ISOLATION AND CHARACTERIZATION OF MOUSE-HUMAN
SOMATIC CELL HYBRIDS WHICH PRODUCE
A LEUKEMIA ASSOCIATED ANTIGEN

BY

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B. Sc., University of British Columbia, 1980

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
(Department of Microbiology)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

July 1985

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ABSTRACT

Interspecific somatic cell hybridisation has the potential not only of producing hybrids expressing antigens of two different species but also of providing genetic information on these foreign antigens. The work presented here, describes the successful interspecific somatic cell hybridisation of a mouse myeloid cell line (WeHi-TG) with peripheral blood leukocytes from a patient with chronic granulocytic leukemia. Hybrid fusion products were screened for their ability to produce a leukemia associated protein (CAMAL) as detected by indirect immunoperoxidase as well as ELISA using a monoclonal antibody specific for CAMAL. Production of this antigen was verified by immunoprecipitation of internally labelled cell lysates from these hybridomas. CAMAL-producing cell hybrids were further tested for the presence of human DNA, using the BLUR 8 "Alu" containing DNA probe and a panel of monoclonal antibodies with specificities to a variety of myeloid antigens. These studies confirmed the presence of human DNA and showed some correlation between cells producing CAMAL and those expressing other granulocyte markers.

Karyotype analysis was carried out on hybridoma lines after long term culture. In all hybrids examined, no recognizable human chromosomes or translocations were detected using either the methods of G-banding or differential G-11 staining.
The development of an in vivo animal model for human leukemia would provide a mammalian system for the testing of various cancer therapies. The ability of our CAMAL expressing hybrids to grow in BALB/C mice provided the rationale for the development of an animal model for cancer therapy. Our preliminary in vitro results showed that CAMAL expressing hybrids could be specifically eliminated using hematoporphyrin conjugated to CAMAL-1.
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<tr>
<td>ALL</td>
<td>Acute lymphocytic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myelogenous leukemia</td>
</tr>
<tr>
<td>ANLL</td>
<td>Acute non lymphocytic leukemia</td>
</tr>
<tr>
<td>BBS</td>
<td>Borate buffered saline</td>
</tr>
<tr>
<td>BLUR 8</td>
<td>BamHI ubiquitous repeat</td>
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<tr>
<td>CGL</td>
<td>Chronic granulocytic leukemia</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myelogenous leukemia</td>
</tr>
<tr>
<td>CAMAL</td>
<td>Common antigen of myelogenous acute leukemia</td>
</tr>
<tr>
<td>DAB</td>
<td>Diamino Benzidine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DME</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunoadsorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorter</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>HAT</td>
<td>Hypoxanthine, Aminopterin, thymidine</td>
</tr>
<tr>
<td>Hp</td>
<td>Hematoporphyrin</td>
</tr>
<tr>
<td>HPRT&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Hypoxanthine phosphoribosyl transferase deficient</td>
</tr>
<tr>
<td>J/m&lt;sup&gt;2&lt;/sup&gt;</td>
<td>joules per meter squared</td>
</tr>
<tr>
<td>LAA</td>
<td>Leukemia associated antigen</td>
</tr>
<tr>
<td>MAbs</td>
<td>Monoclonal Antibodies</td>
</tr>
<tr>
<td>NaDOC</td>
<td>Sodium deoxycholate</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>Rosewell park memorial institute 1640 medium</td>
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</table>
SDS-PAGE - Sodium Dodecyl sulphate polyacrylamide gel electrophoresis.

PBL - Peripheral Blood Leukocytes

PBS - Phosphate buffered saline

(137mM NaCl, 2.7mM KCl, 43mM Na$_2$HPO$_4$, 1.5mM KH$_2$PO$_4$)

PMSF - Phenyl methyl sulfonyl fluoride

RIPA - Radioimmunoprecipitation assay

(0.1% SDS, 0.5% NaDOC, 1% NP40, 1mM EDTA

100mM NaCl, 10mM Tris HCl pH 7.5)

RNA - Ribonucleic acid

SDS - Sodium dodecyl sulphate

SSPE - Standard saline phosphate EDTA

TCA - Tri-carboxylic acid

Tris - Tris (hydroxymethyl) aminomethane

TX100 - Triton X100

WC - WeHi-CGL fusion product

WeHi-TG - Walter and Eliza Hall Institute cell line thioguanine resistant.
ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor Julia G. Levy for her support and guidance throughout this work, Fred J. Dill for his expertise in genetic analysis and helpful discussion, Chris and Sharon for their patience, and experience in karyotyping and cell culture, Stephen Wood and Raymond Poon for their expertise in molecular biology, my fellow colleagues and friends in the lab for technical as well as theoretical assistance and Mary for understanding the trials and tribulations of a graduate student.
INTRODUCTION

HISTORY:

The Hemopoietic (blood forming) system is responsible for the generation of myeloid cells (erythrocytes, platelets, granulocytes, monocytes and macrophages) and lymphoid cells (lymphocytes and plasma cells) which are produced in the bone marrow, spleen, and lymph nodes. Radiation induced chromosomal markers, as well as isoenzyme studies have verified the ability of self renewing pluripotent stem cells to give rise to all cells of the hemopoietic system (1). The appendix outlines the multiple pathways (pluripotential) that a hemopoietic stem cell can follow.

Chronic and acute myelogenous leukemias are myeloproliferative diseases of hemopoietic progenitor cells (ie. stem cells that are predestined for either a myeloid or lymphoid pathway). Chromosomal markers as well as isoenzyme G6PD analysis have provided evidence for the clonality (single cell origin of these cancer types (2,3).

There has been much evidence for a genetic predisposition (either hereditary or acquired) to cancer development. Patients with rare hereditary disorders such as Fanconi's Anemia and Ataxia Telangiectasia are at increased risk to develop leukemia, lymphomas and epithelial tumors (4). It is also known that individuals exposed to radiation such as blast victims of Hiroshima and Nagasaki have an increased risk to leukemia and have a high incidence of chromosome breakage in their peripheral
lymphocytes. Patients with chromosomal disorders such as Down's syndrome (5) also have an increased risk to cancer development. These genetic predispositions are directly related to instability of chromosomes in patients with these various genetic disorders. These unstable chromosomes are more susceptible to spontaneous or epigenetic induced breakage, mutation (insertion, deletions and substitutions), rearrangements, and recombinations (6). Consequently patients with these genetic abnormalities are at a higher risk to cancer. Thus, it is the instability of chromosomes and therefore the susceptibility to chromosomal change that is thought to be responsible for a genetic predisposition to cancer.

**Mechanisms of Neoplasia:**

An oncogene hypothesis for the development of neoplasms has been a favored mechanism for transformation. Oncogenes are evolutionarily highly conserved human cellular gene sequences that are thought to have functions in normal cell growth and differentiation. Oncogenes present within their human host are referred to as c-onc. These oncogenes have a potential for causing cancer if subjected to a indirect or direct change in location or expression. The source of these malignant changes include physical (eg. radiation) (7), chemical (eg. Benzene) (8), and viral (retroviruses) (9,10) agents. These agents are thought to be oncogene activating either directly or indirectly. Chromosomal translocations have recently been implicated in oncogene activation in a number of neoplastic systems (11).
(a) Physical and Chemical Agents:

Physical and chemical agents are thought to exert their neoplastic effects by causing mutation of unstable DNA and indirect or direct activation of cellular oncogenes. These two mechanisms are strongly implicated in cases of leukemia associated with genetic predispositions.

(b) Viral Agents:

A virally related transformation mechanism has a major theme of research for the past several years. Viral gene sequences have long been associated with the maintenance or induction of neoplastic transformation. Transfection studies using transformation causing DNA from bladder, lung and colon carcinomas, neuroblastomas, soft tissue and boney sarcomas, and neoplasms of B & T cell lymphocytes, have revealed homology or extreme similarity to these long established viral gene sequences (12). The ability of retroviruses (type C RNA viruses) to transform, has been found to be the result of the acquisition of human transforming sequences or oncogenes. The presence of these oncogenes is the result of recombination of non transforming replication competent RNA viruses with host cellular oncogenes (these hybrid transforming DNA segments are referred to as \(v\)-onc or viral oncogenes). Transformation has been found to be directly related to the activation of these oncogenes either by insertion of the viral oncogene into a host genome or activation of a viral promotor. In addition, further
examination of bladder and colon oncogene sequences have shown small mutations in cellular genes (and consequently production of an altered gene product) are responsible for the acquisition of transforming properties (13-15).

Thus, neoplastic transformation via oncogene activation can be a result of oncogene mutation to produce altered gene products (via physical, chemical or viral mechanisms) or elevation of oncogene transcription by a promotor (viral or host).

(c) Chromosomal Translocations:

Nowell and Hungerford (1960) first identified that a specific chromosomal abnormality was associated with a specific neoplasm (ie. the philadelphia chromosome ph' in chronic myelogenous leukemia)(16). The chromosomes involved in this translocation were not elucidated until 1973 when it was found that the majority of patient's with CML or CGL carried a reciprocal translocation (t[9;22][q34;q11]) in their bone marrow (17). In 1982, gene mapping techniques were responsible for the discovery that the philadelphia translocation of CML involved the translocation of the human cellular homolog of the transforming sequence of Abelson murine sarcoma virus, c-ABL, (normally on chromosome 9) to chromosome 22 (18). The mapping of the lambda constant region on chromosome 22 was performed by somatic cell hybridization. The translocation of the c-ABL oncogene close to the lambda gene promotor , presumably allows elevation of the c-ABL gene product and presumably leads to neoplasia.
Specific nonrandom translocations associated with CML and CGL are well documented. Unfortunately the picture is not as clear with acute myeloblastic leukemia (AML). The most frequent non random chromosomal translocation is the (t[8;21](q22;q22)) in approximately 17% of M2 AML cases (19). The human homolog to the murine sarcoma virus c-MOS has been mapped to the breakpoint on chromosome 8 of the 8;21 translocation.

Neoplastic transformations have been associated with chromosomal rearrangement, specifically the relocation of human oncogenes to regions of active gene function. Chromosomal translocations appear to have some primary function in the complex steps involved in neoplasia. In the Burkitt's lymphoma system there has been strong evidence for a number of translocation, responsible for promotor and enhancer amplification of the c-MYC gene with coexpression of c-MYC and immunoglobulin proteins (20).

**LEUKEMIA_ASSOCIATED_MARKERS:**

The significance of leukemia associated proteins for the understanding and treatment of myeloproliferative disorders has been a major part of cancer research for the past decade. Myeloid leukemia associated antigens have been described by a number of workers: a 75-80K dalton mol. wt. glycoprotein of pI 7.8 (21) and a glycoprotein of 400K daltons mol. wt. (22) are two antigens isolated from myeloblastic leukemia patient cells.
These antigens have been utilized in diagnostic assays as well as development of immunotherapy and classification of myeloid leukemias. A common antigen of myelogenous acute leukemia (CAMAL) has been the center of much research in our laboratory. CAMAL, a leukemia associated protein is a polypeptide of approximately 68000 daltons and a pI of 7.2 (23). Using immunofluorescence or indirect immunoperoxidase methods, CAMAL has been found to occur at high incidence on the membrane and in the cytoplasm of cells from patients with myeloproliferative disorders (either acute or chronic). Immunoprecipitation of CAMAL from patients with chronic granulocytic leukemia as compared with normal patients reveal one hundred times greater antigen in the case of the former. Furthermore, CAMAL cannot be detected associated with normal granulocyte enriched populations using immunoperoxidase or immunofluorescent assays (24). CAMAL has been used successfully as the leukemia associated target antigen in immunotherapy of CAMAL bearing tumor cells (25).

Somatic Cell Hybridization:

Interspecific somatic cell hybridization procedures (26,27) allow the mapping of specific genes to particular chromosomes (28). These procedures used in conjunction with monoclonal antibodies specific to mapped gene products allow mapping of genes to sections within chromosomes. Recent work by Kessel et al. (29) involving interspecific intralineage fusions, have demonstrated expression of human myeloid proteins on mouse tumor
parents. These methods have allowed the mapping of tumor associated antigens on specific chromosomes (11). The expression of human leukemia associated antigens on somatic cell hybrids have implications for the study of these antigens: (function, mapping, therapeutic use). The ability of these hybrids to grow in their mouse syngeneic systems will facilitate the development of an in vivo model for human leukemia. Clinical immunotherapy studies (immunological methods to cause tumor regression or bone marrow purging to destroy antigen positive cells) could be performed using an in vivo mouse model for human leukemia.

IMMUNOTHERAPY:

The present methods of therapy for the treatment of leukemias involve nonspecific killing of actively dividing cells. Radiation therapy as well as chemotherapeutic agents are two such treatments. The nonspecific killing of cancer cells as well as normal cells results in severe and often dangerous side effects. There has been much effort in the past decade to increase the specificity of cancer therapy. Selective drugs such as the photoactivable chemical hematoporphyrin have a higher affinity for tumor tissue than for normal tissue (30-32). Physical removal of neoplastic cells using gradients (33) or differential agglutination (34) have also been used. These methods have met with limited success.

Immunotherapy refers to the specific killing of cancer cells via the recognition by antibodies of neoplastic specific
or associated antigens. Two immunotherapeutic modalities include anti-tumor antibody plus complement mediated destruction (35) and anti-tumor antibody conjugated to toxins (36).

Our laboratory has shown previously, that a monoclonal antibody with specificity to a transplantable murine tumor could be conjugated to hematoporphyrin (Hp) and be used to effectively deliver toxic levels of Hp to in situ tumors. These Hp conjugates were able to eliminate tumor cells in vivo at levels of Hp less than 10% of that used in clinical trials (37). Similar in vitro studies using a monoclonal antibody directed to CAMAL and conjugated to Hp (CAMAL-1-Hp) showed specific killing of CAMAL bearing tumor cells when the MAb-Hp conjugates were laser-activated (38). The possibilities for the treatment of human leukemias using the CAMAL-1-Hp conjugate although shown effective in vitro would require in vivo testing. Somatic cell hybrids expressing CAMAL and growing in mice would provide an in vivo model for immunotherapeutic treatment of human leukemia.

The work presented in this thesis intended to develop a mouse-human somatic cell hybrid system that constitutively expressed the CAMAL antigen and would ultimately allow the study of human myeloid leukemia in an animal model system.
MATERIALS AND METHODS

Patient Cell Samples:

Peripheral blood leukocyte (PBL) samples from patients with chronic granulocytic leukemia (CGL) were obtained from the Department of Hematology, Vancouver General hospital. Diagnosis of CGL had previously been determined by cellular morphology, biochemical tests (Sudan Black, Periodic acid stain, combined esterases and Acid phosphatase test) and chromosome analysis. Heparinized blood was centrifuged at 1500 RPM through Ficoll Hypaque (Pharmacia, Uppsala Sweden) in order to separate mononuclear leukocytes, granulocytes and RBC (39,40) after which they were washed four times in PBS pH 7.4. Viability counts were made directly using Trypan blue exclusion prior to fusion (41).

Tumor Cell Lines:

The mouse myeloid cell line used as the fusion parent was WeHi-TG a hypoxanthine phosphoribosyl transferase deficient (HPRT-) mutant which was obtained after ultraviolet irradiation (10 J/m²)(42) of WeHi-3B cells (29) followed by culture in RPMI 1640 medium (GIBCO laboratories, Grand Island, New York) plus 10% fetal calf serum (FCS) and 10 ug of
6-thioguanine in a humidified 10\% CO\textsubscript{2} incubator. Cell lines were kindly provided by Dr. D. Bootsma, Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands. Cultures are presently maintained in RPMI 1640 plus 10\% FCS in a humidified 10\% CO\textsubscript{2} incubator.

The human promyelocytic cell line HL60 and the pre-erythroid cell line K562 was provided to us by Dr. Tony Pawson, Dept. of Microbiology, University of B.C. HL60 cells were maintained in DME plus 10\% FCS in a humidified CO\textsubscript{2} incubator. The K562 cell line was maintained in RPMI 1640 plus 10\% FCS in a humidified 10\% CO\textsubscript{2} incubator.

Cell Fusion:

Somatic cell fusions were performed essentially by procedures described by Qi and Herzenberg (43). Briefly, 5 x 10\textsuperscript{7} WeHi-TG cells were washed three times in serum free RPMI 1640 and added to pre-washed 1 x 10\textsuperscript{8} peripheral blood leukocytes from a CGL patient. The mixture of cells was centrifuged at 1500 RPM for 10 min. The cell pellet was warmed to 37\textdegree C for 10 min and then fused by the addition of 1.0ml of warm (37\textdegree) 50\% polyethylene glycol (PEG) 4000 (Serva chemicals) in PBS, with gentle stirring over one min. After one more minute of gentle stirring, 2.0ml of serum free RPMI 1640 was added over two min. Then 8.0ml of serum free RPMI 1640 was added over the next 4 min. Fused cells were gently spun down at 1000 RPM for 5 min. Fusion products were plated in 96 well Linbro (Fisher Scientific) plates at 2 x 10\textsuperscript{5} cells per well.
in 100 ul of RPMI 1640 + 20% FCS + 1xHAT (44) medium. Thymocytes were added as feeders at a final concentration of 5 x 10^5 cells per ml in 100ul. Medium was changed every four days with the removal of 100ul of spent medium and the subsequent addition of 100 ul of fresh RPMI 1640 containing 20% FCS and 25% conA stimulated rat spleen supernatants containing 50mM alpha-methyl-mannoside (Sigma). Viable colonies were transferred to 24 well Linbro plates for expansion for subsequent testing.

Monoclonal Antibodies and Antisera:

Initial screening of hybrid cells for the production of a leukemia associated antigen involved the use of a monoclonal antibody CAMAL-1 and a conventional rabbit antiserum, rabbit anti CAMAL. CAMAL-1 was developed in our lab (45) and was raised against the leukemia associated antigen, CAMAL. CAMAL, a 68000 dalton protein, pi 7.2 was originally isolated from acute non lymphoblastic leukemia (ANLL) cell membrane extracts on 7.5 % Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (23). Similarly, Rabbit anti-CAMAL antisera were produced in young adult female albino rabbits by primary immunization intramuscularly into four peripheral sites with a 1:1 (v:v) mixture of 12.5 ug of CAMAL antigen in a final 50% Freunds complete emulsion, adjuvant and subsequently a secondary immunization four weeks later. Rabbits were bled one week later from the marginal ear vein. The CAMAL-1 monoclonal antibody and the Rabbit anti-CAMAL (R&CAMAL) antisera have been shown to
react specifically with CAMAL antigen present in bone marrow and PBL cells from patients with CGL or ANLL. CAMAL has been shown to be present at low concentrations on normal granulocytes (24). Rabbit anti-Human PBL membrane extract antiserum (R&Hu) was produced by the immunization of young adult female albino rabbits with normal peripheral blood leukocytes membrane extracts produced from a pooled sonicated preparation of normal PBLs. Immunization protocols for production of rabbit anti human antisera was performed as previously described (46).

Immunoadsorbents:

Immunoadsorbents containing 2mg/ml of CAMAL-1 were prepared as follows: Monoclonal CAMAL-1 antibody obtained from a 50% ammonium sulfate ascites salt cut, and purified over an DEAE sepharose column was coupled to cyanogen bromide activated sepharose CL4B (Pharmacia, Upsalla Sweden) (47,48). Immunoadsorbent columns were equilibrated with borate buffered saline (BBS) pH 8.5. Samples were applied to affinity column and cycled over six times. The columns were washed with BBS pH 8.5 until the effluent had no adsorbance at 0.D.280nm. The CAMAL antigen was eluted using a 0.1 N HCl solution. One ml fractions were collected and read on a spectrophotometer at 280nm. and protein fractions were neutralized with 5% Na₂CO₃.

Myeloid Specific Monoclonal Antibody Panel:

The panel of monoclonal antibodies used for this study
include the following: NKHIA was obtained from Dr. Th. Hercend (49) and reacts with an antigen on NK cells and on some AML cells CRIS-6, 94-3D1 and JOAN-1 were obtained from Dr. R. Vilella, Servei d'immunologia, Casanova, Barcelona. CRIS-6 is a monocyte reactive monoclonal antibody which does not react with lymphocytes, normal granulocytes, platelets, erythrocytes, Raji (Burkitts Lymphoma), Daudi, but in our hands reacted with cells of patients with CGL and some ANLL samples. 94-3D1 is reactive with platelets and monocytes but not with lymphocytes, normal granulocytes, erythrocytes, Raji or Daudi. 94-3D1 reacted in our tests with some ANLL specimens, a promyelocytic leukemia HL60 and KG1. JOAN-1 is a pan reactive monoclonal antibody, being positive for T and B, PBL, monocytes, normal granulocytes, erythrocytes, platelets and Raji. In our hands it reacted strongly with all cell types tested which included CGL and ANLL patient material as well as the myeloid lines HL60 and KG1 derived from ANLL cells. M101 was obtained from R.W. Knowles, Sloan Kettering, New York, and is a pan-reactive monoclonal antibody. We found it to react in immunoperoxidase with essentially all patient samples tested as well as HL60 and KG1. KD3 was obtained from Dr. I. Indo, ICRF, University College, London, and reacts with cells of both lymphoid (Daudi, Jurkatt, RAJI, T and B blasts, thymus) and myeloid (monocytes, CGL, ANLL, HL60, KG1) lineages.

GA-1 was obtained from Dr. A. Hiraiwa, Nagoya University School of Medicine and appears to react mainly with granulocytes. GA-1 is presumed to be directed to glycosylated membrane
components since it does not yield identifiable antigens in immunoprecipitation tests. Our test revealed reactivity to CGL, some AML, PBL and HL60. CLB-LFA-V and CLB gran were obtained from Drs. F. Miedema and A. Von Dem Borne, Central laboratory of the Netherlands Red Cross Blood Transfusion service, Amsterdam. CLB-LFA 1/1 inhibits NK activity and CTL activity, recognizes the lymphocyte function associated (LFA) antigen (50) as well as an antigen on T cells, B cells, monocytes, normal granulocytes and some AML cell types. CLB gran reacts with the neutrophil antigen NA1 (present on lymphocytes, monocytes and granulocytes, some AML cell types, HL60 and the pre erythroid line K562. CIPAN-Hu was obtained from Dr. T. de Krester, The Cancer Institute, Melbourne, Australia. This monoclonal antibody is reactive with a pan-human cell surface antigen in higher primates as well as some AML cell types and HL60. All of the monoclonal antibodies were tested originally as part of the Second International Conference on Human Leucocyte Differentiation Antigens in Boston (1984) (51). Because of their reactivity to one of our original interspecific somatic cell hybrids WC-2, they were selected for further testing with our other hybridomas.

Selection of CAMAL Positive Hybridomas:

(a) ELISA (enzyme linked immunoadsorbent assay) on putative CAMAL containing hybrid supernatants. 50 ul of supernatants from fusion products in 96 well plates were transferred to Immulon I microtitre plates (Fisher Scientific) containing 50 ul
of Standard carbonate/bicarbonate ELISA coating buffer pH 9.6. The contents of these plates were mixed and then incubated at 4°C for 16 hours. Plates were washed three times with PBS-TWEEN 20 buffer and 100 ul of a $10^{-4}$ dilution of rabbit anti-CAMAL was added to each well of the plate, further incubated for 2 hours at room temperature and again washed three times with PBS TWEEN 20 buffer. 100 ul of alkaline phosphatase (Sigma type VII) (52) labelled sheep anti-rabbit Ig was then added (1:2000) for a further two hours at room temperature. The plates were washed three times in PBS-TWEEN 20 and once with PBS. Plates were developed with 100 ul p-nitrophenyl phosphate-Na (Sigma 104) to each well for one hour at room temperature. Microtitre plates were read for absorbance at 405 nm on a Titertek Multiskan plate reader (Flow Laboratories, Inglewood California), although positive wells could easily be scored visually.

(b) Cellular ELISA:

The cellular ELISA is a modification of the above procedure. Cells to be tested were washed three times in PBS and subsequently resuspended at $1 \times 10^5$ cells/ml in the standard carbonate/bicarbonate ELISA coating buffer pH 9.6. Aliquots of 100 ul were plated onto Immulon II plates. The plates were spun at 750 rpm for ten minutes in a clinical DAMON IEC centrifuge and cells were incubated for 16 hours at 4°C. Carbonate/bicarbonate buffer was removed and the cells were ultimately fixed by the addition of 1% formalin in PBS for
one hour at 37 degrees celsius. ELISA's were carried out in the standard manner.

(c) Cell Lysate ELISA:

5 x 10^6 cells to be tested were washed three times in PBS pH 7.4 prior to lysis. Lysis was performed on loosened cell pellets by slow addition of 2x lysis buffer (2% Triton X100, .2% SDS and 10 mM PMSF - Sigma, 1% Na DOC - BDH) with gentle agitation. Lysates were spun at 1500 rpm to remove unlysed material, and subsequently spun at 50,000xg (12,000 rpm in the SS 34 rotor Beckman Centrifuge) to remove membranes. Protein concentrations of the supernatant were determined by the Lowry protein assay (54). Lysates were coated on microtitre plates at 1.0 ug/ml in the carbonate/bicarbonate ELISA coating buffer. ELISAs were carried out in the standard manner as described above.

Immunoperoxidase Slide Preparation:

Test cells were washed three times in serum free RPMI 1640 (GIBCO) medium and resuspended with RPMI 1640 to a concentration of 2 x 10^6 cells/ml and loaded (3 drops) into Shandon II cytopsin funnels for slide preparation. Slides were prepared by centrifugation of the three drops of test cells at 700 rpm for five minutes on a Shandon cytopsin II apparatus.
(c) Cell Lysate ELISA:

5 x 10^6 cells to be tested were washed three times in PBS pH 7.4 prior to lysis. Lysis was performed on loosened cell pellets by slow addition of 2x lysis buffer (2% Triton X100, .2% SDS and 10 mM PMSF - Sigma, 1% Na DOC - BDH) with gentle agitation. Lysates were spun at 1500 rpm to remove unlysed material, and subsequently spun at 50,000xg (12,000 rpm in the SS 34 rotor Beckman Centrifuge) to remove membranes. Protein concentrations of the supernatant were determined by the Lowry protein assay (54). Lysates were coated on microtitre plates at 1.0 ug/ml in the carbonate/bicarbonate ELISA coating buffer. ELISAs were carried out in the standard manner as described above.

Immunoperoxidase Slide Preparation:

Test cells were washed three times in serum free RPMI 1640 (GIBCO) medium and resuspended with RPMI 1640 to a concentration of 2 x 10^6 cells/ml and loaded (3 drops) into Shandon II cytopsin funnels for slide preparation. Slides were prepared by centrifugation of the three drops of test cells at 700 rpm for five minutes on a Shandon cytopsin II apparatus.
Immunoperoxidase Staining:

Prepared slides were allowed to age overnight at room temperature before staining was performed as follows: Test slides were fixed for 30 minutes in a methanol - 2% H2O2 solution, and subsequently washed by spraying three times with PBS pH 7.4. The primary antibody (CAMAL-1 or control MA6 at 10 ug/ml, rabbit anti-CAMAL-1 or NRS at 1/400 dilution in PBS) was applied to the wet cytospin slide containing the cell circle, allowed to incubate for 60 minutes at room temperature and washed three times by spraying with PBS pH 7.4. The secondary antibodies were rabbit anti-mouse immunoglobulin linked to horse radish peroxidase (R&MIg-HRP) and swine anti-rabbit immunoglobulin linked to horse radish peroxidase (S&RIg-HRP) (DAKO). R&MIg-HRP was added to slides treated with monoclonal primary antibody treated slides at 1/100 dilution whereas S&RIg-HRP was added to slides treated with rabbit antisera as a primary antibody at a 1/200 dilution. Both secondary antibody treated slides were incubated 1 hour at 20°C in darkness. Slides were histochemically stained for horse radish peroxidase for 10 minutes by immersing the slides in a coplin jar containing 50 ml PBS, 10 mg diaminobenzene, and 100 ul of 30% H2O2. Slides were transferred to coplin jars containing 50 ml PBS, washed for five minutes under tap water, and counterstained with hematoxylin.

Slides were coverslipped using Permount (Fischer Scientific) and examined under light microscopy using the oil immersion 100x objective. If the number of positive cells in a
cell spread was less than 1.0% of the cell sample, at least 400 cells were scored on each slide. If positive reactivity was much less than 10%, between 1000 to 3000 cells were examined for each slide. All slide tests done were carried out at least twice on separate occasions (24).

\[\text{35S Methionine In vivo Labelling of Cell Lines:}\]

Approximately $5 \times 10^6$ cells were isolated and washed three times in sterile PBS pH 7.4. Cells were resuspended in 2.0 ml of RPMI 1640 lacking methionine (Met-, GIBCO) transferred to 35mm petri dishes (FALCON). Cells were subsequently incubated for 2 hours in a 37°C, humidified, 10% CO2 incubator to deplete internal pools of cold methionine. \text{35S methionine (NEN)} was added to the cells to a final concentration of 150 uCi/ml and incubation was carried out overnight in a 37°C humidified 10% CO2 incubator. Cells not adhering to the 35mm dishes were removed and transferred to a 10.0 ml Falcon tube. Plates were washed twice with ice cold PBS pH 7.4 and these wash solutions were transferred to the same falcon tube. Non-adherent cells were spun and washed twice in ice cold PBS. Cells adhering to the 35mm plates were lysed using 2 changes of 0.5 ml RIPA (Sigma: 100mM NaCl, 10mM Tris Base, 1% NP40, 0.5% NaDOC, 0.1% SDS, 1% Aprotinin) buffer. These changes of RIPA buffers were added to the washed non-adherant cell pellet for lysis. The resulting lysates were transferred to 15 ml polycarbonate tubes and spun at 15,000 rpm (50,000g) in a Beckman RC5B with a fixed angle SS34 sorval rotor for 30 minutes.
to remove insoluble membrane fragments and nuclei. TCA precipitable counts were determined on the cell lysis supernatants by the spotting of 5 ul of each supernatant onto separate 2.5 cm Whatman glass microfibre filters, air drying these filters and subsequently washing them on a millipore vacuum filter apparatus with two 3ml volumes of 20% TCA, two 3ml volumes of 5% TCA and one 3 ml volume of 95% ETOH. Filters were allowed to air dry in plastic scintillation vials and then baked for 10 minutes at 90°C. 5.0 ml of scintillation cocktail (4 gm PPO/BIS MSP/1 toluene) was added to the vial and capped. Filters were read on a united technologies Hewlett Packard beta counter to determine cpm/ul.

Immunoprecipitations:
Prespun 35S methionine labelled cell lysates were precleared by the addition of NRS to lysates at a final dilution of 1/250 and the subsequent concentration of these serum treated lysates for 30 minutes on ice. Ten volumes of a 10% suspension of staphlococcus aureus strain Cowan I (IgG sorb; The Enzyme Center) in RIPA buffer was added to lysates for an additional 30 minutes on ice. The lysates containing the nonspecific immune complex was spun on a microfuge (Eppendorf) for 5 minutes. 2 x 10^7 CPM of resultant supernatants were aliquoted into four separate 1.5 ml microfuge tubes for specific immunoprecipitation. All test lysates performed together were normalized for volume prior to addition of primary antibodies. Five microliters of primary antibodies NRS, rabbit anti-CAMAL,
and CAMAL-1 were added separately to each of the three lysate aliquots to give a final dilution of 1/1000 and incubated for 2 hours on ice. Ten volumes (50ul) of a 10% solution of IgG sorb was added to NRS and rabbit anti-CAMAL containing aliquots and ten volumes of a 10% solution of R&MIg - IgG sorb was added to mouse monoclonal antibody containing aliquots and subsequently allowed to incubate for 1 hour on ice with vortexing every 15 minutes. The immune complexes formed were spun down at 12,000 rpm on a microfuge (Eppendorf) resuspended in 1.0 ml of RIPA buffer, transferred to a new microfuge tube, spun on the microfuge and the pellet was washed three additional times with RIPA buffer. The final cell pellets after four washes were resuspended in 50 ul of SDS sample buffer (2% SDS, 5% beta-mercaptoethanol, 10mM Tris base pH 6.8, 10% vol/vol glycerol), incubated in a 37°C incubator for ten minutes to release Ag-Ab complexes from Staphlococcus aureus, and spun down on a microfuge for 5 minutes. Resultant antigen antibody containing supernatant was transferred to a new microfuge tube for heating prior to polyacrylamide electrophoresis (55).
SDS - Polyacrylamide Gel Electrophoresis (SDS - PAGE)

Proteins present in the immune complex generated by the immunoprecipitation procedure were characterized using a discontinuous 7.5% Sodium Dodecyl Sulphate PAGE system. PAGE was performed essentially by the method described by Laemmli (56). Briefly, samples were heated for 3 minutes at 100°C in SDS sample buffer and subsequently applied to a 3% stacking, 7.5% running SDS-PAGE system. Protein samples were stacked at 50 volts and separated at 130 volts using a Hoescht Scientific electrophoresis unit and power pack on constant voltage. Molecular weight standards were coelectrophoresed and included: Bovine serum albumin (67000 daltons), Ovalbumin (43000 daltons), Carbonic Anhydrase (29000 daltons), and Trypsin Inhibitor (20000 daltons). Immunoprecipitation gels were stained with Coomassie blue (57) overnight, destained for four hours using a 41.4% methanol to 5.4% acetic acid solution. Gels were impregnated with a chemical fluor by soaking destained gels for 60 minutes at room temperature in En^Hance (NEN) and precipitating the fluor within the gel by soaking the gel in deionized distilled water for 60 minutes. Gels were dried at 60°C on a BIORAD 1125B slab dryer and subsequently exposed to Kodak XOMAT-AR film.
Labelling of Cells for FACS

Cells were isolated from suspension and from flask bottoms using 0.1% EDTA in PBS washed 3X in serum free RPMI 1640 pH 7.4 and 1 x 10^6 cells were placed in each tube and spun down for antibody labelling. Cell pellets received 0.2 ml of a 1/50 dilution of either: no antibody, no primary antibody, NRS, R&CAMAL or R&Hu. Samples were incubated with these primary antibodies on ice for 1.5 hours with light shaking at 15 minute intervals. Cell samples were then washed 3X in serum free RPMI 1640 and 0.2 ml of fluorescin isothiocyanate linked to goat anti rabbit F(ab')2 fragment of IgG (Cappel) at a 1/20 dilution was added to each cell pellet and samples were allowed to incubate on ice in darkness for 1 hour. In order to detect dead cells in our samples, 1 ul of a 25 mg/ml solution of propidium iodide solution (Becton Dickinson) was added to each test tube and the samples were incubated a further 30 minutes. Cells were then washed twice in ice cold PBS pH 7.4 and cell pellets were finally suspended in 1.0 ml of PBS pH 7.4 + 2% FCS and placed on ice prior to FACS IV analysis.

Fluorescence Activated Cell Sorter Analysis

Fluorescently labelled cells were analyzed on a Becton Dickinson FACS IV using the 448nm wavelength of the Spectra Physics Model 164-05 Argon laser at a power setting of 400mw. The standard filters for FITC analysis was used (520 long pass filter). The FACS IV was standardized by using glutaraldehyde
fixed chicken red blood cells (S8) and fluorescent monodispersed carboxymethylated microspheres (d=1.75m ± .02sd; Polysciences Inc., Warrington, PA). Ten thousand cells were analyzed per test sample, the effect of fluorescent dead cell membrane fragment background was negated by gating out propidium iodide red fluorescence on cells receiving secondary antibody only. The negative control serum (NRS) as well as the hybrid cell parent line were used for gating of FACS for determination of relative positivity to WC cell lines. The head drive frequency was set at 36KHz, and 2000V were applied across the electrostatic deflection plates. The FACS was run at five droplets per deflection pulse.

DNA Dot Blot Analysis

Plasmid Isolation:

The human repetitive "Alu" DNA Sequence (59,60) probe was kindly provided by Dr. S. Wood of the Medical Genetics department, UBC, as a plasmid probe referred to as BLUR 8 (Amersham: "a BamH1 linked ubiquitous repeat constructed by the ligation of a 300 b.p. repeat sequence from human DNA into the BamH1 site of pBR322 using BamH1 linkers"). Plasmid DNA was prepared by the method as described by Davis et. al. (1980)(61). Briefly, Blur 8 containing E. Coli HB101 was inoculated by loop to 10.0ml of L broth in a 37°C shaking incubator containing 50ug/ml of ampicillin (an AmpR plasmid allows only HB101 to
survive) and allowed to grow for four hours before addition of chloramphenicol (180ug/ml to amplify plasmid copy number) and further incubation overnight. The following procedures were performed on ice: cells were spun down at 2000 rpm for 10 min and resuspended in 5.0 ml of ice cold 25% sucrose in 50mM Tris pH 8.0. Cells were lysed by the addition of 1.0ml of lysozyme (5mg/ml in .25M EDTA pH 8.0) and 2.0ml of .25M EDTA pH 8.0. Cells were swirled in this solution for 10 min. Cells were further lysed by the addition of 1.0ml of 10% Triton X100 (10ml Triton X100 in .01M Tris pH 8.0, 25ml 0.25M EDTA pH 8.0, 0.5ml 1.0M Tris pH 8.0 and finally distilled H$_2$O to 100ml final volume). This solution was mixed (Triton allows cell lysis but not cellular DNA release) and incubated on ice for 15 min. Insoluble fragments were spun down at 19000rpm at 4°C in a Beckman RC5B centrifuge with an SS34 rotor (Beckman Instruments, Calif.). Supernatants containing DNA and RNA were separated by ethidium bromide (EtBr) CsCl density centrifugation for 20 hours at 45000rpm. Plasmid DNA covalently closed circles (ccc) were isolated as a lower uv long wavelength fluorescing band using a 5.0ml syringe with a 21 gauge needle. EtBr was removed by repeated extraction with isobutanol saturated with aqueous 5.0M NaCl,10mM Tris and 1mM Na$_2$EDTA (pH 8.5). Two volumes of distilled H$_2$O were added plus six volumes of 95% ethanol in order to precipitate out plasmid DNA. DNA was dried and resuspended in 1ml of 10mM Tris ;1mM EDTA . DNA concentrations were determined spectrophotometrically by ratios of OD$_{260}$/OD$_{280}$ (ratios over 1.7 were considered DNA ) and DNA
concentrations were calculated as O.D. 260/280 extinction coefficient x dilution.

Preparation of Eucaryotic DNA:

The isolation of high molecular weight eukaryotic DNA from cells grown in tissue culture was performed by the method of Blin and Stafford (1976) (62). Briefly, $2 \times 10^7$ cells were washed twice in PBS pH 7.4 and resuspended in 0.2 ml of ice cold 10 mM Tris : 1 mM EDTA. DNA extraction was performed by sequential addition of 0.33ml of 10% SDS and 0.033ml of Proteinase K (10mg/ml) respectively, followed by incubation in a 37°C incubator for 18 hours. The following day the resultant DNA was phenol extracted, phenol chloroform (CHCl₃ 24:1 in isoamyl alcohol) extracted (3:1 then 1:1), chloroform extracted, and finally dialyzed against two changes of Tris 10mM : 1mM EDTA at 4°C. DNA concentrations were determined spectrophotometrically by measuring absorbance at 260nm. RNA contamination was absent as evidenced by 0.3V. agarose gel electrophoresis after treatment of DNA with 100ug/ml Rnase(Sigma).

Nick Translation of pPD8 Alu Probe:

The human repetitive DNA sequence probe pPD8 was radioactively labelled on one strand using nick translation. The following mixture was prepared for nick translation: 0.2ug of pPD8 DNA isolated previously as described, 2.5ul of 10X nick translation buffer (0.5M Tris pH 7.2, .1M MgSO₄, 1mM DTT,
.5mg/ml BSA), 2.5ul of 100uM dXTP (A,T,G), 1.0ul of E. Coli Pol I (possesses a 3' to 5' polymerase + a 5' to 3' exonuclease, approximately 5 units of enzyme) (63), 1.0ul of DNase at 1 mg/ml (in 50mM Tris pH 7.5, 10mM MgCl2, 1mM DTT, 50% glycerol), 60uCi of alpha 32P dCTP (NEN) and distilled water to make a final reaction volume of 25ul. This reaction mixture was microfuged and allowed to incubate for 2 hours at ~12-14°C. TCA precipitable counts were determined prior to stopping translation reaction so that specific activity (10^8 cpm/ug DNA desired) could be determined. The reaction was terminated by the addition of 25ul of nick translation stop buffer (50mM EDTA, 20mM NaCl, 0.1% SDS, 500ug/ml salmon sperm DNA).

Dot Blot Hybridization:

NEN gene screen plus (GSP) hybridization transfer membranes (NEN, Dupont) were prepared for use by presoaking them in distilled water for ten minutes. GSP was then placed concave side up into a 96 well Millipore dot blot vacuum apparatus (Millipore, Bedford, Massachusetts)(aa). Duplicate single stranded DNA quantities (DNA is boiled for 10 minutes and rapidly cooled on ice) of 500, 250, 125, and 50ng were added to wells of this blot apparatus and allowed to soak for 15 mins under light vacuum. Single stranded DNA bound to GSP was baked in a vacuum evacuated dessicator for 1 hour at 65°C. GSP with test single stranded DNA was soaked in 5X SSPE (5mM EDTA, 50mM NaH2PO4, 40mM NaOH, .9M NaCl) + 0.3% SDS for 5 minutes to
remove non-specific binding and then changed to a pre-hybridization mix (5ml of formamide, 2ml of 5M NaCl, 2ml salmon sperm DNA) and allowed to incubate for two hours at 62°C in a heat-sealable bag. 3 x 10^6 TCA precipitable cpm of nick translated pPD8 probe was added and allowed to incubate overnight at 62°C in a shaking water bath. The following day hybridized DNA on GSP was washed free of non hybridized radioactive DNA by four changes of 5X SSPE + 0.3% SDS and subsequently dried under a heat lamp and exposed to Kodak XOMAT-AR film on Dupont Lightning-Plus screens.

Karyotype Analysis

Culture Treatment and Slide Preparation:

Fresh cultures of WeHi-TG and WC hybridomas were prepared the day before karyotype analysis by passing the cultures in RPMI 1640 + 10% FCS at a final concentration of 2 x 10^5/ml. Colcemid (GIBCO) was added to cultures to a final concentration of 250ug/ml and allowed to incubate for 25 minutes at 37°C in order to arrest cells in metaphase by a mechanism of spindle fiber formation inhibition. Cells were spun at 1000rpm for 10 minutes in a v bottom glass tube. Cells were brought up in 10 ml of .075N KCl (for hypotonic lysis) and allowed to incubate for 25 minutes at 37°C. Cells were again spun down at 1000rpm for ten minutes and then brought up in 10 ml of 3:1 methanol: Acetic acid fixative, mixed and allowed to stand for 30 minutes.
at room temperature. Cells were washed 3X with this same fixative solution and slides prepared (65).

Giemsa Staining:

Chromosomes were analyzed using a modification of the G-banding method of Seabright (1971)(66). Briefly, slides prepared with chromosomes were heated for 2 hours at 65°C and trypsinized (2.5 ug/ml solution) for 1 minute, washed in 0.1 M CaCl₂ and allowed to sit in 0.1M CaCl₂ for 1 minute. Slides were washed in distilled water and stained in Harleco Giemsa stain (3ml stain in 40ml dH₂O) for 1 minute and subsequently washed in distilled H₂O and examined microscopically.

Differential Staining:

G-11 differential staining of human and rodent chromosome was done by the method of Bobrow (1974)(67). Briefly, slides prepared with chromosomes were heat fixed for 2 hour at 65°C, wetted with distilled H₂O and placed in freshly prepared (no more than 20 minutes) Harleco Giemsa stain (1/50 dilution in distilled H₂O, pH 11.5) for 8 minutes in a 37°C water bath. Slides were immediately washed in distilled H₂O and heated for 2 minutes at 65°C prior to microscopic analysis.
Preparation of Hematoporphyrin (Hp)-Antibody Conjugates:

Linkage of Hematoporphyrin to monoclonal antibodies as well as antisera has been described and carried out by Mew et al. (1984) (25). Briefly, Hp was conjugated to proteins by the carbodiimide linkage method: to 20mg of Haematoporphyrin dihydrochloride (95%) (Sigma) in 1.25ml water and 0.8ml N,N-dimethylformamide, was added 20mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl in 0.6ml distilled H2O. After 30 minutes, this solution was mixed with 15mg of antibody (monoclonals CAMAL-1 and anti-L1210=L12, R&CAMAL and R&Hu) in 5ml distilled H2O for 5 hours. During this period the pH of this solution was continuously adjusted to 6-7. After the 5 hour incubation period, 50ul of monoethanolamine was added and the solution was left overnight at room temperature. Hp conjugates were dialyzed exhaustively against PBS, following which their Hp concentration was determined spectrophotometrically by taking the absorbance at 505nm (1.0 O.D. is 124ug Hp/ml). Protein concentrations were determined by measuring absorbance at 280nm using a conversion facotor of 1.4 O.D. = 1.0 mg/ml. Cross absorbance contamination was negated by measuring the absorbance of free Hp at 280nm as well as the absorbance of protein at 505nm and calculating the % contribution of each for subsequent subtraction.

Hp conjugates were tested for antibody activity using the ELISA assay directed against their specific antigens and a Hp control. Hp conjugates were also tested for Hp activity by measurement of sheep erythrocyte hemolysis upon exposure to
light in the presence or absence of Hp, Hp conjugates or PBS. Hp conjugates were filter sterilized prior to use in tissue culture systems. Hp concentrations as well as protein concentrations were reevaluated at this time.

In Vitro Cytotoxicity Assays for Hp Conjugate:

Specific Hp conjugate cytotoxicity was determined using a 0.2% trypan blue dye exclusion viability test. 5 x 10^5 prewashed test cells were dispensed in 100ul to 17 x 100mm Falcon test tubes for each test antibody assayed. Primary antibodies not conjugated to hematoporphyrin were added to cell suspensions for 1 hour at 4°C prior to exposure to Hp conjugated antibody (Rabbit anti MIg-Hp as the photoactivatable agent). Hematoporphyrin conjugates were added under minimal light conditions to cells and allowed to incubate in the dark for two hours in a humidified 10% CO₂ incubator. Cells were washed three times in serum free RPMI 1640 and finally resuspended in 13 ml of RPMI 1640 and exposed to an 100W GE 125V incandescent light of intensity 22.5mW/cm², (as measured by a YSI-Kettering model 65 radiometer) at 30cm for 1 hour at room temperature. Cells were then centrifuged, resuspended in 5ml of RPMI 1640 + 10% FCS and incubated in a humidified 10% CO₂ incubator overnight. Relative viabilities were determined as a percentage of cell samples processed in an identical manner but receiving only PBS(25).
3H-Thymidine incorporation Assay

Viable cells were assessed by incorporation of 3H-Thymidine after treatment as above. After removal of cells for 0.2% Trypan blue dye exclusion test, 5 x 10^4 cells in 100μl was plated in six wells of a Linbro microtitre plate (according to original cell numbers) and 50μl of RPMI 1640 + 10% FCS containing 2uCi of 3H-Thymidine (NEN) was added to each well and allowed to incubate 24 hours in a 37°C 10% CO₂ incubator. The cells were then collected on a MASH harvestor as described previously (68) and 3H Thymidine incorporation was measured as cpm on a Hewlett Packard Tri-carb 4550 scintillation counter.
RESULTS

I. Evidence for the Presence of the Common Antigen of Myelogenous Acute Leukemia

(a) Reactivity of Hybrids to Rabbit anti-CAMAL and CAMAL-1 in the Indirect Immunoperoxidase assay:

Interspecific somatic cell hybridization of rodent WeHi-TG (HPRT-) tumor myeloid cells to mononuclear leukocytes and granulocytes from CGL patients resulted in the isolation of 150 hybrids selected after five weeks in culture which showed weak reactivity in ELISA associated with hybrid supernatants. Of these 150 hybrids 6 reacted 100% positively with CAMAL-1 and Rabbit anti-CAMAL antibodies in the indirect immunoperoxidase. These results suggest that these clones arose from a single hybrid cell. Representative photomicrographs are shown in Figure 1. The CAMAL antigen is located on the membrane, nucleus and within the cytoplasm of the hybrid cells in varying degrees. No reactivity was demonstrated with the WeHi control cells.

It was also noted that WC hybrid cells demonstrated differences in growth characteristics as compared to the WeHi parent line. WC lines were capable of growth at higher cell densities and shorter cell cycles (13 hrs vs WeHi 16 hrs) after the initial slow 5 week post fusion growth rate.
Figure 1. Immunoperoxidase Staining of cell lines using CAMAL-1 as the primary antibody. a, WeHi-TG; b, WC 1; c, WC 2; d, WC 4; e, WC 6.
Figure 2. Results of the cellular ELISA on WeHi and WC cell lines using Rabbit anti-CAMAL as the primary antibody.

- - - WC 2; △△△ WC 6; ▲▲▲ WC 1; ■■■ WC 3;

○○○ WC 4; □□□ WeHi-TG.
Figure 3. Results of ELISA's using cell lysates and Rabbit anti-CAMAL as the primary antibody.

- WC 2; 
- WC 6;
- WC 4; WeHi-TG.
(b) Reactivity of Hybrids to Rabbit anti-CAMAL and CAMAL-1 in the ELISA assay:

Immunoperoxidase positive cloned (by limiting dilution) hybridomas were further expanded and tested for the presence of CAMAL using an ELISA procedure with either cell lysates or fixed cells as the solid phase antigen. Results using the cellular ELISA and Rabbit anti-CAMAL antisera are shown in Figure 2. The antiserum reacted strongly with all the selected hybrids and showed only minimal (background) reactivity with WeHi. Similarly, cell extracts of hybrids exhibiting good growth characteristics were tested for the presence of CAMAL. The ELISA results revealed strong reactivity as shown in Figure 3. In these titrations quantitative differences exist between the cell lines in terms of overall reactivity with the rabbit anti-CAMAL polyclonal antibody. It appears that the amounts of CAMAL expressed by the cell lines can be assigned as WC2 > WC6 > WC1 > WC3 > WC4 >> WeHi-TG.

(c) Immunoprecipitation of CAMAL from Hybrids with Rabbit anti-CAMAL and CAMAL-1:

To determine whether apparent expression of CAMAL as detected by indirect immunoperoxidase and ELISA correlated with the production of the 68000 dalton CAMAL protein, immunoprecipitations were carried out on $^{35}$S-Methionine labelled hybrids WC4, WC6 (these hybrids showed the most
consistent growth patterns) and on the parent line WeHi, using both the rabbit anti-CAMAL antibody as well as the monoclonal CAMAL-1. The results shown on Figure 4. clearly demonstrate the presence of a protein of approximately 68000 daltons precipitated from both WC4 and WC6 $^{35}$S-methionine labelled cell lysates using rabbit anti-CAMAL as well as CAMAL-1, whereas the negative control antibody (NRS), does not show a 68000 daltons protein. In addition, no equivalent 68000 dalton protein was immunoprecipitated from $^{35}$S-Methionine labelled WeHi cell lysates by any of the antibodies used.

(d) FACS IV analysis for identification of cell surface CAMAL:
The previous experiments showing the presence of CAMAL antigen associated with our hybrid cell lines do not differentiate between an outer membrane or intracellular location. In order to determine whether there was CAMAL on the outer membrane of our hybrids WC4 and WC6 we performed FACS IV analysis using the rabbit anti-CAMAL antisera. Results in TABLE I show direct comparisons between WeHi and the hybrids and reveal a large number of highly fluorescing cells on hybrids WC4 (72.6%) and WC6 (63.7%) using rabbit anti-CAMAL antisera as compared to the WEHI (8.4%) parent or the non-CAMAL expressing variant of WC2, WC2$^v$ (15.9%) line. These findings suggest that there are the presence of CAMAL molecules on the outer membrane of our cell hybrids WC4 and WC6.
Figure 4. Immunoprecipitation of WC 4, WC 6, and WeHi-TG after 35S-Methionine in vivo labelling, using either CAMAL-1 or Rabbit anti-CAMAL. Lane a, NRS; lane b, Rabbit anti-CAMAL; lane c, CAMAL-1.
TABLE I  Results of FACS IV analysis (using Rabbit anti-CAMAL antiserum) of WeHi-TG directly compared to hybrid fusion products.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Cell Line</th>
<th>Number of Cells</th>
<th>Fluores. Avg.</th>
<th>% Cells Fluorescing* of 2 trials.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)Normal</td>
<td>WeHi-TG</td>
<td>1107</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>WC2\textsuperscript{V}</td>
<td>552</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>WC4</td>
<td>2643</td>
<td>26.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WC6</td>
<td>1252</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>2)Rabbit anti-CAMAL serum</td>
<td>WeHi-TG</td>
<td>837</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WC2\textsuperscript{V}</td>
<td>1587</td>
<td>15.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WC4</td>
<td>7260</td>
<td>72.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WC6</td>
<td>6374</td>
<td>63.7</td>
<td></td>
</tr>
</tbody>
</table>

* % of 10000 cells analyzed per sample.
II. Evidence for the Presence of Human Proteins and DNA

(a). Reactivity of Hybrids to Myeloid Monoclonal Antibodies:

The presence of the CAMAL antigen on hybrid cells and its apparent absence on the tumor parent line WeHi-TG suggested to us that it was a protein obtained from the CGL fusion partner. Because the human fusion partner was of myeloid origin and we were able to demonstrate the presence of the myeloid specific antigen CAMAL on hybrid cells, we decided to examine our hybrids for other myeloid specific human antigens. A group of monoclonal antibodies, reacting with a variety of myeloid cell markers, were tested with the hybrids and WeHi using the indirect immunoperoxidase method. One of the hybrid cell lines WC2 was part of a study on monoclonal reactivity to human differentiation antigens, and the monoclonal antibodies used to test the other WC hybrids were selected because of their strong reactivity with WC2 in this study. The reactivity of these monoclonal antibodies on our hybrid cells is shown in Table I. A number of monoclonal antibodies reacted not only with WC2, against which they were originally selected, but with a number of the other CAMAL producing hybrids. In particular, the monocyte reactive CRIS-6 showed reactivity to all hybrids and not to the parent tumor line WeHi-TG. NKH1A, the NK reactive monoclonal showed very strong reactivity to the hybrid lines WC2 and WC6 which express the largest amounts of CAMAL as suggested by ELISA results. CLB gran, an NA1 reactive monoclonal, shows reactivity
Table II. Reactivity of various MAbs with reactivity for myeloid markers with the somatic cell hybrids. Cytospin preparations were tested.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>NKH1</th>
<th>CRIS-6</th>
<th>94-3D1</th>
<th>JOAN-1</th>
<th>M101</th>
<th>KD3</th>
<th>CA-1</th>
<th>CLB LFA 1/1</th>
<th>CLB gran</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEHI</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>•+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WC-1</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WC-2</td>
<td>++++</td>
<td>•+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>WC-3</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>WC-4</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>•+</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>WC-6</td>
<td>++++</td>
<td>•</td>
<td>-</td>
<td>•</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>-</td>
</tr>
</tbody>
</table>

1. NK cell reactive.
2. Monocyte reactive, not reactive with lymphocytes, platelets or erythrocytes.
3. Monocyte and platelet reactive.
4. Panreactive (myeloid + lymphoid).
5. Panreactive (myeloid + lymphoid).
6. Panreactive (myeloid + lymphoid).
7. Myeloid marker (mainly granulocytes).
8. NK cell reactive, recognizes LFA 1/1.
10. MA1 antigen reactive.
11. * signs indicate intensity of immunoperoxidase staining going from ± to ++++. 
only to the high CAMAL expressing hybrid WC2 (as shown by ELISA) and not to any of the other hybrids or WeHi-TG. Thus, while a high frequency of reactivity was observed with a number of myeloid associated monoclonal antibodies (CRIS-6, JOAN-1, CLB LFA 1/1), no absolute pattern of linkage could be construed from these experimental findings.

(b) Dot Blot Analysis for identification of "Alu" Sequences:

Immunological and biochemical evidence for human myeloid antigens as well as the leukemia associated antigen CAMAL suggested that human DNA was present in our cell hybrids. In order to verify this contention we performed Dot Blot analysis of hybrid DNA using the BLUR 8 plasmid probe which possesses the human repetitive Alu sequence. Results are shown in Figure 5, positive controls pPDB (BLUR 8) hybridized to itself and 8348 hybridized to Alu sequences within the pPDB probes verify the ability of this nick-translated probe to hybridize human DNA sequences as well as pBR322. Since the dotted volumes of specific DNA amounts were normalized across samples, the relative dot diameters and respective intensities of hybridised DNA dots, were able to provide some information on human DNA quantities within hybrid cells. Relative DNA amounts present in hybrids can be ordered in terms of decreasing quantities as follows: WC 6 > WC 4 > WC 3 > WC 1 > WC 5 > WeHi.
Figure 5. Dot blot analysis for detection of human repetitive DNA sequences in WeHi-TG and WC DNA. DNA is spotted in duplicate at 500, 250, 125 and 50 ng. pPD8 refers to the Alu containing plasmid probe and 8348 is a human DNA positive control.
(c) Analysis of Hybrid Cell Karyotypes:

Karyotype analysis was carried out on all hybrid lines to determine whether any of the hybrids contained whole human chromosomes or identifiable human translocations to mouse chromosomes. Karyotype results are shown in Figure 6, WeHi-TG the parent line, exhibited 32 normal mouse chromosomes and 6 distinct marker chromosomes. All hybrid cells karyotyped revealed an increase in chromosome number as well as several new marker chromosomes as listed in Table III. Despite the increase in chromosome number as well as the emergence of new marker chromosomes, no obvious chromosomes or segments of human chromosomes were demonstrated using Giemsa banding or differential staining procedures. Thus, our results suggest that translocation of human DNA to murine chromosomes has taken place (as evidenced by HAT resistance which is a trait mapped to the long arm of the X chromosome, CAMAL production and expression of other human myeloid associated markers) in the absence of identifiable human chromosome material.
Figure 6.  

a) G-Banded karyotype of the WeHi-TG cell line. M1-M6 refer to characteristic markers of this cell line.

b) G-Banded karyotype of the WC1 cell line. M5' is an isochromosome derivative of a segment of M5. M7-M10 are new markers characteristic of WC1. No identifiable human chromosomes or segments are present.
Figure 6.  c) G-Banded karyotype of the WC 2 cell line.

  d) G-Banded karyotype of the WC 4 cell line.
Figure 6. e) G-Banded karyotype of the WC 6 cell line.
<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>TOTAL CHROMOSOME #</th>
<th>WeHi MARKER</th>
<th>NEW MARKER</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>CHROMOSOME</td>
<td>CHROMOSOME</td>
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<td>WeHi</td>
<td>38</td>
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<tr>
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III. Utilization of WC Cell lines as Models for Treatment of Human Leukemias using Hp Conjugates.

The development of an interspecies somatic cell hybrid expressing human leukemia associated antigen on its surface (as shown by FACS analysis) yet being of murine origin had implications to us for the development of an in vivo model for cancer therapy. The ability of WC clones to grow in H-2d Balb/c mice as well as the cell surface expression of CAMAL provided us with a rationale for the use of our hybrids for human leukemia models. Work in our laboratory by Daphne Mew(37) on antibody-Hp conjugated tumor specific destruction in an in vivo mouse system, provided us with the background for our preliminary work.

Determination of Hp conjugate levels for an initial in vitro killing assay was performed by titration of Hp-conjugates (Figure 7) on a fixed number of cells (1 x 10^6), either WeHi or WC 6. The hematoporphyrin level chosen was that which maximized specific killing and minimized toxic effects of hematoporphyrin by itself. Titration revealed that 12 ng Hp/10^6 cells was the optimal level for our hybrid system. Consequently all experiments performed involved the use of Hp-conjugate concentrations of 12 ng/10^6 cells.

Preliminary results demonstrating antigen-specific tumor destruction are shown in Figure 8. High levels of killing occurred in the WC 4 and WC 6 cell lines with both Hp conjugated CAMAL-1 and rabbit anti-Hu whereas none occurred with the other
preparations. The antibody preparations failed to demonstrate any specific killing when used to treat the parent cell line WeHi-TG. In addition, the WC2\textsuperscript{V} cell line, (a non CAMAL expressing variant of WC2) was unaffected by either Hp conjugated CAMAL-1 or rabbit anti-Hu antisera, suggesting complete loss of CAMAL-1 and other human myeloid surface antigens.
Figure 7  a) Titration of hematoporphyrin compounds on WeHi-TG at fixed cell numbers of $1 \times 10^6$ were treated with various amounts of different Hp conjugates. Viability was determined by eosin y exclusion.

(●—●), Rabbit anti Human-Hp;

(■—■), MAb CAMAL-Hp;

(▲—▲), MAb anti L1210-Hp;

(△—△), Hematoporphyrin alone Hp;
Figure 7. b) Titration of hematoporphyrin compounds on WC 6 at fixed cell numbers of $1 \times 10^6$ treated as per a).

(●●●), MAb CAMAL-Hp;

(○○○), Rabbit anti Human-Hp;

(■■■), MAb anti L1210-Hp;

(△△△), Hematoporphyrin alone Hp;
Figure 8. Survival of cells of the WeHi-TG, WC 2V, WC 4 and WC 6 cell lines, following treatment with 12.0 ng/10^6 cells of Hp either alone (——) conjugated to Rabbit anti-Hu Ab (----), MAb CAMAL-1 (-----) or MAb anti L1210 (-----). PBS (-----) control was shown in each case.
DISCUSSION

The expression of human proteins in interspecific (Human-Mouse) hybrids depends not only on the presence of the genes for these proteins but on appropriate intrinsic and/or extrinsic regulatory factors. Intralineage fusion partners presumably provide for this regulatory environment and thus allow expression of certain lineage associated proteins over others (27). In this study we present evidence for the successful fusion of a mouse myeloid cell line and human myeloid cells (from a patient with CGL), and the subsequent expression of human myeloid proteins as well as a leukemia-associated protein. The expression of this leukemia-associated antigen, CAMAL, as well as other myeloid antigens in our hybrids has been verified using biochemical (immunoprecipitations), cytological (immunoperoxidase) and immunological (ELISA, FACS analysis, Hp CAMAL-1 specific cell destruction) methodology. The apparent molecular weight of CAMAL expressed on/in hybrid cells appears to have a value of approximately 68000 daltons. The concurrent presence of a variety of human myeloid markers and the CAMAL antigen suggested to us the possibility of linkage with some of these genes but further study using specific DNA probes must be done to verify this (myeloid as well as CAMAL antigen specific probes).
Somatic cell hybrid clones WC 4 and WC 6 were chosen for the majority of our studies due to their growth stability as well as their consistent expression of CAMAL over the course of their one year in continuous culture. Expression of CAMAL by WC hybrids was shown to be variable by ELISA, immunoprecipitation (WC 6 showing more overall CAMAL as compared with WeHi) as well as FACS IV analysis (WC 4 showing more cell surface CAMAL than WeHi). These results were confirmed when cell surface antigen dependent Hp-CAMAL killing showed WC 4 to be more susceptible to cell destruction as compared with WC 6. Although we have no data on cell surface expression of human myeloid antigens on WC 4, reactivity to human myeloid antigen specific monoclonal antibodies using immunoperoxidase, suggests a large variety of myeloid antigens are present. Presumably this would provide for greater killing of WC4 as compared with WC6 when treated with the positive control R&Hu-Hp conjugates. Some of the hybrids described here (WC 6 for example), produce considerably more CAMAL than do human cell lines derived from human myeloid leukemias, thus providing a suitable cell line for in vivo labeling, CAMAL functional studies and possible cloning of the CAMAL gene.

Dot blot analysis as well as aminopterin resistance and expression of other human myeloid antigens provided strong evidence that human DNA material was present and being expressed in WC hybrids. Contrary to Dot blot findings, karyotypic analysis by Giemsa and differential staining failed to reveal the presence of whole human chromosomes or visible translocations.
Thus it was not possible to determine unequivocally whether or not there were present short segments of human DNA coding for specific gene products within hybrid cells. These results suggest small karyotypically undetectable translocations have occurred within our hybrid lines. Consequently, we were unable to associate or assign the CAMAL gene to a particular chromosome(s).

Somatic cell hybridisation techniques have been frequently used for the purpose of assigning various gene products to specific chromosomes. Recent work by Von Dem Borne has revealed that expression of a neutrophil antigen NA1 previously mapped to chromosome 11 can occur in the absence of karyotypically detectable chromosome 11 (personal communication). These findings suggest that one must exercise caution when assigning specific proteins to specific chromosomes in somatic cell hybrid systems.

The expression of CAMAL antigen in or on hybrid cell lines that apparently lack karyotypically detectable human chromosome material suggest that genes coding for CAMAL antigen may provide a selective advantage to somatic cell fusion products. This contention is supported by changes in growth characteristics (loss of anchorage dependant growth i.e. growth at higher densities and less fibroblastic in appearance), faster growth rates compared with WeHi (13 vs 16 hrs/doubling). Presumably the original fusion products possessed detectable
human chromosomes and throughout the hybrids one year in culture were lost. Surviving cells must have retained that portion of the X chromosome coding for the HPRT enzyme in order for its continued growth in selective medium HAT. The retention of other portions of human chromosomes would logically provide for greater survival. Although the function of the CAMAL protein is at present unknown, one can speculate as to the function of this leukemia associated antigen. CAMAL is present on a major population of CGL cells thus it is conceivable that it may have a positive function in the growth and survival of leukemic cells. The origin and function of the CAMAL gene product is currently being investigated. In addition, analysis of DNA from our hybrid cell lines using DNA probes from a human chromosome library is presently underway, in an attempt to elucidate which human gene sequences are present. Isoenzyme analysis of our hybrids will further verify the presence of human gene segments.

The successful antibody mediated hematoporphyrin destruction of hybrid cells expressing specific antigens has verified the potential use of MAb-Hp conjugate for "magic bullet type" cancer immunotherapy. The specific homing ability of antibodies coupled with the destructive effects of hematoporphyrin, provides for a potentially powerful tool for the treatment of cancer victims. These in vitro results
demonstrated that WC hybrid cells possess characteristics of human leukemia suitable for development into an in vivo mouse model for human leukemia study. Ongoing experiments are presently directed towards development of this animal model system.
HEMOPOIETIC STEM CELL DISORDERS - POSSIBLE SITE OF LESION

**PLURI-POTENT STEM CELL**

Myeloproliferative Diseases, Aplastic Anemia

**MYELOID STEM CELL**

? Acute Myeloid Leukemia (AML)

**ERYTHROID PROGENITOR**

Erythroid Leukemia

- PRIMATIVE BFU
- MATURE BFU
- CFU-E
- RED CELLS
  - Polycythemia Vera (PV)

**PLATELET PROGENITOR**

Myelofibrosis

- CFU-M
- MEGAKARYOCYTES
- PLATELETS
  - Essential Thrombocytosis (ET)

**GRANULOCYTE PROGENITOR**

Granulocytic Leukemia

- CFU-C
- GRANULOCYTES
  - Chronic Granulocytic Leukemia (CGL)

**LYMPHOID STEM CELL**

Acute Lymphoblastic Leukemia (ALL)

**LYMPHOCYTES**

- Hodgkin's Disease
- Lymphoma
- Chronic Lymphocytic Leukemia (CLL)

**PLASMA CELLS**

Multiple Myeloma
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(41) Trypan Blue Exclusion test


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