Haematology and Blood Transfusion Vol. 26
Modern Trends in Human Leukemia IV
Edited by Neth, Gallo, Graf, Mannweiler, Winkler

Genetic and Oncogenic Influences on Myelopoiesis

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A Introduction

An increased understanding of hemopoietic regulation has provided insight into the pathophysiology of leukemia in man and other species. This may be illustrated if we consider the use of leukemic cell lines as models pointing to specific defects which may confer a malignant phenotype. An additional consequence of studying such cell lines has been the insight obtained into the regulatory biology of normal hemopoiesis. I have elected to document this thesis by considering information we have obtained by studying the murine myelomonocytic leukemic cell line, WEHI-3. This myelomonocytic leukemia was detected in a Balb/c mouse which had undergone mineral oil (parrafin) injections intended to induce plasma cell tumor development. (Warner et al. 1969; Metcalf et al. 1969). The tumor was composed of a mixed population of monocytic and granulocytic cells. On transplantation of the tumor, four distinct sublines developed, two of which retained the original chloroma appearance and were distinguishable by karyotype (one diploid and one tetraploid). The other two nonchloroma sublines were also distinguishable karyologically, because one had a hypodiploid 39 stemline. Chromosome marker studies in vivo and DNA-content studies on cells from mice carrying the tetraploid subline confirmed that in this leukemia both the monocytic and granulocytic cells are neoplastic, indicating the existence of a neoplastic stem cell capable of differentiation into both cell series. Serum and urine samples from mice carrying this tumor contained high levels (frequently over 200 μg/ml) of muramidase, and cell suspensions of the solid tumor also contained this enzyme. This tumor therefore fulfills all the criteria applied to human myelomonocytic leukemia and proved a useful laboratory model for this type of leukemia. Tumor cells could proliferate in agar to form mixed colonies of granulocytes and macrophages, and both colony size and plating efficiency were significantly increased in the presence of an exogenous source of colony stimulating factor (Metcalf et al. 1969; Metcalf and Moore 1970). Individual colonies were capable of self renewal upon in vitro recloning and can be considered as leukemic stem cells, since individual colonies implanted in vivo into the spleen or kidney produced progressively growing tumors with the same morphology as the original WEHI-3 tumor (Metcalf and Moore 1970). Recent studies have shown that pure GM-CSF consistently increased the proportion of colonies exhibiting partial or complete differentiation in agar culture (Metcalf 1979). Serial recloning of WEHI-3 colonies in the presence of GM-CSF showed that the colonies differentiated completely and self replication of colony forming cells was suppressed; however, clonal instability was evident, since even in the continuous presence of GM-CSF many colony forming cells still generated cells able to form undifferentiated colonies. The primary tumor and early passage generations could be considered as in a conditioned state due to the dependence of in vitro proliferation upon endogenous or exogenous provision of CSF. The former characteristic of endogenous production of CSF by WEHI-3 cells was evident both in vivo and in vitro (Metcalf and Moore 1970) and has provided one of the most valuable features of this leukemic model. The subsequent history of WEHI-3 follows its adaptation to culture as a continuous cell line and the derivation of this cell line merits

consideration. Sanel (1973) obtained the WEHI-3 subline B (the hypodiploid line) at the 35th in vivo passage and maintained this by intraperitoneal passage in NIH Balb/c mice, reporting that it did not deviate from the original description through a further 50 passages. It is also of interest that Sanel (1973) reported a preponderance of immature C-type particles in WEHI-3 by electron microscopy with high titers of infectious NB-tropic virus of the Friend-Moloney-Rauscher subgroup. (It should be noted that the present WEHI-3 B cell line appears to be devoid of detectable C-type virus). A cell line was developed by Ralph et al. (1976) from the WEHI-3 B subline of Sanel at the 125th passage and all subsequent reports are based on the properties of this cell line which has been maintained in our laboratories at Sloan-Kettering since 1975.

A number of properties of this cell line are shown in Table 1, and what is most striking is the retention of the capacity to produce a wide spectrum of biologically relevant molecules that influence hemopoiesis and immune responses. While it may be argued that production of these various regulatory macromolecules reflects oncogenic transformation, it should be noted that all the features of the cell line are features displayed by subpopulations of macrophages under appropriate stimulation. Indeed, the most neoplastic feature of the cell line is that most of the factors are produced constitutively rather than as a result of lymphokine or adjuvant induction.

Table 1. Properties of the WEHI-3 myelomonocytic cell line

1. Partial retention of ability to differentiate to macrophage and granulocyte.
2. Fc and C receptor positive, phagocytic (Ralph et al. 1977). Thy-1 positive.[a]
3. Produces lysozyme (Ralph et al. 1976) and plasminogen activator.[a]
4. Growth inhibited 50% by 0.004 μg/ml LPS (Ralph and Nakoinz 1977), by $10^{-9}M$ prostaglandin E (Kurland and Moore 1977), and by 5 μg/ml tumor necrosis factor (Shah et al. 1978).
5. Produces GM-CSF (Ralph et al. 1978), macrophage (M) – CSF, neutrophil (G) – CSF (Williams et al. 1978), and eosinophil CSF (Metcalf et al. 1974).
6. Produces megakaryocyte-CSF (Williams et al. 1980) and Burst-promoting activity.[a]
7. Produces LAF (Interleukin I). (Lachman et al. 1977).
8. Produces endogenous pyrogen (Interleukin I?) (Bodel 1978).
9. Produces prostaglandin E (Kurland et al. 1979).
10. Produces mast cell growth factor (Yung et al., to be published).
11. Has receptors for and responds to lactoferrin inhibition (Broxmeyer and Ralph 1977).

[a] Unpublished observations

B. Properties of G-CSF and M-CSF Produced by WEHI-3

Activities in WEHI-3-conditioned medium (WEHI-3 CM) stimulated granulocyte and macrophage colony formation over an approximately 100-fold dilution of an eight fold concentrate of serum-free conditioned medium, and with optimal concentrations of CM approximately 40–50 granulocyte-macrophage colonies could be stimulated per 10^4 cells plated. Partial separation of the activities stimulating the formation of granulocyte and macrophage colonies can readily be obtained by passing concentrated WEHI-3 CM through a DEAE Sephadex (A-25, Pharmacia) column. One type of colony stimulating activity is found in the break through volume and another in the bound fraction which is eluted with 1 M NACl in equilibrating buffer (Williams et al. 1978). The morphology of colony cells stimulated by increasing dilutions of the break through fraction was exclusively macrophage. In contrast, high percentages (greater than 90%) of purely granulocytic colonies were stimulated using low concentrations of the break through fraction. When the break through fraction and eluate were mixed, dose-response curves identical to the unfractionated material were obtained, suggesting that the two entities acted independently in stimulating colony formation. In subsequent studies we have used this semipurified neutrophil colony stimulating activity and will refer to it as "G-CSF".

Prostaglandins of the E Series (PGE) inhibit myeloid colony formation (Kurland et al. 1979; Kurland and Moore 1977), and biosynthesis of PGE by normal or neoplastic macrophages and monocytes may be of significance to hemopoietic regulation. Using WEHI-3 CM as a source of CSF, we have shown that PGE inhibition is selective and that physiologically relevant suppression is restricted to colonies

PGE_1 (Concentration)	Colonies % of control			
	Total	Macrophage[a]	GM-mixed	Neutrophil
$10^{-5}M$	41%	8%	30%	74%
$10^{-6}M$	59%	16%	55%	89%
$10^{-7}M$	76%	41%	85%	95%
$10^{-8}M$	85%	50%	95%	95%
$10^{-9}M$	96%	58%	100%	100%
$10^{-10}M$	94%	83%	100%	100%

Table 2. Effects of prostaglandin E_1 on proliferation and morphology of CFU-c stimulated by WEHI-3 CM

[a] Single colony morphology of 50 sequential colonies

with an exclusively or predominantly macrophage-monocyte differentiation, whereas colonies of an exclusively neutrophil morphology (and dependent on G-CSF) are PGE insensitive (Pelus et al. 1979). Table 2 shows that PGE inhibition of WEHI-3 CM stimulated mouse bone marrow colony formation was selectively directed at macrophage and mixed colony types. Since the biosynthesis of prostaglandin E by normal and neoplastic macrophages is intrinsically linked to their synthesis of and exposure to myeloid colony-stimulating factors (Kurland et al. 1979), we addressed the question of the extent to which different CSF species possessed the capacity to induce macrophages to synthesize PGE. Adherent peritoneal macrophages were exposed to unfractionated WEHI-3 CM and to its DEAE breakthrough (G-CSF) and eluate (M-CSF) fractions. As can be seen in Table 3, WEHI-3 CM induced a striking increase in macrophage PGE biosynthesis within 24 h, and this inducing activity resided in the M-CSF and not in the G-CSF-containing fractions of WEHI-3 CM.

Thus WEHI-3 displays the capacity to synthesize PGE constitutively, but this basal level can be increased 5–10 times by exposing the leukemic cells to an exogenous source of CSF (Kurland et al. 1979). The leukemic cells also produce one CSF species (M-CSF) which stimulates normal and leukemic macrophage PGE synthesis and macrophage colony formation, the latter being sensitive to PGE inhibition. Leukemic cell-derived G-CSF stimulates PGE-insensitive neutrophil colony formation and lacks the capacity to induce macrophage PGE synthesis (Pelus et al. 1979). Finally, WEHI-3 leukemic cells are particularly sensitive to PGE inhibition (Kurland and Moore 1977) which may reflect the preponderance of monocytoid rather than granulocytic differentiation of the cell line.

C. Genetic Restrictions in Response to WEHI-3 CSF

Our laboratory has extensively used WEHI-3 CM as a source of CSF and in doing so we have some striking strain differences in marrow CFU-c incidence which were not apparent when other types of CSF, such as L cell or

CSF dilution	PGE[a]		
	WEHI-3CM	DEAE breakthrough (G-CSF)	DEAE elute (M-CSF)
Control	65± 11	65± 11	65± 11
1:2[b]	2030± 12	186±112	4093±711
1:4	1504±180	19±16	2485± 78

Table 3. Production of PGE by resident murine peritoneal macrophages after stimulation by WEHI-3 colony stimulating activities

[a] Radioimmunoassay measurements of PGE in cell-free 24-h supernates. The results are expressed as mean concentration of PGE (picograms/milliter) ± SE elaborated by adherent macrophages derived from cultures of 2.5×10^5 BDF, peritoneal exudate cells

[b] Concentrations of CSF which maximally stimulates CFU-c proliferation

endotoxin serum, were used. A particular abnormality was evident in NZB marrow cultures, since with unfractionated WEHI-3 CM, CFU-c numbers were consistently low over a broad age span, and when this material was partially purified by passage over DEAE, an even lower response was measured in these mice (Kincade et al. 1979, see also Table 4). In contrast, when either media conditioned by L cells or endotoxin serum were used, NZB marrow cells not only responded well but the plateaus of colony numbers seen with normal mice were not obtained. The incidence of WEHI-3 CSF-responsive cells in NZW mice was normal, and (NZB×NZW)F_1 had an intermediate CFU-c incidence that reflected the influence of both the parental strains (Table 4). Certain other strains besides NZB are very poor responders to WEHI-3 CSF, for example, the NZC strain, which unlike the NZW shares a common origin with the NZB. The C58/J strain also has a low incidence of CFU-c and like NZB produces xenotropic virus and has a high incidence of spontaneous leukemia (Kincade to be published). Horland et al. (1980) recently reported that the RF strain of mice which has a very high spontaneous incidence of granulocytic leukemia also had a marked defect in CFU-c numbers (<1 colony per 10^5 marrow cells). It is of interest that they used WEHI-3 CM as their exclusive source of CSF.

The possible relationship of the defective WEHI-3 CSF response in certain mouse strains to a more fundamental lesion at the pluripotential stem cell level was suggested by published evidence of CFU-s defects in NZB and NZC mice (Warner and Moore 1971) and in RF mice (Horland et al. 1980). To investigate this possibility, we attempted to establish long marrow cultures using a single femoral inoculum of marrow from NZB, NZW, and (NZB×NZW) F_1 mice. The technique was as described by Dexter et al. (1977) with cultures subjected to weekly demidepopulation of suspension cells. Cultures were assayed for CFU-s by injection into lethally irradiated syngeneic or allogeneic DBA/2 mice. (Note that NZB-irradiated recipients are not suitable for CFU-s assay due to their abnormally high incidence of endogenous spleen colonies). CFU-c were assayed using both WEHI-3 CM and L cell conditioned medium. Table 4 shows that CFU-c and CFU-s were produced for 3–4 months at high levels in NZW and F_1 cultures with a normal pattern of myelopoiesis. In contrast, NZB marrow failed to support myelopoiesis. CFU-c responding to either WEHI-3 CM or L cell CSF disappeared rapidly from culture, and CFU-s could not be detected by the 2nd week. Mast cells develop in long-term marrow cultures of most strains, generally some weeks after initiation of cultures. In the present study such cells appeared in NZW and F_1 cultures at 11 weeks but were not observed in NZB cultures at any stage. Refeeding of NZB marrow cultures with a second inoculum of NZB marrow was also unsuccessful in establishing sustained stem cell replication and myelopoiesis in culture.

In order to further define the level of the lesion in NZB bone marrow, marrow coculture studies were undertaken. We have previously shown that bone marrow from genetically

Table 4. Defective response of NZB marrow to WEHI-3 CM and associated defects in continuous marrow culture

Strain	CFU-c/10^5 marrow[a]	Continuous marrow cultures[b]		
		Duration of production (wks)		Mast cells Produced
		CFU-c	CFU-s	
NZB	1± 1	4.5±1.3	1	−
NZW	220±10	12 ±3	11	+
(NZB×NZW) F_1	152±11	15.5±2	12	+

[a] Stimulated by semipurified WEHI-3 G-CSF. L Cell CSF stimulated 290±11 colonies with NZB marrow

[b] Continuous marrow cultures established with a single inoculation of bone marrow without refeeding using conditions as described by Dexter et al. (1977). The maximum duration of CFU-c production determined in cultures stimulated with L cell CSF

anemic WW^v and $S1/S1^d$ mice was defective in vitro, and long-term bone marrow cultures could not be established (Dexter and Moore 1977). However, the addition of $S1/S1^d$ marrow (with normal stem cell function) to adherent bone marrow monolayers of WW^v (with a normal hemopoietic environment) resulted in normal long term hemopoeisis. Coculture studies were undertaken involving NZB bone marrow with either WW^v or $S1/S1^d$ marrow. The results clearly indicated that coculture of NZB and $S1/S1^d$ marrow did not augment the in vitro replication of stem cells of either genotype, whereas coculture of NZB with WW^v marrow showed long-term maintenance of stem cell production and myelopoiesis significantly in excess of that observed with marrow from either strain when cultured alone. These results point to a defect in a regulatory cell population in NZB marrow, and a defect in macrophage function has been proposed (Warner 1978).

D. Conclusion

The linked production of a wide spectrum of hemopoietic growth regulatory factors, as displayed by the WEHI-3 cell line, can be duplicated by pokeweed mitogen stimulation of murine spleen cells (Metcalf et al. 1978). A mitogenic stimulus, T lymphocytes, and adherent cells are required for multifactor production by spleen cells suggesting that induced lymphokines may in turn induce macrophages to elaborate the growth factors. The feature of the WEHI-3 myelomonocytic cells is their ability to produce factors constitutively and thus circumvent control networks implied by mitogen-lymphocyte-macrophage interactions. A second feature is that the leukemic cells produce factors (G^-, GM^-, M^- CSF) to which the leukemic cells can respond by proliferation and differentiation (Metcalf and Moore 1970; Metcalf 1979). Under normal conditions the myelomonocytic progenitor cells respond to GM-CSF but do not produce it; in contrast the neoplastic myelomonocytic CFU-c of WEHI-3 (50%–100% cloning efficiency) both produce and respond to GM-CSF. In this context normal monocytes and macrophages can be induced to produce CSF and can be shown to possess receptors for CSF, since macrophage proliferation and prostaglandin synthesis can be induced by exposure of the cells to exogenous CSF (Kurland et al. 1979. Pelus et al. 1979). Individual WEHI-3 leukemic cells would appear to possess a combination of features possessed by the earliest committed progenitor cells (CFU-c) and their differentiated progeny. The association of growth factor production with the presence of growth factor receptors on the cell surface is rare among tumor cells but has been reported in the case of nerve growth factor and human melanoma cells (Sherwin et al, 1979). It is possible that this represents an opportunity for "autostimulation" of tumor cells by growth factors and may be more universally applicable as we better understand the characteristics of specific growth factors required for different tissues. The model developed by Todaro and De Larco (1978) to explain the mechanism of sarcoma virus transformation mediated by endogenous polypeptide growth stimulatory factors may equally be applicable to leukemogenesis. In this model growth factors are produced by cells that normally do not respond to their own product. Inappropriate production by a target cell of an active factor for which it also has receptors may be sufficient to stimulate cell division, and the persistent production of a growth factor may serve as a continuous endogenous stimulus leading to continued inappropriate cell growth.

The inability of CFU-c from certain mouse strains to respond to G-CSF of a WEHI-3 origin may involve a defect in the CSF molecule, i.e., WEHI-3 G-CSF is a leukemic product similar but not identical to normal G-CSF. Alternatively, or perhaps in addition, an impaired G-CSF response reveals a broader defect involving growth factor receptors back as far as the pluripotential stem cell. Certainly the associated abnormality of NZB, NZC, and C58 mice involving impaired stem cell replication in long term marrow culture would suggest a broader defect. This is supported by the in vivo evidence of multiple defects in G-CSF-unresponsive mouse strains variously involving autoimmunity, endogenous xenotropic virus expression, and high leukemia incidence.

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