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Hooper, William Craig

THE CHARACTERISTICS OF THE IN VIVO AND IN VITRO RESPONSE OF HAIRY CELL LEUKEMIA TO INDUCERS OF DIFFERENTIATION

The Ohio State University

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THE CHARACTERISTICS OF THE IN VIVO AND IN VITRO RESPONSE
OF HAIRY CELL LEUKEMIA TO INDUCERS OF DIFFERENTIATION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
William Craig Hooper, B.A.

*****

The Ohio State University
1985

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Dedicated to my parents
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I am extremely grateful to the National Cancer Cytology Center for awarding a very generous fellowship which allowed me to complete most of my graduate work without any financial hardships.

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Studies in Cell Biology. Professor Ralph E. Stephens
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CHAPTER I

HAIRY CELL LEUKEMIA. AN OVERVIEW OF THE DISEASE
"The existence of leukemic reticuloendotheliosis as an independent entity and its relation to other types of leukemia have excited considerable interest among hematologists." (Bouroncle 1958)

So began, twenty-seven years ago, the opening sentence of a paper, now considered a classic in the field of hematology. This paper detailed the clinical, hematological and histopathological findings of 26 patients who had a distinct form of leukemia (1). Now almost three decades later, and after over 500 reported cases, there still exists "considerable interest among hematologists" and other investigators in leukemic reticuloendotheliosis (LRE) or hairy cell leukemia (HCL). This interest stems from the fact that there is no satisfactory treatment for HCL and that the cell pathognomonic for the disease, is still undefined as to its normal cell lineage.

Since the last review by this author (2), there has been considerable progress made in establishing new treatment modalities for HCL as well as in the biology of the cell. The purpose of this chapter is to provide a comprehensive review of hairy cell leukemia, with particular emphasis on progress made over the past five years.
Introduction

In 1923, Ewald (3) introduced the term "leukemic reticuloendotheliosis" (LRE) to describe a hematological disorder that would now probably be classified as acute monocytic leukemia (4,5). Although there had been earlier reports (5), it was not until 1958 that hairy cell leukemia (HCL), a synonym for LRE, was shown to be a distinctive hematopoietic disorder with unique hematological and clinico-pathological features (1). This report was based on the observations of 26 patients during the preceding 8 years. According to Bouroncle (6), the recognition of the "hairy cell", the neoplastic cell characteristic of HCL, was facilitated by the routine use of supravital staining in the hematology laboratory at The Ohio State University. The use of the stain allowed the "hairy cell" to be easily identified and differentiated from other cell types (6,7). The stain, which also preserved the morphological features of the cell, allowed the cell to be described as being round, polygonal or oval in shape with a serrated border. The nucleus was described as being round or oval and approximately one-half the size of the cell. Usually eccentric in location, the nucleus was observed to have a distinct nuclear membrane, while the chromatin appeared spongy and agranular with a single nucleolus sometimes being present (1). It was also observed that mitochondria, which stained with Janus green, was present throughout the cytoplasm (1). Fresh preparations viewed under a phase contrast microscope illustrated the same characteristics with the "hairs" or microvillus-like projections being more prominent and easier
visualized. The advantage of phase-contrast microscopy was that no staining of the cell was required and Bouroncle has emphasized its value in the recognition of the pathognomonic hairy cell (1, 6, 7).

Although accepted and recognized as a distinct disease entity, leukemic reticuloendotheliosis was initially often confused with other hematological disorders. Part of the confusion stemmed from the disease being referred to by other names such as "histioleukemia", "reticulum cell leukemia", "medullosplenic-histiolympcytosis of primitive appearance" and "lymphoid myelofibrosis" (4). Also since the term leukemic reticuloendotheliosis had been broadly used in describing neoplastic disorders of the reticuloendothelial system, the nomenclature describing this disease remained somewhat misleading (1, 4, 5). In 1966, based on its appearance under phase contrast microscopy, Schrek and Donnely (8), coined the term "hairy cell" in describing the cell characteristic in LRE. Since this term does not suggest cell origin, the name hairy cell leukemia has become increasingly popular when referring to the disease process described by Bouroncle et al (1). In a recent monograph, Cawley et al (5) chose to use the term HCL in concurrence with a statement made by Symmers - "It is difficult to conceive how a disease that has become so widely known as 'hairy cell leukemia' can ever be referred to by any more mundane name." I concur as well, and will use HCL throughout the rest of this review.
Clinical Features

In the original article (1) it was noted that HCL accounted for 2% of all leukemias seen, with a 4.2:1 male-female ratio. The age ranged from 30 to 76 years. It was observed that there were no specific symptoms of HCL and that the onset was usually insidious. The most prominent physical finding was splenomegaly and was present in 25 of 26 patients, in 14 cases the spleen was moderately enlarged and significantly enlarged in the other 11 cases. It also was noted that spleen size in the same patient often fluctuated throughout the course of the disease. Hepatomegaly was seen in 15 patients and lymphadenopathy in only 9 cases. The most frequent complication was infection, which occurred in 58% of the patients, with pneumonia being the most common. Hemorrhagic complications were less common, occurring in only 20% of the patients. Out of the 26 patients, 16 had died when the paper was written (1). Bouroncle et al (1) concluded from this study that the clinical course was quite variable, ranging from acute to chronic. In this series, 46% of the patients had a chronic course. Remarkably, clinical studies throughout much of the western world has revealed a similar clinical course (4,5,9,10,11,12,13,14,15).

Since 1958, Bouroncle has published two articles updating the Ohio State series (6,7), basing her data on 82 and 100 cases respectively. The clinical data compiled in these publications substantiates her original observations. Based on 100 cases, the Ohio State series had a male-female ratio of 4.9:1, with the median age being 55. Age was evenly distributed between 30 and 70 years (6).
In a review of the world literature, Cawley et al (5) found the male-female ratio to be at 3.8:1 with the average age being 51. In agreement with Bouroncle et al (1), they noted that the onset of HCL was insidious and presented with nonspecific symptoms in 75% of the patients. Splenomegaly was the chief physical finding, being documented in 84% of the patients. Lymphadenopathy was undetected in 76% of the patients and hepatomegaly was observed in 40%. Since 1980, these clinical findings were reaffirmed in reviews on 211 patients (11) and 245 patients (9).

Cytological/Hematological Features

Light Microscopy. As previously stated, the cell pathognomonic for HCL is characterized as having a serrated border, or under phase contrast microscopy, long delicate "hairs" or microvillus-like structures protruding from the cytoplasm (1). The nucleus, which is usually eccentric in location, has a spongy chromatin pattern and is sharply defined by the nuclear membrane (1,5). Hairy cells have a diameter between 15-30 μm with a modal cell volume of approximately 450 m³ (5). Thus they are larger than most lymphocytes (5). The nuclear:cytoplasmic ratio is usually between the high ratio of lymphocytes and the low ratios of monocytes and macrophages. In a Romanowsky stain, the cytoplasm appears slate-blue, some "patchy" variations in density are sometimes observed as well as vacuoles (5). Cawley et al (5) observed that the cytoplasm in some cases contains small azurophilic granules. It should, however, be noted that the
hairy cell may not be found in the peripheral blood of some patients with this disease, and when present, can be seen in varying percentages (1,5,7).

Using the morphometric criteria of nuclear area, cytoplasmic area, cytoplasm:nucleus ratio and the nuclear contour index, Meijer et al (16) showed that there is significant differences between HCL and various B cell small lymphomas. They concluded that this type of analysis can be of value in the differential diagnosis of HCL.

Electron Microscopy. In a detailed ultrastructural study, utilizing both transmission and scanning electron microscopy (TEM SEM) Katayama et al (17) examined hairy cells that were found both in the peripheral blood and solid tissues. The blood cells and tissues were fixed immediately after recovery from the patient as to eliminate possible in vitro artifacts. After fixation, hairy cells in the peripheral blood were measured to be 5-8 microns in diameter, exclusive of cytoplasmic projections. TEM showed that nuclear morphology was variable ranging from oval, notched, bilobed to multilobed (17). These lobes were due to deep nuclear indentations. It was observed that the heterochromatin was condensed and one and sometimes two nucleoli were seen (17). They noted that the surface of the hairy cell was covered by two types of cytoplasmic projections. One type referred to as microvilli, measured 50-150 µ in diameter and 0.6 to 1.4 µ in length. They were either straight or tortuous and referred to as slender finger-like processes (17). Pseudopods were the other type of projections. They were described as "broad-based tongue-like processes measuring usually about
1 μ in width and 3 μ in length” (17). As one could expect there was variation in the hairy cell surface morphology. For example, the number of pseudopods and microvilli were about equal in some hairy cells, while others had numerous microvilli but only a few pseudopods (17).

A tubular inclusion body, the ribosomal-lamella complex (RLC) was seen in the cytoplasm of some hairy cells (5,17,18,19). This complex has been referred to by other names such as granulolamellar structures, tubular cytoplasmic inclusions and lamellar bodies (5). Although RLC can be seen in other hematological malignancies, it is more common in HCL and can be considered diagnostic in conjunction with other clinico-pathological findings. Katayama et al (17) described the structure as being "composed of a central space and an outer sheath of multiple parallel lamellae with rows of ribosomes spaced evenly between the lamellae". Cawley et al (5) have noted that the ribosomal granules measure approximately 200 Å in diameter and are identical with the ones in the surrounding cytoplasm, and as evidenced by RNAase digestion, the granules contain RNA. Depending on the plane of the section, the complex may appear as parallel lines, concentric circles or ellipsoids (5). In Katayama's patient series, it was observed that 10 out of 23 HCL patients had the ribosome-lamella complex and in these 10 patients, the percentage of hairy cells in which the RLC was found, ranged from 0.2 to 90. These cells contained one or several RLC (17).

Other ultrastructural cytoplasmic features of hairy cells observed by Katayama et al (17) were pinocytotic vesicles, multiple and medium-sized vesicles, azurophil granules and lysosomal inclusions.
They also documented random distribution of fine fibrils in the cytoplasm, prominent microtubules within the nuclear indentation and both rough and smooth endoplasmic reticulum (17). Cawley et al (5) have found that when properly stained, hairy cells frequently contain glycogen. They noted that the distribution of glycogen correlated with the diffuse and fine granular PAS positivity of hairy cells.

Analysis of spleens, surgically removed from HCL patients by TEM, revealed a significant concentration of hairy cells in the cords and sinuses (17). The morphology of the cells were the same as observed in the peripheral blood. The cytoplasmic projections were usually observed to interdigitate with one another and, in some cases, they surrounded erythrocytes, platelets or segments of basal lamina (17). Similar studies looking at bone marrow revealed increased cellularity with the majority of the cells being hairy cells and erythroid cells. The intercellular space between the hairy cells was occupied by collagen fibrils. The ultrastructure of hairy cells seen in the liver and lymph nodes were similar to those in the peripheral blood and spleen (17).

Hairy cells examined by scanning electron microscopy (SEM) were measured to be 5.8 μ in mean diameter, with a range from 5.0 to 6.8 μ (17). In contrast, normal lymphocytes and chronic lymphocytic leukemia (CLL) cells measured 4.8 microns in mean diameter with a range of 4.0 to 6.0 (17). Monocytes had a mean diameter of 5.5 μ with a range of 4.9 to 7.1. Observations of the cell surface with SEM revealed that hairy cells had numerous cytoplasmic projections that appeared as both short, stub-like and long microvilli, and the membrane appeared
ruffled. Lymphocytes, on the other hand, only had the short stub-like microvilli while monocytes exhibited only a ruffled membrane (17). During the course of this study, normal lymphocytes, from the ultrastructural point of view, were often observed in blood samples taken from HCL patients, but the monocytes rarely looked normal (17). It was also observed that HCL cells in the bone marrow frequently interdigitated with collagen fibrils in the intercellular space (17). Although they were aware that such fibrils are responsible for the dry marrow taps often encountered in HCL patients, they could find no evidence that hairy cells produced these fibrils (17).

Clinical Hematology. Typical hematological findings in HCL patients are anemia, thrombocytopenia and neutropenia (1,4,5,12,13,15,18). Cawley et al (5) has stated that pancytopenia is seen in 68% of the patients. It has also been observed that the white blood cell (WBC) count is the most variable hematological parameter (4,5). In Turner's series, 63 patients were leukopenic, 23 had a normal WBC and 15 had leukocytosis (4). The numbers of circulating hairy cells can also be variable, ranging from 0 to 100%. Cawley et al (5) have noted that the percent hairy cells usually increases as the WBC increases.

Usually the anemia observed is moderate, and is normocytic, normochromic (1,4). Slight to mild anisocytosis, poikilocytosis with an occasional circulating red blood cell is sometimes seen (4,5). Although rare, Bouroncle et al (1) in her original series, found that six patients had hemolytic anemia as evidenced by a positive Coomb's test.
Bouroncle et al (1) have attributed the anemia to the replacement of normal bone marrow by hairy cells. Splenic sequestration of red blood cells and perhaps a dysfunction of erythroid precursor cells may also explain the anemia (4,5,20,21). Cawley et al (5) have stated that a decrease in red blood cell production is a bad prognostic sign.

Berkowitz et al (20) have recently reported a case in which a patient with HCL acquired dyserythropoiesis. This patient was profoundly anemic with macrocytosis. A bone marrow aspirate was diagnostic of hairy cell leukemia and revealed erythroid hyperplasia with an abundance of multinuclear erythroid precursors (20). Characteristic of the abnormal erythroid cells were the nuclei that often showed budding, lobulation, karyorrhexis and internuclear bridging in addition to being multinucleated. As previously stated, the marrow often showed erythroid hyperplasia and this was accompanied with mitotic abnormalities and some degree of megaloblastic change (20). The red blood cells were often macrocytic and contained an increased concentration of fetal hemoglobin (20). Perhaps one of the more interesting features of this case was that the patient had two populations of red blood cells in his peripheral blood, one had a normal mean corpuscular volume (MCV), while the other had a significant MCV. The authors speculated that this latter clone developed as a consequence of HCL (20). Indirectly this hypothesis was supported by normal levels of folic acid, Vitamin B₁₂, no evidence of blood loss and a negative indirect Coombs' test (20).

Orlandi et al (21) carried out a quantitative study of erythropoiesis in 12 HCL patients. These patients were compared to 8
other patients who had aplastic anemia (AA). They stated that their data could argue for a true quantitative defect of erythropoiesis. This argument was based on ferrokinetic data, which illustrated that the final product of erythropoiesis was abnormal in HCL and could thus be traced back to the proliferative capacity of the bone marrow (21). This is in contrast to that of aplastic anemia where there is a stem cell defect. As noted by Orlandi et al (21), some investigators have suggested that other mechanisms may cause dysfunction in erythropoiesis in HCL patients. Brearley et al (22) have hypothesized that the hairy cells themselves can damage erythroid precursors, while Zucker et al (23) suggested that the precursors could be less sensitive to erythropoietin.

Thrombocytopenia is present in well over 80% of HCL patients (4,5,7) and in Turner's series the platelet counts usually ranged from 50-150,000 x 10⁹/l (4). Splenic sequestration and abnormal platelet production are factors in the observed thrombocytopenia (4,5). In many cases, the platelet level returns to normal after splenectomy (4,24,25). In a detailed study, Levine et al (26) examined platelet kinetics in 10 HCL patients, and out of these, 8 patients had qualitative platelet abnormalities. Four of these patients had a prolongation of the template bleeding time, 7 had abnormal platelet aggregation and 1 patient had abnormal platelet adhesiveness measurements (26). Ultrastructural studies generally revealed normal platelet morphology. However, in each of the 4 patients who had a prolonged bleeding time, some platelets had giant granules (26). Golomb et al (27) observed that 6 HCL patients demonstrated marked
reduction of platelet aggregation with epinephrine. In a recent case study, it was reported by Rosove et al (24) that a HCL patient who developed hemorrhagic diathesis in response to platelet dysfunction, experienced a significant clinical improvement after splenectomy. It also was observed that almost all of the platelet abnormalities were corrected as well (24). However it was pointed out by these investigators that not all patients' platelet dysfunction is corrected by splenectomy, thus implying that the spleen is only one factor involved in the dearrangement of platelet hemostasis in HCL (24). It also was correctly pointed out that platelet dysfunction is not unique to HCL, but is seen in other hematological disorders (24).

As previously mentioned, the WBC is perhaps the most variable hematological parameter (4). Turner et al (4) have observed that the WBC can range from less than 1,000 to over 100,000 x10^9/mm^3, with leukopenia however being the most common. Neutropenia and monocytopenia was observed in the majority of her patients (4). Golomb et al (12) have defined a HCL patient as being in the leukemic stage of the disease if the percent of hairy cells is at 50 or greater. Based on his experience, he found that the leukemic phase of the disease is rare at presentation and that the WBC in the majority of his cases ranged from 1,000-20,000 mm^3 and only one patient out of 44 had a count of greater than 25,000 mm^3 (28). Thus, based on his data, he suggested that a WBC over 50,000 mm^3 should make one suspicious of the diagnosis of HCL (28).

In his review, Cawley et al (5) have noted that blood urea, electrolytes and liver functions are usually within the normal range,
as is serum proteins. He found that hypergammaglobulinemia is present in over 40% of the cases while immuno-incompetence is rare. The hypergammaglobulinemia is rarely monoclonal. Serum lysozyme is usually low, beta-2-microglobulin levels were slightly elevated in 2 out of 7 patients tested and the erythrocyte sedimentation rate was quite variable (5). They found that elevated alpha 1 and alpha 2 globulins of the acute phase reactants are sometimes elevated in non-infected patients and that the C-reactive protein was elevated in 1 terminally ill HCL patient (5).

Histopathology

All investigators have stated that the bone marrow is involved in all HCL cases and is diagnostic as well (4,5,7,12,13,14,18,28). Since the majority of bone marrow aspirations are unsuccessful due to the reticulin fibers, biopsies are usually done.

Burke et al (29) recently examined 39 bone marrow biopsies, and found in 21, hypercellularity of greater than 80%. It was observed that the marrow was almost completely replaced with an infiltrate of widely spaced mononuclear cells which on low power microscopy, appeared as a web-like meshwork. A zone of "water"-clear cytoplasm, which was present between the cells, was responsible for the cell spacing or the web-like appearance (29). In the majority of the cases, the nucleus was ovoid in shape with a stippled chromatin pattern. No prominent nucleoli or mitotic figures were observed (29).
In 18 of the 21 biopsies, residual hematopoiesis was observed and associated with a decrease in the hematopoietic cells. Generally, megakaryocytes and islands of erythropoiesis were seen among the infiltrating hairy cells (29). Infrequent indications of granulopoiesis were also observed (29).

Out of the original 39 cases, the cellularity of the marrow was normal in 11 cases, hypocellularity was noted in 4 cases, and in the remaining 3, the biopsies were not diagnostic of HCL (29). The reasons cited for the latter finding were 1) technical and 2) the associated panhyperplasia that can mask the hairy cell infiltrates (29). In biopsies where the cellularity was considered normal, a hairy cell infiltrate was observed that appeared patchy, with an irregular distribution pattern. A greater degree of hematopoiesis also was noted, with the hematopoietic cells randomly dispersed among the hairy cells. A similar finding was noted in the hypocellular biopsies with the exception that the hairy cells were greatly reduced in density.

A finding that was consistent in all 39 biopsies was an increase in stromal reticulin seen in regions of the hairy cell infiltrate (29). Also observed in 16 cases was an increase in red blood cells in the background of the hairy cell areas (29). To the authors, the red blood cells seemed to form small "lakes" similar to what is commonly seen in spleens of HCL patients (29).

Recently, Barthl and co-workers reported that bone marrow biopsies of HCL patients could be prognostic as well as diagnostic (30). Based on nuclear configuration of hairy cells in the bone marrow, they divided the malignant cells into 3 groups; ovoid, convoluted and
indented. In 134 patients examined 47% were of the ovoid morphology, 37% of the convoluted and the remaining 16% fell into the indented group (30). Statistical analysis showed this grouping to be highly significant in predicting prognosis. Median survival for the ovoid, convoluted and indented groups were 56, 12 and 5 months respectively (30). They noted massive splenomegaly was the primary clinical finding of patients who fell into the ovoid group, thus suggesting a relationship between hairy cell nuclear shape and hypersplenism (30).

Spleen. As discussed earlier, splenomegaly is the major clinical finding in HCL (1,4,5,7), with the spleen weight ranging from 350 to 5000 grams (5). The cut surface has been described as having a homogenous dark red appearance with no evidence of tumor nodules (5). Histologically, a unique vascular lesion known as pseudosinuses, are seen in virtually all HCL spleens (31). These lesions appear as dilated sinuses lined with hairy cells and filled with red blood cells thus giving rise to the term "blood lakes" (31). In contrast to normal splenic sinuses, endothelial cells and ring fibers are absent, thereby allowing the pseudosinus to expand under the influence of local hemodynamics (31). This lesion is not seen in normal or in any other pathologically involved spleen (31).

The red pulp is the site of hairy cell accumulation and as the cords within the red pulp begin to expand, the white pulp, which is not infiltrated by hairy cells, becomes atrophic and eventually disappears (32,33). Normal sinuses also become atrophic, and in the process allow the escape of hairy cells into the general circulation (32). In this
process, an accumulation of macrophages occurs, with the subsequent destruction of normal blood cells, thus contributing to the clinical picture of hypersplenism and pancytopenia.

Utilizing histochemical techniques, Nanba et al (32) have confirmed that the cells that line the pseudosinuses are indeed hairy cells by the virtue of TRAP positivity, and by the absence of the endothelial cell marker, naphthyl AS-D acetate esterase.

Ultrastructural studies have generally confirmed the light microscopy observations. Burke et al (29) have reported that hairy projections are readily seen on hairy cells that are found in the distended sinuses. It also was noted that the projections had a tendency to interdigitate, thus giving the appearance of syncytium-like aggregates. In a similar study, Pilon et al (34) have found ultrastructural evidence that the hairy cells can adhere to many splenic cell surfaces, produce endothelial cell injury and death. It was also observed that the hairy cell infiltrate into the subendothelial lymphatics of trabecular veins, formed large clumps that could impede the flow of blood by causing occlusion of the lumen.

Tubbs et al (35) in a study of the antigenic profile of splenic hairy cells in frozen sections from 10 patients, found that B-1 and HLA-DR were expressed in all cases. Markers for monocytes, M1, M02, early B cells, J-5, B-2 and for plasma cells, T10, were not seen. Nor were markers for T cells. These observations suggested to the workers that the hairy cell was in maturation arrest at a point corresponding to normal mature B cells. In another study Meijer et al (36) similarly have shown that splenic hairy cells react with the B cell markers,
Leu-10, Y29/55 and HLA-DR but not with antibodies specific for T cells or macrophages. The hairy cells lining the pseudosinuses were positive for Y29/55, and Leu-10, but negative for the antibodies that normally react with sinus lining cells, Leu-2a and BA-2 (36). The observation was made that unlike hairy cells in the peripheral blood, the malignant cells from the spleen did not react with the OKM-1 monoclonal antibody (36). This suggests that hairy cells, which circulate in the peripheral blood, may acquire monocyte surface markers once they leave the spleen.

Liver and Lymph Nodes. Infiltrates of hairy cells generally are seen in the sinusoids and portal tracts of the liver. This histological appearance is diagnostic of HCL. Delso et al (37) have shown that the hairy cells actually attach to the sinusoidal wall by the virtue of their cytoplasmic processes.

Another distinguishing feature of hairy cell leukemia is the absence of peripheral lymph node enlargement. They are, however, diffusely infiltrated by hairy cells, and in some cases the neoplastic cells appear as focal aggregates in the nodal architecture (5). Total destruction of the inner structure of the node is rarely seen (5).

Cytochemistry

Perhaps the greatest contribution to the laboratory diagnosis of hairy cell leukemia was the observation that isoenzyme 5 of acid phosphatase which is resistant to L(+) tartrate, was largely confined
to hairy cells (38). This observation has provided a quick diagnostic cytochemical test for HCL (2,4,5,7). However, there are several technical factors, which if not considered, could artifactually alter the chemical reaction, thus allowing for a false interpretation of the test (39). It has also been shown that some hairy cells are negative for the tartrate resistant acid phosphatase (TRAP) reaction, and that it can be seen in some non-hairy cell hematopoietic disorders as well (18). This cytochemical reaction thus should be considered to be diagnostic of HCL only in conjunction with other clinical histopathological criteria.

Hairy cells also are positive for nonspecific esterase activity (5), as well as for periodic acid-Schiff (PAS) and β-glucuronidase (5).

Therapy

Chemotherapy has proven to be of little value in the treatment of hairy cell leukemia and in many cases may be detrimental (2,4,5,6,18,28). Drugs which have been valuable in treatment of some patients are chlorambucil (40) and glucocorticoids (5). The only therapeutic endeavor that has proven to be of value when clinically indicated is splenectomy. After this procedure, many patients enjoy a substantial clinical improvement (5,6,7,10,18).

Recently the biological modifier, Alpha interferon, has been reported to be effective in the treatment of HCL (41,42). Patients who were in all stages of the disease responded to the interferon (42). In
a study of 9 patients, Ratain reported that in 8 patients who completed 8 weeks of treatment, 7 had partial responses (45).

The mechanism of action of alpha-interferon is not known, but it has been suggested that it can kill or inhibit the growth of tumor cells, induce differentiation or augment NK activity (41,42).

Cellular Origin

During the past five years, advances in molecular biology and monoclonal antibody technology has allowed investigators to better define the "hairy" cell. The hairy cell has now been almost conclusively placed in the B lymphocyte lineage and is thought to be more mature than chronic lymphocytic leukemia cells. Some investigators have speculated that the maturation defect in hairy cells lies at the isotype switch point (43). This hypothesis was based on the observation that many of the malignant cells had multiple heavy chains with only a single light chain (44).

The first hard evidence which indicated that hairy cells were malignant B cells was the observation that the cells underwent Ig gene rearrangement and could transcribe both heavy and light chain molecules (45). It was also observed that hairy cells possessed the receptor for interleukin-2, which has subsequently been found on certain activated B cells. Extensive studies with monoclonal antibodies have also strongly implied a B cell origin (46,47,48). These studies also indicated that the hairy cells are arrested at a late stage in B cell development.
One group of investigators have gone as far as to suggest that hairy cells are pre-plasma cells (49).

Until recently, attempts to produce monoclonal antibodies specific for hairy cell leukemia have been unsuccessful. Posnett et al (47,50) have reported that two monoclonal antibodies, αHC-1 and αHC2 reacted with hairy cells but were not specific. αHC-1 reacted with endothelial cells but not with other lymphoid cells, while αHC-2 reacted with activated B cells and myeloblasts. Of these two monoclonal antibodies, αHC-1 may be the most useful in the diagnosis of hairy cell leukemia. Schwarting et al (51) have also produced monoclonal antibodies that reacted with hairy cells. The antibodies, αS-HCL-1 and αS-HCL-3 were reported to be useful in the diagnosis of hairy cell leukemia if used in combination (51). They observed that normal B cells and macrophages reacted with αS-HCL-1 and αS-HCL-3 respectively, while only hairy cells reacted with both antibodies. Other hematological malignancies failed to react with this combination (51).

There have been several reports which documented that hairy cells with a B cell phenotype, could acquire the receptor for sheep erythrocytes (E) and lose sIg after stimulation with PHA and autologous T cells (52). This suggested at the time that hairy cells had the ability to express certain T cell antigens in response to external stimuli. But further investigations revealed that normal activated B cells could also express the E receptor (53). It was suggested by these investigators that this observation had two interpretations: 1) normal B cells retain the ability to express T cell antigens and 2) the E receptor is not T cell specific (53).
Several investigators have postulated that HCL and chronic lymphocytic leukemia are closely related (5,45,54). Recent evidence obtained by Caligaris-Cappio et al (54) supports this hypothesis. It was found that after treatment with the tumor promoter, TPA, chronic lymphocytic leukemia cells acquired some morphological and cytochemical characteristics of hairy cells.

Based on these recent findings it can be hypothesized that hairy cell leukemia represents the malignant proliferation of an activated B cell which retains the ability to express cell surface antigens reminiscent of an earlier differentiation state and in some cases, of another cell lineage. But it should be remembered that markers that indicate cell origin or lineage are not absolutely specific, so the biological behavior of the hairy cell may only be unique in that investigators have tried to define it based on known cell antigens, assuming in many cases, that cell markers were cell lineage specific.
CHAPTER II

INDUCERS OF TUMOR CELL DIFFERENTIATION

A BRIEF SYNOPSIS OF THE LITERATURE
Pathologists have long recognized that malignant tumors are composed of cells in various stages of differentiation. Within the spectrum of this observation, tumors have been placed in classification schemes ranging from undifferentiated to well differentiated.

It has only been in the last 25 years that investigators have realized that many undifferentiated tumor cells can be induced to differentiate by various chemical and biological compounds into a more mature phenotype. The implication of this observation is two-fold; 1) tumor cells which can be induced to undergo differentiation could serve as in vitro models of differentiation and 2) clinically, this approach might induce malignant cells to differentiate into non-dividing end stage cells. This therapeutic approach might eliminate much of the cytotoxicity that is associated with conventional cytoreductive chemotherapy.

The leukemias both human and murine, are currently the most commonly used malignant cell type in the study of inductive chemotherapy. The Friend murine erythroleukemia cell line can be induced by a variety of compounds, including DMSO, butyric acid, and hexamethylene bisacetamide (HMBA) to undergo differentiation from a non-producing hemoglobin cell into a mature, non-dividing hemoglobin producing cell (1). Similar observations have been made in other murine erythroleukemia cells which have been infected with a virus different from that of the Friend virus (2). Murine myeloid leukemia can be also induced to differentiate into granulocytes and macrophages by both chemical and biological inducers of differentiation (3).
A similar situation exists in the human. Investigators have shown that fresh myeloid leukemia cells can be induced to differentiate into granulocytes or macrophages with DMSO and TPA (4). The cells become functionally competent, as well as assuming a more mature phenotype (4). Chronic lymphocytic leukemia cells have been shown to undergo plasma cell differentiation after being treated with TPA (5).

Established human leukemia cell lines are the model of choice when studying the mechanism of leukemic cell differentiation. The HL-60 promyelocytic cell line can be induced to differentiate into granulocytes by DMSO, while TPA and ara-C will cause macrophage differentiation (6,7,8). A similar situation has been observed with the histiocytic leukemia cell line, U-937 (9). It also has been shown that some leukemic cell lines can be induced to differentiate into another cell type. For example, the erythroleukemia cell line, HEL, can be induced by TPA to differentiate into a macrophage-like cell. Many of the inducers appear to be maturation stage specific, or in other words, they will not induce differentiation in all differentiative sequences that may be expressed by the cell. This can be observed with TPA induction of differentiation of the KG-1 myelocytic cell line. TPA will induce the parent line to mature into macrophages, but not the less mature subclone, KG-1a. Interestingly enough, the TPA binding affinities of both cell lines are identical (10).

**Biological Inducers.** Recently, physiological inducers or modulators that have been produced by cells have received a great deal of
attention as possible therapeutic modalities for neoplasia. The inducer/modulator that has received the most attention over the past several years has been described under various acronyms such as colony stimulating factor (CSF), colony stimulating activity (CSA), macrophage-granulocyte inducer (MGI), and granulocyte-macrophage colony stimulating factor (GM-CSF) (11). For the sake of simplicity we will refer to this protein factor as CSF.

It is generally accepted that CSF plays an important role in the control of hemopoiesis in vivo. This assumption is based on two broad observations, 1) this factor in vitro stimulates the precursors of granulocytes-macrophages to form mature colonies; it is commonly thought that these precursors are already committed to that particular lineage (12); 2) transplantation of a secreting granulopoietin tumor into a nude mouse greatly enhances granulopoiesis, and this effect can be reversed if the tumor is removed (13).

Investigations into the cellular action of CSF on the proliferating granulocyte-macrophage precursor cells as stated by Burgess et al (11) have revealed:

1) "non-cycling granulocyte-macrophage precursor cells are forced into the S phase of the cell cycle; and lag time from initiation to proliferation in semi-solid cultures is shortened progressively by increasing the CSF concentration;
2) doubling time of the granulocyte-macrophage precursor cells is shortened when the concentrations of CSF is increased and 3) when the granulocyte-macrophage precursor cells are bipotential, increased concentrations of the CSF
favors granulocyte differentiation, whereas a low
collection favors macrophage formation (11)."

Sachs and co-workers have shown that this protein inducer is
essential for the viability, proliferation and differentiation of
normal, myeloid cells (14). They have further demonstrated that
myeloid leukemia cells do not need this factor for viability and
proliferation and as a result they have an advantage over their normal
counterparts when CSF is in limited supply (15). This suggested to
them that a defect in, or an absence of CSF may be the origin of
myeloid leukemia (15).

Recently, another broad class of modulators have been receiving a
great deal of attention. They are collectively known as the
lymphokines or interleukins. The term of choice is interleukin and
they have subsequently been divided into 3 classes, interleukin I,
interleukin II and interleukin III.

The interleukins can be produced by mitogen or antigen activated B
or T lymphocytes in cooperation with the macrophage. The requirement
for the macrophage is essential in the production of T cell derived
interleukins, whereas the B cell, in many cases can elaborate factors
independent of the macrophage. This is probably due to the fact that
the B cell can be stimulated via their C3 or immunoglobulin receptors
(16).

Interleukin I (IL 1), a soluble mediator, is a component of a
macrophage signal cascade that modulates antigen specific activation of
T lymphocytes. This factor is an antigen nonspecific mediator with the
specificity of the immune response being determined by the antigen. The
macrophage is the cell responsible for the production of the low molecular weight peptide (17).

It is thought that IL-1 may possess a dual role in that it stimulates maturation of T cells that in turn amplify the production of IL-1 by macrophages (17). The functional importance of this factor was further underscored when it became evident that the production of IL-2 was dependent on its participation (16,17).

Although the mechanism(s) by which IL-1 causes T cell activation is not yet fully understood, it is thought that one possibility may involve the induction and subsequent synthesis of cell surface antigen receptors (16,17). In fact it has been reported that IL-1 enhances the T cell binding of the synthetic antigen, poly (L-tyr, L-glu-poly (DL-ala)-poly (L-lys) (7). Concomitantly, it also may modulate other surface receptors such as Ly 1, 2, 3, Thy 1, Ia and the E-rosette receptor. The same may also hold true for B cell surface molecules such as C3, Ia sIg (17). As noted earlier the activity of IL-1 in the immune response is dependent upon a second signal, either antigen or mitogen, but this second signal requirement is not necessary for T cell surface modulation (16,17). Thus, according to Mizel (17), it may be possible that IL-1 may cause the T or B cell to partially become activated with the second signal completing the activation cycle. In other words, IL-1 may cause quiescent T lymphocytes to enter into a specific compartment of the cell cycle in which the cells are not committed to synthesize DNA but rather are able to begin synthesis of cell surface receptors and soluble factors necessary for the continuation of the immune response (17).
Interleukin II or T cell growth factor (TCGF) is also a soluble nonspecific mediator that is responsible for maintaining T cell proliferation and growth. Helper T cells produce this factor after mitogen or allogeneic cell stimulation. The assay of choice, when measuring IL-2, is to determine its ability to maintain the growth of cytotoxic T cells even though IL-2 is thought to modulate a host of other biological functions (16,18). As stated earlier, the synthesis of IL-2 is dependent on IL-1 with this process being dependent on the presence of another activating signal such as a specific antigen or T cell mitogen.

Other biological functions thus far attributed to IL-2 are 1) induction of thymocyte proliferation, 2) the generation and proliferation of cytotoxic T cells by alloantigen stimulated T cell populations, 3) acting as the second signal in the stimulation of antigen-specific helper T cells and maintaining these cells in continuous culture and 4) inducing the synthesis of immune interferon (16,18).

Recently, another class of interleukins, IL-3 has been shown to have its own distinct biological characteristics. Like IL-2, IL-3 promotes growth and differentiation of lymphocytes in vitro, but unlike IL-2, this particular interleukin has its primary influence on the various stages of immature lymphocyte differentiation (19). Early in vitro experiments performed with IL-3 and cultured murine splenic lymphocytes have shown that the phenotypes of the resultant cell lines are Thy 1+, Ly 1+2−. These cell lines produce high titers of IL-3 and can be induced to produce IL-2 with the addition of
12-O-tetradecanoylphorbol-13-acetate (TPA) (19). Other experiments have shown that IL-3 can be produced in short term culture from T cells with the same phenotype as above (19).

Although the possible in vivo applications of these physiological modulators have not gone unnoticed, there is relatively little in vivo data. This point was addressed in 1978 by Lotem and Sachs (20) with the use of peritoneal implanted diffusion chambers containing different clones of murine myeloid leukemic cells. In one set of experiments, the cells were induced with CSF in vitro and then placed in the diffusion chambers. They were subsequently analyzed for markers of differentiation, - Fc, C3 and lysozyme, - and in all of the clones examined, these differentiation markers were found. Surprisingly these markers also were present in clones that were resistant to in vitro induced differentiation. These experiments were repeated in cyclophosphamide treated mice, as well as in several strains of nude mice. Differentiation was blocked in these mice with the exception of one clone, which was able to differentiate in the ICR nude mouse but not in the C57BL/6. These experiments suggested to the investigators that different clones might respond to the various molecular species of CSF that may be present in varying amounts in animals (20). They also suggested that the CSF may be regulated in vivo by immune cells since cyclophosphamide treated or nude mice were unable to carry out the differentiation program of CSF induced myeloid leukemia cells (20).
Chemical Inducers of Differentiation. The polar-planar compound dimethylsulfoxide (DMSO) was first shown in 1971 to induce Friend virus-transformed murine erythroleukemia cells to differentiate into hemoglobin-producing end stage cells (1). The induced differentiation program was similar to that normal erythroid differentiation (1). Other polar-planar compounds have also been shown to cause differentiation with hexamethylene bisacetamide (HMBA) being the most potent (21). Reuben et al (21) have stated that this compound can induce approximately 99% of the Friend erythroleukemia to cell differentiate at a concentration 50-fold lower than DMSO.

As previously stated, DMSO and other polar planar compounds can induce in vitro differentiation of some human leukemias and solid tumors. Although the mechanism(s) by which these compounds can re-establish a program of differentiation is not known, there is evidence that a perturbation in the cell cycle may play a role (8,21).

The tumor promoter, 12-0-tetradecanoylphorbol-13-acetate (TPA) as discussed earlier, can also directly or indirectly induce differentiation. The TPA mediated release of growth/differentiation factors could indirectly induce differentiation. It has also been postulated that TPA itself can act as, or mimic, a growth factor, thus providing a needed stimuli for differentiation (22). As with other chemical inducers of differentiation, the mechanism of action of TPA is not known. However, there has been some recent studies which suggest that TPA can exert its effect via the activation of protein kinase C (23). Protein kinase C, or the Ca++ and phospholipid-dependent protein kinase is found in most mammalian tissues and its activation can result
in an increased membrane associated kinase activity (23). Investigators have also shown that TPA can cause a block in $G_1$ of the cell cycle as can DMSO (8,21). But in contrast to the action of DMSO, TPA can induce differentiation independent of DNA synthesis (24). This suggests that differentiation can be induced by different mechanisms, and if this should be the case, then it could be hypothesized that differentiation can normally proceed in one direction by independent mechanisms. Or more simply put, a cellular differentiation program necessarily may not be aborted if one normally used mechanism or stimuli is inoperative, and another one is available. In clinical terms, this hypothesis could be extended to suggest that combination inductive chemotherapy may be as beneficial as has been the combination of cytotoxic drugs. Alternatively, a combination of chemical/biological inducers and cytotoxic drugs may also be of clinical value. In fact it has recently been observed that Ara-c, a routinely used cytotoxic agent, can induce differentiation in vitro and in vivo when administered at low doses (8,25).

In conclusion, both biological and chemical inducers of differentiation could play a dual role in the treatment of neoplasia in that they may not only cause tumor cell differentiation, but could augment the host immune response as well. The clinical experience with both sodium butyrate and alpha interferon tends to support this supposition.
CHAPTER III

PHENOTYPIC AND MOLECULAR CHANGES ASSOCIATED WITH CHEMICALLY INDUCED DIFFERENTIATION OF HAIRY CELL LEUKEMIA CELL LINE
Introduction

The ability of hemopoietic cells to respond to various
differentiative stimuli generally is restricted to the pluriopotent-
ential stem cell, and is progressively lost as the cell becomes committed to a
particular lineage. Recently, it has been proposed that the cell or
cells involved in the hematological disorder hairy cell leukemia (HCL)
have retained, at least in part, the potential to undergo phenotypic
modulation in response to external stimuli (1). In vivo, these stimuli
may be an inductive influence that is exerted on the cell in its
immediate microenvironment. Some investigators have gone as far as to
suggest that HCL may in fact be a manifestation of two diseases, one
involving the spleen and the other, the peripheral blood (2). In vitro
studies have documented that hairy cells with a B cell phenotype can be
modulated with PHA and autologous T cells to display a T cell
phenotype, as evidenced by the disappearance of sIg and the acquisition
of the ability to bind sheep erythrocytes (3,4). However, other
investigators were unable to confirm this finding (5). Phenotypic
modulation has been observed clinically as well. In two studies it was
shown that at various times throughout the clinical course of the
disease, hairy cells exhibited either combined B and T cell features,
or displayed either T or B cell characteristics (6,7).

The JOK-1 cell line was established by Anderson et al (8) from a
patient with hairy cell leukemia. Since this line differed from EBV
positive B cell lines established from the same patient, it was
concluded that JOK-1 represented a pre-hairy cell leukemia line with B
lymphocyte characteristics (Table 2). They also observed that this cell line was tartrate resistant acid phosphatase (TRAP) positive, expressed the κ light chains and had a glycoprotein pattern indicative of an early B cell (8). The presence of the isoenzyme TRAP is usually considered to be diagnostic of HCL although occasionally it is seen in other hematological malignancies and can be absent in HCL as well.

Many investigators have shown that various agents can either induce leukemic cells to differentiate or modulate so that they can re-establish their program of differentiation. One such agent, 12-0-tetradecanoyl-phorbol-13-acetate (TPA) has been shown to have a paradoxical effect on cellular differentiation. TPA inhibits the differentiation of DMSO induced Friend erythroleukemia cells, but stimulates terminal differentiation of the human promyelocytic line HL-60 towards cells that have the characteristics of monocytes and macrophages (9,10). Phorbol ester also has been shown to induce differentiation of freshly obtained cells from patients with myeloid leukemia and common acute lymphoblastic leukemia (12), as well as promoting the production of interleukin-2 (13), and colony stimulating factor (14). Dimethyl sulfoxide (DMSO) also has been shown to be a potent inducer of Friend erythroleukemia cells and HL-60, the latter of which develops along the granulocytic line (15,16).

Based on studies with the JOK-1 hairy cell line, we present evidence that leukemic cells with a B cell phenotype can be modulated by both phorbol acetate and DMSO to express some characteristics that are associated with macrophages, and some morphologic features of plasma cells. It was also observed that after exposure to DMSO and
TPA, the changes in nuclear configuration were consistent with the different hairy cell nuclear shapes described in the literature (17). In this paper data are presented that when combined with those in the literature, suggest that phenotypic plasticity may be an inherent feature of hairy cell leukemia.
Materials and Methods

Cell Line. The JOK-1 cell line was obtained from Dr. Jun Minowada. This line was propagated as a suspension culture in 1640 RPMI media supplemented with 5% fetal bovine serum and 10,000 units/ml of penicillin and 10,000 mcg/ml of streptomycin (complete media). Culture media was changed every 3 days by replacing 1/2 of the old media with fresh media. The cells had a doubling time of every 16-18 hours and reached a saturation density at approximately $2.5 \times 10^6$ cells/ml. They were maintained at 37° in a humidified incubator with 5% CO₂.

Chemicals. DMSO was obtained from Fisher (Scientific, Fair Lawn, New Jersey) and was diluted to the desired concentration in complete media. In early experiments DMSO containing media was resterilized by passing through a 0.2μ Nalge filter (Nalgene, Rochester, N.Y.) and parallel experiments were conducted without resterilization. No bacterial contamination was observed in either case. Later experiments then were conducted without resterilization.

12-O-tetradecanoyl-phorbol-13-acetate (TPA) was obtained from Consolidated Midland Corporation (Brewster, N.Y.) and diluted in acetone to a concentration $10^{-4}$ ng/ml. This stock solution was stored at -20°C, and was later diluted to the desired concentration in complete media. The concentration of acetone was never greater than .001%. Early experiments demonstrated that acetone had no effect on cell growth, morphology and surface changes so later experiments were conducted without acetone. Hexamethylene bisacetamide (HMBA) (Aldrich,
Milwaukee, Wis.) was diluted to the desired concentration in complete media.

**Induction of JOK-1.** Cells were seeded with DMSO, TPA, HMBA or in combination at a concentration of $2-2.5 \times 10^5$ cells/ml in 75 cm flasks or 60mm petri dishes (Corning, N.Y.). After induction cell counts were either performed daily or at designated time intervals. Cell viability was assessed by trypan blue exclusion.

**Immunofluorescence.** Cell surface heavy and light chain immunoglobulins were examined in a direct immunofluorescent assay. Cells were washed 3x in phosphate buffered saline, pH 7.4. The fluorescein isothiocyanate conjugated goat anti-human heavy and burro anti-human light chain antibodies (Kalstead, Austin, Texas) (IgG, IgM, IgA, IgD, $\lambda$, $\kappa$) were added at the proper dilution to $1 \times 10^6$ cells in a volume of 100 $\mu$l. Cells then were incubated for 30-40 minutes at 4°C. The cells were then washed 3x with PBS, fixed with 50% glycerol, mounted on a slide and a minimum of 200 cells were examined with a Zeiss UV FL epi-fluorescence microscope. The monoclonal antibodies, Ia, J-5, B-1 and MO₂ (Coulter Immunology, Hialeah, Fla.), anti-Leu-M1, anti-Leu-M3 (Becton Dickinson, Mountain View, Calif.), anti-human monocyte (Bethesda Research Laboratories, Gaithersburg, MD) and S-HCL-1,2,3 (kindly provided by Dr. Roland Schwarting) were used in the indirect immunofluorescence test. As before, $1 \times 10^6$ cells/ml were incubated with the appropriate antibody dilution and incubated at 4°C for 30-40 min; cells then were washed 3x with PBS, then incubated for an
additional 30-40 min. with FITC conjugated anti-mouse IgG (Miles Scientific, Naperville, IL). The cells again were washed 3 x with PBS, fixed with 50% glycerol and examined as above.

**Determination of DNA Superhelicity by Nucleoid Centrifugation.**

Nucleoid preparation and analysis were as described by Cook and Brazell (18) and Lipetz et al (19). Briefly, neutral sucrose gradients with different concentrations of Ethidium Bromide (EB) (Sigma Chemical Co., St. Louis, MO) were prepared in 7/16 x 2-3/8 in polyallomer (Beckman Instruments) tubes to a volume of 3.6 ml/gradient. JOK-1 cells either untreated or treated with TPA or DMSO were washed 3 x with PBS and resuspended to a concentration of 1 x 10⁷ cell/ml. Prior to the addition of 50 μl of cell suspension to the gradient, 150 μl of lysis solution (2.5 M NaCl, 13 mm Tris-HCl, pH 8.0, 13.3 mm, EDTA, 0.13% Triton-X-100) was layer over the gradient. Nuclei from the lysed cells were distributed by rotating the centrifuge tubes at an angle. Cell lysis was allowed to proceed for 15 min. after which time, gradients were centrifuged at 5000 rpm for 4 hours. Gradients then were fractionated from the bottom into 10 x 75-mm glass culture tubes pre-filled with 1.5 ml of 100 ng/ml DAPI-H₂O. A constant speed pump was utilized to determine fraction size by equal time intervals.

**Flow Cytometry**

**DNA analysis.** Cellular DNA was analyzed using an Ortho Cytofluorograf System 50H with a 2150 Research Data Handling System (Ortho Inst.,
Westwood, MA). Analysis was performed with the Spectrophysics Argon Ion Laser using the 488 nm line at 300 mwatts. Red fluorescence was analyzed at wavelengths greater than 610 nm. Prior to each experiment the machine was calibrated with fluorescent latex particles and glutaraldehyde fixed chicken red blood cells. DNA was stained with propidium iodide (PI). Briefly, 1 x 10^6 cells were fixed with 70% ethanol for 30 minutes at room temperature, washed 3x with PBS and stained with 1 mg/ml of PI for 10-20 minutes at 37°C. This incubation mixture also contained 1 mg/ml of RNAse. After the incubation period, the cells were washed 3x with PBS, placed on ice and analyzed.

**Simultaneous DNA and Total Protein Analysis.** Cells were stained in a 1:1 mixture of PI and fluorescein isothiocyanate at the concentration of 1 mg/ml with 1 mg/ml of RNAse for 10 minutes at 37°C. Prior to staining, cells were fixed as above. After staining, cells were washed 3x with PBS, placed on ice and analyzed. Fluorescein isothiocyanate measured total protein (21) and can be seen on the X axis in Figures 26, 27, 28. Green fluorescence was analyzed at the 510-540 nm wavelength.

The simultaneous staining of surface IgM and DNA was identical to that of DNA staining, except antibody was added prior to fixation (22). Briefly, 1 x 10^6 cells/ml were stained with sIgM as described earlier for 30 min at 4°C. The cells were washed 3 times with PBS and fixed for 30 min with 70% ethanol. The cells were stained with PI as described above.
Assay for cAMP Dependent Protein Kinase I and II

Photoaffinity Labeling of cAMP Dependent Regulatory Subunits. This assay was performed by Mr. Louis Jusenent in the laboratory of Dr. Bruce Zwilling of the Department of Microbiology. Briefly as described by Jusenent (23), up to 100 mg of cytosol protein was added to a volume of 0.1 ml of previously prepared reaction mixture. To determine the specificity of protein kinase regulatory subunit labeling, 50 μm of cold cAMP (Sigma) was incorporated into duplicate reaction mixtures. The subsequent reaction, in order to obtain equilibrium binding, was carried out in the dark at 4°C for 1 hr. A mineralite UVS-11 lamp (Ultra-Violet Products Inc., San Gabriel, CA) was used to photolyze the samples. After the reaction was stopped, the samples were heated at 100°C for 5 min and then subjected to SDS-polyacrylamide electrophoresis. Autoradiography was used to determine the incorporation of radioactivity into the protein bands. The autoradiographs were scanned using an LKB laser densitometer (LKB Instruments, Rockville, MD) interfaced to an Apple II Computer. The Gelscan program developed by Dr. John N. Reeve (The Ohio State University, Department of Microbiology) was used for the integration of the areas under the peaks of optical density tracings. The formula listed below was used to calculate the relative intensity for RI and RII subunits:

\[
\text{Relative Intensity} = \frac{\text{Peak Area of Treated Sample}}{\text{Peak Area of Untreated Control}}
\]

DEAE-Sephacel Column Chromatography. This assay was performed by Mr. Louis Jusenent in the laboratory of Dr. Bruce Zwilling of the
Department of Microbiology. As described by Justement (23), the separation of cAMP dependent protein kinase (cAMPdPK) I and II was carried out on DEAE-Sephacel. Briefly, after equilibration, DEAE-Sephacel was packed into 1.5 x 6 cm columns. To the columns were added 3-6 mg of extensively dialyzed cytosolic protein, after which the columns were washed with 10-20 ml of equilibration buffer. A linear NaCl gradient or a step gradient (0.12, 0.22 and 0.3M successively) in a volume of 60 ml, was used to elute the protein. $^3$H-cAMP binding and protein kinase activity was assayed using 0.75 ml fractions.

**Phagocytosis.** Cells were washed 3 x with PBS and resuspended in 1 ml of complete media or PBS at a concentration of $1 \times 10^6$ cells. Latex beads (0.85μ) in a volume of 2 μl (Sigma Chemical Co., St. Louis, MO) was added to the cell suspension. After a one hour incubation the cells were washed 3 x with PBS. Cells then were examined for latex bead ingestion either under a phase contrast microscope or after a Wrights stain. Cells with 5 or more internalized beads were considered to be positive.

**Histochemistry and Cytogenetics.** Histochemical and chromosomal analysis was performed in the Hematology and Cytogenetics laboratories of University Hospital of The Ohio State University.
Results

Cell Growth. Studies were carried out by continuously exposing JOK-1 cells to either TPA, DMSO or HMBA. These inducers of differentiation were added at time zero when the cells were in an early logarithmic growth phase. At concentrations of $10^{-7}$ through $10^{-11}$ ng/ml of TPA, proliferation was markedly inhibited (Figure 1). TPA at $10^{-11}$ ng/ml was less inhibitory than higher concentrations. Dimethyl sulfoxide at concentrations of 1, 1.5 and 2% also were inhibitory, with 2% having the greatest effect (Figure 2). Hexamethylene bisacetamide (HMBA) and butyric acid at the concentrations of 5 mM and 2.5 mM respectively, also inhibited logarithmic growth (Figure 3). As shown in Figure 3, the effect of 2% DMSO was intermediate to that of HMBA and butyric acid. Figure 4 shows that the combination of 5 mM HMBA and 2% DMSO is additive in their inhibitory ability. Cellular viability, however, was unaffected by treatment and was consistently greater than 90% during exposure to the compounds. As observed by others (24) we noted that TPA had some cytotoxic effect, as evidenced by cellular debris by day 3 or 4, which could explain the downward trend seen in Figure 1. This cytotoxic effect apparently caused immediate cell lysis because few cells took up trypan blue.

Growth inhibition of 2% DMSO is reversible up to day 5, after which it becomes irreversible. Figure 5 depicts the growth of JOK-1 when DMSO is washed out after 3 days of continuous exposure. The previously exposed cells demonstrated a growth pattern that was greater than those of the control. This can be partially attributed to the
fact that the cells received fresh media. In this particular experiment, however, the controls did not receive fresh media. In other experiments when the controls received fresh media at day 3 in parallel with the washed DMSO treated cells, the two groups of cells had similar growth patterns. TPA treated cells showed irreversible inhibition of growth. Figure 6 shows that a 30 minute exposure to $10^{-7}$ ng/ml of TPA had an irreversible effect even though the cells were extensively washed with PBS. This inhibition may be energy independent, since cells that were incubated with $10^{-7}$, $10^{-8}$, $10^{-9}$ ng/ml of TPA at 4°C for one hour, also failed to enter logarithmic growth. As shown in Figure 14, a portion of TPA treated cells became adherent. These adherent cells, as well as the nonadherent cells when extensively washed after a 24 hour exposure to $10^{-7}$ ng/ml of TPA, exhibited no growth. This is depicted in Figure 5. The apparent increase in cell number of the adherent population on day 7 was only temporary since by days 8 and 9, growth was back down slightly from the cell number indicated for day 6.

**Morphology.** The morphological changes of JOK-1 cells following exposure to the inducers are seen in Figures 7-19 as are the control cells. The vast array of pictures shown are necessary to illustrate the varied morphology that occurs after cells have been exposed to DMSO, TPA and HMBA. Figures 7(a,b), 8(a), 11(a), and 12(a) are control JOK-1 cells that have been stained with Wright-Geimsa stain. The cells are primarily lymphoid in appearance, and relatively homogeneous in size and morphology. Figures 8(b), 9(a,b), 10(a), 11(b), 12(b) depict
JOK-1 after exposure to 2% DMSO. Morphologically, these cells exhibited both plasmacytoid and monocytoid features. The nucleus assumed a more eccentric location and was segmented in many of the cells, prominent nucleoli frequently were observed, many of the cells had pink granules, and vacuoles were seen. In contrast to control cells, which generally had an ovoid nucleus, the nucleus in the DMSO treated cells, as well as TPA treated cells, assumed one of three nuclear configurations; indented, convoluted, or remained ovoid. The nuclear shape after induction is consistent with those described in the literature for hairy cells, namely oval, convoluted and indented (16). In many cases the cells became larger as well.

Figures 14(a,b), 15(a,b) and 16 illustrate what is perhaps the most striking feature of the JOK-1 response to TPA and the combination of TPA and DMSO (2%), attachment and subsequent spreading out of the cells. This effect was observed at all concentrations of TPA tested. Generally, cells were attached within 1-3 hours after the introduction of TPA and by 24 hours had developed pseudopodia that became more prominent by 48 hours at which time they were adherent and resembled normal macrophages. When DMSO (2%) and TPA (10^-7 ng/ml) were combined, JOK-1 not only attached, but assumed a more extended configuration with long fine processes (Figure 14a,b). Interestingly enough, if TPA was added 1-7 days after the addition of DMSO, a similar effect was noted. But the addition of DMSO 1-3 days after TPA did not alter the TPA induced morphological changes. Koeller et al (25) as well as other investigators (26) have observed the same response to TPA in several human myeloid leukemia cell lines (HL-60, ML-3, KG-1 and KG-1 clones,
1, 2 and 3). Lockney et al (27) have reported that fresh hairy cells also assumed a similar morphology following exposure to TPA, and we have made a similar observation. A B cell line, RI, (kindly provided by Dr. D. Golde, Department of Medicine, UCLA Medical Center, Los Angeles, CA), failed to exhibit a similar response.

The DMSO treated JOK-1 cells did not attached and spread. They increased in size and formed large cellular aggregates, whereas large aggregates were the exception rather than the rule in control cultures (Figure 13a,b).

Ultrastructural examination by both transmission and scanning electron microscopy (TEM, SEM) revealed further morphological changes. Figures 17(a,b), 18(a,b) and 19(a,b) are SEM photographs which illustrate the surface changes associated with TPA and DMSO induction. A JOK-1 cell from a control culture is depicted in Figure 16a. The cell had a few microvillar projections as well as an area of ruffled membranes, and is consistent with a monocytoid hairy cell described by Katayama et al (28). Figure 17b illustrates JOK-1 cells 3 days after the addition of 2% DMSO. Of the three cells, one had a ruffled membrane with ridge-like projections (28), and several relatively long microvillar like processes (no. 1). This particular cell also resembled a monocytoid hairy cell but with more pronounced features. The other two cells resemble lymphocytes (29) in that they appeared to have a variable number of microvilli, however, cell 2 may be consistent with that of a lymphocytoid hairy cell. Cell 3 is definitely consistent with that of a lymphocyte (29). Figure 18a depicts another JOK-1 cell after a 3 day DMSO exposure. This cell was characterized by
long spindly microvillia projections with some ridge-like projections, and is consistent to that of a lymphocytoid hairy cell (28).

The appearance of JOK-1 3 days after the addition of both TPA ($10^{-7}$ ng/ml) and DMSO (2%) is shown in Figures 18b and 19a. The network of cell processes were readily apparent, and it appeared as if they were crossing other cells and becoming attached in the process. As can be seen many of the cells had blebs and different types of membranous projections. Figure 19b illustrates the effect of TPA alone. The cells shown had long microvillar projections.

When examined by TEM, the DMSO and TPA treated cells had more mitochondria, endoplasmic reticulum, and exhibited a different nuclear configuration (Figures 20,21,22,23,24,25). The TPA treated cells seen in Figures 22 and 23 had a segmented nucleus with a prominent nucleoli. An annulate lamellae could be seen in the cytoplasm of the TPA treated cell in Figure 22. The function of this organelle is unknown and is thought to come from the nuclear envelope (30). One investigator has stated that the only blood cell in which he has seen this structure is the proerythroblast (31), thus suggesting that this is a rare organelle and may only be seen in immature blood cells.

**Cell Surface Changes.** Table 1 summarizes the cell surface markers that were sequentially followed at 3-7 days. The B-1 antigen, which is found on resting B cells, was expressed throughout the period of exposure to both DMSO and TPA. The common acute lymphoblastic leukemia associated antigen (J-5) also was expressed on all JOK-1 cells and was decreased to approximately 50% of control levels after 3 days of
Table 1  
Marker Profiles of JOK-1 Hairy Cells Following Exposure to Dimethyl Sulfoxide or Phorbol Acetate

<table>
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<th>Marker</th>
<th>Days in Culture</th>
<th>Control 0</th>
<th>2% Me2SO 3</th>
<th>5</th>
<th>7</th>
<th>10^{-7}M TPA 3</th>
<th>4</th>
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aThis is a representative experiment, each point was done at least 3 times.

bCells at a concentration of 2-3 x 10^5/ml were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum and 2% Me_2SO and 10^{-7}M TPA. The numbers indicate the percentage of cells showing the marker. ND indicates not done.
continuous exposure to TPA, with no further significant decrease in antigen expression on days 4, 6 and 7. In the case of DMSO, however, the J-5 antigen was completely lost by day 5. Surface IgM, which was present on greater than 80% of JOK-1 cells, dropped to approximately 20% following 3 days of exposure to DMSO and to 0% in the presence of TPA. Expression of the C3 receptor, which was present on 90-95% of cells prior to induction, also was decreased on both DMSO and TPA treated cells. The Ia antigen was strongly expressed throughout the period of exposure. Monoclonal antibodies that recognize monocyte/macrophage determinants M01, M02, anti-human monocyte, Leu-M1 and Leu-M3 did not react with either JOK-1 control or treated cells.

**DNA Supercoiling.** Figure 26 (Panels A and B) demonstrates the effect of DMSO (2%) and TPA (10^-7 ng/ml) on JOK-1 DNA supercoiling. Panels A and B represent cells treated for 7 days with either TPA or DMSO on two different occasions. Control cells were allowed to stay in the same media for 7 days. In both experiments, the control and DMSO treated cells had maximum relaxation of DNA supercoiling at the same ethidium bromide concentration, 4 µg/ml and 2 µg/ml respectively. However, the TPA treated cells exhibited maximum relaxation at 6 µg/ml and 4 µg/ml of EB respectively. Unexpectedly, in panel A, both the control and DMSO treated cells apparently assumed a more supercoiled configuration at 6 µg/ml, then again relaxed at 7 µg/ml after which they both became positively supercoiled. In panel B only the control cells exhibited this phenomenon. Presently, we have no explanation for this observation.
Cell Cycle Analysis

Based on red fluorescence, an indication of DNA intercalation by propidium iodide, cells treated with DMSO and TPA appeared to be arrested in $G_1$ of the cell cycle (Figures 27-32). This became readily apparent by day 5 (Figures 28, 30, 31 and 32). The treated cells also increased in relative size and in total protein. These results can best be seen in Figures 30-32. It can be hypothesized that the increase in total cell protein seen in the treated cells may have been due to the increase in cell size.

The most intriguing observation made can be seen in Figure 27, where both TPA and DMSO treated cells showed a decrease in red fluorescence. This was not seen at day 3, and was only seen in the TPA treated cells on day 7 (Figures 27, 29). Although the observation currently cannot be explained, it can be hypothesized that that the DNA was more supercoiled and thus had less sites available for the intercalation of propidium iodide. Based on this possibility, the data in Figure 29 are of great interest. The data indicate that the DMSO has shifted the cells from an increase in red fluorescence to a position similar to that of the control. But TPA appears to have induced an extended DNA configuration with a considerable portion of the cellular DNA showing a decrease in red fluorescence compared to the control. The exciting thing here is that these TPA data correlate with the supercoiling data shown in Figure 26. As previously discussed, cells treated for 7 days with TPA exhibited an increase in supercoiling
as compared to control and DMSO treated cells. As suggested earlier, a
decrease in red fluorescence may have been an indication of super-
coiling, and if so, TPA treated cells in Figure 29 were supercoiled as
were the cells in Figure 26. If this does turn out to be the case,
then it might be possible to independently verify such data, as well as
providing an alternative method to measure supercoiling.

The relationship of sIgM to the cell cycle as well as its response
to DMSO and TPA, can be seen in Figure 33. Although it appears that
sIgM expression is independent of the cell cycle, it decreased after a
3 day exposure to the inducers.

Cyclic AMP Dependent Protein Kinase I and II

Studies were carried out to determine whether or not the cAMP
dependent protein kinase I could be induced with DMSO, TPA or with the
two combined. As shown in Figure 34, protein kinase I was not induced
nor was protein kinase II altered.

Histochemistry and Phagocytosis. Using histochemical methods, we were
unable to detect TRAP activity either prior to or following induction,
although alpha naphthyl acetate esterase (ANAE) activity was increased
in both TPA and Me₄SO treated cells when compared to controls.
Specific esterase activity was absent in both control and TPA and Me₄SO
treated cells. Phagocytosis of latex beads (0.80μ) was noted in both
the control and treated cells. These results were not unexpected since
hairy cells have been reported to be ANAE positive, and possess
phagocytic activity. It was somewhat surprising that TRAP activity could not be demonstrated on JOK-1 since they originally were described as being positive for this enzyme. However, Stockbauer et al (32) similarly have observed that a hairy cell line established in their laboratory lost TRAP activity after a period of time in culture. Furthermore TRAP is a poor marker for hairy cell leukemia cell lines since human cords lymphocytes are TRAP positive after EBV transformation (33).

Cytogenetics

Anderson et al (8) in describing this cell line noted that it had a modal number of 48 with only one chromosome 6. Our analysis showed there were between 46 and 49 chromosomes with only one chromosome 6 and one chromosome 8. There also were other chromosomal aberrations which can be seen in Figures 35-39. Anderson et al (8) noted some translocations but did not specify what they were.
Discussion

The chemical inducers of differentiation, DMSO and TPA exerted a profound effect on the JOK-1 cell surface phenotype, morphology and cell cycle. The quaternary structure of the DNA was altered by TPA as well. These changes were not associated with any cell surface alterations indicative of differentiation into a more mature or different cell type.

The morphological appearance of JOK-1 following exposure to Me₂SO was similar to that of plasma cells and cells of myelomonocytoid lineage. The expression of B-1, J-5 and Ia, however, argues against these cells being true plasma cells. Furthermore, plasma cells do not have non-specific esterase activity and are unable to ingest latex beads. Myeloid cells however, express the Ia and J-5 antigens, can phagocytize latex beads and can be positive for non-specific esterase, although they usually have specific esterase activity (34). They do not, however, express the B-1 antigen. In addition, the induced cells did not show increased phagocytic activity as would have been expected with myelomonocytoid cells. DMSO treated cells failed to show substrate attachment as was observed with TPA treated cells. The ability to adhere to substrate is characteristic of normal monocyte to macrophage differentiation in vitro (10). Both TPA and DMSO treated cells failed to express surface antigens that are found on macrophages and mature myeloid cells.

The co-expression of the common acute leukemia lymphoblastic associated antigen (cALLa), along with sIgM also is an intriguing
observation. There have been reports of the simultaneous expression of the B-l antigen and cALLa (35), but never with surface immunoglobulin. Some investigators have suggested that cALLa is a stem cell antigen that is lost during normal hemopoietic differentiation. From our own observations, reported herein, we would concur. Several recent reports, however, suggest that this antigen also may be expressed on granulocytes (36). Although we have no adequate explanation for the simultaneous expression of cALLa and sIgM, it may be a reflection of the dearrangement of normal differentiation controls.

Recently, there has been interest in the genomic superstructural changes associated with the neoplastic transformation as well as during chemically induced differentiation. Terada et al (37) and Scher et al (38) found that DMSO induced Friend erythroleukemia cells had a decrease in the sedimentation rate in alkaline sucrose gradients. It was noted that these changes were consistent with the accumulation of single stranded nicks in the DNA (37,38). These investigators hypothesized that genomic changes or single stranded DNA scission may play an important role in the control and commitment in cell differentiation.

Quaternary structure or DNA supercoiling has been shown to regulate gene expression in prokaryotic organisms (39). For example, it has been shown in the T7 bacteriophage, that decreasing DNA supercoiling inhibits the transcription of late but not early genes (39). It has been postulated that supercoiling can modulate gene expression by changing the denaturation of promoter regions, which in turn could create single stranded regions which favor DNA polymerase
binding (39). As DNA supercoiling increases, the stress created, denatures adenine-thymine regions, which is the initiation site of many promoters.

There is no direct evidence that DNA supercoiling can control differentiation in eukaryotic cells. However, data is available that suggest that the superstructure can play an important role in cell differentiation. It has been reported that cells in chronic lymphocytic leukemia (CLL) have an increase in DNA supercoiling (39), and that after treatment with TPA, there was a decrease in supercoiling similar to that of normal lymphocytes. Since TPA has been shown to cause CLL cells to differentiate into plasma cells, it is tempting to speculate that a decrease in supercoiling is associated with differentiation (39). The JOK-1 DNA supercoiling data suggests that control and DMSO treated cells have a sedimentation rate similar to that of normal lymphocytes; while TPA treated cells exhibited a sedimentation rate greater than those of the control or DMSO treated cells and comparable to that of chronic lymphocytic leukemia cells. If TPA did induce differentiation in JOK-1 then this change was not reflected in a decrease in DNA supercoiling. It, however, has been observed that TPA can cause an increase in supercoiling in normal human lymphocytes (39) as well as inducing proliferation.

Both DMSO and TPA induced a change in the cell cycle in the apparent form of a block in the G1 compartment. Although obvious by day 3, the change was more pronounced by day 5, appearing as a significant shift to the left, which is the function of a decrease in red fluorescence. By day 7, only TPA still exhibited the shift;
however, there was also a major shift to the right. Although the significance of these observations are unknown, other investigators have observed changes in the cell cycle after cells have been induced to differentiate (40,41,42). Ross (40) has found that HL-60 cells induced by DMSO had a marked decrease in the number of cells in S and G2, but cells induced by ara-C only exhibited a reduced rate in DNA synthesis, with no concomitant change in the DNA histogram (40). Since these inducers caused cellular differentiation into different end-stage cells, Ross concluded that differentiation may be characterized by different cell cycle events. Boyd et al (41) have shown that all HL-60 cells, which were induced to differentiate by butyric acid, were arrested in G1.

Our studies failed to demonstrate the expression of cAMP dependent protein kinase I in the induced JOK-1 cells. We were prompted to look for this biochemical change by a recent report by O'Dorisio et al (43), in which it was stated that while monocytes only had kinase II, macrophages that underwent differentiation in vitro, exhibited both kinase I and II.

The true nature of the JOK-1 cell line is presently not clear. The cell surface markers sIgM, B-1, the presence of the Epstein Barr nuclear antigen and the morphology suggests a lymphocytic origin. But positivity for non-specific esterase and the ability of 20 to 30% of the cells to phagocytosize latex beads suggest a monocytic-type cell. Furthermore metabolic studies utilizing an assay for the hexose monophosphate shunt (HMPS) revealed that JOK-1 had a pattern that was well within the normal range for monocytes (unpublished observations).
Similar studies on fresh hairy cells have shown an intermediate pattern between that of lymphocytes and monocytes (44). After induction with either TPA or Me₂SO there was no shift of the HMPS activity (unpublished observations).

Our results, when re-examined in the context of several recent reports, have become more clear. Stong et al (45) have reported that the cell line RS4;11, established from a patient with acute leukemia, changed from one of a lymphoid appearance to a monocytoid phenotype after a 5 day exposure to TPA. Functional, as well as immunological changes, characteristic of monocytes, accompanied this morphological shift. Further analysis revealed characteristics of both lymphoid and myeloid lineages. Molecular criteria based on Ig gene rearrangement placed these cells in the B-cell lineage. These cells were positive for both B-cell specific and myeloid specific monoclonal antibodies. Treatment with TPA induced the expression of new myeloid surface antigens but did not decrease the expression of B cell markers. This cell line had the t(4;11) chromosomal rearrangement and it was postulated that the translocation may somehow involve normal myelomonocytic differentiation (45).

Neame et al (46) reported 4 cases of acute leukemia that presented as acute mixed myeloid-lymphoid leukemia. Two of these patients had distinct populations of myeloid and lymphoid blast cells, while the blast cells in the other two patients expressed both lymphoid and myeloid markers on the same cell. They suggested that the population of blast cells that expressed markers of the two different lineages,
may either be a phenotypic reflection of disordered differentiation in
the stem cell or of genomic derepression in a more "mature" cell (46).

Recently investigators have reported that culture conditions may
affect the expression of monocyte antigens (47). It was shown that the
monocyte antigen, FMC17, which is present on substrate adherent
monocytes, rapidly disappeared when monocytes were put into suspension
culture, only to reappear if the cells were allowed to reattach (47).
Since no functional role for this antigen has been found, it was
suggested by the authors (47) that the physical state of the cell may
dictate certain antigen expression. Indirectly, one could say that the
same may be true for the microenvironment, or in other words, the local
cellular environment may induce the expression of an antigen that has
no apparent function. This has obvious implications when trying to
define differentiation by the expression and loss of surface antigens.

Pertinent to our work, was the observations of Ashman et al (48).
They investigated the effects of DMSO and TPA on the B-lymphoblastoid
cell line, BALM-1. Similar to our results, they found that TPA and
DMSO induced morphological changes in the cells, as well as causing a
block in the G1 compartment of the cell cycle.

TPA caused an increase in membrane area and extensive ruffling
was observed as shown by SEM. It also was observed that after 2 days,
TPA treated cells exhibited ridges, blebs and large veil-like
cytoplasmic extensions. In contrast, the control cells were
heterogeneous in size and shape. The cell surface was characterized by
the appearance of microvilli and blebs. DMSO treated cells in SEM
examination demonstrated a monocytoid morphology but failed to react with any monocyte surface markers (48).

Their TEM studies were virtually identical to ours. The cells from control cultures had a high nuclear:cytoplasmic ratio, few mitochondria and a spherical nucleus (48). TPA treated cells had an increase in microvillar extensions and a corresponding increase in cell diameter. Cells treated with DMSO were observed to have an increase in mitochondria with an increase in cytoplasm. The nucleus was usually irregular and sometimes kidney shaped (48).

Cell surface marker studies demonstrated in DMSO treated cells, a decrease in the expression of J-5 and Ia antigens. An increase was noted in the expression of surface immunoglobulin. After induction with TPA, the Ia antigen had an increase in expression and a corresponding decrease in surface immunoglobulin was documented. These investigators concluded that DMSO induced a more mature B cell, while TPA did not initiate a recognizable differentiative process. The authors suggested that the cell was too immature to be completely induced by TPA (48). This hypothesis may be valid since investigators have shown that TPA can bind to less mature cells without inducing differentiation.

The apparent plasticity of the hemopoietic system, especially in the neoplastic state, is beginning to be more fully recognized. This plasticity may either be morphological or antigenic in nature, or represent a discordance between the two. Factors that influence or dictate this feature may be either inherent or acquired, with the microenvironment being the controlling mechanism. The \textit{in vivo} and \textit{in}
vitro biological behavior of leukemia may be explained to a large degree by the concept of plasticity.

In summary, we have shown that a hairy cell line with B lymphocytic surface markers can be induced to lose sIgM and to acquire a completely new phenotype following exposure to either TPA or Me₂SO. This is in agreement with other reports in which hairy cells were phenotypically modulated in vivo to express characteristics of another cell type. Perhaps most importantly, our data may suggest an explanation for the seemingly conflicting views on the origin of the hairy cell. We postulate that an inherent feature of this cell is its ability to undergo phenotypic change as a function of its microenvironment. This perhaps may explain the various nuclear configurations often seen in HCL. Hairy cell leukemia thus may represent a novel hematological malignancy in which the cell has retained enough genomic information that is reminiscent of an earlier state, but that can be phenotypically modified once a change in the microenvironment has taken place.
Table 2
The Comparison of Hairy Cells to Other Cells
With Hairy Cell Characteristics

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\[a^+\] denotes a decrease in expression as compared to controls.

\[^b\]hexose monophosphate shunt
Figure 1

Effects of TPA on the growth of JOK-1 cells. Cells were seeded at a concentration of $2.5 \times 10^5$/ml in 60 mm petri dishes and aliquots were taken at intervals, as indicated. Cell number was determined by counting in a hemocytometer and viability by trypan blue exclusion method. Each experimental point represents the numerical average of 2-3 replicate cultures.

Symbols: ( ) control, ( ) $10^{-7}$ M, ( ) $10^{-8}$ M, ( ) $10^{-9}$ M, ( ) $10^{-10}$ M, ( ) $10^{-11}$ M.
EFFECTS OF TPA ON THE GROWTH OF JOK-1 CELLS

Figure 1
Figure 2

The response of JOK-1 to 1, 1.5 and 2% DMSO. Each point represents the mean of replicates.

Con
1%
+ 1.5%
2%
THE RESPONSE OF JOK-1 TO 1, 1.5 AND 2% DMSO

Figure 2
Figure 3
The effect of various inducers of differentiation on the growth of JOK-1.
Figure 3
Figure 4

The effect of the combination of 2% DMSO and 5 mM HMBA on JOK-1 growth.

Control
DMSO 2%
HMBA 5 mM
DMSO + HMBA
THE EFFECT OF THE COMBINATION OF 2% DMSO AND 5 mM HMBA ON JOK-1 GROWTH

Figure 4
Figure 5

The recovery of JOK-1 after the removal of DMSO. Cells were grown for 3 days in the presence of the chemical, then the cells were washed 3 x with PBS and resuspended in fresh complete media. The effect of DMSO becomes irreversible by day 5.

Control

DMSO

Cells which had the DMSO washed out
THE RECOVERY OF JOK-1 AFTER REMOVAL OF DMSO

Figure 5
Figure 6
The growth of JOK-1 after a 24 hr exposure to TPA. Cells which were exposed to $10^{-7}$ ng/ml of TPA, were subsequently washed 3 x with PBS and placed into fresh complete media. Cells were then allowed to attach for 24 hrs, after which they were divided into adherent and nonadherent groups and reseeded in fresh media. Neither group entered logarithmic growth although viability was above 85% in this particular experiment.

Control cells
Cells which were only exposed to TPA for 30 min

Adherent population

+ Non-adherent population
Figure 6
Figure 7

JOK-1 cells as they appear in continuous cell culture.

A) Control JOK-1 cells 3 days after being freshly seeded in complete media (640 X).

B) Control JOK-1 cells 5 days after being freshly seeded in complete media. These cells appear to be somewhat smaller. Many of the cells have a more eccentric nucleus when compared to the cells seen in A (640 X).
Figure 8

JOK-1 cells before and after a 3 day exposure to DMSO.

A) Control JOK-1 cells (640 X).

B) JOK-1 cells after 3 days of continuous DMSO exposure. Note the size difference within the group as well as the different nuclear configurations (640 X).
Figure 8
Figure 9

JOK-1 cells after 5 and 7 days of continuous DMSO exposure.

A) JOK-1 cells 5 days after the addition of DMSO (640 X).

B) Cells 7 days after DMSO induction (640 X).
Figure 10

The effect of a 5 day exposure to DMSO and HMBA on JOK-1

A) JOK-1 cells treated with DMSO for 5 days (640 X).

B) HMBA (5 mM) for 5 days (640 X).
Figure 11

Control and 3 day DMSO treated JOK-1 cells.

A) JOK-1 cells as they normally appear. These particular cells have a convoluted nucleus (1600 X).

B) Cells that have been exposed to DMSO for 3 days. They are heterogeneous with respect to size. Prominent nucleoli are present (1600 X).
Figure 12

Control and 3 day DMSO treated JOK-1 cells.

A) Control cells (1600 X)

B) Another form of 3 day DMSO treated cells. Note the configuration of the nucleus in the center cell. Also note the size difference (1600 X)
Figure 13

Phase contrast microscopy of JOK-1 and DMSO treated cells.

A) Control JOK-1 cells (120 X)

B) Cells which have been treated with DMSO are more clumped (240 X)
Figure 13
Figure 14

TPA induced JOK-1 adherence.

A) JOK-1 cells after 48 hrs of exposure to $10^{-7}$ ng/ml of TPA (120 X)

B) As viewed at a higher magnification (240 X)
Figure 15

The effect of the combination of DMSO and TPA on JOK-1.

A) JOK-1 cells after 48 hours of exposure to $10^{-7}$ ng/ml of TPA in combination with 2% DMSO (120 X).

B) As viewed at a higher magnification (240 X)

In both photographs, note the extensive dendritic-like processes. These processes are similar to those of neurons.
Figure 16

Wright-Geimsa stain of TPA cells grown in a Lab-Teck chamber (250 X).
Figure 17

SEM appearance of JOK-1.

A) Based on the microvillar projections and an area of ruffled membranes, this cell is consistent to that of a monocytoid hairy cell (7600 X).

B) SEM appearance of JOK-1 cells 3 days after the addition of DMSO (5950 X)

1) this particular cell has a ruffled membrane with ridge-like process; may be considered a monocytoid hairy cell with more pronounced features than the one in A.

2 and 3) these cells resemble lymphocytes in that they appear to have a variable number of microvilli, however, 2) may be consistent with that of a lymphcytoid hairy cell.
Figure 18

SEM appearance of JOK-1 after DMSO and TPA treatment.

A) Another view of JOK-1 after 3 days of DMSO treatment. This cell is characterized by long spindly microvillar projections with some ridge-like projections and is consistent to that of a lymphocyte-like hairy cell (4220 X).

B) The cells were treated with the combination of TPA $10^{-7}$ ng/ml and 2% DMSO. The network of cell processes are readily apparent (1170 X).
Figure 19

SEM appearance of JOK-1.

A) Higher magnification of Figure 17B, the cells appear as lymphocytoid hairy cells (1650).

B) The effects of TPA alone. The cells have long microvillar processes. The cell processes are broader at the base of the cell as compared to the combined DMSO and TPA effect (2370 X).
Figure 20

Transmission electron microscopic depiction of the untreated JOK-1 cell. There is a paucity of mitochondria and endoplasmic reticulum.
Figure 20
Figure 21

Transmission electron microscopic depiction of the JOK-1 cell treated with DMSO for 7 days. There is an increase in mitochondria and endoplasmic reticulum. The nucleus is more eccentric in location.
Figure 22
JOK-1 cells that have been treated with TPA for 3 days. The arrow depicts an annulate lamellae. The function of this organelle is unknown. One investigator has stated that the only blood cell in which he has seen this structure is the proerythroblast. There is an increase in mitochondria and endoplasmic reticulum. The nucleus is indented and is in an extended configuration.
Figure 22
Figure 23

Another form of the JOK-1 cell that has been treated with TPA for 3 days. There are more microvillar processes. An increase in mitochondria and endoplasmic reticulum can be seen in this cell as well.
Figure 23
Figure 24

The purpose of A and B as well as Figure 24 is to illustrate the difference in nuclear configuration after either DMSO or TPA treatment.

A) Control cell (6150 X)

B) 3 day DMSO treatment. Three prominent nucleoli are present. Note the very unique and interesting appearance of the nucleus (6150 X).
Figure 25

3 day TPA treated cell. The nucleus appears to be segmented as in neutrophils (4950 X).
Figure 26

Effects of TPA and Me₂SO on DNA supercoiling. Panels A and B are two separate experiments.

Control
DMSO
TPA
EFFECTS TPA AND Me$_2$SO ON DNA SUPERCOILING

Figure 26
Figure 27

The comparison of cell cycle kinetics in the control DMSO and TPA treated cells after 3 days of treatment. A slight shift to the left on the Y axis can be detected by TPA treated cells.
Figure 28

The comparison of cell cycle kinetics in the control, DMSO and TPA cells after 5 days of treatment. In the experimental cells, there is a decrease in red fluorescence which is depicted on the Y axis.
Figure 28
Figure 29

Comparison of cell cycle kinetics in the control, DMSO and TPA treated cells after 7 days of treatment. The DMSO treated cells have now shifted back to the right on the Y axis, indicating an increase in red fluorescence. The TPA treated cells exhibit a broad range on the Y axis.
Figure 29
Figure 30

Simultaneous analysis of DNA and protein content in control JOK-1 cells. DNA is a function of red fluorescence, green is a measure of protein. Relative cell size can be determined by the blue scatter.
Figure 30
Figure 31

The simultaneous analysis of DNA and protein content in cells treated with DMSO for 5 days. DNA is a function of red fluorescence, while green is a measure of protein. Relative cell size can be determined by the blue scatter. As can be seen DMSO treated cells, as measured by relative size, is larger than control cells.
Figure 31
Simultaneous analysis of DNA and protein content in cells treated with TPA for 5 days. DNA is a function of red fluorescence, while green is a measure of protein. As indicated by blue scatter, TPA treated cells are larger than control cells.
Figure 32

Graphs showing data distribution for different regions.
Figure 33

The relationship of sIgM expression to the cell cycle after 3 days of treatment with DMSO and TPA.

A. Control

B. DMSO

C. TPA
Figure 33
Figure 34

 Autoradiograph illustrating the failure to induce the expression of cAMP dependent protein Kinase I after the induction of JOK-1 with DMSO (2%). TPA (10^{-7} ng/ml) or the combination of the two.

Lanes:

A. SAB cell line

B. Control cells 3 days

C. cAMP only

D. DMSO treated cells 3 days

E. TPA treated cells 3 days

F. The combination of DMSO and TPA 3 days

G. Control cells 7 days

H. cAMP only

I. DMSO treated cells 7 days

J. TPA treated cells 7 days

K. The combination of DMSO and TPA 7 days

L. A positive control - the cell line RAW 264.7
Figure 35

JOK-1 karyotype: 48, X, Y, -3, +3q-, +3p-, 4q+, -6, 7q-, -8, +20, -22, +2 mar
Sex Chromosomes

Figure 35
Figure 36

JOK-1 karyotype: 47, X,-Y, 4q+, -6, 7q-, -8, +3 mar
**Figure 36**

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Figure 37

JOK-1 karyotype: 49, X, Y, 4q+, -6, -8, 14q+, +20, +4 mar
Sex Chromosomes

Figure 37
Figure 38

JOK-1 karyotype: 49, X, Y, 4q+, -5, -6, 7q-, -8, +20, +4 mar
Figure 38
Figure 39

JOK-1 karyotype: 46, X, Y, -3, 4q+, -6, 7q-, -12, -18, -18, +20, +4 mar
Figure 39

Sex Chromosomes

X  Y
CHAPTER IV

THE GROWTH AND PROPAGATION OF THE JOK-1 HAIRY CELL LEUKEMIA CELL LINE IN THE NUDE MOUSE AND ITS RESPONSE TO CHEMICAL INDUCERS OF DIFFERENTIATION
Introduction

The growth and serial propagation of both cultured and fresh human lymphoid neoplasms in the nude mouse has proven to be the most difficult of all the human tumors (1,2,3). This difficulty has hampered the use of the nude mouse as an in vivo tool in which to assess the potential clinical value of chemotherapeutic agents for the treatment of leukemia and lymphoma, an approach which has proven to be invaluable for the screening of drugs for use in the clinical treatment of solid tumors.

Investigators have suggested that natural killer (NK) cells and thymus-independent lymphocytic effector cells play a major role in the rejection of transplanted leukemia/lymphoma cells (2,4). Accordingly, experiments have been carried out in an attempt to circumvent this problem (2,3,5,6,7).

Watanabe et al (2) conditioned adult nude mice with 500 rads of whole body irradiation. After which, both the unirradiated and irradiated mice received an i.v. inoculation of human acute lymphocytic leukemia cells which had been maintained and propagated in the peritoneal cavity of nude mice. In the unirradiated mice, the inoculated leukemia cells completely disappeared in 2 weeks, while tumor cells in the irradiated recipients were observed in the peripheral blood, spleen, lymph node, bone marrow and meninges. This distribution persisted until the mice died approximately 4 weeks after leukemia cell inoculation. The cause of death was due to cachexia, which could probably be attributed to the visceral leukemic
infiltration. These investigators were of the opinion that the sublethal dose of radiation, administered to the experimental mice, reduced the non-T cell mediated immune response to a level that allowed proliferation of the neoplastic cells. They also suggested that radiation damage to the spleen and bone marrow could also provide a less hostile environment in which the cells could settle and begin to proliferate (2).

In a more recent study, Watanabe et al (3) studied the effects of splenectomy and the combination of splenectomy and whole body irradiation on the heterotransplantation of human lymphoid cell lines and fresh specimens of leukemia and lymphoma cells. In an extension of their earlier work, they were able to initiate leukemic cell growth after whole body irradiation, using two T-cell and one B-cell acute lymphocytic leukemia cell lines, as well as the Burkitt-derived Raji cell line and a spontaneously transformed lymphoblastoid cell line. Four cell lines, two established from Epstein-Barr virus (EBV) transformed cord blood cells, a null cell acute lymphocytic leukemia cell line and a line derived from a nodular lymphoma, were only able to grow after the combination treatment of splenectomy and whole body irradiation. Despite the initial success in the growth of these tumors, only limited success was achieved in attempts to serially transplant these tumors. The EBV lymphoblastoid cell lines underwent spontaneous regression after reaching a diameter of 2.5 cm (3). Heterotransplantation experiments conducted with fresh human tumors only had a 29% (7 of 24 cases) acceptance rate, and of these only two could be serially transplanted (3). The cells that did grow in the
nude mice from both cultured and fresh specimens retained their original morphology (3). These investigators noted that the route of transplantation was important in influencing the acceptance of the leukemia cells (3). Their preliminary data indicated the numbers of recoverable cells were greater from tumors grown subcutaneously rather than intracranially. It also was observed that subcutaneous (s.c.) injection of leukemia cells was more effective than either the i.v. or i.p. route. This finding was attributed to the fact that the subcutaneous tissue probably was better vascularized and that the connective tissue or stroma perhaps could prevent tumor cell migration while serving as a framework for cell adherence and proliferation (3). It was further hypothesized that the i.v. or i.p. route might more quickly expose the lymphoid tumor cells to the host defense systems by rapidly activating macrophages and other effector cells (3).

Schaadt et al (6) found that cells from lymphomas, leukemias and lymphoblastoid cell lines could be grown in the intracranial vault of the nude mouse. It was observed that cells from established cell lines had a greater percentage of tumor formation in the brain than did those from primary tumors. A surprising feature of this study was the documentation that normal diploid Epstein-Barr virus transformed lymphocytes were able to grow as well in the brain as did cultured leukemia and lymphoma cells.

The Lozzio's and their colleagues have obtained considerable experience in the growth and serial propagation of human leukemia cells in the nude mouse (5,8,9,10). However, the investigations have largely
been confined to the heterotransplantation of the human myelogenous leukemia cell line, K-562.

One of their early reports detailed the biological characteristics of the initial and subsequent serial growth pattern of K-562 cells transplanted into 1,093 nude mice (5). Growth was initiated by transplanting $5 \times 10^6$ K-562 cells together with a human fibrin clot (5). Previous experiments had demonstrated that the use of the fibrin clot gave greater than a 95% acceptance rate, as compared to a 40-50% rate when only cells were inoculated s.c. (5). They were able to divide the s.c. growth of the K-562 cells into three stages. The first stage, defined as the latent period, was characterized as the time from transplantation to initial signs of growth. During this time period, vascularization of the tumor bed had begun and a rudimentary fibrillar framework was beginning to separate groups of tumor cells in alveolated spaces. This phase varied from passage to passage, but usually consisted from time zero to day 5-7 (5).

The proliferative period, or the second stage, was characterized by numerous tumor cell mitotic figures and rich vascularization. Reticular cells formed a fiber network that supported the growth of the leukemia cells and allowed them to grow as solid tumor cords. Eventually the cells became arranged in nodules surrounded by a delicate reticulin fiber network. At the end of the proliferative period, the tumor had become a large, compact mass composed of pleomorphic neoplastic cells. An acute inflammatory reaction or host lymphocytes in the tumor stroma was never observed (5).
The third stage, or the stable period, was characterized by the beginning of hemorrhage and ischemic necrosis within the tumors. Associated with the vascular changes, was scattered areas of hemorrhage and ischemic necrosis. It was observed that after 40 days, most tumors had extensive areas of necrosis interspersed with groups of seemingly viable neoplastic cells. Spontaneous regression was never observed and the mice usually died 42 days after transplantation from cachexia (5).

Histopathological studies of tumors that had been serially transplanted revealed that at the earliest stage of growth, central areas of necrosis were present. Nevertheless, the tumors grew progressively and were identical to the ones in the primary passage. Autopsy studies revealed no signs of metastases (5,8).

It was noted that the high number of nearly triploid cells in the primary inoculum appeared to be essential for K-562 proliferation in the nude mouse. This was the result of an apparent in vivo clonal selection process, because more than twice the number of triploid cells was found in the primary tumor as compared to the cultured cells. This triploid state was probably also responsible for the successful serial passages (8). The fibrin clot contributed to successful tumor growth by not only serving as a nutrient rich matrix, but also by preventing cell migration and stimulating leukemic cell proliferation by the release of thrombin (5,8).

The importance of the fibrovascular stroma was acknowledged as providing support for the growth of new capillaries, which in turn provided a blood supply and nutrients to the growing tumor (8).
Lozzio et al (8) suggested that the strain background of the mice carrying the nu gene might also be important in determining the success of in vivo leukemic cell growth. It was found that tumor cells inoculated in the NIH-Swiss nude mice had a longer latent period and smaller tumors as compared to an identical inoculum in the BALB/c nude mice.

As previously discussed in the background section, metastases from transplanted human tumors in the nude mouse are infrequent (1). After three years of work, Lozzio et al (8) managed to get the K-562 tumor to metastasize if $5 \times 10^6$ cultured cells were injected s.c. into one day old nude mice. Metastases were found in the lungs, kidneys, lymph nodes and brain. The incidence of metastases was 61%, and appeared as early as 17 days and as late as 84 days of age. This could not be reproduced in the adult nude mouse (8).

Interestingly enough, tumors only occurred at the s.c. injection site in approximately one-third (27%) of the newborn mice, while metastases were noted in 61%. In mice where s.c. tumors were observed, they were preceded by a long lag period as compared to short lag periods in the adults where the K-562 cells were injected with a fibrin clot. Histopathological examination showed that the metastases from the s.c. injection of K-562 cells in the newborn nude were identical to the tumor that arose in the adult after inoculation with K-562 cells and the fibrin clot (8).

The extensive knowledge that has been gained in working with the nude mouse and the K-562 cell line has allowed the Lozzios to use this model to assess various therapeutic agents (8).
In one such study, monkey and goat anti-K-562 sera were used to treat nude mice that carried tumors in the proliferative stage of human leukemic cell growth (9). The antibody was administered i.p. every 3 days for 19 days. It was observed that the anti-sera not only suppressed the growth of the K-562 tumors, but also the growth of HL-60, KG-1, JM, MOLT-4 and RAJI cell lines as well (9). These tumors also were reactive in vitro with the K-562 anti-sera (9). The anti-sera were non-toxic to the animals and did not suppress the growth of transplanted human lung, breast and prostate tumors, which had been propagated in the nude mouse. The mechanism of action, according to Lozzio et al (9), was leukemia cell specific, and probably due to direct antibody cytolysis, as well as antibody dependent macrophage-mediated cytotoxicity. It was found that cytosine arabinoside, daunomycin, vincristine, adriamycin, 5-azacytidine and methotrexate were not effective in treating the K-562 tumor at non-toxic doses. It is noteworthy that these drugs also are ineffective clinically against chronic myelogenous leukemia in terminal blast crisis, the stage from which the K-562 cell line was derived (8).

Machado et al (11) has documented that some hemopoietic cell lines have the capability to undergo differentiation after propagation in the nude mouse. This induced differentiation was probably due to host environmental factors (11). Thus, when one considers using the nude mouse as a vehicle in which to study in vivo induced differentiation of human tumors, the possible biological changes that may be host mediated, should not be confused with changes brought about by inducers of differentiation.
A recent publication has discussed a method that enhanced the acceptance of a transplanted leukemic cell line in the nude mouse (7). Ziegler and his colleagues (7) have used x-irradiated HT-1080 fibrosarcoma cells to promote the growth of the MOLT-4 cell line in the nude mouse. Prior to inoculation, mice were conditioned for 3 weeks with 2000 cGy of whole body irradiation. Under these conditions, 19 of 25 mice developed tumors, while in the control group no tumor growth was observed. It was hypothesized that irradiation reduced the thymus-independent immune response, and the HT-1080 cells produced a factor that promoted neo-vascularization, thus providing optimal conditions for tumor growth (7).

We have utilized the technique described by Ziegler et al (7) to grow a hairy cell leukemia cell line, JOK-1 (12), in the nude mouse for use as a model for inductive chemotherapy. This cell line which has been described in Chapter II, was established from a patient, diagnosed as having hairy cell leukemia, and has been termed a pre-hairy cell leukemia cell line (12). The chemical inducers of differentiation, dimethylsulfoxide (DMSO) and hexamethylene bisacetamide (HMBA) were administered in vivo after the JOK-1 cell line had been successfully propagated for more than three generations. Results of these experiments are given and discussed within this paper.
Materials and Methods

**Mice.** BALB/c nude mice of both sexes were maintained in the nude mouse facility, depicted in Figure 1, of the Battelle Memorial Institute. The facility has been fully accredited by the American Association for the Accreditation of Laboratory Animal Care and adheres to the policy stated by the Department of Health and Human Services on the care of laboratory animals. Mice used were housed in sterile cages on shelves in a room that had 16 complete air changes per hour. Mice were handled only if the worker had on a sterile gown, gloves, and outfitted with disposable masks, hats and footwear.

**Leukemic Cells.** The JOK-1 cell line, derived from a patient with hairy cell leukemia (12), was maintained in suspension culture with a media change every 2-3 days. Cells that were to be inoculated into nude mice were in the early to mid-logarithmic stage of growth.

The JOK-1 cells were either inoculated s.c. in the dorsal region of the mouse, alone at a concentration of $5 \times 10^6$ cells in a volume of 200 l or in combination with $5 \times 10^6$ irradiated (6000 cGy), HT-1080 fibrosarcoma cells. In both cases the contralateral side was injected as well. In some experiments, $2 \times 10^6$ JOK-1 cells were injected in a volume of 200 l i.v. or at a concentration of $5 \times 10^6$ cells i.p.

**Animal Preparation and Tumor Measurement.** Prior to leukemic cell inoculation, mice were conditioned by exposing them to 200 cGy of radiation one time per week for 3 weeks. The mice were irradiated at
the rate of 100 cGy/min in sterile 50 ml centrifuge tubes with perforated air holes. Mice were transported in sterile cages with filter tops to the X-ray machine, and were handled with sterile gloves. During the course of irradiation, the drinking water was supplemented with antibiotics.

Tumor size was measured by calipers 2-3 times per week and tumor weight was calculated using the formula \( \frac{w^2 \times l}{2} \) (w=width, l=length).

The relative increase in size was determined by dividing the tumor weight in milligrams by the initial weight.

**Tumor Transplantation and Serial Propagation.** When the tumor mass was in the logarithmic stage of growth, mice were sacrificed and 1 mm fragments of the JOK-1 tumor were inoculated s.c. in both the left and right dorsal regions of unirradiated nude mice without HT-1080 fibrosarcoma cells.

**In Vitro Growth of Tumor Cell Derived JOK-1 Cells.** JOK-1 tumors were removed from mice that had been sacrificed, and were aseptically teased apart with 18g needles in RPMI 1640 media supplemented with 5% fetal bovine serum (FBS) and 10,000 units/ml penicillin and 10,000 mcg/ml streptomycin (complete media). This was done in a sterile 60 mm culture dish (Corning #25011). Without further manipulation, cells were placed in a 37°C humidified incubator with 5% CO\(_2\) and 95% air. The culture dish was examined daily for signs of cell growth.

Once a cell line was established, the following characteristics and parameters were examined; cell growth, cell surface markers,
response to DMSO and TPA, cytochemistry and the chromosome number.
These procedures are discussed in the materials and methods section of
Chapter III, with the only exception being the enumeration of
chromosome number.

Chromosome Enumeration. Cells that grew from the JOK-1 s.c. tumor were
seeded during logarithmic growth into a 25 cm flask (Corning #25100) at
a concentration of $3-5 \times 10^5$ cells/ml in a total volume of 20 ml of
complete media; 500 μl of colcemid (Gibco #120-5210) were added, and
the cells were then incubated at 37°C in a humidified incubator at 5%
CO$_2$ and 95% air for 30 min. At the end of the 30 min incubation
period, the cells were centrifuged at 2000 rpm for 10 min and the cell
pellet was resuspended in 3 ml of warm (37°C) 1% sodium citrate. This
was allowed to stand at 37°C for 20 min, then without disturbing the
pellet the supernatant was carefully removed and 2 to 3 ml of
acetic-methanol fixative (1 part glacial acetic acid – 3 parts absolute
methanol) were added. After standing at room temperature for 10 min,
the pellet was resuspended in the fixative, centrifuged, and the
fixative process was repeated. Again after standing at room
temperature for 10 min, the pellet was resuspended and centrifuged. A
volume of 250 μl of the fixative was used to resuspend the pellet. A
Pasteur pipet was used to drop 2-3 drops of the cell suspension on
slides held at a 45° angle approximately 2 ft below the pipet. The
slides were immediately passed through an open flame and after drying
were stained with Geimsa for 20 min. Prior to use, the slides were
washed with Cytoclean® and rinsed with distilled H₂O and placed at 4°C in 70% ethanol alcohol.

Administration of DMSO and HMBA. DMSO was administered either parenterally by i.p. surgical implantation of ALZET® osmotic pumps (#2002), seen in Figure 2, or orally via the drinking water. The osmotic pumps are miniature self-powered pumps that continuously deliver a drug at a closely controlled rate. Once implanted, the pumps delivered a volume of 2% DMSO at 0.5 μl/hr for a period of two weeks. The implantation of three pumps was necessary for the continuous delivery of the desired concentration of DMSO. Control mice received three pumps that contained saline. As stated above, in other experiments, mice received 2% DMSO via their drinking water.

HMBA was administered i.p. every 8 hrs for 9 days at the dose of either 400 or 800 mg/kg.

Natural Killer Cell Assay Measured by the Release of ⁵¹Cr. Thirty days after the beginning of HMBA treatment, mice were sacrificed by cervical dislocation. Peritoneal exudate cells were obtained by the lavage of the abdominal cavity with 5 ml of phosphate buffered saline (PBS). First the skin was flooded with 70% ethanol alcohol, PBS was then carefully injected into the abdomen by a 20g needle. The abdomen was carefully massaged with the fingers, then with sterile scissors and forceps, the skin was reflected back to expose the peritoneal wall. A slit was then made in the wall and lifted up with forceps. The PBS was then removed by a sterile Pasteur pipet and placed in a sterile 50 ml
centrifuge tube. Cells from 5 animals were pooled and washed 3x with PBS.

The spleen was aseptically removed and placed in PBS. Mononuclear cells were obtained by placing the spleen on the rough side of a frosted glass slide and making circular motions with the rough edge of another frosted glass slide on top. The cells were collected in PBS. Red blood cells were lysed with tris-buffered ammonium chloride, pH 7.2, by resuspending the splenic cells in 2 ml of the Tris-NH₄Cl and holding at room temperature for 2 mins. The cells then were washed 3x with PBS.

Bone marrow cells were obtained by removing the muscle from the femur and tibia. The epiphysis then was removed by scissors and the marrow flushed out with PBS by a 25 gauge needle attached to a 5 ml syringe into a sterile 35 mm culture dish (Falcon #1008). The cells were washed 3x with PBS and resuspended in complete media.

The YAC-1 cells (kindly provided by Dr. John Hughes, Children's Hospital, Columbus, Ohio), a murine lymphoma cell line, were used as target cells. Two million cells were labeled with 150 μl of ⁵¹Cr (New England Nuclear, Boston, Mass., specific activity 200-900 Ci/g) for 30-40 mins at 37°C in a humidified 5% CO₂ and 95% air incubator in complete media, after which the cells were washed 3x with PBS and resuspended to a concentration of 1 x 10⁶ cells/ml in complete media and incubated for an additional 30-40 min. The cells were washed 1x with PBS, resuspended and adjusted to a concentration of 1 x 10⁵ cells/ml in complete media; 100 μl of the target cell suspension was added to 96 well micro-liter plates (#3596 Costar, Cambridge, MA). In
separate plates, the splenic mononuclear cells, bone marrow cells, or the peritoneal exudate cells were added to triplicate samples of the target cells in a volume of 100 µl at effector-target cell ratios of 100:1, 50:1, 25:1, 10:1 and 5:1. The plate then was centrifuged at 600 rpm for 5 min and incubated for 12 hrs at 37°C in a humidified incubator with 5% CO₂ and 95% air. At the end of 12 hrs the plates were centrifuged at 2000 rpm for 10 min, 100 µl of the supernatant were placed in 12 x 75 mm test tubes, and counted in a Tracor Analytic 1185 gamma scintillation counter. Percent lysis was calculated using the formula:

\[
\frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100
\]

Maximum release of the target cells was determined by incubating them with 0.16 N hydrochloric acid for 12 hrs in conjunction with the NK assay. Similarly the spontaneous release was determined by incubating the target cells with complete media in the absence of HCl or effector cells.

Effect of HT-1080 Cells on JOK-1 In Vitro. HT-1080 cells were irradiated with 6000 cGy of radiation in the log and stationary phases of growth. Fresh complete media was added to JOK-1 cells at a concentration of 2.5 x 10⁵ cells/ml, and daily cell counts and viability determinations of JOK-1 growth were made. Viability was assessed by trypan blue exclusion. HT-1080 supernatant was removed from both unirradiated logarithmic and stationary cultures and centrifuged at 4000 rpm for 20 min. The supernatant was added to
freshly seeded JOK-1 cultures at the concentrations of 10 and 50%. Daily cell counts and viability determinations were made.

**Histopathology.** Tumors were removed, placed in 10% buffered formalin, processed for light microscopy and stained with hematoxylin and eosin by the Histology Section of The Ohio State University Department of Pathology. Organs from autopsied mice were similarly processed.

**Electron Microscopy.** Tumors were cut into small fragments and fixed with 2.5% glutaraldehyde in 0.05 mM sodium cacodylate and processed for electron microscopy by Mr. Terry McBride of the Department of Pathology.
Results

The Growth of JOK-1 in the Nude Mouse. The growth of JOK-1 in the radiation conditioned (200 cGy/week x3) nude mouse with and without the irradiated HT-1080 fibrosarcoma feeder cells is shown in Table 1 and Figure 3. As can be seen, there is a 7 day lag period in measurable tumor growth in the JOK-1 tumor which was implanted without the irradiated HT-1080 fibrosarcoma cells as compared to the tumor which was co-transplanted with HT-1080 cells. Each of the two groups consisted of 5 animals; in the group without the feeder cells, no tumor growth was observed in two of the animals and in one animal, there was a measurable tumor present on day 21, but it subsequently regressed by day 25. In the group with the HT-1080 feeder cells, all 5 animals had measurable tumors eleven days after tumor cell inoculation, but 3 died during the course of the experiment; one died by day 14, one by day 25 and the other by day 36. The histological appearance of the tumor from both groups are shown in Figure 4. There were no morphological differences between tumors from the two groups which histopathologically were undifferentiated lymphomas. Intravenous and i.p. injections of JOK-1 cells failed to produce liquid tumors.

On day 36, a tumor from a mouse in each group was surgically removed in a sterile fashion, cut into 1 mm fragments and transplanted into 2 groups of 4 irradiated mice (200 cGy/week x3). One group received tumor fragments from a mouse that was inoculated with JOK-1 cells alone, while the other from a mouse that received JOK-1 and HT-1080. Neither group received fresh feeder cells. The growth of
these two tumors are shown in Table 2 and Figure 5. There was no significant growth difference between the two groups, however, there was variation in tumor size within the 2 groups. In Table 2 it can be seen that once a tumor was established, it could grow just as well in unirradiated as in irradiated animals.

The gross appearance of the tumor, and as it appeared in the mouse is illustrated in Figures 6 and 7.

In subsequent passages, 1 mm fragments of the JOK-1 tumor was transplanted without HT-1080 feeder cells in the dorsal region of unirradiated mice. Fragments were implanted in the contralateral side as well.

Tumor Histology and Autopsy Findings. Histological examination of the kidneys, lungs, stomach, lymph nodes and spleen failed to reveal any evidence of JOK-1 metastases. Figures 8 and 9 depict the histopathological appearance of the JOK-1 tumor. The morphological appearance is compatible to that of an undifferentiated lymphoma. The necrosis seen in Figures 8 and 9 was widespread and present in all tumors examined. This necrosis could be grossly observed in the central area of the tumor, and in many animals would cause the skin to ulcerate and become necrotic. It could perhaps be argued that this necrosis might have been responsible for the marked variation seen in JOK-1 tumor size within a group of animals. Figures 10 and 11 are electron micrographs of the tumor, which also are compatible with undifferentiated lymphoma.
Figure 12 illustrates lymphocytic infiltration in the JOK-1 tumor, suggesting that there may have been a host inflammatory reaction against the tumor. Lozzio et al (8) has never observed host cell infiltration into the K-562 myelosarcoma.

Effects of HT-1080 Cells and Supernatant on the In Vitro Growth of JOK-1. Since it has been postulated that HT-1080 fibrosarcoma cells produced an angiogenesis factor that could possibly promote leukemic cell growth in vivo via neovascularization, studies were initiated to see what, if any, effect in vitro these cells exerted on JOK-1 cells. As can be seen from Figures 13 and 14 neither the HT-1080 supernatant at the concentration of 10 and 50% or the cells had any appreciable effect on JOK-1 growth. The viability of the JOK-1 cells, as assessed by trypan blue, was never below 90%.

Characteristics of Cells Derived from the JOK-1 Tumor. Cell lines were established from the 3rd and 7th passage of the JOK-1 tumor. In both cases, approximately 2 weeks had elapsed before cell growth was observed. Cells were expanded and characterized as to surface markers and growth characteristics. The cells were positive for 

\[ \text{slgM, B-1, J-5 (cALLa), Ia and S-HCL-1 and negative for S-HCL-3, an identical phenotype to the in vitro maintained JOK-1 cell line.} \]

The morphology also was similar. The growth of tumor cells derived JOK-1 cell line and its response to DMSO (2%) and TPA \((10^{-7} \text{ ng/ml})\) was similar to that of the JOK-1 cell line that had been propagated in vitro. The results of these experiments are depicted in Figures 15 and 16.
Karyotypic analysis of 4 metaphase spreads for the 3rd generation and 5 for the 7th generation, revealed between 46 and 48 chromosomes. This also was similar to that which was observed in vitro and consequently no further analysis was undertaken.

**Inductive Chemotherapy with 2% DMSO.** In the first experiment, 3 Alzet® osmotic pumps were implanted into individual mice in 2 groups; one group received saline and the other DMSO. Multiple pumps were necessary in order to achieve a constant infusion of 2% DMSO. It quickly became apparent that the mice were unable to physically tolerate the pumps. They became emaciated and several died. At the conclusion of the experiment, 3 mice were alive in the experimental group and 2 in the control. Due to the physical condition of the mice, no conclusions were drawn.

Tables 4, 5 and Figures 17, 18 summarize the results of the next 2 DMSO experiments. In these experiments, 2% DMSO was administered via the drinking water and the controls received ordinary water. In the second experiment, there were 4 mice in each group, each of which was followed for 43 days. The experimental group had DMSO in the water throughout this time period. As can be seen in Figure 17, there was a marked difference in tumor size between the experimental and control group. Examination of Table 3 shows that this growth inhibitory effect became apparent by day 33, at which time the mean value of the control tumor weight was \(160 \pm 11.0\) mg compared to \(130 \pm 25\) mg of the DMSO treated tumors. This difference became significant \((p<0.05)\) by day 39. However, there were marked variations in tumor weight within the 2
groups, with it being more pronounced in the control group. In the control group the standard error of the mean (S.E.M.) ranged from 10.0 mg on day 28 to 217.0 mg on day 71, while in the DMSO treated group, it ranged from 20.0 mg on day 28 to 44.0 mg on day 43. Toward the latter stages of the experiment, gross central tumor necrosis was observed in both groups of animals.

The third DMSO experiment had 5 mice in each of the control and experimental groups. This experiment lasted for 36 days, with the experimental animals getting DMSO in their drinking water throughout the course of the experiment. As depicted in Figure 18, the differences between the two groups were not as pronounced as in the previous experiment, but nevertheless, by day 60 a difference was observed. At this point, the mean value of tumor weight (Table 5) in the control was 190 ± 84 mg as compared to 140 ± 58 mg in the experimental group. By day 71, the control weight was 168 ± 93 mg while the mean value of the DMSO treated tumor was 49 ± 37 mg. As in the previous experiments, there were marked variations in tumor weight within the two groups, and due to this, there were no statistically significant differences between the two groups. Gross tumor necrosis also was observed.

The fourth DMSO experiment consisted of 100 mice and was inconclusive because the control tumors as a group displayed very little growth and the standard error of the mean in both groups was huge. The data are depicted in Tables 6 and 7. This particular experiment was conducted using third generation tumors, while tumors in
the second experiment were from the seventh, and the third experiment used tumors from the tenth passage.

Histopathological examination of the DMSO treated tumors revealed no signs of differentiation, but rather extensive areas of necrosis. These observations are illustrated in Figures 19-23.

**Inductive Chemotherapy with HMBA.** Hexamethylene bisacetamide (HMBA) at the concentrations of 400 mg/kg and 800 mg/kg was administered to tumor bearing nude mice, i.p., every 8 hrs for 9 days. The results are summarized in Table 8 and Figure 24. HMBA at the dose of 400 mg/kg had no effect, but at the concentration of 800 mg/kg there was a marked growth inhibitory effect. This was first seen on day 36. The mean value of the control tumor weight was 139 ± 59.0 mg and that of the tumor was 95 ± 20.0 mg. However, due to the large S.E.M., there was no statistically significant differences between the control tumors and those in mice treated with 800 mg/kg of HMBA. Gross tumor necrosis was observed in all 3 groups. The tumors which were used in this experiment were from the fourth JOK-1 passage.

**Nude Mouse Cytolytic Activity.** Initially, mice from the HMBA experiment were examined for splenic NK activity and peritoneal cell mediated cytolysis. These results can be seen in Table 9. Comparing control mice (tumor-bearing) to normal nude mice in Table 9, one can observe that there is a decrease in NK activity at the effector to target cell ratio of 10:1 and 100:1. There was, however, a modest improvement at the ratio of 100:1 in the HMBA treated group as compared
to the control group. Cells isolated from the peritoneal lavage also had an increase in cytolytic activity as compared to both the normal and control mice. Since the spontaneous release of $^{51}$Cr in these experiments was 30%, caution needs to be exercised in the interpretation of these data.

To further clarify these results, similar cytolytic experiments were performed on other mice of the same age, and the mice with tumors were comparable to the tumor bearing mice of Table 9. Table 10 summarizes the NK values found in 6 nude mice carrying the JOK-1 tumors. As can be seen, the mean value of NK activity at an effector to target cell ratio of 10:1 was 14 ± 5.0 and at a 100:1 ratio it was 64 ± 6.0. These were similar to the values found in the tumor bearing mice of Table 9, 10 ± 7.0 and 53 ± 2.0 respectively. The mean value of NK cytolysis in tumor bearing mice in nos. 4-7 of Table 11 have similar levels as well. Mice 1-3 of Table 11 were normal, and their splenic NK activity was 37 ± 17.0 at 10:1 and 79 ± 6.0 at 100:1, values which were close to the ones observed in the normal mice of Table 9, 41 ± 19 and 87 ± 3.5 respectively. The ability of peritoneal exudate cells, unstimulated and stimulated with 10% peptone also were investigated, and the results are shown in Table 11. In this particular experiment, the unstimulated and peptone stimulated cells from the tumor bearing mice had an increase in cytolytic activity as compared to the normal non-tumor bearing mice. To see what effect DMSO had on splenic NK cell activity, mice were given 2% DMSO in their drinking water for 16 days. The results are depicted in Table 12. DMSO had no effect, although
there was a slight decrease in activity in the DMSO group at the ratio of 50:1 and 100:1.
Discussion

One of the greatest obstacles facing investigators who wish to use the nude mouse as an in vivo model in the development and testing of drugs for clinical use against leukemia, has been the inability to consistently grow and propagate malignant human hemopoietic cells in the animal. This difficulty has been attributed to the destruction of the leukemia cells by NK cells, a lack of stromal support that could provide nutrients and prevent cell migration, and an inadequate blood supply.

Recently, investigators have focused their attention on ways that would allow consistent and reproducible leukemic growth in the nude mouse. We have taken advantage of a method reported by Ziegler et al (7) to establish the growth and subsequent serial passages of the JOK-1 hairy cell leukemia derived cell line. Growth was initiated in the nude mouse, after whole body irradiation of 200 cGy one time per week for three weeks, by the co-inoculation of $5 \times 10^6$ JOK-1 cells and $5 \times 10^6$ irradiated HT-1080 fibrosarcoma cells into the subcutaneous tissue of the dorsal region. Although it remains to be firmly established, the HT-1080 cells have been hypothesized to increase the incidence of leukemic growth by the secretion of an angiogenesis factor that could promote neovascularization of the tumor (7). These feeder cells also could promote tumor growth by the production of factors, as well as by the prevention of leukemic cell migration, with the latter decreasing the probability of leukemic cell destruction by NK cells and allowing
the maintenance of the critical mass needed for continued cell proliferation.

In our study, we found that the HT-1080 cells not only increased tumor incidence, but decreased the time it took for the tumors to become palpable. In 5 irradiated mice that were inoculated with both JOK-1 and HT-1080 cells, tumor growth was observed by day 11, whereas in another group of 5 irradiated mice that only received the JOK-1 cells, growth was observed only in two mice by day 18. These observations are similar to those made by Ziegler et al (7) who used the MOLT-4 T cell leukemia cell line. Lozzio et al (8) made a similar observation when nude mice were co-inoculated with K-562 cells and bone marrow derived fibroblasts. It was found that the fibroblastic like cells stimulated K-562 tumor growth thus allowing for a larger tumor. However, in our case, HT-1080 cells only increased the tumor incidence and decreased the lag time from the time of inoculation to the time the tumors became palpable. An increase in eventual tumor size was not observed. When compared to the mice that received only JOK-1, there was no significant size differences noted after day 14 (Table 1). We also found that once the JOK-1 tumor was established, it could be passed into unirradiated mice without the HT-1080 cells. This finding is particularly important in that it reduces the variables that must be considered in the interpretation of the data.

Lozzio et al (5,8) have used a fibrin clot to establish K-562 growth in both the nude and lasat mice. These workers found that this method produced tumors in greater than 95% of the mice, whereas only 40-50% of the mice developed tumors when K-562 cells were inoculated
alone. Similar to what has been postulated for the HT-1080 cells, the fibrin clot is believed to promote leukemic cell growth by providing nutrients and growth factors, as well as preventing the random migration of the K-562 cells (5,8).

More recently, investigators have been able to grow the HL-60 promyelocytic cell line in the nude mice by pretreatment with cyclophosphamide (14). They did not however state whether or not this tumor could be serially propagated, a condition which is essential for the development of the nude mouse as model for the treatment of human leukemia. We were unable, however, to obtain consistent JOK-1 tumor growth by the pretreatment with cyclophosphamide.

The JOK-1 hairy cell leukemia cells grew as a non-invasive subcutaneous tumor and on gross examination, appeared ovoid in shape, fleshy, white in color and firm. This is a significant observation, for many leukemias and lymphomas that have been transplanted s.c. in the nude mouse eventually have appeared as a mass characterized by a soft consistency (3) and subsequently yielded very little interpretable data. These observations, coupled with the histopathological appearance, allowed us to state with confidence that we had established a viable lymphoid tumor and had not invoked a focal inflammatory reaction.

Frequently at the end of logarithmic growth, the JOK-1 tumor began to develop central necrosis that extended from the tumor core to the overlying skin. As a function of time, the necrosis progressed to the point that many of the tumors regressed and were no longer palpable. This process could perhaps be contributed to the fact that the tumor
outgrew its blood supply. Lozzio et al (8) have made a similar observation. In his K-562 tumor model, it was observed that 30 days after transplantation, necrosis was present in large portions of the tumor. They too attributed this to the tumor outgrowing its vascular network. Major problems that were encountered in our JOK-1 tumor model were tremendous variation in tumor weight within a group of mice, and the eventual spontaneous tumor regression; all of which could be partially explained by the extensive necrosis. Another factor that could have played a role was the presence of the Epstein-Barr nuclear antigen (EBNA). It has been reported that a transplanted B cell line that was positive for the Epstein-Barr virus underwent spontaneous regression after reaching a size of 2.5 cm (3).

Both light and electron microscopic examination revealed the JOK-1 tumor to be consistent with that of an undifferentiated lymphocytic lymphoma. Since the JOK-1 cell, by virtue of the expression of cALLa and sIgM with no associated light chain, can be considered an immature B cell, this interpretation is not surprising. Microscopically, the tumor was composed of a monomorphic population of cells that were similar in size, but varied in shape. Nuclei were large, cytoplasm sparse and mitotic figures were frequently seen. There was no evidence of HT-1080 fibrosarcoma cell proliferation. As demonstrated by electron microscopy, the tumor cells were similar to the in vitro JOK-1 cells.

Autopsy examination of the tumor bearing mice revealed no overt signs of metastases either in the peripheral blood or in the lungs, spleen, lymph nodes, kidneys, liver, stomach and pancreas. This
finding was not surprising since it has been reported that the K-562 leukemia cells can metastasize and appear in the blood only if they are injected subcutaneously into newborn nude mice (8). It could be hypothesized that in the adult nude mouse, leukemia cells that are injected either i.p. or i.v. are more accessible to the thymic independent immune effector cells and macrophages. Although the same is probably true in the newborn, the immune system may not be totally effective.

Cells that grew from the JOK-1 tumor were similar to the JOK-1 cells that had been maintained in vitro, and there were no changes to indicate that cells had been modulated by the host's environment. This possibility should always be taken into consideration in reviewing the data, especially if the purpose of an experiment is to induce cellular changes with exogenous agents. Machado et al (11) have provided evidence that suggests that the myeloid leukemia cell line subclone, KG-1a, can undergo differentiation in response to the microenvironment encountered in the nude mouse. Potter et al (13) also have reported that the HL-60 myeloid leukemia may undergo monocytic differentiation if allowed to proliferate in the nude mouse. However, Lozzio et al (8) have found no evidence to suggest that the K-562 tumor underwent spontaneous in vivo differentiation.

Analysis of the chromosome number showed that the JOK-1 tumor had a range of 46-48 chromosomes, a finding characteristic of the continuous in vitro cell line. Lozzio et al (8) found that the K-562 leukemia cells, many of which are near triploid in vitro, have a better in vivo growth rate if the triploid cells are clonally selected during
tumor proliferation. He went as far as to suggest that the number of triploid cells in the initial cell inoculum determined whether or not a tumor would be formed. It was postulated that a tumor with such a make-up could better adjust to the metabolism of the nude mouse. Other investigators have observed as well, that only hemopoietic cell lines that were aneuploid could grow in the nude mouse (3). Based on our data, it can be postulated that the variable growth rate and spontaneous regression of the JOK-1 tumor could have been due to a near normal diploid state.

In contrast to the in vivo situation, the irradiated HT-1080 cells or their supernatant did not enhance or inhibit the in vitro growth of the JOK-1 cells. If in fact, the mechanism of action of the fibrosarcoma cells was the production of an angiogenesis factor, then its failure to enhance in vitro growth is not surprising. Also our in vivo data suggests that these cells increase the tumor incidence in mice, not tumor size. In a similar situation, Lozzio et al (8) noted an opposite effect. He observed that fibroblasts isolated from normal bone marrow enhanced in vivo K-562 growth but inhibited in vitro growth.

When the JOK-1 tumor reached approximately 100 mg and had undergone at least two consecutive passages, mice were given either DMSO (14) or HMBA (15). In our first DMSO experiment, three osmotic pumps were placed in the peritoneal cavity of each mouse. One group received 2% DMSO, and the other saline. Three pumps had to be implanted in order to achieve a continuous infusion equivalent to 2% DMSO administered via drinking water. During the course of the
experiment it became apparent that the mice could not tolerate multiple pumps. The animals became emaciated and several died. As a result we were unable to interpret the experimental results even though the DMSO treated animals had markedly smaller tumors when compared to the controls. In the second, third and fourth DMSO experiments, mice were given 2% DMSO ad libitum in their drinking water. In the second and third experiments a marked reduction in tumor size was noted, with a more pronounced effect in the second experiment. However, with the exception of day 39 in the second experiment, our results were not statistically significant using the Students' t test or analysis of variance. The tremendous intergroup variation of tumor size was responsible for the failure to prove significance. In the fourth experiment, the tumors simply did not demonstrate any consistent growth once they reached 100 mg. The major difference between the tumors in this experiment and the other three, were that they were only in the third generation while the others had been at least in the seventh passage. This particular experiment used tumors at an earlier passage because there had been concern on whether or not the JOK-1 tumor underwent biological changes as a function of the number of successive passages. In vitro studies conducted on cells obtained from the third and seventh passages demonstrated that there was no difference between cells of the two passages and of the in vitro maintained JOK-1 cell line. Currently we have no explanation for the failure of the tumors in the fourth experiment to grow. Histopathologically, no signs of differentiation were noted in these experiments, only indications of extensive necrosis.
A similar situation was noted in mice that were given HMBA via i.p. injections. No differences in tumor growth were noted in the group that received 400 mg/kg of HMBA, but a decrease in growth was observed in the group that received 800 mg/kg. As in the DMSO experiments, there was too much tumor variability for our results to be statistically significant. Microscopic examination revealed no indications of differentiation, only signs of necrosis.

In similar studies, Lozzio et al (8) found that DMSO treatment was ineffective in reducing tumor growth in nude mice that carried the K-562 tumor. However, pretreatment of K-562 cells with DMSO prior to inoculation in the mice did decrease tumor incidence and size. In studies with another chemical inducer of differentiation, sodium butyrate, it was found that i.p. injections resulted in extensive K-562 tumor necrosis, not differentiation. Although doubtful, it could be hypothesized that the necrosis seen in their study, as well as our own, could represent cells that had terminally differentiated and subsequently died. Using other chemical inducers of differentiation in another model system, Preisler et al (16) have shown that the proliferation of murine erythroleukemia cells in the spleen could be significantly inhibited, and to a lesser degree, the same was observed in the bone marrow. These agents only exerted this effect on the erythroleukemia cells that were intravenously injected as opposed to cells that were inoculated subcutaneously. It was suggested that these agents might have altered the microenvironment of MELC, thereby, reducing their proliferation.
Attempts to induce differentiation in the human also have been reported. Recently, a 5 year old child with acute myelogenous leukemia who was refractory to conventional combination chemotherapy was treated during his third relapse with a continuous infusion of 2% sodium butyrate (500 mg/kg/24 hrs) for 10 days (17). By day 15 the blood was clear of myeloblasts and this was followed by the appearance of mature myeloid cells. Bone marrow aspirates indicated a partial remission. Unfortunately, the child relapsed two weeks after the cessation of butyrate infusion and was unresponsive to further treatment with sodium butyrate.

Another possible therapeutic use for chemical as well as biological inducers of differentiation is the augmentation of the host immune system. This in itself would be an invaluable method to reduce the tumor burden as well as possibly preventing the occurrence of secondary malignancies. In our study based on a small sample number, the data suggested that tumor bearing mice had a decrease in splenic NK activity when compared to normal nude mice, and there also was a hint that 800 mg/kg of HMBA induced a modest increase in NK activity at an effector-target ratio of 100:1. A tremendous increase in the cytolytic capability of the unstimulated peritoneal exudate cells was observed at the ratio of 100:1 in mice also treated with HMBA. Although it is tempting to speculate that this agent stimulated or activated peritoneal effector cells, we cannot rule out the possibility that this activation was due instead to substances or products released from the necrotic tumor. It should also be remembered that nude mice can undergo "spontaneous" peritoneal macrophage activation (18). For
example, in our case and alluded to above, macrophages could remove by phagocytosis, necrotic debris from the tumor and in the process become activated (18).

To indirectly test the hypothesis that DMSO and H MBA inhibited tumor growth by augmenting NK activity, the JOK-1 cells were exposed in vitro to either DMSO or H MBA, and after 7-9 days, a 4 hr $^{51}$Cr assay was performed using mononuclear cells isolated from the peripheral blood. These cells, which are normally NK resistant, remained NK resistant. Although we did not use mouse effector cells, a similar inability to kill the JOK-1 cells would probably also be observed thereby suggesting that inductive chemotherapy in some cases might render a cell more susceptible to NK cytolysis. In our particular case, this possibility is remote. The reason for conducting this experiment was the observation that after induction of differentiation, an NK resistant human melanoma cell line became NK sensitive (19).

Chemical inducers of differentiation may also inhibit the immune response. Suthanthiran et al (20) have reported that DMSO, butyric acid and H MBA, at concentrations that induce differentiation in the Friend erythroleukemia cell line, inhibit in vitro human lymphocyte proliferation as well as the generation of cytolytic T cells. Although these observations suggest that these compounds might be of value as immunosuppressive agents, the findings also could be a deterrent for the use of inductive chemotherapy.

Due to the tremendous intragroup variation in tumor weight our in vivo data was not statistically significant.
This variation in tumor size could in part be attributed to its spontaneous regression, and the extensive necrosis seen prior to regression. Another problem with the tumor was that it did not kill the host or become disseminated. Another factor mitigating against its use as a model, was our failure to find a marker in vitro that indicated differentiation. Although these results are a disappointment, the problems associated with the development of a human hemopoietic tumor in the nude mouse are best appreciated when it is remembered that Lozzio et al (8) used 1200 nude mice to develop the K-562 cell line into a myelosarcoma that had reproducible characteristics and was amenable to in vivo manipulations.

We have succeeded in growing and propagating the JOK-1 hairy cell leukemia cell line in the nude mouse thus meeting the sine qua non for the development of an in vivo model for inductive chemotherapy. Due to the variability in tumor size within groups, the validity of our data cannot currently be interpreted. However, it is felt that our observations are of enough interest to warrant further investigations into an in vivo model for inductive chemotherapy.
Figure 1

Floor plan of the barrier facility, for housing nude mice. This facility, located at Battelle Memorial Institute, was the site for our nude mice experiments.
Figure 1

FLOOR PLAN OF THE BARRIER FACILITY FOR HOUSING NUDE MICE
Figure 2

Cross-section of functioning osmotic pump. Three of these pumps are needed to deliver a continuous infusion of 2% DMSO. Mice, however, usually cannot tolerate more than one pump.
CROSS SECTION OF FUNCTIONING OSMOTIC PUMP

Figure 2
Figure 3

Growth of the JOK-1 cells in nude mice. Prior to tumor cell inoculation, mice were irradiated with 2 Gy per week X3.
Figure 4
Light Microscopic of the JOK-1 Tumor. The tumor is composed of a monomorphlc population of cells similar in size but varying in shape. Pathologically, this tumor is consistent with a poorly differentiated lymphocytic lymphoma.
A) JOK-1 grown without the irradiated HT-1080 cells
B) JOK-1 grown with the irradiated HT-1080 cells
Figure 5

First generation growth of the JOK-1 tumor in the nude mouse. After the initial inoculation, the JOK-1 tumor could be passaged without irradiation and the HT-1080 feeder cells.
RELATIVE TUMOR SIZE

FIRST GENERATION GROWTH OF THE JOK-1 TUMOR IN THE NUDE MOUSE

Figure 5

- ▲ ▲ TUMOR ORIGINALLY INITIATED WITH HT-1080 FEEDER LAYER
- ■ ■ TUMOR ORIGINALLY INITIATED WITH ONLY JOK-1 CELLS

DAYS FOLLOWING IMPLANTATION

RELATIVE TUMOR SIZE

- 10^{-1}
- 10^{0}

20 30 40 50 60 70
Figure 6

Cross appearance of JOK-1 growing as a subcutaneous tumor. This particular tumor was 1.1 cm in diameter.
Figure 7

The JOK-1 tumor as it appears in the mouse. This particular tumor showed no gross signs of necrosis.
Figure 8

A and B represent the morphological appearance of the JOK-1 tumor.

Note the areas of necrosis and dying cells.
Figure 8
Figure 9

A and B depict another view of the histopathological appearance of JOK-1. In A, note the well demarcated division between the tumor and necrotic area. B represents a low power view of the tumor. Here the tumor is interspersed among the areas of necrosis.
Figure 9
Figure 10
Transmission electron microscopic view of the JOK-1 tumor. This is comparable to the ultrastructure of an undifferentiation lymphoma (5,700 X)
Figure 11

Transmission electron microscopic view of the JOK-1 tumor (4,750 X)
Figure 11
Figure 12

Lymphocytic infiltration of the JOK-1 tumor.
Figure 12
Figure 13

The effect of HT-1080 supernatant on JOK-1 growth. The supernatant was generated from confluent HT-1080 cultures.

Control ●
10% HT-1080 supernatant ○
50% HT-1080 supernatant □
Figure 13
Figure 14

The effect of irradiated HT-1080 cells on JOK-1 growth. HT-1080 cells were irradiated with 6000 rads, fresh media with JOK-1 cells were then added and daily cell counts were performed. The HT-1080 cells remained attached throughout the course of the experiment.

Control ●

Cells grown with HT-1080 cells ○
Figure 14

(time in culture (days))

(cell number)

10^5

10^6

10^7

1 2 3 4 5 6 7

Figure 14
Figure 15

The effect of DMSO and TPA on JOK-1 cells grown in vitro. This experiment was done in parallel to the one illustrated in Figure 16.

- Control
- 2% DMSO
- 10^{-7} \text{ ng/ml TPA}
Figure 15
Figure 16
The effect of DMSO and TPA on cells derived from the JOK-1 tumor.
Comparing this figure with Figure 15, it can easily be seen that JOK-1 cells grown in the nude mouse does not alter its response to these inducers of differentiation.

Control ●
2% DMSO ○
$10^{-7}$ ng/ml TPA □
Figure 16
Figure 17

Growth of JOK-1 cells in control and DMSO treated nude mice. Each point represents the mean of 4 mice. Treatment was initiated when tumor size reached 100 mg.
GROWTH OF JOK-1 CELLS IN CONTROL AND DMSO TREATED NUDE MICE

Figure 17
Figure 18

Growth of JOK-1 cells in control and DMSO treated nude mice. This experiment is a repeat of the one seen in Figure 17.
GROWTH OF JOK-I CELLS IN CONTROL AND DMSO TREATED NUDE MICE

Figure 18
Figure 19

The appearance of the JOK-1 tumor after DMSO treatment. The necrosis is more pronounced in A. In B, the nuclei are becoming pyknotic.
Figure 19
Figure 20

Low magnification of the JOK-1 tumor after DMSO treatment.
Figure 21

The ultrastructural view of the JOK-1 tumor after treatment with DMSO. The nucleus is more convoluted than observed in the controls (Fig 10, 11). There are however signs of necrosis (5,700 X).
Figure 22

JOK-1 degeneration.

Note the massive vacuolation seen in this electron microscopic view of the JOK-1 tumor. The mitochondria are swollen and extensively damaged (4,750 X).
Figure 23

Ultrastructural depiction of two JOK-1 tumor cells near the epidermis of the mouse. Note the collagen. The cells have a convoluted nucleus and a more defined endoplasmic reticulum system than observed in the controls. This observation is similar to the JOK-1 cells that have been induced in vitro with DMSO (6,650 X).
Figure 24

Growth of JOK-1 cells in control and HMBA treated nude mice. This agent given i.p. every 8 hrs for 9 days. Treatment was started when the tumor weight reached 100 mg.
GROWTH OF JOK-1 CELLS IN CONTROL AND HMBA TREATED NUDE MICE

- Control
- 400 mg/kg
- 800 mg/kg

Days following implantation: 35, 45, 55, 65

Relative tumor size on a log scale.
Table 1

The Growth of JOK-1 in the Nude Mouse, with and without the Irradiated HT-1080 Fibrosarcoma Cells

<table>
<thead>
<tr>
<th>Day</th>
<th>JOK-1</th>
<th>JOK-1 + HT-1080</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td></td>
<td>33.3 ± 9.0 (5)b</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>56.9 ± 13.0 (4)</td>
</tr>
<tr>
<td>18</td>
<td>90.0</td>
<td>108.4 ± 28.0 (4)</td>
</tr>
<tr>
<td>21</td>
<td>163.3 ± 129.0 (3)</td>
<td>153.3 ± 41.0 (4)</td>
</tr>
<tr>
<td>25</td>
<td>303.3 (2)</td>
<td>285.3 ± 57.0 (3)</td>
</tr>
<tr>
<td>29</td>
<td>285.9 (2)</td>
<td>303.3 ± 64.0 (3)</td>
</tr>
<tr>
<td>36</td>
<td>496.9 (1)</td>
<td>293.1 (2)</td>
</tr>
</tbody>
</table>

aTumor weight is expressed in milligrams.

b( ) indicates the number of animals which had measurable tumors.

A total of 5 mice in each group were inoculated.
Table 2
First Generation Growth of the JOK-1 Tumor in the Nude Mouse

<table>
<thead>
<tr>
<th>Day</th>
<th>Mean Value of Tumor Weight + S.E.M.&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JOK-1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>21</td>
<td>32.7 ± 7.0 (3)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>28</td>
<td>68.0 ± 21.0 (4)</td>
</tr>
<tr>
<td>43</td>
<td>129.3 ± 33.0 (4)</td>
</tr>
<tr>
<td>50</td>
<td>153.8 ± 46.0 (4)</td>
</tr>
<tr>
<td>56</td>
<td>162.1 ± 71.0 (4)</td>
</tr>
<tr>
<td>63</td>
<td>215.3 ± 83.0 (4)</td>
</tr>
<tr>
<td>71</td>
<td>340.5 ± 52.0 (3)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Tumor weight is expressed in milligrams.

<sup>b</sup>JOK-1 tumor initiated without the HT-1080 feeder layer.

<sup>c</sup>JOK-1 tumor initiated with the HT-1080 feeder layer.

<sup>d</sup>( ) indicates the number of animals per group. Originally each group consisted of 4 mice which received 200 rads of radiation once a week for 3 weeks.
Table 3
Growth of the established JOK-1 tumor in Irradiated and Non-Irradiated Nude Mice$^a$

<table>
<thead>
<tr>
<th>Day</th>
<th>Irradiated Mean Value of Tumor Weight + S.E.M. $^b$</th>
<th>Non-irradiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>29 ± 14.0</td>
<td>41 ± 15.0</td>
</tr>
<tr>
<td>19</td>
<td>54 ± 25.0</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td>56 ± 25.0</td>
</tr>
<tr>
<td>26</td>
<td>67 ± 37.0</td>
<td>87 ± 54.0</td>
</tr>
<tr>
<td>33</td>
<td>94 ± 58.0</td>
<td>142 ± 78.0</td>
</tr>
<tr>
<td>40</td>
<td>141 ± 95.0</td>
<td>183 ± 109.0</td>
</tr>
</tbody>
</table>

$^a$Tumors were first established using irradiated HT-1080 feeder cells along with the JOK-1 cell line in irradiated mice.

$^b$Tumor weight is expressed in milligrams. Each group consisted of 8 mice.

$^c$Mice were irradiated 200 rads/week x3.
Table 4

Growth of JOK-1 cells in Control and DMSO Treated Mice

<table>
<thead>
<tr>
<th>Day</th>
<th>Controls</th>
<th>DMSO Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor Wt.</td>
<td>Log Relative Size</td>
</tr>
<tr>
<td>28</td>
<td>116.4 ± 10.0</td>
<td>1.0</td>
</tr>
<tr>
<td>33</td>
<td>160.1 ± 11.0</td>
<td>1.4</td>
</tr>
<tr>
<td>36</td>
<td>187.4 ± 27.0</td>
<td>1.6</td>
</tr>
<tr>
<td>39</td>
<td>235.9 ± 30.0</td>
<td>2.0</td>
</tr>
<tr>
<td>43</td>
<td>271.3 ± 50.0</td>
<td>2.33</td>
</tr>
<tr>
<td>47</td>
<td>307.5 ± 84.0</td>
<td>2.64</td>
</tr>
<tr>
<td>50</td>
<td>401.5 ± 90.0</td>
<td>3.45</td>
</tr>
<tr>
<td>55</td>
<td>444.4 ± 145.0</td>
<td>3.82</td>
</tr>
<tr>
<td>57</td>
<td>493.6 ± 143.0</td>
<td>4.24</td>
</tr>
<tr>
<td>60</td>
<td>521.8 ± 156.0</td>
<td>4.48</td>
</tr>
<tr>
<td>71</td>
<td>421.0 ± 217.0</td>
<td>3.62</td>
</tr>
</tbody>
</table>

DMSO was given continuously for 43 days in the drinking water.

Tumor weight is expressed in milligrams as the mean value ± S.E.M. of 4 mice.
Table 5
The Effect of DMSO on the JOK-1 Tumor

<table>
<thead>
<tr>
<th>Day</th>
<th>Control Tumor Wt.</th>
<th>Log Relative Size</th>
<th>DMSO Tumor Wt.</th>
<th>Log Relative Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>100.8 ± 25.0</td>
<td>1.0</td>
<td>115.2 ± 17.50</td>
<td>1.0</td>
</tr>
<tr>
<td>40</td>
<td>125.8 ± 36.0</td>
<td>1.25</td>
<td>147.6 ± 39.0</td>
<td>1.28</td>
</tr>
<tr>
<td>43</td>
<td>147.6 ± 47.0</td>
<td>1.46</td>
<td>159.9 ± 65.0</td>
<td>1.39</td>
</tr>
<tr>
<td>48</td>
<td>163.2 ± 65.0</td>
<td>1.62</td>
<td>168.4 ± 65.0</td>
<td>1.46</td>
</tr>
<tr>
<td>53</td>
<td>161.9 ± 65.0</td>
<td>1.61</td>
<td>197.3 ± 83.0</td>
<td>1.71</td>
</tr>
<tr>
<td>60</td>
<td>190.2 ± 84.0</td>
<td>1.89</td>
<td>140.4 ± 58.0</td>
<td>1.22</td>
</tr>
<tr>
<td>64</td>
<td>180.1 ± 89.0</td>
<td>1.79</td>
<td>101.4 ± 41.0</td>
<td>0.88</td>
</tr>
<tr>
<td>71</td>
<td>168.8 ± 93.5</td>
<td>1.67</td>
<td>49.9 ± 37.0</td>
<td>0.43</td>
</tr>
</tbody>
</table>

DMSO was given continuously for 36 days in the drinking water.

Tumor weight is expressed in milligrams as the mean value ± S.E.M. of 5 mice.
Table 6
The Effect of DMSO on the JOK-1 Tumor

<table>
<thead>
<tr>
<th>DAY</th>
<th>CONTROL TUMOR WT. LOG RELATIVE SIZE</th>
<th>DMSO&lt;sup&gt;1&lt;/sup&gt; TUMOR WT. LOG RELATIVE SIZE</th>
<th>DMSO&lt;sup&gt;2&lt;/sup&gt; TUMOR WT. LOG RELATIVE SIZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>91.9 ± 32.0 1.0</td>
<td>88.8 ± 41.0 1.0</td>
<td>89.9 ± 37.0 1.0</td>
</tr>
<tr>
<td>34</td>
<td>114.9 ± 44.0 1.25</td>
<td>122.2 ± 41.0 1.37</td>
<td>135.0 ± 47.0 1.5</td>
</tr>
<tr>
<td>39</td>
<td>141.9 ± 52.0 1.54</td>
<td>181.9 ± 45.0 2.04</td>
<td>190.0 ± 57.0 2.12</td>
</tr>
<tr>
<td>42</td>
<td>116.7 ± 63.0 1.27</td>
<td>199.3 ± 64.0 2.25</td>
<td>223.5 ± 71.0 2.49</td>
</tr>
<tr>
<td>46</td>
<td>129.3 ± 77.0 1.40</td>
<td></td>
<td>126.0 ± 86.0 1.4</td>
</tr>
<tr>
<td>49</td>
<td>111.2 ± 75.0 1.21</td>
<td></td>
<td>103.3 ± 79.0 1.15</td>
</tr>
<tr>
<td>53</td>
<td>144.4 ± 81.0 1.57</td>
<td></td>
<td>117.25 ± 81.0 1.31</td>
</tr>
<tr>
<td>61</td>
<td>145.7 ± 85.0 1.58</td>
<td></td>
<td>60.75 ± 96.0 0.67</td>
</tr>
</tbody>
</table>

1. DMSO was administered via the drinking water for two weeks
2. DMSO was given for two weeks, the mice were followed for two weeks after DMSO was discontinued
3. Weight is expressed in milligrams
Table 7

The Effect of DMSO on the JOK-1 Tumor

<table>
<thead>
<tr>
<th>DAY</th>
<th>CONTROL</th>
<th></th>
<th>DMSO&lt;sup&gt;1&lt;/sup&gt;</th>
<th></th>
<th>DMSO&lt;sup&gt;2&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TUMOR WT.</td>
<td>LOG RELATIVE SIZE</td>
<td>TUMOR WT.</td>
<td>LOG RELATIVE SIZE</td>
<td>TUMOR WT.</td>
<td>LOG RELATIVE SIZE</td>
</tr>
<tr>
<td>29</td>
<td>91.9 ± 32.0</td>
<td>1.0</td>
<td>59.4 ± 52.0</td>
<td>1.0</td>
<td>120.1 ± 96.0</td>
<td>1.0</td>
</tr>
<tr>
<td>34</td>
<td>114.9 ± 44.0</td>
<td>1.25</td>
<td>75.5 ± 67.0</td>
<td>1.27</td>
<td>141.8 ± 103.0</td>
<td>1.17</td>
</tr>
<tr>
<td>39</td>
<td>141.7 ± 52.0</td>
<td>1.54</td>
<td>74.05 ± 69.0</td>
<td>1.24</td>
<td>187.2 ± 92.0</td>
<td>1.55</td>
</tr>
<tr>
<td>42</td>
<td>116.7 ± 63.0</td>
<td>1.27</td>
<td>127.4 ± 81.0</td>
<td>2.14</td>
<td>179.3 ± 89.0</td>
<td>1.49</td>
</tr>
<tr>
<td>46</td>
<td>129.3 ± 77.0</td>
<td>1.40</td>
<td>150.5 ± 110.0</td>
<td>2.50</td>
<td>213.4 ± 141.0</td>
<td>1.77</td>
</tr>
<tr>
<td>49</td>
<td>111.2 ± 75.0</td>
<td>1.21</td>
<td>147.6 ± 112.0</td>
<td>2.48</td>
<td>220.9 ± 181.0</td>
<td>1.83</td>
</tr>
<tr>
<td>53</td>
<td>144.4 ± 81.0</td>
<td>1.57</td>
<td>131.4 ± 92.0</td>
<td>2.40</td>
<td>244.8 ± 200.0</td>
<td>2.03</td>
</tr>
<tr>
<td>61</td>
<td>145.7 ± 85.0</td>
<td>1.58</td>
<td>114.4 ± 91.0</td>
<td>1.92</td>
<td>212.0 ± 196.0</td>
<td>1.76</td>
</tr>
<tr>
<td>69</td>
<td>21.6 ± 62.0</td>
<td>0.23</td>
<td></td>
<td></td>
<td>237.0 ± 196.0</td>
<td>1.97</td>
</tr>
<tr>
<td>80</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1. DMSO was administered via the drinking water for 4 weeks
2. DMSO was given in the drinking water for 6 weeks
Table 8

Growth of JOK-1 Cells in Control and HMBA Treated Mice

<table>
<thead>
<tr>
<th>Day</th>
<th>Control Tumor Wt. (Mean ± SD)</th>
<th>Log Relative Size</th>
<th>HMBA&lt;sup&gt;a&lt;/sup&gt; Tumor Wt. (Mean ± SD)</th>
<th>Log Relative Size</th>
<th>HMBA&lt;sup&gt;b&lt;/sup&gt; Tumor Wt. (Mean ± SD)</th>
<th>Log Relative Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>139.2 ± 59.0 1.0</td>
<td></td>
<td>130.0 ± 22.0 1.0</td>
<td></td>
<td>95.9 ± 20.0 1.0</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>200.7 ± 92.0 1.44</td>
<td></td>
<td>157.5 ± 44.0 1.2</td>
<td></td>
<td>113.5 ± 23.0 1.18</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>264.8 ± 158.0 1.9</td>
<td></td>
<td>223 ± 61.0 1.72</td>
<td></td>
<td>144.8 ± 33.0 1.51</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>346.2 ± 215.0 2.49</td>
<td></td>
<td>299.5 ± 85.0 2.3</td>
<td></td>
<td>146.7 ± 41.0 1.53</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>403.6 ± 271.0 2.9</td>
<td></td>
<td>327.0 ± 92.0 2.5</td>
<td></td>
<td>152.5 ± 45.0 1.59</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>464.7 ± 337.0 3.3</td>
<td></td>
<td>360.4 ± 124.0 2.77</td>
<td></td>
<td>150.0 ± 46.0 1.56</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>449.0 ± 342.0 3.2</td>
<td></td>
<td>381.0 ± 115.0 2.9</td>
<td></td>
<td>146.5 ± 47.0 1.53</td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>458.9 ± 366.0 3.3</td>
<td></td>
<td>341.6 ± 77.0 2.63</td>
<td></td>
<td>122.5 ± 60.0 1.28</td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>363.3 ± 322.0 2.6</td>
<td></td>
<td>368.1 ± 129.0 2.8</td>
<td></td>
<td>116.9</td>
<td>1.22</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mice were given 400 mg/Kg of HMBA every 8 hrs i.p. for nine days. The tumor weights represent the mean of five control and four treated nude mice. By day 66, all four treated mice were alive, while 3 control mice were left.

<sup>b</sup>Mice were given 800 mg/Kg of HMBA every 8 hrs. i.p. for nine days. This group consisted of five treated mice. By day 66, four mice were left in the treated group. However, only 2 mice had measurable tumors.
Table 9

Nude Mice Cytolytic Activity Against YAC Lymphoma Target Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Splenocytes</th>
<th>PE Cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Bone Marrow Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10:1</td>
<td>100:1</td>
<td>100:1</td>
</tr>
<tr>
<td>Normal (n=2)</td>
<td>41 ± 19.0</td>
<td>87 ± 3.50</td>
<td>15</td>
</tr>
<tr>
<td>Control (n=2)</td>
<td>10 ± 7.0</td>
<td>53 ± 2.0</td>
<td>7.2</td>
</tr>
<tr>
<td>400mg/kg (n=3)</td>
<td>11 ± 2.0</td>
<td>63 ± 3.0</td>
<td>113</td>
</tr>
<tr>
<td>800mg/kg (n=4)</td>
<td>17 ± 5.0</td>
<td>73 ± 9.0</td>
<td>94</td>
</tr>
</tbody>
</table>

<sup>51</sup>Cr release assay was performed 21 days after the last i.p. injection of HMBA.

<sup>a</sup>Peritoneal exudate cells.
Table 10

Splenic Cytolytic Activity in Tumor Bearing Nude Mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>10:1</th>
<th>25:1</th>
<th>50:1</th>
<th>100:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25</td>
<td>34</td>
<td>50</td>
<td>71</td>
</tr>
<tr>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7</td>
<td>16</td>
<td>26</td>
<td>46</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>18</td>
<td>30</td>
<td>49</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>23</td>
<td>52</td>
<td>76</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>18</td>
<td>36</td>
<td>58</td>
</tr>
<tr>
<td>6</td>
<td>33</td>
<td>67</td>
<td>75</td>
<td>85</td>
</tr>
</tbody>
</table>

Mean Values

| ± S.E.M. | 14 ± 5.0 | 29 ± 8.0 | 45 ± 7.50 | 64 ± 6.0 |

<sup>a</sup>Tumor necrotic.

<sup>b</sup>Mouse emaciated and tumor necrotic.
Table 11

Cytolytic Activity in Normal and Tumor Bearing Nude Mice. The Effects of Peptone\textsuperscript{a}

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Splenocytes</th>
<th>PE Cells\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10:1 100:1</td>
<td>100:1</td>
</tr>
<tr>
<td>(1) Normal</td>
<td>65  70</td>
<td>27\textsuperscript{c}</td>
</tr>
<tr>
<td>(2) Normal</td>
<td>25  88</td>
<td></td>
</tr>
<tr>
<td>(3)\textsuperscript{e} Normal</td>
<td>21  81</td>
<td>61</td>
</tr>
<tr>
<td>Mean Value + S.E.M.</td>
<td>37\textsuperscript{\pm}17.0</td>
<td>79\textsuperscript{\pm}6.0</td>
</tr>
<tr>
<td>(4) Tumor</td>
<td>11  66</td>
<td></td>
</tr>
<tr>
<td>(5) Tumor</td>
<td>15  64</td>
<td>43\textsuperscript{d}</td>
</tr>
<tr>
<td>(6)\textsuperscript{e} Tumor</td>
<td>10  59</td>
<td>82</td>
</tr>
<tr>
<td>(7) Tumor</td>
<td>12  60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12\textsuperscript{\pm}1.0</td>
<td>62\textsuperscript{\pm}2.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}1 ml of sterile 10% peptone was injected i.p. 3 days prior to assay.

\textsuperscript{b}PE represents peritoneal exudate cells.

\textsuperscript{c}Represents the activity of cells pooled from mice 1 and 2.

\textsuperscript{d}Represents the activity of cells pooled from mice 4, 5 and 7.

\textsuperscript{e}Received i.p. injections of 10% peptone
## Table 12

Effects of DMSO on Normal Nude Mouse Splenic Cytolytic Activity

<table>
<thead>
<tr>
<th>Mouse</th>
<th>5:1</th>
<th>10:1</th>
<th>25:1</th>
<th>50:1</th>
<th>100:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13</td>
<td>31</td>
<td>43</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>33</td>
<td>74</td>
<td>99</td>
<td>118</td>
</tr>
<tr>
<td>Mean Value</td>
<td>17±4.0</td>
<td>32±1.0</td>
<td>58±15.0</td>
<td>95±3.5</td>
<td>105±13.0</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>41</td>
<td>70</td>
<td>96</td>
<td>117</td>
</tr>
<tr>
<td>4</td>
<td>22</td>
<td>34</td>
<td>54</td>
<td>79</td>
<td>93</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>7</td>
<td>44</td>
<td>27</td>
<td>60</td>
</tr>
<tr>
<td>Mean Value</td>
<td>15±7.0</td>
<td>27±13.0</td>
<td>56±9.0</td>
<td>67±25.0</td>
<td>90±20.0</td>
</tr>
</tbody>
</table>

Mice 1-2 received ordinary drinking water.

Mice 3-5 received 2% DMSO in the drinking water for 16 days.
CHAPTER V

THE ABSENCE OF NATURAL KILLER (NK) CELL ACTIVITY WITH NORMAL ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY IN HAIRY CELL LEUKEMIA PATIENTS. PARTIAL RESTORATION OF NK-LIKE ACTIVITY WITH INTERLEUKIN-2.
Introduction

The major cause of morbidity and mortality in patients with hairy cell leukemia (HCL) are infections. In a recent study of 127 patients, Golomb et al. (62) found that 47 (37%) patients had 111 documented infections (culture positive), 40 (31.5%) had 113 nondocumented infections (no culture results available or culture negative) and the remaining 40 (31.5%) patients had no major infections during the course of their disease. In the group of patients where infections were documented, 24 had pyogenic infections, 8 had nonpyogenic infections and 15 had both. Of the patients who had nonpyogenic infections, 8 were positive for Aspergillus fumigatus, 5 for Mycobacterium Kansasii, and 3 for Pneumocystis carinii (62).

In the analysis of the data, Golomb et al. (62) observed that HCL patients without clinical infections had an excellent prognosis, while patients with documented infections had a significantly graver prognosis, and usually died from infectious complications. Patients with nondocumented infections usually had an intermediate disease course. It was also reported that patients with pyogenic or both pyogenic and nonpyogenic infections had a worse prognosis than did patients with only a nonpyogenic infection (62). Golomb was of the opinion that the primary cause of infections was the prolonged chronic clinical course coupled with pancytopenia.

In a review of 22 patients, Stewart et al. (63) found that 18 patients developed infections which were considered life-threatening, with 9 succumbing to either proven or presumed infection. They found
that a wide range of organisms were capable of causing infections in HCL patients and that splenectomy did not reduce the infection rate (63). This later finding is in contrast to the observations reported by Bouza et al (64). These investigators on the basis of 20 patients, concluded that splenectomy reduced the incidence of infections.

Mackowiak et al (65) suggested that infections in HCL patients were the result of a defect in cell-mediated immunity. He noted that since HCL patients were frequently neutropenic, it was not surprising that these patients developed pyogenic infections. But the predisposition to nonpyogenic infections are less easily explained. These infectious organisms, which includes mycobacteria, fungi and viruses, are usually controlled by the cell-mediated immune system, and their frequency in HCL patients are only matched by individuals with Hodgkins disease. This suggested to Mackowiak et al (65) that the immune defect seen in HCL was more related to that of Hodgkins than that of any other malignant neoplasm (65).

Mackowiak's (65) suggestion may be considered to be a paradox in that HCL patients usually have a normal delayed-type hypersensitivity and the ability to respond to intradermally applied antigens. These investigators, also aware of the discordant observations, suggested that as in animal models, a normal delayed-type hypersensitivity reaction is not necessarily indicative of a healthy immune response (65).

Other immunological disorders have been associated with HCL, with the majority being autoimmune in nature. Dorsey et al (66) in a review of 31 HCL patients, found 4 with an associated immunological disorder.
Two patients had cutaneous vasculitis, one had polyarteritis nodosa and the other an IgA(K) monoclonal gammopathy and amyloidosis of the kidney, liver and small bowel. In a recent report, Westbrook et al (67) has made the observation that autoimmune diseases in HCL patients has been diagnosed with increasing frequency, with vasculitis, polyarteritis, or arthritis being the most common (67). In their UCLA series, it was found that second to infections, autoimmune disease was the most frequent cause of morbidity (67). There has been one report in which a patient developed Kaposi's sarcoma, 6 years after the diagnosis of HCL was made. Concomitantly, the patient developed immune thrombocytopenia, and an opportunistic infection (68).

Immunological investigations revealed normal humoral immunity and the response of the peripheral blood mononuclear cells to mitogenic stimulation was normal as well. However a reversal in the ratio of T helper cells to suppressor cells was found, a condition similar to the one seen in patients with AIDS (68).

Investigations into the immune system of HCL patients has revealed a normal humoral immunity with an impaired cellular immune response (69). Sabbe et al (70) using lymphocyte transformation tests, demonstrated that lymphocytes isolated from 16 HCL patients, had a decreased ability to respond to mitogens. After T cell enrichment, lymphocytes were able to respond to PHA, but not Con-A or PWM. The addition of allogenic monocytes, to varying degrees, augmented the responses to Con A and PWM. These observations suggested to the authors that the low PHA response was due to the dilution of T cells by the leukemic hairy cells, and that monocytopenia, a finding often seen
in HCL patients and documented in their study, was responsible for the poor Con-A and PWM response. Based on these observations, the authors hypothesized that a T cell defect may contribute to infections that are seen in HCL patients.

Subsets of T cells in HCL patients were studied by Worman et al (71) using monoclonal antibodies. They reported that Leu 3a helper cells were either normal or increased in percentage numbers, while the suppressor cells, Leu 2a, were normal or slightly reduced (71). Thus, if there is a T cell defect, it is due to a decrease in the number of T cells. In a follow-up study, these investigators showed that T cell subsets from the peripheral blood at a given time, differed from those found in the spleen. It was also shown that the spleen may influence the circulating T cells (72). In functional studies of T cells in HCL patients, Foa et al (73) showed that they were usually normal as compared to the controls.

We have found that NK but not ADCC activity is markedly decreased in patients with HCL, but following a 3 day in vitro incubation with IL-2, NK-like activity could be partially restored in splenocytes and peripheral blood mononuclear cells obtained from 4 patients with the disease. No marked increase in cytolytic activity was noted in cells incubated for 3 days without IL-2. Since it has been shown that many of the organisms which cause infections in HCL patients can be killed by NK cells, we would like to hypothesize that non-functional NK cells may in part be responsible for infections seen in these patients and that based on our observations, IL-2 may have a therapeutic role in the treatment of HCL.
Background

In the early to mid 1970's it was recognized that a discrete lymphoid subpopulation could induce rapid tumor cell cytolysis (1-3). These cells initially termed spontaneous cell mediated cytotoxicity (SCMC), are now known as natural killer (NK) cells (4,5). The unique feature of these cells is their ability to lyse virally infected or tumor cells in vitro, without prior immunological "knowledge" or any added exogenous factor. The NK cell has been shown to be operative in vivo as well (6). Thus for the past decade, this cell has been undergoing intensive investigation as one of the principle effectors of the immune surveillance network.

Morphology and Cytochemistry. The natural killer cell can be defined as a large granular lymphocyte (LGL) that is devoid of either B or T cell characteristics and primarily is found in the peripheral blood and spleen. In a detailed study of the LGL morphology, Grossi et al (7), described the cell as being a medium size lymphocyte with round or indented nuclei, having clumped chromatin and a usual prominent nucleoli. The cytoplasm was described as being abundant with infrequent mitochondria and "isolated profiles" of rough endoplasmic reticulum. Prominent features of the cytoplasm, according to Grossi et al (7), was (1) an extended Golgi apparatus from which smooth vesicles budded, (2) "coated" vesicles that were usually concentrated in the nuclear notch, (3) unit-membrane bound granules containing a matrix of variable density and (4) an absence of phagolysosomes.
The LGL contains the following acid hydrolases, alpha-naphthyl acetate esterase (ANAE), alpha-naphthyl butyrate esterase (ANBE), acid phosphatase (AP) and beta-glucuronidase (B-Gluc) (7). These enzymes are specific markers for the lysosomal system (7). Electron microscopy has demonstrated that these enzymes are found in the granules and "coated" vesicles (7).

**Tissue Distribution.** In man, the natural killer cell can be detected in the fetal liver at 9 weeks of gestation (8). At birth, NK cells can be found in significant levels in the cord blood (8). Thereafter, they increase to high levels in adulthood and only begin to decrease late in life (8).

As previously stated, most NK activity is found in the peripheral blood and spleen. To a lesser degree, NK activity can also be found in the lymph nodes, bone marrow and thoracic duct (8). No activity has been demonstrated in the thymus (8). Using the NK specific monoclonal antibody anti-Leu-7 (HNK-1+) and the avidin-biotin peroxidase complex technique, Si et al (9) have examined the distribution of NK cells in the peripheral lymphoid tissue of humans. It was observed that the anti-Leu-7 monoclonal antibody reacted strongly with cells in the germinal centers of secondary follicles in tonsils, spleens and lymph nodes (9). It did not react with the majority of lymphocytes in the germinal centers, nor with the surface immunoglobulin (sIg) positive lymphocytes found in the follicular mantles (9). The distribution of Leu-7 positive cells in the spleen correlated with the *in vitro* observation that splenic NK cells have the highest level of cytolytic
activity (9). As defined by this monoclonal antibody, splenic NK cells were primarily located in the red pulp and nodules (9). These investigators made the interesting observation that while the tonsils had approximately 15% Leu-7 positive cells, only marginal NK activity could be demonstrated. The addition of interferon did not increase the cytolytic level of the tonsillar NK cells (9). This suggested to the authors that the NK cells were either suppressed or unable to lyse K-562 target cells.

In acknowledgement of the hypothesis that NK variation among individuals may have a genetic basis, Si et al (9) noted that in 12 tonsils examined, there was a marked increase in the number of Leu-7 positive cells in the germinal centers of some tonsils as compared to the others. They however stated that it was not known whether this difference was a reflection of the activation state or of genetic makeup. Also examined in this study was the in vivo relationship of NK cells to solid tumors. It was found in all cases examined, that Leu-7 positive cells were both around and in tumors (9). This observation supports the hypothesis that NK cells may be involved in host resistance to tumors.

In another study, Banerjee et al (10) noted a similar normal tissue distribution of Leu-7 positive cells. Using double marker studies, it was observed that intrafollicular Leu-7 positive cells in lymph nodes, tonsils and the spleen expressed the pan-T antigen Leu-1, but were negative for Leu-2, Leu-3, Ia and the myeloid-monocytic antigen Ml (10). Leu-7 positive cells in the splenic red pulp, expressed the Ml antigen but not the Leu-1 marker (10). The authors
noted that NK cells with the phenotype Leu-7+, Leu-1+ had a lower
cytolytic capability than did the ones with the phenotype of Leu-7+, M1+. This observation may explain why splenic NK cells have the
highest cytolytic level of the peripheral lymphoid tissue (10). In
examining lymphomas, it was observed that Leu-7 positive cells were
preserved in the lymphomas of follicular origin but were randomly
distributed in the diffuse lymphomas of either B or peripheral T cell
origin (10). It was not known whether or not the NK cells had the
capability to lyse the malignant lymphoma cells (10).

Cell Surface Phenotype. Abo et al (11) in further characterization of
the NK cell, documented that Leu-7 positive or HNK-1+ positive cells
were heterogeneous with respect to the receptors for E-rosettes (ER)
and were distributed in both the ER−sIg−FcR+ (null cell) and ER+FcR+
(T cell) populations. Two color immunofluorescence of the NK cells
showed variable expression of T cell antigens; T1 and T3 were
col-expressed on 20 to 50% of the Leu-7 positive cells while T4, T5 and
T8 were expressed on 10-20% of the cells (11). The HLA-DR antigen was
observed on about 20% of Leu-7 positive cells (10,11). Based on two
color immunofluorescence and functional studies, Abo et al (11)
proposed that the Leu-7,T3 positive cell was a less mature cell than
the Leu-7 positive T3 negative cell. This hypothesis was supported by
the observation that Leu-7,T3 positive cells had low cytolytic
activity, few cytoplasmic granules and were primarily found in the bone
marrow. The Leu-7 positive T3 negative cell population had a high
level of cytolytic activity, multiple cytoplasmic granules and circulated in the peripheral blood (11).

**Nature of NK Target Cells.** Natural killer cell target specificity is currently not well understood. Cells susceptible to lysis by NK cells range from normal undifferentiated cells to virally infected and certain tumor cells. Further complicating the issue is that target cell sensitivity can be modulated in vitro by a variety of biological and chemical agents. The erythromyeloid K-562 cell line, a widely utilized NK target, can be rendered resistant to NK-mediated cytolysis after hemin and sodium butyrate induced differentiation (12,13,14). Similarly, the NK sensitive pro-myelocytic cell line, HL-60, can be rendered NK resistant after TPA and DMSO induced differentiation (14). In contrast, the human melanoma cell line, MeWo, becomes NK-sensitive after theophylline induced differentiation (15). These studies indicated that the change in target susceptibility was due at least in part to a change in the NK-binding ability of these cells (13,14). Biochemical studies have suggested that an increased amount of sialic acid on the cell surface can reduce target cell sensitivity to NK cell mediated lysis (16,17).

Along with NK target structure(s), the metabolic state may be important as well in determining sensitivity or resistance of a target cell to NK-mediated lysis. Kunkel et al (18) have shown that the inhibition of protein synthesis can render a cell more susceptible to NK lysis. They hypothesized that the inhibition of protein synthesis could impair a membrane repair process thus increasing the
susceptibility to lysis (18). One could conceivably visualize this
process occurring after the generation of the superoxide anion, \( \text{O}_2^- \) (8). Once generated, the \( \text{O}_2^- \) could cause membrane damage by initiating lipid
peroxidation. Thus not only could the inhibition of protein synthesis
impair membrane repair, but it could initiate free radical production
as well.

Augmentation of NK Cell Activity by Interferon and Interleukin-2.

Interferon (IFN) and more recently, interleukin-2 have been shown to
significantly augment natural killer cell activity. Studies have shown
that IFN can rapidly enhance the cytolytic activity of NK cells in both
man and rodent (19,20,21). Cell surface marker studies have indicated
that no overt cell differentiation had taken place after exposure to
IFN, although there was a slight increase in the products of HLA A, B
and C on NK cells, but this increase also was seen on all lymphocytes
(22).

The mode of action in the augmentation of NK-mediated cytolysis by
IFN appears to occur via several different mechanisms. Trinchieri et
al (22) and other investigators (23,24) have demonstrated that IFN can
increase cytolysis by enhancing the recycling ability of the natural
killer cell. Trinchieri et al (22) also have given preliminary
evidence that IFN can reverse the inhibition in cytolysis caused by the
interaction of immune complexes with the NK cell. Timonen et al (25)
have shown that IFN can cause an increase in NK target recognition and
binding, thus suggesting that IFN can confer cytotoxic properties on
"pre"-NK cells. However, the increased binding of NK cells to target
cells is seen only when the targets are propagated as a monolayer, not as a suspension culture (25).

In dissecting the mode of action of IFN on NK cells, one must keep in mind that there are multiple species of IFN produced in the body with a wide spectrum of activity. Mononuclear cells isolated from the peripheral blood may produce both alpha and gamma interferon. Both lymphocytes and monocytes can produce alpha IFN when virally infected, but when co-cultured with tumor cells, only the lymphocytes can produce this particular class of IFN (22). Gamma interferon is produced by T lymphocytes in response to an immune stimulus. Although all species of alpha and gamma IFN can augment NK cytolysis, there is considerable difference as to the level of augmentation achieved (19,22). Recombinant IFN can enhance NK activity as well (19).

IFN also can modulate target cell susceptibility to NK-mediated lysis. For example, low doses of IFN can render normal human fibroblasts resistant to lysis (22). In this particular case, NK cells are able to bind to the IFN treated fibroblasts but are unable to initiate cytolysis (22). On the other hand, IFN does not protect virally infected or some tumor cells (22). These observations prompted Trinchieri et al (22) to propose that IFN can protect normal cells in vivo from inappropriate cytolysis.

Studies have been conducted that demonstrate that IFN can augment NK activity in vivo. This has directly been shown by the injection of IFN into an animal, or indirectly by the inoculation of viruses and other agents that induce IFN production (26). The mode of action of
IFN in vivo may be to modulate the differentiation and cytolytic activity of NK cells (26).

Clinically the use of IFN in the treatment of malignant diseases has caused much excitement as well as disappointment. In some trials, the daily intramuscular administration of IFN has caused an immediate and prolonged increase in NK activity, while in other trials, only temporary increases were observed, and in some instances, actual decreases in NK activity were seen (27,28).

As previously stated, IL-2 can augment the NK response as well. This observation is, however, complicated by the following facts: (1) IL-2 preparations could be contaminated with IFN, mitogen or TPA; (2) IL-2 can induce the generation of killer cells that are distinct from NK cells and (3) although a distinct possibility, the action of cytolytic T cells could be mistaken for NK activity.

Henney et al (29) first showed in 1981 that IL-2 could boost NK activity in mononuclear cells isolated from the murine spleen. A similar finding was observed with nude mouse splenocytes (30). Kuribayashi et al (31) have shown that the IL-2 potentiating effect could be eliminated if the lymphokine was absorbed out with IL-2 receptor positive cells, or if IL-2 was precipitated out with an anti IL-2 monoclonal antibody. It was further shown that together IFN and IL-2 were additive (31). This observation led workers to hypothesize that IFN possibly could either induce IL-2 receptor expression or increase the affinity of the receptor (31). It was shown, however, by Abo et al (32), that human NK cells (HNK-1+) did not express the receptor for IL-2 unless they were mitogen stimulated.
Domzig et al (33) have made a similar observation in the human. These investigators observed that low amounts of IL-2 could rapidly augment NK activity in the standard 4 hour assay. The presence of an anti-IL-2 monoclonal antibody during the 4 hour assay significantly inhibited NK activity, thus supporting the hypothesis that IL-2, not any other biological modifier, was responsible for the augmentation of NK activity. Ades et al (34) have demonstrated that IL-2 could boost the NK activity of the Leu-11 positive lymphocytes, but not the Leu-11 negative population.

Presently the mechanism(s) by which IL-2 augments NK activity is not known, but preliminary evidence very strongly suggests that there is a positive relationship between IL-2 and IFN. It has been reported that IL-2 can induce the production of gamma interferon, (35) and that antibodies against gamma IFN will inhibit the NK boosting effect of IL-2 (35). If these observations can be confirmed, then it probably can be said that IL-2 potentiates NK activity in a short term assay via the production of gamma IFN.

Interleukin-2 can induce the generation of another class of cytolytic cells known as lymphokine activated killer (LAK) cells (36). These cells can be generated after incubating autologous lymphocytes for 2-3 days in the presence of IL-2 (37,38), after which they are capable of lysing autologous or allogeneic fresh tumor cells. They can also lyse NK resistant cells (36,37). These cells are distinct from the cytolytic T cells and NK cells. Phenotypically, LAK precursors are negative for Leu-1, OKT-3, Leu-7 and OKM-1, and therefore are neither T (Leu-1+, OKT-3+) nor NK (Leu-7+ OKM-1+) cells. After exposure to IL-2,
the LAK cell is positive for OKT-3 and OKT-8, which suggests some sort of relationship to the T cell lineage (39). These cells are very effective in reducing the tumor burden in tumor bearing mice after adoptive immunotherapy. This observation along with the in vitro lysis of human autologous tumor cells by LAK cells, provides a strong impetus to use these cells in a clinical trial (37,40).

Antibody-Dependent Cellular Cytotoxicity. Cells that mediate antibody-dependent cellular cytotoxicity (ADCC) also have been investigated as a possible effectors in the host defense against tumors. This effector cell, known only from in vitro studies is recognized by its ability to lyse target cells coated with target cell specific IgG antibody (41). This cell, sometimes known as the K or null cell, are thymus-independent lymphocytes that express receptors for the Fc portion of the IgG immunoglobulin (41). A cell that mediates ADCC initiates cytolysis by binding to the Fc portion of the IgG molecule, which has been absorbed on to the surface of a target cell. There is no direct contact between the effector cell, and the cell surface of the target cell.

There is still some controversy as to whether or not the NK cell and the cell that mediates ADCC are identical. It does seem clear, however, that NK cells, as well as other cells, can mediate ADCC as long as they have receptors for the Fc portion of the IgG molecule (42). Furthermore, a subpopulation of cells that normally contains NK activity, can still mediate ADCC even when it has lost its NK activity (43).
NK and ADCC Activity in Disease. The natural killer (NK) cell and the cell(s) that mediates antibody-dependent cellular cytotoxicity (ADCC) have been intensively investigated in individuals who have malignant as well as non-malignant diseases. A decrease in NK cell activity has been observed in patients with the X-linked lymphoproliferative (XLP) syndrome (44), Chediak-Higashi Syndrome (CHS) (45), systemic lupus erythematosus (46), severe combined immunodeficiency (SCID) (47), acquired immunodeficiency syndrome (AIDS) (48,49), chronic lymphocytic leukemia (CLL) (50), lymphomas (51) and malignant solid tumors (52).

Individuals who have the XLP syndrome, are immunodeficient with respect to the Epstein-Barr virus (EBV) (44). Up to 50% of these patients develop fatal infectious mononucleosis, while 15 to 30% develop an acquired varied immunodeficiency. Lymphoreticular malignancies develop in about 40% of these patients. These clinical observations led Sullivan et al (44) to investigate NK activity in XLP patients. It was found that the majority of the patients had a significant decrease in NK activity. But a modest increase in NK activity was observed if the lymphocytes were first preincubated in IFN for 1 hour (44). These investigators suggested that their studies supported the hypothesis that NK cells may play an important role in the immune response to viral infections and in immune surveillance against malignant lymphoid proliferation (44).

The Chediak-Higashi syndrome is a rare autosomal recessive disease. This genetic disorder is characterized by abnormal lysosomal granules, partial oculocutaneous albinism and severe recurrent pyogenic
infections (45). Patients with this disease often survive the complications of infections, only to later die of an aggressive lymphoproliferative disorder (45). Katz et al (45) have shown that these patients have an NK defect that is not due to the lack of cells, but rather to the inability of the NK cells to cause cytolysis after target cell binding (45). There were, however, some NK cells that were able to function properly (45). In vitro treatment with IFN enabled the previously non-lytic NK cells to become cytolytic (45). The authors were also of the opinion that the NK cell defect predisposed CHS patients to lymphoproliferative disorders (45). It can be hypothesized that the lack of cytolytic NK cells is in part responsible for the rampant infections seen in these patients.

The autoimmune disease, systemic lupus erythematosus (SLE) is characterized by a multitude of immune rearrangements including NK deficiency. This defect may be expanded in part by reports that indicate that SLE patients have serum factors and anti-NK cell antibodies that could inhibit NK cell function (46). Tsokos et al (46) observed that highly purified IL-2 could increase the NK levels to that of normal levels. Unexpectedly, these investigators noted that alpha IFN was only partially able to augment the depressed NK level (46). They did, however, note that a similar situation has been observed in patients with AIDS and in children with Nezelof's T cell deficiency (48,53). It also was observed that IL-2 production was impaired (46). This latter point was addressed by Linker-Israeli et al (54). They reported that the removal of OKT-8 (Leu 2a+) and Leu-7+ cells from SLE patients enabled the remaining lymphocytes to produce near normal
amounts of IL-2 after in vitro mitogen stimulation. This was observed in 19 of 21 patients. The removal of OKT-8 cells from normal individuals did not alter IL-2 production (54). Their experiments indicated that IL-2 production was suppressed by the OKT-8, Leu-7+ cells and not passively absorbed (54). These patients as well, have a high incidence of infections and malignancies (54), again implying that the NK cell may play a crucial role in immune surveillance.

The devastating disease, AIDS, is characterized by a profound defect in cell-mediated immunity, including a significant reduction in NK activity (48,49). A recent report has documented that IL-2 can enhance NK activity in patients with this disease (49). These investigators demonstrated that IL-2 was able to do this independently of gamma IFN production (49). The significance of this finding is that IL-2 normally induces the production of gamma IFN from lymphocytes, suggesting that IL-2 indirectly increases NK activity via gamma IFN production (49). But in the case of AIDS, IL-2 can apparently enhance the cytolytic ability of NK cells independently of gamma IFN (49). This suggests that NK cells can be augmented via two separate mechanisms (49). Rook et al (49) have suggested that this observation may have clinical significance. It was noted that patients with AIDS have a significant impairment in the ability to produce gamma IFN in response to IL-2 and recall antigens. Thus combination therapy with both biological modifiers may be clinically beneficial (49).

Natural killer cell defects have been observed in both T- and B cell CLL (43). Pandolfi et al (43) has reported a case of suppressor T-cell CLL in which the patients lymphocytes exhibited ADCC reactivity
but not NK activity. Ziegler et al (50), noting the observation that
CLL patients have a high incidence of second malignancies, investigated
NK activity in B-cell CLL patients. They found a profound deficiency
in these patients, despite the ability of lymphocytes to bind to NK
target cells (50). To eliminate the possibility that CLL cells could
interfere with or dilute out the NK cells, Ziegler et al (50) used a
monoclonal B cell antibody (BA-1) and complement to lyse B cells thus
removing the majority of the malignant cells. This procedure did not
cause a decrease in the NK activity of the controls, nor did it
increase activity in the CLL patients (50). They observed that IFN
augmented NK activity in patients will with early stage CLL, but not in
late stage CLL (50). Pattengale et al (55) have reported that
neoplastic B-cell cells are NK sensitive. This effect was most obvious
when allogeneic effector cells were IFN treated (55). Their
observations were suggestive that CLL cells in the progressive stage of
the disease were more sensitive to NK mediated lysis than cells in the
stable form of the disease (55).

The lymphomas and many of the solid tumors are associated with a
decrease in NK activity as well. As discussed earlier, the treatment
of patients with solid tumors with IFN has had both positive and
negative results (27,28) in terms of both tumor remission and NK
augmentation. Recently, evidence has been generated that suggests that
low NK levels may be present in patients with preleukemia (PL) (56).
Preleukemia is a term that is used to describe a hematopoietic syndrome
which often antedates the development of acute non-lymphocytic leukemia
(57). The clinical presentation can be quite variable, but usually is
associated with pancytopenia, increased cellularity in the bone marrow and maturational defects in the erythrocytic, granulocytic and megakaryocytic progenitor populations (57). In examining the cell-mediated immune parameters of these patients, Porzsolt et al (56) found that although the NK activity was low, it did however fall in the lower range of the corresponding control values. They noted that this decrease could not be correlated with prognosis.

The antibody-dependent cellular cytotoxicity assay often has been used as an in vitro assay to measure the status of the in vivo immune state (41). There has been good correlation between this assay and the immune response to tumors, transplants and autoimmunity (41). For this reason, ADCC has been used in the investigation of the immunobiology of the leukemias, as well as a potential tool in the clinical management of these patients (58,59,60).

Talpaz et al (58) found that there was no differences in NK and ADCC activity between leukemia patients in remission and normal controls. But when the patients were subdivided into early and late remission groups, a significant difference was found. Individuals who had early remission had a significant decrease in both NK and ADCC activity as compared to the controls and the late remission group (58). However, the ADCC was only significant when the targets were cells from the CEM lymphoid tumor cell line. If chicken and human red blood cells were used as targets, there was no significant decrease or increase in cytolysis when the early remission group was compared to the controls and late remission group (58). Although there is still some uncertainty, the different targets that are used in an ADCC assay are
apparently recognized by different populations of effector cells (61). Antibody coated human red blood cells are lysed by monocytes, chicken red blood cells by polymorphonuclear leukocytes, macrophages and sIg negative lymphocytes. The lysis of the CEM cells are probably the result of more than one effector cell population (61).

Holm et al (59) found that cells from patients with acute myeloid leukemia (AML) were unable to mediate ADCC, while cells from individuals who promyelocytic and monocytic leukemia had the capability. Neoplastic B cells also were unable to mediate ADCC. These investigators concluded that this assay may be useful in distinguishing leukemic cells of different origin, the degree of differentiation and may be of value in the more precise classification of the leukemias (59).
NK Cell Preparation: Lymphocytes were obtained from surgically removed spleens or from peripheral blood. In patients 1, 2 and 9 (Tables 1,2) lymphocytes were collected after leukopheresis. The spleens were teased apart with 18 gauge needles in RPMI 1640 media supplemented with 5% fetal bovine serum (FBS) and 10,000 units/ml penicillin and 10,000 mcg/ml streptomycin (complete media). The single cell suspension was washed 1 X with phosphate buffered saline (PBS), pH 7.4 sedimented at 2000 rpm and then resuspended in PBS. The mononuclear cells then were separated from the red blood cells by Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) gradient centrifugation. The cells were washed 3 X in PBS as indicated above, and then resuspended at concentrations ranging from 3 x 10^6 to 1 x 10^7 cells/ml in RPMI 1640 supplemented with 10% heat inactivated FBS, 10,000 units/ml of penicillin and 10,000 mcg/ml of streptomycin. Mononuclear cells were isolated from the peripheral blood by Ficoll-hypaque gradient centrifugation and treated as described for splenic mononuclear cells. Whenever possible, cells were either cryopreserved or placed into culture.

IL-2 in vitro Activation. Splenic mononuclear cells of patient 8 were incubated with 10% IL-2 (Cellular Products, Buffalo, N.Y.) for three days in a 37°C incubator with 5% CO₂ and 95% air. Mononuclear cells from patients 7, 9 and 10 were treated similarly with the exception that they were cultured in 5% heat inactivated human AB serum, while 5% FBS was used in the case of patient 8. IL-2 was diluted to the
concentration of 10% by directly adding it to the culture media in which the cells were to be incubated. As controls, cells from the same patients were treated in an identical fashion with the exception that IL-2 was not present during the incubation period. Viability was assessed by trypan blue exclusion prior to and following each experiment.

NK Assay. The K-562 cell line was used as the target for NK lysis. Two million target cells were labelled with 150 μCi of 51Cr (New England Nuclear, Boston, Mass., specific activity 200-900Ci/g) for 40-50 min at 37°C, 5% CO2 and 95% air in complete media, after which the cells were washed 3 X with PBS and resuspended to a concentration of 1 x 10^6 cells/ml and incubated for an additional 30-40 min. The cells then were diluted in fresh complete media to a concentration of 1 x 10^5 cells/ml and 100 μl of the target cell suspension were added to each of 96 wells of a microtiter plate (#3596 Costar, Cambridge, MA). Freshly prepared mononuclear cells, cells obtained from IL-2 stimulated cultures or control cultures were added to triplicate samples of the target cells in a volume of 100 μl at the effector-target cell ratios of 100:1, 50:1, 25:1, 10:1 and 5:1. The plate then was centrifuged at 600 rpm for 5 min. and incubated for 4 hours at 37°C in a humidified incubator with 5% CO2 and 95% air. At the end of 4 hours the plate was centrifuged at 2000 rpm for 10 min., 100 μl of the supernatant were placed in 12 x 75 mm test tubes, and counted in a Tracor Analytic 1185 gamma scintillation counter. Percent lysis was calculated using the formula:
Experimental release - spontaneous release

Maximum release - spontaneous release \times 100

Maximum release of the target cells was determined by incubating them with 0.17N hydrochloric acid for 4 hours at 37°C in conjunction with the NK assay. Spontaneous release was determined by measuring the amount of $^{51}$Cr released in 4 hours in the absence of effector cells.

**ADCC Assay.** 2–3 drops of human blood was obtained from a finger prick of a normal laboratory volunteer and washed 3x with PBS. The red cell pellet was resuspended in 200 μl of complete media and 150 μCi of $^{51}$Cr was added, with a subsequent 3–6 hr incubation period at 37°C, 5% CO$_2$ and 95% air. The red cells were washed 3x with PBS and resuspended in complete media for an additional 1 hr. Following centrifugation, the red cell pellet was diluted in fresh complete media to a concentration of $1 \times 10^5$ cells/ml and 100 μl of the target cell suspension were added to 96 well microliter plates to which 50 μl of a 1:20 dilution of mouse anti-human red blood antisera (kindly provided by Dr. W-Z Wei) was added. The red cells and antibody were mixed on a Cooke microtiter mixer and allowed to remain at room temperature at 30 min. Freshly prepared mononuclear cells were added to triplicate samples of the target cells in a volume of 100 μl at the effector-target cell ratio of 100:1, 50:1, 25:1, 10:1 and 5:1. The plate then was centrifuged at 600 rpm for 5 min and incubated for 2 hrs at 37° in a humidified incubator with 5% CO$_2$ and 95% air. At the end of 2 hrs, the plate was treated as outlined in the NK assay.
Surface Markers. The following cell surface markers were studied: IgG, IgM, IgA, IgD, \( \kappa \), \( \lambda \), B-1 and Ia. Fluorescein isothiocyanate (FITC) conjugated goat anti-human heavy and burro anti-human light chain antibodies (Kalstead, Austin, Texas) were used in a one step immunofluorescence assay. Briefly, \( 1 \times 10^6 \) cells were incubated with the appropriate dilution of antibody for 30-40 min at 4°C. The cells were then washed 3 times with PBS, fixed with 50% glycerol, mounted on a slide and a minimum of 200 cells were examined with a Zeiss UV FL Epifluorescence microscope. The B-1 and Ia (Coulter Immunology, Hialeah, Florida) monoclonal antibodies were used in the indirect immunofluorescence test. As before, \( 1 \times 10^6 \) cells/ml were incubated with the appropriate dilution of antibody and incubated at 4°C for 30-40 min. Cells were then washed 3 times with PBS, incubated for an additional 30-40 min with FITC-conjugated anti-mouse IgG (Miles Scientific, Naperville, IL). The cells were again washed 3 times with PBS; fixed with 50% glycerol and examined as above.
Results

Patients 1 through 10, listed in Tables 1 and 2 were diagnosed as having hairy cell leukemia by histopathological criteria (74). Eight of these patients were seen at The Ohio State University Hospital and the other two were treated at a neighboring hospital. Patients listed in Table 3 were seen at the University Hospital as well. The cell surface phenotypes of these patients, percent hairy cells and the peripheral leukocyte count are summarized in Table 1. The cell surface markers are indicative of a B cell neoplasia, consistent with that of HCL. Patients 1(a), 2, 4, 5, 7, 8 and 9 all had multiple heavy chain isotypes, with a light chain restriction in patients 2, 4, 7, 8 and 9 (4, 1). These observations are consistent with those made by Jansen et al (75). In contrast, however, patients 1(a) and 5 expressed both the \( \kappa \) and \( \lambda \) light chain, thus suggesting that there may be more than one clone of cells present. The loss of sIgM and the variation of sIgG in patient 1 is not surprising since this patient underwent repeated leukophereses and the percent of surface immunoglobulin (sIg) positive cells has been reported to vary over a period of time within the same patient (76). Patient 3 had no detectable sIg and could be considered a null case of HCL, several of which have been reported (77). However, this patient had strong reactivity for the anti-\( \text{B}-1 \) monoclonal antibody, which recognizes a 30,000 molecular weight glycoprotein that is present on almost all normal B cells (78). This observation would suggest that although hairy cells may be negative for sIg, they still may belong to the B cell lineage if other phenotypic criteria are
Table 1
Cell Surface Phenotypes of Peripheral Blood
Lymphocytes Isolated from Hairy Cell Leukemia Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>IgM</th>
<th>IgG</th>
<th>IgD</th>
<th>IgA</th>
<th>$k$</th>
<th>$\lambda$</th>
<th>Ia</th>
<th>B-1</th>
<th>WBC</th>
<th>ZHC</th>
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<td>ND</td>
<td>ND</td>
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<td>54</td>
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<td>16,250</td>
<td>30</td>
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</table>

^aWBC designates the peripheral leukocyte count. HC designates the percent hairy cells in the peripheral blood.

^bThese values were obtained from the peripheral blood (a) and spleen (b) within 3 days of each other from the same patient.

^cNA Patient's record not available

ND Not done
utilized. In patient 6, the mononuclear cells obtained from the peripheral blood and spleen were identical with respect to the heavy and light chains examined. Natural killer cell activity from HCL patients are summarized in Table 2.

Patient 1 underwent repeated leukopheresis, and as can be seen in b from Table 2, there was a marked increase in NK activity after the second leukopheresis. Four days had elapsed between the first and second treatments, and only 2 days separated the second and third leukophereses. The last leukopheresis was performed 35 days after the first treatment. As can be seen in c-e this increase was only temporary. The variability in NK activity seen in a-e in Table 1 is not completely unexpected, since values from individuals can vary from day to day. The mean values ± S.E.M. for this one patient is stated at the bottom of Table 2. This particular patient was diagnosed as having HCL in 1962 and underwent a splenectomy in 1975. Similarly, in patients 2-9, NK activity also was low. In patient 6, the NK values obtained from both peripheral blood and spleen were low. The values were obtained within 3 days of one another. These observations take on added significance when compared to the NK values seen in Table 3. Values in this table represent NK activity in patients with other hematological disorders (patients 3-6), with non-hematological diseases (patients 1 and 2) and for normal laboratory volunteers (No. 7). For example, as shown in Table 2 the mean percent lysis at a 100:1 effector to target cell ratio for HCL patients was 10.50 ± 7.82 compared to the control value of 62.70 ± 15.82 (Table 3). Statistical analysis using Students' t test revealed that at all effector to target ratios the
Table 2
Natural Killer Cell Activity in Patients with Hairy Cell Leukemia

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Source of Cells</th>
<th>Effector to Target Cell Ratio (% Lysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5:1</td>
</tr>
<tr>
<td>1a</td>
<td>Peripheral Blood</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>e</td>
</tr>
<tr>
<td>2</td>
<td>Peripheral blood</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>6a</td>
<td>Peripheral blood</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>b</td>
</tr>
<tr>
<td>7</td>
<td>Peripheral blood</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Peripheral blood</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Peripheral blood</td>
<td></td>
</tr>
<tr>
<td>Mean Values + S.E.M.</td>
<td></td>
<td>1.17±1.48</td>
</tr>
</tbody>
</table>

*aThis patient underwent repeated leukophereses indicated as a-e, and at each time an NK assay was performed. The values obtained at each ratio per leukopheresis was averaged together to derive a mean value for the patient. These averages were calculated in with the other NK values to obtain the mean for each ratio in the series. The mean values of ± S.E.M. at each ratio for patient 1, a-e are: (5:1) 3.96 ± 5.98, (10:1) 4.57 ± 5.40, (25:1) 13.10 ± 12.08, (50:1) 13.58 ± 13.93 and (100:1) 14.3 ± 14.68.*
Table 3

Natural Killer Cell Activity in Patients with Non Hairy Cell Hematological Disorders and Normal Individuals

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Clinical Diagnosis</th>
<th>Source of cell</th>
<th>Effector to Target Ratio (% Lysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Incidental splenectomy</td>
<td>spleen</td>
<td>10:1 31:1 5:1 10:1 25:1 50:1 100:1</td>
</tr>
<tr>
<td>2</td>
<td>Non Malignant Disease</td>
<td>spleen</td>
<td>15:1 31:1 58:1 73:1 79:1</td>
</tr>
<tr>
<td>3</td>
<td>Idiopathic thrombocytopenic purpura</td>
<td>spleen</td>
<td>7:1 14:1 29:1 51:1 69:1</td>
</tr>
<tr>
<td>4</td>
<td>Non Hodgkins lymphoma</td>
<td>spleen</td>
<td>3:1 7:1 15:1 27:1 46:1</td>
</tr>
<tr>
<td>5</td>
<td>Non Hodgkins lymphoma</td>
<td>spleen</td>
<td>4:1 4:1 10:1 22:1 36:1</td>
</tr>
<tr>
<td>6</td>
<td>Myeloproliferative disorder</td>
<td>spleen</td>
<td>10:1 17:1 34:1 57:1 75:1</td>
</tr>
<tr>
<td>7</td>
<td>Normal laboratory volunteers</td>
<td>peripheral</td>
<td>11:1 17:1 28:1 36:1 64:1</td>
</tr>
</tbody>
</table>

Mean Values + S.E.M.  
8.5±4.19 15±9.48 29.2±15.42 42±18.8 62.7±15

*This represents the mean value of 4 different laboratory volunteers.*
differences between patients with HCL and controls were significant at the level \( p < 0.01 \). Data are presented in Table 4 from patients 7, 8, 9 and 10 from Table 2 whose freshly isolated cells were incubated with IL-2 in vitro for 3 days. In patients 7, 8, 9 and 10 the mononuclear cells from the peripheral blood or spleen had virtually no activity prior to culture (Table 2) or after culture in the absence of IL-2 (Table 4). However, there was a marked increase in NK-like activity following incubation with IL-2. No differences in activity between the untreated and IL-2 treated mononuclear cells were noted at an effector to target cell ratio of 5:1 in patient 7, and only a small difference in patients 9 and 10. In contrast to the untreated cells, however, there was a stepwise increase in cytolysis of the treated cells with progressively higher effector to target cell ratios. These differences could best be appreciated at a 100:1 ratio where there was only 5% cytolysis for the untreated cells and 25% for the IL-2 treated cells in patient 7, 0.8% compared to 17% in patient 9, and −1.1% compared to 21% in patient 10. In patient 8, mononuclear cells were obtained from the spleen and as noted in Table 2, this patient originally had little NK activity. As can be seen from Table 4, after 3 days in culture, the untreated cells showed only a modest degree of activity, but the IL-2 treated cells demonstrated a dramatic increase in NK-like activity. At all effector to target cell ratios there was a marked increase in cytolysis by the IL-2 generated killer cells. For example, at a ratio of 5:1, the untreated cells exhibited a 1% kill, whereas the treated cells had a 14.5% kill; and at 10:1 a 2.5% lysis compared to 35% lysis by IL-2 treated cells. In this particular experiment the cells were
### Table 4
The Effect of IL-2 on Mononuclear Cells Obtained from HCL Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Source</th>
<th>Control</th>
<th>IL-2 Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Of Cell</td>
<td>5:1</td>
<td>10:1</td>
</tr>
<tr>
<td>7a</td>
<td>Peripheral</td>
<td>0.7</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Peripheral</td>
<td>0.25</td>
<td>-1.2</td>
</tr>
<tr>
<td>8b</td>
<td>Spleen</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td>9a</td>
<td>Peripheral</td>
<td>-0.30</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Cells were incubated in the presence of 10% IL-2 diluted in RPMI 1640 media supplemented with 5% human heat inactivated AB serum.

\(^b\) Cells were treated as above with the exception that 5% heat inactivated fetal bovine serum was used instead of human AB serum.
cultured in the presence of 5% fetal bovine serum. This is important because it has been suggested that FBS can cause or enhance non-specific cytolysis (5). However, even if this were the case, the IL-2 treated cultures still showed enhanced activity when compared to the original and untreated cultured mononuclear cells. Cells of patients 7, 9 and 10 were cultured in 5% human AB serum, which is not thought to cause or enhance non-specific killing.

The ADCC values with the corresponding NK levels from 3 HCL patients are displayed in Table 5. Patient 1, as previously described, underwent repeated leukophereses and as can be seen, had a marked increase in ADCC activity, as compared to the NK levels. The ADCC values of patient 1 approach the values obtained from normal laboratory volunteers seen in Table 6. This particular patient was not monocytopenic, which is in contrast to the majority of HCL patients, and since HRBC have been reported to be lysed by monocytes (61) this observation is not surprising. In patients 2 and 3, there was also marked ADCC activity as compared to the NK levels. Although we were unable to determine whether or not the patients were monocytopenic, the level of ADCC activity was not surprising since Braylan et al (79) have reported that monocytes are often sequestered in the spleens of HCL patients. Comparing these splenic ADCC values to the ones depicted in Table 6, are difficult due to the variation that is seen in the 4 patients who had a non hairy cell hematological disorder.
Table 5

ADCC Activity in Patients with Hairy Cell Leukemia

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Source of Cells</th>
<th>Effector to Target Cell Ratio (% lysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5:1</td>
</tr>
<tr>
<td>1a</td>
<td>Peripheral blood a'</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(0.6)b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Spleenc</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.5)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Spleenc</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.19)</td>
<td></td>
</tr>
</tbody>
</table>

a This patient underwent repeated leukophereses indicated as a-c and at each time an ADCC and NK assay was performed.

b ( ) indicates the NK value found.

c The viability of the splenic mononuclear cells were 68 and 71% respectively.
## Table 6

ADCC Activity in Patients with Non Hairy Cell Hematological Disorders and Normal Individuals

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Clinical Diagnosis</th>
<th>Source of Cells</th>
<th>Effector to Target Ratio (% lysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5:1</td>
</tr>
<tr>
<td>1</td>
<td>Incidental splenectomy</td>
<td>Spleen</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(10)a</td>
</tr>
<tr>
<td>2</td>
<td>Nonmalignant disease</td>
<td>Spleen</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(15)</td>
</tr>
<tr>
<td>3</td>
<td>Idiopathic thrombocytopenic purpura</td>
<td>Spleen</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(7)</td>
</tr>
<tr>
<td>4</td>
<td>Lymphoma</td>
<td>Spleen</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3)</td>
</tr>
<tr>
<td>5</td>
<td>Normal lab volunteers</td>
<td>Peripheral blood</td>
<td>7+3.0</td>
</tr>
<tr>
<td></td>
<td>(Mean value + S.E.M. of 4 individuals)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a( ) indicates the NK value found
Discussion

We have shown that natural killer cell activity from both the peripheral blood and splenocytes of HCL patients was extremely low. This deficiency was more pronounced when the data were compared to NK values obtained from spleens of non-HCL patients. Perhaps more importantly, we have shown that IL-2 can partially restore in vitro NK-like activity of mononuclear cells from four patients who were studied. It was also shown that ADCC values in 3 patients studied, were near normal levels.

While our work was in progress, several other recent studies also have documented that there is a marked decrease in NK values for hairy cell leukemia patients. Hersh et al. (80) noted a low level of NK activity in the peripheral blood of several HCL patients. It also was noted by these investigators that the lower the activity, the poorer the prognosis. At the present time all but two of the patients in our series are still alive. Ruco et al. (81) have reported that NK activity was virtually absent in splenocytes from 3 HCL patients. It was further reported that NK activity of peripheral blood cells of 10 HCL patients was low (82). In this latter study, it was shown that the hairy cell did not dilute out with the NK effector cells. This conclusion was based on data from 4 patients who had less than 5% circulating hairy cells but who still exhibited a low NK response. The possibility that this was due to NK suppressor cells in the peripheral blood was ruled out (82). Cells from 3 of these patients showed enhanced NK activity after a 3 hour in vitro incubation with 1000 units
of fibroblast interferon (82). This finding is in contrast to that observed in patients with late stage chronic lymphocytic leukemia (CLL) where natural killer cell activity was not enhanced following exposure to interferon (50). NK activity, however, was enhanced by interferon in patients who were in the early stages of CLL (50).

In a more recent report, Smith et al (83) found that out of 11 HCL patients studied, 4 had the normal percentage of NK cells, as defined by morphology and monoclonal antibody analysis. However, these cells were unable to lyse K-562 cells (83). Five other patients had a combined deficiency in both NK cells and function, and the remaining 2 were normal with respect to both the NK cell number and function. These observations suggest that in at least some patients there may be a mechanism present to prevent effective functional activity of NK cells. A similar situation is seen in patients with the Chediak-Higashi syndrome (45).

We would like to suggest that the spleen may exert a regulatory effect on NK activity. Since it has been shown that splenic NK cells are primarily localized in the red pulp (9), and that hairy cells disrupt the microenvironment of this region, it can be hypothesized that this pathological change might produce a dysfunction in NK homeostasis. Ruco et al (81) have suggested a similar hypothesis. They noted that in some cases normal hemopoiesis was restored in HCL patients following splenectomy and that 2 of 10 patients experienced a substantial increase in NK activity following splenectomy. This increase was associated with the restoration of normal hemopoiesis.
Although it has been established that interferon can augment NK cell activity (19-23), it only recently has been reported that IL-2 also can enhance NK cytolysis and generate lymphokine-activated killer (LAK) cells (29-40). Clinical trials with IL-2 in AIDS patients and in one child with Nezelof's syndrome indicate that the lymphokine has some in vivo effect on T cells (53). Also IL-2 has been shown to either restore or enhance NK activity in vitro in patients who had systemic lupus erythematosus or AIDS (46,48,49).

In our study, IL-2 was unable to augment NK activity in either a 4 or 12 hour assay. But rather we saw a restoration of NK-like activity after 3 days of continuous incubation with IL-2. This is in contrast to the effect that IL-2 has on normal NK cells. Domzig et al (33) have found that partially purified IL-2 could enhance NK activity in a 4 hour assay. This augmentation could take place with only a small amount of IL-2, thus suggesting to the investigators that the NK cells were already "spontaneously active NK cells" (33). This idea was supported by the finding that if IL-2 was removed by monoclonal antibodies, then the same amount of IL-2 would enhance only a low level of NK activity (33). On the other hand, the LAK cells can only be generated from peripheral blood after a 2 to 3 day incubation period with IL-2 (36). These cells, distinct from the NK cells and cytolytic T cells, are able to lyse autologous tumor cells as well as NK resistant target cells (39).

The IL-2 generated killer cells that were observed in this study were not phenotypically examined, so it is not known whether or not we truly restored the "classical" NK cell or in fact, induced another type
of "killer" cell. The antibody dependent cell cytotoxicity assay was performed on the peripheral blood obtained via leukophereses from one patient and on splenic mononuclear cells from two other HCL patients. The target cells for this assay, antibody coated human red blood cells (HRBC), have been reported to be lysed by monocytes (61). In contrast to the results reported by Hersh et al (80), patient no. 1, Table 5, exhibited near normal ADCC values in the 3 times studied as compared to the mean values found in normal laboratory volunteers (Table 6). In the study conducted by Hersh and colleagues, they found that 15 of 20 patients had no ADCC activity against HRBC and less than 5% activity was observed in the other patients. This discrepancy can perhaps be explained by the monocytopenia that was found in Hersch's patients, while the patient in our study was not monocytopenic. The splenic mononuclear cells in the other two cases had moderate ADCC activity as compared to the non hairy cell leukemia controls in Table 6. Hersch et al did not look at ADCC activity mediated by splenic mononuclear cells, nor has anyone else as far as we know, so comparison with results obtained from another laboratory is not possible. In any event, it is not surprising to find ADCC activity in spleens from HCL patients since it has been suggested that they selectively sequester monocytes (79). The wide variation seen in the non hairy cell leukemia spleens (Table 6) makes it difficult to state whether or not ADCC activity in the HCL spleens are within the normal range. Our small sample size also is prohibitive in allowing us to draw any firm conclusions regarding ADCC activity in HCL patients.
Infections are the primary cause of morbidity and mortality in HCL patients (62-65). These infections have been attributed to monocytopenia, pancytopenia or to a cell mediated immune dysfunction. Since it has been shown that NK cells are cytotoxic against Cryptococcus neoformans, cytomegalovirus, Listeria monocytogenes, Herpes virus and other pathogenic organisms (84-87), it can be hypothesized that an NK cell defect could contribute to the increased susceptibility of patients with HCL to opportunistic infections. Based on the apparent augmentation of NK activity in vitro, it might be of interest to initiate a clinical trial with IL-2 in patients with hairy cell leukemia. Promising results with alpha interferon have been reported in HCL patients (88), suggesting that biological response modifiers may have an important role in the treatment of this disease.
CHAPTER VI

THE EFFECT OF SUPERNATANT GENERATED FROM THE JOK-1 HAIRY CELL LEUKEMIA DERIVED CELL LINE AND THE IRRADIATED JOK-1 CELLS ON MITOGEN INDUCED LYMPHOCYTE PROLIFERATION
Infection, which frequently are encountered in lymphoid malignancies, have often been attributed to nonspecific immunosuppression. Factors that have been thought to cause immunosuppression are prostaglandins (1), viruses (2), suppressor cells (1), tumor produced hormones and soluble factors (3,4,5).

Soluble suppressor factors have been reported to be released by both tumor cells, as well as host cells responding to the influence of the tumor (1). Such soluble factors have been found to be released by both cultured and fresh leukemic cells as well as by established lymphoid cell lines. These factors generally have been detected by measuring the ability of the supernatant to inhibit mitogen induced lymphocyte proliferation (6,7,8).

Hairy cell leukemia (HCL), a malignant proliferation of hemopoietic cells of uncertain origin, is associated with pancytopenia and infections. It has been hypothesized that the cell pathognomonic for this disease can suppress normal hemopoiesis as well as the stimulatory capacity of normal B cells (9). If further has been suggested that hairy cells can suppress T cell proliferation. To further investigate this hypothesis, we have added either cells or supernatant generated from the JOK-1 cell line to the mitogen induced human lymphocyte proliferation assay in order to assess whether or not an inhibitory factor was present. This cell line was derived from a patient with hairy cell leukemia, and it has been described as a "pre-hairy" cell leukemia cell line (10). In this chapter preliminary
data are presented that indicate that both JOK-1 cells and their supernatant can inhibit mitogen induced lymphocyte proliferation.
Materials and Methods

Leukemic Cell Lines. The JOK-1 cell line was obtained from Dr. Jun Minowada and maintained at 37°C, 5% CO₂ and 95% air in RPMI 1640 supplement with 5% fetal bovine serum (FBS), 10,000 units/ml of penicillin and 10,000 mcg/ml of streptomycin (complete media). The HL-60, K-562 (kindly provided by OSU Cell Service), YAC-1 (kindly provided by Dr. John Hughes), IS, RE and WB-25 (established in our laboratory) were maintained in a similar fashion. Cells were split and media was changed every 3 days. The JOK-1 cells had a doubling time of every 16-18 hours and reached a saturation density at approximately 2.5 x 10⁶ cells/ml.

Cell Preparation. JOK-1 cells which were to be used in the mitogen assay, were irradiated with 60 Gy at the rate of 1 Gy/min. They were placed in the microtiter plate at the desired cell number.

Generation of JOK-1 Supernatant. Supernatants were collected from the JOK-1 cell line at confluency, in logarithmic growth and 24 hours after the addition of fresh media. The collected supernatant was centrifuged at 4000 rpm for 45 min and then immediately used in the assay or stored at 4°C.

Mitogen Assay. Human mononuclear cells were obtained from the peripheral blood of normal laboratory volunteers by means of Histopaque (Sigma, St. Louis, MO 1077-1) gradient centrifugation. The lymphocytes
were washed 3 x in PBS and resuspended at the desired concentration in complete media. The lymphocytes in a volume of 200 μl was added to a 96 well microtiter plate (#3596 Costar, Cambridge, MA). PHA-M (Difco, Detroit, MI #0528-57) and Con-A (Difco, Detroit, MI #3351-57) were added at the final concentration of 10 μg. PWM (Sigma, St. Louis, #L-9379) was added at the final concentration of 2.5 μg. All three were added in a volume of 10 μl. After the addition of mitogens, either the JOK-1 cells at the desired cell number or JOK-1 supernatant at the desired volume was added. The plate then was incubated for 3 days at 37°C, 5% CO₂ and 95% air. Sixteen hours prior to the termination of the incubation period, 1 μCi of either ³H-thymidine (Amersham Sp. Act. 43 Ci/mmol) or ³H-lysine (ICN, Irvine, Calif. Sp. Act. 40-60 Ci/mmol) was added. At the end of 72 hrs the cells were harvested on a Skatron cell harvester (Skatron Inc, Sterling, Va.) onto glass fiber filtermat (Skatron #7031). The filtermat was allowed to dry, and the disks were placed into mini-vials and then scintillation cocktail (Research Products International Corp., Mount Prospect, IL #3a20) was added. The vials were counted on a Beckman LS 7800 scintillation counter (Beckman, Irvine, Calif).

**Viability Determination.** The viability of the lymphocytes after exposure to either the JOK-1 cells or supernatant was assessed by trypan blue exclusion.
Results

The effects of the supernatants from 8 confluent cultures are shown in Table 1. Inhibition of mitogen induced lymphocyte proliferation was observed in supernatants from the JOK-1, YAC-1, K-562 and HL-60 leukemic cell lines, while augmentation of proliferation was seen in the lymphoblastoid cell lines RE and IS. This augmentation was most apparent in the unstimulated and in the Con-A treated cells.

The potency of this factor is illustrated in Table 2. As can be seen, a volume of 5 µl markedly decreased lymphocyte proliferation; at the volume of 20 µl and 50 µl there was very little difference in the potency of the factor. When the factor was diluted in fresh complete media to the titer of 1:100, the inhibitory factor can no longer exert its effect. The data suggest that the effect of the factor was concentration dependent. It also can be seen from this table that 10% IL-2 (Cellular Products, Buffalo, N.Y.) could not reverse the inhibitory effect. Table 3 shows that this factor did not affect the uptake of lysine, although uptake in the Con-A treated cells was slightly lower than the controls. This indicates that cell viability was unaffected by the inhibitory factor. Also in support of this, viability, as determined by trypan blue exclusion, was greater than 90%.

Table 4 illustrates that supernatants taken from JOK-1 cells freshly seeded at different cell concentrations 24 hours earlier, were also able to inhibit lymphocyte proliferation. The data shown here imply that JOK-1 cells do not need to be confluent in order to secrete
the factor. Cells which were seeded at the concentrations of $1.36 \times 10^5$ cells/ml and $5 \times 10^5$ cells/ml, produced enough factor in 24 hours to significantly inhibit lymphocyte proliferation with supernatants from the latter cell concentration, having the greatest effect. In Table 5, it is shown that this factor, in a 96 hour mitogen assay, can inhibit proliferation up to 48 hours after the addition of mitogen. There is no difference in the inhibitory activity of the factor when added at time zero and at 24 hours after the addition of mitogens. There is markedly less inhibition when added 48 hours after mitogen, and no inhibition after 72 hours. Table 7 shows that a small number of irradiated JOK-1 cells can inhibit lymphocyte proliferation as well. It can be hypothesized from this table that only a relatively small number of cells is required for factor production.
Discussion

Preliminary data, presented in this chapter, show that the JOK-1 hairy cell leukemia derived cell line produced a factor(s) that inhibited PHA, Con-A and PWM induced lymphocyte proliferation. These observations give credence to the hypothesis that a factor in HCL may be responsible for the poor mitogen induced lymphocyte proliferation as well as the disruption of normal hemopoiesis (9).

Davey et al (9) and other investigators (11) have demonstrated that mononuclear cells from both the peripheral blood and spleen responded poorly to mitogen stimulation; however, once separated, the T cells responded normally to PHA, whereas the non-T-cell fraction only responded to PWM and Con-A if allogeneic monocytes were added. From these data, Davey (9) concluded that hairy cells may either suppress or fail to stimulate a proliferative response in T cells, or alternatively render B cells incapable of appropriately stimulating T cells. In support of this, our preliminary data on the JOK-1 cells show that the irradiated cells not only will inhibit lymphocyte proliferation but will also fail to stimulate proliferation. These observations were surprising since the JOK-1 cell was strongly Ia positive and investigators have reported that leukemia cells can be stimulatory in a mixed lymphocyte reaction (MLR) (12). However, the JOK-1 cell is an Epstein Barr nuclear antigen positive cell line with B cell characteristics (sIgM, B-1, Ia, S-HCL-1, see chapter III), and it has been reported that the ability of B cells to stimulate in an MLR probably depends on their activation state. It has been reported that
EBV transformed B cells can stimulate autologous T cells to proliferate and that EBV positive established lymphoid cell lines can present soluble antigens. David (13) has stated that B cells are not good stimulators in an MLR unless they are pretreated with neuraminidase. Similarly Crow et al (14) have reported that mitogen activated B cells are better stimulators than resting B lymphocytes. It also was noted that a decrease in surface immunoglobulin was associated with an increase in the ability to stimulate in an MLR. In our study, JOK-1 cells failed to stimulate in an MLR after treatment with neuramidase as well as after induction with DMSO and TPA (data not shown). Since we have shown that JOK-1 cells lose their sIgM after DMSO and TPA treatment, it was somewhat surprising in view of Crow's work that we failed to observe stimulation. However, our cell line may be considered a relatively immature B cell line that may be hypostimulatory under any condition.

In contrast to inhibitors of proliferation secreted by other lymphoid cell lines, our inhibitory substance can be produced by proliferating cells. This is an important observation that suggests that the production of the factor can be produced in a situation where the metabolic stress on the cell is not as great as would be expected in a stationary culture. In a stationary culture it is possible that a normal degradation product of the cell line may inhibit proliferation. However, our observation tends to suggest that the factor is intrinsically produced by the cell line, and not artificially produced. This factor was specific in that it did not block the in vitro proliferation of HL-60 and K-562 cells. It also was observed that this
factor did not provide autostimulation for the JOK-1 cells, which is in
contrast to reports which state that many leukemia cell lines secrete
factors that promote "self" growth when the cells are seeded at low
densities (15,16). Nor did the factor inhibit JOK-1 growth. Other
investigators have reported that inhibitory factors released from other
lymphoid cell lines failed to inhibit growth of that particular line
(8,17). Our factor can be added up to 48 hours after the addition of
mitogen and still cause a significant reduction of proliferation. Hersh
et al (8) have stated that the inhibitory factor released from their
lymphoid cell line must be present for at least 24 hours before
inhibition can occur. Other investigators have concurred (17). It
also has been suggested that cells in their proliferative phase are to
to a certain degree resistant to the action of the inhibitor (8,17).

The JOK-1 inhibitor as illustrated by our data, inhibits the
uptake of $^3$H-Thymidine, but not $^3$H-lysine. Other inhibitors from
lymphoid cell lines have been shown to inhibit both DNA and protein
synthesis or to selectively inhibit DNA replication (8,17). As with
other inhibitors, the JOK-1 inhibitor appears to be non-toxic
(4,6,8,17).

In studies conducted with other lymphoid and leukemic cell lines
(Table 1) inhibition of mitogen induced lymphocyte proliferation was
observed in supernatants from the JOK-1, YAC-1, K-562, and HL-60 cell
lines, while augmentation of proliferation was seen in the EBV positive
cell lines RE and IS. This augmentation was most apparent in the
unstimulated and in the Con-A treated cells. It can be hypothesized
that this apparent enhancement may either be due to the production of
the EBV virus by these cells or by the ability of these cells to
stimulate in a one way MLR (12). The virus has been shown to induce a
mitogen-like proliferation in human lymphocytes (18). It was not
surprising that YAC-1, K-562 and the HL-60 cell lines inhibited
lymphocyte proliferation. Investigators have shown that supernatant
generated from the YAC-1 Lymphoma cell line can inhibit the
proliferative response of murine splenocytes to both Con-A and
allogeneic cells (19). Other workers have demonstrated that the K-562
cell line can suppress normal bone marrow colony formation (20). The
fact that the promyelocytic cell line HL-60 inhibited lymphocyte
proliferation was expected since the human histiocytic lymphoma cell
line, U-937 has been reported to do the same (21). No marked effect
was observed with the WB-25 cell line.

Due to the preliminary nature of our studies, we cannot speculate
on the mechanism of action of our factor. Other investigators even
after extensive biochemical studies, have failed to find a concrete
mechanism for the inhibitory nature of their factors (3,22). Recently
however, it has been shown that gangliosides that are shed from the
YAC-1 lymphoma cell line, had the ability to inhibit Con-A induced
lymphocyte proliferation (19). Merritt et al (23) has suggested that
ganglioside inhibition of mitogen induced proliferation may be due to
the inhibition of interleukin-2 stimulated proliferation of T-cells.
This is an interesting possibility and if this were to be the case in
our system, then it would have to occur by a mechanism that does not
inhibit the expression of the IL-2 receptor, because we have found that
this factor does not decrease the expression of the TAC antigen or IL-2 receptor in PHA stimulated lymphoblasts.

The effects that the JOK-1 supernatant and cells have on human lymphocyte proliferation closely resembles the \textit{in vivo} situation and thus may serve as a model in which to study the immune defects that are frequently seen in this disease.
Table 1
The Effect of Supernatants Obtained from Leukemic Cell Cultures on Mitogen Induced Lymphocyte Proliferation as Measured by \( ^{3}\text{H}-\text{Thymidine} \) Uptake

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Unstimulated</th>
<th>PHA</th>
<th>Con-A</th>
<th>PWM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2364 ± 126</td>
<td>245982 ± 6597</td>
<td>90564 ± 5907</td>
<td>67171 ± 1400</td>
</tr>
<tr>
<td>JOK-1(^{a})</td>
<td>7668 ± 366</td>
<td>9407 ± 434</td>
<td>9350 ± 590</td>
<td>10496 ± 1065</td>
</tr>
<tr>
<td>WB-25</td>
<td>4796 ± 587</td>
<td>268008 ± 9545</td>
<td>133673 ± 16665</td>
<td>89197 ± 4944</td>
</tr>
<tr>
<td>RE(^{b})</td>
<td>58391 ± 4709</td>
<td>290330 ± 23076</td>
<td>256128 ± 5384</td>
<td>117293 ± 5814</td>
</tr>
<tr>
<td>IS(^{b})</td>
<td>59544 ± 11143</td>
<td>253930 ± 7721</td>
<td>184404 ± 21681</td>
<td>112528 ± 1350</td>
</tr>
<tr>
<td>YAG-1(^{a})</td>
<td>3691 ± 709</td>
<td>6280 ± 3563</td>
<td>7261 ± 553</td>
<td>5991 ± 1345</td>
</tr>
<tr>
<td>HL-60(^{a})</td>
<td>7910 ± 999</td>
<td>15474 ± 922</td>
<td>6338 ± 851</td>
<td>5992 ± 638</td>
</tr>
<tr>
<td>K-562(^{a})</td>
<td>6251 ± 1478</td>
<td>10658 ± 1210</td>
<td>9175 ± 402</td>
<td>6910 ± 3440</td>
</tr>
</tbody>
</table>

50 microliters of supernatant from each cell line were added to individual wells of the microtiter plate.

\(^{a}\)The inhibition was significant at \( p<0.05 \) using a two tailed student's \( t \)-test.

\(^{b}\)The enhancement of the unstimulated, Con-A and PWM was significant at \( p<0.05 \)
### Table 2
The Effect of Different Concentrations of JOK-1 Supernatant on Mitogen Induced Lymphocyte Proliferation as Measured by $^3$H-Thymidine Uptake

<table>
<thead>
<tr>
<th>Row</th>
<th>Unstimulated</th>
<th>PHA</th>
<th>Con-A</th>
<th>PWM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4369 ± 367</td>
<td>253928 ± 6860</td>
<td>68907 ± 14052</td>
<td>38044 ± 4464</td>
</tr>
<tr>
<td>5</td>
<td>4013 ± 2161</td>
<td>52966 ± 2811</td>
<td>24534 ± 4106</td>
<td>17620 ± 260</td>
</tr>
<tr>
<td>10</td>
<td>5598 ± 144</td>
<td>16675 ± 1272</td>
<td>16670 ± 3308</td>
<td>19306 ± 1069</td>
</tr>
<tr>
<td>20</td>
<td>5025 ± 2554</td>
<td>12320 ± 909</td>
<td>11846 ± 3701</td>
<td>11514 ± 870</td>
</tr>
<tr>
<td>30</td>
<td>7083 ± 1009</td>
<td>14890 ± 5031</td>
<td>10746 ± 2121</td>
<td>13202 ± 954</td>
</tr>
<tr>
<td>50</td>
<td>7454 ± 2493</td>
<td>10572 ± 2081</td>
<td>8839 ± 609</td>
<td>10121 ± 625</td>
</tr>
<tr>
<td>1:5</td>
<td>4814 ± 2611</td>
<td>27724 ± 920</td>
<td>14801 ± 1470</td>
<td>13651 ± 1402</td>
</tr>
<tr>
<td>1:10</td>
<td>4507 ± 560</td>
<td>65093 ± 4782</td>
<td>19022 ± 3887</td>
<td>17367 ± 288</td>
</tr>
<tr>
<td>1:20</td>
<td>4324 ± 654</td>
<td>66796 ± 1880</td>
<td>36299 ± 6461</td>
<td>25930 ± 2514</td>
</tr>
<tr>
<td>1:50</td>
<td>12932 ± 5135</td>
<td>159498 ± 2516</td>
<td>52841 ± 2834</td>
<td>40427 ± 1550</td>
</tr>
<tr>
<td>1:100</td>
<td>5799 ± 3866</td>
<td>288575 ± 14598</td>
<td>93557 ± 14627</td>
<td>40169 ± 3802</td>
</tr>
<tr>
<td>1:500</td>
<td>11144 ± 7635</td>
<td>285042 ± 8419</td>
<td>64798 ± 3549</td>
<td>31961 ± 3896</td>
</tr>
<tr>
<td>1:1000</td>
<td>979 ± 223</td>
<td>280344 ± 9809</td>
<td>56457 ± 8042</td>
<td>43426 ± 4205</td>
</tr>
<tr>
<td>50</td>
<td>17764 ± 13043</td>
<td>9926 ± 1566</td>
<td>20489 ± 11376</td>
<td>10953 ± 1704</td>
</tr>
</tbody>
</table>

*a The volume added to each well from a confluent JOK-1 culture.

*b The dilution of the supernatant.

*c Inhibition was significant at p<0.05.
Table 3
The Effect of $^3$H-Lysine Uptake in Mitogen Induced Lymphocyte Proliferation by JOK-1 Supernatant

Mean Values of Triplicates + S.E.M.

<table>
<thead>
<tr>
<th>Row</th>
<th>Unstimulated</th>
<th>PHA</th>
<th>Con-A</th>
<th>PWM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2836 ± 486</td>
<td>108812 ± 6923</td>
<td>31303 ± 2122</td>
<td>11821 ± 1045</td>
</tr>
<tr>
<td>50 μl</td>
<td>2844 ± 146</td>
<td>115804 ± 5456</td>
<td>20422 ± 2056</td>
<td>10423 ± 1123</td>
</tr>
<tr>
<td>1:10</td>
<td>2403 ± 823</td>
<td>120681 ± 6205</td>
<td>16334 ± 3344</td>
<td>11131 ± 1646</td>
</tr>
<tr>
<td>1:50</td>
<td>3111 ± 436</td>
<td>101397 ± 5436</td>
<td>24041 ± 1016</td>
<td>10385 ± 926</td>
</tr>
<tr>
<td>1:100</td>
<td>5711 ± 603</td>
<td>115852 ± 4362</td>
<td>18000 ± 1803</td>
<td>12206 ± 1045</td>
</tr>
<tr>
<td>1:1000</td>
<td>2679 ± 363</td>
<td>111844 ± 7015</td>
<td>19290 ± 926</td>
<td>16826 ± 1916</td>
</tr>
</tbody>
</table>
### Table 4

The Effect of Supernatant from Freshly Seeded JOK-1 Cells on $^3$H-Thymidine Uptake in Mitogen Induced Lymphocyte Proliferation

<table>
<thead>
<tr>
<th>Amount added</th>
<th>Unstimulated</th>
<th>PHA</th>
<th>Con-A</th>
<th>PWM</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>797 ± 96</td>
<td>204482 ± 6960</td>
<td>20669 ± 4035</td>
<td>40481 ± 4265</td>
</tr>
<tr>
<td>$a_{5 \mu l}$</td>
<td>2454 ± 125</td>
<td>48080 ± 2050</td>
<td>13814 ± 2011</td>
<td>10559 ± 857</td>
</tr>
<tr>
<td>$a_{50 \mu l}$</td>
<td>10318 ± 909</td>
<td>16202 ± 1606</td>
<td>9194 ± 1012</td>
<td>9017 ± 986</td>
</tr>
<tr>
<td>$b_{5 \mu l}$</td>
<td>4168 ± 625</td>
<td>10685 ± 1234</td>
<td>5343 ± 686</td>
<td>7816 ± 432</td>
</tr>
<tr>
<td>$b_{50 \mu l}$</td>
<td>5570 ± 903</td>
<td>11785 ± 3707</td>
<td>6899 ± 926</td>
<td>9751 ± 1045</td>
</tr>
<tr>
<td>$c_{5 \mu l}$</td>
<td>3128 ± 263</td>
<td>13397 ± 4216</td>
<td>6653 ± 643</td>
<td>6922 ± 965</td>
</tr>
<tr>
<td>$c_{50 \mu l}$</td>
<td>6730 ± 360</td>
<td>12658 ± 3256</td>
<td>4793 ± 426</td>
<td>8366 ± 1114</td>
</tr>
<tr>
<td>$d_{5 \mu l}$</td>
<td>2960 ± 195</td>
<td>34122 ± 5092</td>
<td>7657 ± 1012</td>
<td>13936 ± 1246</td>
</tr>
<tr>
<td>$d_{50 \mu l}$</td>
<td>4000 ± 400</td>
<td>19465 ± 3262</td>
<td>7261 ± 726</td>
<td>9373 ± 842</td>
</tr>
</tbody>
</table>

$^a$Cells were seeded at $1.36 \times 10^5$ cells/ml and the supernatant was harvested at 24 hr and stored at -80°C until use.

$^b$Cells were seeded as in (a), but harvested 72 hr later.

$^c$Cells were seeded at $5 \times 10^5$ cells/ml and the supernatant was harvested at 24 hr and stored at -80°C until use.

$^d$Cells were seeded as in (c) but harvested 72 hr later.
Table 5

The Effects of Time on the Inhibition of Lymphocyte Proliferation by JOK-1 Supernatant. As measured by $^3$H-Thymidine in a 96 hour Assay

<table>
<thead>
<tr>
<th>Row</th>
<th>Unstimulated</th>
<th>PHA</th>
<th>Con-A</th>
<th>PWM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1297 ± 126</td>
<td>268720 ± 6892</td>
<td>84252 ± 10145</td>
<td>67892 ± 8062</td>
</tr>
<tr>
<td>5 µl</td>
<td>2374 ± 412</td>
<td>11905 ± 986</td>
<td>6364 ± 986</td>
<td>5309 ± 202</td>
</tr>
<tr>
<td>5 µl(24)$^a$</td>
<td>1553 ± 536</td>
<td>11611 ± 978</td>
<td>4176 ± 245</td>
<td>5101 ± 1470</td>
</tr>
<tr>
<td>5 µl(48)$^a$</td>
<td>2883 ± 124</td>
<td>48885 ± 4126</td>
<td>10220 ± 565</td>
<td>10502 ± 2245</td>
</tr>
<tr>
<td>5 µl(72)$^a$</td>
<td>1949 ± 210</td>
<td>310486 ± 7910</td>
<td>54395 ± 1096</td>
<td>67973 ± 4262</td>
</tr>
</tbody>
</table>

$^a$Indicates time the factor was added after the addition of mitogen.
Table 6
The Effect of Irradiated JOK-1 Cells on Mitogen Induced Lymphocyte Proliferation as Measured by $^3$H-Thymidine Uptake

<table>
<thead>
<tr>
<th>Row</th>
<th>Unstimulated</th>
<th>PHA</th>
<th>Con-A</th>
<th>PWM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>294 ± 95</td>
<td>107561 ± 8055</td>
<td>ND</td>
<td>15068 ± 348</td>
</tr>
<tr>
<td>$^a1 \times 10^4$</td>
<td>1862 ± 301</td>
<td>2362 ± 176</td>
<td>ND</td>
<td>2450 ± 207</td>
</tr>
<tr>
<td>$^a2 \times 10^4$</td>
<td>2759 ± 601</td>
<td>2523 ± 370</td>
<td>ND</td>
<td>2873 ± 311</td>
</tr>
</tbody>
</table>

$^a$The number of irradiated JOK-1 cells added per microtiter well.

The suppression is significant at $p<0.05$
CHAPTER VII

ESTABLISHMENT OF A HAIRY CELL LIKE LEUKEMIA CELL LINE FROM A PATIENT WITH PRIMARY SPLENIC T CELL LYMPHOMA, AND THE ESTABLISHMENT OF A HAIRY CELL LIKE CELL LINE WITH MONOCYTIC FEATURES FROM A PATIENT WITH B-LYMPHOCYTIC HAIRY CELL LEUKEMIA.
Introduction

Hairy cell leukemia (HCL) is a well-defined clinico-pathological entity (1), the cellular origin of which has yet to be fully determined. The cell pathognomonic for this disease, has been reported to phagocytize latex beads, adhere to glass, contain hydrolytic enzymes, express Fc receptors and have a ruffled membrane. These characteristics, along with the visceral distribution of hairy cells and the associated monocytopenia, have suggested a monocytic origin, (2,3). These cells, however, have been reported to produce endogenous antibody that express multiple isotypes with a single light chain present on the cell surface (4). More recently, hairy cells have been reported to undergo immunoglobulin gene rearrangement and to have receptors for interleukin 2 (5,6). These findings have almost conclusively placed the hairy cell in the B cell lineage despite the aforementioned monocytic features. From these seemingly conflicting observations some investigators have suggested that hairy cells may represent either the proliferation of a new cell type or be a monocyte-lymphocyte hybrid (7).

Clinically it has been postulated that HCL comprises two diseases, one that is primarily manifested in the spleen, and the other in the peripheral blood (8). There also have been reports that at various times throughout the clinical course of the disease, hairy cells have exhibited the features of either B or T cells or both (9). We believe that the hairy cell is responsive to its immediate microenvironment, thereby giving it plasticity that usually is not observed with other
types of leukemic cells (10). In support of this hypothesis, we report the establishment of two phenotypically different hairy cell like leukemia cell lines from the spleen of two patients.

One cell line designated as WB-25 is an EBNA negative B cell line, with hairy cell characteristics that was derived from the spleen of a patient diagnosed as having a primary splenic T cell lymphoma. The characteristics of this cell line, WB-25, which were not seen either in the patient's peripheral blood or spleen, are hair-like projections and positivity for tartrate resistant acid phosphatase (TRAP). Furthermore, WB-25 not only exhibits B cell markers, but also expresses the activated B and T cell marker Tac. The other cell line, SAB was established from a patient who presented with "typical" hairy cell leukemia. Unlike the fresh hairy cells that were phenotypically B lymphocytes, the cell line which was derived from this patient was positive for the following myeloid surface markers, Leu-M1, Leu-M3, anti-monocyte1 and the Ia antigen. A small percentage of the cells also were positive for Tavcand S-HCL-3. Strong positivity for tartrate resistant acid phosphatase (AP) TRAP and PAS was also observed. This line is sensitive to natural killer cell mediated cytolysis as well. A detailed description of these two lines follows.
Case History

A 24 year old white male presented with a six week history of fever and abdominal pain. On physical examination he was found to be thrombocytopenic with petechiae, was jaundiced, and had a spiking fever. There was splenomegaly but no peripheral lymphadenopathy. Pertinent laboratory values were as follows: Hb 9.5 gm/dl; Hct 28%; WBC 3,500/cu.mm; platelets 12,000/cu.mm; and bilirubin to 6 mg/dl. Differential showed 2% myelocytes, 40% segs, 48% lymphocytes and 10% monocytes. Examination of the bone marrow showed erythroid and myeloid hyperplasia with megaloblastic changes in the erythroid series. There was no evidence of lymphoma. A splenectomy was performed. Gross examination of the spleen revealed a homogenous, beefy red surface without nodularity or capsular thickening suggesting that there was diffuse involvement of the red pulp. Histologically, the red pulp showed marked enlargement due to dilatation of the sinusoids and widening of the cords, which were infiltrated by large mononuclear cells (Figure 1a). These had large round to indented vesicular nuclei and usually a single prominent nucleolus. These same cells were found in the liver along the sinusoids. As seen by electron microscopy, the cells had large round to convoluted nuclei and usually a single, prominent nucleolus (Figure 1b). Cytoplasm was scant with few organelles and no cellular attachments or microvilli. These morphologic findings were consistent with a diagnosis of lymphoma. The patient's disease progressed and he died six months later. Autopsy revealed that the lymphoma had entered a leukemic phase with
involvement of multiple organs, including bone marrow, lungs, liver and kidneys. The cell line WB-25 was derived from this patient.

The case history of the patient from which SAB was derived was typical of that of hairy cell leukemia (1). The patient's chart was unavailable for further review.

Cell Culture. A portion of the surgically removed spleen was finely minced in Phosphate Buffered Saline (PBS) pH 7.4. Red cells were lysed by adding 50 ml of tris-ammonium chloride (pH 7.65) to the cell suspension for 2 minutes. The cells were centrifuged, washed 3 times in PBS, and then their concentration was adjusted to $3 \times 10^6$/ml in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS), 10,000 units/ml of Penicillin and 10,000 µg/ml of streptomycin. Twenty-five ml of the cells were seeded into 75 cm² flasks and maintained at 37°C in a humidified incubator with 5% CO₂. After two weeks the cultures, which showed an outgrowth of fibroblast-like monolayers, were split and fresh media was added. Approximately two months later, clumps of cells were noted in one flask, and thereafter the cells were split every 4-5 days for WB-25 and 3-4 days for SAB.

Surface Markers. The following cell surface markers were studied: IgG, IgM, IgA, κ, λ, B-1, MO₁, J-5 (cALLa), OKT-3, OKT-4, OKT-8, OKT-11, Tac, Leu-M1, Leu-M3, anti-monocyte.1, Ia and the formation of E-rosettes. Fluorescein isothiocyanate (FITC) conjugated goat anti-human heavy and burro anti-human light chain antibodies (Kalstead, Austin, Texas) were used in a one step immunofluorescence assay.
Briefly $1 \times 10^6$ cells were incubated with the appropriate dilution of antibody for 30-40 min at 4°C. The cells then were washed 3 times with PBS, fixed with 50% glycerol, mounted on a slide and a minimum of 200 cells were examined with a Zeiss UV FL Epifluorescence microscope. The B-1, MO, J-5, Ia (Coulter Immunology, Hialeah, Florida), OKT-3, OKT-4, OKT-8 and OKT-11 (Ortho Diagnostic Systems Inc. Raritan, NJ), Leu-M1, Leu-M3 (Becton-Dickinson, Mountain View, Calif.) were used in the indirect immunofluorescence test. The anti-Tac antibody was kindly provided by Dr. T. Waldmann, Metabolism Branch, National Cancer Institute. As before, $1 \times 10^6$ cells/ml were incubated with the appropriate dilution of antibody and incubated at 4°C for 30-40 min. Cells then were washed 3 times with PBS and incubated for an additional 30-40 min with FITC-conjugated anti-mouse IgG (Miles Scientific, Naperville, IL). The cells were again washed 3 times with PBS, fixed with 50% glycerol and examined as above.

**Cytogenetics.** For chromosomal analysis, the karyotyping and subsequent interpretation were performed in the Cytogenetics Laboratory of the Department of Pathology.

**Electron Microscopy.** The cells were fixed in 2.5% glutaraldehyde in 0.05M sodium cacodylate and processed for electron microscopy by Mr. Terry McBride of the Department of Pathology.
Results

Cell Lines. The cell line, designated WB-25, grew in a fashion similar to that described by Keusch et al. (11), in that initially biphasic growth was observed. Two cell types were observed, one forming a monolayer that resembled macrophage or fibroblast-like cells, and the other consisting of clumps of cells that appeared to be lymphoid. Only two flasks showed both cell types, and it was from one of these that WB-25 was established. This cell line has been propagated for over two years. Growth is characterized by both free floating cells and aggregates. The saturation density is approximately $1.5 \times 10^6$ cells/ml and the doubling time, 30-40 hours. The cells do not grow in serum depleted medium.

SAB also grew in a similar fashion, however, growth initially was observed occurring in clumps with later cell growth characterized by round single cells with very little clumping. Out of 8 flasks seeded with cells isolated from the spleen, growth was observed in only one. This line has been propagated for more than a year, with the saturation density ranging from 1.5 to $2 \times 10^6$ cells/ml. The doubling time is from 24-36 hours.

Morphology. Figures 2 and 3 demonstrate the morphologic features that are characteristic of WB-25. The most striking observation is the presence of "hairs" or microvillous like projections, although these are not present on all cells. Other noteworthy features, as seen in Figures 2 and 3, are a prominent nucleolus and a large nuclear
cytoplasmic ratio. There is a paucity of endoplasmic reticulum, no discernible Golgi apparatus, numerous mitochondria and a well defined nuclear membrane (Fig. 3). Wright's stain revealed a rather agranular basophilic cytoplasm, with serrated cell borders and prominent tufts or "hairs" (Fig. 2).

SAB is shown in Figure 4. These cells are heterogeneous with respect to size. A prominent nucleolus is sometimes seen. Many of the cells have blebs and vacuoles. Examination by phase contrast microscopy revealed no "hairs" and electron microscopy was inconclusive due to cell destruction by the fixative process.

Membrane Markers. Table 1 summarizes the surface membrane markers that were expressed on the original splenic cells, and the progeny that have been propagated in vitro as a continuous cell line. Perhaps the most important feature of this cell line are the absence of the Epstein Barr Nuclear Antigen (EBNA), and the presence of the Tac antigen. This strongly suggests that WB-25 does not represent an outgrowth of transformed B cells. As can be seen from Table 1, the Ia, B-1 and the J-5 antigens, as well as the kappa light chain, are either newly expressed or found in a higher percentage on WB-25. The κ light chain, B-1, Ia antigen and surface IgM define this as a B lymphocytic line. The acquisition of J-5 should be interpreted with caution, however, since it recently has been described on neutrophils (12). The presence of this antigen suggests that the line may be composed of cells of varying degrees of maturation or B cell differentiation. The presence of the Tac antigen was surprising since it has been reported to be the
Table 1
Phenotypic Markers of Splenic Cells and WB-25

<table>
<thead>
<tr>
<th>Marker</th>
<th>Spleen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>WB-25 Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIgG</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>sIgM</td>
<td>ND</td>
<td>34%</td>
</tr>
<tr>
<td>sIgA</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Total Ig</td>
<td>21%</td>
<td>ND</td>
</tr>
<tr>
<td>λ</td>
<td>7%</td>
<td>0</td>
</tr>
<tr>
<td>κ</td>
<td>15%</td>
<td>80%</td>
</tr>
<tr>
<td>B-1</td>
<td>6%</td>
<td>100%</td>
</tr>
<tr>
<td>J-5</td>
<td>0</td>
<td>25%</td>
</tr>
<tr>
<td>Ia</td>
<td>8%</td>
<td>98%</td>
</tr>
<tr>
<td>E rosettes</td>
<td>91%</td>
<td>0</td>
</tr>
<tr>
<td>OKT 3</td>
<td>57%</td>
<td>0</td>
</tr>
<tr>
<td>OKT 4</td>
<td>3%</td>
<td>0</td>
</tr>
<tr>
<td>OKT 8</td>
<td>35%</td>
<td>0</td>
</tr>
<tr>
<td>OKT 11</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>HC-1</td>
<td>25%</td>
<td>0</td>
</tr>
<tr>
<td>HC-2</td>
<td>18%</td>
<td>51%</td>
</tr>
<tr>
<td>Anti-Tac</td>
<td>ND</td>
<td>33%</td>
</tr>
<tr>
<td>EBNA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>Neg.</td>
</tr>
<tr>
<td>TRAP</td>
<td>Neg.</td>
<td>Positive</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>ND</td>
<td>Positive</td>
</tr>
<tr>
<td>HTLV&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Neg.</td>
<td>Neg.</td>
</tr>
</tbody>
</table>

<sup>a</sup>Epstein Barr Nuclear Antigen

<sup>b</sup>Human T Leukemia Virus

ND designates "not done"
IL-2 receptor and to be present on activated T cells (13). However, it recently has been reported that Tac was expressed on hairy cells that otherwise have B cell markers (5). It has also been found on certain in vitro activated B cells (14).

The monoclonal antibodies HC-1 and HC-2, kindly provided by Dr. David Posnett, recognize membrane antigens expressed on hairy cells. HC-1 appears to be specific for only hairy cells, while HC-2 not only reacts with the majority of hairy cells, but in some instances with myeloblasts or myelomonoblasts from patients with acute non-lymphocytic leukemia (15). Table 1 shows that while both HC-1 and HC-2 were present on splenic cells, only HC-2 was expressed by WB-25.

Fresh splenic mononuclear cells from which SAB was derived, was a predominately B cell population. The cells were positive for IgG (77%), IgA (48%), κ (38%) and λ (77%). The kappa light chain, however, was only faintly expressed. The cells that constitute the SAB cell line are negative for both heavy and light chains and are positive for Leu-M1 (77%), Leu-M3 (26%), anti-monomocyte.1 (25%) and the Ia antigen (23%); all of these markers define SAB as belonging in the myeloid lineage. A small percentage of the cells, 2-12%, are positive for both Tac and S-HCL-3 (kindly provided by Dr. Roland Schwarting). S-HCL-3 is a monoclonal antibody that recognizes hairy cells as well as monocytes. In parallel, with S-HCL-1 positivity, a B cell marker, S-HCL-3 positivity can be diagnostic of HCL (16). However, SAB was negative for S-HCL-1. This data is summarized in Table 2.

The isoenzyme that perhaps is most characteristic of HCL, tartrate resistant acid phosphatase (TRAP), was demonstrated in WB-25 (Figure 5)
Table 2
Phenotypic Markers of Splenic Cells and SAB

<table>
<thead>
<tr>
<th>Marker</th>
<th>Spleen</th>
<th>SAB Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIgG</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>sIgM</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sIgA</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>( \lambda )</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>( \kappa )</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>Leu-M1</td>
<td>ND</td>
<td>77</td>
</tr>
<tr>
<td>Leu-M3</td>
<td>ND</td>
<td>26</td>
</tr>
<tr>
<td>Anti-monocyte.1</td>
<td>ND</td>
<td>25</td>
</tr>
<tr>
<td>Ia</td>
<td>ND</td>
<td>23</td>
</tr>
<tr>
<td>Tac</td>
<td>ND</td>
<td>2-12</td>
</tr>
<tr>
<td>S-HCL-1</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>S-HCL-3</td>
<td>ND</td>
<td>2-12</td>
</tr>
<tr>
<td>TRAP</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>positive</td>
<td>positive</td>
</tr>
</tbody>
</table>
but was not evident in cells derived from the fresh spleen. The pattern of acid phosphatase positivity was polar (Figure 7), which is similar to that of T cells. This isoenzyme also was present in SAB (Figure 6) and in contrast to WB-25, also was seen in the patient's spleen. SAB is negative for non-specific esterase but is PAS positive.

The chromosome complement of WB-25 was normal, 46, X, Y (Figure 7). However, SAB is hyperdiploid (Figures 8 and 9). The trisomy 12 present is sometimes seen in HCL and CLL.
Discussion

There have been two major problems in trying to define the nature and origin of HCL, failure to obtain a homogeneous population of fresh hairy cells and the inability to obtain an in vitro cell line that consists of true biological hairy cells.

The inability to obtain a pure population of fresh hairy cells can be attributed to the difficulty in separating them from lymphocytes and monocytes. All three share enough characteristics as to make this virtually impossible with the currently available methodology. In vitro cell lines that have been established usually are positive for the Epstein-Barr virus, thus making it impossible to determine whether or not it is a hairy cell line or represents the outgrowth of transformed B cells. The isoenzyme TRAP, is not a good in vitro marker because it can be expressed by cord blood lymphocytes following transformation by EBV (17).

We report the establishment of two cell lines with hairy cell like characteristics from two different patients, one with a primary splenic T cell lymphoma and the other from a HCL patient. These lines with emphasis on WB-25, are discussed in context of the immunobiology of hairy cell leukemia.

The hairy cell like line, designated WB-25, was established from a patient diagnosed as having a primary splenic T-cell lymphoma. The distinguishing features of this cell line, which were not noted in either tissue sections or cell suspensions of the original spleen, were positivity for TRAP and microvillous projections. Furthermore, this
line is EBNA negative, and has the IL-2 receptor, thus implying that WB-25 does not represent an outgrowth of EBV transformed B cells. It is noteworthy that the initial tissue diagnosis that this patient carried was primary splenic T cell lymphoma. The diagnosis of hairy cell leukemia was included in the differential because of the gross appearance of the spleen and the distribution of neoplastic cells in the spleen and liver. There was no peripheral blood involvement, abdominal lymph nodes were not enlarged, splenic cells were negative for TRAP, no hair-like projections were observed, and other than myeloid hyperplasia, the bone marrow appeared to be uninvolved. These findings, taken together with the formation of 90% E rosettes and the splenic ultrastructure lead to a diagnosis of primary splenic T cell lymphoma.

This is a particularly important point since there have been several cases reported in which B cell lymphomas initially were diagnosed as HCL (18). There also has been a report of HCL co-existent with histocytic lymphoma (19). Correct diagnosis of these cases has important therapeutic implications since patients with HCL usually respond poorly to chemotherapy, while lymphoma patients may respond favorably.

The demonstration of a specific hairy cell antigen, HC-1, and the in vitro growth of spleen-derived, EBNA negative, Tac positive B cells, with hairy cell characteristics, raise some interesting questions. First, the splenic T cell lymphoma in this patient might have represented a T cell variant of HCL, of which there have been several reported cases (20,21). Second, the patient might have had a T cell
lymphoma that was evolving into HCL, or conversely HCL that was evolving into a lymphoma with the target organ being the spleen. Third, WB-25 could represent the clonal expansion of a previously postulated but undocumented, normal counterpart to the malignant hairy cell. Fourth, and probably the least likely, HCL and the lymphoma could have been two separate but co-existent malignancies. Although it is not possible to unequivocally rule out these alternatives, it is tempting to speculate that the spleen was the site of lymphomatous to leukemic cell transition during the malignant phase of this patient's disease. Such a transition could very well have lead to several phenotypic variants, which only could be expressed under certain micro-environmental conditions. In fact, transformations from one cell type to another are not unusual in hemopoietic malignancies (22). For example, chronic myelogenous leukemia can evolve into acute lymphocytic leukemia, or non-Hodgkin's lymphoma can develop into acute lymphoblastic leukemia. These morphological and immunological transformations, however, may be part of the original malignancy rather than the expression of a new neoplasm (22). The cell line WB-25 conceivably could represent either a phenotypic variant of the original malignant cells, or cells that were able to express a new phenotype once removed from the splenic microenvironment.

Similarly the establishment of SAB, a hairy cell like line with monocyte characteristics, from a patient with a B-cell proliferation of hairy cells also suggests that once removed from microenvironmental constraints, the neoplastic cell may undergo a phenotypic shift. It is not surprising that this hairy cell line has monocyte characteristics
since it has been hypothesized and shown that hairy cells are sometimes more monocytic than lymphocytic (2).

On this basis, we would like to revise an earlier hypothesis in which it was postulated that hairy cells represented the clonal expansion of a transient stem cell whose genome was influenced by the immediate microenvironment (10). It was implied in this hypothesis that plasticity was inherent to hairy cells and because of this they were able to respond to multiple differentiative stimuli thus enabling them to either differentiate along multiple pathways or to undergo phenotypic modulation. We would like to suggest that instead of a transient stem cell, aberrant gene expression allows the hairy cell more latitude in which to respond to various differentiation inducers or modifiers. It has been suggested many times that the phenotypic diversity of leukemias and solid tumors could be the result of aberrant gene expression, but we would like to go one step further and suggest that in certain cases this inappropriate expression allows the cell to respond to and acquire characteristics of another cell type. For example, one could envision hairy cell acquiring enough interleukin-2 receptors so as to allow them to override their B cell commitment and to acquire T cell characteristics. Such a hypothesis would account for the phenotypic diversity that often is a hallmark of HCL. There have been several recent reports that lend credence to this hypothesis. Guglielmi et al (23) have shown that hairy cells with a B cell phenotype assumed a T cell phenotype following stimulation with PHA. Worman et al (24) confirmed and extended these observations and further showed that T cell contact was essential for phenotypic transformation.
Reaction with monoclonal anti-T cell sera, however, could not be demonstrated. Further support for the plasticity of hairy cells comes from clinical observations. In a recent study of 10 HCL patients, 6 of them had a phenotypic reversal from sIg+, Fc+, E-, to sIg-, Fc- and E+ (25). In five of these patients, this reversal was accompanied by marked clinical improvement and may have been indirectly related to splenectomy. This phenotypic reversal was not considered to be a sign of remission since cytoplasmic IgM was present in cells with the phenotypic markers sIg-, Fc and E+ (25). Jansen et al (9) recently have reported two cases in which hairy cells in the peripheral blood possessed both T and B cell characteristics, while the cells in the spleen had only B cell characteristics. They further noted that most of the hairy cells in the bone marrow had both surface IgA and IgM, whereas in the spleen, IgG was the predominant isotype (9). Several alternative interpretations were suggested to explain the discordance of membrane markers expressed on cells from the blood and spleen. Most favored was the selective sequestration of "B" hairy cells in the spleen. It was reasoned that the bone marrow probably produced both B+ and B+/T+ hairy cells and that the spleen selectively captured the B+ population (9). Parenthetically, it might also be argued that the spleen was the target organ for the B+ population. Alternatively, WB-25 and SAB could represent the clonal expansion of a cell that normally is present in the spleen but in very low numbers. If this should prove to be the case, then these cell lines might be the normal counterpart of the hairy cell and as such might provide a useful in vitro model to study the immunobiology of the hairy cell.
Figure 1

Splenic histopathology: (a) The red pulp showed marked enlargement due to dilatation of the sinusoids and widening of the cords, which were infiltrated by large mononuclear cells. These had large round to indented vesicular nuclei and usually a single prominent nucleolus (250 X). (b) As seen by electron microscopy, the malignant cells had large round to convoluted nuclei and usually a single, prominent nucleolus (7600 X).
Figure 2

Microscopic view of WB-25.

A) Light microscopic view of WB-25. Note the serrated cell borders.

Two mitotic cells are shown. The ingestion of latex beads can also be seen.

B) Phase contrast microscopy of WB-25. The hairs can be readily seen.
Figure 3

WB-25 as illustrated by transmission electron microscopy. There is a paucity of endoplasmic reticulum, no discernible Golgi apparatus, numerous mitochondria and a well defined nuclear membrane.
Figure 3
Figure 4

Light microscopic view of SAB.
Figure 4
Figure 5

WB-25 cytochemical reaction.

A) The demonstration of acid phosphatase activity in WB-25. Most of the activity is polar.

B) Tartrate resistant acid phosphatase activity in WB-25, this too is polar.
Figure 6
SAB cytochemical reaction.
A) Acid phosphatase activity in SAB
B) Positivity for tartrate-resistant acid phosphatase. As illustrated, this particular cell line is strongly positive for the cytochemical reaction.
Figure 7

WB-25 karyotype: 46, X, Y
Sex Chromosomes

Y

X

22 21

18 17 16

15 14 13

12 11 10 9

8 7 6

5 4 3 2 1
Figure 8
SAB karyotype: 61, X,-Y, +1, +2, del(3)(q1), +4, inv(6)(p21, p23), +10, +12, +14, +15, +16, 19 q+, +19, del(20)(q1) +22, +8 mar
Sex Chromosomes

Figure 8
Figure 9

SAB karyotype: 64, X, Y, del (3) (q1), inv (6) (p21, p23), del (11) (p1), 19q+, del (20) (q1), +10, +1, +2, 7, -9, -9, +11, +12, +14, +15, +16, +17, +9 mar
Sex Chromosomes

Figure 9
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