Modification of the Proliferative and Differentiation Capacity of Stem Cells Following Treatment with Chemical and Viral Leukemogens

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1. Introduction

Following treatment with a leukemogen, a variable length of time, from weeks to years, elapses before the onset of clinically apparent leukemia. The events occurring during this time are still a mystery, but as one approach to this problem we have been investigating the mechanism(s) whereby leukemogens modify the behaviour of the haemopoietic stem cells and the consequences of such alterations in terms of the proliferation and differentiation potential of such treated populations. Since the stem cells are probable "targets" for many leukemogens, our understanding of leukemogenesis, in many respects, lies in the answer we can give such questions.

These studies have been greatly facilitated by the development, in the last few years, of suitable systems whereby the pluripotent haemopoietic stem cell (CFU-S) can be maintained in vitro for several months (Dexter and Testa, 1976; Dexter et al., 1977) and where the progeny of such stem cells can be induced to undergo proliferation in soft-gel media to form the variety of haemopoietic elements – the lymphocytes, granulocytes, megakaryocytes and erythroid cells (Metcalf et al., 1975b; Sredni et al., 1976; Bradley and Metcalf, 1966; Pluznik and Sachs, 1966; Metcalf et al., 1975a; Stephenson et al., 1971). The development of these systems, and the demonstration that under appropriate conditions haemopoietic cells can be readily transformed in vitro (Rosenberg et al., 1975; Rosenberg and Baltimore, 1976; Dexter and Lajtha, 1976; Dexter, Scott and Teich, 1977) provide valuable tools in elucidating both normal and abnormal haemopoiesis. In this respect we show the effects of several chemical and viral leukemogenic agents upon haemopoietic stem cell proliferation and differentiation in bone marrow cultures and demonstrate that such treatments can induce a variety of abnormal haemopoietic conditions in vitro.

2. Materials and Methods

2.1. Bone Marrow Cultures: These have been described in detail elsewhere (Dexter and Testa, 1976; Dexter et al., 1977). The content of a single mouse femur are flushed into culture flasks containing 10 ml of Fisc[illegible]'s medium

+25% horse serum (Flow Labs.). No attempt is made to obtain a single cell suspension and the cultures are maintained at 33°C or 37°C (as indicated) and fed weekly by demi-depopulation. After three weeks, the adherent layer established is inoculated with growth medium containing a further 10^7 syngeneic marrow cells. These are considered the cultures proper, and are subsequently maintained on a feeding regime of demi-depopulation at weekly intervals. The non-adherent cells removed in the growth medium are assayed for stem cells and the "committed" progenitor cells using established procedures (Dexter and Testa, 1976).

2.2. Mice: BALB/C, C57BL/6, DBA/2 and (C57BL/6×DBA/2) F_1 (BDF_1) mice were used. Cultures were established using femoral bone marrow of mice 6–8 weeks of age.

2.3. Chemical Leukemogen Treatment: 6–8 week old BDF_1 mice were treated with a single i.v. injection of methylnitrosourea (MNU) dissolved in ice cold 0,9% NaCl. Three weeks later, some mice were killed, and adherent layers established from the femoral bone marrow cells. Three weeks later a further batch of the same mice were killed, the femoral marrow cells removed and 10^7 cells inoculated onto the established adherent layers.

2.4. Viral Leukemogen Treatment: These included the NB-tropic pool (F-B strain) of Friend leukemia virus complex, provided by F. Lilly. Abelson virus (provided by E. Lennox) and the FBJ osteosarcoma virus (provided by Dr. C. Reilly). Infectivity was measured by established techniques (Rowe et al., 1970; Scher and Siegler, 1975).

2.5. Leukemogenic Capacity of Cultured Cells: Non adherent cells removed at the time of refeeding were injected into syngeneic adult mice. Occasionally neonates were used. The animals were palpated twice weekly for signs of splenomegaly or lymphodenopathy. When leukemia was suspected, the animal was killed – tissue was removed for microscopic examination – and the suspected leukemic cells were retransplanted.

3. Results

3.1. Characteristics of Normal Bone Marrow Long Term Cultures: These have been described in detail elsewhere (Dexter et al., 1977). Cultures are characterised by maintenance of proliferation of stem cells for 10–12 weeks, and concomitant production of CFU-C. Initially there is an extensive granulopoiesis, followed by production of mononuclear phagocytic cells. The cells do not produce leukemia when injected into adult or neonatal syngeneic recipients.

3.2. Culture of MNU-treated Bone Marrow Cells: The characteristics of these cultures are shown in Table 1. An initial granulopoiesis is followed by an ac-

Table 1. Characteristics of long term cultures of MNU treated bone marrow cells

Weeks cultured	Cell Count $\times 10^5$	CFU-S	Morphology B	G	Mo
1	32.0	1000	16	80	1
5	20.0	370	11	85	2
7	12.0	190	30	66	4
10	8.0	234	37	61	3
17	7.0	150[a]	25	0	75
22	5.0	0	20	0	80

[a] Atypical, small undifferentiated
B = Blast cells G = Granulocytes (all stages) Mo = Phagocytic mononuclear cells

cumulation of blast cells (for at least 22 weeks) and mononuclear phagocytes. Apparently normal CFU-S are maintained for the first 10–12 weeks (able to form erythroid, granulocytic and megakaryocytic spleen colonies and protect mice from potentially lethal radiation) – but at later times (12–17 weeks) the spleen colonies formed contain undifferentiated blast cells. Injection of cells cultured for 0–12 weeks does not produce leukemia, whereas injection of cells cultured for 13–22 weeks will regularly produce a transplantable leukemia (Table 2). These leukemias, which are of donor origin (chromosome analysis), show no detectable θ antigen or surface Ig, and are best classified as null-cell type.

Table 2. Culture of MNU treated bone marrow cells. Leukemogeneic potential

Weeks cultured	Virus production[a]	Leukemic mice/ mice injected	Average latent period (weeks)
0	ND	0/30	—
1–5	$8\times 10^1 - 2\times 10^3$	0/59	—
6–12	$1\times 10^2 - 2\times 10^3$	1/20	26
13–17	$4\times 10^2 - 8\times 10^2$	10/21	22[b]
18–24	$2\times 10^2 - 2\times 10^3$	9/17	22[b]

[a] PFU/ml culture medium
[b] θ and sIg negative, TdT positive Donor origin (chromosome analysis)

The cultures were regularly assayed for the production of infective virus and found to be low level producers from the first weeks. However, there was no correlation between the production of infectious particles and the ability of the cultured cells to produce leukemia.

3.3. Infection of Marrow Cultures with Leukemia Viruses: The results of these studies are summarised in Table 3. Infection of genetically susceptible BDF_1 cultures with Friend leukemia virus complex generates three biologically distinct "variants", depending upon whether the whole viral complex (SFFV and LLV) or only the LLV component, is replicated. When both viral

Table 3. Biological effects of infection of long term bone marrow cultures with various murine leukemia viruses

			Characteristics				
Cultured cells	Virus inoculated	Replication	Left[a] shift	Extended CFU-C production	Transformed agar CFC	Extended CFU-S production[b]	Leukemic potential
BDF_1	FLV	1. SFFV LLV	+	+	–	+	Viral (Erythroleukemia)
		2. LLV	+	+	–	+	Non-leukemic
		3. LLV	+	–	+	–	Myelomonocytic leukemic
C57	FLV	LLV	–	–	–	–	Non-leukemic
BALB/c	Abelson	Abelson	–	–	+	–	Lymphoid leukemic
DBA/2	FBJ	FBJ	+	–	+	–	Myelomonocytic leukemic

[a] increase in "promyelocytes"
[b] Atypical-limited differentiation

components are replicated the cultures are characterised initially by an extensive granulopoiesis, followed by a shift to the left (especially an increase in promyelocytes), an extended maintenance of CFU-C (normal in their responsiveness to colony stimulating activity – CSA) and prolonged production of CFU-S. For the first 8–10 weeks these CFU-S are apparently normal-forming erythroid, megakaryocytic and granulocytic colonies, but after 10 weeks they become limited in their differentiation capacity and form spleen colonies containing *only* granulocytes and megakaryocytes. Injection of the cultured cells into syngeneic mice gives rise to an erythroleukemia of host origin (presumably representing infection and transformation of "erythroid" host cells by the viral complex liberated from the cultured cells).

Sometimes, following FLV infection, only the LLV component is replicated causing: –

a) an initial granulopoiesis, followed by a shift to the left, loss in CFU-S, production of transformed CFU-C (which grow independent of CSA and form compact myelomonocytic colonies) and injection of the cells into adults or neonates produces a rapidly progressing leukemia of myelomonocytic type. The cells grow autonomously of the adherent layer and can be established as permanently growing cell lines.
b) Initial extensive replication of normal CFU-S and CFU-C followed by a shift to the left, extended production of normal CFU-C, and proliferation of CFU-S, again limited in their differentiation capacity. These cells will *not* produce leukemia (even when injected into neonates) – and can also be established as permanent cell lines.

Infection of C57BL/6 cultures with FLV, although leading to extensive LLV replication, does not produce the biological effects described above.

As previously reported (Teich and Dexter, 1978) infection of susceptible cells with Abelson virus leads to a rapid transformation and accumulation of leukemic blast cells. Initially, the growth of these cells is dependent upon the marrow derived adherent population – but subsequently the cells develop autonomously, and will grow in the absence of known stimulating factors.

Finally, we have recently found that infection of DBA/2 cultures with FBJ osteosarcoma virus may give a situation similar to that seen after FLV infection *i.e.* a shift to the left and the development of a leukemic myelomonocytic cell population.

4. Discussion

The results demonstrate that following treatment with leukemogenic chemicals or viruses a series of changes occur in the proliferative and differentiation capacity of stem cells – often terminating in leukemic transformation. *All* cultures initially (first few weeks) show normal granulocyte maturation and production of normal CFU-S and CFU-C. Injection of the cells, at this time, *will not produce leukemia.* Subsequent changes depend upon the treatment given and – in the case of viruses – the response of the cells to the leukemogen. MNU treated marrow cultures show a production of atypical

"CFU-S" and an accumulation of undifferentiated blast cells. These changes in the culture seem to parallel the ability of the cells to produce leukemia, since maximal leukemic activity is demonstrated by cells cultured for 13 to 24 weeks. This indicates that a period in culture is "essential" for some change to occur in a potentially leukemic cell population. However, the long latent period in vivo indicates that after culture "conditioning", an essential part of the leukemic process is "host-conditioning". Of some interest is that the leukemia inducing ability of the cultured cells is not simply related to the expression of endogenous virus.

In the Friend virus infected cultures, in particular those showing only replication of the LLV component, proliferation of apparently normal CFU-S and CFU-C is followed by either a myelomonocytic leukemic transformation or production of "atypical" CFU-S with extensive replication capacity in vitro. While these cells do not produce leukemia in neonates, little is so far known of their capacity to reconstitute (in the long term) the haemopoietic systems of mice. Will they, for example, produce a chronic granulocytic leukemia after several weeks? A major obstacle in such studies is their limited differentiation capacity (being defective for erythropoiesis – and probably for lymphopoiesis – and the consequent difficulty in keeping reconstituted irradiated mice alive) but studies currently underway using hypertransfused mice, housed under sterile conditions, should give us some information on this point. Nonetheless, FLV cultures (as with MNU cultures) again show this pattern of initial normal non-leukemic haemopoiesis fallowed by the development of leukemic cell populations or production of atypical stem cells. Similarly when infection of cultures with Abelson leukemia virus or FBJ osteosarcoma virus is performed.

It will be of great interest to determine whether these changes are brought about by virally induced malfunction in the adherent haemopoietic microenvironment or in the stem cells themselves. Using appropriate combinations of genetically resistant environments and susceptible stem cells, such studies are now possible. Also, we need to define more clearly the role of viruses in such transformation. Is the original virus active in transformation or do such events represent a recombinational process between the virus and part of the host cell genome (xenotropic virus?) as suggested by Troxler et al., 1977. Certainly, increasing evidence for the role of such recombinations has emerged (Elder et al., 1977) and analysis of any recombinants produced in the long term marrow cultures is currently being performed.

Of particular interest is our finding – and others previously reported (Moore and Dexter, 1978) of the progressive changes occurring in the cultures prior to transformation. Hopefully, these are a reflection of similar processes operating in vivo, but which can now be analysed in our in vitro system.

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