion induced polycythemia suppressed the number of colonies formed in the mouse spleen by one third. A greater than usual number of granulopoietic colonies were observed. No erythropoietic colonies were seen. A possible interpretation is that some colony forming cells are sensitive to erythropoietin and fail to develop in its absence. However, the Canadian group feels that the colony forming cell is not the erythropoietin sensitive cell and that other explanations are more likely.

Distribution of colony forming cells after intravenous injection:

The spleen colony technique is a very useful method of studying the kinetics and the radiobiology of cell proliferation and differentiation. As mentioned earlier, the colony forming cell and the hemopoietic stem cell responsible for protection of the fatally irradiated mouse are probably one and the same cell. About 17 % of the injected colony forming cells lodge in the spleen (). The exact distribution of the remainder is not known with precision because it is not technically feasible to determine the quantitative distribution. However, the studies of Ilbery and Micklem et al. utilizing the T-6 chromosome clearly have demonstrated that marrow derived cells penetrate into the thymus, lymph nodes and the bone marrow. The totipotential stem cell(s) are found only in the

marrow. Thymic and lymph node cells only have the capacity to penetrate into and repopulate their own tissues to a limited extent. The capability of marrow stem cells to repopulate all hemopoietic tissues vigorously and permanently in the mouse appears to be dependent in part upon the presence of a functioning thymus.

Is it feasible to dissociate immunological functions from the radioprotective properties of suspensions of marrow cells?

The importance of bone marrow in altering radiation injury was first demonstrated by Fabricius-Moller who showed that shielding of a leg of a guinea-pig prevented thrombopenia and radiation hemorrhage. The subsequent studies of Jacobsen et al., Lorenz et al., Cole et al., Barnes et al. and van Bekkum et al. clearly demonstrated that spleen cells (if the spleen has myeloid tissue as it does in mice) and bone marrow cells will protect against otherwise fatal whole body irradiation when injected intravenously shortly after the exposure. If the protecting cells were syngenic or autologous, the protection is long lasting. The protected animals experience a life shortening and other late effects of radiation, but rarely develop leukemia. If the protecting cells are allogenic in origin, the protection is generally of lesser degree, and at variable times the protected animals may develop a usually fatal wasting disease that has been called secondary disease, allogenic disease, and graft-versus-host disease (GvH).

The GvH disease is believed to be caused by an immunological attack of the grafted cells upon the host. Experimental facts that are pertinent appear to be the following. The bone marrow contains relatively few immunologically competent cells as demonstrated by Tyan and Cole. The most convincing evidence for there being cells in the marrow that can proliferate and differentiate into cells capable of launching immunological reactions is the complete restoration of immunological competence of irradiated animals by syngenic marrow cells and the later production of GvH disease by allogenic marrow cells in the irradiated recipient (either parent to F₁ or mongrel donor recipient combinations). If one uses lymph node or spleen cells in addition to marrow cells, the GvH disease appears earlier and is much more severe, producing deaths within two weeks at times despite some marrow regeneration. This accelerated secondary disease from lymph node or splenic cells can be explained on the basis of the injection

of more immunologically competent cells (ICC) than are present in the marrow alone. However, in addition to the number of ICC present in the marrow is the fact that the potentially immunologically competent cells of the marrow must proliferate and differentiate into ICC before the GvH disease can be launched by them. There is also competition for the progeny of transplanted stem cells for differentiation and repopulation of many hemopoietic cell lines. Apparently the body economy gives precedence to re-establishment of granulopoiesis, erythropoiesis and megakaryocytopoiesis over immunological competence. The latter in fact returns later. If the former were not restored quickly, death would ensue from the sequelae of marrow aplasia.

Next, from the work of Duplan and the Canadian group it appears that immunologically competent cells or antigen sensitive cells in the Canadian terminology have a limited capacity to divide as compared to the colony forming cells. In similar time periods the antigen sensitive cells divide 4 to 6 times, whereas colony forming cells divide at least 20 times. The difference may be due to only limited self-replication of the former. Antigen clearly initiates division, and continued presence of antigen may either further differentiate cells to a non-dividing stage (plasma cells?) or kill the cells.

The facts so far as the natural history of allogenic marrow treated irradiated rats is concerned are as follows. Marrow is regenerated by the donor cells and hemopoietic death is prevented. The time and severity of secondary disease apparently depends upon the number of ICC injected or the rate of their production by the donor marrow cells. The secondary disease either kills the recipient or the recipient in some manner adjusts to the immunological attack upon its tissue. Perhaps a state of immunological tolerance develops. Perhaps the immunologically competent cells die out because they have a limited capability of self-replication and are also consumed in the GvH disease. In any case, the GvH disease does frequently subside. If the host's own immunological system regenerates, one would expect it to arise from the totipotential stem cell of the host and then re-establish the bone marrow also and thus eliminate the chimaeric state.

In principle one can postulate the following degrees of stem cell committedness:

1. Totipotential stem cell with the following properties:

- a) Unlimited self-replication
- b) Capability of differentiation into
 - (i) Erythropoiesis

- (II) Granulopoiesis
- (III) Megakaryocytopoiesis
- (IV) Immunologically competent cells
- c) Size conscious
- 2. Committed stem cells with limited capability to self-replicate
 - a) Erythropoietin sensitive cells
 - b) Granulopoietic committed cells
 - c) Immunologically competent cells
 - d) Other lines

In this scheme the radioprotective cell would be the totipotential stem cell.

The work of Mathé, van Bekkum and our own studies clearly show that certain treatments can diminish the number of immunologically competent cells in the bone marrow and marrow lymph node cell mixtures (van Bekkum). If the scheme presented above is true and the immunologically competent cells truly are limited in capability to self-replicate, the management and/or prevention of secondary disease is twofold. First, reduce the numbers of ICC in the marrow suspensions. Second, kill ICC as they develop from totipotential cells of the donor marrow, and third, attack the differentiation of totipotential stem cells into ICC. Of course, the latter may be a double edged sword since if the goal is attained the recipient would be an immunological cripple.

The problem is most complicated and vexing. It is primarily one of trying to encourage differentiation and proliferation along certain cellular pathways and suppressing it along others or enabling tolerance to develop.

It is clear that the incidence of secondary disease has been altered by various treatments of marrow and mixed marrow lymph node spleen suspensions by Mathé, van Bekkum and others, but the mechanism of suppression of the secondary disease is not at all clear.