Summary

Normal function of hemopoietic tissues depends on a continuous supply of functional mature cells through maturation of progenitor cells and stem cells. Leukemia is believed to be resulted from a disturbance in this maturation process. In mouse, the stem cells are measured by spleen colony technique (CFU-S measurement) and the progenitor cells by in vitro culture technique (CFU-C measurement). Sachs and his colleagues reported that human leukemic cells can be induced to mature in the in vitro culture system in the presence of colony-stimulating activity (CSA). These findings were partially confirmed and extentioned to many leukemic cases. Our attempt to establish a relationship between the type of leukemia and nature of leukemic cell maturation in culture was not successful. Using RLV induced leukemia in mice as a model system, the effect of RLV infection on the number of CFU-C and CFU-S was studied. Our results showed that there was an early transitory depression of CFU-S and a long-term stimulation of CFU-S following RLV infection. These results suggest that the earliest event following RLV infection is the commitment of CFU-S to CFU-C and therefore CFU-S are target cells of RLV. However, these conclusions do not exclude the possibility that the other cell types could also be target cells.

The mature granulocytes derived from leukemic cells show budding virus particles. The budding virus particles were also found in mature granulocytes and anucleated erythrocytes obtained from RLV infected mice.
I. Concept of a Stem Cell

The normal function of hemopoiesis is carried out by various kinds of mature blood cells, such as erythrocytes, granulocytes, megakaryocytes and lymphoid cells. The life span of these mature cells are relatively short. Therefore, a continuous supply of these mature cells is essential for the normal function of the hemopoietic tissue. These mature cells are derived from a class of early undifferentiated cells through a sequence of differentiation and proliferation. This class of early undifferentiated cells are termed "stem cells" (1, 2). It is important to emphasize that the stem cells are defined according to their functions. The morphology of this class of cell is not known.

A stem cell should have the following properties. It should be capable of 1) proliferation, 2) differentiation, 3) self-renewal upon proliferation and 4) response to physiological demands. If a stem cell is capable of giving rise to more than one type of differentiated cells, it is termed a multipotent stem cell whereas if it is capable of giving rise only to one type of differentiated cell it is termed unipotent stem cell. A multipotent stem cell by definition is a precursor of a unipotent stem cell (progenitor cell). Their relationship is diagrammatically illustrated in Fig. 1. This figure depicts the transition from stem cell to progenitor cell and the transition from progenitor cell to differentiated cell which are dependent on the presence of specific factors. The production of these regulatory factors presumably are under physiological control.

Any cause which disturbs the process of maturation shown in Fig. 1 will naturally impair the normal function of hemopoietic tissue. Leukemia is believed to result from a disturbance of this maturation process (3–8). There are three possible outcomes that could result from this disturbance (see Fig. 2). These are: (A) blockage of differentiation (4–8), (B) aberration of differentiation and (C) reversal of differentiation. In (A) and (C), the leukemic cells should represent some cell types present during the course of normal differentiation and in (B), the leukemic cells should be different from any of the known cell types. The models in this figure also indicate that the target cells of leukemogenesis (with respect to their stage of differentiation) are not well defined. So far there is no conclusive evidence to support or rule out any of these possibilities or to implicate a particular stage of cell differentiation as the target cell for the initiation of leukemogenesis.
II. Measurement of Stem Cells

Currently there are two functional assays available, one for stem cells, the other for progenitor cells. Both of them are based on clonal methods. They are summarized in Table 1. In mice, stem cells can be assayed by the spleen colony technique (2). The assay is performed by injecting an adequate number of nucleated cells into a lethally irradiated recipient. After a period of 8–10 days, the spleen is removed. The macro-nodules which develop on the surface of the spleen are then counted. These nodules are called spleen colonies. Each colony was shown to derive from a multipotential stem cell as judged by a chromosomal analysis using radiation induced chromosomal aberrations as markers (9) and by studying the cellular composition of each colony (10, 11). Apparently, the development of a spleen colony represents a response of a stem cell to a physiological demand after irradiation and is regulated by the microenvironment (12) which contain cells that produce short-term mediated humoral factors (13).

The progenitor cell for granulocytic cells can be assayed by an in vitro method originally developed by Bradley and Metcalfe (14) and by Pluznik and Sachs (15). A single cell suspension is prepared in a semi-soft medium (either 0.3 % agar [14, 15] or 0.8 % methyl cellulose [16]) in the presence of colony stimulating activity (CSA). The CSA is supplied either in the same layer as the cell suspension (16) or in a separate layer of 0.5 % agar containing conditioned medium or feeder cells (14, 15). The conditioned medium was generally prepared by growing cell population containing factor producing cells in culture medium (either in the presence or in the absence of

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CSA = colony-stimulating activity for granulocytic colonies.
CFU-S = Colony forming unit on spleen.
CFU-C = Colony forming unit of granulocytic cells in culture.
CFU-E = Colony forming unit of erythropoietic cells in culture.
MODELS OF LEUKEMIA AS A DISEASE OF DIFFERENTIATION

1. BLOCKAGE OF DIFFERENTIATION

2. ABERRATION OF DIFFERENTIATION

3. REVERSAL OF DIFFERENTIATION

Fig. 2: Abbreviations:
S, P, and D are the same as those of Figure 1; L: leukemic cells; solid arrow, direction of normal differentiation; open arrow, mode of disturbance of normal differentiation.
fetal calf serum) for 4 to 6 days (15). The number of colonies is generally recorded 8 to
10 days after plating. At this stage, the colony size may be as large as $10^4$ cells. The
colonies are classified as granulocytic or macrophage colonies depending on the
composition of cells in the colonies. Both of these colony types contain cells at various
stages of maturation. If erythropoietin is present in this culture system the second day
after plating, some very small colonies containing erythropoietic cells (8–100 cells)
may develop (17, 18). In mice, colony formation is strictly dependent on the addition
of CSA. CSA for assaying mouse colony forming cells can be obtained from mouse
sera (19), human urine (20), medium from cultured fibroblasts (21), and from
embryonic primary culture cells (22). CSA are glycoproteins which can be purified by
use of concanavalin A sepharose chromatography. The molecular weight estimates of
this activity have indicated heterogeneity ranging from 10,000 to 190,000 (23). It is
not known whether CSA is a population of heterogenous glycoproteins or a complex
protein containing many subunits.

This in vitro culture assay for progenitor cells has been successfully applied to
human blood cells (24–27). Similar to colonies derived from mouse cells, the colonies
derived from human blood cells (CFU-C) also contain granulocytic cells at various
stages of maturation, but in general, the colony sizes of human origin are much smaller
(50–1,000 cells) than those of mouse origin. The specificity of CSA required for the
in vitro colonies of human origin also differs from that of mouse origin. In most cases,
CSA prepared from human cells can stimulate the growth of colonies of both human
and murine origin, but the CSA prepared from mouse cells stimulates only mouse
colonies. One difficulty in obtaining a quantitative measurement of human colony
forming cells is the lack of an absolute requirement for exogenous CSA for colony
formation. This is due to a contamination of factor-producing cells in the human
blood cell population. A successful attempt to separate the factor producing cells
from the colony forming cells has recently been reported (28). Obviously, separation
of these two cell populations is essential for a successful study of the maturation
process.

III. Maturation of Human Leukemic Cells in Culture

One of the most important observations made during the course of studies of
human progenitor cells in culture is that apparently some leukemic blast cells can be
induced to mature in culture in the presence of appropriate CSA to apparently mature
normal appearing granulocytes (25, 29). The evidence that these mature cells are
derived from leukemic cells is based on some chromosomal analyses (30, 31). But the
argument is not conclusive, because one still cannot rule out the possibility that the
mature cells are derived from normal cells which contains the same chromosomal
aberration. However, since the disclosure of maturation of human leukemic cells in
culture, many studies have been carried out to establish a pattern as to which type of
leukemic cells seems to be “inducible” to mature. The result of these studies are also
inconclusive. A summary of such studies carried out in our laboratory in the past year
is shown in Table 2. The capacity of colony formation varies with various types of
leukemia and also varies within the same type of leukemia. This variation might be due
either difficulty in assuring reproducibly standardized in vitro colonies assay
Table 2. Differentiation of Leukemic Cells in Culture

<table>
<thead>
<tr>
<th>Cell Source</th>
<th>Colony Forming Ability</th>
<th>Differentiation of Colony Cells</th>
<th>Factor Producing Ability</th>
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<tr>
<td>Normal</td>
<td>++</td>
<td>yes</td>
<td>+</td>
</tr>
<tr>
<td>AML</td>
<td>0 - +++</td>
<td>yes, no</td>
<td>N. T.</td>
</tr>
<tr>
<td>AML (Remission)</td>
<td>++</td>
<td>yes</td>
<td>N. T.</td>
</tr>
<tr>
<td>AMML</td>
<td>++</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>CML</td>
<td>+</td>
<td>yes</td>
<td>N. T.</td>
</tr>
<tr>
<td>CML (Blast Crisis)</td>
<td>0 - +</td>
<td>no, yes</td>
<td>±</td>
</tr>
<tr>
<td>ALL</td>
<td></td>
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1. All of cell sources are bone marrow.
2. Yes: presence of mature granulocytic cells in culture.
   No: absence of mature granulocytic cells in culture.
3. N. T.: Not Tested

systems for human blood cells or a lack of precise standards for classification of leukemia (classifications presently being based almost entirely on morphology). For example, leukemic cells from some patients with acute myelocytic leukemia (AML) are not able to form any colonies while some are able to produce many more colonies than cells derived from the blood or bone marrow of normal individuals. In general, the number of colony forming cells from AML blood is significantly lower than that from normal individuals. Moreover, colonies derived from AML cells sometimes do not contain mature granulocytes. In remission, the efficiency of colony formation and maturation of colony cells returns to normal. The remission is defined as the return of blood cell count to a normal level and disappearance of blast type leukemic cells. In acute monomyelocytic leukemia (AMML), the leukemia cells behave similarly to normal cells with respect to colony formation. In chronic myelocytic leukemia, the leukemia blood cells contain less colony forming cells than that of normal blood cells, and they contain even less colony forming cells when blast crisis occurs. At this stage, most cells in colonies are not mature (however, there are exceptions), patients with acute lymphocytic leukemia do not form any colonies. This is not surprising if the in vitro CSA dependent colonies are, in fact, derived from granulocytic cells. These findings are more or less similar to those reported from other laboratories, e.g., Robinson (32).

We have examined human leukemic cells not only for their ability to respond to factor (CSA) but also for their ability to produce CSA. These results are also shown in Table 2. Leukemic cells are poor producers of CSA. Since this information in the table is still incomplete and since one cannot rule out the possibility of inhibitors of CSA released by leukemic cells, this conclusion is tentative.

It would seem that both colony forming (factor responding) cells (CFC) and factor producing cells (FPC) are required to achieve maturation in vitro and probably in vivo. At the cellular level the defect in leukemia may be of two general types: one, a defect
in factor production, and the other, a defect in response to factor stimulation. Leukemias might be classified in this manner and further, according to the degree of responsiveness to CSA. Reclassification of leukemia based on detectable physiological functions may be helpful in obtaining meaningful conclusions from these in vitro cell maturation systems.

IV. RNA Oncogenic Viruses and Human Leukemogenesis

The apparent inducement of human leukemic cells to develop into seemingly normal mature cells supports the idea that leukemia is due to a disturbance in the maturation process. The cause of this disturbance is not known. However, there are now strong reasons for suspecting a role for type-C RNA tumor viruses or information derived from these viruses in the pathogenesis of the disease. Expression of viral genomes in human leukemic cells either from endogenous genetic information or from an exogenous infection has been recently supported from results of extensive studies in searching for the footprints of viral information in human leukemic cells by Gallo and colleagues and Spiegelman and his colleagues. The evidence for the existence of viral information in human cells is as follows: 1) Virus-like reverse transcriptases have been isolated from some human leukemic cells but not from normal proliferative cells. These enzymes have the biochemical characteristics like most of known mammalian viral reverse transcriptases (33-35). For example, this enzyme activity is sensitive to RNase. The enzymes are able to transcribe the heterogenous part of 70S RNA isolated from primate and mammalian viruses. They prefer (dT)\textsubscript{12-18} (rA)n as template-primer over (dT)\textsubscript{2-18} (dA)n and are able to use (dG)\textsubscript{2-18} (rC)n. These are the characteristics of viral reverse transcriptase (35, 36); 2) The leukemic reverse transcriptase is immunologically related to reverse transcriptase of primate RNA tumor viruses (37, 38). (They were inhibited by antibodies against viral reverse transcriptase especially of primate, but also of murine RNA tumor viruses [37, 38]. This suggests that the human leukemic enzyme shares a common antigenic determinant with primate and murine viral enzymes,); 3) Human leukemic cells contain an RNA sequence homologous to DNA products prepared from an endogenous reaction of mammalian and primate RNA oncogenic viruses (39, 40, 41); 4) The size of the RNA template-primer for the leukemic reverse transcriptase appears to be the same as viral RNA (42, 43); 5) The reverse transcriptase and associated RNA are present in a post-mitochondrial cytoplasmic particle which retains morphological integrity on repeated high speed centrifugation and has a density of 1.15 to 1.18 (43), characteristics typical of type-C RNA tumor viruses. It now appears unequivocal that these particles are viral-related. This information has been recently reviewed in detail elsewhere (44).

V. RNA Oncogenic Viruses and Murine Stem Cells

With the above background in mind, it is evident that studies designed to determine the affect of animal type-C viruses on normal hemopoiesis are of immediate interest. The data described below was derived from our beginning attempts in this direction in mice. One advantage of an animal system is that one can follow a defined course of leukemic development, especially the early events following viral infection. Informa-
Fig. 3: Experimental design for the effect of RLV infection on CFU-S and CFU-C compartments. Eight-week old NIH Swiss mice were used for this experiment. RLV was injected intravenously through the tail vein. To prepare cell suspensions, at least five uninfected and infected mice were used, respectively, Spleen colony technique was performed as described previously (2). The CFU-C was measured according to the procedures described by Pluznik and Sachs (15), except that CSA was prepared from primary culture of BALB/C embryonic cells.

Fig. 4: Effect of RLV infection on CFU-S from marrow and spleens. The spleen colony assay was carried out as described previously (2). The spleens were fixed in Bouin solution. Each point is an average of 7 spleens. Day 0 started with injection of RLV.
of this experiment for spleen colony formation is shown in Figure 4. There is an initial dip in the number of colony forming units (CFU-S) per unit cell number injected both from spleen and marrow cells on the second and third days after injection of RLV. However, the CFU-S per unit cell number then gradually recovered to a normal level. A short period of "overshoot" was observed on the fourth and fifth day after RLV injection. Since the cell number increased during the course of leukemia development, the total CFU-S, in fact, increased more than 10 fold by one month following the development of leukemia.

The effect of RLV infection on the progenitor compartment of marrow is shown in Figure 5. Soon after viral infection, the number of CFU-C (granulocytic progenitor cells) per $10^5$ nucleated cells increased about 10 fold. This level was maintained for a few weeks and then gradually decreased by the fifth week after infection. We did not determine whether CFU-C eventually decreases to a normal or a subnormal level since infected animals started to die by the eighth week after inoculation with RLV. This rise and decline of CFU-C following leukemic development might provide an explanation for the variation in measuring CFU-C in human leukemic cell populations.

Fig. 6: Effect of RLV infection on CFU-C from spleens of uninfected and infected mice. The procedures are the same as those described in Figure 5.
The number of CFU-C may be a function of the stage of leukemic development. Similar results were obtained when spleen cells were used to study the effect of CFU-C upon viral infection. The result of this experiment is shown in Figure 6. This graph was plotted as CFU-C per unit cell number as a function of time. Since spleen cell number increased at least 20 fold during the development of leukemia, the total CFU-C increased at least 120 fold. From a practical point of view, we wonder whether this increase of CFU-C in early infection might be useful as a diagnostic tool for pre-leukemia or an early stage of leukemia.

The effect on the number of CFU-C and CFU-S by RLV were not simply due to an antigenic stimulation since heat-inactivated viruses did not cause such an effect. Some agents such as Freund's complete adjuvant (45), pertussis vaccine (46), phenylhydrazine (47), and endotoxin (48) are able to enhance the number of CFU-C and CFU-S in vivo. However, these effects are transitory and, therefore, are different from the long-term effect of RLV.

Our observation on the initial decrease of CFU-S by RLV infection is in agreement with that reported by Seidel (49) as is our finding on the late effect of CFU-S by RLV infection (49) and this late effect is also similar to that reported by Okunewick, et al. (50). Many interpretations can account for the initial decrease of CFU-S accompanying the increase of CFU-C. One interpretation is that the CFU-S is a target cell of RLV. Upon infection, the stem cells are affected such that their capabilities for spleen colony formation are lost. Now, these infected stem cells are still capable of forming colonies in culture as evidenced by an increase of CFU-C. Perhaps this occurs by a commitment of the stem cell to granulocytic progenitors. However, our results did not rule out the possibilities that the other cell compartment could also be a target for RLV infection.

VI. Properties of Granulocytic Colonies Derived from Murine Leukemic Cells

1) The colonies contain apparent mature granulocytes and colony formation is absolutely dependent on addition of exogenous CSA. We found type-C RNA tumor viruses bud from the membrane of the mature granulocytes. In addition, on examining the other cell types from infected mice, we found that besides early immature cells, both mature granulocytes and anucleated erythrocytes show budding type virus particles. The phenomenon of the budding of virus particles from mature erythrocytes is interesting and puzzling.

2) With the H3-thymidine suicide technique (51, 52), we found that progenitor cells from uninfected and leukemic mice have the same fraction of cells in cycle, — 50% of CFU-C. Thus, the leukemic cell population apparently does not contain a larger fraction of cells in cell cycle. This is in agreement with the notion that leukemia results from a block in leukocyte maturation. This observation could be important not only from a physiological point of view, but also from a chemotherapeutical point of view.

3) Similar to normal granulocytic colonies, the granulocytic colonies which originated from leukemic populations can only be transferred in culture 2 to 3 times. Our attempts to establish a clonal line of colony forming cells has not been successful.
4) An injection of mature colony cells originating from a leukemic cell population induced leukemia in the recipient mice. However, it is not known whether this transplantability of leukemia is due to an infection by viruses associated with the cells or due to the leukemic nature of the transplanted cells. It is worthwhile to emphasize that we noted that several transplantable murine tumor cells all contain type-C virus particles. These cells are L1210 cells (53), M1 cells (54), and leukemic cells from Rauscher leukemia virus infected mouse (data not shown). These viruses may play an important role in the transplantability of these cells.

VII. Conclusion

Leukemia appears to result from a disturbance of the normal differentiation of hemopoietic stem cells. This disturbance might be a blockage, aberration or reversal of differentiation. In some cases, the blockage process of some cells can apparently be removed in culture as evidenced by apparent induction of leukemic cells to produce seemingly normal mature granulocytes in culture in the presence of CSA. This dependence of CSA on the maturation of leukocytes may cast some light on the process of leukemogenesis. The phenomenon of CSA stimulation may provide avenues for studying the process of leukocyte maturation at the molecular level and a potential new approach for the therapy of human leukemia and other blood illnesses. In this respect, however, it is surprising and puzzling that so far there are no reports evaluating in vivo results of treatment of leukemic mice with CSA.

Acknowledgements

This work was in part supported by a contract from cancer therapy area, National Cancer Institute. The authors wish to thank the technical assistance of Craig Snyder and Ellen Hambleton.

References

47. Stohlmann, F. (1973) personal communication.