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Tumor Cell Rejection Through Terminal Cell Differentiation

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Leukemic cells cultured in the presence of various conditioned media differentiate into macrophages. This finding suggested that the maintenance of undifferentiated state and self-renewal in vivo may be related to the inability of the host to generate an appropriate level of differentiation factor (DF). Evidence for this hypothesis was derived from experiments in vitro and in vivo with myeloid leukemia of rat. The following results were obtained: (i) in vitro, the percentage of cell differentiation at a fixed concentration of DF was inversely related to the concentration of cells; (ii) leukemic cell inoculates that were lethal to 7-day-old rats were rejected by 21-day-old rats; (iii) leukemic cells in diffusion chambers underwent differentiation in 21-day-old rats but not in 7-day-old rats; (iv) organs from 21-day-old rats contained more DF activity than those of 7-day-old rats; (v) treatment of rats with DF in diffusion chambers resulted in leukemic cell differentiation inside the chamber; and (vi) the development of leukemia in 7-day-old rats was aborted by treatment with DF. These results show that the differentiation of rat leukemia cells requires the appropriate level of DF. The proliferation of transplanted leukemia cells in 7-day-old rats goes unchecked because of inadequate generation of DF. Conversely, in the 21-day-old rats, rejection is accomplished by differentiation of the transplanted cells.

INTENSE INTEREST IS CURRENTLY FOCUSED on the role of colony-stimulating factor in leukemic cell differentiation and its potential applicability to the therapy of human myelogenous leukemia. In both the murine and human systems, factors derived from various conditioned media have been identified that induce the terminal differentiation of leukemia cells in vitro (*1–3*). Preparations containing granulocyte colony-stimulating factor (G-CSF) have been shown to suppress self-renewal of myeloid leukemia stem cells in vitro and the leukemogenicity of treated myeloid leukemic cells in vivo (*4–10*). Although the responsiveness of the leukemic cell to differentiation factors (DFs) relative to normal stem cells is uncertain, the studies cited above have clearly established that the leukemic cell can be induced to undergo terminal differentiation in response to physiological factors. This raises a number of questions, perhaps most important among them: What maintains the leukemic cell in the undifferentiated state in vivo? In vitro and in vivo observations reported herein on the myelogenous leukemia of rat suggest that the maintenance of the undifferentiated state and self-renewal may be related to the host's inability to generate the appropriate level of DF. Rejection of a leukemic cell transplant in 21-day-old recipient rats is accomplished by terminal cell differentiation of the transplanted cells.

The experimental model we have used in these studies is the chloroleukemia of rat (*11, 12*). This is a myelogenous leukemia with many similarities to the human disorder. It was originally produced by gastric instillation of 20-methylcholanthrene and later successfully transferred by the injection of chloroleukemia cells into newborn rats (*12, 13*). In 1975, the establishment of a permanent cell line, MIA C51 (*14*), from this tumor was reported. MIA C51 is maintained in suspension culture in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (DMEM-FCS). It has a doubling time of 12 hours and maintains many of the characteristics of the parent tumor, including transferability to newborn rats.

It has long been known from the work of Shay *et al.* (*12*) and Moloney *et al.* (*13*) that the successful transfer of rat chloroleukemia depends on the age of the recipient animal (best in newborn) and the number of leukemic cells injected. This is generally interpreted as a tendency to immune rejection as the rat matures. An alternative explanation was initially suggested to us by our in vitro observations. When C51 cells were cultured at increasing cell density (from 1×10^4 to 8×10^4 cells per milliliter) at a fixed level of

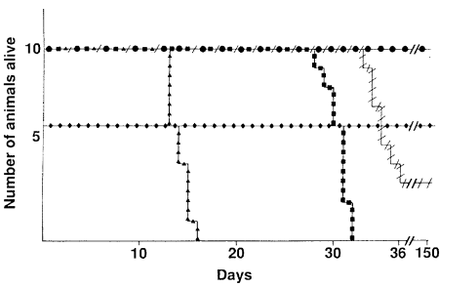

Fig. 1. Relation of survival to the age of recipient animal and to the number of transplanted leukemia cells. C51 cells in log phase were suspended in serum-free DMEM at 5×10^5 cells per milliliter. Appropriate dilutions were made and the indicated numbers of cells were injected intraperitoneally in 0.1 ml volume: (▲) 7-day-old rat with 1×10^5 cells injected; (■) 7-day-old rats, with 2.5×10^4 cells injected: (+) 7-day-old rats with 2×10^4 cells injected; (●) 7-day-old rats with 10^4 cells injected; and (◆) 21-day-old rats with 2.5×10^4 cells injected. At death, the abdomen of each animal was opened through an incision and inspected for leukemic ascites and for macroscopic leukemic infiltrates.

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rat lung–conditioned medium (RLCM) as a source of DF, the percentage of cells that underwent terminal differentiation to macrophages at 48 hours was inversely related to the number of cells in the initial inoculum (Table 1). Thus at 1×10^4 cells per milliliter there was 100% differentiation with 98% viability, and the cell number remained unchanged (no growth). Conversely, when the inoculate was 8×10^4 cells per milliliter, cell growth was comparable to control (100% growth) with virtually no differentiation. When the number of cells in the inoculate was fixed at 1×10^4 cells per milliliter and the amount of DF was varied, a similar pattern was observed—for example, full growth with no differentiation at 50 units of DF per milliliter versus 98% differentiation and no growth at 500 units per milliliter. These results indicated that an appropriate amount of DF was needed for terminal differentiation of a given number of leukemic cells; in the presence of inadequate amount of DF, proliferation outstripped differentiation, and there was leukemic cell "escape."

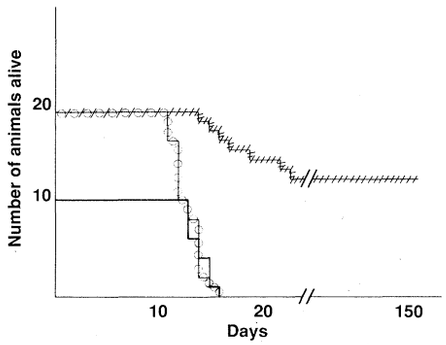

Fig. 2. Effect of treatment with RLCM on the survival of recipient rats after injection of 1×10^5 C51 cells intraperitoneally. For details see text. (ǂ) Group I, treated with RLCM; (○) group II, treated with inactivated RLCM; (——) group III, treated with serum-free medium.

A similar phenomenon may account for rejection or successful "take" in the transfer of rat chloroleukemia. To examine this hypothesis, we first determined the relation of successful transferability of MIA C51 cells to the number of cells injected and to the animal's age. Forty 7-day-old rats were randomized into four groups of ten rats each. Rats in group I each received 1×10^5 cells intraperitoneally, and those in groups II, III, and IV received 2.5×10^4, 2×10^4, and 1×10^4 cells, respectively (Fig. 1). All rats in group I had died of leukemia within 16 days. All rats in group II had died of leukemia within 32 days. Of the ten rats in group III, seven had died of leukemia within 36 days, and the other three showed no signs of disease. None of the rats in group IV developed leukemia. Six 21-day-old rats each received 2.5×10^4 cells intraperitoneally; none developed leukemia. This is in sharp contrast to the 100% of the 7-day-old rats dying of leukemia after receiving a similar number of cells (group II). These results indicated that the maximum leukemia cell load that the 7-day-old rats can "reject" is around 10^4 cells and the minimum number of cells that would bring about 100% death from leukemia is 2.5×10^4 cells, a cell load that is rejected by the 21-day-old rats.

To determine whether rejection correlates with terminal cell differentiation, we placed the C51 cells to be transferred into diffusion chambers intraperitoneally instead of injecting them directly. When diffusion chambers containing 10^4 cells were placed in 7-day-old rats (Table 2), 78% of the cells were differentiated at 72 hours, with only 50% increase in the total number of cells. When the cell load in the diffusion chamber was raised to 2.5×10^4 cells, the cell number increased to 7.8×10^5 (31-fold) and only about 5% of the cells were differentiated. By contrast, no cell growth was observed in diffusion chambers containing 2.5×10^4 cells placed in 21-day-old rats, and over 90% of the cells were differentiated. These results indicated that the 21-day-old rat was capable of generating the required level of DF to effect total differentiation of the leukemic cell load and thus reject the graft. On the other hand, 10^4 cells appeared to be the maximum leukemic cell challenge that the 7-day-old rat could successfully reject by terminal differentiation of the injected cells.

That the 21-day-old rat is able to generate more DF was shown by the following experiment. Conditioned media were prepared from organs of 7-day-old and 21-day-old rats, six animals each (60 mg of tissue per milliliter of serum-free DMEM), and the total DF activity generated was determined as described in Table 1. The combined total DF activity of conditioned media from lungs, kidneys, liver, spleen, and thymus of 21-day-old rats was 32,018 units ± 363 SEM compared to only 4,585 units ± 199 SEM for 7-day-old rats. Even when adjusted to total body weight, the 21-day-old rats had twice as much DF activity.

From these data, one would predict that the development of leukemia from a given chloroleukemia cell load in the rat can be aborted by treatment with DF. Forty-eight 7-day-old rats were each injected with 10^5 cells intraperitoneally and randomized into three groups. Group I (19 rats) received 0.2

Table 1. MIA C51 cells cultivated as previously described (*14*) were plated at the indicated cell number in 1 ml of medium at a fixed level of RLCM (*15*) (500 units of differentiation activity). At 48 hours the cells were counted and cytospin slide preparations were made and stained with Wright Giemsa for morphological examinations. Differential counts were made on 200 cells. Viability was by the trypan blue exclusion method. Phagocytosis of latex beads was assessed as described (*16*). Each value represents the mean of six cultures ± SEM. Differentiation activity was assayed as follows: The C51 cells were seeded in 35-mm petri dishes (500 cells per plate) containing 1 ml of 0.3% agar in DMEM-FCS. After 3 days of incubation, assay samples diluted to 0.2 ml were added to the plates as overlays, and incubation was continued for another 4 days. The numbers of colonies were counted and the numbers of dispersed colonies were scored separately. A unit of differentiation activity represented one dispersed colony per 100 colonies.

Cells	Number plated (cell/ml)	Cell count at 48 hours	Growth inhibition (%)	Differentiation (%)	Viable cells (%)
Control	10,000	$1.4 \times 10^5 \pm 9{,}000$		0	100
DF added	10,000	$1.1 \times 10^4 \pm 1{,}500$	92.5 ± 3	100	98 ± 1
Control	20,000	$3.5 \times 10^5 \pm 15{,}000$		0	100
DF added	20,000	$1.7 \times 10^5 \pm 7{,}000$	52 ± 5	60 ± 3	99 ± 0.5
Control	40,000	$6.1 \times 10^5 \pm 25{,}000$		0	100
DF added	40,000	$5.5 \times 10^5 \pm 21{,}000$	10 ± 2	5 ± 2	98 ± 1
Control	80,000	$1.3 \times 10^6 \pm 55{,}000$		0	100
DF added	80,000	$1.2 \times 10^6 \pm 57{,}000$	8 ± 2	1 ± 0.5	98 ± 1

Table 2. The relation of percent cell differentiation in diffusion chambers to animal age and the initial number of cells in the chamber. The technique of diffusion chamber implantation (*17*) was briefly as follows. Diffusion chambers were filled with the indicated number of C51 cells in 0.1 ml of serum-free DMEM and then implanted in the abdomen (one chamber per rat). At 72 hours, the animals were killed, chambers were removed, and the chamber contents were collected as described earlier (*17*). Cell counts, viability, and phagocytosis were carried out as described in Table 1. Each value represents the mean ± SEM.

Age of animal (days)	*N*	No. of cells in chamber	Cell count at 72 hours	Differentiation (%)	Phagocytic cells (%)	Viable cells (%)
7	7	25,000	$7.8 \times 10^5 \pm 61{,}000$	5.5 ± 2	3 ± 1	100
7	6	10,000	$1.5 \times 10^4 \pm 1{,}200$	78 ± 4	65 ± 3.5	98 ± 1
21	6	25,000	$1.8 \times 10^4 \pm 1{,}350$	92 ± 5	90 ± 2.5	100

ml of crude RLCM (10,000 DF units) intraperitoneally, beginning 8 hours after cell injection and continuing every 8 hours for a total of 14 doses. Group II (19 rats) received RLCM that had been rendered inactive by being heated at 70°C for 2 hours, and group III (10 rats) received serum-free medium. All rats in groups II and III died of leukemia within 16 days (Fig. 2). Autopsy in each animal showed leukemic ascites and grossly visible diffuse peritoneal leukemic infiltrates. Three animals in group I died of leukemia in 16 days; another four were dead at 23 days; and 12 animals (63%) were alive at 150 days without evidence of disease. Thus the development of leukemia was aborted in 63% of the rats by treatment with RLCM.

Finally, in order to demonstrate that RLCM exerts its action by inducing differentiation of the transplanted C51 cells, we placed diffusion chambers containing 1×10^5 C51 cells each in twelve 7-day-old rats and randomized them into two groups of six rats each. Group I received 0.2 ml of RLCM intraperitoneally every 8 hours beginning 8 hours after placement of diffusion chambers and continuing for a total of five doses. Group II received serum-free medium and served as controls. At 48 hours 94% of C51 cells in group I were differentiated with >90% viability but without growth, whereas cell number in the control rats increased approximately sevenfold with no evidence of differentiation. These results confirmed that RLCM exerted its action in vivo by inducing terminal differentiation of the transplanted leukemic cells. However, since differentiation is accompanied by loss of proliferative capacity, the presence of an additional independent growth-inhibiting activity in the RLCM cannot be excluded.

The results described herein strongly suggest that in the rat chloroleukemia model, the success or failure of transplantability of a given tumor load in young rats (≤21 days old) is a function of the amount of DF which the animal can generate. Rejection of the tumor can be accomplished through terminal differentiation of the injected cells. The relevance of these observations to human myelogenous leukemia remains uncertain at present. However, given the fact that human leukemic cells respond to DF in vitro, our observations in this rat model suggest the possibility that maintenance of the undifferentiated (leukemic) state in vivo may at least in part be due to the host's limited capacity to generate the appropriate level of DF. It is possible that some CSFs act primarily as proliferation factors in vivo whereas others like G-CSF stimulate differentiation. Proliferation predominates if the ratio of proliferation to differentiation activity is increased—particularly if the leukemic cell is less responsive to DF, thus requiring higher DF levels. A more attractive hypothesis involves a key role for the leukemic cell itself. The leukemia cell may help maintain itself in the undifferentiated state (and therefore at a proliferative advantage) by feedback inhibition of differentiation, perhaps through the production of soluble mediators. The soluble mediators could exert their action at the leukemia cell receptor level to competitively inhibit the action of DF. In this regard, when concentrated conditioned medium from C51 cells is added to cultured C51 cells along with DF, it does not block differentiation. However, this does not exclude the possible production of blocking factors in vivo. Alternatively, soluble factors from leukemic cells could act on DF-producing cells to block DF production. The nature of factors produced by the host and the leukemic cells and the interplay among the factors remain to be defined.

Whatever the exact mechanism involved in the maintenance of the undifferentiated (leukemic) state in vivo, our observations suggest that the administration of DF with or without cytoreductive chemotherapy is a promising treatment of human myelogenous leukemia.

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Signal for Attachment of a Phospholipid Membrane Anchor in Decay Accelerating Factor

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Decay accelerating factor (DAF) belongs to a novel group of membrane proteins anchored to the cell surface by a glycophospholipid membrane anchor that is covalently attached to the carboxyl terminus of the protein. The last 37 amino acids of membrane DAF, when fused to the carboxyl terminus of a secreted protein, are sufficient to target the fusion protein to the plasma membrane by means of a glycophospholipid anchor. This approach provides a novel means of targeting proteins to the cell-surface membrane.

WHILE MOST INTEGRAL MEMbrane proteins are anchored to the lipid bilayer by a hydrophobic polypeptide transmembrane domain, a small class of proteins is held in the plasma membrane by an unusual mechanism involving covalent attachment of a complex phospholipid anchor to the carboxyl terminus of the protein. Proteins anchored in this way include Thy-1 (*1, 2*), the variant surface glycoproteins of African trypanosomes (*3*), acetylcholinesterase (*4*), 5′ nucleotidase (*5*), and decay accelerating factor (DAF) (*6, 7*). Attachment of the anchor, which contains glycosylated phosphatidylinositol (PI) and ethanolamine, apparently occurs after proteolytic removal of 17 to 31 carboxyl-terminal residues from the protein (*8, 9*). The signal that dictates processing and attachment is not known. In this report we show that the last 37 amino acids of DAF fused to the carboxyl terminus of a secreted protein (i) direct attachment of a PI-anchor and (ii) target the fusion protein to the plasma membrane. Use of this signal provides a novel means of anchoring a protein to the outer cell membrane in such a way that the entire protein projects into the extracellular space.

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