Isolation and Characterization of a Vulvar Carcinoma in Tissue Culture: Studies of the Tumor Biology and Tumor-Host Interaction

Melvin G. Dodson
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ISOLATION AND CHARACTERIZATION OF A VULVAR CARCINOMA IN TISSUE CULTURE:
STUDIES OF THE TUMOR BIOLOGY
AND TUMOR-HOST INTERACTION

by
Melvin G. Dodson

A Dissertation Submitted to
the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

May
1980
ACKNOWLEDGEMENTS

The most difficult aspect of any work, especially one of this magnitude and duration, is the adequate recognition of all those who helped to make it possible. It is both humbling and gratifying to reflect on the many hours, countless discussions and monumental patience of those whose special efforts have contributed to the ultimate success of this work.

To Dr. Charles Lange, my advisor, my deepest appreciation is extended for his courage in accepting this naive and over-zealous student; for his persistent coaching, guidance, and unwavering assistance through what I'm sure were trying times; for his tolerance and forbearance of this student who was consistently late for registration and generally on the short end of one rule or another. Dr. Lange is an exceptional scholar and scientists, and I consider it a privilege to have been his student.

Dr. Irene Check has been and continues to be the model of a superlative yet practical scientist; always current and comprehensive in her knowledge while simultaneously providing its realistic and direct application. If I someday learn one-half of what Dr. Check already knows, I will consider myself a scientist.
For the values that both good science and strong friendship bring to the things that make life better, my sincerest respect and gratitude are extended to Dr. Eugene Major. He listened to and discussed LT-2 endlessly; sharing all the many frustrations and failures as well as the few but meaningful triumphs. From Dr. Major I have learned the significance and validity of the statement, science means hard work.

Dr. Mira Menon selflessly relinquished her laboratory, time, knowledge and patience to teach me tissue culture techniques. She shared with me all of the skills which produce sound research, while exemplifying the greater qualities of meaningful science.

The epitome of intellectual pursuit and utter enthusiasm, Dr. Marvin Stodolsky has provided the inspiration and opportunity to break out of the constraints of realism and revel in the fun of "pure science." I enjoyed and needed our many brainstorming sessions and appreciated his guidance and expertise, particularly in mathematics and genetics.

This work also reflects the tireless efforts of Mel Klegerman and John Slota, my technical assistants, who are both exceptionally capable in the laboratory and who helped and often taught me the tricks of the trade.

My special thanks to Maria Weber for teaching me many laboratory techniques and in general for putting up with me
in "her" laboratory. Without the perseverance and good humor of Carol Friis, this dissertation would still be on little scraps of paper, and I thank her.

My lasting thanks to these and so many others who doggedly helped this old student learn new tricks.

· Mel Dodson
VITA

The author, Melvin G. Dodson III, was born September 19, 1940 in Boston, Massachusetts.

Educated in private schools in Savannah, Georgia and Miami, Florida, he graduated from Archbishop Curley High School, Miami, Florida in 1959. His undergraduate education was pursued at Tulane University and The University of Florida at Gainesville.

In September, 1963, he entered the University of Miami Medical School where he was subsequently awarded two Summer Fellowships for work in transplantation immunology and epidemiology of glomerulonephritis. He received his Doctor of Medicine degree in 1967.

He served both an internship and residency in Obstetrics and Gynecology; achieved Board Certification by the American Board of Obstetrics and Gynecology in 1973; and is a Fellow of the American College of Obstetricians and Gynecologists.

In 1973, he was awarded the Joint Service Commendation Medal after serving as a Major and Section Chief in the United States Army Medical Corp from 1971-1973.
During his two years in private practice, he was also a consultant to the University of Miami, Department of Family Medicine; Chairman, Obstetrics and Gynecology and member of the Executive Committee, Miami-Dade General Hospital.

He has been an Assistant Professor, Obstetrics and Gynecology, Loyola University Stritch School of Medicine since 1975.

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The following is a list of his published works:


ABSTRACT

Melvin G. Dodson
Loyola University of Chicago

ISOLATION AND CHARACTERIZATION OF A VULVAR CARCINOMA IN TISSUE CULTURE: STUDIES OF THE TUMOR BIOLOGY AND TUMOR-HOST INTERACTION

LT-2, a squamous cell carcinoma, represents the third vulvar cancer isolated in tissue culture.

Immunological evaluation of the host revealed markedly decreased absolute numbers, percentages of peripheral blood lymphocytes, T-cell, active T-cell, and B-cell. A response was noted to only one of five bacterial recall skin test antigens, which subsequently became negative when re-tested 6 weeks later. No response was noted to primary immunization with DNBCB, but the patient became PPD positive following intralesional BCG therapy. The patient became anergic even to PPD within 2 months. No clinical response was noted to BCG treatment, although a "bone marrow" sparing effect with an increase in lymphocytes occurred. No response occurred to immunization with an autologous tumor extract. Lymphoblast responses to PHA were markedly depressed and no lymphoblast response occurred to autologous tumor extracts.
Cytotoxicity studies using autologous lymphocyte and tumor cells revealed no specific cytotoxicity and non-specific cytotoxicity of patient lymphocytes to an unrelated tumor were depressed. A significant increase in both specific cytotoxicity and non-specific cytotoxicity was noted following a blood transfusion.

LT-2 has continued to grow in vitro through 70 plus subcultivations during a 4 year period. It grows as a strict monolayer with no piling up of cells and a plating efficiency of about 40%. The doubling time of 34 hours and the saturation density of $1 \times 10^5$ cells/cm$^2$. LT-2 requires serum for growth. In time-lapse cinematography, LT-2 is motile and small colonies are noted to move together with no contact inhibition of cell movement (topoinhibition), although strong lateral cell to cell adhesions were noted. Cell divisions were also noted in the center of large cell colonies of LT-2 demonstrating a lack of contact inhibition of cell division despite monolayer growth. LT-2 produces the β-chain of hCG and CEA in tissue culture and was agglutinated by lectins. It retained its ABH antigens, but expressed a heterophile antigen and was agglutinated by normal rabbit serum. This heterophile antigen was different from the heterophile antigen expressed in infectious mononucleosis infections.

Karyotyping of LT-2 demonstrated aneuploidy with marker chromosomes and considerable variation in chromosome numbers.
LT-2 was noted to divide in soft agar increasing the number of cells in agar cultures by about 60%, but no clones were produced. Experiments were performed to determine if a soluble stimulant or inhibition of anchorage independent growth could be detected. Co-cultivation of an anchorage independent cervical carcinoma cell line with LT-2 resulted in stimulation of cell growth with increased cloning efficiency. No "feeder effect" was often noted after 2 weeks. Mitomycin C treated AU-471 and WI-38 were also capable of "feeding" AU-471. The "feeder effect" was transferable with conditioned media and was generally dose dependent.

Co-cultivation of LT-2 with mitomycin C or protein extracts of AU-471 did not stimulate anchorage independent growth of LT-2.

Anchorage independence could not be stimulated by enzyme treatment with carbohydrates, neuraminidase, culturing in agarose or with DEAE or dibutyrl cyclic GMP.

Fibronectin markedly increased cloning efficiency of AU-471, but not LT-2 or "normal" cell strains like M.S. or WI-38, that do not clone in soft agar. BHK-21 soft agar cloning was stimulated by fibronectin. Antifibronectin inhibited cloning efficiency of AU-471, but not BHK-21. Injecting LT-2 in nude mice resulted in tumor formation. Tumor nodules were relatively well differentiated epithelium with keratin formation toward the center of tumor nodules.
Chromosomal analysis of in vitro cultivated nodule cells indicated marked aneuploidy. Soft agar cultures of LT-2 nude mouse nodule cells did not produce anchorage independent growth. No metastases were noted.
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<tr>
<td><strong>A</strong></td>
<td>Angstrom(s)</td>
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</tr>
<tr>
<td><strong>A°</strong></td>
<td>Blood group antigens: A, B, and H</td>
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<tr>
<td><strong>ABH</strong></td>
<td>Blood group antigen</td>
<td></td>
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<tr>
<td><strong>ADCC</strong></td>
<td>Antibody dependent cell-mediated cytotoxicity</td>
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<tr>
<td><strong>Anti-A</strong></td>
<td>Blood group antigen</td>
<td></td>
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<tr>
<td><strong>Anti-B</strong></td>
<td>Blood group antigen</td>
<td></td>
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<tr>
<td><strong>Anti-H</strong></td>
<td>Blood group antigen</td>
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<td><strong>Anti-RhC</strong></td>
<td>Blood group antigen</td>
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<td><strong>Anti-Rho</strong></td>
<td>Blood group antigen</td>
<td></td>
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<tr>
<td><strong>AT-264</strong></td>
<td>A lung carcinoma cell line</td>
<td></td>
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<tr>
<td><strong>AU-471</strong></td>
<td>A human cervical carcinoma cell line</td>
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<p>| <strong>B</strong>                       | Syngeneic strains of mice |
| <strong>BALB/C, C3H &amp; C57/BL</strong>    | <strong>Bacillus Calmette-Guerin</strong> |
| <strong>B CG</strong>                    | <strong>Beta chain of human Chorionic Gonadotropin</strong> |
| <strong>BH K-21</strong>                 | <strong>A baby hamster kidney cell line</strong> |</p>
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<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>C</td>
<td>Degree(s) Centigrade</td>
</tr>
<tr>
<td>oc</td>
<td>Plus or minus complement</td>
</tr>
<tr>
<td>C₃ + or −</td>
<td>Corynebacterium parvum</td>
</tr>
<tr>
<td>C. parvum</td>
<td>Sub-division of RNA tumor viruses</td>
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<td>C-type</td>
<td>Cytochalasin B</td>
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<tr>
<td>CB</td>
<td>Strain of syngeneic mice</td>
</tr>
<tr>
<td>CBA</td>
<td>Carcinembryonic antigen</td>
</tr>
<tr>
<td>CEA</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Curie(s)</td>
</tr>
<tr>
<td>Ci</td>
<td>Cold-insoluble globulin</td>
</tr>
<tr>
<td>CIG</td>
<td>Centimeter(s)</td>
</tr>
<tr>
<td>cm</td>
<td>Complete medium</td>
</tr>
<tr>
<td>CM</td>
<td>Complement mediated antibody dependent cytotoxicity</td>
</tr>
<tr>
<td>CMAD</td>
<td>Cell mediated cytotoxicity</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethyl aminoethyl</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNBCB</td>
<td>Dinitrochlorobenzene</td>
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<td>DTH</td>
<td>Delayed-type hypersensitivity reaction</td>
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EDTA  Ethylenediaminetetraacetic acid
EGF  Epidermal growth factor
Exp.  Experiment

F  Fibronectin
FBN  Crystallizable fragment of Papain enzyme digestion of IgG
FCS  Fetal calf serum
FGF  Fibroblast growth factor
F.L.  Floating cells
ft  Foot/feet

G  Gram(s)
g  Gravity
G phase  Portion of the cell cycle
GAFS  Goat antifibronectin sera
GMP  Guanosine monophosphate

H  Tritium thymidine
3H-T  Major histocompatibility antigens of the mouse
H-2  Human Chorionic Gonadotropin
hCG  High powered microscopic field
Hp  Hour(s)
hr.  Hour(s)
IgG  Class of immunoglobulin
IgM  Class of Immunoglobulin
Im + and -  Infectious mononucleosis positive and negative antisera
I.U.  International unit(s)
I.V.  Intravenous
IVP  Intravenous pyelogram

K  Killer cells
K cells  Killer cells
Ki-NRK  Kirsten virus infected rat kidney
KRV  Kilham rat virus

L  Liter(s)
L  Liter(s)
LCMV  Lymphocytic choriomeningitis virus
LETS  Large external transformation sensitive protein
LH  Luteinizing hormone
LMC  Lymphocyte-mediated cytotoxicity
LNM7  Nude mouse line - 7
log N_t  Log of the number of cells at time t
log N_0  Log of the number of cells at time o
LT-2  Squamous cell carcinoma of the vulva
LT-4  Normal human fibroblast cell strain
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram(s)</td>
</tr>
<tr>
<td>min.</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>mI.U.</td>
<td>Milli International Unit(s)</td>
</tr>
<tr>
<td>ml</td>
<td>Milli liter</td>
</tr>
<tr>
<td>M.L.</td>
<td>LT-2 patient's initials</td>
</tr>
<tr>
<td>mm</td>
<td>Milli meter</td>
</tr>
<tr>
<td>mM</td>
<td>Milli Molar</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>MN</td>
<td>Red blood cell antigen</td>
</tr>
<tr>
<td>mos.</td>
<td>Month(s)</td>
</tr>
<tr>
<td>M-RNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>A diploid fetal fibroblast cell line cultured from an abortion</td>
</tr>
<tr>
<td>ND</td>
<td>Not done</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram(s)</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NMNC</td>
<td>Nude mouse nodule cultures</td>
</tr>
<tr>
<td>No.</td>
<td>Number</td>
</tr>
<tr>
<td>NRK</td>
<td>Normal rat kidney</td>
</tr>
<tr>
<td>NRS</td>
<td>Normal rabbit serum</td>
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xxvii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.S.</td>
<td>Not significant</td>
</tr>
<tr>
<td>NSILA</td>
<td>Nonsuppressible insulin-like activity</td>
</tr>
<tr>
<td>nu</td>
<td>&quot;Nude gene&quot; specifying thymic agenesis</td>
</tr>
<tr>
<td>nu/nu</td>
<td>Homozygous for the nude gene</td>
</tr>
<tr>
<td>O</td>
<td>Chemical used for embedding tissue in order to cut frozen histologic sections</td>
</tr>
<tr>
<td>O.C.T.</td>
<td>Chemical used for embedding tissue in order to cut frozen histologic sections</td>
</tr>
<tr>
<td>P</td>
<td>Term used in statistics to express the probability of significance. A P value of less than .05 is generally considered significant.</td>
</tr>
<tr>
<td>Pas.</td>
<td>Passage</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Plating efficiency</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PM-10</td>
<td>Membrane used in ultrafiltration with a pore size retaining molecules with a molecular weight greater than 10,000 daltons</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>Polyninosinic-polyctidylic acid</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative used in skin testing for tuberculosis</td>
</tr>
<tr>
<td>R</td>
<td>Rate</td>
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</tbody>
</table>

xxviii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>rad</td>
<td>Radiation absorbed dose(s)</td>
</tr>
<tr>
<td>RFC</td>
<td>Rosette forming cells</td>
</tr>
<tr>
<td>Rh</td>
<td>Blood group antigen</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Nutrient media used in feeding culture</td>
</tr>
<tr>
<td>RX</td>
<td>Treatment/Reaction</td>
</tr>
<tr>
<td>S</td>
<td></td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneously</td>
</tr>
<tr>
<td>SCMC</td>
<td>Spontaneous cell mediated cytotoxicity</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>S.E.</td>
<td>Standard error</td>
</tr>
<tr>
<td>S.I.</td>
<td>Stimulation index</td>
</tr>
<tr>
<td>SMAF</td>
<td>Specific macrophage arming factor</td>
</tr>
<tr>
<td>SRBC</td>
<td>Sheep red blood cell</td>
</tr>
<tr>
<td>Std.</td>
<td>Standard</td>
</tr>
<tr>
<td>SV-40</td>
<td>Simian virus</td>
</tr>
<tr>
<td>T</td>
<td></td>
</tr>
<tr>
<td>t</td>
<td>Time</td>
</tr>
<tr>
<td>T</td>
<td>Student statistical T test used to calculate significance of statistics</td>
</tr>
<tr>
<td>3T3</td>
<td>Regimen used for feeding and sub-culturing cells</td>
</tr>
<tr>
<td>T-30</td>
<td>Small culture flask</td>
</tr>
<tr>
<td>xxix</td>
<td></td>
</tr>
</tbody>
</table>
T-250
TC
TCA
tD
T/E
T-neg.
tntc
TPB

Large culture flask
Tissue culture
Trichloroacetic acid
Doubling time
Method of harvesting monolayer cell cultures. Cultures are washed with EDTA, then EDTA with trypsin
Non-thymus
Too numerous to count
Tryptosephosphate broth

U
μ
Unit(s)
μC
Micro curie
μg
Microgram(s)
UV
Ultraviolet

V
V.
Viable

W
WBC
WGA
WI-38
White blood count
Wheat germ agglutinin
A near diploid "normal" lung fibroblast cell strain

xxx
CHAPTER I

INTRODUCTION

Many approaches have been undertaken in research directed towards understanding and treating cancer. Some studies have investigated one cancer characteristic, or used one assay in many different cancer types, while others have intensely studied one protein or one phenotype or characteristic associated with cancer. This dissertation has by both necessity and design used a large number of clinical and experimental assays to better appreciate the complex interaction between the tumor and the tumor host. From an in-depth study of a tumor cell, new insights into the behavior and characteristics of the cancer cell might be achieved. The cancer cell model studied was an epithelial carcinoma originating from the vulva which clinically progressed to the death of the patient, confirming its biological potential.

Tumor immunology has generated considerable enthusiasm as a potential method for the treatment of cancer. Evidence of immune responses to tumor cells have come from a wide range of clinical and experimental observations in research. The participation of the immune response in controlling tumors can be discussed under the general concept
of "immune surveillance". In order to have an immune response, tumor cells must contain a "tumor antigen". The host must be "immunocompetent" and capable of responding to such an antigen. In order to bring the question of immune responses from the clinical bedside to the laboratory for closer scrutiny, in vitro "cytotoxicity" studies have been developed using host lymphocytes and tumor cells in culture. Chapter 2, Section A. will summarize pertinent aspects of these "four topics" under the appropriate headings.

A study of tumor immunobiology cannot divorce itself from a basic understanding of tumor cell physiology. Although, on a clinical level, cancer is relatively easy to define by its biological behavior, the exact criteria of tumorigenicity on a cellular level in vitro are much more difficult. Considerable advancements have been made in culturing tumor cells in vitro, and a number of cell phenotypes have been found to be associated with tumorigenicity. A brief review of these cancer phenotypes as pertinent to this discussion appears in Chapter 2, Section B.

Reported here is the establishment and characterization of a squamous cell carcinoma of the vulva in tissue culture (designated LT-2), including evaluation of its malignant potential, cytomorphology, growth properties, secretory products, antigens, in vitro behavior, and immunological studies of the host, clinical course, therapy, and some aspects of the tumor-host relationship.
The following briefly reviews the findings and clinical course of the patient from which the tumor LT-2 was isolated.

A 60-year-old white woman was seen in June of 1975 with a two-week history of vulvovaginal itching, swelling, and discharge. Extensive induration of the external genitalia with ulcerative lesions involving the introitus and anterior vaginal wall was noted. Groin nodes on both sides were enlarged. Biopsy revealed squamous cell carcinoma. Chest x-ray, intravenous pyelogram (IVP), and liver and bone scans were normal. The patient refused surgery and was treated with radiotherapy consisting of 6,000 rads in 30 fractions over 56 days through a single perineal port. Additional therapy to the inguinal nodes consisted of 4,550 rads in 26 fractions over 36 days. Some regression of the vulvar lesions was noted. The inguinal nodes remained enlarged and were treated with an additional 1,050 rads in six fractions. She did well until December, 1975, when she noted increasing pelvic discomfort and pain. By January, 1976, the inguinal nodes had ruptured through the skin, producing a fungating lesion. She was started on dactinomycin, 0.8 mg intravenously, daily for five days and bleomycin, 20 units intravenously every other day for three days. She experienced considerable nausea and vomiting and refused further chemotherapy. She received two units of blood one
month later because of anemia. Adjuvant immunotherapy consisting of $2 \times 10^6$ Bacillus Calmette-Guerin (BCG) organisms was administered intraleisonally. She was started on Cytoxan, 50 mg four times daily, depending on the leukocyte count, and received three additional BCG immunizations during breaks in chemotherapy. In vivo tests of immunocompetence consisted of skin testing with bacterial recall skin test antigens, dinitrochlorobenzene (DNCB), and autologous tumor extracts. In vitro tests of immunocompetence included total and percent peripheral blood T-cells, active T-cells, B-cells and phytohemagglutinin (PHA) stimulation of lymphocytes. The tumor-host relationship was evaluated by autologous lymphocyte-tumor cytotoxic reactivity as measured by a chromium release assay, and lymphoblast transformation in the presence of tumor extracts and mitomycin C-treated tumor cells as described in the text. The patient died in September, 1976.
CHAPTER II

REVIEW OF RELATED LITERATURE

A. TUMOR IMMUNOBIOLOGY

1. IMMUNE SURVEILLANCE

In a general discussion of delayed hypersensitivity, Thomas suggested in 1959, that it is a "universal requirement of multicellular organisms to preserve uniformity of cell type and to prevent mutant cells from colonizing and flourishing," and that "the phenomenon of homograft rejection will turn out to represent a primary mechanism for natural defense against neoplasia" (1). This concept was expanded by F.M. Burnet into the concept of immunological surveillance which suggests that neoplasms frequently occur and contain new tumor antigens and subsequently are rejected by the immune system (2). According to this concept, it is the immune system that prevents the host from developing cancer.

Observations that have been used to support the concept of immune surveillance according to Moller et al (3), include the following: a) The incidence of neoplasia in the elderly is increased simultaneously with a decrease in the efficiency of immune responses. Old mice cannot reject
small numbers of sarcoma cells that can be readily rejected by younger mice. Old mice die and young mice are immunized.

b) The incidence of malignancies diagnosed histologically is much higher than the known clinical incidence; for example, autopsied infants have 50 times greater neuroblastomas diagnosed histologically than the known clinical incidence. Similarly, the incidence of histologically malignant thyroid nodules noted in autopsy specimens is 2%, but the clinical incidence of thyroid cancer is only 6 per $1 \times 10^6$ individuals. It has been suggested that the immune system accounts for these differences. c) The increased incidence of tumors in some genetic immunodeficiency disorders like Ataxia Telangiectasia (i.e., 14 tumors in 200 reported cases), Chediak-Higashi (11/50) and Wiskott-Aldrich (11/90) has also been used to support the concept of immune surveillance. Gatti (4) has reported that the incidence of malignancy in patients with primary immunodeficiencies is 10,000 times that of the general age-matched population. d) The increased incidence of tumors in immunosuppressed individuals, for example in patients receiving kidney transplants, also confirms the role of the immune systems in tumors. In 4,000 renal allograft recipients, 37 developed tumors, which is estimated to be 50x the expected incidence. Israel Penn (5) collected data on 47 kidney
transplant recipients who received organs from donors with cancer; 36% developed cancers histologically identical to that present in the donor. Immunosuppressive therapy was discontinued in 3 patients with known metastasis, and the tumors regressed and disappeared. In 432 kidney recipients, reviewed by Penn, 24 developed neoplasms, an incidence of 5.6%, which is approximately 100x greater than the incidence in the general population in the same age range. Penn also reported on 48 patients receiving immunosuppressive therapy for a number of medical problems like psoriasis, systemic lupus erythematosus, rheumatoid arthritis and ulcerative colitis, suggesting a possible increased incidence of cancers in such patients.

The occasional clinical observations of tumor regression have also been used to support the concept of immune surveillance. Everson and Cole reviewed 176 examples of spontaneous tumor regression between 1900 and 1964 (6). The implication was that the tumor regressed because of the immune system.

We might add to these observations the occasional report of dramatic tumor regression using immunotherapy like BCG and C. Parvum. In addition to these arguments supporting the concept of immune surveillance, there have been extensive animal experimental data implicating the immune system as an important participant in tumor progression.
prehn has summarized the data against immune surveillance, and feels that the case for immunological surveillance has been "exaggerated" (7).

prehn first notes that some "classes of tumors have many members with little or no detectable immunogenicity". This lack of tumor antigens would preclude any immunological surveillance mechanism. If immunoselection was responsible for the lack of tumor antigens in spontaneous neoplasia, then neoplasms arising in immuno-deficient environments should be highly antigenic. Prehn tested this hypothesis by growing tumors in intraperitoneal diffusion chambers, and found relatively little immunogenicity. Also mouse embryo cells, grown in tissue culture and treated with chemical carcinogens yield tumors showing relatively little immunogenicity. Very small inocula of immunogenic tumor cells sometimes survive and kill the host (a phenomenon first described by Humphreys et al, and is termed "Sneak Through" (8), even though large inocula result in effective immunization and inhibition of tumor growth. This "sneak through" phenomenon suggests the immune system is sometimes ineffective even with immunogenic tumors (9). Moller and Møller have also pointed out that in several diseases with pronounced immunosuppression there is no increased tumor incidence (Leprosy, Sarcoidosis), and that immunodeficient patients develop a very restricted range of neoplasms,
generally of lymphoid origin, rather than the common tumors seen clinically (10).

Further negative data come from animal studies. Nude mice which lack T-cells and cannot reject foreign grafts, have a very low spontaneous rate of tumor development. Rygaard and Povlsen reported on 11,000 nude mice without a single example of a tumor developing (11). Simpson and Nehlsen used antilymphocyte sera to suppress the immune system in CBA mice and also found no increase in the incidence of spontaneous tumors (12). Moller and Moller also point out that immune surveillance should produce a high incidence of tumors in immune privileged sites like the anterior chamber of the eye, cheek pouch of the hamster and brain. Yet, these are not common sites of spontaneous malignancy.

The data implicating the potential for involvement of the immune system in cancer are impressive, particularly if the experimental animal data are accepted as a valid comparison to human cancers. From a pragmatic point of view, the question remains one of degree. How important is the immune system in the prevention of tumors, not theoretically but practically?

Experimental systems must be viewed with caution since many such tumors are induced by viruses or strong chemical carcinogens (mutagens) which have repeatedly been
demonstrated to express viral or highly antigenic mutational antigens. Therefore, immunological rejection of such tumors may only reflect the involvement of the immune system in tissue graft rejection or the killing of virus infected cells. Klein and Klein have suggested that immune surveillance may, in fact, be mainly involved in the prevention of viral tumors (13). Prehn's note of caution regarding tumor antigens would appear to be central to the controversy (14).

2. **TUMOR ANTIGENS**

The first convincing evidence that malignant cells have antigens not present in the normal precursor cell came from studies using tumor transplants by Gross (15) in 1943 with syngeneic mice and chemically induced tumors. Prior to that time, most reports of tumor antigens were thought to have resulted from transplantation or histocompatibility antigens. Prehn and Main (16) confirmed these findings in 1957 when they showed that skin grafts from tumor donors were accepted while the tumor itself was rejected, indicating the presence of antigens on the surface of tumor cells which are absent on the normal skin of the tumor donor. These new antigens, which were responsible for tumor rejection in the syngeneic host, were originally termed tumor-specific transplantation antigens, since they were
demonstrated by rejection of a grafted tumor. Results of further studies by Prehn (17) revealed that two such chemically induced tumors arising at different sites on the same mouse had different tumor-specific antigens. Most chemically induced tumors have tumor neoantigens. New antigens may also arise from spontaneous mutations occurring in any dividing cell population.

Some viruses also induce malignant growth. The first virus-induced tumors were demonstrated by Ellermann and Bang (18) in 1908 and by Rous (19) in 1911. The presence of virus-specific tumor rejection antigens was described by Sjogren and associates (20) and independently by Habel (21) in polyoma virus-induced mouse tumors.

A different type of tumor antigen was demonstrated in 1965 when Gold and Freedman (22) reported an antigen which was associated with human carcinoma of the colon, and was also present in fetal tissues. This antigen, termed carcinoembryonic antigen, was later found to be associated with many different types of human tumors and some non-malignant diseases, as well as normal fetal tissues. The initial enthusiasm for carcinoembryonic antigen as a diagnostic test for carcinoma of the colon waned because of its lack of specificity and its inconsistency of expression in tumors. Since these limitations have been appreciated, carcinoembryonic antigen has become a useful screening tool
in suspected instances of malignant disease, and is used to monitor therapy in some patients with cancer (23).

The presence of fetal antigens in malignant tumors was suggested in 1906 by Schone (24), but the significance of these antigens was not fully appreciated. Schone immunized mice with extracts of fetal tissue and noted resistance to transplantation of a sarcoma, suggesting the presence of fetal antigens in the sarcoma.

In addition to recognition of antigens resulting from chemical or viral transformation or expression of repressed fetal antigens in tumors, there is now a better appreciation of the large number of antigens expressed by a normal cell. The closer normal cell antigens are observed, the more complex and varied are the antigenic determinants noted. For example, there have been over 379 recognized normal red cell antigens reported (25). An increase or decrease in the number of normal cell antigens may occur following malignant transformation (26, 27), and some normal antigens initially have been confused with tumor-associated antigens (28, 29).

New antigens in tumor cells may also result from enzymatic change or synthesis. Such structurally altered antigens have been noted in studies of both the ABH and MN blood group system.
Not all antigens expressed on a tumor cell can be explained by a single mechanism of formation. Any given transformed cell may express a variety of new antigens, including those arising from mutation, modulation of normal cell macromolecules, derepression of fetal genes or recessive alleles and faulty macromolecular biosynthesis.

3. IMMUNOCOMPETENCY

Host immunocompetency can be assessed both by in vivo and in vitro testing. In vitro methods of evaluation of immunocompetence in tumor patients include total lymphocyte counts, T and active T cell subpopulations, lymphocyte response to mitogen stimulation and specific lymphocyte response to tumor cells or tumor extracts. Stefani and colleagues (30, 31) and Kerman and associates (32), have shown depression of total T cells, active T cells, and mitogen stimulation in patients with certain solid tumors.

In vivo host immunocompetence is frequently evaluated by delayed hypersensitivity skin test responses - specifically to tumor antigens prepared from autologous or allogeneic tumors or nonspecifically through recall (secondary) responses to microbial skin test antigens and response to primary sensitization with dinitrochlorobenzene (DNCB). It has been found that 90 to 100 per cent of normal individuals will respond to at least one of a series of
five ubiquitous microbial skin test antigens and 96.5 per cent will respond to primary sensitization with DNCB (33,34,35). Patients with a wide variety of tumors have shown depressed responses to both recall antigens and primary immunization. Nalick and co-workers demonstrated depressed responses by both parameters in cervical, uterine, ovarian, and vulvar cancers (36). Eilber and Morton reported a correlation of DNCB responsiveness to prognosis (37). Ninety-six per cent (50 of 52) of patients who were DNCB non-reactive were either inoperable or developed recurrent cancer within 6 months.

In some tumors, immunocompetence may correlate with prolonged survival and/or stage of disease (38). However, reports vary considerably as to the prognostic value and dependability of different immune parameters. Even when a correlation exists for a tumor of a given histologic type between immunocompetence evaluations and state of disease or prognosis, it cannot be easily extrapolated to other types of tumors. In addition, even in studies showing statistical correlation between immunocompetence and prolonged survival, any individual patient may show good immune response in the face of tumor progression, deteriorating clinical condition, and death or poor immune response and prolonged survival. Eilber and Morton noted two patients who were DNCB positive despite widespread
metastasis and they remained positive until their death (39).

Attempts have been made to use immune stimulation for tumor therapy. Bacillus Calmette-Guerin (BCG) has been used extensively as the primary mode of immunotherapy. Generally, limited success has been noted, although occasional good responses have been reported in individual cases. The rationale for BCG immunotherapy includes stimulation of cellular immunity, possibly resulting in enhanced sensitization and consequent immune response to tumor antigens leading to tumor regression. A bone marrow sparing effect has been reported with BCG, increasing the number of peripheral blood lymphocytes giving the potential for delivery of increased doses of chemotherapeutic agents that produce leukopenia (40).

Of even more interest is the response of cancer patients to extracts of their own tumor. Herberman (41) noted that skin tests for delayed hypersensitivity to tumor extracts have two principal objectives: a) a means of detecting antigens on tumor cells, and b) as a mechanism for assaying host cellular mediated immune responses to neoplastic disease, and correlation of such responses to resistance to tumor growth, clinical status, prognosis, and for monitoring patients during therapy.
Delayed-type hypersensitivity reactions (DTH) have been reported in 72% of patients to extracts of leukemias and lymphomas (42). Fass noted positive DTH in 61% (8/13) of patients with Burkitt's Lymphoma, and noted a correlation with clinical status (43). Stewart found specific DTH responses to autologous tumor extracts in 26% of a group of 134 patients with lung cancer (44). Fass, et al, also reported positive skin reactivity to autologous tumor extracts in 3 patients with malignant melanoma with localized disease, and an absence of reactivity in 5 patients with advanced disease (45).

Herberman suggested that in vivo skin testing to autologous tumor extracts had several advantages over in vitro testing of host cellular mediated immunity: its simplicity, elimination of the need for tissue cultures, availability of better controls using normal tissues which are more difficult to grow in vitro, and a better reflection of results of in vivo skin testing with the immune status of the patient. The disadvantages of in vivo skin testing are the difficulty of precise quantitation, the necessity of patient cooperation, and the fact that such testing involves the use of tumor tissue (extracts) in human subjects.

Care must be used in interpretation of autologous tumor DTH, since skin test reactivity to extracts of normal tissue has been reported. Bacterial contamination may also be responsible for false positive skin test (46,47).
4. CYTOTOXICITY

Cytotoxicity may result from a wide variety of biological, physical and chemical agents. Three general "types" of immunological cytotoxicity are recognized: a) complement mediated antibody dependent cytotoxicity (CMAD), b) antibody dependent cell mediated cytotoxicity (ADCC), and c) cell mediated cytotoxicity (CMC).

a. COMPLEMENT MEDIATED ANTIBODY DEPENDENT CYTOTOXICITY (CMAD)

Kalfayan and Kidd (48) in 1953 noted tumor cell swelling in the presence of antibody and complement, and Gorer and O'Gorman (49) noted tumor cell killing in vitro in 1957 as judged by vital dye exclusion (trypan blue) after incubation with isoantibodies and complement. Green, et al reported cellular leakage of radioactive molecules from ascites cells in the presence of antibody and complement, and suggested that complement induced "holes" in the cell membrane (50). These "holes" when visualized by electron microscopy, are uniform, stable structures 100 Å in diameter; and result from insertion of complement components into the cell membrane, forming a doughnut-like hole (51). Although IgM, IgG₁, IgG₂ and IgG₃ antibodies can "fix" complement with resulting cytolysis, IgM is 800 times more effective than IgG (52).
Despite the undeniable fact that antibody and complement can result in cytotoxicity, the role of such humoral immunity to tumors remains quite controversial. Tumor cells very considerably in their susceptibility to cell lysis by CMAD (53), and evidence has even been presented suggesting that antibodies might "protect" tumors from cell-mediated cytotoxicity resulting in tumor enhancement (54).

The existence of antibodies to tumor-associated antigens has variably been reported, but in the opinion of Herberman, not adequately proven (55). Multiple reports have probably adequately confirmed the existence of anti-tumor antibodies in patients with at least three different types of tumors: 1) Burkitt's Lymphoma, 2) malignant melanoma, and 3) sarcomas. Theofilopoulos in 1977 reported immune complexes in about half of patients with melanoma, colon carcinoma, and osteogenic sarcomas (56). Hoffken et al 1978, noted circulating immune complexes in the initial stages of chemically induced carcinomas (3-methylcholanthrene) that returned to normal with tumor excision and also decreased in concentration with continued tumor progression (57). Antibody responses also occurred in viral induced tumors (Mammary Tumor Virus in BALB/cfC3H female mice), with specificities against not only viral antigens, but also against a wide variety of unique specificities (58).
Hellstrom reported an enhancement of tumor growth by hyper-immune serum (59). This effect was initially thought to result from blocking antibodies (60). The effect of serum in tumor growth enhancement was found to be specific. Others have also reported tumor cell enhancement (61,62). The tumor growth enhancement could be removed by absorption with specific tumors (Moloney sarcoma), but not with control cells (63). The serum effect was initially thought to be due to antibodies that "blocked" cell-mediated cytotoxicities (CMC). Such CMC blocking was confirmed by Sjögren et al (64) and by Baldwin (65). The concept of "blocking antibodies" was based on the observation that: 1) blocking activity could be removed by absorption with specific tumor cells, and 2) blocking occurred if target cells were incubated with serum, but not if lymphocytes were incubated with serum. The concept of "blocking antibodies" had to be modified when the blocking effect was noted to disappear shortly after tumor regression or surgical removal of tumors. Sera from such tumor-free animals was found to be "unblocking" and the blocking activity was then felt to result from antibody-antigen complexes, and unblocking from antibodies (66). The blocking effect is reported to occur at either the target cell or effector cell level by antigen-antibody complexes. Free antigen blocked at the effector cell level. These
observations were partially substantiated by Baldwin using papain solubilized tumor antigens (67). Neither antigens nor antibodies to the same antigens blocked CMC, but complexes in the right proportion were capable of blocking at the level of the target cell, and both complexes and free antigens were found to be capable of blocking at the level of the effector cell. CMC reactivity using patient lymphocytes against histologically similar tumor cells was also used as evidence of histological type specific reactivity, and suggested similar antigens in patients with histologically similar type tumors (68).

The importance of the observation in vitro is suggested by the correlation between blocking factors against CMC and melanoma growth. Hellstrom noted a correlation between blocking factors against CMC and melanoma growth (69). Bansal and Sjorgen confirmed these findings in vivo using a polyoma viral induced tumor in rats (70). Murray reported a 15,000 dalton blocking factor that bound both to Con A and to melanoma antibodies, and which was also found in the supernatants and membranes of melanoma cells in tissue culture (71).

How these observations integrate with more recent observations regarding ADCC is not clear. The lack of normal reactivity (spontaneous cell mediated immunity, SCMC or natural cytotoxicity) in these studies is very
questionable (72). The histologic type-specific reactivity has also been seriously questioned (73). Takasugi, Mickey and Terasaki reported on cytotoxicity reactions involving peripheral blood lymphocytes in a variety of cancer patients using 45 different human tumor cell lines with 9294 different lymphocyte-tumor combinations and found no correlation between CMC and "histologic types" of cancers (74).

b. **ANTIBODY DEPENDENT CELL MEDIATED CYTOTOXICITY (ADCC)**

Like CMAD, ADCC requires antibody; however, in ADCC, lysis results from the interaction of an effector cell with antibody coated target cells rather than complement. Therefore, ADCC is complement independent. ADCC has been thought to be exclusively a function of IgG (all subclasses), although one report has indicated that IgM may also be capable of initiating ADCC (75). Effector cells bind to the Fc portion of the antibody, and do not have to be previously sensitized. The specificity of ADCC resides in the antibody. Effector-target cell interaction is required. Effector cells must be viable in order to participate in ADCC, although DNA synthesis is not required. A wide variety of cells has been capable of participating as effector cells in ADCC including polymorphs, macrophages,
mononuclear cells, K cells, and even fetal liver cells (76).

c. CELL-MEDIATED CYTOTOXICITY

Kidd and Toolan in 1950 noted that tumors or tumor cells mixed with lymph node cells when injected into recipient animals resulted in tumor growth and eventually host death, but tumor cells mixed with specifically sensitized lymphocytes resulted in tumor prevention or at least retardation of tumor growth (77). In 1955, using allografts, Mitchison noted that in vitro cells could mediate cytotoxicity (78). Graft rejection could be adoptively transferred with lymphocytes, but not with serum. Winn in 1960, noted reduction of allogeneic tumors when specifically sensitized lymphocytes were injected along with the tumor cells (79). Govaerts reported in vitro cytotoxicity using dog cell monolayers following in vitro incubation with specifically sensitized lymphocytes (80).

Following these early observations, a large number of experiments has been performed to evaluate in vitro cell-mediated cytotoxicity against tumors.

In vitro CMC studies involve the use of an effector cell responsible for the cytotoxic action, a target cell, and a mechanism of measuring the cytotoxic effect. A wide variety of effector cells has been used in the past
including monocytes, macrophages, polymorphonuclear leukocytes and lymphoid cells from lymph nodes, spleen, thymus or peripheral blood. Likewise, a wide variety of normal and tumorigenic target cells has been used. Frequently, allogeneic tumor cells of the same histological type as the lymphocyte donor have been used in assessing tumor patient cytotoxicity. Occasionally syngeneic or autochthonous tumor lymphocyte combinations have been used. More recently, cytotoxic effects of non-immunized mononuclear cells against normal and even self tissue have been recognized (81). A wide range of techniques has been used to measure and quantitate the effector-target cytotoxic interaction including: early experimental observations of cytopathic changes in monolayer plaques; exclusion of supravital dyes by target cells; cell counting; colony inhibition or colony formation in soft agar; release of radiolabeled proteins, amino acids, $^{32}$P or $^{51}$chromium (82). The effector target interaction may occur in allogeneic or autologous serum, and length and condition of incubation vary as well as manipulations in the processing of effector and target cells.

The mechanism of cytotoxicity is not known, although Ferluga et al reported cytotoxicity resulting from plasma membranes of peripheral blood lymphocytes (83).
CMC is antibody and complement independent and may be specific (directed against target cells expressing specific antigens) or non-specific (resulting from release of cytotoxic soluble substances or from the action of nonsensitized but activated effector cells). As noted above, a variety of effector cells can produce cytotoxic reactions including macrophages, T-lymphocytes, B-lymphocytes, and natural killer (NK) cells.

Two general types of macrophage cytotoxicity have been described. One type, resulting from "Activated" macrophages is not immunologically specific, the other is immunologically specific, and is a function of "armed" macrophages.

Armed macrophages demonstrate immunological specificity. The mechanism of "arming" is not known. Armed macrophages may be generated in vitro or in vivo by exposure of immunized lymphoid cells to antigen. Evans reported a specific T-cell product (specific macrophage arming factor (SMAF) that armed macrophages (84). The possibility of cytophilic antibodies has also been suggested as "the arming agent", since treatment of macrophages with trypsin or heat inactivation of serum at 56°C for 30 minutes results in loss of specific cytotoxicity which could be restored by immune serum. Armed macrophages (specific reactivity to a particular antigen like SRBC) can be activated (with non-
immune inhibition of tumor cell growth) by exposure to specific antigen (SRBC) (85).

Macrophages may be activated by factors from T cells or by substances like endotoxin, peptone, starch, glycogen, BCG, double-stranded RNA, or exposure to intracellular pathogens like toxoplasma (86). Activated macrophages may also be obtained by exposing "armed" macrophages to specific antigens. The cytotoxic action is non-specific, but reportedly only abnormally growing cells are killed (87). The recognition mechanism is nonimmune and not based on H-2 foreign target cell antigens. Instead, the cytotoxic action is based on recognition of cell properties associated with abnormal growth characteristics. Contact-inhibited nontumor 3T3 fibroblasts are spared, but transformed (SV-40) 3T3 fibroblasts which have lost contact inhibition are killed (88).

The mechanism which macrophages use to distinguish cancer from a normal cell is not known. Inhibitors of protein synthesis (puromycin, cycloheximide), DNA synthesis (mitomycin C, actinomycin D) or inhibitors of phagocytosis (cytochalasin B) have no effect on the cytotoxic capabilities of activated macrophages. The mechanism of killing is thought to result from "secretion" of lysosomal enzymes into the target cells (89), because it is inhibited by hydrocortisone (which also inhibits lysosomal
exocytosis), and trypan blue (inhibits lysosomal enzyme action). No cytotoxic factor(s) is/are noted in cultured media of activated macrophages suggesting that cell-cell contact is needed for the cytotoxic effect (90).

d. **SPONTANEOUS CELL MEDIATED CYTOTOXICITY (SCMC)**

More recently, cytotoxicity of non-sensitized effector cells has been recognized and termed spontaneous or natural cytotoxicity. The effector cells have been called the natural killer or NK cell. Early reports suggested a non-T, Fc+, C₃⁺ cell as the responsible effector cell (91). The NK cell has been characterized by Eremin as a T-neg. (non-thymus cell), Fc+, C₃⁻, Ig⁺ cell in peripheral blood (92), similar or identical to the K cell of ADCC (a B-cell, but not requiring antibody for cytotoxic effect). In contrast, the NK cell in tonsils and lymphnodes was found to be an Ig⁻, Fc⁻, C₃⁻, T⁺ cell. Vessella reported data suggesting that cells mediating SCMC were a heterogenous population composed of Fc⁺ lymphocytes both with and without C₃ receptors (93). Vessella also suggested that the cells responsible for ADCC and SCMC are identical although the mechanisms are different. Hersey has suggested that an "activated" T-cell is responsible for SCMC (94). Removal of the Fc bearing cell decreased responsiveness, although depletion of phagocytic cells did not affect SCMC activity. Oehler noted
an increase in NK cytotoxicity following intraperitoneal injection of *C. Parvum*, Poly I:C (Polyinosinic-Polycytidylic Acid), LCMV (lymphocytic choriomeningitis virus) and KRV (Kilham rat virus) (95). Treatment with hydrocortisone, X-irradiation or cyclophosphamide suppressed SCMC in vivo, but Poly I:C following such treatment restored cytotoxicity. Silica also reduced SCMC which could not subsequently be restored with Poly I:C. Thymectomy had no effect on SCMC. Oehler used these observations to suggest that phagocytosis plays a role in SCMC, although T-cells do not, and implicates interferon as possibly playing an important role in SCMC (96). Menon et al have demonstrated a decrease in SCMC in tumor patients, and noted a correlation between PHA responsiveness and SCMC (97). PHA is a non-specific stimulator of T-cells.
B. TUMOR CELL PHYSIOLOGY

1. TISSUE CULTURE

HeLa was the first human carcinoma cell line to be established in vitro. It was isolated by Gey (98) from a squamous cell carcinoma of the cervix in 1951, and since then has been maintained in continuous culture. Established gynecologic tumor cell lines include carcinomas of the cervix, ovary, uterine endometrium and chorion. No exact record of the number of gynecologic tumor cell lines is available. Fogh and Trempe (99), Gall (100), Pattillo (101), Nelson-Rees (102), DiSaia (103), and the American Type Culture Collection (104), collectively have listed 15 cervical, 13 ovarian, and three uterine tumor cell lines, but not all of these have been characterized. Other such lines may also now exist. The in vitro cultivation of vulvar carcinoma cell lines has been reported by Giard and associates (105), in 1973 and Fogh and co-workers (106) in 1977.

Ironically, the proliferation of established human carcinoma cell lines may not be as abundant as previously believed, since many cell lines have been found to be contaminated with HeLa or even animal cells (107,108). Other cell lines used in various studies have not been well-characterized, and even the origin of some lines is
vague. When a tumor is cultured in vitro, it is important to determine that the cells that establish themselves are in fact malignant. The capability of continual subcultivation in vitro, abnormal karyotype, growth in soft agar suspension, and the ability to grow and form tumors in immuno-suppressed or immunodeficient animals are frequently used criteria of malignancy (109-114).

A number of continuous malignant cell lines have been used in studies involving the biochemical, biological, genetic, and ultra-structural properties of cancer cells. In addition to studies of tumor cells per se, cultured cells have been used to evaluate the host's immune response (immunocompetence) to the tumor.

2. CONTACT INHIBITION: SATURATION DENSITY

The behavior of cancer cells and normal cells has been compared in tissue culture and correlated with the in vitro potential for tumor growth. Normal cells grow in strict monolayers, but cancer cells pile up often forming multiple layers. Abercrombie and Heaysman initially explained the tendency of fibroblasts to remain as monolayers on the substratum as contact inhibition (115). Middleton notes that although monolayering is commonly used to designate contact inhibition, there are other potential explanations for monolayer growth (116). Steinberg has emphasized the
point that contact inhibition can be used to designate several phenomena: contact inhibition of cell movement implying cessation of cell movement secondary to cell-cell contact; contact inhibition of growth implying inhibition of cell growth and division with cell-cell contact. The term may indicate the behavior of one cell when it collides with another cell in which there is a cessation of locomotion in the direction that brought about the collision; or the term may even be applied to the behavior of whole populations of cells and imply monolayer growth (117). Monolayer growth resulting from contact inhibition can readily be appreciated in vitro on inspection of confluent cell cultures. The phenomenon can be quantitated by measuring the cell density of confluent cell cultures and is termed saturation density. Pollack et al, reported a correlation between contact inhibition by saturation density, and in vitro tumorigenicity (118).

Although the mechanism of contact inhibition is not known, Abercrombie (119) has suggested that it might result from: the inability of the dorsal surface of one cell to provide a substratum for locomotion for another cell, or the development upon cell-cell contact of strong lateral adhesions such that further movement in the direction of the adhesions is impossible. Studies by Middelton demonstrated that epithelial cells, layered over a
confluent monolayer of the same cell type failed to spread, suggesting that the dorsal surface of the normal cell does not provide an adequate substratum for attachment and locomotion for another cell of the same type (120).

Also, Steinberg has noted cell movement in time-lapse studies from confluent monolayer cultures with cells in mutual contact on all sides (121). Steinberg concluded that the avoidance of overlapping rather than the inhibition of locomotion was responsible for monolayer growth.

3. **SERUM DEPENDENCE**

Almost all normal cells require serum for growth in vitro. Tumors have been noted to have a reduced requirement for serum factors (122). This has been demonstrated by Holley using SV-40 transformed 3T3 cells (123). Holley noted that the active material in the serum is a 100,000 dalton, non-dialyzable factor which is destroyed by pronase treatment. Smith and Temin have suggested that tumor cells might adopt a serum free culture condition by producing their own growth factor (124). The growth factor in serum must be produced by some cells within the body, and since cells of the same origin all have the same genetic material, this would be conceivable. Smith and Temin, have in fact, isolated such a growth promoting factor from conditioned media from a cell line that is serum
independent. Gospodarowicz et al have reviewed the mitogen and growth factors found in serum and plasma, and note that there is a complex mixture of substances including epidermal growth factor (EGF), fibroblast growth factor (FGF), nerve growth factor (NGF), insulin, nonsuppressible insulin-like activity (NSILA), colony-stimulating factor (CSF), somatomedins (125). More recently, Guinivan et al, using normal rat kidney (NRK) and Kirsten sarcoma virus transformed NRK cells (Ki-NRK) reported that cells were stimulated by EGF and expressed high concentrations of EGF receptors. Ki-NRK cells were unresponsive to EGF and no receptors were detected. Cross feeding experiments demonstrated the production of a heat-stable substance from Ki-NRK cells that stimulated DNA synthesis in NRK cells, independently of the presence of serum or epidermal EGF (126).

Despite the good correlation of in vitro serum independence of cells with in vivo tumor formation, Shin has noted that serum independence can be disassociated from in vivo tumorigenicity (127).

Interestingly, Stanbridge et al (128) were able to disassociate anchorage independent and nude mouse growth as separate genetic phenotypes using HeLa and normal fibroblast fusion hybrids. Their findings suggest that the properties of serum independence, anchorage independence,
loss of contact inhibition (density dependent limitation of growth) and lectin agglutination do not by themselves or in concert endow a cell with tumorigenic potential.

Stanbridge suggested that the in vitro transformation phenotypes are dominant traits whereas malignant behavior in vivo is a recessive trait.

4. **LOCOMOTION**

Both normal cells and tumor cells have been reported to show locomotion in tissue culture. In normal cells, contact inhibition is important in controlling cell movement in vitro (129). Collisions between cells lead to the development of stable adhesions (130). Not all tumor cells show motility in vitro. Hosaka et al, studied the motility of four strains of rat ascites tumor cells and noted no locomotion in two of these strains (131). Tickle et al, tried to correlate cell movement and invasiveness using a variety of tumor cells implanted into developing chicken wing buds. The results suggest that cell movement may not be a common factor in all invasive tumors (132). Trophoblasts, sarcoma 180, and cultured hamster fibroblasts did invade the mesenchyme, but appeared to do so by different mechanisms. Trophoblasts destroyed adjacent host tissue. Even cells that showed contact inhibition were capable of movement and tissue invasion.
5. **KARYOTYPE**

Aneuploidy is a common finding in cancer cells; it has almost become an integral part of any definition of cancer—a cellular criterion of malignancy. Boveri in 1941 noted that malignant cells are cells with genetically unbalanced chromosomal complements (133). Boveri's intuition regarding chromosomes and cancer was confirmed in 1952 by Makino (134). Atkin has reviewed the subject, and has noted that data fail to disclose any simple chromosomal change common to all cancers, and that considerable variations occur in different cancers (135).

Spriggs has posed a particularly pertinent question: Do chromosomal changes occur before or after malignant changes? (136). If chromosomal changes are seldom or inconsistently present in precancerous stages of tumors, then they can be dismissed as inessential features, but if chromosomal changes are regularly found in lesions preceding cancer by many years, then these changes may play an integral part in the carcinogenic process. Not enough precancerous lesions have been studied to confidently answer this question to date, but Spriggs' review of cervical dysplasia and carcinoma in situ would suggest that many of these lesions do in fact have abnormalities in chromosomal numbers and/or abnormal marker chromosomes long before invasion and metastasis occur. Indeed, this
supports the suggestion that aneuploidy does play a central role in the development of cancer.

Most carcinogens, including viral, chemical or radiation are known to produce chromosomal breaks, and a number of inherited disorders known to be associated with chromosomal breaks like Bloom's Syndrome and Fanconi's Anemia are also associated with an increased incidence of cancer (137).

Katayama and Woodruff have noted aneuploidy in both carcinoma in situ and invasive carcinoma of the vulva (138).

6. HISTOLOGY

Morphological changes in cells have in the past been the most widely used criteria of malignancy, and have served clinical medicine well as a means of distinguishing normal tissue from its malignant counterparts. Despite the developments in oncogenic pathology and cytology, exceptions do occur, as well recognized in the difficulty in distinguishing between benign and malignant thyroid tissue. The criteria and morphological hallmarks of malignancy sometimes vary in different tissue types. Morphological criteria for spontaneous chemically induced tumors have also been studied in experimental animal systems. Morphological changes which have been reported to correlate
with neoplastic transformation include: pleomorphism in cell and/or nuclear size and shape; increased nuclear-cytoplasmic ratio; prominent enlarged or numerous nucleoli; basophilic cytoplasm; abnormal mitosis; reduced intercellular cohesiveness with piling up of cells; and reduced cytoplasmic spreading. The cytoplasmic criterion which Montesano et al noted to correlate best with tumorigenicity was increased nuclear cytoplasmic ratio, and basophilic cytoplasm (139). Cytodiagnosis was reliable in 94% of 18 cultured rat liver tumor cell lines.

Electron microscopic studies of multiple tumor systems have not shown any specific or universal patterns in cancers (140). Mao, Nalcao and Angrist studying grade 1 adenocarcinomas could not distinguish malignant from normal or hyperplastic tissues (141). Ultrastructure differences are generally quantitative rather than qualitative.

7. ANCHORAGE INDEPENDENT GROWTH

The physiological and biochemical alterations which result in anchorage independent growth of tumor cells are not known. Tucker et al, noted that decreased cytoplasmic spreading resulted in increased growth in suspension, and also noted variation in cloning efficiency from cell line to cell line by a 200 fold range (142). Variations in cloning efficiency at different inoculum sizes were also
noted. A decrease from $10^5$ to $10^4$ cells in soft agar resulted in an increase in cloning efficiency of from 6 to 1000 fold. Kimball et al, also reported that increasing concentrations of cells decreased soft agar cloning efficiency (143). Mavligit et al, reported that a large percentage of non-viable cells could inhibit in vitro growth in soft agar (144). MacPherson and Bryden noted an increase in cloning efficiency if responder cells were co-cultured in soft agar with x-ray or UV-radiated or mitomycin C treated "feeder" cells (145). A 4 fold increase in cloning efficiency was noted in the range of $0.5 - 1.0 \times 10^5$ feeder cells, but a 38% reduction in cloning when feeder cells were reduced to $0.1 \times 10^5$ cells. No feeder effect was noted when as few as $0.05 \times 10^5$ feeder cells were used. Dextran sulfate and heparin have been reported to decrease soft agar cloning reportedly because of their strong negative charges. Conditioned media, collagen, increased serum, insulin, DEAE-dextran and a variety of viruses have all been reported to increase soft agar growth (146). Cells grown in agar which form colonies may be subcultured on conventional culture plates by removing colonies from the agar with finely drawn pipettes producing a colony of cells derived from a single cell. Such cells when grown as monolayer cultures in vitro and replated in soft agar show a high plating efficiency between 40% and
Even cells that do not form colonies remain viable in soft agar suspension as manifest by their ability to absorb neutral red after 10 days (147). Salmon and Buick have described a methodology for fixing colonies in soft agars and drying them onto microscopic slides for permanent preservation and staining (148).

Normal cells do not multiply in liquid or semi-solid suspension cultures. Cancer cells and some cell lines derived from normal tissue but carried through many generations will grow in suspension. Also, some virus "transformed" cells will grow in semi-solid suspensions (149). MacPherson, in 1964, first used this property of growth in suspension as an in vitro test of malignancy by culturing cells in soft agar (150). Since that time, many investigators have reported a good correlation between biological potential of malignancy as manifested by growth and tumor formation in an appropriate animal host and anchorage independent growth and colony formation in soft agar. Colburn et al, studied transformation in BALB/C mouse epidermal cells following treatment with chemical carcinogens, and reported a strong correlation between anchorage independent growth and tumor formation in syngeneic mice (151). Montesano et al compared cytology, production of plasminogen activator and soft agar growth of 22 tumors and non-tumorigenic rat liver epithelial cultures
There was a poor correlation of tumorigenicity with plasminogen activator production, but a good correlation with anchorage independent growth, although Marshall et al, reported two tumors derived from human bladder carcinoma that were nontumorigenic in nude mice, but which grew in soft agar, and one cell line that grew in the nude mouse, but would not clone in soft agar (153). Dexter et al, reported a complete loss of soft agar growth when cells were plated in the presence of polar solvents (N,N-Dimethyl-formamide) (154). No tumors were produced in nude mice inoculated with treated cells. Removal of polar solvents from the culture media resulted in appearance of anchorage independent growth. Courtenay et al attempted to culture 40 fresh human tumors from a variety of tissues in soft agar. Colonies were noted in 22 soft agar cultures with a plating efficiency varying from 0.02 to 15% (155). More recently, Salmon et al has used soft agar growth as a mechanism of quantitation of differential sensitivity of human tumor cells to anticancer drugs (156).

8. FIBRONECTIN

In 1948 Morrison et al isolated a cold-insoluble globulin (CIG) from human plasma. Since that time, CIG has been noted to have a wide variety of biological activities, and it has acquired a considerable number of synonyms.
including cell surface protein, large external transformation sensitive protein (LETS), and fibronectin, which is derived from the Latin "fibra" meaning to bind or tie, and which characterizes its biofunctional properties (157).

Fibronectin is a major cell surface glycoprotein with a molecular weight of 220,000 daltons (158), and exists as a dimer cross-linked by a disulfide bridge. It constitutes about 3% of the total cell protein (159). The plasma and cell forms differ slightly. Fibronectin is found in the human plasma at a concentration of 0.3 mg/ml.

The carbohydrate portion of the molecule consists of 4-6 oligo-saccharides with terminal galactose, sialic acid and fucose linked by N-glycosidic bonds to asparagine. It is not necessary for synthesis, processing or secretion, but appears to protect the molecule from rapid degradation. The half life of fibronectin is 36 hours, and cell concentration is highest in the G phase of the cell cycle, and lowest during mitosis. The diffusion constant for fibronectin is \(5 \times 10^{-12} \text{ cm}^2/\text{sec.}\), indicating a relatively immobile molecule on the cell surface (160).

Fibronectin is an integral part of adhesion plaques forming an interface between the cell and the substratum (161), and its secretion by the cell is both time and temperature dependent.
Fibronectin is responsible for cell-cell and cell-substrate interaction, for maintenance of normal cell morphology (162). It will also agglutinate sheep erythrocytes in vitro. Agglutinating activity is destroyed by proteases, chelating agents or boiling. Tumorigenicity of cells transplanted into nude mice also correlates with decreased fibronectin (163).

There is a decrease in m-RNA for fibronectin, and therefore a decreased biosynthesis in transformed cells, although Yamada and Olden reported no decrease in fibronectin in 12 of 89 transformed cell lines (164). Transformed cell lines and permanent (immortal) cell lines required plasma fibronectin for substratum adhesion, but cell strains attached and spread in the absence of exogenous fibronectin (165). Addition of fibronectin restores cell spreading and normal morphology to transformed cells. This restoration of more normal cellular behavior and morphology is prevented by the presence of anti-fibronectin (166).

9. **IN VIVO GROWTH IN THE NUDE MOUSE**

In addition to the progress made in in vitro propagation of human tumors, progress has also been made in the in vivo establishment and propagation of such tumors. Several in vivo methodologies have been explored, including
the use of thymectomized and radiated mice or the treatment of recipient animals with antilymphocyte serum. A particularly useful mechanism was described in 1969 by Rygaard and Povlsen using a congenitally athymic mutant "nude" mouse (167). Since their original description, numerous reports have appeared using the nude mouse as a mechanism for establishing or serially passing human or animal tumors. Shimosato et al, histologically confirmed an initial transplant "take" in 22 of 91 human tumors, and successfully serially transplanted 14 (168). Fogh et al reported growth of 127 cultured human tumors in the nude mouse (169). Stiles noted that every tumor cell line that was tumorigenic in some other animal host or of neoplastic origin was also tumorigenic in the nude mouse. Some human tumors that have been particularly difficult to establish in tissue culture have been established in the nude mouse (170). Bastert reported growth in the nude mouse of 85% of 44 human breast cancers (171).

Morphology of the original tumor is generally maintained, (172), although scirrhous tumors such as stomach and breast tend to become medullary with a decrease in the amount of tumor stroma. Some tumors may exhibit a higher degree of differentiation in the nude mouse than in the original tumor (173). Production of tumor products such as mucin in gastric carcinomas or melanin in malignant
melanomas as well as ectopic hormone production (adrenocorticotropic) is maintained in the nude mouse transplants (174). Tumor antigenicity and karyotype are also maintained (175). Although normal tissue (skin, etc.) can readily be transplanted onto the nude mouse, normal cells or grafts do not produce tumors (176). Stiles suggests that this is a response to host mediated growth-regulatory signals (177). The "Nu" gene has been successfully bred into a number of different genetic backgrounds including BALB/C, C3H and C57/BL with a similar effect in the acceptance of heterotransplants (178).

The nude mouse is apparently not without some defense in terms of tumor metastasis, despite its T-cell and humoral immunodeficiency. Despite the growth of heterotransplanted tumors in the nude mouse, metastases are uncommon (179). Shimosato (180) noted metastases in only one of 22 transplanted human tumors, and Kullander (181) noted no metastases in 10 tumors in nude mice. Graham noted metastases following injection of human T-cells into nude mice with human malignant transitional bladder carcinomas or prostatic adenocarcinoma (182). Control animals not receiving T-cells did not develop metastasis. Warner reported a radiation sensitive non-T-cell surveillance system against lymphoid tumors in the nude mouse (183). Rygaard noted a considerable cytotoxic antibody response to
heterograft stimulus, but not to allografts. Yet, heterografts were more readily accepted than allografts (184). Cheers reported an augmented macrophage response in the nude mouse (185).

Another interesting apparent paradox of the nude mouse is the relative sparsity of spontaneous neoplasms. Machado noted one spontaneous neoplasm in over 1000 nude mice, in an animal that had received a neonatal thymus transplant, and Rygaard noted no neoplasms in over 11,000 nude mice (186). This lack of malignant neoplasms in an animal lacking a T-cell mediated immunologic system has been used as a strong argument against the immunosurveillance theory as proposed by Thomas.

C-type particles that have been repeatedly noted in other mice strains are also present in the nude mouse. Tralka reported a spontaneous lymphosarcoma in a nude mouse with no C-type particles visualized in the primary tumor by electron microscopy, but abundant C-type particles were present in the in vitro cultured cells (187). Tumors developed in nude mice injected with tumor, but again no C-type particles were noted in the transplanted tumor suggesting some type of in vitro suppression or control of virus expression in vivo. Suzuki reported the presence of C-type particles in 6 of 9 human tumors transplanted into the nude mouse. Other human tumors could be readily infected by co-cultivation (188).
Growth of tumor in the nude mouse or other immunodeficient or syngenic (to the tumor) animals is unquestionably the most stringent criterion of tumorigenicity for in vitro cultured cells. The development of the nude mouse is a significant advancement in tumor biology.
CHAPTER III

MATERIALS AND METHODS

A. CELL CULTURES

1. PREPARATION OF PRIMARY CULTURES

Finely minced pieces of tissue, obtained from a biopsy of a metastatic lesion in the right inguinal lymph node were placed in complete medium, CM, (RPMI 1640, KC Biological; Lenexa, KS; or Grand Island Biological Co. supplemented with 15% fetal calf serum, 2 mM L-glutamine (GIPCO), 100 U Penicillin/ml (GIPCO), 100 μg streptomycin/ml (GIPCO), and/or 100 μg kanamycin/ml), and incubated at 37°C in 5% CO₂, 95% air. Fibroblast growth was antagonized by periodic scraping of the flask under microscopic visualization with a sterile cotton-tipped Pasteur pipette. Repeated mechanical removal of fibroblasts eventually allowed the development of a confluent epithelial monolayer. Cells were cultured in 25 cm² (T-30) and 75 cm² (T-250), Corning tissue culture flasks (Corning Glass, Corning, N.Y.). Feeding consisted of CM (15 ml for 75 cm², 5 ml for 25 cm²), when cultures became acidic as indicated by a color change in the media (phenolphthaleine) - generally every three days. The epithelial cell monolayer isolated was designated LT-2.
2. SUBCULTIVATION

Media was removed; the monolayers washed with 5 ml of Ca++ and Mg++ free PBS; followed by 3 ml of 0.02% EDTA (Mallincrodt); followed by incubation for 20 to 30 minutes with 2 ml of 0.02% EDTA and 2 ml of 0.25% trypsin (GIPCO, Grand Island, N.Y.). Once the cells became detached 5 ml of CM were added and the cells were centrifuged at 1200 rpm for 10 minutes (400 x g, Model TJ-6, Beckman Instruments, Fullerton, CA), resuspended in CM and placed in culture flasks. The flasks were labeled, dated, and the passage number recorded. A confluent 75 cm² monolayer generally yielded between 5-14 x 10⁶ LT-2 cells. During subcultivation these cells were generally split into two flasks with 15 ml CM each.

3. CELL COUNT AND VIABILITY

Washed cells were obtained as above, suspended in 5 to 10 ml of complete media, and a 0.05 ml aliquot mixed with 0.05 ml of 0.4% trypan blue in normal saline (Matheson, Coleman and Bell; Norwood, OH) and placed in a hemocytometer counting chamber (Scientific Products; McGraw Park, IL). The viable (non-staining cells) were counted, and the number of cells/ml calculated as follows: No. cells counted/mm² (at least 100) x 10⁴ x dilution factor = No. cells/ml.
4. FREEZING AND THAWING

Cells from confluent flasks (75 cm\(^2\)) were collected as above, washed with Ca\(^{++}\) and Mg\(^{++}\) free PBS, and resuspended in 1 ml of CM with 10% DMSO (Fisher Scientific Co.; Fairlawn, NJ). Cells were placed in 1 ml vials (Wheaton Scientific, Millville, NJ) and sealed using a propane torch. The cells were then slowly frozen (about 1°C/min.) in the freezer core in the vapor phase (BF-5 biological freezer assembly) of a LD-30 Linde liquid nitrogen container (Union Carbide Co., Linde Products, Indianapolis, IN) and stored in the liquid nitrogen tank at -70°C.

Cells were thawed by placing frozen vials in a water bath at 37°C with shaking until melting occurred. The glass vial neck was cleansed with alcohol, broken and the contents placed in 10 ml of pre-warmed (37°C) CM, centrifuged (1200 RPM x 10 min.) and the pellet resuspended in 10 ml of CM and placed in culture flasks. Viability was generally about 80%.

5. PLATING EFFICIENCY

Monolayer cultures of LT-2 were harvested (T/E), washed in CM, centrifuged, resuspended in CM and plated at 6 x 10\(^5\) V. cells in 5 ml CM per flask in 25 cm\(^2\) tissue culture flasks. Cells were harvested at 2, 4 and 6 hour intervals after washing x 2 with PBS without Ca\(^{++}\) and Mg\(^{++}\), 2 ml
versene followed by 2 ml Versene with 2 ml trypsin, incubated for 60 minutes at 37°C, neutralized with CM, centrifuged and an aliquot diluted 1:1 with trypan blue and counted on a hemocytometer. The procedure was repeated at 2, 4 and 6 hours. A repeat experiment plated 4.25 x 10^6 V. LT-2 cells per flask, processed as described above, except that cells were incubated for 30 minutes at 37°C with or without 100 mg/ml mitomycin C per flask; were harvested at 2, 4, 6 and 24 hours and counted.

6. **ACETO-ORCEIN STAINING OF CELLS**

LT-2 cultures were tested for the presence of Mycoplasma by evaluation of aceto-orcein (GIPCO) staining of Leighton tube (Wheaton Scientific, Millville, NJ) monolayers subjected to hypotonic treatment as described by Fogh (189).

7. **OTHER CELL LINES AND STRAINS**

Other cell lines and strains used in studies include: AU-471, a human cervical carcinoma cell line obtained from Doctor Walter Nelson-Rees, Naval Biomedical Research Laboratory, Oakland, CA; WI-38, a near diploid "normal" lung fibroblast cell strain obtained from The American Type Culture Collection, Rockville, MD; AT-264; a lung carcinoma cell line kindly provided by Doctor Mira Menon, Hines
Veteran Administration Hospital, Chicago, IL; MS, a diploid fetal fibroblast cell line cultured from an abortion at Loyola University, Stritch School of Medicine, Chicago, IL; BHK-21, a baby hamster kidney cell line kindly provided by Doctor Eugene Major, Loyola University, Stritch School of Medicine, Chicago, IL; LT-4, a normal human cell strain isolated at Loyola University, Stritch School of Medicine, Chicago, IL. Cell numbers in all experiments represent viable cells determined by trypan blue exclusion.
B. TUMOR-HOST INTERACTIONS: IN VITRO

1. ISOLATION OF LYMPHOCYTES

Peripheral blood was collected by venipuncture into heparin containing tubes which were immediately mixed. Five (5) ml of blood were gently layered on top of 3 ml of Ficoll-Hypaque (Lymphoprep, Nyegaard Ans. Co.; Oslo, Norway) in sterile tubes and centrifuged at 400 x g. (1400 rpm) for 20 minutes (190). The supernatant plasma was removed and frozen, and the interface layer of lymphocytes was removed with a Pasteur pipette. The lymphocytes were washed with Ca++ and Mg++ free PBS, and re-centrifuged 1200 x g. for 5 minutes. The cell pellet was resuspended in 5 ml of PBS and an aliquot counted in a hemocytometer.

2. DETERMINATION OF T AND B CELL POPULATIONS

Sheep red blood cells (SRBC) were stored at 40°C in Alsevar's Solution (1:1), and used within 7 to 10 days of delivery. Before use, the cells were washed x3 and adjusted to a 0.5% suspension in PBS. Two populations of T-RFC were measured: the first termed the total T-RFC, representing all T-cells in the peripheral blood; the second called the active T-RFC, representing a subpopulation of the total T-cells that 1) may have an active surveillance function, and/or 2) be an index of cellular immunity (191).
T cells were evaluated by the formation of spontaneous rosettes by mononuclear peripheral blood lymphocytes (PBL) with unsensitized SRBC. As a measure of the total T-RFC population, 0.25 ml (5 x 10^5) PBL were mixed with 0.25 ml of 0.5% unsensitized SRBC (a final SRBC: PBL ratio of 40:1) and centrifuged at room temperature for five minutes at 200 x g, and then incubated in an ice-water bath at 4-8°C for 60 minutes. After the cell pellet was gently resuspended, a drop of suspension was placed onto a hemocytometer and the number of rosettes (3 or more SRBC surrounding a lymphocyte) were counted. All tests were performed in duplicate and 200 or more lymphocytes were counted to determine the percentage of total T-RFC.

To measure the active T-RFC, a modification of the procedure of Wybran et al (192), was used: 0.25 ml (5 x 10^5 PBL) was mixed with 0.25 ml of 0.5% unsensitized SRBC and centrifuged immediately at room temperature for five minutes at 200 x g. The cell pellet was gently resuspended and a drop of the suspension was placed onto a hemocytometer and the number of active T-RFC counted.

B-cells were determined by an erythrocyte-antibody-complement (EAC) rosetting technique. Five ml of a 5% solution of washed unsensitized SRBC was incubated for 30 minutes at 37°C with 5 ml of rabbit anti-SRBC sera diluted 1/2500 in PBS. The cells were
washed three times, and resuspended in 5 ml of PBS. Five ml of a 1/10 dilution of mouse sera was added, and the suspension was incubated for 30 minutes in a 37°C water bath. The sensitized SRBC were washed, and adjusted to a 0.5% final concentration in PBS containing 0.01 M Na₃HEDTA.

For the B-RFC assay, 0.25 ml (5 x 10⁵) PBL was mixed with 0.25 ml of 0.5% sensitized SRBC, centrifuged at 200 x g. for two minutes at room temperature, and then incubated for 30 minutes in a 37°C water bath. The cell pellet was resuspended by vigorous mixing, and the B-RFC were counted as described before. When unsensitized SRBC were tested as controls, no or few rosettes were formed.

3. **LYMPHOCYTE-MEDIATED CYTOTOXICITY (LMC)**

LMC of patient and control lymphocytes versus cultured tumor cells was determined by the chromium release assay (CRA) described by Brunner, et al (193).

Briefly, 5 x 10⁵ patient or control lymphocytes in 0.5 ml HEPES buffered complete medium with 15% fetal calf serum, patient plasma or control plasma were incubated with 10⁴ ⁵¹Cr-labeled tumor cells in 0.5 ml complete medium with the corresponding serum or plasma (50:1 effector/target) 5 to 7 hours at 37°C in a CO₂ incubator. Total count and blank tubes contained labeled tumor cells in a 1 ml complete medium with the appropriate serum or plasma without
lymphocytes; 0.1 ml 10% Triton X-100 was added to each total count tube. All determinations were made in triplicate or quadruplicate.

After incubation, tubes were centrifuged at 400 x g. for 10 minutes and an aliquot of the supernatant was assayed for radioactivity in an automated gamma spectrophotometer. Lymphocyte-mediated cytotoxicity of samples was determined by the expression:

\[
\%\text{LMC} = \frac{\text{CPM Sample} - \text{CPM blank}}{\text{CPM total count} - \text{CPM blank}} \times 100.
\]

4. DETERMINATION OF LYMPHOBLAST TRANSFORMATION

Lymphocytes were obtained from heparinized blood as described above and 5 x 10^5 of these cells were incubated in complete medium with 15% fetal calf serum, patient plasma or control plasma with 0.1 ml PBS, phytohemagglutinin (PHA-P, Difco Labs; full-strength or 1:10 dilution), a cell KCl extract (100 mcg or 1 mcg), or 10^4 mitomycin C-treated LT-2 cells at 37°C in a CO_2 incubator.

After 48 hours incubation 1 μCi ^3H-thymidine (2 Ci/mM, New England Nuclear Corp.) was added to each tube. After an additional 24 hours incubation, the reaction was stopped by transferring the tubes to a refrigerator, DNA was
precipitated with trichloroacetic acid (TCA), the precipitate was washed with PBS, centrifuged, and resuspended in NCS solubilizer (Amersham/Searle Corp., Arlington Heights, IL) and the resultant solution was transferred to scintillation vials in 10 ml Bray's solution with Triton X-100. Radioactivity in the vials was assayed in a Packard Tri-Carb automatic refrigerated liquid scintillation counter.

Lymphoblast transformation was ascertained from the relative $^3$H-thymidine uptake by lymphocytes, represented by a stimulation index calculated from the ratio of CPM in sample vials to CPM in PBS control vials. Quench was found to be invariable among samples with 5%. Samples were assayed in triplicate or quadruplicate.
C. TUMOR-HOST INTERACTIONS: IN VIVO

1. SKIN TESTS

General and specific delayed type hypersensitivity of the patient was determined by intradermal injection on the flexor aspect of the forearms of the patient with 0.1 ml streptokinase/streptodornase (50 units with respect to streptokinase in 0.1 ml (Lederle Lab., Pearl River, NY), dermatophytin (0.1 ml of a 1/30 dilution, Hollister-Stier Lab., Spokane, WA), dermatophytin-0 (0.1 ml of a 1/100 dilution, Hollister-Stier Lab.), PPD (0.1 ml intermediate strength, Parke Davis, Detroit, MI), mumps (0.1 ml, Eli Lilly, Indianapolis, IN), and 0.1 ml LT-2 KC1 extract (sterilized by filtration; 100 μg and 1 μg protein/0.1 ml). DNCB (2000 and 50 μg in acetone, Eastman Organic Chemical Co., Rochester, NY) challenge was accomplished by allowing test samples to evaporate on a 25 to 30 mm³ area of skin on the forearm. At least 10 mm of induration at 48 hours after challenge with microbial antigens was considered a positive skin test response. For DNCB the reaction was evaluated by retest at 14 days. A flare at both the 2000 μg and 50 μg sites was scored as a 4+ reactivity; at only the 2000 μg site as 3+; a positive flare to only a repeat application of 50 μg on the opposite forearm evaluated at 48 was scored 2+. In
2. **PREPARATION OF KCl EXTRACTS**

LT-2 monolayers consisting of between 6 to $14 \times 10^6$ cells were harvested using either 3M KCl or 0.081% EDTA in 25 mm HEPES buffer, followed by centrifugation and resuspension in 3M KCl. The KCl suspensions were rocked at 4°C overnight and then centrifuged at 163,000 x g. for 60 minutes at 4°C in a Beckman Ultra-centrifuge. The supernatants were filtered, concentrated (PM-10 membranes), dialyzed against three changes of 200 volumes of normal saline at 4°C for 24 hours, centrifuged at 1,500 x g. for 20 minutes at 4°C, lyophilized and reconstituted in 2 ml of PRS and the protein concentration measured by the Lowrey method. The extract was filtered through 0.2 μm pore size Gelman filters and stored in glass vials at -75°C. Skin testing consisted of the intradermal injection of 0.1 ml of the above preparation, and 0.1 ml of PBS on the volar skin of the forearm. The autologous tumor skin test was read at 48 and 72 hours.

3. **BCG IMMUNOTHERAPY**

The patient received four BCG immunizations over a five month period, commencing 46 days after the lesion in the
right inguinal lymph node was biopsied. For the first immunization, 3 to 5 x 10^8 lyophilized viable Chicago strain organisms were reconstituted with 0.5 ml sterile saline and 0.2 ml (1.2-2.0 x 10^6 viable organisms) was injected into one site of the lesion.

Three additional immunizations were given, administered at one month intervals following the first injection. For the second and third immunizations, the contents of one ampule were reconstituted with 1 ml sterile water followed by a 100-fold dilution; 0.2 ml (0.6-1.0 x 10^6 viable organisms) was injected intraleisonally, in six sites for the second immunization and in one site for the third immunization. For the final immunization, one ampule was reconstituted with 0.5 ml sterile water and this suspension was spread over an area of the anterior right thigh: 72 puncture wounds were made in this area by applying a 36-tine instrument to two adjacent sites.
D. CELL CHARACTERIZATION AND TUMOR PROPERTIES

1. ELECTRON MICROSCOPY

A portion of a nude mouse nodule (see below) and cells harvested from monolayer cultures were fixed in 2.5% glutaraldehyde in PBS at 40°C overnight and post-fixed in osmic acid one hour at room temperature. The fixed specimens were then carried through graded alcohol dehydration and embedding in Araldite. Electron microscopy procedures were performed by Doctor Emilio Orfei, Department of Pathology, Loyola University Medical Center, Chicago, IL.

2. KARYOTYPING

Karyotyping was done by a modification of the method of Hsu (194). Briefly, colchicine 10^-6M (BBL) in CM was added to an LT-2 monolayer and incubated for five hours at 37°C. An EDTA-HEPES-harvested single-cell suspension was counted, centrifuged at 300 x g. for 10 minutes and resuspended in hypotonic KCl (71mM) at a concentration of 10^4 cells/ml. Following a 20 minute incubation at 37°C the cells were again centrifuged and resuspended in a 10 ml freshly prepared absolute methanol-glacial acetic acid (3:1). The cells were centrifuged, the supernatant decanted, the pellet left overnight at 4°C, and resuspended
in a 2 ml methanol-acetic acid fixative. The cell suspension was dropped onto slides, framed, allowed to dry, and stained with Giemsa (for 7 to 9 minutes). Mitotic figures were observed and photographed through a Zeiss microscope at 1000 x magnification.

3. **DETERMINATION OF GENERATION TIME**

Two confluent 75 cm² culture flasks (approximately 17.6 x 10⁶ cells, 96% V.) were harvested, washed and resuspended in CM and distributed equally among twenty 25 cm² culture flasks (8.5 x 10⁵ cells per flask). Adherent cells were harvested from quadruplicate flasks after 27 hours, 46.5 hours, 72 hours, 97 hours and 118 hours. The single-cell suspension was centrifuged at 300 x g. for ten minutes and the pellet was resuspended in 0.5 ml phosphate buffered saline (PBS), diluted with an equal volume of 0.4% trypan blue stain in normal saline and counted in a hemocytometer. The log of the number of viable cells (N) was plotted against time in hours. Based on the relation log N = \( \frac{0.301t}{t_D} \) + log N₀. The doubling time (t_D) during the logarithmic growth phase of the cells was determined from the slope \((0.301/t_D)\) of the linear growth curve.
4. **TIME-LAPSE CINEMATOGRAPHY**

Time-lapse cinematography was done using a Nikon model M inverted phase contrast microscope with Matthias time-lapse control unit and Bolex camera and incubation chamber with heater and a Precision Scientific air/CO₂ blender. This equipment was used courtesy of Doctor George Willbanks, Chairman, Department of Obstetrics and Gynecology, Presbyterian-St. Lukes Medical School, Chicago, IL. Kodak Plus-X reversal film 7276 (400 ft., 16mm-B wind) was exposed for one second at two frames per minute at 200 x magnification, (about 40.18 frames/ft.). Two separate filmings of LT-2 and one of AU-471 were done.

5. **SATURATION DENSITY**

LT-2, AU-471 and WI-38 monolayer cultures were harvested; washed and resuspended each in 5 ml PBS; the cells counted, centrifuged, and adjusted to a concentration of $5 \times 10^5$ cells/ml in RPMI-1640 with 15% FCS; and plated in 24 well cluster dishes, Costar, (0.1 ml/well with $5 \times 10^4$ viable cells). Only the inner eight wells of each 24 cell cluster plate were used for culture. The outer wells were filled with sterile H₂O. Each well has a 16 mm diameter or an area of 2.011 cm²/well. Three cultures (24 total wells) were used for each cell type. Cultures were fed periodically with CM (1 ml/well). One well from each of
three plates was harvested serially, and the cells counted, and scored for viability. The cell density was expressed as the number of cells per cm$^2$.

6. **DENSITY DEPENDENT INHIBITION OF CELL DIVISION OF LT-2, AU-471 and WI-38 CELL LINES**

Eighteen Leighton tubes with cover slips (Wheaton Scientific, Millville, NJ) were autoclaved. Three groups of six tubes each were seeded with $5 \times 10^4$ viable cells per tube with LT-2, AU-471 and WI-38. When the cover slips were noted to be partially confluent, two tubes from each set were harvested, fixed in acetone for five minutes, stained with aceto-orcein for five minutes and counterstained with Giemsa for five minutes. The remaining four cultures were then refed with colchicine $10^{-6}$ M in CM, and two tubes harvested at 24 and 48 hours, fixed and stained as above. Photographs were taken of appropriate areas to locate the position of dividing cells in moderate sized colonies.

7. **SURFACE ANTIGENS: ABH**

LT-2 was harvested (Pas. 30, 92% V.), washed, counted and resuspended at $5 \times 10^6$ cells/ml. Twenty-five $\mu$l aliquots were mixed with 0.025 ml of an agglutinogen consisting of: Anti-Rho, Anti-RhC, Anti-A, Anti-B, Anti-H substance. The antiseras and cells were incubated at both
25°C and 32°C for ten minutes. Controls with LT-2 and 0.025 ml of PBS were also used to assay for "spontaneous" agglutination.

8. HETEROPHILE ANTIGEN

LT-2 monolayers were washed with PBS without Mg++ and Ca++ and x3 with 5 ml of Versene. They were incubated with Versene until cells became detached or harvested in a routine manner (as described above) using trypsin and EDTA. They were washed with PBS, centrifuged and resuspended at 5 x 10^6 cells/ml in PBS with Mg++ and Ca++. AU-471 was processed similarly.

Infectious mononucleosis positive and negative human serum was obtained (courtesy of Doctor Ken Thompson, Loyola University Clinical Microbiology Laboratory, Chicago, IL), and normal rabbit serum were mixed (0.025 ml). Each were incubated at 70°C for 1 and 2 hours, and evaluated for agglutination.

9. ASSAY FOR CEA AND hCG

Two days after nutrient media changes, spent media from LT-2 cultures (subsequently harvested with 0.01% Na_2EDTA/0.125% trypsin for counting of single cells) was filtered through 0.2 micron pore size Nalgene disposable filter units. Most of this filtered spent media was concentrated to approximately 1/7th or 1/4th of its original
volume through a PM-10 membrane in an Amicon ultrafiltration unit. An equal quantity of unused complete media was treated in an identical fashion. Samples of spent media, 7-times concentrated spent media, 14-times concentrated spent media, and corresponding samples of unused complete media were assayed for CEA, hCG, and β-hCG-subunit.

Carcinoembryonic antigen (CEA) in the above samples was determined by the Foster G. McGaw Hospital Nuclear Medicine Department, Chicago, IL, under the direction of Doctor Robert Henkin, using the CEA-Roche test kit (Roche Diagnostics, Nutley, NJ). Radioimmuno-assay for hCG/LH and β-hCG-subunit was performed by the John I. Brewer, Choriocarcinoma Research and Treatment Center, Northwestern University Cancer Center, Northwestern University Medical Center, Chicago, IL. The rate, r, of β-hCG or CEA production (ng/10⁶ cells/day) by LT-2 was approximated as

\[ r = \frac{(\text{ng/ml assayed})(\text{concentration factor}) \times 10 \text{ ml/flask}}{(3 \times 10^6 \text{ cells/flask average}) \times 2 \text{ days}} \]

10. **SERUM DEPENDENCE**

Serum dependence was evaluated for LT-2, AU-471 and WI-38. Two separate experiments were performed. In the
first experiment LT-2, AU-471 and WI-38 were plated in 10%, 1%, 0.01% and 0% FCS at $1.5 \times 10^5$ viable cells per well in 24 cluster plates (Costar, Cambridge, MA) in triplicate. Only the inner eight wells of any given 24 well plate were used. Duplicate plates with each cell type were plated in 15% FCS, incubated overnight, and the media aspirated. Culture wells were washed once and then refed with CM with the varying concentration of FCS as noted above. Plates were harvested (T/E) four days later, and cells counted using a Coulter counter (Coulter Electronics Inc., Elk Grove Village, IL).

A similar experiment was repeated using triplicate cultures plated in the appropriate serum concentration using AU-471 and LT-2 with cultures harvested six days after plating and then counted on a hemocytometer.
E. GROWTH IN SOFT AGAR: ANCHORAGE INDEPENDENT GROWTH

1. EPIGENETIC FACTORS

Tumor cell growth in soft agar is a commonly used test for in vitro malignancy. It is based on the observation that nonmalignant epithelial cells do not grow in soft agar or in suspension, but require attachment to a surface before cell division will occur. Therefore, growth in soft agar (anchorage independence for cell division) is an "abnormal" property for a cell, and suggests a malignant biological potential.

Soft agar assays were adopted from protocols of MacPherson and Montagnier (195) and also from Major and di Mayorca (196). Briefly, a "feeder agar" consisting of 0.5% Difco Bacto agar in RPMI-1640 with 10% tryptosephosphate broth (GIPCO), and antibiotics (consisting of either penicillin, 100 units/ml and streptomycin 100 μg/ml or kanamycin 100 μg/ml or gentamycin 100 μg/ml) was poured into 60 mm x 15 mm petri dishes (Flacon No. 1007) and allowed to solidify. Over this "hard" or "feeder agar" was poured 1.5 ml of 0.33% "soft agar" (prepared as for 0.5% agar above, but with less agar). Test cells were suspended in the soft agar. The cultures were maintained at 37°C in an atmosphere of 5% CO₂, 95% air for 3 to 4 weeks, until visible colonies were scored and the test terminated.
2. SOLUBLE STIMULANTS OR INHIBITORS

Anchorage independent growth in soft agar suspension is a frequently used in vitro criterion for malignancy as noted above. Yet, under usual conditions LT-2 would not clone in soft agar, but did fulfill most other criteria for malignancy including: continued growth in culture, growth and tumor formation in nude mice, abnormal karyotype, (see below). Experiments were performed to determine whether LT-2 produced a soluble inhibitor that would prevent the growth of other tumors in soft agar culture, and conversely determine if growth in soft agar could be stimulated by a soluble substance from tumor cells that grow well in soft agar as manifest by a high soft agar cloning efficiency. LT-2, AU-471 and WI-38 were used in these experiments.

3. THE EFFECT OF LT-2 ON THE SOFT AGAR GROWTH AND CLONING OF AU-471

In four experiments, viable LT-2 cells were treated with mitomycin C (100 μg/ml CM), and incubated for 40 minutes at 37°C with shaking, washed 2 times with PBS and re-suspended in CM. These mitomycin C treated LT-2 cells served as "feeder cells" to test for the presence of soluble factors that might stimulate or inhibit cloning in soft agar. Mitomycin C is an antibiotic produced by Streptomyces Verticillatus and is converted in a cell by reductive acti-
vation to an alkylating agent that selectively inhibits DNA synthesis (197). AU-471 served as test cells, and were plated at $5 \times 10^2$ or $2 \times 10^3$ cells per plate in soft agar in different experiments and mixed with increasing concentrations of LT-2 "feeder" cells ($1 \times 10^4$ to $1 \times 10^5$ LT-2).

AU-471 controls were plated at the appropriate concentration without "feeder" cells to serve as a standard control, and LT-2 mitomycin C or nonmitomycin C LT-2 without AU-471 served as negative controls. LT-2 preplated onto the bottom of 60 mm culture plates with agar poured on top of the culture served as a LT-2 growth and viability control, and were scored negative or positive for growth and viability. AU-471 test plates and controls were scored on two occasions.

Two additional experiments were done using untreated LT-2 (non-mitomycin C) as "feeder" cells taking advantage of the fact that LT-2 will not clone in soft agar. In one experiment, ten plates of $2 \times 10^3$ and $2 \times 10^4$ viable LT-2 cells per plate (Pas. 21, 85% V.) were used as controls, and were also co-cultivated with AU-471 (AU-471:LT-2 at 1:1 and 1:10 cells per plate ($2 \times 10^3$ AU-471:2 $\times 10^3$ LT-2 or $2 \times 10^3$ AU-471:2 $\times 10^4$ LT-2). Colonies were scored at day 14 and 24. The other experiment used ten plates each of $2 \times 10^3$ and $2 \times 10^4$ viable AU-471 per plate (Pas. 121, 90% V.) co-cultivated in soft agar with 1:5 or 1:50 ratio of AU-
471:LT-2 per plate. Controls of ten plates of 2 x 10^3 and 2 x 10^4 AU-471 without LT-2 and 10^5 LT-2 (Pas. 18, 99% V.) without AU-471 served as an LT-2 control.

4. EFFECT OF AU-471 ON SOFT AGAR GROWTH OF AU-471

Since an increase in cloning efficiency or "feeder effect" was noted in LT-2:AU-471 and also in WI-38:AU-471 co-cultivation experiments, the effect of mitomycin C treated AU-471 on the cloning efficiency of untreated AU-471 was evaluated. The experiments were performed using 5 x 10^2 (Pas. 112, 87% V.) and 2 x 10^3 (Pas. 126, 89% V.) AU-471 test cells co-cultivated in soft agar with increasing concentrations (2 x 10^3 to 1 x 10^5 viable cells in different experiments) of mitomycin C treated AU-471 "feeder" cells. Controls consisted of untreated AU-471, mitomycin C treated AU-471 (no untreated cells) and pre-plated AU-471.

5. EFFECT OF MITOMYCIN C TREATED AU-471 CO-CULTIVATED WITH LT-2

The reverse experiment used five plates each of 2 x 10^4 and 1 x 10^5 mitomycin C treated AU-471 "feeder" cells (Pas. 122, 84% V.) with a comparable number of untreated LT-2 cells (Pas. 28, 78% V.). Controls consisted of five plates
each of $2 \times 10^4$ and $1 \times 10^5$ LT-2 without AU-471 and $2 \times 10^4$ and $1 \times 10^5$ mitomycin C treated (100 µg/ml CM for 40 minutes at 37°C with shaking) AU-471.

6. EFFECT OF WI-38 ON THE SOFT AGAR GROWTH AND CLONING OF AU-471

A similar "feeder" experiment was done in order to determine the stimulatory or inhibitory effect of co-cultivation of varying concentrations of "normal" cells on the soft agar cloning efficiency of AU-471, to serve as a "normal" control. Five plates each of WI-38 (Pas. 26, 95% V.) with cell concentration of $2 \times 10^4$ and $1 \times 10^5$ viable WI-38 cells per plate were co-cultivated with $2 \times 10^3$ viable AU-471 (Pas. 121, 84% V.) in soft agar. Colonies were scored on days 14 and 22. Cloning efficiency was compared to ten plates of $2 \times 10^3$ control AU-471 per plate. Five plates each of WI-38 at $2 \times 10^4$ and $1 \times 10^5$ without AU-471 served as controls.

7. FIELD COUNT EXPERIMENTS AND THE EFFECT OF MITOMYCIN C AND CYTOCHALASIN B ON LT-2 GROWTH AND CLONING IN SOFT AGAR

Since LT-2 had been noted during time-lapse cinematography to be highly motile in tissue culture, experiments were performed to determine whether LT-2 was dividing in agar (or agarose), and the daughter cells
moving apart forming single cells rather than developing into clones. To test this hypothesis, LT-2 was plated in agar suspension (or agarose), and the number of cells per high power microscopic field (150x) were scored for five random fields in each of five agar culture plates. Similar "field counts" were repeated on two additional occasions one and two weeks later.

One experiment consisted of five plates of $4 \times 10^4$ viable LT-2 and five control plates of $100 \mu g$ mitomycin C/ml with field counts on days 1, 8 and 23. Additional experiments were done to evaluate the effects of cytochalasin B on soft agar growth and cloning of LT-2. Cytochalasin B is known to inhibit cell movement, phagocytosis, and under appropriate conditions may produce cell enucleation. The probable mechanism of action is through disruption of microfilaments (198). Cytochalasin B was used at concentrations of $1 \mu g/ml$ and $5 \mu g/ml$ CM and compared with similar field counts of the same plates at different time periods and/or to control plates of untreated or mitomycin-treated ($100 \mu g$ mitomycin C) LT-2 agar plates. Five $\mu g$ cytochalasin B has been shown to cause cell cytoplasmic retraction of spread cells with cell rounding and inhibition of cell movement (199).
Heparin and other highly negatively charged molecules have been reported to reduce cloning efficiency in soft agar. Cell membranes are negatively charged and contact between normal cells generally results in contact inhibition of cell division. That ions in contact with the cell membrane in soft agar might result in a form of "contact inhibition" preventing cell division was considered. This hypothesis was evaluated using agarose. Agarose is a purified form of agar with many of the negatively charged ions removed. Diethylaminoethyl dextran which complexes with positively charged ions was also used to evaluate the effects of decreased positively charged ions.

The concentration of the agar is important in evaluating anchorage independence since even "normal" fibroblasts will sometimes produce colonies in agar when the concentration of the agar is increased. Agar concentrations of 0.33% give a semi-solid consistency. Apparently, cell growth and colony formation results from giving the cells a more solid surrounding for contact with the cell membrane.

The consistency of pure agarose was noted to be somewhat different from that of agar (0.33% agar has a
semiliquid consistency, but .033% agarose has a more solid consistency). Dilutions of agarose were made and their consistency (solid to semi-solid to liquid) determined at various concentrations of agarose. A consistency of agarose of about 0.275% was chosen for the top agar with a feeder layer of 0.300% agarose giving a consistency similar to 0.3%/0.5% agar. Other experiments used 0.33% top agarose with 0.5% feeder agarose giving a more solid consistency similar to experiments with 1% DIFCO agar, or 0.1% top agarose with 0.15% bottom agarose giving a much more fluid agarose than the standard 0.33% "soft agar."

The effect of cloning LT-2 in "soft agarose" was evaluated by plating five 60 mm TC plates with 1 x 10^5 viable LT-2 cells per plate (Pas. 29, 80% V.) in 1.5 ml of 0.275% agarose with 5 ml of 0.3% bottom or feeder agar. Five comparable control plates were preplated and overlayed with agarose.

One arm of the experiment combined both agarose and DEAE and consisted of three 60 mm TC with 1 x 10^5 viable LT-2 cells per plate (Pas. 31, 44% V.) with 22 μg DEAE per ml top agar in agarose.

A "hard agarose" experiment consisted of five 60 mm TC plates with 2 x 10^4 viable LT-2 per plate (Pas. 31, 44% V.) in 0.33% top agarose with 0.5% bottom agarose. Two additional 60 mm TC plates were plated with 1 x 10^5 viable LT-2 as above.
A "softer" agarose experiment used 0.10% top agarose with 0.15% bottom agarose. Four 60 mm TC plates were each plated with $0.7 \times 10^5$ viable LT-2 (pooled flasks, 27% V.). Comparable preplated viability controls were overlaid with agarose.

Two experiments were done to evaluate the effect of DEAE on the soft agar cloning efficiency of LT-2. These experiments consisted of five 60 mm TC plates each with $1 \times 10^5$ viable LT-2 cells (Pas. 30, 38% V.) and (Pas, 23, 60% V.) plated with 30 μg DEAE per ml soft agar as test plates with five comparable untreated control plates. In addition five plates were preplated with LT-2 as viability controls.

9. **EFFECT OF HEPES BUFFER ON LT-2 GROWTH IN SOFT AGAR**

The effect of HEPES buffer in cloning efficiency of LT-2 was evaluated using five 60 mm TC plates each with $1 \times 10^5$ viable LT-2 (Pas. 31, 53% V.) with 30 mm HEPES with scoring for colony formation on days 16 and 23.

10. **EFFECTS OF CYCLIC GMP ON LT-2 GROWTH IN SOFT AGAR**

The effects of cyclic dibutyrlyl GMP (cGMP) on cloning of LT-2 in soft agar was evaluated using five 60 mm TC plates each with $1 \times 10^5$ viable LT-2 per plate (Pas. 32, 62% V.) with concentrations of cGMP of $10^{-9}$M, $10^{-10}$M, $10^{-11}$M with scoring for colonies on days 16 and 23.
11. THE EFFECT OF ENZYME TREATMENT OF LT-2 ON CLONING IN SOFT AGAR

The effect of digestive enzyme treatment of membrane molecules of LT-2 on the cloning efficiency using neuraminidase trypsin and a variety of carbohydrates was evaluated.

a. NEURAMINIDASE

A monolayer culture of LT-2 was harvested, (T/E), washed with PBS and the cells resuspended in 4 ml of PBS without Ca++ and Mg++ (4.35 x 10^6 cells; Pas. 31, 85% V). Two milliliter aliquots were placed in separate tubes, centrifuged and resuspended in 0.2 ml of PBS or PBS with 60 units neuraminidase/ml and incubated with shaking for 60 minutes at 37°C. Two 60 mm culture flasks with 1 x 10^5 viable non-treated LT-2 (44% V. post-incubation) in soft agar served as controls. Three cultures contained .91 x 10^5 neuraminidase treated LT-2 (53% V. post-incubation) and three additional soft agar cultures contained .91 x 10^5 viable LT-2 treated with neuraminidase as above, but with the addition of 0.22 μg DEAE per/ml of soft agar. All neuraminidase cultures also contained 50 units neuraminidase per/ml of soft agar.
**b. CARBOHYDRASES**

A soft agar experiment was performed in order to determine the effect of a variety of enzymes specific for carbohydrate substrates on anchorage independent growth. LT-2 was harvested (T/E), neutralized with CM, centrifuged, counted (Pas. 24, 71.4% V.) and resuspended in 2 ml of PBS (without Ca\(^{++}\) or Mg\(^{++}\)), passed through a 50 \(\mu\) mesh, counted on a hemocytometer with trypan blue and resuspended at 5 x 10\(^{6}\) viable cells per ml. Fifty microliter aliquots (2.5 x 10\(^{5}\) viable cells) were incubated at 37\(^{\circ}\)C for one hour with 0.2 ml of either: 0.6 or 6 mg B-glucosidase, 0.55 mg \(\alpha\)-D-glucosidase, 0.75 and 0.075 mg chitinase, 1.25 units (2.75 mg/ml) \(\alpha\)-L-Fucosidase, 3 units (18 units per mg) \(\alpha\)-D-mannosidase or a mixture of all of the above enzymes. The incubated mixtures were plated in soft agar. Final concentrations in top agar were 1 \(\mu\)g glucosidase, 1 and 10 \(\mu\)g B glucosidase, 1 and 10 \(\mu\)g chitinase, 0.25 \(\mu\)g L Fucosidase; 1 \(\mu\)g mannosidase per ml; and 1 \(\mu\)g of a mixture of all of the above per ml top agar.


The effect of LT-2:AU-471 cell contact or LT-2 cell lysis was compared in mitomycin C treated and untreated LT-2 feeder cells. Six plates of AU-471 with 2 x 10\(^{3}\)
viable cells per plate were used as a control to determine cloning efficiency. Three plates were each co-cultured with: $2 \times 10^3$ viable AU-471 and $10^3$ or $10^5$ untreated or mitomycin C treated LT-2 (100 μg/ml incubated for 40 min. at 37°C); $10^3$ or $10^5$ untreated or mitomycin C treated LT-2 pelleted with AU-471 responder cells and then incubated for 60 min. at 37°C in 5% CO₂; $10^3$ or $10^5$ untreated or mitomycin C treated LT-2 lysed by freeze thawing (zero percent viability as checked by trypan blue exclusion). Two prelate controls consisted of $10^5$ V. LT-2 or $1.2 \times 10^3$ V. AU-471 overlaid with 1.5 ml of 0.33% soft agar. A growth control for LT-2 consisted of $10^5$ LT-2 plated alone in soft agar without AU-471.

A repeat experiment was done as above except $10^4$ and $10^5$ LT-2 cells were used instead of $10^3$ and $10^5$ in each of the arms of the experiment as described above.

13. CONDITIONED MEDIA

Filtered (0.2 μm Nalgene) conditioned media taken from partial confluent monolayer cultures of LT-2 and AU-471 at 24 hours and from MS at six days was used as "feeder" media. Five cultures of $1 \times 10^3$ viable AU-471 (Pas. 125, 83% V.) without conditioned media served as controls. Five plates each with 30% of .15% conditioned media from AU-471 (Pas. 125), LT-2 (Pas. 50), and MS (Pas. 6) were substituted for
fresh media and were added to 1 x 10^3 viable AU-471 cells in soft agar. Concentrations of tryptose phosphate broth, fetal calf serum and total media content (RPMI-1640) were kept constant. Two preplate controls of 1 x 10^3 viable AU-471 without conditioned media or with 30% conditioned media from AU-471, LT-2 or MS were also done. A total of 43, 60 mm soft agar cultures were done.

14. **PROTEIN EXTRACT FROM AN ANCHORAGE INDEPENDENT CELL LINE**

Monolayer cultures of AU-471 were treated with 10 ml of 3M KCl for 10 minutes at 37°C; the flask content decanted into 50 ml tubes and rocked overnight at 4°C; centrifuged at 25,000 x g. for 90 min. at 4°C. The two supernatants were pooled and concentrated using an Amicon PM-10 membrane, the retentate dialyzed with normal saline x 3, centrifuged at 1500 x g. at 4°C for 120 min. and filtered using 0.2 Nalgene filters. Protein content was estimated using the Wadell method. Three plates each with 1 x 10^3 viable LT-2 (Pas. 43, 84% V.) were mixed with 20, 310 and 1,000 μg per plate of the protein extract with 12% PBS.

Two AU-471 plates of untreated LT-2 controls and two preplate cultures were also done. A comparable set of cultures (13 plates) were done without FCS.
15. CELL CLUMPS IN SOFT AGAR

LT-2 had been noted to be relatively sensitive to plating at low cell densities. Therefore, studies were done in order to determine if cell-cell contact was necessary to initiate or stimulate anchorage independent growth in soft agar. Five separate experiments were performed consisting of 30 cultures and 8 preplate controls to evaluate cell viability. Two culture plates contained no cells to insure that as the agar dried that precipitants did not form visible clumps in agar. $10^5$ viable LT-2 cells were plated in each culture as previously described, except that cells were not passed through 100 micron mesh filters to eliminate clumps. Small clumps of cells noted in the agar were circled and photographed serially. Photos were then compared to evaluate for enlargement of cell clumps indicating growth in soft agar.

16. FIBRONECTIN

The effect of fibronectin (FBN) and antifibronectin on anchorage independent (soft agar) cell growth and cloning was evaluated. The cancer cell lines AU-471 (clones in soft agar) and LT-2 (no clones in soft agar) were used. Three "normal" cell lines were also used: BHK-21 (clones in soft agar), WI-38 and MS (no clones in soft agar).
AU-471 was used to evaluate the effects of FBN on the cloning efficiency of a malignant tumor cell line that demonstrates anchorage independent growth. Four experiments were done. AU-471 (Pas. 121, 126, 127) monolayer cultures were harvested, washed, passed through an 80 μ nylon mesh and counted. Cells were divided into two aliquots and incubated for 30 minutes at 37°C with 100 μg per ml FBN or PBS control in 2 ml volumes. The cells were then centrifuged, resuspended in PBS and four cultures were each plated with $2 \times 10^3$ V. AU-471 in 100 g/ml FBN or PBS per ml soft agar. One or two preplate cultures with or without FBN were also used as cell viability controls. Colonies were scored twice, generally at 2 and 4 weeks.

The effect of FBN on soft agar cloning of LT-2 was cloned as an example of a malignant cell line (defined by tumor production in nude mice) that does not demonstrate anchorage independent cloning. Three experiments were done using LT-2 (Pas. 49, 41, 61). Monolayer cultures were harvested, washed, passed through 80 μ nylon mesh cultures, counted and incubated for 30 minutes at 37°C with 100 μg of FBN or PBS in 2 ml volumes. The cells were centrifuged, resuspended in PBS and four plates each were seeded with $1 \times 10^4$ V. cells in 100 μg per ml FBN or PBS of soft agar. One or two preplates were also done and cultures scored as above.
The effect of FBN on soft agar cloning of BHK-21 was done using BHK-21 as an example of a "benign" cell line that does demonstrate anchorage independent growth. The experiments were done using BHK-21 as described above.

The effects of FBN on soft agar cloning of WI-38 and MS were done using these cell lines as examples of "benign" cell lines that do not demonstrate anchorage independent growth. The experiments were done as described above.

Heat inactivated (57°C for 60 minutes) Goat antifibronectin sera (GAFS) was used to determine the effect on anchorage independent growth and cloning of AU-471 and BHK-21. AU-471 (Pas. 123) and BHK-21 cells were harvested, washed, passed through an 80 μ nylon mesh, counted and scored for viability, and 4 plates each with 2 x 10^3 V. AU-471 or BHK-21 were seeded in soft agar with GAFS or normal goat sera (heat inactivated as above) at the same protein concentration (9.0 mg protein per culture). Additional controls consisted of four plates each of AU-471 and BHK-21 in soft agar without treatment and six preplate controls seeded at 8 x 10^5 V. cells for cell viability (one preplate without treatment, one with FBN and one with NGS each for AU-471 and BHK-21). Cultures were scored for colonies at 14 days and 27 days.
Monolayer cultures of AU-471 show piling up of cells (lack of contact inhibition) that results in high cell saturation densities (cell/cm²) in confluent cultures. Many cells can be found floating in the media even in cultures of cells that grow as strict monolayers without piling up of cells (low saturation density). Evaluation of the cloning efficiencies of floating cells, loosely attached cells and firmly adherent cells of an anchorage independent cell line (AU-471) was done.

Media was decanted from culture flasks and constituted the "floater" fraction. The flasks were gently washed once with PBS, then 20 ml of PBS was added and the flasks shaken to obtain loosely attached cells. The flasks were washed again with PBS and the remaining cells harvested (T/E). Cell number and viability were determined for each fraction. Four cultures of each of the three AU-471 fractions were plated in soft agar at both $2 \times 10^3$ V. and $2 \times 10^4$ V. cells per culture.

The number and viability of "floaters", loosely attached and firmly attached LT-2 cells were also determined. Since LT-2 had been shown not to clone in soft agar in over 1000 cultures, and since the floater and loosely attached fraction represented a minor fraction of dead cells they were not plated in soft agar (see Results).
F. NUDE MOUSE GROWTH

1. GROWTH OF TUMOR CELL SUSPENSIONS IN NUDE MICE

Four nude (nu/nu) mice were kindly provided by Doctor Donald Rowley, LaRabida Children's Hospital, Chicago, IL, and 18 additional nude mice were obtained from Northwestern University Animal Care Facilities, Chicago, IL. The mice were maintained in sterilized filter top cages in a laminar flow hood. All food, H₂O and bedding were sterilized, and the water was adjusted to a pH of 2.5 with HCl. Two mice were injected subcutaneously (s.c.) with 4 x 10⁶ viable LT-2 cells in 0.1 ml PBS into the upper dorsal thoracic area. (Pooled passages 21, 22, 23, 24, 26 and 27 with viability of 89-95%). Four mice received 8 x 10⁶ LT-2 cells s.c. (pooled passages 91% V.) and four animals received 10 x 10⁶ viable LT-2 (Pas. pooled 26-29, 81-100% V.). Ten animals were injected intravenously with LT-2 via the dorsal tail vein; three with 5 x 10⁶ viable cells in 0.1 ml PBS and three with 1 x 10⁶ cells (Pas. pooled 21-27, 89-95% V.), and four additional animals received 1 x 10⁶ viable LT-2 in 0.05 ml intravenously (Pas. 22, 95-99% V.). Two additional animals were injected with 2 x 10⁶ viable AU-471 cells in 0.05 ml PBS intravenously (Pas. 119, 79% V.). Subcutaneously injected cells that resulted in nodule formation were harvested at 3 months, 4-1/2 and 7 months.
Nodules were cut into four small pieces. One portion was frozen in O.C.T., one was fixed in paraformaldehyde, and one portion was fixed in formalin and imbedded in paraffin blocks, cut and stained with hematoxalin and eosin. The last piece was chopped into small fragments and placed in culture.

2. **INTRAVENOUS INJECTED NUDE MICE**

One animal convulsed apparently suffering a pulmonary embolus from injection of tumor cells. The remaining animals (twelve) survived and were sacrificed or died from day 33 to day 200 post-injection. The animals were autopsied, and no tumor tissue was noted in the peritoneal or pleural cavity. Lungs, spleen and livers were fixed in formalin and processed for hematoxylin and eosin staining.

3. **NUDE MOUSE CULTURES**

Nude mouse nodules were maintained in culture as noted above until partial confluent flasks were obtained. Two nude mouse nodule cultures (NMNC) were successfully propagated in tissue cultures. Nude mouse nodule cells and nude mouse skin fibroblasts were karyotyped using the procedure described above. The two successfully propagated nude mouse nodule cultures (NMNC) were later cultured in soft agar to determine if successful passage through nude
mice would alter their anchorage independent culture properties.

4. **SOFT AGAR EXPERIMENTS WITH NUDE MOUSE NODULES IN CULTURE**

Five 60 mm culture plates were seeded with $1 \times 10^5$ viable (58% V.) LT-2, (NMNC culture No. 5) in soft agar, and five additional cultures with $1 \times 10^5$ viable LT-2 overlaid with agar (controls). Another soft agar experiment compared NMNC No. 5 and NMNC No. 7. Each were cultured in five 60 mm culture plates with $1 \times 10^5$ viable LT-2 cells per plate in soft agar. There were five "preplate controls" with agar poured over the cells for both NMNC 5 and 7.

5. **KARYOTYPE OF NUDE MOUSE CELLS**

Cells from nude mouse nodules that were again established in vitro were karyotyped as described above.
CHAPTER IV

RESULTS

A. CELL CULTURES

1. GROWTH AND HISTOLOGY OF LT-2 IN TISSUE CULTURE

LT-2, a vulvar carcinoma cell line has been successfully maintained in tissue culture for 4 years through 50 passages, probably representing between 100-200 generations considering a plating efficiency estimated at 40% (Section 2: Plating Efficiency). The plating efficiency has improved during the four years of observation. Viability of freeze-thawed LT-2 cells was generally good (average 87.1% V.). This is the third vulvar cancer established in tissue culture. Cell monolayers, grown on cover slips in Leighton tubes, and also single cell suspensions evaluated by Papanicolaou staining were both cytologically malignant. Cells are predominately mononuclear with multiple prominent nucleoli, weakly basophilic cytoplasm, and a large nuclear/cytoplasm ratio (Figure 1). Both cells and nuclei are pleomorphic with occasional giant forms. Frequent mitoses are noted. During the four years of observation, LT-2 has continued to grow as a very strict monolayer with no piling up of cells
Figure 1. LT-2 monolayer from Leighton tube culture, fixed in absolute ethanol. (Giemsa stain x 300.)
as frequently noted in other tumor cells in tissue culture (Figure 2). Electron micrographs show both dense and fibrous nucleoli and numerous mitochondria, rough endoplasmic reticulum, light-staining vacuoles, and tonofibrils characteristic of epithelial cells. No viral particles were noted. Using aceto-orcein staining of Leighton tube cover slip monolayers, no mycoplasma were noted.

2. PLATING EFFICIENCY

Following plating, LT-2 cells attached to the plastic substrate of the culture flask and began to "spread". One difficulty in determining plating efficiency was that cells continued to attach to the substratum over a period of time after plating. If cells were counted too early, before all potential plating cells attached, an inaccurately low value might be obtained. Likewise, if plating efficiency was not determined soon after plating, cell division might give an inaccurately high value. The use of both mitomycin C treated cells and untreated cells helped determine a more accurate plating efficiency. Plating efficiencies

\[
\frac{\text{No. cells harvested}}{\text{No. cells plated}} \times 100 \text{ varied between 11.3 and 36% at 2 hours and cell attachment leveled off between 2 and 4 hours.}
\]

Mitomycin C treated cells showed a 20.5% attachment efficiency at 2 hours which leveled off to 40.6% by 4
Figure 2. Comparison of cell growth in vitro: top, LT-2, note the strict monolayer growth; bottom, AU-471, note the piling up of cells.
hours. No further increase in cell attachment to the sub­stratum was noted after 4 hours. By 12 hours, a considera­ble variation in the number of cells harvested was noted be­tween the mitomycin C treated and untreated cultures with plating efficiency increasing to 61.8% in the untreated cul­tures indicating cell growth in culture. These experiments indicate an average plating efficiency for LT-2 of about 40% with cell attachment plateauing between 2 and 4 hours. Cell division was noted shortly after plating (Figure 3).
Figure 3. Plating efficiency of LT-2. Experiment 1, ⊗, no treatment. Experiment 2, □, treatment. Experiment 2, x, with mitomycin C.
B. TUMOR-HOST INTERACTIONS IN VITRO

1. LYMPHOCYTES

Total white blood count (WBC) was consistently elevated (10-30,000 WBC per mm$^3$) during the 15 months of observation, yet the total peripheral blood lymphocytes (PBL) percentages and absolute numbers were depressed. Total and active T-RFC percentages and absolute numbers were also depressed. An increase in PBL, total and active T-RFC % and absolute numbers occurred following BCG immunization; confirming a bone marrow sparing effect of this form of therapy. B-RFC percentages were also depressed below normal levels, and also increased following BCG therapy. All values returned to their previously repressed values over a period of several months despite continued BCG injections (Figure 4).

2. CYTOTOXICITY

Chromium release cytotoxicity assays were done on two occasions using patient and control lymphocytes versus autologous tumor or an unrelated malignant cell line (AT-264). Cytotoxicity was assayed separately in autologous and control plasma.

The first assay (Exp. 1) revealed no patient lymphocyte cytotoxicity against her own tumor in autologous or in
Figure 4. Patient M.L. immunoevaluation and treatment during a 15 month period. Shaded areas indicate normal range. ↑, dermatophytin-o positive; φ, PPD positive.
control plasma or against the lung carcinoma cell line (AT-264). Control lymphocytes also showed no specific cytotoxicity although some "spontaneous or natural" cytotoxicity was noted against control target cells in both control and patient (M.L.) plasma (9.12 & 10.07% LMC). Control LMC against LT-2 cells was more varied showing only 0.63% cytotoxicity in patient plasmas and 11.73% LMC in control plasma. (Although no statistically significant conclusions can be drawn from this variation because of the large standard deviations of samples in control plasma). The patient's "natural" cytotoxicity was depressed against both her own autologous tumor and AT-264 cells in autologous plasma, although some (4.70 ± 1.56% LMC) spontaneous cytotoxicity was noted against AT-264 in control plasma.

During the first and second cytotoxicity assays and until the patient's death two months later her general health and nutritional status continued to deteriorate. All skin tests, including skin tests to autologous tumor extracts, were negative at that time. DNCB skin testing was negative, and total PBL, T-RFC, active T-RFC and B-RFC percentages and absolute numbers were depressed. Yet, despite her general state of anergy, and her considerable tumor burden at that time, a significant degree of specific cytotoxicity (P< 0.001) was noted in both autologous and
control plasma (15.93 & 11.44% LMC, respectively) against her autologous tumor. Also a small increase in "natural" cytotoxicity (4.64 & 6.37% LMC) was noted against AT-264 in both control and autologous serum. Control lymphocytes again showed the expected degree of "natural" cytotoxicity (8.09 & 9.40% LMC) against AT-264 similar to the first experiment. Again no natural cytotoxicity was noted against LT-2 using control lymphocytes, but a very large variation in standard deviation in cytotoxicity was noted in control plasma (S.D. ±22.38).

This increase in cytotoxicity with patient's lymphocytes against her own tumor occurred 2 days following several blood transfusions. It is interesting to speculate that this increase in cytotoxicity was an example of the "allogenic effect" or a more remote possibility is that it occurred because of immune reactivity of viable transfused lymphoid cells.

The patient's sister's lymphocytes were also assayed for cytotoxicity against LT-2 in control plasma and none was noted. (Table 1.)

3. **LYMPHOBLAST TRANSFORMATION**

PHA induced blastogenesis was assayed on four occasions and remained markedly depressed throughout the period of observation. No increased response was noted.
<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>Targets</th>
<th>% LMC</th>
<th>± S.D.</th>
<th>No.</th>
<th>Plasma</th>
</tr>
</thead>
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<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>AT-264</td>
<td>9.12</td>
<td>3.00</td>
<td>4</td>
<td>Control</td>
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<tr>
<td>Control</td>
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<td>M.L.</td>
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<td>3.49</td>
<td>4</td>
<td>M.L.</td>
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<tr>
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<td><strong>Experiment 2</strong></td>
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<td>8.09</td>
<td>2.35</td>
<td>3</td>
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</tr>
<tr>
<td>Control</td>
<td>AT-264</td>
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<td>M.L.</td>
<td>AT-264</td>
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<td>1.66</td>
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<td>M.L.</td>
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<td>22.38</td>
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<td>Control</td>
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<td>LT-2</td>
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<td>5.92</td>
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Despite the increase in PBL, T-RFC and active T-RFC following BCG. Also no increase in blastogenesis was noted following blood transfusions similar to the increase in LMC noted above. Blast transformation to KCl extracts of LT-2 cells (1 μg or 10 μg protein) showed no antigenic recognition by the patient, or by a close household contact (the patient's sister), or by a non-tumor control patient's lymphocytes. No blast transformation was noted to KCl extracts with mitomycin C treated patient, sister or control lymphocytes (control for non-specific thymidine uptake) or using a KCl extract of a normal human fibroblast cell line (control for non-specific lymphocyte stimulation). (Table 2.)
TABLE 2

LYMPHOBLAST TRANSFORMATION

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>Antigen</th>
<th>Stimulation Index</th>
<th>± S.D.</th>
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<tr>
<td></td>
<td>PHA</td>
<td>6.80*</td>
<td>1.01</td>
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<tr>
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<td>PHA 1/10</td>
<td>14.39*</td>
<td>0.97</td>
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<tr>
<td></td>
<td>LT-2 KCl (10 μg)</td>
<td>1.14</td>
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<td>LT-2 KCl (1 μg)</td>
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<td>0.24</td>
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<td>LT-2 + mc</td>
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<td>LT-2 + mc (M.L. plasma)</td>
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<td>LT-4 KCl</td>
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<td>67.06</td>
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<td></td>
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<td>LT-2 KCl (1 μg)</td>
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<td>LT-2 + mc</td>
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<td>PHA</td>
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<td>LT-2 KCl (1 μg)</td>
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<td></td>
<td>LT-4 KCl</td>
<td>0.99</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Significantly lower than PHA stimulation of control lymphocytes (P < 0.01)
+ Not significantly different from normal population (S.I. = 116±26; P > 0.1)

PBS: 0.01 ml phosphate buffered saline
PHA: Phytohemagglutinin (Difco PHA-P), undiluted or diluted 1:10
LT-2 KCl: KCl extract or cultured LT-2 cells (1 μg or 10 g protein in 0.1 ml PBS)
LT-2 + mc: 10^4 mitomycin C-treated LT-2 cells in 0.1 ml PBS
LT-4 KCl: KCl extract of normal human fibroblasts (10 μg protein in 0.1 ml PBS)
C. TUMOR-HOST INTERACTION: IN VIVO

1. SKIN TESTS

Initially, the patient (M.L.) responded to only one of the five recall bacterial skin test antigens (dermatophytin-0). She was anergic to all other skin test antigens including PPD. No response was noted to DNCB. The patient received BCG intralesional immunotherapy (see below), and was again skin tested. At that time, she responded to PPD, but was now anergic to dermatophytin-0. Two months later, she was again re-tested, and she was then totally anergic to all recall skin test antigens including PPD despite the fact that she had received three BCG immunizations and was previously PPD skin test positive. Still no response was noted to DNCB. She remained totally anergic when again re-tested six weeks later. The patient was also anergic to skin testing with autologous KCl tumor extracts.

2. BCG

Following the first BCG intralesional immunization, the patient became hyperthermic for 2 days. No decrease in the size of the right inguinal lesion was noted, and by the time of the third intralesional injection the mass had doubled in size (3 months). No beneficial clinical response was noted to BCG injections, although an increase in PBL's occurred as noted above.
D. CELL CHARACTERIZATION AND TUMOR PROPERTIES

1. ELECTRON MICROSCOPY

As pointed out by Bernhard, the ultrastructural differences between normal and malignant cells are quantitative rather than qualitative. Well differentiated cancer cells generally resemble the tissue of origin and contain organelles characteristic of differentiation. There is no universal ultrastructural pattern characteristic of cancer (200). Electron micrographs of LT-2 revealed numerous pinocytotic vacuole and lysosomes with prominent tonofilaments. The presence of tonofilaments is characteristic of epithelial cells or undifferentiated tumor cells (201). No keratonyalin was noted. Mitochondria appeared normal in numbers and appearance. The nucleus showed large prominent nucleoli. No virus particles were noted.

2. KARYOTYPING

Karyotyping of 38 cells revealed marked aneuploid variation with chromosome numbers varying from 47 to 81 with no single modal number, although a tendency toward a triploid hypertriploid modal range was noted. Abnormal marker chromosomes and ring chromosomes were noted. Karyotyping four years later still revealed aneuploidy with
marked variations in chromosome numbers. (Table 3 and Figure 5).

3. GENERATION TIME

The doubling time was determined using the relationship \( \log N = \frac{0.301t}{t_D} + \log N_0 \) and was calculated to be 32 hours, during the initial log phase of growth from 27 to 72 hours in culture (Figure 6). Pirt has noted that the validity of such calculations are based on the assumption of constant exponential growth as noted with procaryotes and constant environment conditions (202). Fractions of non-growing cells resulting from cell differentiation, production of inhibitors, etc., would invalidate the assumption of exponential growth. Cell generation time calculated by time-lapse cinematography (see below) was highly varied with one multinucleated cell dividing twice in only 88.5 minutes (from one cell division to another) while other cells did not divide at all.

4. TIME-LAPSE CINEMATOGRAPHY

The doubling time of individual cells (cell cycle time) was calculated by observing the interval between two consecutive cell divisions and counting the number of frames during that interval and dividing by the number of frames per minute (2 frames per minute). The interval between
TABLE 3

FREQUENCY OF LT-2 CHROMOSOME DISTRIBUTION OF 38 CELLS

<table>
<thead>
<tr>
<th>Cells:</th>
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<td>Chromosomes:</td>
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<th>3</th>
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<tbody>
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<td>65</td>
<td>68</td>
<td>69</td>
<td>72</td>
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</tr>
</thead>
<tbody>
<tr>
<td>Chromosomes:</td>
<td>75</td>
<td>78</td>
<td>81</td>
</tr>
</tbody>
</table>
Figure 5. LT-2 karyotype showing 69 chromosomes and abnormal marker chromosome.
Figure 6. Doubling time of LT-2 in culture. Initial plating at 0.85 x $10^6$ cells per culture.
divisions was noted to vary considerably between cells with some cells dividing twice while other cells within the same colony did not divide at all.

Cell division did not necessarily appear to correlate with cell size. Some small cells were noted to divide while much larger cells with abundant cytoplasm did not divide. Also cell division did not necessarily occur with equal division of the cytoplasm. As noted above, the interval between two cell divisions of one multinucleated cell was only 88.5 minutes. Some other cells within the same colony did not divide at all during the period of observation - about 4 days. Considerable movement or membrane ruffling was noted by many cells. Individual cells were noted to move in and out of the photographic field, and one small colony consisting of about 10 cells was also noted to move as a group, demonstrating a total lack of cell to cell contact inhibition of cell movement-topoinhibition (Figure 7).

Cell to cell adherence was demonstrated in one film. As a cell moved away from a small colony, a long cytoplasmic stream attached to an adjoining cell was "left behind" pulling out the membrane of the stationary cell. When the cells finally separated, both cells rapidly recoiled in a fashion similar to a rubber band that had been stretched out and then released - suggesting rather firm cell to cell contact (Figure 8).
Figure 7. Time lapse cinematography showing movement of a whole colony. Cell a. moving away from colony.
Figure 8. Time lapse cinematography showing cell to cell adhesion with cell b. moving away from colony.
5. **SATURATION DENSITY**

AU-471 reached confluent growth with a maximum saturation density of $10.4 \times 10^5$ cells/cm$^2$ at 11 days in culture (average $8.68 \times 10^5$ cells/cm$^2$ with a range from 7.76 to $1.04 \times 10^5$ cells/cm$^2$ from day 9-14). LT-2 reached a maximum saturation density of $1.20 \times 10^5$ cells/cm$^2$ at 14 days in culture (average of $1.10$ cell/cm$^2$ with a range from 1.02 to $1.20 \times 10^5$ cells/cm$^2$ from day 10-16). WI-38 reached a maximum saturation density of $0.074 \times 10^5$ cells/cm$^2$ at day 29 in culture (average $0.0639 \times 10^5$ cells/cm$^2$ with a range from $0.0522$ to $0.074 \times 10^5$ cell/cm$^2$ from day 15 to 35). Even after 35 days, WI-38 cultures were not totally confluent.

AU-471 which grew with "heaped-up" colonies reached a maximum saturation density about 8.6 times higher than LT-2 and 140 times greater than WI-38. Saturation density for LT-2 was 16 times greater than WI-38. Both LT-2 and WI-38 grow as a strict monolayer without "heaped-up" cells as noted with AU-471, and both have a much lower saturation density (Table 4).
**TABLE 4**

SATURATION DENSITY OF LT-2, AU-471 AND WI-38 CELLS IN VITRO

<table>
<thead>
<tr>
<th>LT-2</th>
<th>Saturation Density</th>
<th>AU-471</th>
<th>Saturation Density</th>
<th>WI-38</th>
<th>Saturation Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time In Culture Days</td>
<td>No. cells/cm² x 10⁵</td>
<td>Time In Culture Days</td>
<td>No. cells/cm² x 10⁵</td>
<td>Time In Culture Days</td>
<td>No. cells/cm² x 10⁵</td>
</tr>
<tr>
<td>10</td>
<td>1.02</td>
<td>8</td>
<td>3.86</td>
<td>15</td>
<td>0.0662</td>
</tr>
<tr>
<td>11</td>
<td>1.09</td>
<td>9</td>
<td>7.76</td>
<td>17</td>
<td>0.0552</td>
</tr>
<tr>
<td>14</td>
<td>1.20</td>
<td>10</td>
<td>8.81</td>
<td>21</td>
<td>0.0721</td>
</tr>
<tr>
<td>15</td>
<td>1.17</td>
<td>11</td>
<td>10.4</td>
<td>24</td>
<td>0.0522</td>
</tr>
<tr>
<td>16</td>
<td>1.06</td>
<td>14</td>
<td>7.66</td>
<td>29</td>
<td>0.0741</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>5.22</td>
<td>32</td>
<td>0.0652</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>35</td>
<td>0.0622</td>
</tr>
</tbody>
</table>

* Average of three cultures
6. DENSITY DEPENDENT INHIBITION OF CELL DIVISION

Review of stained Leighton tube cover slips of LT-2 revealed mitotic figures both in the center of large colonies with cell contact on all sides of dividing cells and on the periphery of the colonies (Figure 9). AU-471 also demonstrate cell division throughout large colonies (Figure 10). WI-38 did not form discrete colonies. Instead, cells were noted to form anastomosing columns with individual cells and small groups spread over the slide. Fewer cell divisions were noted compared to LT-2 or AU-471 cultures and only rare cell divisions in the center of large colonies were noted by cells completely surrounded and in contact on all sides with other cells (Figure 11).

7. SURFACE ANTIGENS: ABH

Agglutination of LT-2 was noted using anti-A. Since the patient (M.L) was known to be blood type A, this normal cell marker has remained invariant in her tumor. No agglutination was noted with anti-Rho or anti-RhC (patient was Rh positive, but the Rho antigen is generally expressed only on red blood cells). No agglutination occurred with anti-B, anti-H substance or PBS. LT-2 agglutinated in the presence of normal rabbit serum. Heterophile positive human serum (from a patient with infectious mononucleosis) did not agglutinate LT-2, but did agglutinate SRBC's. Normal
Figure 9. Colonies of LT-2 demonstrating cell division (arrows) by cells in the center of a colony with cell contact on all sides.
Figure 10. Colonies of AU-471 demonstrating cell division (arrows) by cells in the center of a colony with cell contact on all sides.
Figure 11. WI-38 with cell division (arrows) by cells separated from other cells. Cell division was uncommon compared to LT-2 or AU-471 cultures. Only rare cell division was noted by a cell in the center of a large colony.
human control serum did not agglutinate SRBC's or LT-2. Infectious mononucleosis positive serum did not agglutinate AU-471 nor was a heterophile-like antigen noted since no agglutination with normal rabbit serum occurred (Table 5).

8. FETAL ANTIGENS: CEA, hCG

Spent media from cultured LT-2 cells concentrated 14.5 times as described in the section on Materials and Methods was found to contain 21 ng./ml CEA per milliliter at passage No. 3, corresponding to 0.877 ng. per 10⁶ cells per day. Fetal calf serum used in cell cultivation was also found to contain CEA immunoactivity corresponding to 6.15 ±0.83 ng. CEA per milliliter fetal calf serum or 0.922 ±0.124 ng. CEA per milliliter complete medium or 13.4 ng. CEA per milliliter of 14.5 times concentrated medium. Thus, in vitro production of CEA by LT-2 was significantly elevated above levels expected in concentrated complete media with 15 percent fetal calf serum (P < 0.001). CEA activity attributable to LT-2 production decreased with continued culture and was undetectable at passage No. 11. Spent media was also found to contain 179 I.U. β-hCG per liter, corresponding to an output of approximately 20 mI.U. (4 ng.) per 10⁶ cells per day. Immunoreactive hCG (cross-reactive with LH) in this concentrated medium was found to be only 9 I.U. per liter, at the lower level of sensitivity of
TABLE 5

DEMONSTRATION OF HETEROPHILE ANTIGEN ON LT-2
AND DIFFERENTIATION FROM INFECTIOUS MONONUCLEOSUS HETEROPHILE ANTIGEN

<table>
<thead>
<tr>
<th>Sheep RBC</th>
<th>LT-2</th>
<th>AU-471</th>
<th>Human Blood Type A RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>*NRS</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>**Im+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>***Im-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*NRS = Normal Rabbit Serum
**Im+ = Infectious Mononucleosus Positive Antisera
***Im- = Infectious Mononucleosus Negative Antisera
the radioimmunossay, and did not increase when the medium was concentrated (immunoreactive β-hCG did increase in the medium with concentration), indicating preferential secretion of the beta subunit of hCG by LT-2 cells.

9. SERUM DEPENDENCE

LT-2 was plated at 5 x 10⁴ cells per well in 10%, 1%, 0.1%, .01% and 0 serum and was harvested at 4 days, resulting in recovery of 4 x 10⁴ cells from 10% serum, 2.3 x 10⁴ cells from 1% and progressively fewer cells in less serum, but some cells survived even when cultured in 0 serum (1.7 x 10⁴ cells at 4 days). Plating LT-2 in 15% serum and changing to lower serum concentrations as noted above after 24 hours, did not consistently produce increased growth.

In a repeat experiment, LT-2 was plated at higher concentrations (1.5 x 10⁵ cells per well) and harvested later (6 days). High yields were noted with 10% and 1% serum (2.5 and 3.6 x 10⁵ cells per well), indicating growth in 10% and 1% serum, but lower cell yields were noted with 0.1, 0.01 and 0 serum (1.2, 0.8, 1.2 x 10⁵ cells per well) indicating cell survival in limited serum, but not growth. These data indicate serum growth dependence by LT-2. In contrast, AU-471 demonstrated serum independence with growth at both 10% and 0 serum, but much greater growth at the higher serum levels (1.5 x 10⁵ cells per well plated with
yields of 20.5, 12.2, 5.1, 2.6, 2.6 in 10%, 1%, 0.1%, 0.01% and 0 serum, respectively). Plating in 15% serum for 24 hours prior to feeding with various serum concentrations resulted in much better growth in all cultures compared to cultures initially plated in lower serum concentrations. These data indicate serum independence but also response to serum, suggesting retention of receptors for growth stimulating factors in serum by AU-471. Interestingly, WI-38, a "normal diploid" cell line also demonstrated serum independence when harvested at 4 days but with better growth when plated in higher serum concentration.
Preliminary studies revealed that LT-2 did not clone in soft agar. This lack of anchorage independent growth by LT-2 was of particular interest since most tumors in tissue culture clone in soft agar (Figure 12). In fact, soft agar anchorage independent growth is currently the most reliable assay of in vitro malignancy. Although Marshall has reported a human malignant bladder carcinoma that did not clone in soft agar, but was tumorogenic in the nude mouse (203). Smith and Temin had demonstrated that serum independence by tumors results from production by the tumor of growth stimulating factors that can replace serum (204). The possibility that a soluble inhibitor or stimulant was responsible for anchorage dependent or independent growth was considered. Experiments were done to determine whether anchorage independence was a function of a soluble product produced by cells capable of growth in soft agar and/or whether non-cloning cells produced a soluble inhibitor.

Two co-cultivation experiments using untreated LT-2 cells cultured with AU-471 showed an increase in soft agar plating efficiency of AU-471 when colonies were scored at about two weeks. One experiment showed a dose response
Figure 12. Clones of AU-471 growing in soft agar.
increase (1:10>1:1) in plating efficiency (control mean 55.0 ±10.87; 77.3 ±11.69 co-cultured with 2 x 10³ LT-2 giving an LT-2:AU-471 ratio of 1:1; and 129.2 ±26.16 with 2 x 10⁴ LT-2 giving an LT-2:AU-471 ratio of 1:10) (P<0.001). After 24 days, no significant increase in cloning efficiency was noted at the lower (1:1) feeder to response cell ratios, but a significant (P<0.001) response was still noted at the higher (1:10) cell ratios (Table 6.) This "loss" of feeder effect by 24 days in the 1:1 co-cultivation may have resulted from the much greater increase in cloning efficiencies occurring in control plates (3.32 x increase) from day 14 to day 24 compared to test plates (2.52 x for 1:1 and 2.22 x for 1:10). Similar results were seen in other experiments using AU-471 co-cultivated with mitomycin AU-471 (see below).

Another experiment used low and high cell ratios of responder and feeder cells. (1:5 and 1:50 AU-471:LT-2 ratios with 2 x 10³ "responder" AU-471 as above, but also with 2 x 10⁴ responder cells). Significant (P<.001) increases in cloning efficiency were noted when cultures were scored at both 15 days and 26 days, and at both low and high AU-471:LT-2 ratios and with low and high numbers of responder AU-471 cells (Table 7). Plating efficiency of control AU-471 was greater with 2 x 10⁴ AU-471 (4.97%) compared to 2 x 10³ AU-471 (2.36%) suggesting a self-feeder effect.
### TABLE 6

**ENHANCEMENT OF AU-471\(^a\) GROWTH IN SOFT AGAR BY LT-2**

<table>
<thead>
<tr>
<th>LT-2(^d) No. x 10(^3)</th>
<th>No. of Colonies (Mean ± S.D.)</th>
<th>Increase In Cloning Efficiency:</th>
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<tbody>
<tr>
<td></td>
<td>Day 14 PC</td>
<td>Day 21 PC</td>
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<tr>
<td>0</td>
<td>55 ± 10</td>
<td>183 ± 8</td>
</tr>
<tr>
<td>2</td>
<td>77 ± 11 .001</td>
<td>195 ± 23 N.S.</td>
</tr>
<tr>
<td>20</td>
<td>129 ± 26(^b) .001</td>
<td>295 ± 30(^b) .001</td>
</tr>
</tbody>
</table>

\(^a\) = 2 \times 10^3\) AU-471, 75% viable

\(^b\) = Significant increased number of colonies compared to 2 \times 10^3 LT-2 cultures, \(P < .001\)

\(^c\) = P values compare significance to cultures with 0 LT-2 cells

\(^d\) = No growth in LT-2 soft agar control cultures
## TABLE 7

**ENHANCEMENT OF AU-471 GROWTH IN SOFT AGAR BY BOTH HIGH (1:50) AND LOW (1:5) AU-471:LT-2 CELL RATIOS**

<table>
<thead>
<tr>
<th>AU-471 No. x 10^3</th>
<th>LT-2a No. x 10^3</th>
<th>No. of Colonies (Mean ± S.D.)</th>
<th>Day 15</th>
<th>pC</th>
<th>Day 26</th>
<th>pC</th>
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<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>47 ± 18</td>
<td>-</td>
<td></td>
<td>129 ± 11</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>154 ± 34</td>
<td>.001</td>
<td></td>
<td>256 ± 30</td>
<td>.001</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>995 ± 243</td>
<td>-</td>
<td></td>
<td>1700 ± 428</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>1000</td>
<td>Sig. 4120 ± 868</td>
<td>.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a = No growth in LT-2 soft agar control cultures

*b = Plating efficiency calculated after counting only a portion of the total plate

*c = P values compare 100 x 10^3 LT-2 cultures to 0 LT-2 with comparable numbers of Au-471

Sig.= Obviously significant, but could not be calculated
These experiments demonstrated stimulation of cloning efficiency or growth of an anchorage independent cell by an anchorage dependent cell rather than inhibition which was expected. The response is dose dependent and more evident when cultures are scored early (2 weeks) rather than late (3-4 weeks).

2. **SOLUBLE STIMULANTS & INHIBITORS: CO-CULTIVATION, MITOMYCIN C TREATED LT-2:AU-471**

Three additional experiments used increasing concentrations of mitomycin C "feeder" LT-2 cells. Mitomycin "feeder" cells were used to insure that the increased cloning noted by AU-471 in previous experiments was not the result of clone formation by LT-2. One experiment confirmed a significant (P< .05 - .01) "feeder effect" with cloning efficiency increasing directly with increasing numbers of feeder cells at day 18 (Table 8). No difference between test and control cultures was noted by day 24 except for one set of cultures with $1 \times 10^4$ feeder cells (P< 0.05). In another experiment no "feeder effect" was noted at either 15 or 22 days, but the cloning efficiency of control plates was 25% by 15 days and 64% by 22 days. This cloning efficiency of controls in this experiment was much higher than noted in other experiments in which "feeder effect" was clearly demonstrated (Table 9).
**TABLE 8**

**DOSE DEPENDENT ENHANCEMENT OF AU-471 CLONING EFFICIENCY WITH VARIATIONS IN MITOMYCIN C TREATED LT-2 CELLS**

<table>
<thead>
<tr>
<th>LT-2 No. x 10⁴</th>
<th>No. of Colonies Mean ± S.D.</th>
<th>Day 18</th>
<th>Day 14</th>
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<tr>
<td>0</td>
<td>51 ± 6</td>
<td>-</td>
<td>74 ± 15</td>
</tr>
<tr>
<td>1</td>
<td>49 ± 7</td>
<td>N.S.</td>
<td>62 ± 9</td>
</tr>
<tr>
<td>2</td>
<td>59 ± 4</td>
<td>.05</td>
<td>61 ± 6</td>
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<tr>
<td>5</td>
<td>63 ± 9</td>
<td>.05</td>
<td>76 ± 16</td>
</tr>
<tr>
<td>10</td>
<td>77 ± 9</td>
<td>.01</td>
<td>92 ± 6</td>
</tr>
<tr>
<td>50</td>
<td>74 ± 6</td>
<td>.01</td>
<td>83 ± 5</td>
</tr>
</tbody>
</table>

a = 500 AU-471 per culture, 5 cultures per group

b = No growth of LT-2 soft agar control cultures

c = P values compare significance to cultures with 0 LT-2 cells
TABLE 9

LACK OF LT-2 FEEDER EFFECT ON AU-471 CLONING EFFICIENCY
IN SOFT AGAR CULTURES WITH HIGH SPONTANEOUS CLONING EFFICIENCYa

<table>
<thead>
<tr>
<th>LT-2c</th>
<th>No. x 10³</th>
<th>No. Colonies (Mean ± S.D.)b</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 15</td>
<td>Day 22</td>
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<tr>
<td>0</td>
<td></td>
<td>505 ± 41</td>
<td>1239 ± 214</td>
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<td>1</td>
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<td>506 ± 49</td>
<td>1267 ± 295</td>
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<td>2</td>
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<td>524 ± 28</td>
<td>1212 ± 102</td>
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<tr>
<td>5</td>
<td></td>
<td>498 ± 25</td>
<td>1226 ± 197</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>550 ± 30</td>
<td>1232 ± 163</td>
<td></td>
</tr>
</tbody>
</table>

a = No significant differences noted between co-cultured and control cultures cloning efficiency
b = Cloning efficiency 25%
c = No growth in LT-2 soft agar control cultures
The last experiment showed no "feeder effect" (Table 10). In fact, inhibition of soft agar cloning efficiency was noted with 5 and \(10 \times 10^5\) feeder LT-2 cells. This experiment had a very low viability of mitomycin C treated LT-2 feeder cells (35%) which may have accounted for the inhibition (Table 10). These experiments using mitomycin C treated LT-2 demonstrated "feeder effect", but generally only when colonies were scored early (usually 2 weeks) in the experiment and when the cloning efficiency of control cultures was low (generally less than 20%). The last experiment suggested that large numbers of dead mitomycin C treated feeder cells might decrease cloning efficiency.

3. **SOLUBLE STIMULANTS & INHIBITORS: CO-CULTIVATION, AU-471 FEEDING AU-471**

Experiments were done to determine whether AU-471 could stimulate its own cloning efficiency in soft agar using mitomycin AU-471 "feeder cells". In two co-cultivation experiments using mitomycin C treated AU-471 cells as feeder cells and untreated AU-471 responder cells, a significant increase (\(P<.05 - 0.01\)) in AU-471 soft agar cloning efficiency was noted when cultures were scored at about two weeks, and the efficiency increased directly with the number of feeder cells (Table 11 and Table 12). Evaluation of cloning efficiency at 4 weeks was more variable with one ex-
### TABLE 10

**INHIBITORY EFFECT OF HIGH NUMBERS OF CO-CULTIVATED LT-2 CELLS ON THE CLONING EFFICIENCY OF AU-471**

<table>
<thead>
<tr>
<th>LT-2&lt;sup&gt;b&lt;/sup&gt; No. x 10&lt;sup&gt;3&lt;/sup&gt;</th>
<th>No. of Colonies (Mean ± S.D.)</th>
<th>Day 17</th>
<th>Day 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>297&lt;sup&gt;a&lt;/sup&gt; ± 39</td>
<td></td>
<td>473 ± 48</td>
</tr>
<tr>
<td>10</td>
<td>315 ± 23</td>
<td>N.S.</td>
<td>494 ± 59</td>
</tr>
<tr>
<td>20</td>
<td>276 ± 37</td>
<td>N.S.</td>
<td>427 ± 45</td>
</tr>
<tr>
<td>50</td>
<td>242 ± 34</td>
<td>.05</td>
<td>360 ± 16</td>
</tr>
<tr>
<td>100</td>
<td>80 ± 29</td>
<td>.005</td>
<td>110 ± 26</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Cloning efficiency 15%

<sup>b</sup> = No growth in LT-2 soft agar control cultures

<sup>c</sup> = P values compare significance to cultures with 0 LT-2 cells
**TABLE 11**

DOSE RESPONSE INCREASED CLONING EFFICIENCY OF AU-471 CO-CULTURED WITH MITOMYCIN C TREATED AU-471a

<table>
<thead>
<tr>
<th>AU-471 No. Cells x 10⁳</th>
<th>No. of Colonies (Mean ± S.D.)</th>
<th>Day 14</th>
<th>p&lt;&lt;.01</th>
<th>Day 23</th>
<th>p&lt;.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>46 ± 6</td>
<td>&lt;.01</td>
<td>128 ± 12</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>72 ± 8</td>
<td>&lt;.01</td>
<td>130 ± 13</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>88 ± 9</td>
<td>&lt;.01</td>
<td>143 ± 17</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>92 ± 3</td>
<td>&lt;.01</td>
<td>182 ± 10</td>
<td>&lt;.01</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>87 ± 7</td>
<td>&lt;.01</td>
<td>210 ± 40</td>
<td>&lt;.01</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>104 ± 12</td>
<td>&lt;.01</td>
<td>181 ± 20</td>
<td>&lt;.01</td>
<td></td>
</tr>
</tbody>
</table>

a = 500 viable AU-471 per culture, 89% viable

b = significant increased number of colonies compared to 0 LT-2, P<.01
<table>
<thead>
<tr>
<th>AU-471 No. Cells x 10³</th>
<th>No. of Colonies Day 16</th>
<th>(Mean ± S.D.) Day 16 pC</th>
<th>No. of Colonies Day 28</th>
<th>(Mean ± S.D.) Day 28 pC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>339 ± 51</td>
<td>-</td>
<td>612 ± 44</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>398 ± 58</td>
<td>N.S.</td>
<td>608 ± 61</td>
<td>N.S.</td>
</tr>
<tr>
<td>20</td>
<td>430 ± 37</td>
<td>.01</td>
<td>592 ± 41</td>
<td>N.S.</td>
</tr>
<tr>
<td>50</td>
<td>474 ± 105</td>
<td>.05</td>
<td>622 ± 135</td>
<td>N.S.</td>
</tr>
<tr>
<td>100</td>
<td>564 ± 57</td>
<td>.01</td>
<td>660 ± 53</td>
<td>N.S.</td>
</tr>
<tr>
<td>500</td>
<td>624 ± 37</td>
<td>.01</td>
<td>668 ± 49</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

a = 2000 viable Au-471 per culture, 87% viable

b = 72% viable, no colony formation in control mitomycin C treated cultures

c = P values compare significance to cultures with 0 mitomycin C treated AU-471 cultures
periment showing no significant difference between feeder cultures and controls, and the other experiment showing significant differences \((P>0.01)\) only with higher numbers of feeder cells \((10, 50 \text{ and } 100 \times 10^3 \text{ feeder cells})\), but not at lower feeder cell proportions \((2 \text{ and } 5 \times 10^3 \text{ feeder cells})\). Similar results were noted (see above) in some LT-2:AU-471 feeder experiments suggesting that the "feeder effect" increases cell cloning efficiency early in experiments resulting in more recognizable (scorable) clones, but when cultures are scored later (3-4 weeks) non-feeder cultures had a greater proportional increase in cloning efficiency often eliminating any noticeable "feeder effect" on cloning efficiency. Control cultures (Table 12) increased by 1.8 x while "feeder cultures" demonstrated a small increase in the number of scorable colonies \((1.5x, 1.1x, 1.4x, 1.2x, 1.1)\).

4. **SOLUBLE STIMULANTS & INHIBITORS: CO-CULTIVATION, WI-38:AU-471**

Experiments were done using a "normal" near diploid cell strain that does not clone in soft agar as "feeder" cells to determine if cloning stimulating efficiency was specific for tumor lines or was also produced by "normal cells". Co-cultivation of WI-38 with AU-471 also demonstrated a "feeder effect" with significantly increased colony formation at both 14 and 22 days with AU-471; WI-38
cell ratios of 1:10 and 1:50 (P<.0005) (Table 13). No colonies were noted with 2 or 100 x 10^3 WI-38 cells plated alone in soft agar (controls). Cloning efficiencies of control cultures were 1.6% at 14 days and 6.39% at 22 days indicating a 3.97 x increase while feeder cultures had only a 3.50 x and 1.76 x increase.

A larger increase in cloning efficiency occurred in the control cultures between day 14 and 22 than in "feeder" cultures with the smallest percent increase occurring in the group with the highest number of feeder cells. Similar results were seen in other experiments and suggest "self feeding" with increased time in culture of control plates with resulting stimulation of growth of controls later in experiments (after 2 weeks). Similar results were noted in previous experiments (see above).

5. SOLUBLE STIMULANTS & INHIBITORS: CO-CULTIVATION, LT-2 WITH MITOMYCIN C TREATED AU-471

No significant stimulation of soft agar growth by soluble products from AU-471 was noted in co-cultivation experiments using mitomycin C treated AU-471 to "feed" untreated LT-2 (Table 14).
TABLE 13

ENHANCEMENT OF AU-471<sup>a</sup> CLONING EFFICIENCY BY CO-CULTIVATION WITH A "NORMAL DIPLOID" CELL LINE WI-38

<table>
<thead>
<tr>
<th>WI-38 No. x 10&lt;sup&gt;4&lt;/sup&gt;</th>
<th>No. of Colonies (Mean ± S.D.)</th>
<th>INCREASE IN CLONING EFFICIENCY:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 14</td>
<td>Day 21</td>
</tr>
<tr>
<td></td>
<td>(p&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>(p&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
<tr>
<td>0</td>
<td>32 ± 9</td>
<td>128 ± 30</td>
</tr>
<tr>
<td>2</td>
<td>68 ± 10 (.0005)</td>
<td>239 ± 20 (.0005)</td>
</tr>
<tr>
<td>10</td>
<td>230 ± 20 (.0005)</td>
<td>405 ± 14 (.0005)</td>
</tr>
</tbody>
</table>

<sup>a</sup> = 2 x 10<sup>3</sup> viable AU-471, 84% viable

<sup>b</sup> = P value compares significance with cultures with 0 WI-38 cells
**TABLE 14**

LACK OF ANCHORAGE INDEPENDENT GROWTH STIMULATION OF CO-CULTIVATION WITH MITOMYCIN C TREATMENT

<table>
<thead>
<tr>
<th>LT-2</th>
<th>AU-471</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. $x 10^3$</td>
<td>No. $x 10^3$</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Results: No colony formation in all cultures on day 14 or day 21
Since mitomycin was being used to treat "feeder" cells, an experiment was designed to evaluate mitomycin C in intact and lysed cells and when feeder and responder cells were in "close contact" following centrifugation and incubation.

Control AU-471 (no co-cultivation with LT-2) had a cloning efficiency in soft agar of 1.6% at 20 days (Table 15). Feeder cultures with 1:1 untreated LT-2:AU-471 showed no significant stimulation or suppression of cloning. Feeder cultures with $10^5$ untreated LT-2 (1:50 ratio) resulted in total inhibition of AU-471 anchorage independent growth. Gross speckling of culture plates was noted as the agar dried which microscopically represented lysed and dead cells, cell debris and a few 2 and 3 cell clusters (probably of LT-2 origin), but no colonies. Similarly, centrifugation of LT-2:AU-471 with incubation for 60 min. at 37°C (cell contact) resulted in no significant change at 1:1 ratios, but total inhibition of AU-471 cloning at 1:50 ($10^5$ LT-2) ratios. Significant suppression was noted with $10^5$, but not $10^3$ lysed LT-2 cells at 20 days.

Comparable mitomycin C treated LT-2 cells showed significant depression ($P<0.05$) of AU-471 co-cultured with $10^5$ LT-2, but no other demonstrated suppression or stimulation of colony formation by day 20.
TABLE 15

EVALUATION OF LT-2:AU-471 CELL CONTACT CELL LYSIS AND MITOMYCIN C ON THE CLONING EFFICIENCY OF AU-471\textsuperscript{d}

Experiment No. 1

<table>
<thead>
<tr>
<th>No. of LT-2</th>
<th>Description</th>
<th>Mitomycin C Treatment of LT-2</th>
<th>Colony Count (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mc-</td>
<td>mc+</td>
</tr>
<tr>
<td>0</td>
<td>Control</td>
<td>32 ± 6</td>
<td>164 ± 32</td>
</tr>
<tr>
<td>10\textsuperscript{3}</td>
<td>Co-Culture</td>
<td>30 ± 4</td>
<td>143 ± 32</td>
</tr>
<tr>
<td></td>
<td>Cell Contact</td>
<td>30 ± 5</td>
<td>158 ± 11</td>
</tr>
<tr>
<td></td>
<td>Lysed</td>
<td>23 ± 6</td>
<td>128 ± 6\textsuperscript{a}</td>
</tr>
<tr>
<td>10\textsuperscript{5}</td>
<td>Co-Culture</td>
<td>0\textsuperscript{a}</td>
<td>0\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Cell Contact</td>
<td>0\textsuperscript{a}</td>
<td>0\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>Lysed</td>
<td>11 ± 4\textsuperscript{a}</td>
<td>8 ± 1\textsuperscript{a,c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} = Significant suppression of cloning efficiency of AU-471 compared to control cultures, \textit{P} \textless .01

\textsuperscript{b} = Significant difference between groups labeled "b" \textit{P} \textless .01

\textsuperscript{c} = Significant difference between groups labeled "c", \textit{P} \textless .005

\textsuperscript{d} = 2 x 10\textsuperscript{3} viable AU-471
Mitomycin C treated and lysed LT-2 showed significant suppression with $10^3$ but not $10^3$ LT-2 at 30 days. Findings were similar at 30 days with suppression of AU-471 soft agar cloning efficiency with $10^3$ co-cultured, and cell contact LT-2, but in addition, $10^5$ co-cultured LT-2 cells showed suppression ($P<0.001$) at 30 days. No suppression was noted with $10^5$ cell contact or lysed LT-2 groups at 30 days. Significant suppression was noted in all mitomycin C treated groups except in $10^5$ lysed and cell contact groups at 30 days.

Comparison between mitomycin C and non-treated groups revealed significant suppression between the $10^5$ lysed groups at 30 days, and between $10^5$ co-cultured and cell contact groups at 30 days. This experiment indicates suppression of AU-471 soft agar cloning with high numbers of untreated and co-cultivated LT-2 cells at both 20 and 30 days, but not with low numbers of untreated LT-2 cells. Untreated lysed cells also inhibited AU-471 cloning. No feeder effect was noted in this experiment with either mitomycin C or untreated cells.

A repeat experiment (Table 16) identical to the above was done except $1 \times 10^4$ and $1 \times 10^5$ mitomycin C treated and untreated LT-2 were used instead of $1 \times 10^3$ and $1 \times 10^5$ and colonies were scored at 2 and 4 weeks. The increase to $10^4$ instead of $10^3$ LT-2 was used to maximize the possibility of
TABLE 16

EVALUATION OF LT-2:AU-471 CELL CONTACT, CELL LYSIS AND MITOMYCIN C
ON THE CLONING EFFICIENCY OF AU-471

Experiment No. 2

<table>
<thead>
<tr>
<th>Description</th>
<th>Day 14</th>
<th>Day 27</th>
<th>Day 15</th>
<th>Day 27</th>
<th>Mitomycin C Treatment of LT-2 Colony Count (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 ± 3</td>
<td>39 ± 8</td>
<td></td>
<td></td>
<td>mc-</td>
</tr>
<tr>
<td>Co-Culture</td>
<td>31 ± 3b,c,g 302 ±64b</td>
<td>7 ± 2c</td>
<td>33 ± 16</td>
<td></td>
<td>mc+</td>
</tr>
<tr>
<td>Cell Contact</td>
<td>29 ± 3b,d,h 254 ±15b,e,j 11 ± 3d 60 ± 23e</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysed</td>
<td>6 ± 1g,h 60 ±18j 5 ± 2 50 ± 24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co-Culture</td>
<td>0a</td>
<td>0a</td>
<td>14 ± 3</td>
<td>98 ± 9a</td>
<td>mc-</td>
</tr>
<tr>
<td>Cell Contact</td>
<td>0a</td>
<td>0a</td>
<td>33 ±10</td>
<td>165 ± 21b,f</td>
<td>mc+</td>
</tr>
<tr>
<td>Lysed</td>
<td>8 ± 3</td>
<td>41 ± 7</td>
<td>13 ± 3</td>
<td>71 ± 9b,f</td>
<td>mc-</td>
</tr>
</tbody>
</table>

a = Total suppression of AU-471 cloning efficiency compared to control
b = Significant enhancement of AU-471 cloning efficiency compared to controls, P<.01
c = c,d,e,f,g,h,j: Significant difference between groups with the same letter P<.01
"feeder effect", since other experiments generally noted a greater "feeder" when larger numbers of feeder cells (up to $1 \times 10^5$) were used. In this experiment AU-471 cloning efficiency was 0.49% at 14 days and 1.9% at 27 days (much lower than generally noted). A marked feeder effect was noted in the $10^4$ non-mitomycin C treated co-cultured and contact groups ($P<0.002$), and a total suppressive effect with $10^5$ co-cultured or cell contact groups at both 14 and 27 day. Lysed cells produced no suppressive effect in any group at day 14 or 27. No significant effects, suppressive or stimulatory were noted with mitomycin C treated cells at day 14. The mitomycin C treated groups showed "feeder effect" with $10^5$ LT-2 in all three groups - co-cultured, contact and lysed at 27 days, but no significant difference in all three groups with only $10^4$ LT-2 at 27 days. Comparisons between mitomycin C and untreated and unlysed groups revealed stimulation by non-mitomycin C treated LT-2 compared to mitomycin C treatment with $10^4$ LT-2 at both 14 and 27 days, and marked suppression with $10^5$ non-mitomycin C treated LT-2 at both 14 and 27 days. No difference between mitomycin C treated and non-treated lysed cells was noted.

These experiments indicate a complex series of growth stimulation and inhibition that is dependent on both feeder cell number, duration of cells in culture and treatment with mitomycin C. In both experiments $10^5$ untreated non-
lysed LT-2 suppressed cloning of AU-471. No effect was noted with $10^3$ LT-2 and a feeder effect (increase in cloning efficiency) was noted with $10^4$ LT-2. Similar results have been suggested in other experiments. Low numbers of LT-2 cells generally have no effect "feeder" or inhibitory, but stimulation occurs with higher numbers of cells and finally inhibition occurs with large numbers of feeder cells. Suppression with large numbers of cells might result from competition for nutrients, direct toxic effect was suggested in the first experiment using untreated lysed LT-2 cells, and was not noted with $10^5$ mitomycin C lysed cells (Table 15). Direct toxic effect was also not confirmed in the second experiment with either untreated or mitomycin C treated lysed cells. Nutrient competition might explain the marked suppression in cloning with $10^5$ untreated LT-2 cells in both experiments, but this "competition" is eliminated by mitomycin C treatment.

7. CONDITIONED MEDIA

The effect of conditioned media rather than intact cells or lysed cells was evaluated. A cloning efficiency of 3.8% (32 colonies) was noted at 17 days and a final efficiency of 5.9% (59 colonies) by 28 days (Table 17). No increase was noted in cloning efficiency with 15 or 30% conditioned or spent media (SM) from AU-471, but a significant
TABLE 17

DOSE DEPENDENT TRANSFER OF INCREASED CLONING EFFICIENCY BY CONDITIONED MEDIA FROM LT-2 AND M.S.a

<table>
<thead>
<tr>
<th>Source of Conditioned Media</th>
<th>Percent Conditioned Media</th>
<th>Colony Count (Mean ± S.D.) Day 17</th>
<th>P</th>
<th>Colony Count (Mean ± S.D.) Day 28</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>32 ± 10</td>
<td>.</td>
<td>59 ± 7</td>
<td>.</td>
</tr>
<tr>
<td>AU-471</td>
<td>30</td>
<td>31 ± 6</td>
<td>N.S.</td>
<td>55 ± 12</td>
<td>N.S.</td>
</tr>
<tr>
<td>AU-471</td>
<td>15</td>
<td>26 ± 4</td>
<td>N.S.</td>
<td>53 ± 9</td>
<td>N.S.</td>
</tr>
<tr>
<td>LT-2</td>
<td>30</td>
<td>74 ± 11</td>
<td>.001</td>
<td>101 ± 14</td>
<td>.001</td>
</tr>
<tr>
<td>LT-2</td>
<td>15</td>
<td>51 ± 11</td>
<td>.02</td>
<td>71 ± 15</td>
<td>N.S.</td>
</tr>
<tr>
<td>M.S.</td>
<td>30</td>
<td>81 ± 14</td>
<td>.001</td>
<td>77 ± 47</td>
<td>N.S.</td>
</tr>
<tr>
<td>M.S.</td>
<td>15</td>
<td>70 ± 8</td>
<td>.001</td>
<td>100 ± 18</td>
<td>.005</td>
</tr>
</tbody>
</table>

a = Good growth and viability of AU-471 control cultures on the bottom of culture plates with 30% conditioned media from all three cell lines and overlaid with soft agar...
increase (P<0.001) was noted with 30% LT-2 SM at both 17 and 28 days. A significant increase was also noted with 15% LT-2 SM (P<0.02) at 17 days, but not by 28 days. A significant increase (P<.001) was also noted with both 15 and 30% SM from M.S. at 17 days, but only with the 15% SM by day 28. Good growth and colony formation was noted in all preplated controls showing cell viability and growth capabilities or a substratum.

This experiment indicates that "feeder effect" can be obtained with spent media from LT-2 or M.S. Feeder effect was more apparent when cultures were scored early rather than later as noted in co-cultivation experiments, and a dose response increase was noted with LT-2 conditioned media at both day 17 and day 28. No inhibitory effect was noted.

8. SUMMARY OF AGAR FEEDER EXPERIMENTS

Stimulation of anchorage independent soft agar growth in co-cultivation experiments was noted with a variety of cell types both benign and malignant - AU-471, LT-2, WI-38 and M.S. Co-cultivation stimulation could generally be noted by two weeks, but results by 4 weeks were more varied suggesting that the "feeder effect" stimulated growth early, but that un-fed cultures eventually caught up, perhaps by conditioning their own media or production of growth stimulant factors. Control cultures generally demon-
strated a greater proportional increase in cloning efficiency between early (2 weeks) and late (3-4 weeks) readings. "Feeder effect" was not noted when control culture showed high cloning efficiencies (>23%) by two weeks. Effects of very low (2000 cells) numbers of co-cultivated cells showed no "feeder effect". High concentrations of LT-2 cells sometimes produced decreased cloning efficiency. Feeder effect was most consistently noted with 10,000-50,000 feeder cells. Mitomycin C treated and lysed LT-2 cells were generally not inhibitory, but high numbers of untreated lysed LT-2 cells were inhibitory. Mitomycin C treated LT-2 cells were less effective as feeders and generally did not suppress.

9. **LT-2 CELL PROLIFERATION IN SOFT AGAR AND THE EFFECTS OF CYTOCHALASIN B**

Four experiments were done to evaluate the number of cells in soft agar versus time in culture and the effects of cytochalasin B (an inhibitor of all movement). The period of observation was generally two to three weeks. Counts of the numbers of cells per 150x microscopic field for each of five fields in (generally) five plates were recorded on day one of each experiment and repeat counts were recorded at approximately weekly intervals. The initial experiment compared untreated LT-2 cells with mitomycin C
treated LT-2 cells (Table 18). A significant increase in the number of cells per field (16.5 on day one to 26.1 by day 23 was noted (P<0.0001), but no increase in mitomycin C treated LT-2 controls.

A preliminary experiment suggested cytochalasin B treated LT-2 cells also proliferated in soft agar. Microcolonies consisting of aggregates of 8 - 12 cells were also noted, but cannot be attributed to the effect of cytochalasin B since similar microcolonies were noted in control untreated LT-2 plates and had on occasion been noted in other LT-2 soft agar experiments. Another experiment confirmed LT-2 proliferation (P<0.0001) in the presence of cytochalasin B, but no colony formation was noted (Table 19). No proliferation of untreated LT-2 was noted. Pre-plated control cells were noted to be healthy and proliferating on day 1 and day 14.

A final experiment, compared untreated, mitomycin C treated, and cytochalasin B treated LT-2, and showed a significant increase in the number of cells per Hpf between day 1 and day 8 or 18 in untreated and cytochalasin B treated LT-2 cells (P<0.0001), but no increase in the number of mitomycin C treated (control) cells (Table 20). The increase in cell number occurred early in the experiment (between day 1 and day 8) since no further increase was noted between day 8 and day 18.
TABLE 18

TIME-INDEPENDENT INCREASE IN THE NUMBER OF DISCRETE LT-2 CELLS
IN SOFT AGAR CULTURES WITHOUT COLONY FORMATION

<table>
<thead>
<tr>
<th>LT-2 Treatment</th>
<th>Cell Counts&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 8</td>
</tr>
<tr>
<td>0</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td>100 µg + mc/ml</td>
<td>19</td>
<td>19</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Mean count of number of cells per 5 high power microscopic fields from 5 plates (25 values)

<sup>b</sup> = Analysis of variance by Waller-Duncan K-ratio T test
TABLE 19

EFFECT OF CYTOCHALASIN B ON THE GROWTH OF LT-2 CELLS IN SOFT AGAR

<table>
<thead>
<tr>
<th>Treatment of LT-2</th>
<th>Cell Counts&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 7</td>
<td>Day 16</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 μg CB/ml</td>
<td>20</td>
<td>26</td>
<td>30</td>
<td>.0001</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 μg CB/ml</td>
<td>56</td>
<td>70</td>
<td>89</td>
<td>.0001</td>
</tr>
<tr>
<td>5 μg CB/ml</td>
<td>50</td>
<td>75</td>
<td>91</td>
<td>.0001</td>
</tr>
<tr>
<td>0</td>
<td>54</td>
<td>50</td>
<td>51</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Mean count of number of cells per 5 high powered microscopic field from 5 plates (25 values)

<sup>b</sup> = Analysis of variance by Waller-Duncan K-ratio T test

CB = Cytochalasin B

N.S. = Not significant
TABLE 20

EFFECT OF CYTOCHALASIN B AND MITOMYCIN C ON THE GROWTH OF

LT-2 CELLS IN SOFT AGAR

<table>
<thead>
<tr>
<th>Treatment of LT-2</th>
<th>Day 1</th>
<th>Day 8</th>
<th>Day 18</th>
<th>p&lt;sub&gt;b&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>38</td>
<td>61</td>
<td>55</td>
<td>.0001</td>
</tr>
<tr>
<td>1 μg CB/ml</td>
<td>44</td>
<td>64</td>
<td>60</td>
<td>.0001</td>
</tr>
<tr>
<td>100 μg + mc/ml</td>
<td>44</td>
<td>44</td>
<td>43</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Mean count of number of cells per 5 high powered fields from 5 plates (25 values)

<sup>b</sup> = Analysis of variance by Waller-Duncan K-ratio T test

CB = Cytochalasin B

+mc = Mitomycin C

N.S. = Not significant
In summary, these experiments indicate that LT-2 may in fact divide early in soft agar, although only a 68.2% to 73.0% increase in total cell number was noted. This small increase in cell number may only represent cell division of LT-2 cells already committed to cell division prior to plating in soft agar. Cytochalasin B did not inhibit this cell division, but approximately the same per cent increase in cell number was noted (67.1, 63.1, 55.1%). No colonies were noted with cytochalasin B treatment.

10. SOLUBLE PRODUCTS: PROTEIN EXTRACT FROM AU-471

Experiments were done to evaluate protein extracts of AU-471 on soft agar cloning of LT-2.

No LT-2 soft agar colonies were noted when cultured with 20, 310 and 1000-g per culture of KC1 protein extracts from AU-471 with and without FCS. Preplate controls showed good viability with an estimated 75% of cells attached to the surface with 50% of cells spreading on the substratum. A few small 4-6 cell colonies were noted indicating cell growth in the FCS(+) plates. Preplates without FCS(−) showed good viability and attachment, and only 10% of cells flattened out on the surface. No toxic effect was noted from the AU-471 protein extract, but no soft agar colonies were noted.
11. **CELL CLUMPS IN SOFT AGAR**

Attempts were made to determine if small aggregates of LT-2 cells, could proliferate in agar using the cell clump as a point of anchorage. The possibility that a small aggregate of cells might "condition" the media in the local microenvironment of the clump was also considered.

Review of serial photographs of small clumps of cells in soft agar showed no change in size indicating any additional significant growth in soft agar when plated in cell clumps (see Figure 13, 14, and 15.).

12. **ENZYME TREATMENT: CARBOHYDRASES, NEURAMINIDASE**

Many cell functions are controlled by membrane glycoproteins. Enzyme treatments were used to alter the membrane surface glycoproteins. Experiments were done to determine the effect of such enzyme treatments on anchorage independent growth.

Preplate controls overlaid with agar showed some colony formation indicating cell viability and growth capabilities following enzyme treatment. Light "speckling" consisting of microcolonies generally in the range of 10-15 cells per colony were noted in control untreated plates and also in treated plates. A few microcolonies consisting of 10-15 cells were noted in both α and β glucosidase plates. Speckling was frequently noted even in untreated culture.
Figure 13. Cell clump of LT-2 plated in soft agar and serially photographed. Top Day 1; Middle Day 7; Bottom Day 14.
Figure 14. Cell clump of LT-2 plated in soft agar and serially photographed. Top Day 1; Middle Day 7; Bottom Day 14.
Figure 15. Cell clump of LT-2 plated in soft agar and serially photographed. Top Day 1; Middle Day 7; Bottom Day 14.
plates after 4 weeks when seeded with $10^5$ LT-2 cells. This speckling generally consisted of dead cells and cell debris noted when the agar began to dry, making interpretation difficult. The microcolonies noted did not appear to consist of healthy or viable cells.

No anchorage independent colony formation was noted following incubation and plating of LT-2 with neuraminidase. No definite conclusions can be drawn from these experiments since several possible explanations exist including the possibility that important membrane glycoproteins receptors are important in anchorage independent growth, but are buried in the cell membrane or some manner protected from the actions of the enzymes used.

13. **EFFECTS OF cGMP AND HEPES BUFFER**

No anchorage independent growth of LT-2 was noted in separate experiments using cGMP or Hepes buffer.

14. **EFFECTS OF AGAROSE AND/OR DEAE**

No colonies (25 cells/colony) were noted with LT-2 cells plated in agarose or with DEAE. Small clusters (microcolonies) consisting of 5-15 cells were noted in both test and control plates of agarose and DEAE experiments, and no enhancement of anchorage independent growth could be attributed to elimination of negatively charged ions. The
presence of some "microcolonies" was frequently noted where LT-2 was plated in high concentration. (1 x 10^5 cells per 60 min. soft agar plate.)

Agar contains contaminating negatively charged ions which might inhibit anchorage independent growth. Increased cloning efficiency has been reported using agarose (205). No effect was noted using agarose. No clones were noted using the chelating agent DEAE. Heparin (negatively charged molecule) has been reported to decrease cloning efficiency in soft agar (206).
F. FIBRONECTIN

Fibronectin has been noted to restore normal cellular behavior and morphology of in vitro tumors. Experiments were designed to evaluate the effect of fibronectin on anchorage independent growth (Table 21). Fibronectin significantly increased (P<0.01) the cloning efficiency of AU-471 in soft agar. A more pronounced effect was noted when cultures were scored early in experiments (2, 5, 6, and 7 fold increases) compared to later (0, 1.9, 2 and 3.1 fold increases).

Increased cloning efficiency following FBN treatment was also noted with BHK-21, but again the increase was noted only early in experiments similar to results obtained with "feeder" experiments (Table 22).

No clones were noted with FBN treatment with cell lines that do not clone in soft agar (LT-2, WI-38, M.S.).

Antifibronetin treatment of AU-471 resulted in complete suppression of soft agar colony formation. No toxicity was noted in preplate controls plated with antifibronectin and cell attachment was noted. Normal goat serum added in comparable concentrations was not statistically significantly different from control plates without NGS (Table 23).

Antifibronectin treatment of BHK-21 resulted in
TABLE 21

ENHANCED CLONING EFFICIENCY OF A MALIGNANT CELL LINE - AU-471 BY FIBRONECTIN

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatmentb</th>
<th>Day 16</th>
<th>Colony Count (Mean ± S.D.)</th>
<th>Day 28</th>
<th>Pa</th>
<th>Pa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>12 ± 2</td>
<td>-</td>
<td>108 ± 17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+FBN</td>
<td>84 ± 20</td>
<td>.03</td>
<td>200 ± 8</td>
<td>.007</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 13</td>
<td>P</td>
<td>Day 31</td>
<td>P</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>12 ± 3</td>
<td>-</td>
<td>91 ± 4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+FBN</td>
<td>61 ± 6</td>
<td>.001</td>
<td>284 ± 17</td>
<td>.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 14</td>
<td>P</td>
<td>Day 31</td>
<td>P</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>41 ± 8</td>
<td>-</td>
<td>272 ± 18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+FBN</td>
<td>83 ± 5</td>
<td>.008</td>
<td>264 ± 16</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 13</td>
<td>P</td>
<td>Day 30</td>
<td>P</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>12 ± 1</td>
<td>-</td>
<td>168 ± 6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+FBN</td>
<td>71 ± 9</td>
<td>.008</td>
<td>335 ± 19</td>
<td>.004</td>
<td></td>
</tr>
</tbody>
</table>

a = P values compare significance to cultures with 0 FBN
b = Good growth was noted by untreated and +FBN cells plated on the bottom of culture plates and overlaid with agar
TABLE 22

ENHANCED CLONING EFFICIENCY OF AN ANCHORAGE INDEPENDENT "NORMAL" CELL LINE - BHK-21 BY FIBRONECTIN

Experiment 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Colony Count (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 14</td>
</tr>
<tr>
<td>0</td>
<td>146 ± 5</td>
</tr>
<tr>
<td>+FBN</td>
<td>191 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Experiment 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Colony Count (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 13</td>
</tr>
<tr>
<td>0</td>
<td>412 ± 21</td>
</tr>
<tr>
<td>+FBN</td>
<td>881 ± 20</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a = P values compare 0 FBN with +FBN cultures
TABLE 23

SUPPRESSION OF SOFT AGAR CLONING EFFICIENCY OF AU-471

BY ANTIFIBRONECTIN

<table>
<thead>
<tr>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>Anti-FBN&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NGS&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Colony Count (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 14</td>
</tr>
<tr>
<td>0</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Anti-FBN&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>NGS&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Colonies too small to score</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Anti-FBN - antifibronectin

<sup>b</sup> = NGS = normal goat serum

<sup>c</sup> = No statistical significant difference between control cultures with PBS or NGS

<sup>d</sup> = All cells demonstrated good viability when incubated with PBS, Anti-FBN or NGS and plated on the bottom of culture dishes and overlayed with soft agar
significant suppression of cloning efficiency at 14 days
\( p = 0.0008 \) and day 26 ( = .007), but normal goat serum
also produced significant suppression (\( p = 0.009 \) at day 14,
\( p = .008 \) at day 26). There was no statistical significance
between antifibronectin treated and normal goat serum
treated. The reason for clone suppression by normal goat
serum was not evident. (Table 24).
## TABLE 24

**SUPPRESSION OF SOFT AGAR CLONING EFFICIENCY OF BHK-21 BY ANTIFIBRONECTIN**

<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Colony Count (Mean ± S.D.)</th>
<th>Day 14</th>
<th>Day 26</th>
<th>P&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>198 ± 4</td>
<td>-</td>
<td>215 ± 9</td>
</tr>
<tr>
<td>NGS&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>138 ± 19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>.009</td>
<td>133 ± 23&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anti-FBN&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>129 ± 8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>.0008</td>
<td>153 ± 15&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> = NGS = normal goat serum  
<sup>b</sup> = Anti-FBN = antifibronectin  
<sup>c</sup> = No statistical significant difference between Anti-FBN and NGS at day 14  
<sup>d</sup> = No statistical difference between Anti-FBN or NGS at day 26  
<sup>e</sup> = All cells demonstrated good viability when incubated with PBS, Anti-FBN or NGS and plated on the bottom of culture dishes and overlayed with soft agar  
<sup>f</sup> = P values compare significance to 0 treatment control groups
G. CELL SUBSTRATUM AND ANCHORAGE INDEPENDENCE

AU-471 grows as heaped up or piled up cells, indicating lack of contact inhibition and has a high saturation density. Cells harvested from flasks indicate that floating cells composed 8% of the total AU-471 cells, and were 85.2% viable. Loosely attached cells composed 74% of total cells and were 91.0% viable. Substratum attached cells accounted for only 18% of the total cell population and were 88.2% viable.

These findings were in marked contrast with LT-2 which grows as a strict monolayer where floating cells composed 5% of the total with only 7.3% viable. Loosely attached cells composed only 1.28% of total cells and were all dead. Substratum attached cells constituted 93.7% of total cells and were 62.2% viable.

Soft agar plating of floaters, loosely attached and substratum attached AU-471 cells at $2 \times 10^3$ cells per plate revealed a significant difference in cloning efficiency at 14 days of floaters compared to loosely attached ($P = .0002$) and substratum attached cells ($P = .0001$). There was no difference between loosely attached and substratum attached cells (Table 25).

Similar results were noted at 26 days, with $20 \times 10^3$ cells per culture significant differences were also noted
<table>
<thead>
<tr>
<th>Type of Cells</th>
<th>Number Viable Cells x 10³</th>
<th>Colony Count (Mean ± S.D.) Day 14 P</th>
<th>Day 26 P</th>
</tr>
</thead>
<tbody>
<tr>
<td>F.L.</td>
<td>2</td>
<td>59 ± 2  -</td>
<td>177 ± 2  -</td>
</tr>
<tr>
<td>L.A.</td>
<td>2</td>
<td>58a± 3 .0002  86b± 9 .002</td>
<td></td>
</tr>
<tr>
<td>S.A.</td>
<td>2</td>
<td>11a± 2 .0001  83b± 5 .0001</td>
<td></td>
</tr>
<tr>
<td>F.L.</td>
<td>20</td>
<td>1400 ± 50  -</td>
<td>2603 ± 45  -</td>
</tr>
<tr>
<td>L.A.</td>
<td>20</td>
<td>598c ± 16 .0006  2057d± 82 .004</td>
<td></td>
</tr>
<tr>
<td>S.A.</td>
<td>20</td>
<td>430c ± 2 .0001  1072d± 5 .0001</td>
<td></td>
</tr>
</tbody>
</table>

F.L. = Floating cells
L.A. = Loosely attached cells
S.A. = Substratum attached cells

a = N.S. (No significance) between groups lettered "a"
b = N.S. (No significance) between groups lettered "b"
c = N.S. (No significance) between groups lettered "c"
d = Significant at P = .0002 between groups lettered "d"
between floaters and loosely attached cells (\( P = .0006 \)) and substratum attached (\( P = .0001 \)) and similar findings were noted at 26 days. No differences were noted between loosely attached and substratum attached cells at 14 days, but a significant difference was apparent by day 20 (\( P = .0002 \)).
H. NUDE MICE

The ultimate test of tumorigenecity of an in vitro cultured cell line is growth and tumor production in an appropriate animal host like the nude mouse. Experiments were done to evaluate LT-2 tumorigenecity in nude mice.

Tumor nodules were noted in one of two nude mice injected s.c. with $4 \times 10^6$ V. LT-2 (Table 26). This nodule was harvested after seven months observation. One of two animals receiving $8 \times 10^6$ V. LT-2 cells s.c. developed two nodules which were harvested at three months and were $3 \times 4$ mm and $2.5 \times 2.5$ mm respectively. Three of four animals inoculated with $10 \times 10^6$ V. cells s.c. were sacrificed after four and one half months with nodules measuring $4$ mm, $3$ mm and $2$ mm (Figure 16.). The exact interval from injection to first appearance was difficult to evaluate. In one animal receiving $10 \times 10^6$ V. LT-2 cells, the s.c. "bleb" produced by injection never fully disappeared before enlargement began. Once a visible lesion was definitely discernable $1/2 - 1$ mm, growth was very slow. No metastasis was noted in animals receiving s.c. injections that developed tumor nodules. Histologic section revealed a relatively well-differentiated epithelial tumor (Figure 17) compared to the original tumor (Figure 18). Injected cell suspensions had organized themselves into a well recognizable
### TABLE 26

#### NUDE MICE

<table>
<thead>
<tr>
<th>No. Mice</th>
<th>No. Cells</th>
<th>Cell Type</th>
<th>Route of Injection</th>
<th>Date Injected</th>
<th>Date Harvested</th>
<th>Duration</th>
<th>Nodule</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>$4 \times 10^6$</td>
<td>LT-2</td>
<td>s.c.</td>
<td>4/28/78</td>
<td>11/29/78</td>
<td>7 months</td>
<td>1/2</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>$8 \times 10^6$</td>
<td>LT-2</td>
<td>s.c.</td>
<td>8/05/78</td>
<td>10/11/78</td>
<td>approx. 2 mos.</td>
<td>2/4*</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>$10 \times 10^6$</td>
<td>LT-2</td>
<td>s.c.</td>
<td>7/17/78</td>
<td>11/29/78</td>
<td>approx. 4-1/2 mos.</td>
<td>3/4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>$5 \times 10^6$</td>
<td>LT-2</td>
<td>I.V.</td>
<td>4/28/78</td>
<td>6/20/78</td>
<td>33 days</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7/06/78</td>
<td>49 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>$1 \times 10^6$</td>
<td>LT-2</td>
<td>I.V.</td>
<td>4/28/78</td>
<td>7/26/78</td>
<td>89 days</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8/8/78</td>
<td>102 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>$1 \times 10^6$</td>
<td>LT-2</td>
<td>I.V.</td>
<td>5/12/78</td>
<td>11/28/78</td>
<td>200 days</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>$2 \times 10^6$</td>
<td>AU-471</td>
<td>I.V.</td>
<td>5/02/78</td>
<td>6/20/78</td>
<td>49 days</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7/06/78</td>
<td>65 days</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Two separate nodules noted in one mouse. First noted at 7 weeks.
Figure 16. Nude mouse showing growth of tumor nodule.
Figure 17. Histology of nude mouse nodule showing organization into an epithelium with differentiation reflected by keratini toward the center of the nodule. (Hematoxylin and eosin.)
Figure 18. Patient M.L. biopsy of right inguinal lymph node. (Hematoxylin and eosin x 125.)
epithelium. All nude mouse nodules demonstrated central keratinization with cells becoming flattened and hyalinized as they progressed toward the center of the nodule indicating cell differentiation as they progressed inward in the tumor nodule (Figure 19). Some cells demonstrated bi-or-tri-polar mitotic plates. Some mononuclear inflammatory cells were noted to surround some nodules suggesting a host (nude mouse) immune reaction.

Two nude mice tumor nodules were successfully cultured in vitro. Cell morphology remained similar to LT-2 with strict monolayer growth and no piling or heaping up of cells. No change in growth characteristics or morphology could be noted.

Subsequent soft agar cultures of the two in vitro cultured nude mouse nodules showed no anchorage independent growth. Karyotyping of these cells indicated that they were of human origin with metacentric, submetacentric and acrocentric chromosomes present. The cells were aneuploid with considerable variation in chromosomal number similar to karyotyping of LT-2 prior to nude mouse injections (Table 27). Control nude mouse skin cells were also placed in culture and revealed only telocentric chromosomes characteristic of mouse cells.

No metastasis to liver, spleen, kidney or other peritoneal cavity was noted in 10 mice injected
Figure 19. Cross section and high-power magnification of nude mouse nodule showing epithelial formation with keratinization.
<table>
<thead>
<tr>
<th>Cells:</th>
<th>2</th>
<th>2</th>
<th>1</th>
<th>1</th>
<th>2</th>
<th>1</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomes:</td>
<td>57</td>
<td>59</td>
<td>61</td>
<td>62</td>
<td>63</td>
<td>64</td>
<td>65</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cells:</th>
<th>2</th>
<th>2</th>
<th>1</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomes:</td>
<td>67</td>
<td>69</td>
<td>70</td>
<td>71</td>
<td>73</td>
</tr>
</tbody>
</table>
intravenously with $1 \times 10^6$ or $5 \times 10^6$ V. LT-2 cells or in the mice injected with $2 \times 10^6$ V. AU-471. Duration of observation ranged from 33 days to 200 days.
CHAPTER V

DISCUSSION

Vulvar carcinomas comprise 3-4% of gynecologic malignancies (207). These squamous cell carcinomas differ from other skin cancers arising elsewhere in the body in that they are particularly malignant with an overall five year survival rate of only 60-70%, even with very aggressive therapy. Isolation of LT-2 in tissue culture afforded a good opportunity to study in detail an uncommon malignancy. Such studies have revealed some particularly interesting properties.

The only absolute test of malignancy for an in vitro cell line is its biological behavior in the autologous host. Because such testing is impossible, a variety of alternate test systems and criteria have been developed to evaluate the malignant potential of cultured cells.

Based on extensive study of the growth properties of human embryonic and adult lung fibroblasts, Hayflick (208) postulated the following criteria for malignancy in vitro:

1. Heteroploidy
2. Morphology (histologic criteria of malignancy)
3. Indefinite growth (ability to be propagated continually in culture).
Other criteria of malignancy include growth in an immuno-deficient test animal (nude mouse) and/or anchorage independent growth in soft agar (209, 210, 211, 212). These criteria have been accepted and confirmed to some extent by many investigators, but some apparently "normal" cells have also been established in continual cell culture. Although most normal human cells in tissue culture undergo senescence and will not proliferate past 50-70 generations, occasional normal cells undergo an "immortality event", generally with a change in cell growth kinetics resulting in a continuous proliferating cell line (213, 214). Although initially diploid these cells generally convert to a heteroploid karyotype in tissue culture. "Normal" diploid cells in culture can also mimic the morphologic appearance of malignant cells. While mimicking some of the properties of malignant cells, these immortal or established presumably non-malignant cell lines have not been found to produce clones when cultured in soft agar, and do not produce tumors in 8-10 weeks when injected into an appropriate animal model like the nude mouse (215).

Therefore, the definitive criteria of malignancy remain 1. the ability of the cells in question to proliferate into progressively growing tumors in syngeneic, immunosuppressed, or immunodeficient animals, and 2. anchorage-independent cell growth, such as in soft agar suspension.
Cultured LT-2 cells amply satisfy Hayflick's criteria of malignancy in vitro, with a heteroploid chromosome complement, abnormal cytology, and continued growth in culture beyond 70 generations. Most important, LT-2 cells satisfy the criteria of Shin et al (216) for tumorigenicity in the nude mouse, consisting of the appearance of a palpable nodule at the site of injection within 10 weeks, and colony formation in soft agar suspension. Although such criteria are the most stringent currently available, they are not infallible. For instance, malignant tumor cells taken directly from a cancer may not grow in a nude mouse because of metabolic considerations. Unfortunately, no single cellular property has been found which adequately defines the malignant state. The present criteria of malignancy of in vitro cells are based on a variety of parameters which include the heterogeneous properties of cancer cells, but exclude normal cells.

Concern has been expressed regarding the use of cultured cells as models of malignancy, because of possible selective pressures occurring in vitro with resulting alterations in antigenicity or metabolism compared to the original tumor. Such concerns are valid and alteration in any given parameter may occur in vitro within the limits imposed by the genetic information of the cells. On the other hand, many cell lines maintained in culture for long peri-
ods have continued to express properties of the original tumor type. For example, the BeWo cell line (a choriocarcinoma) has continued to secrete hCG despite 11 years in tissue culture (217). Isoenzyme phenotypes in cultured cells have also been found to remain stable, even in the face of karyotype instability, treatment with mutagens, and malignant transformation (218, 219). In studies of karyotype stability in long-term cultures, Fraccaro (220) noted that abnormal karyotypes do not necessarily predispose to instability, and long-term cultures may accurately reflect the tissue of origin. The RPMI 2650 cell line was reported to show very little change in karyotype over a period of one year in culture. (221).

Other studies of cell lines have noted a numerical drift in chromosome complement in culture (222). Analogous exceptions to the stability of other malignant cell properties during continual culture have also been reported (223). Consequently, interpretation of observations using any cultured tumor cell line must be made in view of alterations that may result from continual cell propagation in vitro.

However, alterations in cell properties may not be limited only to malignant cells in vitro. Altered subclones may also occur in vivo in any large tumor cell population as a result of spontaneous mutations or selective pressures.
occurring in the expanding tumor cell population. Even a one centimeter tumor represents nearly 30 cell doublings and more than one billion cells, giving ample opportunity for development of subclones. In fact, Baumal and Scharff (224) noted subclones of MOPC 173 myeloma cells produced by in vivo passage, as judged by their immunoglobin secretory products. In vivo development of subclones is also indicated by non-CEA producing primary tumors with CEA producing metastases. Harvey et al (225) demonstrated heterogeneity of immunoreactive CEA extracted from various tumors, showing differences in carbohydrate composition and molecular weight. No immunoreactive CEA was found in breast carcinomas (an epithelial tumor), but CEA activity was found in metastases in the liver, verifying subclonal variation of tumors in vivo. Similarly, a CEA producing tumor recurring following treatment may no longer express CEA activity. Thus, alteration in karyotype or phenotypic expression in malignancies may occur both in vivo and in vitro.

Interestingly, after 4 years in tissue culture and after passage through nude mice and reculturing in vitro, the karyotype of LT-2 still revealed a wide separation in chromosome numbers with no modal number (variations from 57 to 73 chromosomes in 18 karyotypes). Despite the presence of many subclones and markedly variable chromosomal number and continued growth and subcultivation in tissue culture,
LT-2 was still capable of some degree of differentiation as noted in the nude mouse tumor nodule. A single cell suspension was injected subcutaneously on the back of nude mice, resulting in the formation of a tumor nodule. This nodule was not a mass of unorganized cells or tissue, but instead formed into a recognizable skin with cells further differentiating towards the center of the nodule forming keratin. This remarkable cell differentiation would appear to be ploidy-independent, since the cells injected and recovered consisted of many subclones with variable chromosomal numbers and morphology.

Similarly, over the years of observation with close to one thousand soft agar cultures using LT-2, no anchorage independent growth could be demonstrated. (Small clusters of cells were frequently noted, but no colonies over 25 cells.) LT-2 also continued to grow as a strict monolayer with minimal heaping-up of cells as noted with most malignant cells grown in culture. The stability of these cell phenotypes despite the wide karyotypic variability suggested that these phenotypes may also be ploidy-independent. Although there may be a variety of explanations, the work of Jacob and Monod regarding control of gene expression would appear to be pertinent (226). Studies of the induction of the B-galactosidase (lac) gene in bacteria revealed a repressor gene product which controlled expression of B-ga-
lactosidase. Mutations in the regulator gene resulted in constitutive expression. There are only about 10 soluble repressor molecules for the lac gene in Escherichia-coli (227). The balance between the availability of repressors and binding sites on the structural gene operon (operator) is critical. If a similar repressor control system is present and operational in eukaryotic cells, it is not unreasonable to expect the ratio of repressor molecules to operator binding sites to be important. Variations in chromosomal number may result in disturbances of such a delicate ratio when the repressor and operator are physically located on different chromosomes. Repression or derepression of gene function might consequently result from variation in chromosomal number.

Cancer cells with karyotypic variations might therefore result in variations in expression of ploidy-independent phenotypes, while ploidy-independent phenotypes and functions would remain inviolate. Obviously all genes have a degree of ploidy-independence in that the total lack of a gene results in an absence of expression. Yet, much of the cell metabolism and basic housekeeping functions of cells in culture can occur despite considerable variation in karyotype and even some degree of differentiation, as clearly demonstrated by LT-2. These basic cell functions for all intent and purpose, would appear to be ploidy-independent.
On the other hand, consistent cell regulation and control as evident in normal cells in the total organism would appear to be much more sensitive to variations in chromosomal number. Most embryos with abnormal karyotypes are aborted and one has only to consider the many problems of the Down's Syndrome child born with 3 No. 21 chromosomes, to appreciate the effects of even small changes in the delicate balance of gene dose.

A definition of an in vitro ploidy-independent phenotype could be the following: a consistent expression of a phenotype or function despite considerable karyotypic variation in a large number of cells. Also important in such consideration is the stability of the karyotypic variations. If cell chromosomal sorting at the time of mitosis is highly variable, the potential of a whole spectrum of cells with wide karyotypic variation and consequently ploidy-independent phenotypes would be realized. Ploidy-independence arising from karyotypic variation might require repressor and operator genes to reside on different chromosomes or at least different loci on translocated chromosomes. Undoubtedly large tumor populations could potentially express new (derepressed) genes, secondary to loss of environmental controls (soluble regulators) or from mutation of repressor genes on the same chromosome with selection for such positive cells.
The patient M.L., discussed in this report, was found to have a serum CEA concentration of 9.8 ng/ml, despite the epithelial origin of her vulvar tumor. Subsequently, cultured cells LT-2 derived from a lymph node metastasis of this tumor were found to produce .877 ng CEA/10^6 cells/day (above fetal calf serum CEA levels). The presence of CEA activity noted both in vivo and in vitro and subsequent loss with passage in culture reflect the dynamic state of tumor cells which may occur both in the test tube or in the patient. These vagaries are the realities which must be considered by both cancer researchers and therapists.

Sensitization to CEA in some cancer patients has been reported. Gold (228) noted circulating antibodies against CEA, and Costanza et al (229) noted CEA antibody complexes in a patient with nephrotic syndrome and colon cancer. The secretion of CEA by cells in tissue culture may explain some cases of specific lymphocyte mediated cytotoxicity or lymphoblast transformation in CEA sensitized patients. Fetal calf serum (FCS) should not be used in either test (LMC or lymphoblast transformation) since sensitized patient lymphocytes could potentially respond to the CEA in FCS.

An intriguing line of investigation suggested by properties of the LT-2 line is the apparent relationship between human malignancy and cellular production of the beta subunit of human chorionic gonadotropin (β-hCG). McManus et
al (230) have demonstrated $\beta$-hCG on the cell surfaces of 25 or 28 different tumors using peroxidase-labelled antibody staining. Active tumor cell production of $\beta$-hCG in vitro has also been noted, not only in this study of LT-2 which secretes about 4 ng/10^6 cells/day (0.1% of the $\beta$-hCG production noted in the choriocarcinoma cell line BeWo), but also by the cervical carcinoma cell line CaSki (231) and an ovarian carcinoma cell line (232).

The only non-malignant source of biologically active hCG known is trophoblastic tissue, which actively invades the uterine endometrium during early pregnancy. Serum concentration of hCG begins to decline during gestation at about the same time that active endometrial invasion by trophoblasts ceases (233). (A recent report suggests low-level hCG production by liver and colon of normal individuals, but this hCG is apparently non-functional) (234).

LT-2, a relatively well-differentiated cell line derived from a metastatic lesion also produces and secretes the beta subunit of hCG. Further research will be needed to determine whether the expression of $\beta$-hCG by such a wide variety of tumors as noted by McManus et al is integrally involved in the cancer process or simply the random expression of a normally repressed gene. (235).

LT-2 retained its original ABH blood group type. Many tumors have been reported to change their blood group types (236).
Despite some of the malignant properties of LT-2 like continued growth in culture, abnormal karyotype, lectin agglutination, production of fetal antigens like CEA, hCG and most importantly growth in vivo in the nude mouse, LT-2 has retained some normal cell phenotypes. LT-2 grows as a very strict monolayer in culture and this is reflected by the relatively low saturation density compared to AU-471 at the same serum concentrations. Low saturation density and monolayer growth are frequently used criteria for contact inhibition. Yet, frequent cell division can be noted within large colonies of cells despite cell contact on all sides. Time lapse cinematography has clearly confirmed a lack of contact inhibition of both cell division and locomotion by LT-2. Abercrombie has suggested that contact inhibition can result from the development of strong lateral adhesions to adjacent cells during cell-cell collisions or the inability of the dorsal surface of one cell to provide an adequate substratum for locomotion for another cell. (237)

Studies by Middelton (238) in which cells were plated on confluent monolayers suggest that the latter phenomenon is more important. Time lapse studies by Steinberg also suggest avoidance of overlapping as the main mechanism for monolayer growth (239). Both mechanisms might apply to monolayering by LT-2. Our time lapse studies have demonstrated the formation of strong lateral adhesions resulting
from cell-cell collision, although these lateral adhesions were not sufficient to prevent a colliding cell from moving away from a cell colony. Avoidance of overlapping was also noted. LT-2's low saturation density would therefore appear to result not from contact inhibition per se, but from a strong tendency to monolayer growth, and may therefore result from either the formation of strong lateral adhesions and/or the inability to use the dorsal surface of another cell for adhesions. A preferential strong adhesion potential for the plastic substratum rather than another cell might also be a possible explanation.

Holley has noted that the phenotype of contact inhibition is serum dependence (240). Cell growth continues with an increase in saturation density when fresh serum is added to contact inhibited monolayer cell cultures. Holley isolated a factor from serum, and Temin (241) isolated a growth promoting factor from contact inhibited media of a cell line that demonstrated such serum independence. Lipkin (242) has also reported a factor produced by contact inhibited cells that restores contact inhibition to malignant melanocytes. LT-2 requires growth factors found in serum for stimulation of growth, but not for metabolism and cell survival as demonstrated by a lack of cell division in the absence of serum, but good cell survival. The necessity for an additional factor (not in serum) produced by LT-2 for
stimulation of cell division is suggested by the inability to clone LT-2 in small wells in Cuprak dishes even with conditioned media, and the requirement for as many as 500-1000 cells per well in a 96 well cluster dish to obtain colonies and cell growth. The presence of some types of growth stimulation is also suggested in the soft agar feeder experiments in which LT-2 increased anchorage independent cloning efficiency of AU-471. WI-38 also increased cloning efficiency of AU-471 in soft agar and is known to produce a growth factor unin vitro (243). Since neither cell (LT-2 nor WI-38) clones in soft agar, this growth promoting factor would appear to stimulate cell division, but not anchorage independent growth per se. Conditioned media could also transfer the growth stimulation. Anchorage independence could not be produced by co-cultivation of AU-471 with LT-2, suggesting that these phenotypes are either not controlled by a soluble mediator or the mediator is in such low concentration or so labile that it is not efficient in stimulation of anchorage independent growth by LT-2.

Spandidos and Siminovitch were able to transfer anchorage independent growth by means of metaphase chromosomes (244).

Field count experiments showed a small increase in the number of LT-2 in soft agar cultures suggesting that a few cells were able to divide in soft agar. These may be cells
already committed to cell division before plating in the soft agar. Such anchorage independent growth suggests that cell division can occur in an anchorage independent environment even by a cell that will not normally clone without anchorage, and the absence of cell division under the conditions of anchorage independence results from the control of the initiation of cell division rather than the inhibition of the division process itself. Stanbridge (245) has noted a separation of in vitro transformation phenotypes like absence of contact inhibition and lack of density-dependent inhibition of growth, lectin agglutination, serum independence, and anchorage independence from malignancy in vitro using HeLa and normal fibroblast hybrids. HeLa-fibroblast hybrids retained the in vitro transformation phenotypes, but lost their in vitro malignancy potential. LT-2 also clearly demonstrates that these phenotypes are independent, but in contrast, LT-2 grows in nude mice demonstrating its malignant potential, but retains many normal in vitro phenotypes that are usually regarded as associated with malignancy.

Fibronectin increased cloning efficiency in soft agar of AU-471, but had no effect on LT-2 or WI-38. Anti-fibronectin had a marked inhibitory effect on anchorage independent growth of AU-471, but not BHK-21. These experiments suggest that fibronectin is necessary but not sufficient for
anchorage independent growth. These findings were surprising since fibronectin has been associated with restoration of normal phenotypes and monolayer growth in transformed cells.

Although tumor nodules could be repeatedly produced by subcutaneous injection of LT-2, no tumors were produced following intravenous injection of either LT-2 or AU-471. Variation in tumor formation with route of injection is well recognized (246), and might result from differences and local microenvironmental conditions or from an active process of cell destruction by macrophages or other cells in the nude mouse. Interestingly metastases are uncommon even for massive subcutaneous tumors in the nude mouse, suggesting some controlling mechanism.

The patient M.L.'s lack of specific tumor antigen recognition, ascertained by lymphocyte-mediated cytotoxicity, lymphoblast transformation and in vivo autologous tumor antigen skin test response, may reflect a lack of antigenicity of her tumor cells or a specific suppression of immune response to tumor antigens. However, the absence of general immune responses to both microbial recall skin test antigens and DNBC is more consistent with a state of total immune suppression. Lymphocyte numbers, total T-cell, active T-cell, and B-cell fractions, and mitogen stimulation of lymphocytes are also consistent with a total suppression of
the patient's immune system. A spontaneous (nonspecific) LMC of the patient's lymphocytes versus unrelated malignant cells significantly lower than normal lymphocytes is consistent with recent observations that spontaneous LMC is depressed in cancer patients. (247, 248).

The repeated re-evaluation of both in vivo and in vitro immuno-competency over a 15 month period of observation clearly demonstrates the dynamic interaction of the host with its tumor and disease process. The presence of a positive DTH to a bacterial recall skin test antigen (dermatophytin-O) which became negative a little over a month later at the same time that the patient converted from a negative to a positive PPD skin test demonstrates the rapid development of anergy while retaining the ability to respond to a primary immunization with PPD. The response to PPD immunization occurred despite the fact that the patient did not respond to a primary immunization with another antigen - DNCB. Likewise, the marked suppression in cytotoxicity discussed above which increased to a statistically significant degree shortly before the patient's death also testifies to the dynamic equilibrium between energy and immune responsiveness in the cancer patient. It suggests caution in interpretation of studies of immunocompetency in cancer patients where observations are made at only a single point in time. The variations in responsiveness to PPD and DNCB
are another example of the vagaries of the immune systems in cancer patients.

Any immune responses on the part of a tumor host to its autochthonous tumor require either the presence of a "foreign" or "new antigen" expressed by the tumor or a breakdown in the patient's normal tolerance recognition system with a resulting response to self antigens expressed by the tumor. Both types of responses have been reported in tumor patients. No immune responsiveness was noted in this study as evaluated by in vivo skin testing with autologous tumor extracts and in vitro by lymphocyte blast transformation in the presence of extracts of the tumor (LT-2). The 15% specific cytotoxicity noted shortly before the patient's death suggests that a tumor antigen might be present on LT-2. In vitro studies have demonstrated that the tumor has retained its original blood group type with no expression of Rh antigens (normally restricted to red blood cells and not seen on epithelial cells), but did express CEA which is unusual for an epithelial tumor. The expression of β-hCG (which is normally expressed only by trophoblastic cells), and lectin binding (indicating changes in carbohydrate composition of the cell membrane which are generally noted in tumor cells but not in normal cells), and reactivity with normal rabbit serum also suggest the presence of a "new antigen". All of these findings indicate an "abnormal
or unusual" antigen expressed on LT-2 which could have been responsible for the cytotoxicity noted.

Levin, et al, (249) have reported responses of household contacts of cancer patients to allogenic tumors using a cytotoxicity assay. No such antigen exposure of the patient's sister could be demonstrated using the sister's lymphocytes in a blast transformation assay to LT-2 tumor extracts in this study.

The cytotoxicity assay used in this study involved the use of an in vitro cultured autologous tumor. Most studies in the past have involved the use of allogeneic tumor cells or on occasion, fresh tumor tissue. The rationale for the use of allogeneic tumor is based upon the concept of a shared tumor antigen between histologically similar tumor types. Unfortunately, the same studies which are used for supporting the concept that histologically similar tumors share the same common tumor antigen are used as proof of cytotoxicity to tumor cells. Proof of a shared "tumor antigen" in most histologically similar tumors in humans is poor at best. Dodson and Menon (250) have recently reviewed the subject of "tumor antigens", and their possible mechanism of production and have emphasized the vagaries of antigen expression between different tumors and even between cells of the same tumor and note that except for viral antigens, most "tumor antigens" are a reflection of the
karyotype and metabolic vagaries of the tumor cell. Although such "tumor antigens" have been amply demonstrated in viral induced tumors in experimental animals, and have been suggested in humans in Burkitt's Lymphoma and possible malignant melanoma, their presence in most human tumors is questionable.

The great bulk of tumor cytotoxicity studies are also questionable because of the recent observation by Zinkernagel and Daugherty (251) regarding the major histocompatibility restrictions between the cytotoxic effector cell and the target cell. In order for cytotoxic T-cells to kill virus infected cells expressing new (viral) antigens, the lymphocyte and infected cell must share the same histocompatibility antigens. This cytotoxicity system - killing self cells expressing new antigens (viral in the case of infected cells) would appear to be a similar system to that proposed for immune surveillance in which cancerous self cells expressing new antigens are destroyed. Allogeneic cells with viral antigens are not killed in this system. Undoubtedly, the body contains a mechanism for rejecting foreign tissue as demonstrated by graft rejection, but it would not appear to be the same cytotoxicity system that checks self cells for new antigens since allogeneic cells are not affected in the system illustrated by Zinkernagel and Daugherty. A lack of a tumor antigen in histologically
similar tumors, and a lack of histocompatibility between target and effected cells in allogeneic cytotoxicity systems results in serious questions regarding the great majority of such tumor studies currently in the literature.

The use of fresh tumor avoids the problem noted above, and may give some significant data, but it also raises other questions. The general method of processing tumors involves mincing the tissue into small pieces and sometimes treatment with enzymes to disperse the cells. Such a process results in the presence of many fibroblasts. As a consequence, it is difficult to compare values since a low cytotoxicity score may simply reflect a high percentage of fibroblasts rather than a lack of cytotoxicity between lymphocyte and target cells. Enzymes may modify or destroy surface antigens, also raising questions regarding cytotoxicity data when tissue is processed in this manner.

Undoubtedly, the use of culture cells can also be criticized on the basis that cells may change their phenotype, karyotype or other properties while in tissue culture. Although such changes may occur in vitro, they may also occur in the tumor mass in vivo.

These questions of techniques are not simply academic. Cytotoxicity studies have suggested that tumors possess new "tumor antigens", and that the immune system is capable of destroying tumor cells. Yet the data are in conflict with
common clinical observations that tumor progression is com-
mon and immunological tumor regression is exceptionally
rare. Enthusiasts of immune surveillance suggest that tu-
mors are common, and that it is the immune system that
kills most of these tumors very early, before they are
clinically evident. Unfortunately, the theory has been
difficult to test clinically, and cytotoxicity data which
has been used to support the concept of immune surveillance
is questionable for the reasons discussed above. Perhaps
there is an immune surveillance system with cytotoxic lym-
phocytes despite the problems with experimental design of
many cytotoxicity studies previously published. Additional
research will be needed to answer this question, but should
be done with autologous tumors and lymphoctyes if relevant
data are to be obtained.

A particularly interesting finding in this study is the
dissociation of the phenotypes of in vivo malignancy and
anchorage independent growth by LT-2. Only a few tumors
have been reported that fulfill the stringent criteria of
tumor formation in the nude mouse, but that will not clone
in soft agar. The studies and experiments presented here
raise serious questions regarding the use of anchorage inde-
pendent growth as the ultimate in vitro criterion of malig-
nancy.
LT-2 is capable of cell division in an anchorage independent environment as revealed by field count experiments in this work, but colony formation does not occur. The control of soft agar cloning would therefore appear to involve initiation of cell division rather than a limitation of the capacity for mitosis. This is not surprising since even cells growing in monolayers "round-up" during cell division and are already "rounded" in shape when plated in soft agar.

Fibronectin increases anchorage independent growth of malignant cell lines, but does not initiate soft agar growth by LT-2. Anti-fibronectin decreases anchorage independent growth. Therefore, fibronectin is necessary but not sufficient for anchorage independent growth. No soluble stimulants released by an anchorage independent cell line could be demonstrated, but LT-2 was capable of both stimulation and suppression of colony formation by an anchorage independent cell line depending on dose and time in culture. These effects were less apparent when LT-2 was treated with mitomycin C, suggesting that limitation of cell division was an important factor in both processes.

Single cell suspensions of LT-2, injected into nude mice, were capable of aggregating and forming an easily recognizable skin with differentiation as reflected by keratin formation. These findings suggest that LT-2 retains receptors for cell aggregation and the capacity for differen-
tiation despite its malignant nature and markedly variable and aneuploid karyotype. Cells re-cultured in vitro from such nude mice also demonstrate markedly variable aneuploid karyotype indicating that some capabilities of LT-2 to organize into an epithelium and differentiate and form keratin are ploidy independent. Soft agar cultures of such cells again demonstrated a lack of anchorage independent growth confirming the separation of the phenotypes of anchorage independence and nude mouse growth even in cells passed through nude mice.
REFERENCES


10. Möller, G., Möller, E.: Considerations of some current concepts in cancer research.3


37. Eilber, F.R., Morton, D.L.: Impaired immunologic reactivity and recurrence following cancer surgery.34
38. Eilber, F.R., Morton, D.L.: Impaired immunologic reactivity and recurrence following cancer surgery.34
41. Herberman, R.B.: Delayed hypersensitivity skin reactions to antigens on human tumors. (Cancer 34, No. 4:1103-11, 1974).
46. Herberman, R.B.: Delayed hypersensitivity skin reactions to antigens on human tumors.41 pp 1469.
47. Stewart, T.H.M.: The presence of delayed hypersensitivity in patients toward cellular extracts of their malignant tumors.44


60. Hellström, I., Hellström, K.E.: Cell-mediated immune reactions to tumor antigens with particular emphasis on immunity to human neoplasms. (Cancer 34, No. 4:1461-1468, 1974).


63. Hellström, I., Hellström, K.E.: Cell-mediated immune reactions to tumor antigens with particular emphasis on immunity to human neoplasms. 60


66. Hellström, I., Hellström, K.E.: Cell-mediated immune reactions to tumor antigens with particular emphasis on immunity to human neoplasms. 60


68. Hellström, I., Hellström, K.E.: Cell-mediated immune reactions to tumor antigens with particular emphasis on immunity to human neoplasms. 60

69. Hellström, I., Hellström, K.E.: Cell-mediated immune reactions to tumor antigens with particular emphasis on immunity to human neoplasms. 60


71. Murray, E., Ruygrok, S., Milton, G.W., Hersey, P.:


86. Alexander, P.: In Immunobiology of the Tumor-Host Relationship.85

87. Henney, C.S.: Mechanisms of tumor cell destruction.52


89. Hibbs, J.B.: Macrophage nonimmunologic recognition: Target cell factors related to contact inhibition.88

90. Henney, C.S.: Mechanisms of tumor cell destruction.52


100. Gall, S.: Personal communication.


lines HBC and BrCa 5 and other cell cultures. (Science 195:1343, 1977).


118. Pollack, R.E., Green, H., Todaro, G.J.: Growth con-


121. Steinberg, M.S.: Cell movements in confluent monolayers: A re-evaluation of the causes of "contact inhibition", in Ciba Foundation Symposium 14.


125. Gospodarowicz, D., Moran, J.S.: Growth factors in mammalian cell culture.


138. Kataymama, K.P., Woodruff, J.D., Jones, H.W., Pre-


148. Salmon, S.E., Buick, R.N.: Preparation of permanent
slides of intact soft-agar colony cultures of hematopoietic and tumor stem cells. (Cancer Res., 39:1133-1136, 1979).


150. MacPherson, I., Montagnier, L.: Agar suspension culture for the selective assay of cells transformed by polyoma virus.147


152. Montesano, R., Drevon, C., Kuroki, T., Vincent, L.S., Handleman, S., Sanford, K.K., Defeo, D., Weinstein, I.B.: Test for malignant transformation of rat liver cells in culture: Cytology, growth in soft agar, and production of plasminogen activator.139


176. Rygaard, J., Povlsen, C.O.: The mouse mutant nude does not develop spontaneous tumors.11

177. Stiles, C.D., Desmond, W., Chuman, L.M., Sato, G., Saier, M.H.: Growth control of heterologous tissue culture cells in the congenitally athymic nude mouse.170


192. Wybran, J., Carr, M.C., Fudenberg, H.H.: Effect of
serum on human rosette forming cells in fetuses and adult blood. 191


214. Todara, G.J., and Green, H.: Quantitative studies of the growth of mice embryo cells in culture and

215. Fogh, J., Fogh, J.M., Orfeo, T.: One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice.\textsuperscript{106}

216. Shin, S.I., Freedman, V.H., Risser, R., Pollack, R.: Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth in vitro.\textsuperscript{127}

217. Pattillo, R.: Personal communication.\textsuperscript{101}

218. Gartler, S.M.: Apparent HeLa cell contamination of human heteroploid cell lines.\textsuperscript{107}


232. DiSaia, P.: Personal communication.103


237. Abercombie, M., Heaysman, J.E.M.: Observations on the social behavior of cells in tissue culture, 1. Speed of movement of chicken heart fibroblasts in relation to their mutual contact.115


240. Holley, R.W., Kiernan, J.A.: "Contact Inhibition" of cell division in 3T3 cells.


251. Zinkernagel, R.M., Doherty, P.C.: Restriction of in
vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. (Nature 248:701-702, 1974).
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