CHARACTERIZATION OF CELL LINES DERIVED FROM THE PROMYELOCYTIC LEUKEMIA OF THE BROWN NORWAY RAT

by

Simone GLYNN

PHYSIOLOGY

MC GILL UNIVERSITY, MONTREAL

November 1983

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Derivation and Characterization of Rat Leukemic Cell Lines

ABSTRACT

In 1977, Hagenbeek et al reported the induction of leukemia in the Brown Norway rat after injection of 9,10-dimethyl-1,2,- benzanthracene. The cells of promyelocytic morphology produced a syndrome in the animal similar to the human disease with invasion of the liver, spleen and marrow, suppression of normal blood elements and a coagulopathy. However, attempts to establish these cells in long term culture have been unsuccesstul requiring in vivo passage for maintenance. In the present study, a subpopulation of cells capable of proliferation in vitro was obtained from leukemic rats . Although they appear to be less mature than the parent, they retain a partial capability for maturation when returned to in vivo conditions. Using these cells as immunogens six murine monoclonal antibodies were obtained that appear to be myeloid related. These cell lines and antibody markers provide a unique model for studying the control of early stages of cellular regulation in myeloid leukemia.

RESUME

En 1977, Hagenbeek développait une leucémie dans le rat "Brown Norway" après leur avoir injecté du 9,10-dimethyl-1,2-benzanthracène. Ces cellules de type promyélocytaire produisent chez l'animal un syndrome similaire á celui observé chez l'homme c.a.d. invasion du foi, de la rate et de la moelle osseuse, suppression des éléments normaux du sang et une coagulopathie. Cependant, il n'a pas été possible jusqu'à présent de maintenir ces cellules en culture continuelle, ces dernières demandant à être injectées dans le rat pour survivre. Dans cette étude, une souspopulation de cellules obtenue de rats leucémiques est capable de vivre en culture. Ces cellules bien que moins differenciées en vitro, peuvent évoluer au stage promyélocytaire en étant reinjectées dans le rat. Enfin, le développement de six anticorps monoclonaux de souris permet une plus ample caractérization de ces cellules. Ces lignées de cellules ainsi que les anticorps obtenus forment un model unique qui permet l'étude du control necéssaire aux premiers stades de différenciation dans la leucémie myeloïde.

AKNOWLEDGMENTS

The author wishes to aknowledge the support of Dr. Philip Gold who first welcomed her to the physiology program at McGill University.

Her personal thanks and most special recognition are given to Dr. Arthur Sullivan. Without his excellent guidance, individual concern and support, this study would not have been possible.

Mr. Gilles Methot is also to be recognized for his help in learning the necessary laboratory techniques. Appreciation is also extended to the other students and staff members of the Mc Gill Cancer Centre and Physiology department.

This work was supported by the National Cancer Institute of Canada.

Table of Contents

List of Photographs

List of Figures

INTRODUCTION

"En me rappelant que j'avais fréquemment observé un état analogue dans le sang d'individus chez lesquels on ne pouvait pas soupçonner la présence du pus, je suis plus porté à croire aujourd'hui que l'excès des globules blancs tient plutôt au défaut de transformation de ces globules en globules rouges, à une sorte d'arrêt dans l'évolution du sang, qu'à la présence de globules d'une nature étrangère, comme ceux du pus..... D'après la théorie que j'ai donnée de l'origine et du mode de formation des globules sanguins, la surabondance des globules blancs n'aurait rien que de naturel en pareille circonstance; ce ne serait, encore un fois, que le résultat d'un arrêt de développement dans ces particules transitoires." Alfred Donné (32)

Since Donné's description of the aberrations of white blood cells in 1844, the observations made on leukemia have progressed considerably. Cronkite (28), can now give a more appropriate definition: "Leukemia is a disease of aberrant white cell proliferation characterized always by defective cell maturation and divergence from steady state cell proliferation which may, depending on the stage of the disease, result in over or underproduction of leukemic and normal cells". An understanding of the etiology and pathogenesis of leukemia has not yet been reached. The nature

of the exact mechanisms underlying cell differentiation should be studied in order to understand the block in cell maturation that occurs in leukemia. Valuable information on the various stages of cell differentiation can be gathered with *in vitro* models. This research has been directed more particularly towards the study of acute myeloblastic leukemia (AML). First, an acute myeloblastic cell line was derived from an acute promyelocytic leukemia in the Brown Norway rat grown *in vivo*. The stage of differentiation of these cells and their response to maturation inducing agents were then determined. Finally, myeloid cell surface antigens were identified using murine monoclonal antibodies. These cells now extend the limited number of myeloid lines available and potentially are a useful model in which to study the controlling events in early myeloblast maturation.

The first part of this thesis will outline the process of normal granulopoiesis as well as discuss the two relevant maturation blocks ocurring in acute myeloblastic and promyelocytic leukemia. The few human myeloid cell lines available then will be described followed by a description of the model studied in this thesis i.e. the Brown Norway rat promyelocytic leukemia . The second part will describe the development of the *in vitro* cell line. Finally, the properties and antigenic characterization of the cell line will be discussed.

CHAPTER I

I) Granulopoiesis

The major organs of the haemopoietic system are the bone marrow, spleen and lymph nodes. Mature blood cells are highly differentiated but short lived. They all arise from multipotential hemopoietic stem cells which have high self-renewal and regenerative capacity. Such cells give rise to others programmed to differentiate in a certain lineage. With maturation, their capacity for growth and change is diminished until, as mature peripheral blood cells, they completely lose the ability to proliferate (98,28). The spleen colony method, described by Till and McCulloch (120) is an assay for mouse pluripotent hemopoietic stem cells, CFU-S. Under proper conditions in the spleen, these CFU-S cells can reproduce as well as give rise to cells differentiating along the erythrocyte, granulocyte, monocyte or megakaryocyte pathways. The commitment of hemopoietic stem cells to certain lineages may depend on a specific hemopoietic inductive microenvironment in the bone marrow and spleen, as well as on specific regulators (88,125). For instance, CFU-S proliferates *in vitro* with prostaglandin E produced by

macrophages (23). One of the progeny of CFU-S, CFU-GM, gives rise in vitro to both granulocytes and mononuclear phagocytes in the presence of several factors (glycoproteins) collectively called colony stimulating factor (CSF) (92). CSF binds to marrow cells with subsequent internalization and degradation (102). As well, it may be an important factor in vivo for proliferation and differentiation of CFU-GM. Hence, under control of specific regulators and in certain hemopoietic niches, CFU-GM will give rise to myeloblasts, the first cells entirely committed to become neutrophils .

Neutrophil Differentiation Stages

1) The MYELOBLASTS constitute between 1-5% of normal bone marrow and are never present in circulating blood under normal circumstances. These cells have a high nuclear to cytoplasmic ratio and 1-8 nucleoli. They do not contain granules and they lack myeloperoxidase .

2) The PROMYELOCYTES have a round nucleus and visible nucleoli. Primary or azurophil granules are now present (500 nm); the cells are highly peroxidase positive (Fig 1A).

3) The MYELOCYTES have a smaller nucleus with minimally discernible nucleoli. The presence of peroxidase-negative specific or secondary granules (200 nm) characterize this stage (Fig 1A).

4) The METAMYELOCYTES, BANDS, and mature NEUTROPHILS are non dividing cells and accumulate glycogen particles. The nucleus becomes deeply indented. The specific granules are

then twice as abundant as the azurophilic granules. The neutrophil will acquire sequentially its functions i.e. a) phagocytosis, b) microbial killing, c) random locomotion, and d) chemotaxis (43), so as to become a major participant in the body's defense against invasion by microorganisms. The above description of haemopoiesis and more specifically granulopoiesis (8) is summarized in Fig 2 and Fig 3.

Leukemia can be grouped by morphology, chromosomal aberrations, surface glycoprotein patterns (5), glycolipids profile(72) as well as by enzymatic activities (61). From the various classes of leukemia, only acute myeloblastic leukemia (FAB classification: M1 with no maturation or M2 with some maturation) and acute promyelocytic leukemia (FAB classification : M3) will be considered.

II) Acute Myeloblastic Leukemia

In this leukemia, a block in cell maturation results in accumulation of myeloblasts containing very small amounts of receptors for IgG fragments or receptors for C1 and C2 complement components (58) in the blood and bone marrow. This accumulation is accompanied by failure of normal haemopoiesis. The effect of leukemic myeloblasts on normal myelcid colonies is not clear, although suppression of normal hemopoiesis by

leukemic cells has been shown in some cases (114). Leukemogenesis due to activation of oncogenes or to spontaneous or induced maturation seems to occur at the level of the pluripotent stem cell (22,81,13,89). Studies with Glucose-6-phosphate-dehydrogenase heterozygotes implies that acute myeloblastic leukemia is a clonal disease i.e. leukemogenesis occurs in a single stem cell. This leukemic cell is capable of self replication but cannot mature completely to the functional neutrophil stage and appears to be blocked between the myeloblast and promyelocyte stages. The clonal origin of leukemia is also supported by specific chromosomal changes. A translocation (8;21) is often found in myeloblastic leukemia with maturation (10,11).

Leukemic blast cells can be divided: 1) a rapidly proliferating pool located in the bone marrow where they form 15-35% of marrow leukemic blasts and 2) a quiescent pool consisting of cells that will die, cells that may partially differentiate and cells that will remain in a long G1 cell cycle phase (Fig 1B). This last pool is the most difficult to eradicate with standard therapy (69) . Finally, acute myeloblastic leukemic cells do not form <u>in vitro</u> colonies in a fashion similar to normal cells (86a). Autologous antibodies directed against leukemic myeloblasts of patient's sera in remission have been reported (66). Some patients with AML have circulating immune complexes, an observation usually associated with fewer and shorter remissions (19). Until now, intensive chemotherapy (Cytosine Arabinoside, Daunorubicin) to produce bone marrow aplasia followed by maintenance therapy is the usual treatment for AML (105). Fifty to seventy

percent of patients with AML can achieve remission. Long term disease free survival is attained in 15% of patients.

III) Acute Promyelocytic leukemia (APL)

In this disease, most of the leukemic cells do not mature past the promyelocyte stage. Hence, abnormal promyelocytes accumulate and constitute 40-60% of cells on the peripheral blood film . APL , comprising 10-15% of acute non-lymphocytic leukemia, is associated with a bleeding tendency (petechiae, ecchymoses, gingival bleeding, intracranial and pulmonary hemorrhage). A prolongation of prothrombin time is present in 70-90% of cases as well as increased fibrinogen catabolism with reduced platelet survival (121). Characteristic fibrinolytic enzymes have not yet been found in leukemic promyelocytes (46). As in the case of acute myeloblastic leukemia, APL often is associated with a cytogenetic abnormality t(15;17) (q25 or26; q22?) (10,11,29,40). Treatment is similar to that for AML.

IV) Description of the available human myeloid leukemic lines

Four major human myeloid leukemic lines (monoclonal in origin) have been derived (75) : HL60, KG-1,K562, and ML-2.

1) HL60

HL60 cells were derived from peripheral blood leukocytes from an adult female with APL (24). The cells are promyelocytes and contain large azurophilic granules; they do not express Ia antigen on their surface since promyelocytes seem to lose this structure (74). Granulocyte maturation of the HL60 cells can be induced by agents such as dimethyl sulfoxide, retinoic acid, butyric acid and triethylene glycol. HL60 cells elaborate several factors:

a) a factor of apparent molecular weight 13,000 seems to stimulate their own growth, hence explaining the optimal growth rates obtained at high cell density (17).

b) a factor of apparent molecular weight 500,000 called leukemia associated inhibitor could be involved in inhibition of normal granulopoiesis (99). Finally, HL60 cells as well as K562 cells (descrited below) seem to have higher specific activities of cytosol cAMP-independant casein kinase and lower activation by cAMP of their cytosol histone kinase compared to the normal myeloid cells studied (36).

2) KG-1

The KG-1 line was derived from the bone marrow of a man with erythroleukemia. Most of these cells are at the myeloblast and early promyelocyte stages; the majority express the Ia antigen (74). A variant subline KG-1a has been described by Koeffler et al (73b) which consists mostly of blast cells which do not express the Ia antigen and do not respond to CSF (unlike the parent cell line).

3) K562

K562 was derived from the pleural fluid of a patient with chronic myeloid leukemia in blast crisis. They are blast cells (peroxidase negative) with no Ia antigen on their cell surface and have been suggested by some to be early pronormoblasts.

4) HL-2

HL-2 is an acute promyelocytic cell line derived by Minowada et al (93).

The cell surface markers of these 4 different cell lines can be seen in Fig 4. These markers also can be found on normal hemopoietic cells at various stages of the granulocyte lineage; no leukemia specific antigen has been found on the cell surface of the available cell lines, so far. This

antigen, if it exists, may be present in very small quantities and therefore hard to detect with antiserum. The use of the hybridoma technique which permits the derivation of antibodies specific against only a few amino acids should lead to further characterization of the leukemic cells as well as to the possible identification of a leukemia specific or associated antigen.

These 4 in vitro models are useful to study the cytology of myeloid leukemic cells but because of their human origin, extrapolation of in vitro findings to the conditions in the whole animal usually is not possible.

On the other hand, in vivo and in vitro animal models of the same disease can be derived. These permit direct comparisons between the in vivo and in vitro systems as well as characterization of each system. Such models can be derived in the rat.

VI Rat Models

1) Haemopoiesis in the rat

Haemopoiesis in rats closely corresponds to the description given above for the human. Spleen, lymph nodes, and bone marrow histology is also similar except for the more limited regenerative capacity of the bone marrow in rats (116). The morphology of rat granulocytes is similar to human

except that some mature forms of granulocyte contain a ring shaped nucleus and less visible granules in their cytoplasm (116). Percentages of cells found in the bone marrow and peripheral blood are given as references (Fig5). The normal peripheral white blood cell count is about 10,000/mm3. Haemopoietic activity is found in the spleen of young rats and usually declines with age as it becomes preponderant in the bone marrow. It may reappear with infections or with myeloid leukemia. In the later case, the bone marrow will rapidly attain its maximum cell (blast) capacity resulting in the immigration of normal hemopoietic stem cells as well as other blasts to the spleen (which can attain 10 times its normal size) and other organs such as the liver and lymph nodes. Hence, in the spleen, myeloid cells can be seen at various stages in their development. The peripheral white blood cell count in myeloid leukemia usually will be from 50,000 to 200,000/mm3. The granulocytic or myeloid leukemic cells usually have complement receptors on their surface but none of the other markers used by Moriuchi et al (94) to classify various other rat leukemias.

2) Development of Rat Models

Rat models for granulocytic or myeloid leukemia have been derived either from spontaneous growth or by induction. It may occur spontaneously with age in various strains of rat (independently of sex). For example, 3% of Osborne Mendel rats, 14% of WN rats will develop granulocytic leukemia as

they age (116). However, most of the existing rat models have been artificially induced.

Irradiation and viruses cause the formation of a few granulocytic leukemias with very low incidence . The major producers of rat granulocytic leukemia are chemical carcinogens such as N-2-fluorenylacetamide, N-nitrosobutylurea, fluorenylamine, benzpyrene, methylcholanthrene, 2-acetylaminophenanthrene and 7,12-dimethyl(a)anthracene (116), (Fig 6). These agents may act indirectly or directly to transform the genetic material of some hemopoietic precursor thereby causing granulocytic leukemia (111). Most of these agents must undergo reduction, hydrolysis, or oxidation to be active. For example, oxidation can occur during the prostaglandin synthesis when free radicals are produced (lipoxygenase may also play a role) (4) or by the cytochrome P450 monooxygenase systems. Benz(a)anthracenes and in particular 7,12-dimethylbenz(a)anthracene (7,12-DMBA), are some of the more commonly used carcinogenic inducers. Newman (97) proposed that benz(a)anthracenes (e.g. 7,12-DMBA) can either be metabolized at position 7 or at position 5. The less rapid interaction at position 5 seems to cause cancer. Hence, an agent with the 7 position unavailable and the position 5 available will be carcinogenic. This is indeed the case, since 7-methylbenz(a)anthracene is carcinogenic while 5-fluoro-7-methylbenz(a)anthracene is not. The strongly carcinogenic effect of 7,12-DMBA is caused by the crowding effect of the 2 methyl groups; the molecule is thereby under strain and will be even more reactive to external activating

agents. 7,12-DMBA will damage cells synthesizing DNA. Huggins et al (64), found that 3-4 pulse doses of 7,12-DMBA injected i.v. in Long Evans rats led to the development of leukemia (diffuse hepatic, myelogenous, lymphoblastic and thymic leukemia) in a high percentage of rats. On the other hand, a single dose of 7,12-DMBA totalling the same amount as the several pulse doses, led mostly to the development of mammary cancer.

3) Attempts To Derive An In Vivo Model For AML In ACI Rats

In the present study, eleven ACI rats (160 gms each) were injected three times with 2mg 7,12-DMBA (Sigma) into 0.2mls Pristane i.v. three to four days apart. Development of a granulocytic leukemia in this particular strain would have been useful since a large panel of histocompatibility reagents are available for further characterization of the leukemia. Four months after initial injection, and over a period of eight months, six of the ACI rats developed solid leg tumors and one rat developed ascites. Reinjection of part of the ascites was unsuccessful. In none of the animals were any peripheral blood abnormalities or liver and spleen enlargment noted. Hence, pulse doses of 7,12-DMBA do not cause development of leukemia in the ACI rats with a high frequency. This may be strain-related.

4) A Model For Acute Promyelocytic Leukemia In The Brown Norway Rat

a) General Description

In 1977, Hagenbeek et al (48,123), reported the induction of a promyelocytic leukemia in the Brown Norway rat (BNML), after three injections of 2 mg of 9,10-dimethyl-1,2-benzanthracene Spontaneous occurrence of myeloid leukemia in aged Brown Norway rats (BN) is very rare. The commonest tumor in aged BN rats is pituitary adenoma (37). Hence chemical induction is necessary to establish a model for AML or its subtype AEL in the BN rat.

The leukemic cells of this transplantable in vivo model are promyelocytes 11-16 um in diameter. The nucleus is large with rather loose chromatin; large primary granules (peroxidase positive) are present in the cytoplasm. Enzymatic characteristics of these cells are given in Fig 7. The cell cycle values for BNML derived by Hagenbeek (51) in the spleen are : Tc=14h, TG1=0.8h, TS=10h and TG2 + 1/2M=3.2h. These BNML cells contain double stranded RNA (as found in human AML) as well as 2.3n DNA. Association between chromosomes 2 and 7 and sometimes between chromosomes 1 and 11 or 1 and 12 seem to characterize these cells. No viral particles yet have been found .

Similar to human AML, the BNML cells do not form in vitro colonies using agar or a fetal fibroblast layer (122); these BNML cells must be transplanted in vivo to survive and replicate. Cellular transfer of 10^3 cells and 10^7 cells i.v. in

BN rats led to leukemic death in 44 and 24 days respectively. (48). In the present study, BN rats died after 27 days with 10^7 BNML cells i.p., 29 days with 10^6 BNML cells i.p, and 24 days with 10^8 BNML cells i.p.. The dose survival curve (Fig 8), determined by Bacenbeck shows that a maximum tumor load of 10^{12} cells is theoretically possible even though 10^{10}-10^{11} cells is the usual tumor load at death. The number of cells required to produce disease in 50% of animals was observed to be 25. Peripheral blood smears were made and spleens were palpated every week to characterize the evolution of BNML.

b) Kinetics

Once injected into the BN rat, the promyelocytes migrate predominantly to the bone marrow, spleen and liver. Cellular locomotion is one of the factors enabling dissemination of leukemic cells (103). However, BNML cells are non-locomotive with on spot mobility (47). Colchicine causes loss of microtubules and a change in BNML locomotion thereby showing the importance of microtubules in BNL locomotion (47). Since cellular locomotion does not seem to be involved in dissemination, it has been hypothesized that a lytic action of acid phosphatase present in BNML cells on various tissues is involved in BNML organ infiltration (103).

As in human AML, 2 pools of leukemic cells have been distinguished: a rapidly exchangeable pool of cells (blood) and a slowly exchangeable tissue pool. The latter increases in size as the leukemia progresses and the exchanges of leukemic cells between organs and blood decreases (52).

As mentioned above, the target organs for BNML

predominantly are the bone marrow, spleen and liver. Hagenbeek determined the sizes of these two pools described above in the spleen, liver, bone marrow and lungs (Fig 10). Six days after injection of 10^7 cells i.v., Martens (86) reported a broadening of the G1 peak (DNA content measured by pulse cytophotometry) corresponding to the appearance of BNML cells in the bone marrow. There is progressively a decrease in the cellularity in the bone marrow (femur) while the dead cell percentage increases from 17 to 40% (86b). After day 18, the bone marrow is heavily infiltrated with leukemic cells as is the liver, lymph nodes, lungs, kidneys and spleen. In the present study, after injection of 10^6-10^7 cells ip, the spleen attained 0.9% of the total body weight in terminal stages of the disease. The red and white pulp were completely infiltrated by BNML cells at this stage. Finally, the thymus has been observed to atrophy during the progression of the leukemia while the CNS appeared unaffected (50).

c) Haemopoiesis

As in human ANL, normal hemopoiesis is decreased in the BNML model. Hagenbeek, Martens et al. (55,53), separated normal hemopoietic stem cells from BNML stem cells by velocity sedimentation and density gradients. This method permitted a better analysis of the kinetics of both groups of cells. The decrease in normal hemopoietic activity could be explained by several factors.

Normal hemopoietic stem cells are redistributed from the bone marrow to the spleen and liver as the leukemia progresses. These stem cells represent only 45% of the

original population and cycle less effectively than in the bone marrow (54). An inhibitory factor could be released by BNML cells to inhibit proliferation of normal stem cells (similar to HL60 cells); however such a factor has never been found. Neither has direct inhibition of stem cells been demonstrated. The major variable present in this leukemia seems to be the new environment to which the hemopoietic stem cells are displaced by the BNML cells. The BNML cells when injected, infiltrate the bone marrow especially in the subendosteal region (104,124,49), where the necessary hemopoietic microenvironment for normal stem cells seems to be located. The stem cells, being displaced from this favorable environment, do not replicate as well and usually are out of cycle. Hence, redistribution of normal precursors from their natural niches seems to be a major cause of decreased hemopoiesis in this model.

d) Coagulopatny

In agreement with studies made on human AML, the BNML model posesses blood coagulation abnormalities. Thrombocytopenia and hypocoagulability are present as are increased levels of fibrin degradation products . A pattern similar to disseminated intravascular coagulation has been found. However, the BNML promyelocytes posess procoagulant activity but do not activate fibrinolysis (60,119,95).

e) Antigenic Characteristics

Williams et al (126) showed that some F1 hybrids of BN and other strains survive longer after injection of BNML

cells than do the homozygous animals. Furthermore, these same F1 hybrids are more resistant to BN bone marrow grafts. (Usually, F1 hybrids accept grafts from either their parent.) Finaly, pretreatment of F1 hybrids with BN bone marrow (59) prolonged even further the life of hybrids injected with BNML cells. Hence BNML cells seem to express certain antigens also present on bone marrow cells.

f) Chemotherapy

Since the BNML model is very similar to human acute myeloid leukemia, many researchers have concentrated their efforts to find a successful chemotherapeutic regimen . Arabinoside-cytosine (Ara-C) followed by Adriamycin caused remission in most rats injected with 10^7 cells i.v. The resulting bone marrow aplasia was reversed by isogeneic bone marrow transplantation (26,27). The efficacy of high dose cyclophosphamide followed by total body irradiation is very high in the early stages of the disease curing 90-100% of all animals (57,56,112). Ara-C and Methotrexate have been used separately in the chemotherapy of BNML (1,2,3). The alkylating agent analog 4-Hydroperoxycyclophosphamide can destroy the BNML cells without seemingly destroying the normal hemopoietic stem cells (113). All of these studies showed that the drug effect depended on the cell kinetics.

CHAPTER II

•DERIVATION OF AN IN VITRO CELL LINE IN THE BN RAT

Note: this paper is published (44)

I) Abstract

Cells from the spleens of Brown Norway rats made leukemic with the in vivo-passaged promyelocytic line BNML have been adapted to in vitro culture in RPMI-1640 medium supplemented with 4% rat serum and 6% fetal bovine serum. The presence of rat serum, which also was associated with suppression of fibroblasts, appeared to be required for initial growth. In contrast to the parent, this new cell line (BNML-RS) was predominantly myeloblastic under standard conditions of culture with only 4-10% of cells showing granules or stainable peroxidase. However, when passaged through an animal, 60-70% contained both granules and peroxidase. Neither parent nor established line evolved to the polymorph stage when assessed for terminal maturation after exposure to dimethyl sulfoxide or retinoic acid. These cells now extend the limited number of myeloid lines available and potentially are a useful model in which to study the controlling events in early myeloblast maturation.

II) Introduction

The above (Chapter I) description of the BNML model points out the numerous similarities to human AML and, more specifically, to its subtype human APL. However, that model has been limited by its dependence upon in vivo passage in the Brown Norway rat for propagation. Reported here, is the establishment into long term culture of sublines of these BNML cells. Now, complementary studies of the physiologic control of these leukemic cells may be performed both in vivo and in vitro. Furthermore, these derived lines of myeloblastic morphology show major differences from the parent in the level of maturation expressed in culture.

III) Materials and Methods

An initial sample of BNML cells (48), originally developed at the Radiobiological Institute, Rijswijk, The Netherlands,was obtained through Dr. R. Michael Williams (Cancer Centre,Northwestern University, Chicago, USA) and was maintained by in vivo passage in Brown Norway rats (obtained from Microbiological Associates or bred from this stock in the McGill animal facility).

Female Bn rats were injected intraperitoneally with $2x10^6$ BNML cells. When blastemia and palpable splenomegaly had

developed, usually during the fifth week, the enlarged spleens were removed, minced and gently pressed through a stainless steel mesh under sterile conditions. The resulting cell suspension, typically containing over 90% leukemic and less than 10% lymphoid cells, was distributed into 24 well flat bottom plates (Costar No.3524, Cambridge, Mass.,USA.) at 5×10^5 cells/well in RPMI-1640 medium supplemented with either 10% fetal calf serum (FCS) or with 4% rat serum (RS)/6% fetal calf serum. All trans retinoic acid (Sigma) was then added to these solutions to obtain concentrations of 0, 1, 2, 5 and 10 μM . Five replicate wells for each concentration , containing either 4%RS/6%FCS or 10%FCS were plated (see the appendix). The rat serum was obtained locally by cardiac puncture of larger (250 gms) animals or by salvage of blood from normal animals sacrificed for other reasons and filtered before use through a 0.22 μm filter (Millex-GS, Millipore Corp.). Initially, the cells were fed at one and two weeks and then twice weekly with the starting medium. The contents of wells which showed replicating cells were expanded into larger cultures; one of which called RNHL-RS was selected for further study. These established suspension cultures required passage thrice weekly.

For purposes of analysis or derivation of sublines, aliquots from these larger cultures were suspended in 0.25% agar containing 6%FCS/ 4%RS in RPMI 1640. When the resulting clones became macroscopically visible, they were enumerated, picked, placed in 96 well plates and again expanded.

Standard methods were used for May-Grunwald-Giemsa and peroxidase stains (127). Unless otherwise noted cultures were

sampled for analysis in early log phase of growth. The nitroblue tetrazolium test was performed by the method of Koeffler et al (73a). Immunofluorescence with the monoclonal antibodies W3/13 (T subset and granulocytes), W3/25 (helper T cells), Ox8 (suppressor T cells), Ox7 (Thy1.1), Ox1 (leukocytes), and Ox6 (Ia) was performed according to the method described in the appendix.

IV) Results

1) Behaviour of early cultures

During the first week of culture, most of the cells that were plated initially had degenerated but in some wells unattached round cells of a diameter similar to BNML were noted to be growing in the periphery of cultures containing 4%RS/ 6%FCS but not in those with 10%FCS alone. Also observed in the wells that contained the RS/FCS mixture, were a decreased number of fibroblastoid cells. A decrease in the number of fibroblasts with increasing concentration of retinoic acid was also noted. This pattern of proliferation of adherent cells occurred also in control cultures of normal spleen cells in which the unattached round leukemic cells did not appear as well as in spleen cell cultures from over eight BNML-injected rats of which three yielded long term growths. By the fourth week, there was a heavy growth of BNML-like cells in the RS-containing wells but only adherent and

macrophage like cells in the others (Photograph 1). There was no correlation between the number of macrophages and the concentration of retinoic acid; however, the number of macrophages present in the wells correlated with the number of blasts (Fig 9). The capacity of rat serum to support further growth once established appeared not to be strain dependent since there was no difference in growth rate between that obtained using BN or Lewis varieties. When active cultures were placed in 10%FCS alone, the viability decreased over the next few passages and eventually they died. However, progressively decreasing the rat serum content over the period of a month enabled adaptation to growth in 10%FCS alone.

2) Behaviour of replicating cells

Cells from the more rapidly replicating cultures were expanded for further analysis. Established lines plated at $2-3x10^5$ cells/ml reached a plateau after three days at $1-1.2x10^6$ cells/ml after which viability decreased unless subcultured. They have been maintained in continuous culture for over two years and have the capacity to regrow *in vitro* after recovery from the frozen state in liquid nitrogen. Intravenous injection of 10^7 cultured cells into BN rats resulted in leukemic changes in blood, liver and spleen, with death of 50% at day 20 (N=14); similarly, 50% of animals injected with parental cells had died by day 20 (N=16). Cells obtained from the enlarged spleens of these animals grew immediately when returned to culture without the delayed period noted with

the original explant. The number of alive and dead cells in culture over a period of five days without feeding is recorded in Fig 11.

3) Cellular Characterization

Shown in Photograph 2 are spleen cells from animals made leukemic by injection of parental BNML cells compared to those adapted to long term culture (BNML-RS). The parental spleen cells showed prominent azurophilic granules throughout the cytoplasm of which over 75% stained intensely for peroxidase activity. In contrast, of the cells in culture (BNML-RS) only 4-10% bore stainable granules or peroxidase. However when they were injected into animals and leukemia had evolved, both granules and peroxidase appeared in the centrisomal area in 60-70% of the spleen cells examined; only occasional cells showed these organelles dispersed throughout the cytoplasm. Similar analysis of a subclone (BNML-RS/D) of this line revealed that it appeared to be even less differentiated with less than 2% of cells showing detectible granules or peroxidase under light microscope. It has been noted on occasion with some pools of BN rat serum that both of these lines may show up to fifty percent with peroxidase-staining granules. Immunofluorescence studies demonstrated the absence of T or B cell-related antigens assessed by monoclonal antibodies (W3/13, Cx8, W3/25, Ox7, Ox6, and Cx1); no surface Ig or intracytoplasmic Ig were present on the cells (Fig 12). These studies clearly show that the cells found in culture are

not activated T or B cells.

4) Response of BNML cells to inducers of maturation

Attempts to induce either the parent or BNML-RS to further maturation were unsuccessful. Neither lines matured to neutrophils when incubated with 1.25% dimethyl sulfoxide or 1, 2, 5 and 10 μM retinoic acid (15,25). Likewise, examination of unstimulated cultured BNML cells showed only blasts and a few promyelocytes with no spontaneous evolution to more mature forms. As well, the NBT test initially was slightly positive (0.4%) and increased only to 3-6% after 5 days incubation with 1 and 5μM Retinoic acid and remained zero with dimethyl sulfoxide.

V) Discussion

1) Role of several factors in primary cultures

These studies demonstrate that established cell lines can be adapted from the in vivo passaged BN promyelocytic leukemia if they are cultured in a suitable environment. Recently, Lacaze et al (79) succeeded in deriving a long term culture of BNML cells using the bone marrow liquid culture system

described by Dexter (30). With this system, Dexter was able to maintain haemopoietic stem cells and their derivatives in culture. This depended on the establishment of a bone marrow derived feeder layer consisting of endothelial cells, fat cells and macrophages. These three cell types interact with each other to form a microenvironment necessary for the survival of early cells. By plating bone marrow from leukemic BN rats in 5% horse serum 5% fetal calf serum, Lacaze observed proliferation of BNML cells. No attempt was made to clone or freeze these cells or to adapt them to 10%FCS. Transplantation into BN rats was successful but the bone marrow cells of the transplanted rats did not grow more easily when returned to culture than did the parental cells. Finally, these cells were promyelocytes with the same morphological and cytochemical features as the parental line. Hence, the in vitro model derived by Lacaze's group is entirely different from the model derived in this study. The differences between these two models could be due in part to the different original microhemopoietic environments used i.e. spleen or bone marrow.

In this study, although the significance of many of the critical variables that contribute to in vitro growth remains undetermined, the presence of a component of rat serum appears to be essential in the early stages. It is uncertain whether its action is direct or mediated through secondary effects such as a soluble stimulator of growth, support of an accessory cell or suppression of inhibitory mechanisms.

Although it may be an epiphenomenon, the observation that there were significantly fewer fibroblastoid cells in the

cultures containing rat serum suggests that the latter possibility might have occurred. Since this effect of rat serum was noted also in spleen cell cultures from normal rats it was not due to the leukemic status alone. A selection of a stable cellular subpopulation and not merely a reversible adaptation to culture is indicated by the fact that when the BNML-RS were recovered from spleens of rats made leukemic with them, they regrew immediatly without the lag period. The role of different batches of BN rat sera in stimulating partial maturation to promyelocyte is unclear. This variable also affects the growth rate and viability of the culture which then might influence the cells capability to form peroxidase containing granules.

Serum is known to contain granulocyte-macrophage colony stimulating factor i.e. GM-CSF or MGI (macrophage-granulocyte inducer). MGI can be produced by myeloid, erythroid and lymphoid leukemic cells (101) . Metcalf's work with CSF (18,91) has clarified the role of these factors in vitro. GM-CSF acts directly on granulocyte-macrophage cells to cause their proliferation and differentiation. Other factors such as serum, prostaglandin E, endotoxins, lymphoid cells as well as many other undetermined factors can modify the response of GM cells to GM-CSF. In the mouse,a 23,000 dalton glycoprotein corresponds to the GM-CSF activity described above and present in serum. A 23,000 to 54,000 dalton glycoprotein also has been found in the mouse corresponding to a macrophage only stimulating factor. It is possible also that a granulocyte only stimulating factor exists.

Many experiments have shown that acute and chronic

myeloid leukemic cells depend on stimulation by GM-CSF to proliferate in vitro (92). Some leukemic cell lines do respond to GM-CSF similarly to normal cells while others respond only partially.

Hence, working with inbred mice, Sachs (109,110) found three different types of myeloid leukemic clones. The first type called MGI+/D+ can differentiate normally to mature granulocytes or macrophages in the presence of MGI (CSF). During the differentiation period (6 days), an identical number of changes in the rate of synthesis of 217 specific proteins occurred in MGI+/D+ and normal myeloblasts (83). However, contrary to normal myeloblasts, these clones do not require MGI to replicate. In fact, two different MGI proteins are involved. Normal myeloblasts grow and secrete MGI-2 under the influence of MGI-1. MGI-2 in turn, induces maturation of the myeloblasts. Once differentiated, the cells need MGI-1 to be viable. Hence MGI+/D+ responds normally to MGI-2 but not to MGI-1 i.e. the cells do not need MGI-1 to replicate or to be viable; neither do they secrete MGI-2 under the influence of MGI-1. However, once they have differentiated via MGI-2, they need MGI-1 to be viable in culture (85).

The second type called MGI+D- can only partially differentiate with MGI-2 while the last type MGI-D- cannot differentiate at all with MGI-2. A smaller number of changes in the rate or synthesis of proteins occurred in MGI+D- than in MGI+D+ and an even smaller number in MGI-D- (83). Changing the serum type or removing it, seems to modulate the response of MGI-D- to MGI-2 i.e. permissive factors in some sera permit differentiation of MGI-D- with MGI-2 (117). None of these

leukemic clones require exogeneous MGI-1 to replicate and thus have lost the normal control over their replication. Sachs has proposed that this "uncoupling of controls for growth and differentiation " in AML is at the origin of the leukemia. Since many protein changes occur, many genes are expressed simultaneously under the influence of MGI. Progressive cytogenetic changes due to loss or translocation of murine chromosomes 2 and 12 (7) may permit a continuous biochemical state which, in normal cells, occurs under normal conditions only when more cells are needed by the haemopoietic system.

In this study, the leukemic stem cells could be partially sensitive to MGI factors present in the serum. The presence of an MGI-2 like factor could permit differentiation to the myeloblast stage where the cell no longer would respond to the inducers of differentiation . . An MGI-1 like factor in the serum could lead to survival of these cells in culture. The presence of MGI factors could also explain the correlation observed in primary cultures between the number of macrophages and blasts. Under the influence of MGI, spleen cells in culture (possibly CFU-GM) could differentiate to macrophages and blasts (blocked in their differentiation to neutrophil). Macrophages in turn, can produce MGI, thereby regulating further cellular interactions in culture. Macrophages also could interact directly with leukemic cells to enhance proliferation (68). Such an interaction did not seem essential to BNML cell growth since they survived <u>in vitro</u> without the presence of these accessory cells. Finally, the leukemic cells are very specific in their interaction with rat MGI since fetal calf MGI present in FCS does not lead to

initial growth. It also is possible that FCS contains an inhibitory factor to rat blast growth. As well, rat serum could also contain factors suppressing possible inhibitory mechanisms in vitro.

In several systems a role has been suggested for the organ stroma in the control of parenchymal cell growth. It has been reported that in irradiated rats reconstituted with normal bone marrow the spleen is markedly less permissive to myeloid than to erythroid colony formation (106). Thus, it is conceivable in the present observations that a component of rat serum might have suppressed an inhibitory stromal element (fibroblasts) and allowed sufficient BNML cells to survive from which those capable of growth in culture expanded. A similar observation has been made in other systems where fibroblasts were shown to inhibit the growth of human colonic epithelial cells (41). Also, Kaye et al have suggested that the pericryptal fibroblast sheath may control the growth and differentiation of the overlying tissue (67).

The role of retinoic acid when present is not clear. Lacroix et al (80) observed a decrease in the exponential growth rate of human fibroblasts in culture three days after addition of 10^{-5} M retinoic acid. A progressive decrease in the number of fibroblasts with increasing concentrations of retinoic acid was also noted in this study. Such a decrease did lead to a slight increase in blastic growth (4+ versus 3+) in 4%RS/ 6%FCS. This latter observation supports the possibility that fibroblasts are inhibitory stromal elements. As a second observation, no correlation was noted between retinoic acid and the number of macrophages, even though

retinoic acid is known to regulate the function of macrophages (107). Hence macrophage Fc receptor-mediated binding and phagocytosis are inhibited by retinoic acid while arginase production (a macrophage tumoricidal enzyme) is increased by retinoic acid (107). Such a regulation , if it exists, was not observed in BNML-RS primary cultures.

Finally , Douer et al (33) observed an increase in the number of colonies of normal human myeloid progenitor cells when cultured with 3x10 M retinoic acid. These authors suggest that retinoic acid enhances the responsiveness of GM-CFU to the action of MGI present in the medium since retinoic acid does not actively cause the production of MGI by cultured cells. Retinoic acid does not have any MGI activity itself. Hence , if this hypothesis is correct, the slight increase in the number of blasts observed could be due to a higher responsiveness of the blasts to rat serum MGI.

In summary, factors such as MGI present in the rat serum may be needed to support the growth of the leukemic cells. The removal of an inhibitory stromal element, the fibroblast, also seems to have supported the growth in primary cultures. Undefined cellular interactions between the stromal elements (mostly macrophage and fibroblast) themselves, and between these latter and the leukemic cells, appear to be involved. Further study will be necessary to better understand the various factors and interactions present in such a culture.

2) Primary Characterization of the cultured cells

The process of generation of enzyme-containing granules is finely regulated and its morphologic manifestations have formed a basis for organizing concepts of myeloid maturation (8). These rat cells with the peroxidase staining in the centrisomal position appear to be early promyelocytes as described by Bainton (8). With the light microscope used in this study, it is not possible to state whether this activity is of the type that is yet diffuse in the Golgi cisternae, limited to small vesicles or enclosed in fully developed primary granules that have not yet migrated to the peripheral cytoplasm. Ultrastructural studies are in progress to assess these possibilities.

3) Induction Studies

The clear sequence of changes that occur as granulocytes mature provides a means of comparison of events among different studies. The human promyelocytic line HL60 is able to mature to functioning granulocytes after stimulation by a variety of syntnetic agents. Under similar conditions, the BNML lines do not show this phenomenon. Before discussing in more detail the response of BNML cells to these agents, a description of some of these inducers and their effects will be given.

a) Retinoids

Fat soluble vitamin A is extremely important in growth,

differentiation of epithelial tissues, visual function and reproduction. Three important vitamin A compounds exist: retinol, retinal and retinoic acid (Fig 5). Retinoic acid can promote normal growth but, unlike retinol it does not have any effect on vision or reproduction . Retinoic acid is transported in plasma as its carboxylate anion bound to serum albumin. It then binds with a 100% efficiency to a cellular binding protein, and in this form could mediate its biological function (100). Retinoic acid is formed from the oxidation of retinal in the intestinal wall from B carotene and retinol , or is a metabolite of various exogeneous vitamin A analogues. Retinoic acid then undergoes various reactions such as isomerization from all trans to 13-cis-retinoic acid, esterification, metabolism of the side chains, decarboxylation and conjugation with glucuronic acid (100). Retinoic acid usually is present in very small amounts in the body and does not seem to be stored. It and its analogues (etretinate or RO10-9359) have therapeutic effects in dermatology and also seem to have various effects on cellular differentiation and carcinogenesis. They have therapeutic effects on chemically induced benign and malignant epithelial tumors (87). They inhibit skin papilloma formation and the tumor promoting phorbol diesters induction of ornithine decarboxylase activity. Retinoids also, like phorbol diester, stimulate deacylation of cellular lipids and prostaglandin production in MDCK cells (82). Retinoic acid also induces terminal differentiation of various leukemic cells.

The site of action of retinoids could be not only at the membrane level but also at the level of the nucleus of the

target cell and could resemble the steroid hormone mechanisms. Retinoic acid enters the cytoplasm and forms a complex with the cytosol binding proteins (9). This complex could then act on the nucleus.

Retinoic acid induces lysozyme activity in M1 (a mouse myeloid leukemic cell line) but inhibits induction of phagocytic and migrating activities and morphological changes. It induces formation of an inhibitory factor by M1 cells (118) as well as stimulates production of prostaglandins E2 or D2 (which in turn stimulate lysozyme activity).

HL60 also can be induced to mature into neutrophils in a dose dependent manner with all trans retinoic acid (62). Phagocytosis, morphology and NBT reduction are used to measure maturation. Maximal response occurs at 1 μM retinoic acid (15) and continuous exposure to retinoic acid is needed for optimal effect. All trans-retinoic acid and 13-cis-retinoic acid are equally effective although retinol, retinal and retinyl acetate were 10^{-3} less potent. Further studies showed that retinoic acid induces maturation of unestablished primary leukemic cells at the promyelocytic stage but not before this stage, since myeloblasts did not mature with retinoic acid (16).

b) Dimetnyl Sulfoxide

Dimethyl Sulfoxide (DMSO) has anti-inflammatory activity and has effects on analgesia, nerve blockade, bacteriostasis, diuresis ,analgesia, vasodilation, rheumatoid arthritis and muscle relaxation. DMSO 1.3% applied over nine days induced HL60 maturation (96). The rate of O2 production was

increased, the hexose monophosphate shunt activity rised. Finally, ingestion of paraffin oil droplets opsonized with complement or Ig increased , and degranulation (release of lysozyme, peroxidase and B glucuronidase) reached a maximum after six days in DMSO and then decreased. Furthermore, Bonser et al (14) showed that DMSO could induce production of a phospholipase and of cyclooxygenase. Indomethacin, a cyclooxygenase inhibitor did not block DMSO-induced increase in hexosemonophosphate shunt activity nor the increase in chemotactic peptide receptor binding. Bonser et al also suggest a link between phospholipase absence (no release of arachidonic acid) and the absence of functions characteristic of mature granulocytes. Finally, HL60 membrane fluidity decreases with progressive DMSO induced differentiation, the ratio of membrane cholesterol to phospholipid increasing 37% on day five of induction (65).

c) Phorbol Esters (TPA)

In the same way, TPA could induce KG-1, HL60 and ML-3 to terminally differentiate to cells with some features of macrophages. However, TPA like retinoic acid could not induce KG-1a and K562 to differentiate (76). This suggests that the ability of an inducer to provoke differentiation may depend upon the stage of myeloid commitment of the cells.

d) Alkyl Lysophospholipids

M1 (mouse) and HL60 (human) were induced to differentiate into mature granulocytes and macrophages by o-alkyl-lysophospholipids (63).

e) 2-Mercaptoethanol

In the same way, 2-mercaptoethanol was used to induce blast cells of an acute myeloid leukemia patient into neutrophils (70).

Hence, many agents can induce maturation of myeloid cell lines at a certain stage of development. For example,. retinoic acid can induce terminal differentiation of leukemic promyelocytes into neutrophils as measured by increased adherence, increased phagocytosis, NBT reduction . These agents could interact with the membrane (decreased fluidity, arachidonic acid metabolism) or act directly at the level of the nucleus.

In this study, incubation with 1.25% DMSO or 1,2,5 and 10 µM retinoic acid did not cause maturation of either the parent or BNML-RS cells. Thus, the parent in vivo-passaged BNML is more analogous to the human ML-2 line (78) which clearly is promyelocytic but appears to be blocked at that stage and incapable of further maturation. Intermediate to the promyelocyte and the "undifferentiated" state appears to be the late passage form of the KG-1 (108) human myeloblast which contains few granules, lacks the Ia-like antigen and is unable to form granulocytes in suspension culture. The BNML-RS line in vitro appeared similar to KG-1 showing only minimal features of maturation but in vivo was able to progress to

early promyelocyte . The controlling stimuli for maturation from blast to early promyelocyte appear to have been lacking under the usual conditions of culture but partially were reconstituted in the natural host. Thus, comparison of these rat cells with other human leukemic cell lines suggests that there may exist recurring patterns of phenotypic expression that reflect " quantum-like" levels of regulation at the blast and promyelocyte stages.

VI) Conclusion

In summary, these BNML lines, which appear to be arrested at the myeloblast or early promyelocyte level of maturation, extend the range of animal models available for study of how leukemic cells interact with host regulatory mechanisms. This is so especially at the early stages before the promyelocyte when the leukemogenic factors may be active. However, alterations in gross morphology and peroxidase in themselves are not sufficient proof of differentiation status. They must be confirmed by markers capable of finer discrimination (i.e. hybridoma reagents) that also have the potential to denote the normal equivalent in the non-diseased tissue.

CHAPTER III

ANTIGENIC CHARACTERIZATION OF BNML CELLS

Structures present on the cell membrane can be identified and purified using antibodies directed against them. Such antigens potentially could characterize the differentiation stage of the various BNML cell lines and then could be used as markers for each stage. Antibodies also could be obtained against a tumor specific or tumor associated antigen present on BNML cells, if such an antigen exists.

Three approaches were used to try to develop such antibodies:

1) Syngeneic immunization of BN rats with BNML cells to determine possible serologic reactivity.

2) Xenogeneic immunization of rabbits with BNML cells to obtain a hetero-antiserum.

3) a) Xenogeneic immunization of mice to generate hetero-monoclonal antibodies by the hybridoma technique.

b) Syngeneic immunization of BN rats to obtain autologous monoclonal antibodies by the hybridoma technique.

I) Determination of the Antibody Activity of Sera from BN Rats

1) Materials and methods

Sera from 24 BN rats injected once either with $10^{7}-10^{8}$ irradiated (600 rads) parental BNML cells i.p. (N=10), or with $10^{6}-10^{7}$ parental BNML cells i.p. (N=14) were collected and decomplemented at 56 degree centigrades for 30 minutes. These sera were then analysed by immunofluorescence (see the appendix) against parental BNML cells using a mixture of fluorescein conjugated goat anti-rat IgG and goat anti-mouse IgM which cross reacts with rat IgM. Controls used were normal BN sera.

2) Results

In none of the sera was there measurable antibody activity against parental BNML cells compared to controls.

3) Discussion

Hence, if a significant humoral immune response is not elicited by BNML cells injected into BN rats, it is not clear

if any tumor-specific antigen is present on BNML cells. However, such an antigen may not be present in large quantities or may not be constituted of molecules eliciting a high immune response. Finally, the technique employed may not be specific enough to measure an antibody of very low concentration or affinity.

II) Xenogeneic immunization of rabbits with BNML cells in order to obtain a heterocantiserum

1) Materials and Methods

Two rabbits (Mc Gill animal facility) were injected twice subcutaneously with 10^7 BNML spleen cells containing 10% residual lymphoid cells in complete Freunds adjuvant. Sera were obtained (ear puncture) 2-5 weeks after the last injection. To remove unwanted specificities, they were adsorbed twice with normal BN spleen cells.

Immunofluorescence studies with fluorescein conjugated goat anti-rabbit IgG (Fab')2 were performed to determine specific reactivity with parental BNML cells. Rabbit sera reacted with:

- A leukemic spleen suspension where parental BNML cells constituted 90% of the suspension and lymphocytes 10%. Red blood cells were lysed using 0.2% cold NaCl followed by 1.6%

cold NaCl.

-A suspension containing 90% BNML cells and 10% T cells. Nylon wool columns were used to remove macrophages and B cells from the original spleen cell suspension (see the appendix). Red blood cells were lysed as described above.

- A normal BN spleen suspension as control. Red blood cells were lysed.

2) Results

The resulting sera reacted with both BNML and normal spleen cells. However, after absorption with normal BN spleen cells it did not react against BN rat normal spleen cells, as expected, nor did it show any reactivity above background against parental BNML cells.

3) Discussion

Heteroantisera of rabbits injected with parental BNML cells did not react significantly against these cells as determined by immunofluorescence. Most of the antibodies present reacted against normal antigenic constituents of spleen cells; these antigens must cause a higher immune response than any antigen specific to BNML cells. This phenomenon clearly indicated that monoclonal antibodies should

be derived to obtain any degree of reactivity against BNML cell antigens.

III) Derivation of Monoclonal Antibodies against BNML Cells

1) The Hybridoma Technique

Conventional antisera are a heterogeneous mixture of various classes of immunoglobulins reacting against different antigenic determinants at different concentrations. Hence, the advent of a new technique leading to the production of a monospecific antibody against a specific determinant of an antigen overcomes most of these problems. This technique developed by Kohler and Milstein (77) was based on Burnet's clonal selection theory which predicts that one plasma cell produces only one antibody.

Hybridomas can produce up to 100 µg antibodies/ml in culture and up to 10 mg antibodies/ml in serum or ascites fluid of tumor bearing mice (31). Chromogenic assays now exist (35) which enable the detection of solubilized cell surface antigens which react with monoclonal antibodies and facilitates their purification.

The use of monoclonal antibodies is now widespread in many areas of Biology : probes against proteins, hormones, drugs, tumor associated antigens,etc... Hence monoclonal

antibodies have tended to replace conventionnal antisera because of their greater specificity.

2) The experiment

In this present study, the hybridoma technique was used to derive monoclonal antibodies against BNML cell antigens.

3) Materials and Methods

* = see p 82a, 82b for abbreviations

1) Balb/c mice (Mc Gill animal facility) were injected twice i.v. with 10^7 BNML-ES cells. Three days following last injection, the spleens were removed, minced and pressed through a stainless steel mesh under sterile conditions. The spleen cells were fused with mouse P3* myeloma cells at a 1:10 myeloma:spleen ratio by the method described in the appendix.

The resulting suspensions of hybridoma and parental cells were plated in 96 well flat bottom plates (Flow) at $2x10^5$ myeloma cells/ml in RPMI-1640 medium (Flow) containing 15% FCS. RPMI-1640 medium supplemented with HAT* (Flow) and 15% FCS was added the next day to the plates. The hybridomas were then fed three more times with 15% FCS HAT medium at 2-3 days interval. Afterwards, the cells were fed with RPMI-1640 supplemented with HT* (Flow) and 15% FCS when needed.

Supernatants of wells with clonal expansion were tested

by enzyme linked immunosorbent assays (ELISA ; method described in the appendix) against BNML-BS cells. The resulting positive supernatants were then checked by fluorescence (method described in the appendix) against BNML-BS and normal spleen cells using fluorescein conjugated goat anti mouse IgG, heavy and light chains, selected for low cross reactivity with rat Ig (Cappel). Positive hybridomas were selected, cloned and recloned in 0.25% agar (see the appendix) containing 10% FCS in RPMI-164C. Each clonal supernatant was retested by ELISA against BNML-BS cells. Ascites were then induced in Balb/c mice for each positive cloned hybrid by injecting 10^7 hybrid cells i.p. seven days after a prior Pristane injection. The ascites were drained 7-28 days afterwards; the resulting ascitic fluids were tested by fluorescence against BNML-BS cells, BNML-BS/D cells, BNML-BS/FCS adapted cells , parental BNML cells, normal spleen cells and normal bone marrow cells.

2) 10 BN rats were injected 3-4 times with a leukemic spleen cell suspension containing 10^7-10^8 dead parental BNML cells; cells were killed by irradiation (600 rads) or by being frozen thawed twice. The parental BNML cells constituted 90% of the spleen cell suspension while the lymphocytes constituted 10% of the suspension. The last injection was i.v. Three days after the last injection, spleen cell suspensions were obtained as for the mice. Six fusions with mouse P3 myeloma cells, three fusions with mouse NS1* myeloma cells and one fusion with mouse SP2* myeloma cells at a 1:10

myeloma to spleen cells ratio were performed by the method described in the appendix. The same steps as for the mouse x mouse fusions descrited above were then followed.

4) Results

1) Six 'Balb/c spleenxP3' hybrids were obtained, cloned and recloned and designated BN3,BN6,BN18,BN27,BN35 and BN129. Each of these hybrids produced a monoclonal antibody reacting against BNML-RS cells as demonstrated by ELISA and by fluorescence. Furthermore, all hybrids except for BN6 produced antibodies binding to culture adapted lines (BNML-RS, BNML-RS/D and BNML-FCS) and the parental in vivo BNML cells in a similar fashion. However, in each cell line culture tested, there was some heterogeneity in the proportion of antibody bound to BNML cells except for BN3 and BN6. Indeed, BN3 antibody bound similarly to all cells of a given cell line culture as assessed by visual fluorescence microscopy. Finally,none of these antibodies except for BN6 reacted against normal spleen cells. This antibody appeared to react against macrophages present in normal spleen cells. On the other hand, the BN6 antibody bound less to parental in vivo BNML cells than to any of the cultured BNML cells. There was no heterogeneity in the proportion of BN6 bound to each cell line. Finally, these six antibodies all reacted with a high percentage of normal bone marrow cells, the identity of which remains to be determined and with less than 5% of normal spleen cells.

2) The 'BN spleen x mouse myeloma ' fusions all failed to produce hybrids reactive with BNML cells

5) Discussion

1) Six hybrids producing antibodies against BNML cells were isolated.

a) Antibodies BN18, BN27, BN35 and BN129 all reacted similarly against all cultured cell lines and parental BNML cells. These antibodies must have reacted against molecules present also on normal counterparts since they bound to bone marrow cells of non-leukemic rats. For instance, such antigens could be present on normal myeloblasts and promyelocytes as well as on BNML cells. These antigens, however, were not found on most B or T lymphocytes, fibroblasts, macrophages or red blood cells which are all constituents of normal spleen against which there was no significant reactivity. Since there was heterogeneity in binding amongst cells of the same cultured line, these antigens may be expressed only at some stages in their cell cycle. It is not clear if these antibodies all react against the same antigen or if they react against several antigens. Isolation and chemical characterization of them will answer this question.

b) Antibody BN3 bound to a structure found not only on rat leukemic myeloblasts and promyelocytes but also on some normal bone marrow counterparts. There was, however, less heterogeneity of reactivity against the cultured cells. Hence this antibody appears to react against an antigen expressed independently or the cell cycle.

c) Antibody BN6 reacted against an antigen which seems to

be expressed mostly at the myeloblast stage of leukemic cells. Although present also at the promyelocyte stage it is either less expressed or less available on the cell surface to antibody binding. Addition of terminal oligosaccharides, for instance , could conceal the antigenic binding site. This antigen is also present on early normal bone marrow cells, possibly myeloblasts. BM1 also slightly reacted against a subpopulation of spleen macrophage like cells. This binding could be due on one hand to cross reactivity with some similar determinants of a molecule present on macrophages; on the other hand, macrophages could express on their cell surface an antigen similar or analogous to the one present on myeloblasts but in small quantities i.e. an antigen common to both myeloblasts and macrophages. The possibility that the reactive lymphoid subpopulation are natural killer cells should be explored.

Relevant discussion and comparison of human myeloid antigens to the BNML antigens would be premature at this time and will require a more complete biochemical characterization of the antigens found in this present work.

6) Conclusion

Six stable mouse to mouse monoclonal antibodies have been developed against at least three different antigenic structures present on normal and leukemic rat myeloblasts and/or promyelocytes . Futhermore , one of these antibodies

(BN6), reacts more specifically against myeloblasts. These antibodies might be used as markers of certain cell stages in the rat myeloid lineage. Chemical characterization of these antigens is now possible. It may be found that analogous antigenic structures are present on human myeloblasts and/or promyelocytes. Hence, these markers lead to a better understanding of the normal and leukemic myeloblast-promyelocyte maturation.

As stated above, the 'BN spleen to mouse myeloma' fusions were unsuccessful. The development of a hybrid antibody from these fusions, reacting against BNML cells would have indicated the possible presence of a leukemia-specific or associated antigen on the cell surface of BNML cells. Indeed, the BN rats did not react against structures e.g. histocompatibility antigens, present on normal myeloblasts and promyelocytes.

CONCLUSION

The preexisting model for acute promyelocytic leukemia in the Brown Norway rat was limited in its applications . Indeed, the cells could proliferate only *in vivo*. Cell lines derived from this *in vivo* model now have been maintained in culture for over two years. These lines consist of myelcblasts and early promyelocytes instead of the promyelocytes found in the *in vivo* model. This apparent block in maturation is removed partially when the *in vitro* cells are grown in the animal.

The study of these new lines leads to a better understanding of the various factors and cellular interactions controlling granulopoiesis. For example, the role of macrophage-granulocyte inducers as well as of possible fibroblast-blast or macrophage-blast could be studied. Lacaze's BNML cell line could be compared to the cell line derived in the present study, thus revealing the differences between two microhemcpoietic environments: the bone marrow and the spleen. This model also permits physiological comparisons between *in vivo* and *in vitro* conditions. Furthermore, antigenic characterization of leukemic cells at the myeloblast-promyelocyte stages is possible. In this study, six monoclonal antibodies have been derived against at least three antigenic structures present on these leukemic cells.

Further chemical characterization of these antigens now can be performed. This model will permit an investigation of the cell maturation block occurring in acute myeloid leukemia.

REFERENCES

1. Aglietta M., and Sonneveld P., The relevance of cell kinetics for optimal scheduling of 1-B-D arabinofuranosyl cytosine and methotrexate in a slow growing acute myeloid leukemia (BNML)., Cancer Chemother. Pharmacol., 1, 1978, pp 219-223.

2. Aglietta M. and Colly L., Relevance of recruitment synchronization in the scheduling of 1-B-D arabinofuranosyl cytosine in a slow growing acute myeloid leukemia of the rat., Cancer Res., 39, 1979, pp 2727-2732.

3. Aglietta M. and Sonneveld P., The acute myelcid leukemia of the BN rat : a model for cell kinetics scheduling of antileukemic drugs., Exp. Hematology, 1979, p 137 (abstract).

4. Alexander P., Prostaglandins and cancer., Nature, 295, 1982, p 185.

5. Andersson L.C., Gahmberg C.G., Siimes M.A., Teerenvohi L. and Vuopio P., Cell surface glycoprotein analysis : a diagnostic tool in human leukemias., Int. J. Cancer, 23, 1979, pp 306-311.

6. Astaldi G.C.B, Janssen M.C., Lansdorp P.H., Zeiflemaker

W.P., and Williams C., Human endothelial culture supernatant (HECS): evidence for a growth promoting factor binding to hybridoma and myeloma cells., The J. of Immunology, 126, No 3, 1981, p 1170.

7. Azuma J. and Sachs :., Chromosome mapping of the genes that control differentiation and malignancy in myeloid leukemic cells., Proc. Natl. Acad. Sci. USA, 74, No 1, 1977, pp 253-257.

8. Bainton D., Differentiation of human neutrophilic granulocytes: normal and abnormal. In the Granulocyte: Function and Clinical Utilization, Greenwalt T. and Jamieson G.A., Eds. Alan R. Liss ,Inc.,New York, 1976, p 1.

9. Banerjee C.K. and Sani B.P., Cellular uptake of retinoic acid in vitro., Biochem. J., 190, 1980, pp 839-842.

10. Berger R., The chromosomes in hematology., Cancer Genetics and Cytogenetics, 4, 1981, pp 69-88.

11. Berger R., Bernheim A., Daniel M.T., Valensi F., and Flandrin F., Karyotype and cell phenotypes in primary acute leukemias., Blood cells, 7, 1981, pp 287-292.

12. Blann A.D., Cell hybrids : an important new source of antibody production., Medical Laboratory Sciences, 36, 1979,

pp 329-338.

13. Boggs D.R., Clonal origin of leukemias: site of origin in the stem cell hierarchy and the significance of chromosomal changes., Blood Cells, 7, 1981, pp 205-215.

14. Bonser G.M, Siegel M.I., Mc Connell R.T., and Cuatrecasas P., The appearance of phospholipase and cyclooxygenase activities in the human promyelocytic leukemia cell line HL60 during dimethyl sulfoxide induced differentiation., Biochim. Biophys. Res.Commun., 98, No 3, 1981, pp 614-620.

15. Breitman T.R., Selonick S.E., and Collins S.J., Induction of differentiation of the human promyelocytic leukemia cell line (HL60) by retinoic acid., Proc. Natl. Acad. Sci. USA, 77, 1980, p 2936.

16. Breitman T.R., Collins S.J., and Keene B.F., Terminal differentiation of human promyelocytic cells in primary cultures in response to retinoic acid., Blood, 57, No 6, 1981, p 1000.

17. Brennan J.K., Abboud C.N., Dipersio J.F., Barlow G.H., and Lichtman M.A., Autostimulation of growth by human myelogenous leukemia cells (HL60)., Blood, 58, No 4, 1981, p 803.

18. Burgess A.W., and Metcalf D., The nature and action of

granulocyte-macrophage colony stimulating factors., Blood, 56, No 6, 1980, p 947.

19. Carpentier N.A, Piere D.M., Schuh D., Lange G., and Lambert P.H., Circulating immune complexes and the prognosis of acute myeloid leukemia., The New England Journal of Medicine, 307, No 4, 1982, p 1174.

20. Caskey C.T., and Kruh G.D.,The HPRT locus, Cell, 16, 1-9, 1979, p 1.

21. Chang T.H., Steplewski Z. and Koprowski H., Production of monoclonal antibodies in serum free medium., J. of Immunological Methods, 39, 1980, pp 369-375.

22. Cline M.J. et al., Acute leukemia : biology and treatment., Annals of Internal Medicine, 91, 1979, pp 758-773.

23. Cline M.J., and Golde D.W., Cellular interactions in haematopoiesis, Nature, 277, 1979, p 177.

24. Collins S.J., Gallo R.C. and Gallagher R.E., Continuous growth and differentiation of human myeloid leukemic cells in suspension culture., Nature, 270, 1977, p 347.

25. Collins S.J., Ruscetti F.W., Gallagher R.E., and Gallo R.C., Terminal differentiation of humans promyelocytic

leukemia cells induced by dimethyl sulfoxide and other polar compounds., Proc. Natl. Acad. Sci. USA., 75, 1978, p 2458.

26. Colly L.P., Tcns A., and Hagenbeek A., Experimental chemotherapy in the BN myelocytic leukaemia., Leuk. Res., 1,No 2/3, 1977, pp 247-250.

27. Colly L.P., Tons A., The effect of tumor load reduction in the BN myelocytic leukemia by means of recruitment and synchronization on normal hemopoiesis., Exp. Hematology, V7S6, 1979, p 79 (abstract).

28. Cronkite E.P., Ieukemia revisited, Blood cells, 7, 1981, pp 11-30.

29. De La Chapelle A., Knuutila S., Elonen E., and Vuopio P., Chromosomal abnormalities in acute promyelocytic leukaemia., Scand. J. Haematol., 26, 1981, pp 57-60.

30. Dexter T.M., Haemopoiesis in long term bone marrow cultures., Acta Haemat., 62, 1979, pp 299-305.

31. Diamonds B.A., Yelton D., and Scharff M.D., Monoclonal antibodies : a new technology producing serologic reagents., The New England Journal of Medicine, 304, No 22, 1981, p 1344.

32. Donne A., Cours de microscopie complementaire des etudes

medicales. A Paris chez J.B. Baillieres, 1844.

33. Douer D., Koeffler H.P., Retinoic acid enhances colony stimulating factor induced clonal growth of normal human myeloid progenitor cells in vitro., Experimental Cell Research, 138, 1982, pp 193-196.

34. Douillard J.Y., Hoffman T., and Heberman R.B., Enzyme linked immunosorbent assay for screening monoclonal antibody production: use of intact cells as antigen., J. of Immunol. Methods, 39, 1980, p 309.

35. Eisenbarth G.S., Rankin R.B., Haynes B.F. and Fauci A.S., A visual assay to monitor purification of cell surface antigens reacting with monoclonal antibodies., J. of Immunol. Methods, 39, 1980, pp 387-392.

36. Elias L., Li A.P., Longmire J., Cyclic adenosine 3'5' monophosphate dependent and independent protein kinase in acute myeloblastic leukemia. Cancer Res., 411, 1981, pp 2182-2188.

37. Feldman J.D., and Hoda R.A., Pathology and tumor incidence in aged Lewis and BN rats., Clinical Immunology and Immunopathology 15, 1980, pp 331-343.

38. Fisher A.G., and Brown G., A rapid method for determining

whether monoclonal antibodies react with the same or different antigens on the cell surface., J. of Immuno. Methods, 39, 1980, pp 377-385.

39.Fox P.C., Berenstein E.H., and Siriganian K.F., Enhancing the frequency of antigen specific hybridomas. ,Eur. J. Immunol., 11, 1981, pp 431-434.

40. Fraser J., Hollings P.E. and al, Acute Promyelocytic Leukemia: cytogenetics and bone marrow culture., Int. J. Cancer, 27, 1981, pp 167-173.

41. Friedman E.A., Higgins E.J., Lipkin M., Shinya M., and Gelb A., Tissue culture of human epithelial cells from benign colonic tumors., In vitro, 17, 1981, p 632.

42. Gerter M.L., Margulies D.H., and Scharff M.D., A simple method for polyetnylene glycol promoted hybridization of mouse myeloma cells., Somatic Cell Genetics, 3, No2, 1977, pp 231-236.

43. Glasser L., Leukemic cells as probes for sequential functional differentiation of the human granulocyte., American Journal of Clinical Pathology., 79, No 1, 1983, p 45.

44. Glynn S., and Sullivan A.K.,. In vitro lines of the BN rat promyelocytic leukemia that differ from the parent.,

Leukemia Research, 7, 1983, p557.

45. Goding J.W., Antibody production by hybridomas., J.of Immuno. Methods, 39, 1980, pp 285-308.

46. Groopman J., and Ellman L., Acute promyelocytic leukemia, Am. J. of Hematology, 7, 1979, pp 395-408.

47. Haemmerly G., and Felix H., Motility and cytoplasmic filamentous structures of L522 and BNML leukemia cells., Leuk Res., 1 ,No 2/3, 1977, pp 209-218.

48. Hayenbeek A., Introduction of the BN myelocytic leukaemia., Leuk. Res., 1. No 2/3, 1977. pp 85-90.

49. Hayenbeek A., Colly L.P., and Van Bekkum D.W., Growth regulation in the BN myelocytic leukaemia., Leuk. Res., 1, No 2/3, 1977, pp 149-151.

50. Hayenbeek A., and Hartens A., Organ invasion and the kinetics of intercompartmental distribution in the BN myelocytic leukaemia., Leuk. Res., 1,No 2/3, 1977, pp 117-121.

51. Hayenbeek A., Hartens A.C.H., Van Bekkum D.W., Hermens A.F., Zaat G.S., and Hoogeven-Van Beugen E., Proliferation kinetics of the BNML leukaemia in vivo., Leuk. Res., 1, No 2/3, 1977, pp 99-101.

52. Hagenbeek A. and Martens A.C.M., Functional cell compartments in a rat model for human acute myelocytic leukaemia., Cell Tissue Kinetics, 12, 1979, pp 361-377.

53. Hagenbeek A., and Martens A.C.M., Separation of normal hemopoietic stem cells from clonogenic leukemic cells in a rat model for human acute myelocytic leukemia . II. Velocity sedimentation in combination with density gradient separation., Exp. Hematol., 9, No 6, 1981, pp 573-580.

54. Hagenbeek A., and Martens A.C.M., The proliferative state of normal hemopoietic stem cells during progression of leukemia studies in the BN acute myelocytic leukemia (BNML)., Leuk. Res., 5, No 2, 1981, pp 141-148.

55. Hagenbeek A., Martens A.C.M., and Van Bekkum D.W., Separation of normal hemopoietic stem cells from clonogenic leukemic cells in a rat model for human acute myelocytic leukemia .I. Velocity Sedimentation., Leuk. Res., 5, No 4/5, 1981, pp 421-428.

56. Hagenbeek A., and Martens A.C.M., High dose cyclophosphamide treatment of acute myelocytic leukemia: studies in the BNML rat model., Br. J. Cancer Clin. Oncol., 18, No 6, 1982, pp 763-769.

57. Hagenbeek A., and Martens A.C.M., Efficacy of high dose

cyclophosphamide in combination with total body irradiation in the treatment of acute myelocytic leukemia : studies in a relevant rat model., Cancer. Res., 43, 1983, pp 408-412.

58. Harlozinska A., Fotonski J., Nowowiska A., Becker M., and Richter R., Fc and C3 receptors as membrane differentiation markers of acute myelogenous leukaemia cells., Scand. J. Haematol., 25, 1980, pp 205- 213.

59. Haynor D.R., Singer D.E., and Williams R.E., Resistance to BN myelogenous leukemia in LBNF1 rats preinjected with BN bone marrow., Fed. Proc., 38, #4451, 1979, p 1069 (abstract).

60. Hilyard P., Coagulation studies in the BNML rat leukaemia., Leuk. Res., 1, No 2/3, 1977, pp 175-176.

61. Hoffbrand A., and Janossy G., Enzyme and membrane markers in leukaemia : recent developments., J. Clin. Pathol., 34, 1981, pp 254-262.

62. Honma Y., Takenaga K., Kasuba T, Hozumi M., Induction of differentiation of cultured human promyelocytic leukemia cells by retinoids., Biochemical and Biophysical research communications., 95, No 2, 1980, pp 507-512.

63. Honma Y., Kasukabe T., Hozumi M., Tsushima S., and Nomura H., Induction of differentiation of cultured human and mouse

myeloid leukemia cells by alkyl-lysophospholipids., Cancer Res., 41 1981, pp 3211-3216.

64. Huggins C.B., and Suciyama T., Induction of leukemia in rat by pulse doses of 7,12 dimethylbenz(a)anthracene., Proc. Natl. Acad. Sci. USA., 55, 1966, p 74.

65. Ip S.H.C., and Cooper E.A.., Decreased membrane fluidity during differentiation of human promyelocytic leukemia cells in culture., Blood, 56, No 2, 1980, p227.

66. James S.E., Dean C.J. and Alexander P., Failure to detect autologous antibodies in the remission sera of patients with AML: complications introduced by the presence of rheumatoid factor., Brit. J. Cancer., 42, 1980, p 385.

67. Kaye G.I., Pascal R.R., and Lane N., The colonic cryptic fibroblast sheath : replication, migration and cytodifferentiation of a mesenchymal cell system in adult tissue., Gastroenterology, 60, 1971, p 515.

68. Keller R., Regulatory functions of macrophages., AAS 7, Birkhauser Verlag, Basel, 1980, pp 90-99.

69. Killmann S.A., Review on proliferative kinetics in human acute leukemias with special reference to the relevance of both rat leukemia models., Leuk Res., 1, No 2/3, 1977.

70. Kirshner J., and Goldberg J., In vitro differentiation of myeloblasts from a patient with acute myeloid leukemia., A.J.C.P., 75, No 4, 1981, p 617.

71. Klebe R.J., and Mancuso M.G., Chemical which promote cell hybridization., Somatic cell Genetics, 7, No 4, 1981, pp 473-488.

72. Klock J.C., Macher B.A., and Lee W.M.F., Complex carbohydrates as differentiation markers in malignant blood cells : glycolipids in the human leukemias., Blood cells, 7, 1981,pp 247-255.

73. a) Koeffler H.P., Bar Eli M., and Territo M.C., Phorbol ester effect on differentiation of human myeloid leukemia cell lines blocked at different stages of maturation., Cancer Res., 41, 1981, p 519.

b) Koeffler H.P., Billing R., Lusis A.J., Sparkes R., and Golde D.W., An undifferentiated variant derived from the human acute myelogenous leukemia cell line (KG-1)., Blood, 56, No 2, 1980, p 265.

74. Koeffler H.P., Billing R., Sparkes R., and Golde D.W., Antigens present on human myeloid leukemia cell lines., Leuk. Res., 4, No 1, 1980, pp 69-77.

75. Koeffler H.P., and Golde D.W., Human myelcid leukemia cell lines : a review., Blood, 56, No 3, 1980, p 344.

76. Koeffler H.P., Benasbe B.E., and Territo M.C., Phorbol ester effect on differentiation of human myeloid leukemia cell lines blocked at different stages of maturation., Cancer Res., 41, 1981, pp 919-926.

77. Kohler G. and Milstein C., Continuous culures of fused cells secreting antibody of predefined speciticity., Nature, 256, 1975, p 495.

78. Kubota K., Preisler H.D., Lok M.S., and Minowada J., Lack of effect of colony stimulating activity on human myeloid leukemic cell line (ML-2) cells., Leuk. Res.,5,1981,p 311.

79. Lacaze M., Gombaud-Saintonge G., and Lanotte M.,Conditions controlling long term proliferation of Brown Norway rat promyelocytic leukemia in vitro: Primary growth stimulation by microenvironment and establishment of an autonomous Brown Norway leukemic stem cell line., Leuk. Res., 7, No 2, 1983, pp 145-154.

80. Lacroix A., Anderson G.D.L., and Lippman M.E., Retinoids and cultured human fibroblasts., Experimental Cell Research, 130, 1980, pp 339-344.

81. Lajtha L.G., Which are the leukemic cells?.,Blood cells, 7, 1981, pp 45-62.

82. Levine L., and Ohuchi K., Retinoids as well as tumor promoters enhance deacylation of cellular lipids and prostaglandin production in MDCK cells., Nature, 276, 1978, p 274.

83. Liebermann D., Hoffman-Liebermann D., and Sachs L., Molecular dissection of differentiation in normal and leukemic myeloblasts: separately programmed pathways of gene expression.,Developmental Biology, 79, 1980, p 46-63.

84. Littlefield J., Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants., Science 145, 1964, p 709.

85. Lotem J. and Sachs L., Mechanisms that uncouple growth and differentiation in myelcid leukemia cells: restoration of requirement for normal growth inducing protein without restoring induction of differentiation inducing protein., Proc Natl. Acad. Sci. USA, 79, 1982, pp 4347-4351.

86a. Marie J.P., Izaguarre C.A., Civin C.I.,Mirro J. and McCulloch E.A., Granulopoietic differentiation in AML blasts in culture, Blood 4, 1981,p670.

86b. Martens A.C.M., and Lagenbeek A., Pulse cytophotometry of the BN myelocytic leukemia during development and during treatment with cytostatic drugs., Leuk. Res., 1, No 2/3, 1977, pp 103-106.

87. Mayer H., Bollag W., Hann R., and Huegg R., Retinoids, a new class of compounds with prophylactic and therapeutic activities in oncology and dermatology., Experientia, 34, 9, pp 1105-1246.

88. Mc Culloch E.A., Till J.E., Stem cells in normal early haemopoiesis and certain clonal haemopathies., Recent Adv. in Hematol.,2, 5, 1977, p 4.

89. Mc Culloch E.A., Buick R.N., Curtis J.E., Messner H.A. and Senn J.S., The heritable nature of clonal characteristics in acute myeloblastic leukemia., Blood, 58, No 1, 1981, p 105.

90. Melchers F., Potter E., and Warner N.L., Preface , Current Topics in Microbiology and Immunology 81, Lymphocyte Hybridomas, Springer Verlag Berlin, Heidleberg, New York, 1978,p IX.

91. Metcalf D., Hemopoietic Colony stimulating factors., Tissue Growth Factors. Ed: Baserga R. Springer Verlag Berlin Heidleberg, New York.,1981, Ch 12.

92. Metcalf D., Regulatory control of the proliferation and differentiation of normal and leukemia cells. National Cancer Institute Monograph, No 60, 1982, p 123.

93. Minowada J., Immunology of leukemic cells. Leukemia 4th ed. Ed. by Gunz and Henderson, Grune and Stratton., 1982, p 119.

94. Moriuchi T., Kasai M., Yamaguchi H., and Ichayashi H. Characterization and classification of rat leukemias and lymphomas by membrane markers., Cancer Res., 41, 1981, pp 1938-1942.

95. Mussoni L., Bertoni M.F., Curalolo L., Poggi A. and Donati M.B., In vitro interactions of L 5222 and BNML leukemia cells with fibrin : a preliminary report. Leuk. Res., 1, No 2/3, 1977.

96. Newburger P.E., Chovaniec M.E., Greenberger J.S. and Cohen H.J., Functional changes in human leukemic cell line HL60., J. Cell Biology, 82, 1979, pp 315-321.

97. Newman M.S., Hypothesis concerning the carcinogenic activity of benzanthracenes. The Biochemistry of Disease Vol4, Chemical carcinogenesis Part A, ed. Tso P.C.P. and Dipaolo J.A., ch6, 1972, p 177.

98. Ogawa M., Porter E.B., and Nakahata T., Renewal and Commitment to differentiation of hemopoietic stem cells (an interpretive review)., Blood, 61, No 5, 1983, pp. 823-829.

99. Olofsson T., and Olsson I., Suppression of normal granulopoiesis in vitro by a leukemia associated inhibitor (LAI) derived from a human promyelocytic cell line (HL60)., Leuk. Res., 4, No 5, 1980, pp 437-447.

100. Ott D.B., and Lachance P., Retinoic acid-a review-., Am. J. Clin. Nutr., 32, 1979, pp 2522-2531.

101. Paran M., Ichikawa Y., and Sachs L., Production of the inducer for macrophage and granulocyte colonies by leukemic cells., J. Cell Physiology, 72, 1969, pp 251-254.

102. Pigoli G., Waheed A., and Shadduck R., Observations on the binding and interaction of radioiodinated colony stimulating factor with murine bone marrow cells in vitro., Blood, 59, No 2, 1982, p 408.

103. Pourreau-Schneider N. and Strauli P., In vitro investigation of the penetrative capacities of leukemia cells., Leuk. Res., 1, No 2/3, 1977.

104. Prins M.E.F., and Van Bekkum D.W., Comparison of the distribution pattern of Brown Norway myeloid leukemia cells

and L 4415 lymphatic leukaemia cells in rat femoral bone marrow after i.v infusion., Leuk. Res., 5, No 1, 1981, pp 57-63.

105. Bai K.R. et al., Treatment of acute myelocytic leukemia: a study by cancer and leukemia group B., Blood,58, No 6, 1981, p 1203.

106. Rauchwerger J.R., Gallagher M.T., and Trentin J.J., Role of the hemopoietic inductive microenvironment (HIM) in xenogeneic bone marrow transplantation., Transplantation, 15, 1973, p 610.

107. Rhodes J. and Oliver S., Retinoids as regulators of macrophage function., Immunology, 40, 1980,p467.

108. Ruscetti F., Collins S.J., Woods A.E. and Gallo R., Clonal analysis of the response of human myeloid leukemic cell lines to colony-stimulating activity., Blood 58, 1981, p 285.

109. Sachs L., Constitutive uncoupling of pathways of gene expression that control growth and differentiation in myeloid leukemia: a model for the origin and progression of malignancy., Proc. Natl. Acad. Sci. USA., 77, No 10, 1980, pp 6152-6156.

110. Sachs L., Constitutive gene expression and restoration of

the normal phenotype in malignant cells: A model for the origin and evolution of leukemia., Blood Cells, 7, 1981, pp 31-44.

111. Setlow R.B., Damages to DNA that result in neoplastic transformation., Advances in Biological and Medical Physics, 17, 1980, p 99.

112. Sharkis S.J. and Santos G.W., Bone marrow transplantation in a BN rat model of acute myelogenous leukemia (AML). Leuk. Res., 1, NO 2/3, 1977.

113. Sharkis S.J., Santos G.W. and Colvin M., Elimination of acute myelogenous leukemic cells from marrow and tumor suspensions in the rat with 4-hydroperoxycyclophosphamide., Blood, 55, No 3, 1980, p 521.

114. Spitzer G. et al., Human myeloid leukemic cell interactions in vitro with normal myeloid colonies., Br. J. Cancer, 43, 1981, p 149.

115. Suter L., Bruggen J. and Sorg C., Use of an enzyme linked immunosorbent assay (ELISA) for screening of hybridoma antibodies against cell surface antigens., J. of Immuno. Methods, 39, 1980, pp 407-411.

116. Swaen G.J.V. and Van Heerde P.V., Tumors of the

haematopoietic system., Pathology of Tumors in Laboratory Animals Vol1 : Tumors of the Rat Part I. International Agency for Research on Cancer. Ed. Turusov V.S., 1973, p 185.

117. Symonds G. and Sachs L., Modulation of cell competence for induction of differentiation in myeloid leukemic cells., J. Cell Physiology., 111, 1982, pp 9-14.

118. Takenaga K., Honma Y., Okabe-Kado J., and Hozumi M., Production of differentiation inhibiting factor in cultured mouse myeloid leukemia cells treated with retinoic acid., Cancer Res., 41, 1981, pp 1948-1953.

119. Ten Cate J.W., Haemostatic defects in human acute leukaemia with reference to the two rat leukemia models., Leuk. Res., 1, No 2/3, pp 185-187.

120. Till J.E. and Mc Culloch E.A., A direct measurement of the radiation sensitivity of normal bone marrow cells. Rad. Res., 14, 1961, p 213.

121. Uchida T., Yui T., Muroi S. and Kariyone S., Shortened platelet survival in acute promyelocytic leukemia. Tohoku. J. Exp. Med., 129, 1979, pp 205-206.

122. Van Bekkum D.W., Van Oosterom P. and Dicke K.A., In vitro colony formation of transplantable rat leukemias : comparison

with human acute myeloid leukemia. Cancer Res., 36, 1976, pp 941-946.

123. Van Lekkum D.W. and Hagenbeek A., Relevance of the BN leukemia as a model for human acute myeloid leukemia., Blood cells, 3, 1977, pp 565-579.

124. Van Lekkum D.W., Prins M.E.F., and Hagenbeek A., The mechanism of haemopoiesis in acute leukemia. Blood Cells, 7, 1981, pp 91-103.

125. Von Melchner H. and Metcalf D., Differential seeding of injected hemopoietic precursor cells in the bone marrow and spleen of irradiated mice., Leuk. Res., 4, No 5, 1980, pp 393-397.

126. Williams R.H., Singer D.E., Rodday P., and Bennett M., F1 hybrid resistance to BN rat myelogenous leukemia parallels resistance to transplantation of normal BN bone marrow. Leuk. Res., 4, No 2, 1980, pp 261-264.

127. Williams W.J., In Hematology, Williams W.J., Beutler E., Erslev A., and Rundles R.W., Eds. Mc Graw-Hill Ecck Co., New York, NY., 1977, p 1587.

128. Yelton D.E., Diamond E.A., Kwan S.P. and Scharff M.D., Fusion of mouse myeloma and spleen cells. Current Topics in

Microbiology and Immunology 81. Lymphocyte Hybridomas, Springer Verlag, Berlin, Heidleberg, New York, 1978, p 1.

129. Köhler G., Howe S.C. and Milstein C., Fusion between immunoglobulin-secreting and nonsecreting myeloma cell lines, Eur. J. Immunology, 6 , 1976, pp 292-295.

130. Shulman M., Wilde C.D. and Köhler G., A better cell line for making hybridomas secreting specific antibodies, Nature, 276 , 1978 , p 269.

ABBREVIATIONS

AML : Acute myeloblastic leukemia

APL : Acute promyelocytic leukemia

Ara-C : Arabinoside-cytosine

BN : Brown Norway

BNML : Brown Norway myeloid leukemia

CSF : Colony stimulating factor

7,12-DMBA : 7,12-dimethylbenz(a)anthracene

DMSO : Dimethyl sulfoxide

ELISA : Enzyme linked immunosorbent assay

FCS : Fetal calf serum

GM : Granulocyte-macrophage

HAT : Hypoxanthine (680.60mg/l), aminopterin (8.81mg/l) and thymidine (193.80mg/l) for 50 x HAT (Flow). Add 10 mls 50 x HAT to 500 mls of RPMI 1640 (Flow).

HPRT : Hypoxanthine guanine phosphoribosyl transferase

HT : Hypoxanthine (680.60mg/l) and thymidine (193.80mg/l) for 50 x HT (Flow). Add 10 mls 50 x HT to 500 mls of RPMI 1640 (Flow).

Ig : Immunoglobulin

MGI : Macrophage-granulocyte inducer

NBT : Nitroblue tetrazolium test

NS1 : P3/NSI/1-Ag4-1 (129)

P3 : P3/X63-Ag8 (77)

RA : Retinoic acid

RS : Rat serum

SP2 : SP2/0-Ag14 (130)

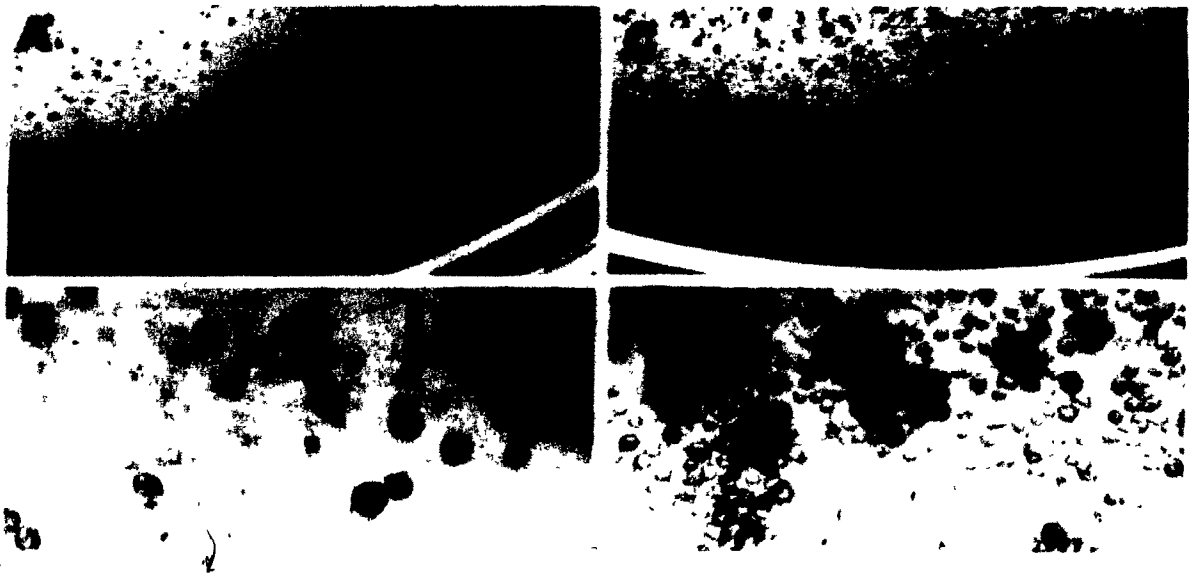
TK : Thymidine kinase

TPA : 12-o-tetradecanoylphorbol-13-acetate

EHCTCGRAPHS

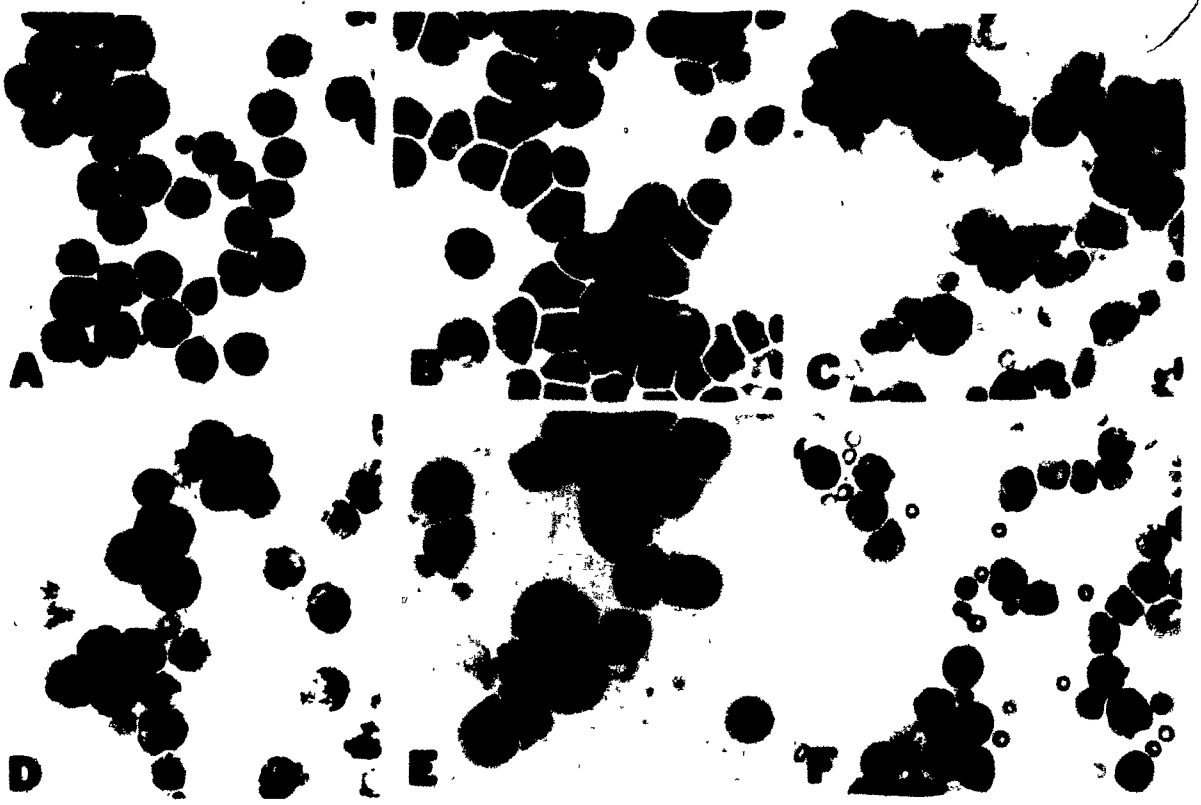
Legend to Photograph I

Photographs of spleen cells of rats made leukemic with the parent line after three weeks in culture. A, B: wells contained cells suspended in medium supplemented with 10% fetal calf serum; C,D: contained 4% rat serum/6% fetal calf serum. A,C: photographed at 20x magnification; B,D: at 160 x.



Legend to Photograph II

Photomicrographs of cytocentrifuge preparations of BNML cells. A, D : spleen cells of rats made leukemic with <u>in vivo</u>-derived parent line. B,E : BNML-RS cells established in culture. C,F : spleen cells of animals made leukemic with cultured BNML-RS cells. A,B,C are stained with May-Grünwald-Giemsa; D,E,F are stained for peroxidase activity and counterstained with safranin O. Preparations photographed at 400x magnification.


A
B
C
D
E
F

FIGURES

Enzymes	Primary granules	Secondary granules
Peroxidase	+	-
Acid hydrolase	+	-
Lysozyme	+	+
Collagenase	-	+
Alkaline phosphatase	-	+

Fig 1a : Enzymes present in primary and secondary granules.

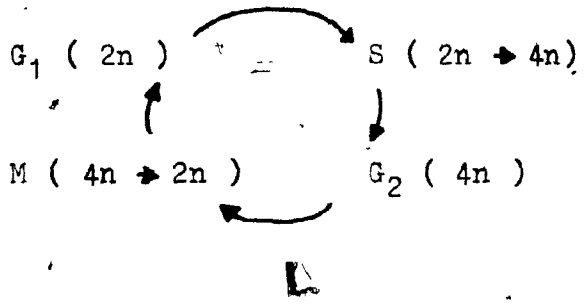


Fig 1b : The cell cycle

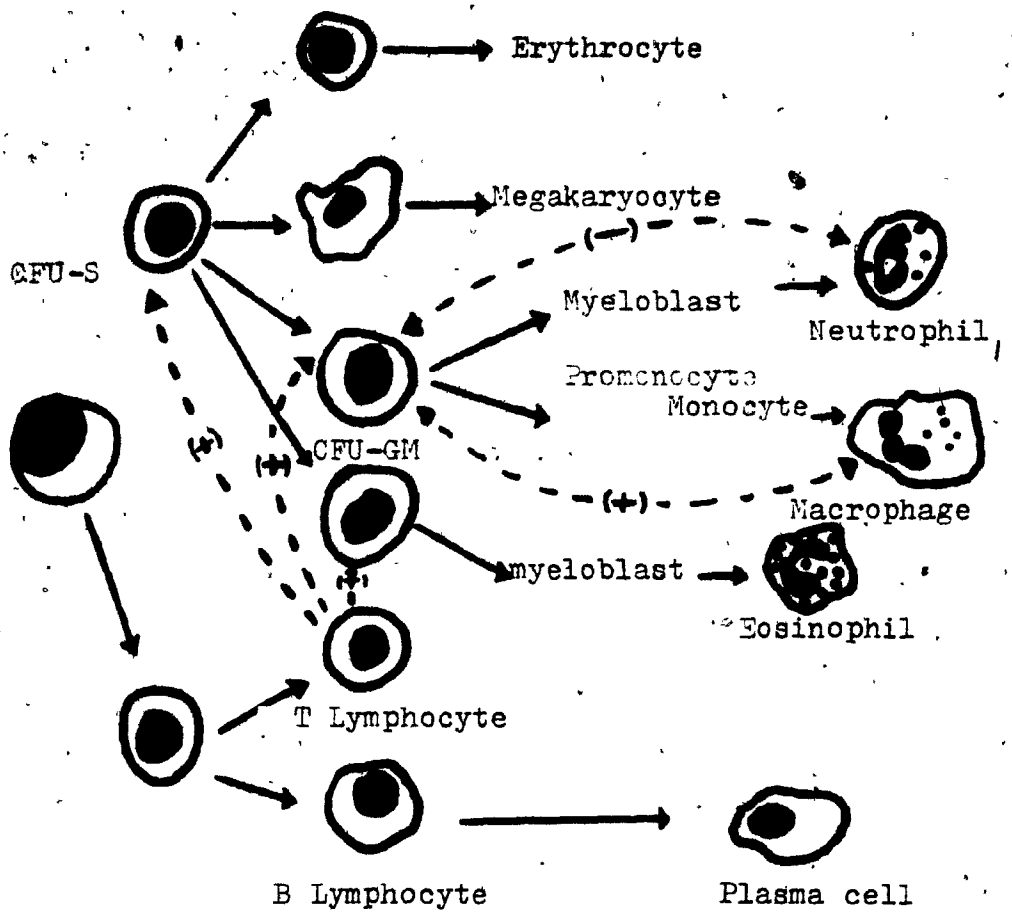


Fig 2 : Postulated lineages and relationships of Hematopoietic progenitor cells. From Cline M.J., Golde D. W. (1979), Ref 23 .

(Stimulatory interactions are denoted by (+) and possible inhibitory interactions by (-).)

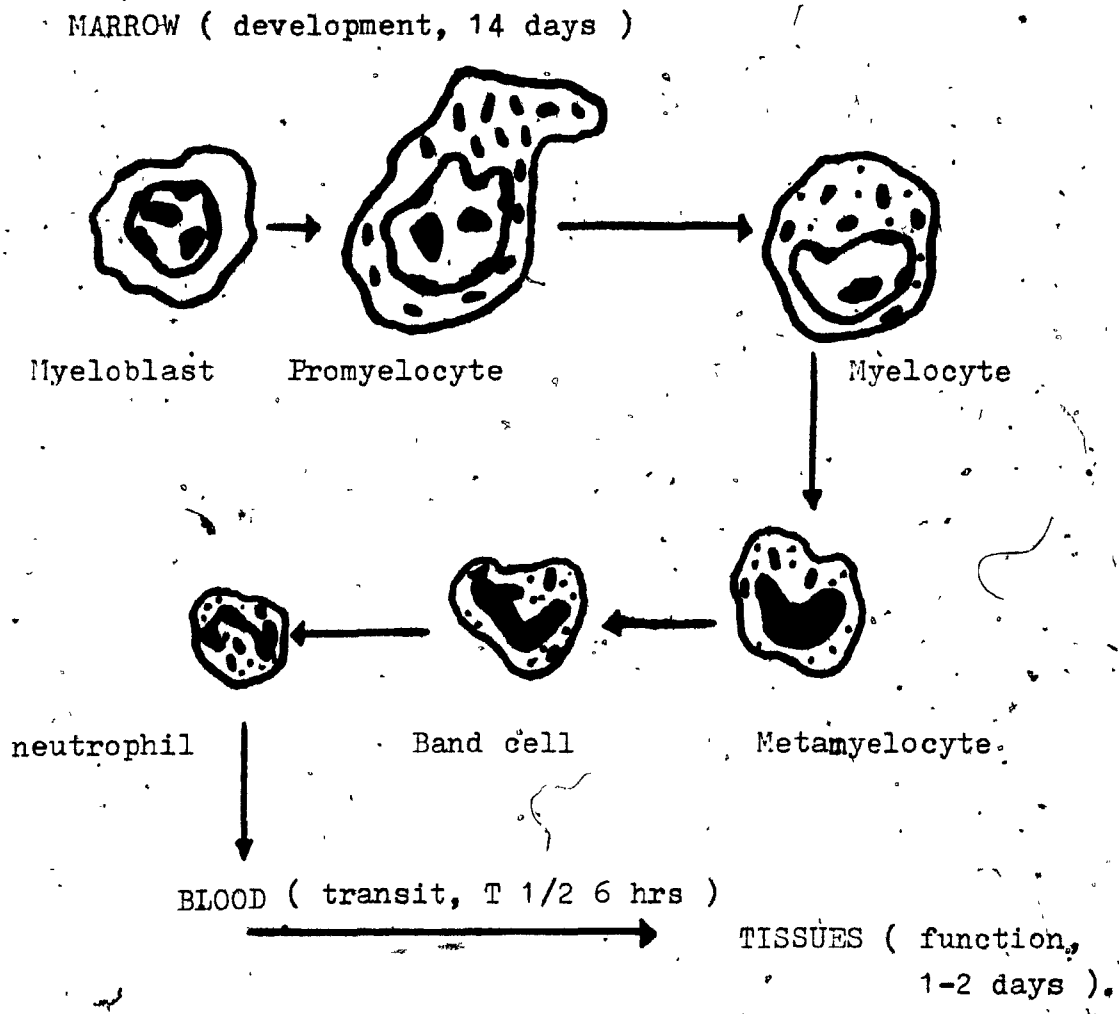


Fig 3 : Diagrammatic representation of neutrophil life-span and stages of maturation. From Bainton D. F. (1976), Ref 8.

Cell Line	Markers									
	E	EA	EAC	SmIg	T-Ag	Ia	cALL	MAg-I	TdT	EBV
K-562	-	+	-	-	-	-	-	-	-	-
HL 60	-	+	+	-	-	-	-	+	-	-
ML-2	-	+	-	-	-	-	-	+	-	-
KG-1	-	+/-	-	-	-	+	-	+	-	-

note: All cell lines have for marker: MLC-S .

Fig 4 : Markers present on K562, HL 60, ML-2 , and KG-1 . From Minowada J. (1982), Ref 93.

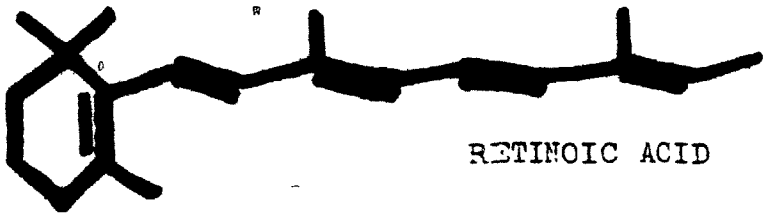
a) Bone marrow

Haemocytoblasts	0.36%	Pro-erythroblasts	1.27%
Promyelocytes	2.58%	Erythroblasts	37.95%
Myelocytes	5.41%	Normoblasts	7.75%
Metamyelocytes	14.94%	Lymphocytes	1.03%
Stab nuclear forms	25.67%	Monocytes	0.04%
Polynuclears:		Reticulum cells	0.32%
- Neutrophils	0.42%	Megakaryocytes	0.08%
- Eosinophils	1.98%		
- Basophils	0.57%		

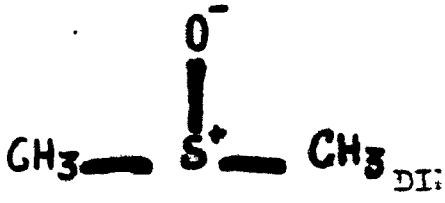
b) Peripheral blood

	Male (%)	Female (%)
Lymphocytes	48.16	54.30
Neutrophils	21.41	16.70
Large Lymphocytes	12.75	9.00
Monocytes	8.66	8.30
Eosinophils	4.75	4.12
Immature cells	3.80	3.80
Basophils	0.83	1.30

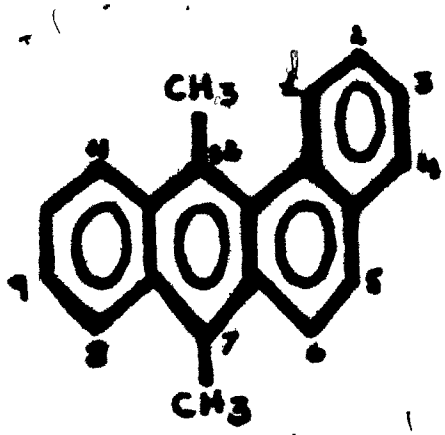
Fig 5 : Percentages of hemopoietic cells present in rats bone marrow and normal blood . From Swaen G.J.V. and Van Heerde P.V. (1973), Ref 116 .



RETINOIC ACID



DIMETHYL SULFOXIDE



7,12, DIMETHYL
BENZANTHRACENE

Fig 6 : Chemical structures of Retinoic Acid, Dimethyl Sulfoxide and 7,12 Dimethyl Benzanthracene.

Reaction	Response
Peroxidase (Graham-Knoll)	++
Esterases:	
- Naphthol-AS-D-chloroacetate	+
- a-Naphthyl acetate	+
Sudan Black	+++
Periodic acid Schiff	+
Phosphatases:	
- Acid	++
- Alkaline	-

Fig 7 : Cytochemical Characterization of the BN Myelocytic Leukaemia. From Hagenbeek A. (1977), Ref 48.

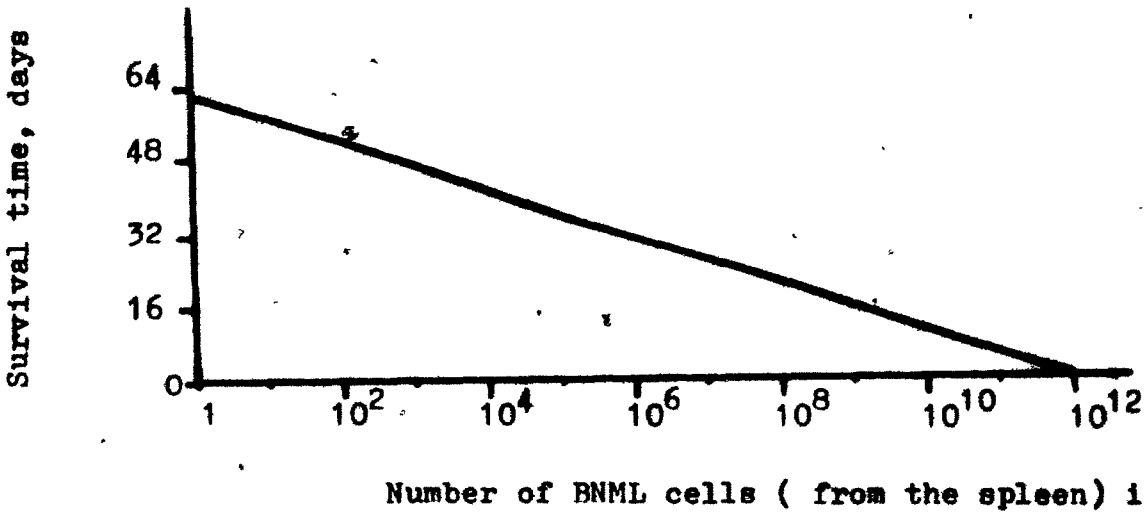


Fig 8 :Dose-survival curve for BNML cells taken from the spleen. From Hagenbeek A. (1977), Ref 48.

Cells/ Solution	10% FCS	10% FCS 1 uM RA	10% FCS 2 uM RA	10% FCS 5 uM RA	10%FCS 10uMRA
Blasts (arbitrary units)	+	++	++1/2+	0	0
Macrophages (arbitrary units)	0	0	+	+	0
Fibroblasts (arbitrary units)	+++	+++	+++	++	++

cells/ solution	4% RS 6% FCS	4% RS 6% FCS 1 uM RA	4% RS 6% FCS 2 UM RA	4% RS 6% FCS 5 uM RA	4% RS 6% FCS 10uMRA
Blasts (arbitrary units)	+++	++++	+1/2+	++++	++++
Macrophages (arbitrary units)	+	++++	++	++++	++1/2+
Fibroblasts (arbitrary units)	++	++	++	+	+

Fig 9 : Blasts, Macrophages and Fibroblasts in 14 days culture

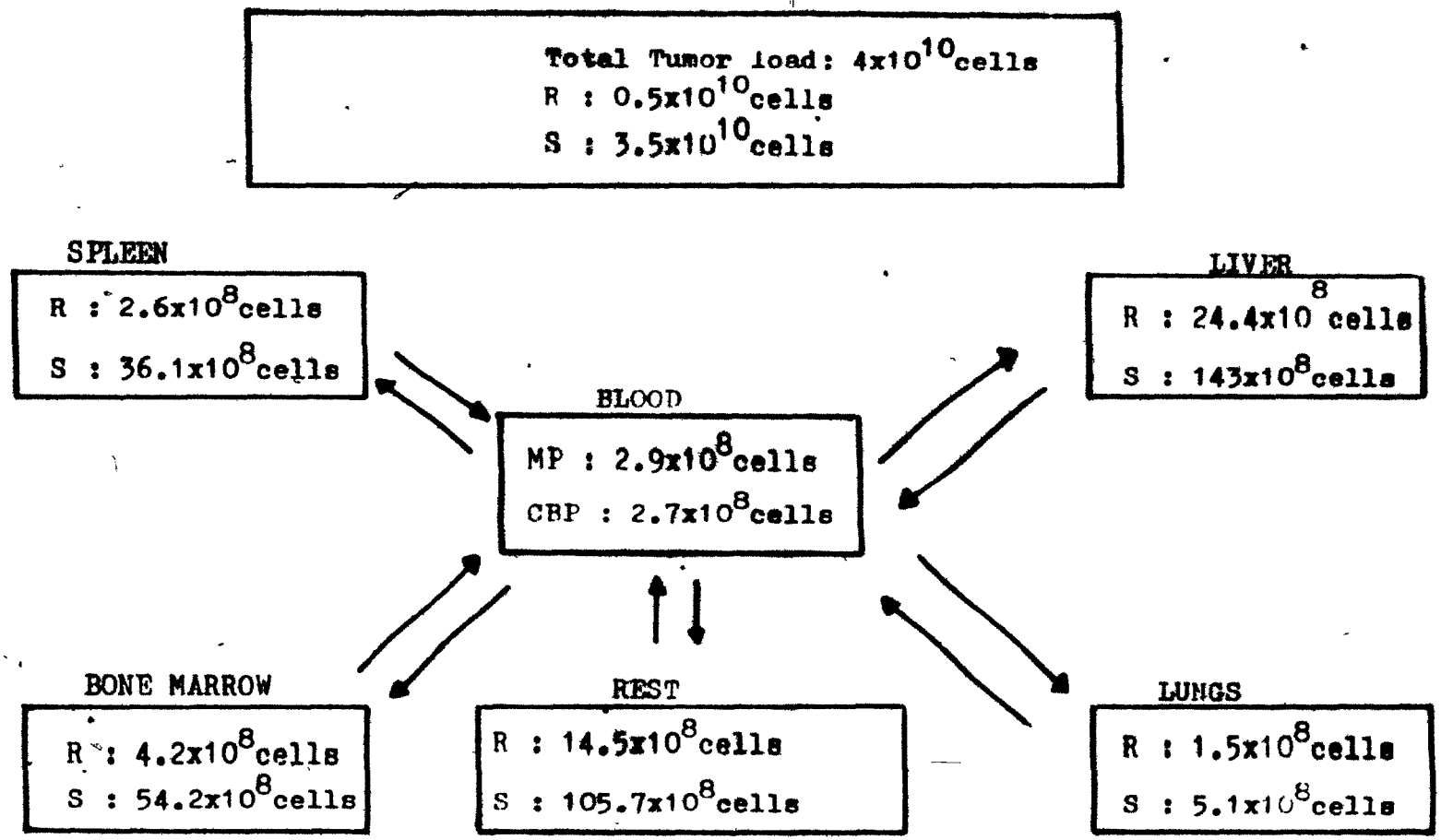


Fig 10 : Sizes of functionnal compartments at the terminal stage of the BNML (day 28 after 10^7 BNML cells i.v.). R=rapidly exchangeable pool. S=slowly exchangeable tissue pool. From Hagenbeek A. and Martens A. (1977), Ref 50.

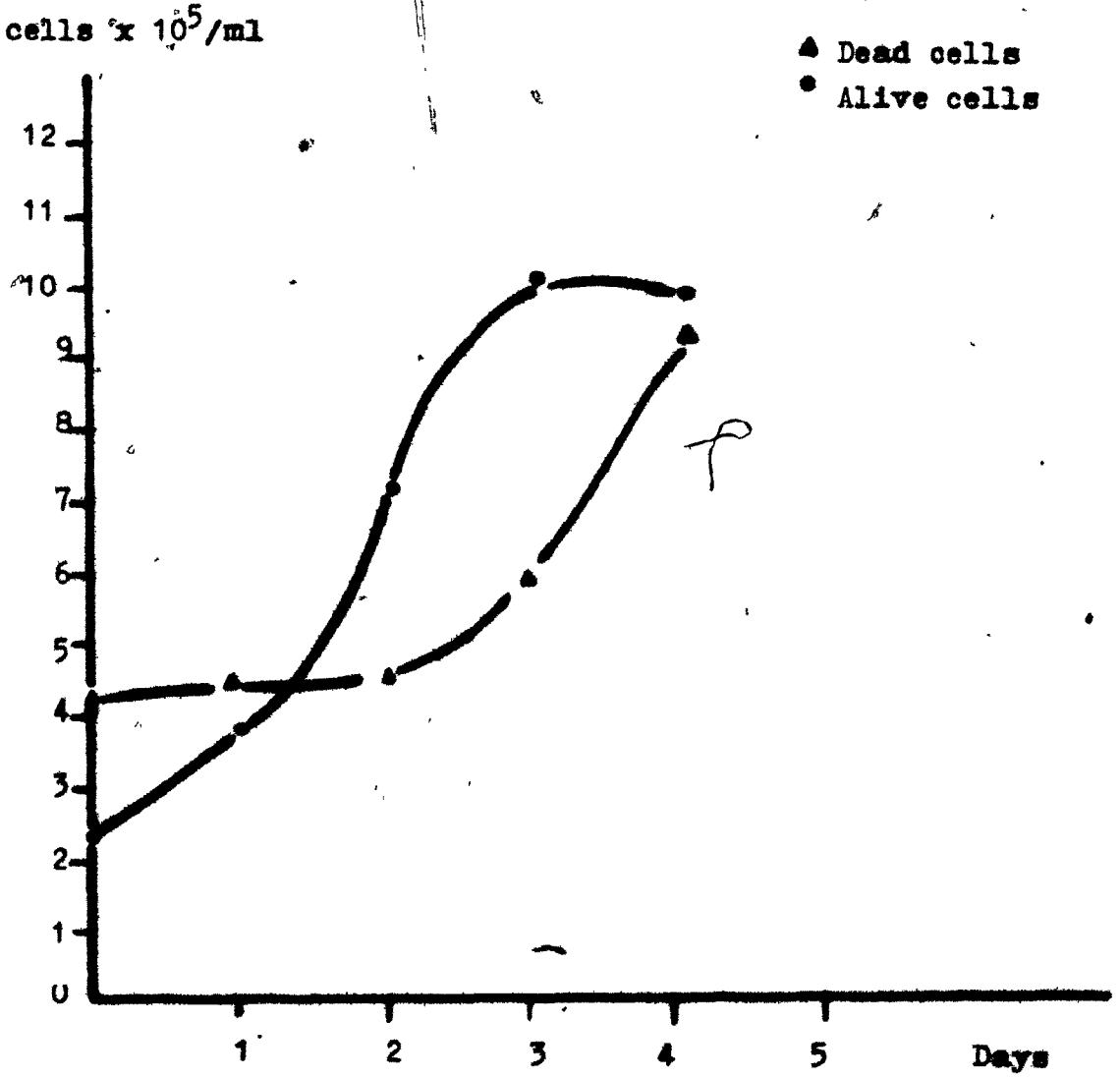


Fig 11 : Number of alive and dead cells as a function of time.

	Parent line	Adapted line
Promyelocytic morphology	+	-
Peroxidase	75 +	4-10% +
Surface Antigens		
Ox-8 (anti T supressor)	-	-
W3/13 (anti T cells and anti granulocytes)	- to 2+	- to 2+
W3/25 (anti T helper cells)	-	-
Ox-6 (anti Ia)	-	-
Ox-1 (anti-leukocytes)	100% +	100% +
Ox-7 (anti-Thy 1.1)	-	-
Surface Ig	-	-
Intracytoplasmic Ig	-	-

Fig 12 : Summary of characteristics of leukemic line

APPENDIX

Note: uM = μM

Agar

1) Prepare 1% Agar (0.25gms Agar (Gibco) in 25 mls double distilled H2O)

2) Autoclave agar and then keep in 56 degree centigrade bath.

3) Prepare 2 x concentrated medium :

- 1 package RPMI 1640 medium (Flow) for 1 liter

- 500mls double distilled H2O

- 5mls glutamine

- 5mls penicillin streptomycin

- 2gms sodium bicarbonate

- 4 mls HCl (1M)

4) Mix 25 mls 2 x medium and 25 mls 1% agar to obtain a 0.5% agar solution.

5) Add 15 mls RPMI 1640 medium (Flow) containing cells to 15 mls of 0.5% agar to give 30 mls of 0.25% agar.

6) Plate 5 mls/well in a 6 ml flat bottom well plate (Costar).

Cell Thawing

1) Take cells out of the -70 degree centigrade freezer or from liquid nitrogen, in dry ice.

2) Add 7 mls of FCS to 15 mls sterile tubes.

3) Thaw each vial in a 37 degree centigrade bath, until only a few crystals remain, and pour into a FCS containing tube.

4) Spin the tubes at 1200 rpm for 5 minutes.

5) Remove the supernatant and wash cells twice with RPMI-1640 medium (Flow). Count cells before the last spun, and reconstitute to the desire volume in RPMI-1640.

Enzyme Linked Immunosorbent Assay (ELISA)

1) Coat Dynatech microtiter plates (Fisher) with polylysine (Sigma):

- add 100 ul of a 50 ug polylysine/ml solution to each well.
- let sit at room temperature for 60 mns.

2) Wash twice in PBS.

3) Add 10^5 cells in 100 ul PBS to each well; cells should be previously washed three times in PBS.

4) Incubate at room temperature for 60 mns.

5) Gently add 100 ul of a 0.050% glutaraldehyde (Sigma) solution to each well.

6) Let sit 10 mns.

7) Wash three times with PBS

8) Add 200 ul of a 200 ug gelatin (BDH)/ml solution to each well.

9) Let sit for 60mns. The plates may be stored in this form at 4 degree centigrade.

10) Wash the plates twice with PBS before use.

11) Add hybridoma supernatants in 50 ul or 100 ul depending on the number of lines to test and availability.

12) Incubate at room temperature for 1-2 hours.

13) Shake out supernatants and add 200 ul of a 1% Bovine serum albumin (Sigma) in PBS solution.

14) Prepare diethanolamine buffer:

- 800 mls dd H2O
- 97 mls diethanolamine
- 0.2 gms NaN3
- 100 mgs MgCl2.6 H2O
- add 6 M HCl until PH=9.8.
- make up to a total volume of 1 liter with dd H2O.
- store at 4 degree centigrade in the dark.

14) Wash the plates three times with PBS.

15) Add affinity purified goat anti-mouse alkaline phosphatase * at 1/1000-1/2000 dilution in 1% bovine serum albumin in PBS.

16) Incubate 30 mns

17) Wash with 200 ul 1% bovine serum albumin 0.05% Tween 20 in BPS.

18) Wash three times with 0.05% Tween 20 in BPS.

19) Add 100 ul substrate (p-nitrophenyl-phosphate-disodium (Sigma)) made up at 1mg/ml in room temperature diethanolamine buffer .

20) Sit at room temperature 1-5 hours to develop colour.

*. In other experiments, goat anti-mouse IgG conjugated with peroxidase (34) or alkaline phosphatase conjugated protein A (115) have been used.

Other screening assays

i) Radioimmunoassays

Adsorb the antigen on polyvinyl plates. Wash the plates and add hybrid supernatants. Wash the plates a second time and add radioactive iodine affinity purified heterologous anti-immunoglobulin. Wash the wells and count each well individually in a y counter (45).

Using radioimmunoassay techniques, Fisher et al (38) showed a simple way to determine whether certain monoclonal antibodies react against the same or very close epitopes or against widely different regions of the antigen. The experiment consists in comparing the saturation binding level for each antibody with the saturation binding level when both antibodies are added in the same well. If competition occurs, the saturation binding level will about equal the individual saturation binding level proving that both antibodies react against the same or very close epitope.

ii) Cytotoxicity Assay

Cytotoxicity assays for cell membrane antigens can also be used:

Incubate radioactive Cr labelled cells having antigens on their membranes with supernatant and complement. If the monoclonal antibody present, reacts against the antigen, and if it is a complement binding antibody, then lysis of cells occurs. Hence, measure the radioactive Cr released

Clone and reclone the positive cell hybrids in soft agar.

Freezing Cells

Freezing medium:

For 50 mls :

- 30 mls serum free RPMI-1640 (Flow).
- 6 gms α D Glucose , MW= 180.2.
- 20 mls dimethyl sulfoxide.

per vial:

- 1.2 mls FCS containing 1-2 x 10^7 cells
- 0.4 mls freezing medium.

Prepare freezing medium , incubate at 37 degree centigrade for glucose to dissolve, and filter it. Add 0.4 mls to each labelled vial and put in ice.

Add 1.2 mls FCS containing the cells to each vial, and put in ice for 20 mns.

Put the vials 2 hours at 4 degree centigrade and then at -70 degree centigrade for 2 hours.

Put vials in liquid nitrogen.

Fusion

1) Put 50% PEG 1000 (BDH) in a 37 degree centigrade bath.

2) Spin myeloma cells. Wash them twice with RPMI-1640 (Flow), for 5 mns at 1400 rpm.

3) Kill animal. Pass spleen pieces through a stainless steel mesh under sterile conditions to get a cell suspension.

4) Wash spleen cells twice with RPMI-1640, for 7 mns at 1500 rpm.

5) Count cells during the last wash and spin them together at a ratio of 3:1 to 10:1 spleen cells : myeloma cells, for 8mns at 1800 rpm.

6) Take off supernatant with a pipet without disturbing the pellet.

7) Use a long tip sterile pasteur pipet to get a film of cells; do not completely loosen the pellet. Incline the tube at an angle and add:

- 1 ml 50% PEG 1000 over 1 mn
- 2 mls RPMI-1640 over 1 mn
- 3 mls RPMI-1640 over 30 seconds
- 5 mls RPMI-1640 over 30 seconds

8) Fill up the tube to 50 mls with RPMI-1640.

9) Spin for 6 mns at 1200 rpm.

10) Add 15% FCS or HS RPMI-1640 to have 2 x 10^5 myeloma cells/ml.

11) Put 2 drops of this solution from a 10 ml pipet in each

well of a 96 flat bottom well plate (Linbro-Flow Lab).

12) Add 2 drops HAT 15% FCS or HS in each well, next day.

13) 2 and 4-5 days after feed again with HAT 15% FCS or HS medium.

14) 10 days after, add HT 15% FCS or HS medium.

As in any new method, many of the technical problems have not yet been solved. Fusion of cells depends on many parameters such as the myeloma cell lines available, the selection media, and the "fusagen" agent (12).

Usually, an animal is immunized with the appropriate antigen concentration ,boosted i.v. one or more times after a certain period and killed three to four days after the last boost. This seems to ensure that:

-the immunological response is at a maximum

-B lymphocytes are in activated blast stage, a stage seemingly preferred for good fusion results (90). The spleens are then harvested , washed; spleen pieces are processed and the resulting spleen cells (red blood cells and lymphocytes) solution is ready to be fused with the appropriate number of myeloma cells (1:2 to 1:10 ratio)

a) Myeloma Cell lines Available

Myeloma cells are malignant plasma cells secreting immunoglobulin proteins in an irregular manner. Non secreting variants can be obtained (NS1 derived from P3) and used in fusions. Myeloma cells, being cancer cells, can be propagated indefinitely in vitro in an anchorage-independant growth

manner. The hybrid cells resulting from fusion of a myeloma cell and of a B lymphocyte conserve the malignant pattern of growth of the myeloma cell (12). Myeloma cells must be in logarithmic growth phase to be used in fusion and are therefore split several times before fusion in the appropriate medium. Most myeloma cell lines have defective hypoxanthine guanine phosphoribosyl transferase (HPRT; see selection). They are killed in HAT medium, but can be complemented by fusions with HPRT+ cells (spleen cells) (128).

b) The Fusogen agent

Early fusions used inactivated Sendai virus as a fusion promoter (77). Many more membrane active agents having fusion capacities have been found, but the most commonly used agent is now polyethylene glycol 1000-1500 Mw, pH=7.55 (PEG). PEG was much more effective than inactivated Sendai virus to promote cell hybridization. Klebe and Mancuso (71) compared hybridogens to non hybridogenic compounds. Comparison of the structure of several hybridogens and non hybridogens showed that:

i) One of the two terminal hydroxyls of PEG may be blocked without loss of hybridogenic activity.

ii) Hybridogenic compounds do not need to be electrically neutral nor do they need to be linear.

iii) However, if a methyl group (Poly propylene glycol) is substituted for a hydrogen in PEG or if a nitrogen replaces the oxygen in PEG, hybridogenic activity is lost.

iv) Very high or very low molecular weights PEG are inactive.

c) Selection Medium

In fusions, it must be ensured that only hybrids will grow i.e. the parent cells should not survive in the selective medium. Littlefield (84) devised a method to select for hybrids. In usual growth medium, cell lines can synthesize de novo the necessary purines. However, if aminopterin, a folic acid analogue, is added to the medium, folic acid reductase is blocked. Since the de novo pathway needs folic acid as a source of hydrogen the purine and pyrimidine synthesis by the de novo pathway is blocked. A preformed purine source is then needed for the cells to synthesize new DNA via the salvage pathways. These pathways depend on thymidine kinase (TK) which can transform thymidine to its nucleotide , and on hypoxanthine guanine phosphoribosyl transferase (HPRT) which can convert hypoxanthine to its nucleotide. The two enzymes HPRT and TK must be present for the cells to survive in the presence of thymidine, hypoxanthine and aminopterin (HAT medium) (45). HPRT catalyzes the transfer of phosphoribose from 5 phosphoribosyl 1 pyrophosphate to hypoxanthine or guanine bases to form 5'IMP or GMP and pyrophosphate. HPRT is an enzyme composed of identical subunits (24-26000 MW in human; 27000 MW in mouse) and may exist under a tetrameric form. HPRT may contribute to the flow of hypoxanthine into the cellular nuclectide pool and is mostly present in brain,ovary, red blood cells and white blood cells. It is coded by an X linked gene residing on the long arm of the X chromosome ,between G bands q22-qter. This enzyme is deficient in Lesch Nyhan syndrome, an X linked recessive disorder (20).

Mutant myeloma cells lacking TK or HPRT are produced by

cytotoxic drugs utilizing this pathway for internalization. Since, the HPRT locus is on the X chromosome, it is easier to obtain a deficient mutant than for TK. Resistance to 8-azaguanine,6-thioguanine,6-mercaptopurine and 6-azahypoxanthine often is accompanied by HPRT deficiency. Thioguanine can be incorporated into DNA via HPRT and cause cell death thus selecting for HPRT-mutants. If such an HPRT- or TK- mutant is fused with a spleen cell (HPRT+TK+), the only surviving product should be a hybrid which can use HPRT or TK from the spleen parent (45).

Other selective medium exists such as the hypoxanthine-aminopterin-5-methyldeoxycytidine medium but the HAT medium is the most widely used.

Once hybrids are obtained, cloned and reclosed, Chang et al (21) has shown that some hybrids may be cultured in serum free medium containing insulin (5 ug/ml) and transferrin (5 ug/ml). Absence of any of these two constituents leads to none or decrease proliferation. The absence of serum can help purify the antibodies.

d) Expected Hybrid Frequencies

If a spleen contains 10^8 lymphocytes and at best one hybrid forms per $2-5 \times 10^4$ cells (90), 300-500 hybrids then could be generated (31). It is often necessary to clone and reclone frequently to prevent overgrowth of non producers and to maintain the stability .

Feeder layers may enhance the frequency and growth of hybrids. Astaldi et al (6) found that human endothelial cell supernatant contains a soluble growth factor that promotes

growth and proliferation of hybrids. Fox et al (39) described two ways to increase the frequency of antigen specific hybrids from mouse to mouse fusions:

i) spleen cells were cultured with the antigen in vitro three to four days, followed by fusion.

ii) primed spleen cells were injected i.v. into sublethally irradiated animals; animals then were boosted with the antigen i.p. and spleen cells fused three to four days later.

These two methods increased the frequency of antigen specific hybrids from 7% to 40-58% and may prove very useful to derive monoclonal antibodies against very weak antigens (poor immunogens).

Immunofluorescence

1) In a 0.5mls polypropylene Eppendorf tube, add 3 x 10^5 cells washed 3x with PBS.

2) Spin and aspirate down to pellet.

3) Add 50 ul of primary antiserum.

4) Incubate 30-60 mns on ice.

5) Wash : x 1 with PBS 5% BS

x 2 with PBS alone

6) Aspirate to pellet.

7) Add 25 ul of conjugate (goat anti mouse IgG heavy and light chains -no cross reactivity with rat, fluorescein conjugated, Cappel, was used for mouse to mouse fusions).

8) Incubate 30 mns on ice.

9) Wash as for (5).

10) Aspirate to pellet.

11) Add 20 ul PBS. Put 1/2 on slide.

12) Add coverslip and examine under fluorescence microscope.

Induction Solutions

Prepare:

1) 45 mls 10% FCS RPMI-1640 (Flow)

2) 45 mls 4% BS/ 6% FCS RPMI-1640

3) Solution A: 20 ul all trans-retinoic acid (Sigma) 10 mM in 2 mls 10% FCS RPMI-1640 (F).

4) Solution B: 20 ul all trans-retinoic acid 10 mM in 2 mls 4% BS/ 6% FCS RPMI-1640 (FR).

Add:

- 1.5 mls solution A to 13.5 mls F to obtain a 10 uM retinoic acid F solution.

- 1.5 mls solution B to 13.5 mls FR to obtain a 10 uM retinoic acid FR solution.

Add:

- 6.5 mls of the 10 uM retinoic acid F solution to 6.5 mls F to obtain a 5 uM retinoic acid F solution.

- 6.5 mls of the 10 uM retinoic acid FR solution to 6.5 mls FR to obtain a 5 uM retinoic acid FR solution

Add:

- 4.8 mls of the 5 uM retinoic acid F solution to 7.2 mls F to obtain a 2 uM retinoic acid F solution.

- 4.8 mls of the 5 uM retinoic acid FR solution to 7.2 mls FR to obtain a 2 uM retinoic acid FR solution.

Add:

- 4 mls of the 2 uM retinoic acid F solution to 4 mls F to

obtain a 1 uM retinoic acid F solution.

- 4mls of the 2 uM retinoic acid FR solution to 4 mls FR to obtain a 1 uM retinoic acid FR solution.

Prepare:

- 8 mls F for a 0 uM retinoic acid F solution
- 8 mls FR for a 0 uM retinoic acid FR solution

Add 1.5 mls of each solution to each well of a 24 flat bottom well plate (Costar No 3524, Cambridge, Mass., USA) to end up with 5 replicates of each solution containing each 5 x 10 cells.

Nylon Wool Column

From Greaves M. and Brown G., J. of Immunology, 112, No1, 1974, p 420.

1) Dry 300 mgs of washed nylon fibers.

2) Pack fibers in a 10 ml seringue up to the 4 mls mark.

3) Wash several times with warm RPMI-1640 (Flow) so that most of the fibers are washed. Leave some medium in and incubate at 37 degree centigrade for 30 mns.

4) Add 2 mls 10% FCS RPMI-1640 medium with 10^7-5 x 10^7 cells on top of the syringe. Incubate syringe upright at 37 degree centigrade for 30 mns.

5) Add 40-45 mls RPMI-1640 medium on top and let drain through a 25 G butterfly needle the cells . The macrophages and B cells will stick to the column. The fibers should never get dry.