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MOUSE SPLENIC CELL IMMUNOCOMPETENCE IN POLYOMA AND NONPOLYOMA TUMOR SYSTEMS

DISSEPTION

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

by

Orval L. Mullen, B.S., M.S.

* * * * *

The Ohio State University
1972

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Introduction

Tumors induced by polyoma virus in mice, rats, hamsters, or any other susceptible species all possess a common polyoma-specific transplant antigen. Presence of this antigen is detected by demonstrating that animals immunized with either polyoma virus or cells carrying the transplant antigen will reject subsequent challenge doses of polyoma tumor cells that would induce tumors in normal animals. For this reason it is believed that host immunity directed against tumor specific transplant antigens plays an important role in deciding the course of tumor disease. Evidence suggests that although humoral mechanisms may play some cytotoxic role in this immune response (Wilson, 1965), it is primarily through cell-mediated mechanisms that the tumor is rejected (Billingham, et al., 1954).

What role antibodies do play in tumor disease is generally thought of as one of "enhancement" (Hellstrom and Hellstrom, 1970). That is to say, antibodies specific for the tumor cells seem to facilitate their growth. It is thought that antibodies bring this about by interfering with cell-mediated cytotoxicity for tumor cells.

The cell-mediated cytotoxic response was first studied in vitro by Rosenau and Moon (1961). They detected
morphological alteration and cell death of target cells in
the presence of sensitized lymphoid cells and in the absence
of specific antiserum. This work involved an allogeneic
system and, as such, was an *in vitro* model of graft rejection.
However, by 1966 Rosenau and Morton had demonstrated *in vitro*
cytotoxicity to cells with tumor specific transplant antigens
in a syngeneic system.

In the few years since these early investigations, a
number of tests have been perfected for quantitation of
*in vitro* target cell destruction. Alteration in release of
Cr-51 from target cells and colony inhibition procedures are
just a couple of these. It is interesting to note that the
latest *in vitro* method for detecting cell-mediated cytotoxicity,
the microtiter assay (Takasugi and Klein, 1970), is
based on the same basic principle as the method originally
used by Rosenau. In each instance the monolayers are washed
after treatment with lymphoid cells and the number of target
cells remaining counted. Since dead or injured target cells
are removed with the wash, this method provides information
concerning the percentage of target cells destroyed by the
test lymphoid cells.

To date, a number of investigators have considered
*in vitro* cell-mediated destruction of nonpolyoma target tumor
cells employing a number of techniques used in this
investigation. In addition, some have considered serum-
mediated cytotoxicity for polyoma tumor cells (Hellstrom and Sjogren, 1965). With one exception, however, no investigators have employed these *in vitro* tests in a consideration of the cell-mediated response directed against the polyoma specific transplant antigen. This work was carried out by Burstein (1970) in a rat system.
Polyoma Mouse Oncogenesis

Polyoma virus will induce tumors in such animals as mice (Stewart, et al., 1958), hamsters (Eddy, et al., 1958), ferrets (Harris, et al., 1961), rabbits (Eddy, et al., 1958), guinea pigs (Eddy, et al., 1960), rats (Eddy, et al., 1959), and mastomys (Rabson, et al., 1960). Mice injected with polyoma virus and rabbit anti-polyoma serum do not develop tumors as readily as mice injected with polyoma virus alone (Stewart, 1962). On the other hand, rabbit antivirus serum will not passively transfer the capacity to reject transformed cells which induce tumors in normal adult animals. Spleen, thymus, lymph node, and bone marrow cells from virus and tumor-cell-immunized animals are capable of doing this (Habel, 1962). Adult mice will not develop tumors if injected with polyoma virus unless previously irradiated (Stewart, 1962 and Sjogren, 1964). Tumors develop at a large variety of sites in the polyoma-infected mouse, but especially in the salivary glands with parotid gland tumors usually being the first detected.

Polyoma In Vitro Cultivation

In vitro, the most obvious response of mouse embryo monolayers to polyoma infection is a cellular degeneration termed cytopathic effect (CPE). Mouse embryo CPE usually
becomes grossly evident 11-14 days post-infection. By six to nine days post-infection clear areas may develop in the monolayers where the cells have undergone lysis (Bereczky, et al., 1961). The virus replicates so well in mouse cells that mouse cell monolayers usually are used to grow stock virus. Polyoma virus replication has also been observed in mouse L-cells (Henle, et al., 1963), Syrian and Chinese hamster cells, and *Bacillus subtilis* (Bayreuther and Romig, 1964).

**In Vitro Correlates of Polyoma Oncogenesis**

Mouse cells surviving CPE give rise to transformed clones. These transformed cells appear to be in vitro correlates of tumor cells arising in vivo. Tumors will develop when polyoma transformed cells are injected into syngeneic mice. Defendi, et al., (1967) determined through karyotypic analysis that the cells of such a tumor are of donor origin. The 4198 cell line was derived originally from a C3H/He parotid cell grown in vitro and transformed with polyoma virus. Since then the cell has been carried both in vivo and in vitro (Ting and Law, 1965).

**Polyoma Cell Associated Antigens**

Polyoma-induced tumor cells of all species possess a common polyoma-specific transplant antigen on their surfaces.
Presence of this tumor-specific transplant antigen (TSTA) may be readily demonstrated by immunization of an animal with polyoma-transformed heterogeneic tumor cells followed by challenge with syngeneic polyoma-transformed tumor cells. In this situation the syngeneic tumor cells which would normally grow in the recipient are rejected, demonstrating transplant antigen common to both tumor cells (Sjogren, 1964). Mice injected with polyoma virus likewise demonstrate resistance to polyoma tumor cells (Defendi, 1963). Both adult mice and hamsters can be made resistant to an isologous transplantable polyoma tumor by an inapparent infection with polyoma virus (Habel, 1962). Various strains of polyoma virus differ in their ability to produce transplantation antigen (Hare, 1967). Specificity of the reaction against TSTA was demonstrated by Defendi (1963) and Koch and Sabin (1963). No cross reactivity was seen by Defendi between the simian virus 40 (SV\textsubscript{40}) and polyoma systems. Animals infected with one type of virus or cell did not show resistance against the other cell type. Koch found that infection with adenovirus types 12 or 7A, herpes virus, or polio virus type 1 produced no increase in polyoma tumor cell resistance. Loss of the tumor-specific transplant antigen (TSTA) from the surface of a variant hamster cell that originally carried the polyoma transplant antigen coincided with development of contact inhibition, but did not coincide with loss of the intracellular tumor T antigen.
(Rabinowitz and Sachs, 1970). Hare (1967) also found that presence of the polyoma TSTA does not depend on presence of the T tumor antigen.

New antigens can also be detected on surfaces of cells infected with polyoma virus by immunofluorescence (Malmgren, et al., 1968; Ting and Herberman, 1970; and Irlin, 1967) or by complement fixation (Habel, 1965). The complement-fixing antigen is apparently a different structure than either the viral or transplantation antigens, for it is known that animals infected with polyoma virus may show transplant resistance without showing complement-fixing antibody in their sera (Habel, 1965). The antigen demonstrated by immunofluorescence may be more closely related to polyoma-induced transplant antigens. Serum used to detect antigen by immunofluorescence contains antibodies to polyoma-induced transplantation antigens (Irlin, 1967). It is also known that two cell lines, 4198 and the 4198V variant of the 4198 cell, which contain different amounts of antibody-binding surface antigen, differ in their ability to form tumors (Ting and Herberman, 1970). The 4198 V cell, having more antigen, does not grow as well as the 4198 cell. The cells grow equally well in immunosuppressed animals.

Intracellular antigens associated with polyoma virus infection include the T antigen or tumor antigen which can usually be demonstrated by immunofluorescence in association
with the nucleus of an infected cell. This antigen has been
detected as early as twenty hours post-infection (Winocour
and Sachs, 1960). A polyoma variant is known which will
infect hamster cells leading to the appearance of this
antigen in the cytoplasm prior to its appearance in the
nucleus (Hare, 1970).

In vitro cytotoxicity testing

Of increasing interest in recent years has been a
consideration of the immune response directed against TSTA
and the ability of that response to prevent tumor growth.
Although humoral mechanisms may play some role in tumor
rejection (Wilson, 1965), evidence suggests that it is
primarily through cell-mediated immune mechanisms that a
tumor is rejected (Morton and Malmgren, 1968; Hellstrom and
Hellstrom, 1969; Billingham, et al., 1954). Over the past
several years techniques originally developed to demonstrate
host cellular immunity to allografts have been applied to
the study of TSTA immunity.

In particular a number of in vitro methods are
presently being used to quantitate in vitro target cell
destruction by lymphoid cells. Rosenau and Moon (1961)
reported the first reproducible in vitro assay of immune
cell-mediated cytotoxicity, in this case directed against
allogeneic histocompatibility transplant antigens of target
L cells. Morphological alterations in stained coverslip monolayers and nuclear counts recording target cell nuclei remaining in the tube cultures after rinsing were employed in this study of cell-mediated target cell destruction. Loss in target cell viability as measured by trypan blue dye uptake (Taylor, 1963), decreased C-14 labelled amino acid uptake (Granger and Williams, 1968), Cr-51 release (Peter, 1971), and altered morphology, as followed by cinematography and electron microscopy (Able, et al., 1970), have also been employed in the L cell system. Other cytotoxicity tests include the monolayer spot test (Ramming and Pilch, 1970), the colony inhibition test (Hellstrom, 1967), and the microassay procedure (Takasugi and Klein, 1970).

The L cell system has played an important role in elucidating the mechanisms involved in cell-mediated target cell destruction. Rosenau (1961) ascertained that allogeneic target cell destruction was occurring in the absence of complement and/or any detectible specific antiserum. Taylor and Culling (1963) noted that since mouse lymphoid cells were 70 percent nonviable after only 12 hours in culture, the L cell-lymphoid cell interaction must occur very early. It was demonstrated that lymphoid cells lost all cytotoxic capacity when they were killed by freeze-thawing, heating, or sonication. Since hydrocortisone (Rosenau and Moon, 1962), cortisone, and X-rays (Rosenau and Moon, 1966) block the cell-
mediated response but do not block serum-mediated cytotoxicity, Rosenau concluded that cell-mediated lysis is related to functional modification of lymphocytes and not due to adsorbed cytotoxic antibodies. A ribosomal suspension but not purified RNA was found by Gerughty (1966) to be capable of transferring L cell sensitivity to nonsensitized lymphoid cells. Granger's pioneering work with lymphotoxin (Granger and Williams, 1968) demonstrated that a soluble factor was responsible for target cell destruction in the L cell system. This lymphotoxin does not bind to target cells, acts nonspecifically, and is blocked by polyanions of similar charge (Williams, 1969).

**Chromium Retention Assay**

Test systems for cytotoxic reactions have been reported using C-14 (Klein and Perlmann, 1963), P-32 (Forbes, 1963), and Cr-51 (Goodman, 1961) isotopes. Isotopic assay procedures have a number of advantages over other cytotoxicity testing procedures. They are less subjective than either the colony inhibition or spot test procedures. Samples taken during the course of the experiment do not have to be counted immediately, but may be saved for a more suitable occasion. Many of the virtues of the Cr-51 technique and parameters of this procedure were considered in a work by Wigzell (1965). He compared chromium retention
to trypan blue dye exclusion in serum-mediated cytotoxicity. Brunner, et al. (1968) compared cytotoxicity as measured by Cr-51 retention and plating efficiency in mouse tumor cells subjected to sensitized spleen cells. Boyle (1968) described a red blood cell (RBC) lysis technique which could be used to free a target cell suspension of contaminating RBC's which interfere with the Cr-51 test by taking up the label. Cytotoxin will induce Cr-51 release from mouse L cells, and a number of antimalarial drugs and anti-inflammatory steroids will block activity of the cytotoxin and release of the label (Peter, 1971). Release of Cr-51 from labelled lymphoid cells (Hayry and Defendi, 1970) was used to detect the effector phase of a mixed lymphocyte reaction occurring when two normal allogeneic mouse peripheral lymphoid cell populations were mixed. The sensitizing phase was measured in the same system by uptake of tritiated thymidine.

**Colony Inhibition Assay**

The colony inhibition assay measures plating efficiency and colonial growth of plated target cells, cells damaged by serum or competent lymphoid cells showing a decrease in plating efficiency or colonial growth. Bases (1964) looked for polyoma tumor antigen on mouse tumor cells by quantitating the plating efficiency of tumor cells incubated with antipolyoma serum prepared in rabbits. Later
the same technique was used by Hellstrom and Sjogren (1965) to detect both H-2 isoantigens and the polyoma transplant antigen in a mouse system with complement and serum. In the first instance of use of this test to detect cell-mediated immunity, Hellstrom (1967) demonstrated that either sensitized lymphoid cells or normal lymphoid cells in the presence of phytohemagglutinin (PHA) would effectively prevent colony formation. Since that time, the Hellstroms have studied cellular immunity and serum inhibition of cellular immunity detected by colony inhibition in a Moloney virus-mouse system (Hellstrom and Hellstrom, 1969) and more recently has devoted efforts to studying the same phenomena in neuroblastoma and other histological types of human tumors (Hellstrom, et al., 1971). An extensive review article is available on this serum-blocking effect called "immunological enhancement" (Hellstrom and Hellstrom, 1970).

Detection of cell-mediated immunity to polyoma TSTA using the colony inhibition technique was accomplished in a rat tumor system (Burstein, 1970). He found that lymph node and spleen cells, but not serum, were effective in reducing plating efficiency of polyoma rat tumor cells.

**Noncytotoxicity Testing Procedures**

The capillary tube method for studying migration inhibition of lymphoid cells has been used to detect reactivity of lymphoid cells against TSTA and transplantation
antigens in animal (Ferraresi, et al., 1970) and human (Anderson, et al., 1970) tumor systems. The test antigen can either be cell-bound or a soluble preparation. This reaction is mediated by a soluble factor called macrophage inhibition factor (MIF) that is produced by sensitized lymphocytes when they are stimulated by specific antigen or by normal or sensitized lymphocytes stimulated by such nonspecific agents as PHA.

Lymphocyte blast cell transformation procedures will also detect lymphocyte reactivity to soluble or cell-associated antigens. In the past this test procedure has been used extensively in human but little in mouse systems due to difficulties encountered in maintaining mouse lymphoid cell viability for the duration of the test. Mangi and Mardiney (1970), who routinely maintain viable mouse lymphoid cells for 9 days in TCF supplemented with 20 percent mule serum, detected both H-2 and weaker histocompatibility differences. He found the antigen-specific response to be maximal at 6 days, whereas maximal PHA response was seen as early as 3-4 days. Neonatal mouse thymectomy will effectively eliminate both the PHA and antigen-specific responsiveness of spleen cells (Takiguchi, et al., 1971).
Target Cell Destruction by NonSensitized Lymphoid Cells

Normal lymphoid cells will destroy allogeneic target cells in the presence of PHA (Ax, et al., 1968; Hellstrom and Hellstrom, 1968) or xenogeneic antibody (Granger and Williams, 1968). Granger studied this phenomenon with mouse and rat lymphoid cells mixed with genetically dissimilar (allogeneic) target cells and surmised that the steps involved in target cell destruction must be: 1) attachment of the nonsensitized lymphoid cell to the target cell by PHA or xenogeneic antibody, or attachment of the sensitized lymphoid cell without these aids; 2) interaction of the two cell membranes and recognition of "self" or "nonself"; 3) and triggering of aggressor lymphocytes to release soluble cytotoxin factor which nonspecifically destroys both target cell and lymphoid cell if "nonself" is recognized. Granger and Williams (1968) later found, however, that PHA or xenogeneic antibody in the presence of normal lymphoid cells and absence of target cells caused lymphoid cells to produce factors which would destroy target cells. Thus, interaction with the target cell membrane and recognition of "self" or "nonself" are evidently not necessary in the PHA or xenogeneic-induced response. Allogeneic cells such as erythrocytes or Burkitt's lymphoma cells, have no cytotoxic activity against target cells in
the presence of PHA (Holm, 1967). Brunner (1968) found that normal allogeneic spleen cells in the absence of stimulation by antibody or PHA did not cause target cell destruction as measured by plating efficiency or Cr-51 release.

**Lymphocyte Harvest**

A wide variety of techniques are presently available to isolate pure lymphoid cell preparations from blood and spleen tissues. In some systems these purified cell preparations are a necessity. For instance, granulocytes are inhibitory to lymphocyte growth *in vitro* (Walker and Fowler, 1965) and mixed lymphocyte reactions are blocked by red blood cell contamination greater than 1RBC/1WBC (Hayry and Defendi, 1970).

It should be noted that there are inherent dangers involved in working with highly purified cell preparations, because synergism with other cell types might be necessary for reactivity to be expressed. Twomey, et al., (1970) found that 1% macrophages were needed to get a mitotic response in mixed allogeneic lymphoid cultures. No response was noted if pure lymphocyte preparations were used. In spleen cell subpopulations obtained by albumin gradient (Eugster, 1970), many of the subpopulations of lymphocytes could not mount a graft-versus-host response, whereas the whole spleen population and some of the subpopulations were
able to do so. Cantor and Asofsky (1970) found synergy between mouse spleen and thymus cells necessary in graft-versus-host reactions. In contrast to Eugster he found that neither spleen nor thymus cell populations alone would bring about a graft-versus-host response.

The first step in many blood separation techniques involves sedimentation of the heparinized blood and removal of the plasma layer. This type of cell-plasma preparation contains most of the platelets, monocytes, and granulocytes of the original blood, whereas defibrination prior to the sedimentation step removes most of the platelets and some of the granulocytes. VonMelen and Unger (1969) found that platelets and granulocytes clumped and could readily be separated from the lymphocytes after centrifugation of the plasma layer and resuspension of the cell button in 0.5 ml. of the plasma supernate.

Agents such as Plasmagel may be added to speed RBC sedimentation (Festenstein, 1968). Hayry and Defendi (1970) got increased lymphocyte yields from mouse blood with Plasmagel if they injected supernate fluid from a Bordetella pertussis culture into the mouse prior to removing the blood. Holm, et al. (1964) followed up a simple sedimentation technique with a glass adherence step. When leukocyte preparations were incubated over glass, the granulocytes were left on the glass and the lymphocytes, red blood cells,
and monocytes removed in the TCF. Red blood cells were then lysed by 0.35% saline. This glass adhesion technique was also used by Wildy and Ridley (1968) who claimed that both monocytes and neutrophils were left attached to the glass.

A number of column techniques for lymphocyte isolation have appeared since the original work by Walker, et al. (1961) who used cotton-containing columns for their column separations. Tarnvik (1970) used a technique combining nylon fiber column filtration with centrifugation in colloidal silica-polyvinylpyrolidone. This yielded a WBC preparation that was 99.5 percent lymphocytes. Zaponin was used if necessary to lyse RBC's.

A number of density gradient techniques are also used to isolate lymphocytes from blood. Albumin was first used for this purpose by Vallee, et al. (1947). An interesting technique used by Thierfelder (1964) involves removal of neutrophils from a preparation with a permanent magnet after the neutrophils have ingested iron particles. Neutrophils from blood with citrate, oxalate, or EDTA anticoagulant did not ingest iron, whereas neutrophils from heparinized blood did. Lymphocytes can be removed from spleen cell preparations as well as from blood by an Isopaque-ficoll gradient centrifugation technique (Boyum, 1968). Various combinations of differential centrifugation (Granger, et al. (1970) and hypotonic lysis (Janowsky, et al., 1964) have
also been widely employed to obtain purified lymphoid cell suspensions from spleens.

**Cell Freezing and Storage**

Cryoprophylactic agents have been employed to protect both viable cells (Vaitkevicius, *et al.*, 1961) and tissue slices (Malinin, 1966). An isotonic glucose (5.3%) solution was used successfully by Kiseliva (1961). Hauschka, *et al.* (1959) found glycerol effective at 10-15% in freeze-storing a large variety of cell types. Polyvinylpyrrolidone at a 10% concentration will freeze protect bone marrow cells, but not as well as glycerol (Bersidsky and Richards, 1964). Dimethylsulfoxide, originally used in the nineteenth century as a solvent in the wood pulp industry, protects cells frozen and stored at -78°C (Bouroncle, 1965) and has been used successfully in chemically defined TCF to store suspension cells in liquid nitrogen (Brown and Nagle, 1965).

Cells thawed after extended freeze storage usually demonstrate properties similar to those of the cells prior to freezing. Persidsky and Richards (1964) found that freeze-stored mouse bone marrow cells retained the ability to protect lethally irradiated isologous mice. Hauschka, *et al.* (1959) checked 82 different types of normal and tumor cells from man, mouse, rabbit, and hamster and found that
after 1-2 years' storage at -78°C, there was no evident change in chromosome number, morphology of marker chromosomes, H-2 isoantigens, strain specific histocompatibility, or drug response. In vitro transforming capabilities of frozen human lymphoid cells (Mangi and Mandiney, 1970) and cytotoxic activity of frozen lymphoid cells (Mullen and Dodd, 1971) have both been demonstrated.
Materials and Methods

Culturing Polyoma Virus

Remove the tissue culture fluid (TCF) from semi-confluent primary mouse embryo monolayers (Winocour, 1960) growing in 30 ml. plastic tissue culture flasks (Falcon Plastics, Oxnard, California). The tissue culture fluid is Minimum essential medium with Earle's balanced salt solution (Grand Island Biological Company, Grand Island, New York), supplemented with 10% heat-inactivated calf serum (Flow Laboratories, Rockville, Maryland), 1X vitamins, 1X L-glutamine, and 1X amino acid mixture (all from Microbiological Associates, Bethesda, Maryland). Antibiotics are Potassium penicillin G, 100 units/ml. (Pfizer Laboratories, New York, New York), streptomycin sulfate, 100 micrograms/ml. (Eli Lilly and Company, Indianapolis, Indiana), and mycostatin, 50 units/ml. (E. R. Squibb and Sons, New York, New York).

Wash the monolayers twice with 2 ml. aliquots of Earle's balanced salt solution (Microbiological Associates). Add 0.5 ml. of virus stock suspension (Polyoma Virus LID-1--ATCC VR No. 252, American Type Culture Collection, Rockville,
Maryland) to each monolayer and adsorb for two hours at 37°C with occasional tilting. After adsorption, add 5 mls. TCF containing 2% calf serum to each flask. Incubate at 37°C until maximal viral cytopathic effect is observed (8-10 days) and harvest. Change the TCF at 4-5 days.

**Harvesting Polyoma Virus**

Shake the culture vessels to remove the cells from the plastic. Pool the contents of all virus-containing flasks alternately freeze and thaw the virus pool three times. Warm to 37°C and adjust the pH to 8.0 before centrifuging at 1500 G's for 15 minutes. This will insure maximal release of the virus from the receptor sites. If greater yields of virus are desired, receptor destroying enzyme (neuraminidase) may be added prior to centrifugation (Crawford, 1969). Collect the supernate, dispense into small sterile vials, and store frozen at -20°C until use.

**Hemagglutination Titration**

The procedure used is a modification of procedures described by Winocour and Sachs (1960) and Fogel and Sachs (1959). Four mls. of guinea pig blood are collected by cardiac puncture and mixed with 4 mls. of modified Alsever's solution (Campbell, 1964). The blood should be used on the same day that it is collected. Centrifuge the cell
suspension at 1000 G's for 15 minutes and discard the supernate. Wash the packed red blood cells (RBC) three times (700 G's for 15 minutes) in isotonic saline in which the pH has been adjusted to 6.8-7.2 with 0.3N NaOH. The washings are carried out with 10 volumes of saline in 12 ml. conical centrifuge tubes.

Resuspend the final button to 1.0% by adding approximately 1 ml. (slight excess) of the packed cells to 99 mls. saline. Finally, the cell suspension is adjusted to 6.6x10^7 RBC/ml by counting on a hemocytometer. The virus is diluted in saline in 10 X 75 ml. tubes (Sever, 1962) making doubling dilutions in 0.5 ml. amounts. If the titer is high an initial dilution of 1:1000 may be made. Add 0.15 ml. of the 1% RBC suspension to each tube. Cover the tubes with Parafilm (American Can Company, Marathon Products, Neenah, Wisconsin) and refrigerate overnight at 4°C. Read the tubes for patterns of viral agglutination while still cold. The number of agglutinating units in the initial suspension is the reciprocal of the highest dilution which causes complete agglutination (Salk, 1944).

Concentrating Polyoma Virus by Centrifugation

Centrifuge the TCF containing the virus particles 2-1/2 hours at 4°C in a model L-2 Beckman centrifuge with
a 25.3 SW head at 25,000 RPM (average G force of 95,753). The cellulose nitrate tubes (Spinco Division of Beckman Instruments, Inc.) are sterilized by ultraviolet irradiation. The pellet is then resuspended in the desired volume of supernate.

Inoculating Neonatal Mice

Neonatal DBA/2J, C3H/HeJ, or C57Bl/6J mice (Jackson Laboratories, Bar Harbor, Maine) less than 24 hours old are injected with 0.02 to 0.1 ml. of virus suspension (volume depends on mouse strain and virus titer). To do this, a 25 gauge needle is introduced subpannicularly from the base of the tail and the virus deposited in the neck region.

Maintaining Mouse Tumor Cell Lines by Cell Culture

The mouse tumor cell lines L-M (Giles, et al., 1966), 4198, and 4198V (Ting and Herberman, 1970) are grown in Eagle's minimum essential medium in Earle's balanced salt solution with 10% fetal calf serum (Microbiological Associates), 2X L-glutamine, and with or without antibiotics. Antibiotics, if used, are 100 units of penicillin, 100 micrograms of streptomycin, and 50 units of mycostatin per ml. of TCF.

The cells are grown in monolayer at 37°C in a 5% CO2 incubator in 8 oz. prescription bottles or in Falcon
plastic tissue culture flasks. Monolayers are subcultured twice weekly. The L-M cells are subcultured by scraping with a rubber policeman and using a pipet to dissociate cell clumps. The 4198 and 4198V cells are subcultured by trypsinizing with 0.25% trypsin (Microbiological Associates) in TCF, Earle's balanced salt solution (BSS) or Mg\textsuperscript{2+} and Ca\textsuperscript{2+} free phosphate buffered saline (Merchant and Kahn, 1967). In each instance one bottle is used to seed two new bottles.

**Maintaining Mouse Tumor Cells by Serial Transplantation**

The 4198 and 4198V cells can be carried by serial transplantation in male C\textsubscript{3}H/HeJ mice. Mice 2-6 months of age are used. To do this, cell suspensions are obtained either from monolayer cultures or from other tumors by techniques outlined in the next section. The animals are etherized and suspended head down by both rear legs. The cell suspension (0.1-0.5 ml.) is injected subpannicularly into the right rear leg using either a 15 gauge or 18 gauge needle on a tuberculin syringe. Cell numbers used for routine transfer of tumors are \(1 \times 10^6\) 4198 and \(1 \times 10^7\) 4198V cells.

**Harvesting Tumor Cells**

Dilute diabutal (Diamond Laboratories, Inc., Des Moines, Iowa) to 6 mg./ml. with diluent that is 10% ethanol,
20% propylene glycol, and 70% water. Anesthetize the tumor-bearing animal by injecting intraperitoneally 0.1 ml. diabutal per 10 gms. of mouse body weight. When the animal no longer possesses any eye reflexes (5-10 minutes) moisten the tumor area with 70% ethanol and remove the tumor tissue aseptically. Place the tissue in a sterile petri dish containing chilled TCF with no fetal calf serum. Cut the tissue into several pieces with scissors and transfer them to a second and third petri plate, each containing about 5 mls. of TCF in order to wash the tissue free of red blood cells. Thoroughly mince the tissue in the third plate with scissors and transfer it to a 100 ml. bottle or trypsinizing flask containing 50-100 mls. of 0.25% trypsin that has been prewarmed to 37°C. Place a sterile teflon-coated magnetic stirrer in the trypsinizing container and trypsinize at 37°C with stirring until the tissue dissociates (30 minutes to 2 hours, depending on the tissue, activity of the trypsin, and pH). Decant the cell suspension carefully, leaving the undissociated clumps of tissue in the bottle. Centrifuge the cell suspension at 200 G's for 5 minutes. Discard the supernate and resuspend the cell pellet in TCF containing no trypsin. Determine cell concentration with a hemocytometer and adjust the cell density to the desired level.
Karyotyping to Validate
Identity of Cell Line

To induce metaphase arrest, TCF on a L-M cell monolayer (30 ml. Falcon flask) is replaced with TCF containing 0.2 X 10^{-3} M colchicine. At the end of a 3-hour 37°C incubation period the colchicine solution is removed and 2.5 ml. of a 0.25% trypsin-TCF is added. After the cells round and leave the surface of the flask, the cell suspension is placed in a 12 ml. Kimax conical centrifuge tube and centrifuged at 100 G's for 5 minutes. The trypsin-TCF is decanted and the cells resuspended in the small quantity of fluid that remains. Five mls. of hypotonic saline prewarmed to 37°C is added. This hypotonic saline is prepared by diluting one part of stock solution in 39 parts of double distilled water. The stock reagent contains 90 gms. of NaCl, 13.65 gms. of Na₂HPO₄, and 2.149 gms. of NaH₂PO₄·H₂O in one liter of solution. The cell hypotonic saline suspension is incubated at 37°C for 30 minutes and centrifuged at 100 G's for 5 minutes.

To fix the cells most of the supernate is discarded, the cells are resuspended in the remaining fluid and 1 ml. of cold freshly prepared fixative (12 ml. glacial acetic acid in 30 ml. absolute methanol) added rapidly. A second ml. of fixative is added and the cells centrifuged at 100 G's for 5 minutes. The supernate is discarded and 2 mls.
of fixative added a second time. The fixative suspended cells are refrigerated at 4°C for 10 minutes or overnight. After refrigeration the cell suspension is again centrifuged, the fixative discarded, and 2 mls. of freshly prepared cold fixative added. After this cell suspension is centrifuged and the fixative discarded, the cells are resuspended in 0.2-1.0 ml. of cold fixative (volume depends on the amount of cells in the button) for application to cold slides. The acid-cleaned slides are placed in doubly distilled water in the freezer. As ice crystals begin to form, the slides are removed for use in the staining procedure. Five or six drops of the cloudy cell-fixative suspension are dropped from a capillary pipet onto a cold wet slide held at a 45° angle to the table top. The slide is drained and waved back and forth in the air until almost dry (Rothfels and Siminovitch, 1958).

The slides are then stained by the following procedure:

a. 5 minutes in distilled H₂O
b. 30 minutes in stain solution*
c. 1-1/2 minutes in 95% ethanol
d. 10 dips in absolute ethanol
e. 10 dips in absolute ethanol
f. 3 minutes in xylene
g. 3 minutes in xylene

h. 3 minutes in xylene

*Stain solution—30 ml. 3% basic fuchsin in 70% ethanol, 180 ml. 5% carbolic acid in distilled water, 27 ml. glacial acetic acid, and 27 ml. formaldehyde are mixed and allowed to stand 24 hours. Filter just before use with a #1 Whatman filter paper.

Finally, the slides are dried and coverslipped with Permount (Fischer Scientific Company, Fairlawn, New Jersey).

Monitoring for PPLO Contamination of Cell Lines

Plates of PPLO agar are prepared using Bacto PPLO agar supplemented with 1% PPLO serum fraction (Difco, Detroit, Michigan). Inoculate the plates either with suspect TCF or cell suspension from monolayer cultures by placing a drop on the surface of the agar and allowing it to dry without spreading. Invert and incubate the plates at 37°C in moist aerobic, anaerobic, and CO₂ environments using BBL Gas Pak jars and envelopes (BBL Division of Bioquest, Cockeysville, Maryland) for 7-10 days. The plates are examined microscopically at this time for PPLO colonies. Dienes' stain (North American Biologicals, Rockville, Maryland) may be used to facilitate identification of the PPLO colonies.

Staining Procedure:

a. With a cotton swab coat a cover glass lightly with Dienes' stain and air dry.
b. Using a wire loop cut out a small circular block of agar containing the suspected PPLO colonies and transfer to a clean glass slide. Place the cover glass, stain side down, on the agar square. Do not press or crush.

c. Examine microscopically after a few minutes and again at the end of one hour. The stain will diffuse into the colonies. Mycoplasma colonies will stain permanently, appearing with deeply stained centers and lighter peripheries. Living bacteria also stain but are completely decolorized after about 30 minutes.

**Immunizing Mice with Tumor Cells**

Harvest tumor cells with either 0.25% trypsin (4198 or 4198V cells) or a rubber policeman (L-M cells). These cell suspensions are centrifuged in 15 ml. screw cap tubes and the cells in the pellet washed 3 times with 5-10 mls. cold FCS-free TCF to remove any FCS from their surfaces. All centrifugations are at 200-300 G's for 5 minutes. The immunization schedule consists of two intraperitoneal injections of $6 \times 10^6$ viable tumor cells in 1 ml. of TCF on days 1 and 6 and one intrasplenic injection of $2 \times 10^6$ viable tumor cells in 0.05 ml. TCF.
Harvesting Splenic Lymphoid Cells
by Differential Centrifugation

This procedure is a modification of procedures outlined by Granger and Williams (1968) and Janowsky, et al. (1964). About 8 mouse spleens (more or less depending on the experiment) are removed aseptically from decapitated normal or sensitized mice by making a longitudinal incision on the left dorsal aspect of the animal. The spleens are weighed and placed in a sterile petri dish containing about 5 ml's of cold TCF (chilled TCF is used throughout this procedure). Mince the tissue with scissors and, using a 15 gauge needle, withdraw and expel tissue repeatedly with a 10 ml. syringe. When the tissue passes freely into the syringe, replace the needle first with an 18 gauge and then a 20 gauge needle each time repeating the procedure. The tissue will be completely disaggregated by the time it passes freely through the 20 gauge needle. Centrifuge the spleen cell suspension at 400 G's for 5 minutes in 15 ml. screw cap tubes, discard the supernate, and resuspend the cell button in 10 ml's. of cold 0.35% sterile saline. This will lyse the red blood cells. After one minute add 1.2 ml. of 5% cold sterile saline to restore isotonicity. Centrifuge the suspension at 400 G's for 5 minutes and resuspend the
button in 5 mls. of TCF. Again centrifuge for 5 minutes at 400 G's. Resuspend the washed spleen cells in 10 mls. of TCF and subject them to the following differential centrifugation procedure:

a. Centrifuge the cell suspension at 75 G's for 1 minute.

b. Transfer the supernate to a fresh tube and centrifuge at 135 G's for 1 minute.

c. Transfer this supernate to a fresh tube and centrifuge at 400 G's for 3 minutes.

d. Suspend the resulting cell button in 10 mls. of TCF. Refrigerate the suspension until cell counts can be made and the cells used to set up assay procedures or frozen.

This procedure routinely yields about 5 X 10^7 cells/spleen, with 95 percent or more being small lymphocytes. Viability as measured by trypan blue is usually 96 percent or higher. A large spleen taken from an "immune" mouse will often yield as many as 2.5 X 10^8 cells.

Harvesting Splenic Lymphoid Cells with an Isopaque-ficoll Single-Step Gradient Centrifugation Technique

Centrifuge the spleen cell suspension for one minute at 75 G's to remove cell clumps and connective tissue. Using
TCF with 10% FCS, bring the volume of the cell suspension up to a final volume determined by the quantity of spleen tissue being processed (5 mls./0.1 gm. original spleen tissue weight). Carefully layer 7.5 mls. of the above spleen cell suspension over each 3 mls. of Isopaque-ficoll separation fluid in 15 ml. siliconized screw cap tubes. Centrifuge at 1200 G's for 7 minutes (1 minute acceleration, 5 minutes at full speed, and 1 minute deceleration). After centrifugation the hazy band of cells located at the interface between the TCF and the separation fluid is removed with a Pasteur pipet and placed 5 mls. per tube into 15 ml. screw cap tubes. To remove the lymphoid cells from the separation fluid, add 11 mls. of TCF to each tube, mix, and centrifuge at 600 G's for 8 minutes. Resuspend the cell buttons in 15 mls. of TCF and centrifuge at 400 G's for 5 minutes to wash the lymphoid cells. Resuspend the cell button in an appropriate volume of TCF and count.

**Freezing and Storing Spleen Cells in Liquid Nitrogen**

To freeze the cells adjust the spleen cell concentration to 2-10 X 10^7 cells/ml. in cold TCF. Prepare an 18% solution of methyl-sulfoxide (DMSO) in cold TCF. Add the DMSO (Eastman Organic Chemicals, Rochester, New York) slowly to prevent an excessive rise in temperature and denaturation of proteins in the tissue culture fluid. This
DMSO is sterilized by autoclaving in 15 ml. screw cap tubes at 115°C for 15 minutes. While agitating constantly, slowly add one part DMSO to one part cell suspension. Using a 5 or 10 ml. syringe with a 1-1/2 inch 20 gauge needle, dispense 1 ml. portions of the DMSO-cell suspension into sterile 1 ml. prescored freezing ampules (Scientific Products, Columbus, Ohio). Do not allow the fluid to fall on the neck region of the ampules. Seal the ampules using a propane torch. Check the quality of the seal after the ampules have cooled by dipping them upside down in cold 95% alcohol. A bad seal will allow alcohol to enter the ampule, causing a visible precipitation of protein. Cool the ampules slowly according to the following schedule:

a. 1 hour at 4°C.
b. 1 hour at -20°C.
c. 1 hour to overnight at -70°C.
d. into liquid nitrogen for storage.

The ampules should be thawed rapidly (less than 45 seconds) by vigorously agitating them in a 37°C water bath. Dip the ampule into 95% ethanol and break it in the neck region by applying pressure on the ampule wrapped in a sterile towel or gauze. Remove the cell-DMSO-TCF suspension from the ampule using a 1 ml. syringe with a 20 gauge needle. Place this cell suspension in a 15 ml.
screw cap tube and add approximately 10 mls. of TCF. Centrifuge at 400 G's and resuspend to the desired volume with TCF.

The mouse tumor cell lines L-M, 4198, and 4198V were freeze-stored in liquid nitrogen as outlined above for spleen cells except at 6 X 10^6 cells/1 ml. ampule in MEM containing 10% FCS and 9% DMSO.

Preparing Tissues for Hematoxylin and Eosin Staining

To prepare fixative dissolve 40 gms. of mercuric chloride in 800 mls. warmed distilled water. Add 50 gms. glacial acetic acid and 200 mls. of 40% formalin. A precipitate may form. Shake before using so the precipitate is mixed. The preparation is stable for up to two years.

Remove the tissue from the animal and wash it in saline or BSS to remove the blood. Fix the tissue for 4 hours if the smallest dimension is greater than 5 mm. or less; 8-12 hours if the smallest dimension is greater than this. Use care not to overfix. After fixation trim one face of the tissue with a razor blade to facilitate subsequent sectioning. Dehydrate the tissue by passing it through a graded alcohol series:

70% ethanol, 12-24 hours
70% ethanol, 12-24 hours
80% ethanol, 12-24 hours (may leave tissue in this for extended time)

95% ethanol, 12-24 hours

To begin the embedding procedure, transfer the tissue from the 95% ethanol to methyl benzoate. Leave it in methyl benzoate until the tissue sinks (a few hours to overnight). The tissue can be stored for up to a week in this chemical. Place the tissue into fresh methyl benzoate again for a few hours to overnight. Embed with parlodion by carrying the tissue through two changes each of 1%, 2%, and 4% parlodion. In each instance the tissue remains in the change of parlodion until it sinks (a few hours to overnight). Drain the tissue on a paper towel so excess parlodion will be absorbed. This will facilitate later sectioning. The tissue is then placed in benzene for 12-24 hours until initiation of the paraffin imbedding step.

Melt 1 lb. of Pathology Embedding Mass (The Ohio State University Lab Stores) and 1/4 lb. of paraffin in a 56°C incubator oven or in a water bath and take the tissue through three changes of this wax mixture (2-3 hours each). The melted wax mixture is poured into a flat glycerine-coated metal container to a depth three times the thickness of the tissue. Place the tissue in the wax trimmed face down. Secure it by rubbing an ice cube over the bottom of the container. After all of the blocks of tissue have been positioned and labeled, let the wax harden to a semi-solid
state and then place the container in a 4°C refrigerator. After the wax has completely cooled and hardened it may be cut and trimmed for sectioning.

Sectioning and Staining with Hematoxylin and Eosin

Prepare 5-7 micron sections of the embedded tissue and float them on the surface of warm water (50°C). Collect the sections on slides that have been coated with a thin film of 1:1 horse serum and water. Dry the slides in a 50-55°C oven for 20-30 minutes. This flattens the sections and helps them to adhere to the slides. Decerate and hydrate the sections by carrying them through three changes of xylene and one change each of 95%, 80%, and 70% ethanol. Each step is 5 minutes long. The yellow sections are placed into 5% sodium thiosulfate until they whiten, indicating the removal of metallic salts. A 5 minute wash under tap water follows. The slides are then stained as follows:

a. Weigert's Hematoxylin 1  3-6 minutes
b. Rinse under tap water
c. 1% aqueous HCl  10-15 dips
d. Rinse under tap water
e. 70% ethanol  5 minutes
f. 80% ethanol  5 minutes
g. Eosin$^2$ 10 minutes
h. 95% ethanol 10-15 dips
i. 95% ethanol 10-15 dips
j. Absolute ethanol 10 dips
k. Absolute ethanol 10 dips
l. Carbo-xylol 5 minutes
m. Clear in xylene for 5 minutes or more
n. Mount with Permount

1Hematoxylin—The working solution is equal parts of stocks A and B. Stock A is 1% Hematoxylin in 95% ethanol. Stock B consists of 4 mls. of 29% aqueous ferric chloride, 1 ml. of 8N HCl, and 95 mls. H$_2$O. The working solution should be prepared immediately before use.

2Eosin—Dissolve 0.2 gm. eosin Y, 0.2 gm. erythrocin, and 0.1 gm. orange G 200 mls. 95% ethanol and 2 mls. glacial acetic acid.

Colony Inhibition Testing

The day before lymphocytes are to be tested for immunocompetence set up the assay plates (60 X 15 mm., Falcon Plastics, Oxnard, California) by adding 8 mls. of TCF, containing enough cells to form 100-150 colonies in the control plates, to each plate (300-500 viable L-M cells or 500-800 viable 4198 cells per plate). Set up four to six plates for each sample. For example, 18 plates might be set up if six were to receive lymphocytes from control normal animals, six were to receive lymphocytes from immunized animals, and six were to serve as controls,
receiving no lymphocytes. Incubate the plates in a CO₂ incubator with 5% CO₂ at 37°C overnight. Add the lymphocytes to be tested in a small volume (less than 1 ml.) directly to the plates without removing the original TCF (5 X 10⁶ viable lymphocytes/plate). Swirl the plates to assure even distribution of the lymphoid cells. Incubate the plates for 3-5 days or until the colonies of target cells are large enough to be counted with a dissection scope at 10X-20X magnification.

At harvest, swirl each plate to suspend loose cells and pour off the TCF. Stain each plate with 3 mls. of crystal violet stain solution (1% crystal violet in 50% ethanol) for 3 minutes. Pour off the stain and gently rinse the plates three times with tap water. Invert the plates to dry and count the colonies at any convenient time.

Monolayer Spot Testing

The day before the assay is to be conducted, semi-confluent monolayers of the tumor target cell are set up by adding 5 mls. of TCF containing approximately 2.5 X 10⁶ viable tumor cells to each 30 ml. Falcon flask. The lymphoid cells are suspended in TCF to a concentration of 4 X 10⁷ viable cells/ml. and 0.25 ml. of this suspension is carefully dropped through the overlaying TCF onto each monolayer. Perform this operation with a 1 ml. tuberculin syringe
equipped with a 20 gauge needle. Move the flasks sparingly after the test has been set up so that the lymphoid cells will be confined to a small area of the monolayer. Check the monolayers at 24-hour intervals for macroscopic or microscopic evidence of target cell destruction. Once target cell destruction is evident (24-72 hours), shake the flasks and pour off the liquid. Air dry the monolayers and stain with crystal violet as in the colony inhibition assay.

Trypan Blue Dye Exclusion

The day prior to the lymphocyte assay prepare the tumor target cell suspension (1.25 X 10⁵ viable cells/ml.) in TCF and dispense 2 mls./tube into sterile pyrex Leighton tubes (Scientific Products, Columbus, Ohio) using an Aupette unit (Clay Adams, Persippany, New York) with a 1 ml. syringe. Incubate the tubes overnight at 37°C in 5% CO₂ at a 15° incline. The final target cell monolayer is about 10 X 30 mm. Remove the TCF from the tubes and add 2 mls. of the appropriate lymphocyte cell suspension. The number of lymphocytes added depends on the system under study. In this investigation, 1 X 10⁶ to 1.2 X 10⁷ viable lymphoid cells/tube are used in various assays. The lymphocyte to target cell ratios are from 7:1 to 20:1 at the time the lymphocytes are added to the tubes. Tube tests are set up in
triplicate. Tubes contain target cells only, lymphocytes only, target cells plus "normal" lymphocytes, or target cells plus "sensitized" lymphocytes. To score target cell viability (12 to 72 hours after the tests are set up) the adhering target cells are first suspended in the TCF with a rubber policeman or trypsin, and then 0.4 ml. of the cell suspension is added to a 10 X 75 mm. Kimax tube containing 0.1 ml. of trypan blue (Grand Island Biological Company). Counts are performed 5 to 15 minutes after the cells are added to the trypan blue. Two counts are performed on samples from each of the three tubes (at least 200 cells/count).

Morphological Alterations in Target Cells

Coverslip cultures are used to check for morphological alterations of target cells in Leighton tube test cultures. At sampling times the coverslips with the target and lymphoid cells in place are removed from the Leighton tubes with forceps and allowed to air dry. These coverslips are then Giemsa stained by the following procedure:

Place the coverslips first in Giemsa stain\(^1\) freshly diluted 1:50 in pH 7 buffer solution\(^2\) for 45 minutes and then into buffer solution for 3-5 minutes. Air dry and mount the coverslips to slides with Permount.

\(^1\)Giemsa stain—Dissolve 0.5 gm. stain powder (Certified, Matheson Coleman and Bell) in 33 ml. glycerol by heating at 55-60°C for 1-1/2 hours. Add 33 mls. of methyl alcohol.
Buffer--Add 61.1 ml. of molar/15 disodium phosphate (Na$_2$HPO$_4$) to 38.9 ml. molar/15 monosodium or monopotassium phosphate (NaH$_2$PO$_4$·H$_2$O or KH$_2$PO$_4$) and bring the volume up to 1 liter with distilled H$_2$O. Store this working buffer in pyrex glassware in the refrigerator no longer than one month before use.

Oxygen Uptake Determinations

Target cells are grown in Leighton tubes, incubated overnight, and lymphocytes added the following day as in the trypan blue test above. The only modification is that 2.5-5.0 $\times$ $10^5$ viable target cells are originally added to the tubes in 3 mls. of TCF. A model 53 YSI biological oxygen monitor (Yellow Springs Instrument Company, Inc., Yellow Springs, Ohio), a model FÈ Haake constant temperature circulator (Polyscience Corporation, Evanston, Illinois), and a model EUW-20A Servo recorder (Heath Company, Benton Harbor, Michigan) are used in this study.

After calibration of the oxygen electrode and recorder (see below) assay for O$_2$ consumption by suspending the cells in the TCF using a rubber policeman or trypsin and placing 3 mls. of this cell suspension into the closed O$_2$ electrode chamber. Allow three minutes for temperature equilibrium at 37°C. A constant temperature circulator is used to maintain 37°C in the O$_2$ electrode chambers.

To calibrate the oxygen electrode and recorder, using distilled water at 37°C equilibrated with air in the chamber, the recorder-electrode combination is set at some
arbitrary level, say 50 units. At 1 atmosphere pressure (760 mm. or 29.92 in. Hg) 5.019 microliters of oxygen is dissolved in each ml. of H₂O equilibrated with air at 37°C. If ambient pressure is 30.66 in. Hg, it follows that:

\[
\frac{30.66}{29.92} \times 5.019 \text{ microliters/ml.} \times 3 \text{ ml./chamber} = 15.4 \text{ microliters O}_2/\text{chamber}
\]

are present in the enclosed oxygen electrode chamber. Thus, 50 chart units are equivalent to 15.4 microliters O₂ in 3 ml. chamber fluid, or a drop of 1 unit is equivalent to consumption of 0.307 microliters O₂.

Record oxygen consumption by the cell suspension for 5-15 minutes or until the slope of the recorder readout graph can be determined. Record data as microliters O₂ consumed/10 minutes/3 ml. cell suspension.

**Chromium-51 Retention**

To label the target cells resuspend 5 \( \times 10^7 \) viable cells in 5 ml. TCF and add a volume of Cr-51 (Chemical form Na₂Cr⁵¹O₄ in saline, 1 millicurie/ml., New England Nuclear, Boston, Massachusetts) containing 20-40 microcuries of label. Incubate at 37°C for 30 minutes in a stoppered tube, tipping the tube several times during this period to keep the cells suspended. Sediment the cells (200 G's for 5 minutes) and wash the button five times with 5 ml. TCF. Save the supernates for counting. Resuspend the final cell button in 30 ml. TCF and count the cell number using a hemacytometer.
To set up the assay tubes, adjust the cell concentration to $0.5 \times 10^6$ viable cells/ml. and add 2 mls. to each Leighton tube. At this time freeze and thaw three Leighton tubes three times, remove the cells and cell debris by centrifugation at 1500 G's for 10 minutes, and count the supernate on the gamma counter (Automatic gamma well counting system, model 4216, Nuclear-Chicago Corporation, Des Plaines, Illinois) to insure that label has attached to the target cells. If desired, this step may be performed just prior to lymphocyte addition by pouring off the TCF, adding 2 mls. fresh TCF, and then freeze-thawing. The latter method will correct for label nonspecifically released from the target cells during the time between setting up the tube cultures and addition of the lymphocytes. Values obtained after TCF change represent 100% lysis of target cells as measured by gamma counting, and other values may be compared to this value.

The following day remove the TCF and replace it with 2 mls. TCF containing $3.5 \times 10^6$ lymphocytes/ml. (more or less as required with the particular system). Save some of the TCF removed to check for release of Cr-51. To sample tube cultures, remove tubes in triplicate from the incubator and pour the TCF into 15 ml. screw cap tubes. Centrifuge at 1500 G's for 10 minutes and decant the supernate into vacutainer tubes. Stopper the tubes and refrigerate until the end of the run when counts are made on all tube samples in the gamma well counter.
Results

The original intent in this study was to detect and perhaps quantitate in vitro immunocompetence of mouse lymphoid cells for the polyoma virus tumor specific transplant antigen. Tumor cells displaying the transplant antigen, lymphoid cells from tumor-cell sensitized mice, and in vitro assay procedures are the elements necessary for such an investigation.

In the initial phases of the study neonatal C57BL/6J, DBA/2J, and C3H/HeJ mice were injected with polyoma virus to induce tumor formation. It was anticipated that cell culture, freeze-storage, or serial transplantation of this tumor once obtained would provide a ready source of polyoma tumor cells. The C57BL/6J and DBA/2J mouse strains were selected for study in light of their marked difference in susceptibility to tumor formation with polyoma virus, the DBA/2J strain readily developing tumors and the C57BL/6J strain rarely doing so. The C3H/HeJ strain was brought into the study after investigations began with the L-M cell because this strain has the same H-2 antigens as the L-M cell.

During the time elapsing between the injection of polyoma virus and the formation of tumors in the mice, the
nonpolyoma C3H mouse L-M cell line was studied to perfect the assay techniques to be employed later in the polyoma system. This cell line was selected because it had been successfully employed earlier by others in lymphocyte-target cell interaction.

Mice of strains DBA/2J, C57BL/6J, and C3H/HeJ were immunized by three intraperitoneal and one intrasplenic injection with this cell. The interaction of sensitized and normal splenic lymphocytes with the L-M target in vitro was then studied. Target cell destruction as measured by trypan blue dye uptake, morphological alterations, Cr-51 release, colony inhibition, and the monolayer spot test were all investigated. In addition, records were kept of splenomegaly in all the test animals.

Target cell destruction was maximal as expected when major H-2 histocompatibility barriers were crossed in the immunization schedule (L-M cells into either DBA/2J or C57BL/6J animals). Less marked although still readily observable target cell destruction occurred within the syngeneic system (L-M cells into C3H/HeJ animals). Nevertheless, it was this syngeneic system that was of the most interest because it was actually measuring immunocompetence of splenic cells against L-M cell specific (Tumor specific?) transplant antigens.
Since the immunization schedule was lengthy and since enough lymphoid cells were obtained in one harvest to run many more tests than time allowed, a technique for freeze-storing the competent lymphoid cells was perfected. It was found that spleen cells stored for extended periods in liquid nitrogen retained their immunocompetence against the L-M cell.

When polyoma tumors were finally obtained (from DBA/2J mice only) maintenance of the cells for study by freeze-storage, transplantation, or cell culture did not prove feasible. Thus, the established cell lines 4198 and 4198V were obtained from C.C. Ting. These polyoma transformed cells were selected because they are from a C3H/He male mouse, as is the L-M cell line, and they have the added advantage of growing as tumors in the C3H/HeJ mouse. The L-M cell line will not do so. According to Ting, the two lines carry vastly different amounts of the polyoma specific transplant antigen. The DBA/2J tumor cells from the polyoma-injected mice were studied briefly by using cells directly from viral induced tumors whenever such tumors were available.

Polyoma Virus

The hemagglutinating titers of polyoma virus stocks grown in this study on mouse embryo monolayers were low. These low titer virus preparations were ultracentrifuged
and resuspended to 1/16 their original volume in tissue culture fluid. Titers by hemagglutination before and after centrifugation corresponded very well with the actual decrease in volume of the virus suspensions, so very little virus was lost during the concentration procedure (see Table 1).

**TABLE 1**

**CULTIVATION, HARVEST, AND TITRATION OF POLYOMA VIRUS STOCK**

<table>
<thead>
<tr>
<th>Virus Stock</th>
<th>Cell Monolayer and Virus Inoculum</th>
<th>Onset of Cytopathic Effect</th>
<th>Harvest Time and Observations</th>
<th>Hemagglutination Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6-day old primary and secondary C57BL/6J mouse embryo LID-1</td>
<td>5 days</td>
<td>8 days Gross Cytopathic Effect</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2-day old primary Swiss Mouse embryo O Virus</td>
<td>5 days</td>
<td>7 days No gross Cytopathic Effect</td>
<td>16</td>
</tr>
<tr>
<td>B</td>
<td>3-day old primary Swiss Mouse embryo A Virus</td>
<td></td>
<td>10 days Gross Cytopathic Effect</td>
<td>16</td>
</tr>
<tr>
<td>C</td>
<td>Virus stocks A and B concentrated 16-fold by ultracentrifugation</td>
<td></td>
<td></td>
<td>256</td>
</tr>
</tbody>
</table>
Three strains of mice were injected with polyoma virus (Table 2). Of these only the DBA/2J strain developed tumors (referred to as POD tumor tissue or cells). Strain variation in susceptibility to polyoma virus oncogenesis is well documented, with the C57BL/6J being particularly resistant and the DBA/2J particularly sensitive to the virus (Jahkola, 1965). The POD tumors which developed in the DBA/2J mice were salivary gland tumors and appeared 5 months after the initial virus injection (see Plates 1-3). No signs of other types of tumors were seen in these mice. DBA/2J mice will normally develop tumors 2-4 months after injection with a sufficient quantity of virus. Longer time intervals are required when small amounts of virus are injected, as was the case in this study.

In an attempt to maintain POD tumor cells for later studies, POD tumor tissue from DBA/2J mice was injected back into DBA/2J mice, grown in cell culture, and frozen in liquid nitrogen. Adult mice injected with $5 \times 10^6$ or $1 \times 10^7$ POD cells failed to develop tumors. Likewise, tumors were never seen in young DBA/2J neonates injected with $2 \times 10^6$ viable cells or male testis injected with $5 \times 10^6$ viable tumor cells. The rationale had been that either the young animals with their immature immune systems or the immunologically privileged testis would provide a good site for POD cell growth. The only method that met with any degree of success was subpannicular implantation of 5 mm
<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Number of Neonates Injected</th>
<th>Virus Dosage/neonate</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HeJ</td>
<td>11</td>
<td>0.1 ml. Stock A</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.1 ml. Stock C</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.05 ml. Stock C</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.02 ml. Stock C</td>
<td>--</td>
</tr>
<tr>
<td>DBA/2J</td>
<td>17</td>
<td>0.1 ml. Stock C</td>
<td>Tumors at 5 months post-injection</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0.05 ml. Stock C</td>
<td>--</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>14</td>
<td>0.1 ml. Stock C</td>
<td>--</td>
</tr>
</tbody>
</table>

"--" No tumor formation during a 12-month observation period.
pieces of tumor tissue. These enlarged initially, but even these had completely regressed inside of two months. Further attempts to initiate transplantation of this tumor should probably utilize irradiated animals.

Recovery of viable POD cells from liquid nitrogen storage was fairly successful, with about a 50% recovery of the cells initially frozen.

In vitro maintenance of viable tumor cells for up to one month as primary cultures was possible. The monolayers seeded with trypsinized cells grew out to near confluency in one week. Degeneration of most of the smaller (probably non-tumorous from their appearance) fibroblasts had occurred by the end of the month. The remaining cells could not be removed from the plastic surface by trypsinization for sub-culture.

Plate 1--DBA/2J mouse with salivary gland POD tumor.
Plate 2--DBA/2J salivary gland tumor, Hematoxylin and Eosin stain, 450X magnification.

Plate 3--Normal DBA/2J salivary gland, Hematoxylin and Eosin stain, 450X magnification.
Serial Transplantation of Tumor Cells

As pointed out above, successful transplantation of the POD tumor tissue was never achieved. The 4198 and 4198V cells were routinely carried subpannicularly in the right rear leg of C3H/HeJ male mice (Plates 4-6).

From the results summarized in Table 3, it is apparent that animal passage of the 4198 cell resulted in an increase in tumor-forming capability. The number of tumor cells required to induce tumor formation decreased with each animal passage. Note that primary monolayer 4198 cells did not bring about tumor induction in all animals injected with $1 \times 10^5$ (1 of 3 animals) or even $1 \times 10^6$ (5 of 6 animals) 4198 cells, but $1 \times 10^5$ 4198 cells were sufficient to induce tumors in all of the animals injected, if the 4198 cells were taken from tissue that had been through two animal passages. Indeed, even as few as $1 \times 10^4$ viable cells from secondary tumor tissue induced tumors in 2 of 3 animals.

Tumors required longer to reach palpable size if monolayer or primary tumor cells were injected than if later animal passage cells were used. Note in Table 3 that $2 \times 10^6$ 4198 cells from a $3^0$ tumor grew out to palpable size in less than one week, whereas a comparable number of monolayer cells required about three weeks, and primary and secondary tumor cells about two weeks.
TABLE 3

TUMOR FORMATION IN C3H/HeJ MICE INJECTED WITH 4198 CELLS

<table>
<thead>
<tr>
<th>Source of Tumor Cells</th>
<th>Number of Animals</th>
<th>4198 Cells/Animal</th>
<th>Days to Palpable Tumors</th>
<th>Animals Developing Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monolayer Cells</td>
<td>6</td>
<td>1.0 x 10^6</td>
<td>19</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0 x 10^5</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0 x 10^4</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>1° Tumor</td>
<td>6</td>
<td>2.0 x 10^6</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0 x 10^6</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.0 x 10^5</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>2° Tumor</td>
<td>3</td>
<td>1.0 x 10^6</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0 x 10^5</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0 x 10^4</td>
<td>24</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.0 x 10^3</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>3° Tumor</td>
<td>2</td>
<td>2.0 x 10^6</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>
Plate 4 -- C₃H/HeJ mouse with 4198 leg tumor.

Plate 5 -- Section of 4198 tumor showing invasion of muscle tissue by tumor cells. Note that tumor cells grow along muscle fascia (mf). Hematoxylin and Eosin, 100X magnification.
Plate 6—Enlargement of field in Plate 5. Note telephase cell (T) and numerous prophase cells (P) indicating active tumor growth, Hematoxylin and Eosin, 450X magnification.

Animals that did not develop tumors after injection with low numbers of 4198 cells showed increased resistance to subsequent injection of large numbers of tumor cells. In one such instance, six animals that had failed to develop tumors after injection of $1 \times 10^4$ or $1 \times 10^5$ 4198 cells in the right rear leg were injected in the left rear leg with $1 \times 10^6$ 4198 cells from a primary tumor. Only two of the six animals developed tumors, whereas even half this number of primary tumor cells brought about tumor formation in all injected untreated animals. When the four remaining tumor-
free mice were injected one month later in the right leg with $1 \times 10^6$ secondary tumor cells, one of them developed a tumor.

Where the 4198V cells were concerned, $1 \times 10^6$ monolayer cells were not enough to induce tumors in any injected C3H/HeJ mice and $1 \times 10^7$ viable cells only induced tumors in about 60% of the animals injected.

**Tumor Cell Harvest**

The three types of polyoma tumor tissue available in this study were POD tissue from DBA/2J mice injected with polyoma virus, and 4198 and 4198V tumor tissues, maintained by serial transplantation.

Viable cell suspensions were readily obtained from POD tumor tissue by the standard trypsinization procedure. A small tumor less than 1 cm. in diameter would routinely yield at least $5 \times 10^7$ and up to $2 \times 10^8$ tumor cells with a viability of 90% or better.

Routine harvest of viable single-cell suspensions from 4198 and 4198V tissues did not prove as successful. Initially a mechanical procedure similar to that used successfully with spleen tissue was tried. This proved very unsatisfactory, yielding a cell suspension with only 10% viability (only $6 \times 10^5$ total viable cells from the tumor). Trypsinization of primary tumors yielded high numbers of cells (up to $2 \times 10^8$ cells from a one cm. tumor.
with viability of 80-90%). As the tumor was serially transferred through C3H/HeJ mice, however, it became increasingly difficult to harvest viable single-cell suspensions from the tissue without encountering formation of a mucous-like material which entrapped a majority of the cells. This material formed after the trypsinized cells were centrifuged to remove the trypsin and the cells were resuspended in tissue culture fluid (TCF). A number of variations in procedure were used in an attempt to avoid this problem, but if the cells were then resuspended in MEM, extensive clumping invariably occurred. Madden and Burk (1961) found that formation of this glairy mucous-like material could be prevented by adding a small amount of DNAase to the cell suspension. This procedure was not tried; but instead, whenever feasible, attempts were made to harvest cells from primary tumor tissue. If later passage tumor tissue was used, the tumor cell harvest procedure was modified so that all steps, including the final cell suspension, were carried out in Ca++ and Mg++ free phosphate buffered saline (PBS). With this modified technique, it was generally possible to obtain at least $5 \times 10^7$ viable tumor cells in a preparation with 80% viability.
Ca++ and Mg++ free PBS Procedure used to avoid cell clumping problem:

Tumor tissue into Wash 2-3 times Trypsinize 
cold PBS and cut into small fragments 
→ in this cold PBS → 1/2-2 hours 
in 0.25% Clumping by 20 min. If remains 
trypsin in PBS at room temperature 
↓

Resuspend Wash 2X Resuspend in Centrifuge 
in cool PBS← with cold← cool PBS ← at 200G for 
and count PBS 5 min.

For trypsinization, 2.5% trypsin in BSS with or 
without EDTA (ethylenediaminetetraacetic acid) was diluted 
out to 0.25% with TCF, Mg and Ca++ free PBS, Mg++ and Ca free 
BSS, or TCF with 10% FCS. Except with difficult tissues as 
noted above, these all proved equally satisfactory. Slightly 
lower total cell yields were perhaps obtained when FCS was 
present in the original trypsinizing flask, but interestingly 
enough, the viability seemed to be better and the clumping 
problem reduced by its presence.

Karyotyping

To insure correct identity of the L-M cell, it was 
karyotyped before work was initiated with it. Chromosomal 
counts of 15 intact metaphase spreads are summarized in 
Table 4. The modal number of total chromosomes per cell is 
58, modal number of biarmed chromosomes 15, and the modal 
number of telocentrics 42. Ten of the 15 spreads, or 66%,
<table>
<thead>
<tr>
<th>Total Chromosome Count</th>
<th>Biarmed Chromosomes</th>
<th>Telecentric Chromosomes</th>
<th>Marker Chromosome Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>16</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>56</td>
<td>15</td>
<td>40</td>
<td>+</td>
</tr>
<tr>
<td>56</td>
<td>15</td>
<td>40</td>
<td>+</td>
</tr>
<tr>
<td>56</td>
<td>13</td>
<td>42</td>
<td>+</td>
</tr>
<tr>
<td>57</td>
<td>15</td>
<td>42</td>
<td>+</td>
</tr>
<tr>
<td>57</td>
<td>13</td>
<td>43</td>
<td>+</td>
</tr>
<tr>
<td>57</td>
<td>14</td>
<td>43</td>
<td>+</td>
</tr>
<tr>
<td>57</td>
<td>14</td>
<td>42</td>
<td>+</td>
</tr>
<tr>
<td>58</td>
<td>16</td>
<td>42</td>
<td>-</td>
</tr>
<tr>
<td>58</td>
<td>16</td>
<td>42</td>
<td>-</td>
</tr>
<tr>
<td>58</td>
<td>15</td>
<td>42</td>
<td>+</td>
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<td>58</td>
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<td>42</td>
<td>+</td>
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<td>16</td>
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<td>60</td>
<td>15</td>
<td>44</td>
<td>+</td>
</tr>
<tr>
<td>60</td>
<td>14</td>
<td>46</td>
<td>-</td>
</tr>
</tbody>
</table>

58 15 42 Modal Counts
 possessed a marker chromosome similar to the E or F marker characteristic of the L-M cell. The karyotype shown in Plate 7 contains 15 biarms, 38 telecentrics, and the marker chromosome.

No karyotypes were performed on the 4198 or 4198V cells because these cells were obtained directly from their original discoverer and there was less chance of mistaken identity.

Plate 7--Karyotype of L-M cell. Note a) telecentrics, b) biarms, and c) marker chromosome, 1000X magnification.
Applying to PPLO Contamination of Cell Lines

Tests for presence of PPLO showed that the L-M cell line carried PPLO. The 4198 and 4198V cells were tested by Ting just prior to shipment to us and found to be negative. Tylosine treatment of the L-M cell reduced the PPLO level, but did not completely rid the cell line of the contaminant. The treatment consisted of three changes of TCF containing 500 micrograms/ml. tylosine (Grand Island Biological) on days 1, 3, and 5.

Various culture media tried for testing purposes included Difco's PPLO agar with added Serum Supplement and Grand Island's prepoured plates and broth. Plates were incubated at 37°C under aerobic, anaerobic, and high CO₂ environments for 8-10 days. Only the Difco plates incubated under anaerobic conditions showed evidence of PPLO growth.

Spleen Cell Harvest-Comparison of Isopaque-ficoll and Hypotonic Lysis Techniques

Two different techniques were used to isolate lymphoid cells from mouse spleens. The Isopaque-ficoll technique did not and the differential centrifugation technique did involve a hypotonic lysis step to rid the cell suspension of red blood cells.
Each of these techniques yielded comparable numbers of lymphoid cells per gram of original wet spleen weight and cell viability invariably fell within the 95-99% range for cells harvested by either of these techniques. In both techniques yields were improved if fewer spleens were processed at one time. This probably is a reflection of initial incomplete tissue disaggregation and tube overload during centrifugation when too many spleens were processed at one time.

Table 5 summarizes results obtained on four different occasions when normal C3H/HeJ mouse spleens were harvested using either Isopaque-ficoll or hypotonic lysis. Using either technique, we could usually recover about $5 \times 10^7$ lymphoid cells from each spleen processed unless the spleen showed signs of splenomegaly in which case the yield per spleen was considerably higher.

In excess of 90% of the cells of the final preparation were medium or small lymphocytes. Other cell types noted in the final cell preparation were monocytes, neutrophils, plasma cells, and a very few myeloid cells. Under ideal conditions, the Isopaque-ficoll preparations contained less than one percent red blood cells. Some red blood cells were seen with the hypotonic technique if the spleen cell button was not completely resuspended in the hypotonic saline before restoration of isotonicity. In the Isopaque technique,
## Table 5

**Lymphoid Cell Harvest from Spleen**

**Comparison of Two Procedures**

<table>
<thead>
<tr>
<th>Type of Procedure</th>
<th>Number of Spleens</th>
<th>Total Wet Weight of Tissue in Grams</th>
<th>Total Viable Lymphoid Cell Yield</th>
<th>Yield in Lymphoid Cells per gm. Spleen</th>
<th>Yield in Lymphoid Cells per Spleen</th>
<th>Percent Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypotonic Lysis</td>
<td>8</td>
<td>0.96</td>
<td>$3.3 \times 10^8$</td>
<td>$3.4 \times 10^8$</td>
<td>$4.0 \times 10^7$</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.40</td>
<td>$2.5 \times 10^8$</td>
<td>$6.3 \times 10^8$</td>
<td>$6.4 \times 10^7$</td>
<td>99%</td>
</tr>
<tr>
<td>Isopaque-Ficoll</td>
<td>6</td>
<td>0.70</td>
<td>$3.9 \times 10^8$</td>
<td>$5.6 \times 10^8$</td>
<td>$6.5 \times 10^7$</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1.26</td>
<td>$2.9 \times 10^8$</td>
<td>$2.3 \times 10^8$</td>
<td>$3.1 \times 10^7$</td>
<td>97%</td>
</tr>
</tbody>
</table>
overloading the gradient layer before centrifugation with too many cells or mixing contents of the tube after centrifugation both led to greater red blood cell contamination.

In terms of number of cells per spleen, more spleen cells were consistently harvested from enlarged spleens. On the other hand, less lymphoid cells per gram spleen weight were harvested from enlarged than from normal spleens. This indicates that splenomegaly observed was in part due to an increase in fluid volume of the spleens and in part due to an increase in lymphoid cells.

Freeze-Storage of Splenic Lymphoid Cells

Splenic lymphoid cells once isolated were either used directly in assay procedures or freeze-stored for later testing.

To determine optimum freeze-thaw-wash conditions, mouse splenic lymphoid cells isolated from the spleens of Swiss albino mice by the hypotonic lysis procedure were frozen in 5% glycerol, 10% glycerol, 9% DMSO, and 4.5% DMSO. Viabilities were checked on 12 vials of cells immediately after removal from liquid nitrogen and thawing. Glycerol at 10% and DMSO at 9% provided more effective protection than either 5% glycerol or 4.5% DMSO (see Figure 1). The three
Figure 1--Viability of Mouse Spleen Cells with Varying Concentrations and Rates of Removal of Cryoprophylactic Agents.
vials from each of the four groups were then washed free of the protective agent by one of the three techniques differing in the rate of removal of the DMSO or glycerol.

A. Rapid Removal--Add contents of vial directly to 4 mls. TCF, centrifuge at 400G for 5 minutes and resuspend in TCF.

B. Intermediate--Rate of Removal--Add 2 mls. TCF slowly to contents of vial over 60 seconds, centrifuge, resuspend cell button by adding 2 mls. TCF slowly, centrifuge, and resuspend button rapidly in TCF.

C. Slow Removal--Add 0.5 ml. TCF slowly to contents of vial. After 1 hour add 1.0 ml. TCF slowly. Wait 20 minutes, centrifuge, and resuspend in TCF.

No decrease in viability of DMSO protected cells was seen regardless of the wash technique employed. With the glycerol protected cells, however, there was a decrease in viability, especially with the 10% glycerol vials. In each instance, the glycerol-containing cells maintained their viability best when the slowest wash procedure was used. Briefly then, 10% glycerol with a very slow wash or 9% DMSO regardless of the wash method allowed for the best
recovery of viable cells. It was decided that 9% DMSO was the method of choice to avoid the inconvenience of the slow wash procedure necessary with 10% glycerol.

Splenic lymphoid cells frozen in 9% DMSO in 1 ml. vials at cell concentrations from 1.5 to $5.0 \times 10^7$ cells/ml. all yielded cell preparations of equal viability upon thawing. Therefore, these concentrations were used interchangeably throughout this study at whichever concentration proved most convenient. It is likely that these cells could be frozen at considerably higher concentrations if the need should arise.

Recovery of splenic cells after extended liquid nitrogen storage is comparable to recovery of the same cells frozen for shorter periods. For instance, the viability of spleen cells thawed 7 days after freezing was 79%, compared to 80% viability after 70 days' storage.

**Freeze-Storage of Tumor Cells**

Methylsulfoxide (DMSO) at 9% proved to be a better cryoprophylactic agent than glycerol at a 5% concentration for the three cell lines used in this study (see Table 6). In 5% glycerol the 4198 cell withstood the freeze-thaw process much better (19% loss in viability) than either the L-M (70% loss) or 4198V cell (53% loss). In 9% DMSO all three cell lines showed about the same viability loss.
### TABLE 6
**RECOVERY OF TUMOR CELL LINES AFTER FREEZE-STORAGE IN LIQUID NITROGEN**

<table>
<thead>
<tr>
<th>Cell and Concentration in Cells/ml. (vial)</th>
<th>Cryoprophylactic Agent</th>
<th>Length of Time Frozen</th>
<th>Percent Viability loss in Freeze-Store Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-M cell, 3.0 X 10^6/ml</td>
<td>5% glycerol</td>
<td>12 days</td>
<td>66%</td>
</tr>
<tr>
<td>4198 cell, 2.0 X 10^6/ml</td>
<td>5% glycerol</td>
<td>12 days</td>
<td>18%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62 days</td>
<td>20%</td>
</tr>
<tr>
<td>4198V cell, 2.0 X 10^6/ml</td>
<td>5% glycerol</td>
<td>12 days</td>
<td>49%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62 days</td>
<td>53%</td>
</tr>
<tr>
<td>L-M cell, 6.0 X 10^6/ml</td>
<td>9% DMSO</td>
<td>7 days</td>
<td>10%</td>
</tr>
<tr>
<td>4198 cell, 6.0 X 10^6/ml</td>
<td>9% DMSO</td>
<td>7 days</td>
<td>11%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14 days</td>
<td>10%</td>
</tr>
<tr>
<td>4198V cell, 6.0 X 10^6/ml</td>
<td>9% DMSO</td>
<td>7 days</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>190 days</td>
<td>7%</td>
</tr>
</tbody>
</table>
With the glycerol-protected cells it was found that the viability of the cells taken directly from the freezing vial was about 10% higher than that of cells after washing and resuspending in TCF. This loss in viability was probably due to too rapid a removal of the 5% glycerol. There was no similar decrease in viability of the DMSO-protected cells when they were washed free of the protective agent.

Varying storage time in liquid nitrogen had little effect on viability of either 5% glycerol or 9% DMSO frozen cells. For instance, 4198 cells frozen for 62 days in 5% glycerol showed a loss of viability of 20%, an increase of only 2% over cells thawed after 12 days' storage in liquid nitrogen, and 4198V cells frozen in 9% DMSO for 190 days showed a loss of 7% viability after thawing as compared to a 5% loss at 7 days.

Usually to culture the thawed cells the contents of one freezing ampule (1 ml. cell suspension) were seeded into one small 25 cm² Falcon flask. No problems were encountered in growing out any of the cell lines previously stored with either protective agent.

Splenomegaly

During the course of this study, splenic enlargement, or splenomegaly, was observed in tumor cell-sensitized
animals. The spleen index is an expression of the amount of splenomegaly, the value always being greater than one. This figure is obtained by dividing the relative spleen weight of sensitized animals by the relative spleen weight of normal animals. The relative spleen weight is the quotient of a spleen's weight in mgs. divided by the animal's weight in gms.

In Figure 2, it should be noted that there is little difference between splenomegaly observed when major histocompatibility barriers are crossed (SI = 1.6) and when the sensitizing cell is of the same H-2 type as the responding animal. C3H/HeJ mice sensitized with L-M or 4198 cells show a SI of 1.4. The most marked splenomegaly was seen in animals bearing large tumors. In each instance the difference between normal and sensitized spleen groups was significant with a P value less than 0.05 as determined by the Student t test. Data are based on four experimental groups of animals, with approximately eight animals per group. The figures are, however, representative of results seen with up to 40 animals of each of the four types.

Histological sections of normal and enlarged spleens (Plates 8 and 9) revealed a marked increase in both small lymphoid cell numbers and intercellular space in the enlarged spleens from sensitized animals. These spleens no longer possessed the normal architecture with red and white pulp
Figure 2-Splenomegaly in Tumor Cell Sensitized Mice.
Plate 8--Spleen from a normal C3H/HeJ mouse. Note clearly delineated red pulp (RP) and white pulp (WP) areas. Hematoxylin and Eosin stain, 100X magnification.

Plate 9--Spleen from sensitized C3H/HeJ mouse. Note homogeneous appearance and intercellular space. Hematoxylin and Eosin, 100X magnification.
areas, but appeared as a rather homogeneous tissue consisting of lymphoid cells with very few red blood cells or red blood cell precursors evident.

**Monolayer Spot Tests**

Using the monolayer spot test, it was possible to check for immunocompetence of lymphoid cells against any or all of the tumor target cells used in this study. This made it possible to determine something about the specificity of the reactions observed. Results are summarized in Table 7.

Normal splenic lymphoid cells rarely caused spot formation on any of the monolayers in this study. C₃H/HeJ animals sensitized with 4198 cells were competent against 4198 cells, but not against POD or L-M cells. Spleen cells from C₃H/HeJ animals with large 4198 tumors or from C₃H/HeJ animals with large 4198 tumors removed one week earlier were not competent against either the 4198 or V cells. This is true even though this group showed marked splenomegaly (see Plate 10). C₃H/HeJ animals with small intraperitoneal tumors resulting from a single injection of 4198 cells 10 days earlier showed a positive reaction against the V and a negative reaction against the 4198 cell, possibly reflecting the greater amount of polyoma-specific antigen on the V cell surface. Finally, it is of interest to note that spleen cells from a DBA/2 animal with salivary gland tumor,
<table>
<thead>
<tr>
<th>Animal Source of Lymphoid Cells</th>
<th>L-M Cell</th>
<th>4198 Cell</th>
<th>4198V Cell</th>
<th>POD Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal C3H/HeJ Mouse</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C3H/HeJ Sensitized with 4198</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C3H/HeJ with large (2 cm) 4198 leg tumor</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C3H/HeJ with large (2 cm) leg tumor removed one week earlier</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C3H/HeJ with small intra-peritoneal 4198 tumor</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBA/2 with bilateral salivary gland tumor induced by polyoma injection</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

+Destruction of monolayer by test lymphoid cells and formation of a "spot."

-No apparent monolayer target cell destruction by test lymphoid cells.
Plate 10--L-M cell monolayers spotted with normal (N) and L-M sensitized (S) C₃H/HeJ spleen cells.
by neonatal injection of LID-1 polyoma virus, were found to be competent against C₃H/HeJ 4198 and V cells, but not against the C₃H/HeJ L-M cell. This is supporting evidence for the presence of polyoma-specific transplant antigen on the 4198 and V cell surfaces and for its absence on the L-M cell surface.

Colony Inhibition Testing

Many investigators claim that higher than normal serum concentrations and/or conditioned TCF are necessary when growing cells in low densities such as those used in colony inhibition plates. With this point in mind, we tested a variety of different TCF's prior to initiating our studies with the colony inhibition test procedure. Parameters measured were plating efficiency and colony growth of the tumor target cells L-M and 4198.

Plating efficiency is obtained by dividing the number of colonies present in the plate at harvest time by the total number of viable cells originally plated. The plating efficiency for L-M cells in TCF containing 10% fetal calf serum (FCS) was 44%, whereas the corresponding value for 15% FCS was only 24%. In 10% FCS, 4198 tumor cells had a plating efficiency of 12%. This value dropped to 2% in 20% FCS. With both the L-M and 4198 cells then, TCF containing 10% FCS proved superior to TCF containing higher concentrations.
Colony growth was followed by scoring the average number of cells per colony at selected times. In Figure 3 it is evident that conditioned TCF (TCF containing 80% fresh TCF and 20% TCF removed from two-day-old cell monolayers and filter sterilized) and the higher serum concentrations were not as satisfactory as nonconditioned TCF containing 10% FCS. Thus, to optimize plating efficiency and colony growth, nonconditioned TCF containing 10% FCS was used for all colony inhibition testing. Table 8 summarizes colony inhibition test results. The percentage values were obtained by dividing the number of colonies per test plate by the number of colonies per control plate, the control plates containing target cells only. Note that lymphoid cell immunocompetence was detectible with $5 \times 10^6$ cells in the C57BL/6J--L-M and C$_3$H/HeJ--L-M systems but not in the C$_3$H/HeJ--4198 system. Lower numbers of lymphoid cells were tried in the C57BL/6J--L-M system and it was found that $2 \times 10^6$ lymphoid cells showed a positive test once and a negative test another time. Less than $2 \times 10^6$ lymphoid cells never showed colony inhibition in any system tested. One problem encountered with $5 \times 10^6$ spleen cells was background which often made it difficult to get accurate colony counts.
Figure 3—Colony growth with various tissue culture fluids.
### TABLE 8

**COLONY INHIBITION TEST RESULTS**

<table>
<thead>
<tr>
<th>Sensitized Lymphoid Cell</th>
<th>Target Tumor Cell</th>
<th>#Lymphoid cells Plate</th>
<th>Normal Lymphoid Cells</th>
<th>Sensitized Lymphoid Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>L-M cell</td>
<td>2 x 10^6</td>
<td>85%</td>
<td>83%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 x 10^6*</td>
<td>91%</td>
<td>62%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 x 10^6*</td>
<td>56%</td>
<td>17%</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>L-M cell</td>
<td>5 x 10^6*</td>
<td>95%</td>
<td>80%</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>4198 cell</td>
<td>5 x 10^6</td>
<td>83%</td>
<td>85%</td>
</tr>
</tbody>
</table>

* Differences between Normal and Sensitized lymphoid cell plates were significant to a P value < 0.05 as tested by the Student $t$ test.

**Tube Cytotoxicity Testing--Dye Exclusion**

Figure 4 depicts trypan blue viability test results obtained when L-M target cells were subjected to C57BL/6J splenic lymphoid cells from animals sensitized with the L-M cell. This experiment was conducted in tubes initially containing 2.35 X 10^5 L-M cells/ml. and 3.3 X 10^6 C57BL/6J lymphoid cells/ml. in 2 mls. of TCF. Since this interaction crosses major histocompatibility lines (H-2 type of L-M cell is k, whereas H-2 type of the C57BL/6J mouse is b), the
Figure 4--Cytotoxicity as measured by Dye Uptake. Allogeneic (C57BL/6J--L-M) system.
early appearance of a large difference in viability between tubes containing normal and sensitized lymphoid cells was not unexpected. The nonspecific cell death seen in normal and control (L-M cells only) cultures after 24 hours resulted from high initial cell concentrations and crowding of the tube cultures as cell numbers increased. This effect was not seen as long as the initial inoculum of cells was held to about $1.25 \times 10^5$ target cells/ml. Note that this nonspecific cell death decreased the magnitude of the difference in viability between the tubes containing normal and sensitized lymphocytes so that the 24-hour samples showed a maximum difference between the normal and sensitized lymphocyte cultures (see Plates 11-13).

Plate 11--Control L-M monolayer (48-hour), Giemsa stain, 200X magnification.
Plate 12--L-M monolayer (48-hour), subjected to C57BL/6J spleen cells from L-M sensitized mice, Giemsa stain, 200X magnification.

Plate 13--L-M monolayer (48-hour), subjected to normal C57BL/6J spleen cells, Giemsa stain, 200X magnification.
Cell Free TCF as a Mediator of Cell Destruction

TCF from the tubes in the previous C57BL/6J--L-M experiment was centrifuged at 400 G's for 5 minutes and frozen at -20°C until a later date when it was filter sterilized using a 0.45 micron millipore filter. This TCF was then added undiluted (1 ml./tube) to Leighton tube cultures containing 6.0 x 10^4 viable L-M cells/ml. At 72 hours these tubes were scored for viability. The results are shown in Figure 5. It is interesting to note that the 12 and 24-hour supernates showed cytotoxic activity, whereas the 48 and 72-hour supernates did not. Since this effect is due to a rising viability in tube cultures with the "sensitized" supernate, either the cytotoxin is being inactivated or destroyed in the initial tube cultures after reaching a peak concentration between 12 and 24 hours. It should also be noted that the highest level of cytotoxic activity, seen at 12 hours, correlates with the maximal rate of cell destruction as seen in Figure 4.

Concentration Dependence of Magnitude of Response

To determine what effect, if any, altering the cell concentrations would have in the onset and magnitude of target cell destruction, an experiment was conducted in which this parameter was varied. Results are summarized in Figure 6.
Figure 5--Cytotoxin Mediated Target Cell Destruction. L-M Target Cell.
Figure 6--Cell concentration dependence of the tube cytotoxicity test. Allogeneic (C57BL/6J--L-M) interaction. Values represent viability differences between normal and sensitized cultures.
Three different cell concentrations were initially placed into the tubes, each concentration being approximately one-half the previous one. The ratio of 7 lymphocytes/1 target cell was maintained and all of the lymphoid cells were from the spleens of L-M sensitized C57BL/6J mice. It can be seen that initiation of the response begins at 0, 12, and 24 hours as the numbers of interacting cells decrease. The response reaches its maximum at 24 hours with the highest cell concentrations, 48 hours with the intermediate cell concentrations, and at 72 hours with the lowest concentrations of cells. Thus, it should be pointed out that the ideal time for detection of this response is dependent upon the initial number of cells in the culture tubes. If tube samples were taken only at 24 hours from the group of tubes containing $1.0 \times 10^5$ target cells and $7.0 \times 10^5$ lymphoid cells, there would be no difference detected between tubes containing normal and sensitized lymphoid cells, whereas samples taken at 72 hours would show an entirely different picture.

**Syngeneic vs. Allogeneic Response**

If other variables are controlled and lymphoid cells from C57BL/6J mice sensitized with L-M cells, C₃H/HeJ mice sensitized with L-M cells, and C₃H/HeJ mice sensitized with 4198 cells are all tested for immunocompetence against the
respective sensitizing target cell, results similar to those depicted in Figure 7 are obtained. Both the L-M and 4198 cells are of C₃H/HeJ origin and still carry the histocompatibility antigens characteristic of that strain of mouse. Thus, the two C₃H/HeJ interactions represent syngeneic responses and the reaction observed is specific for tumor or other non-H-2 antigens on the surface of the 4198 and L-M cells. It is reasonable that the C₃H/HeJ response should be greater against the L-M cell than against the 4198 cell, since the L-M cell has been out of the animal of origin for a longer period of time than has the 4198 cell. This correlates with the observation that 4198 cells will and L-M cells will not grow as a tumor in C₃H/HeJ mice. The C57BL/6J response against the L-M cell is greatest in magnitude because this represents an allogeneic response against H-2 antigens.

Viability of Splenic Lymphoid Cells

One advantage to the dye exclusion tube test is that it is possible to follow not only target cell viability but lymphocyte viability as well. Note in Figure 8 that most of the mouse lymphoid cells are no longer viable by 24 hours. The lymphocytes lose viability most rapidly in tubes containing no target cells. Tubes containing target cells plus sensitized lymphoid cells show a more rapid decline in
Figure 7—Syngeneic versus allogeneic response. Values represent viability differences between normal and sensitized cultures.
Figure 8--Lymphocyte viability by trypan blue.
lymphocyte viability than do tubes containing target cells plus normal lymphoid cells. In this particular test, the lymphoid cells were from L-M sensitized DBA/2J mice (9.5 \times 10^5/ml.) and the target cells were L-M cells (1.4 \times 10^5/ml.).

**Total Target Cell Count**

Data collected in cytotoxicity tube tests for percentage viability can also be used for calculation of total target cell count. Figure 9 depicts typical results for such calculations. In this particular test, L-M sensitized C^3H/HeJ spleen cells were interacting with L-M cells in low concentrations (9.3 \times 10^4 target cells and 6.5 \times 10^5 lymphoid cells/ml.). The low cell concentrations explain the small difference in viability observed between the normal and sensitized cultures (compare this viability curve with that depicted if Figure 7 where 1.5 \times 10^5 target cells and 1.5 \times 10^6 lymphoid cells per ml. were used).

Note that after 24 hours the total number of L-M cells/ml. in tubes containing normal lymphocytes was greater than total L-M cells/ml. in control tubes. This is representative of results seen in other similar runs where tubes also contained low cell densities. The total cell count was lowest in the tubes containing sensitized lymphoid cells.
Figure 9--Total target cell count.
Cytotoxicity in the Absence of Fetal Calf Serum

Since target cells used to sensitize mice were grown in TCF containing 10% FCS, some might argue that the response seen in vitro was a result of sensitization of the experimental animals with FCS. If this were the case, then the target cells would not be playing an active role in the reaction but being destroyed as bystander cells when the FCS sensitized lymphoid cells were stimulated in the tube cultures by FCS in the TCF.

Even though tumor cells were washed thoroughly in serum-free TCF before using them to sensitize the test mice and no anti-FCS was detected in the serum of target cell sensitized mice, one experiment was performed in the absence of FCS to insure that the response observed was indeed a reaction directed against the tumor target cells. Figure 10 shows that not only was cytotoxicity present in the absence of FCS, but target cell destruction in tubes without FCS was more pronounced than in tubes with FCS. This test was conducted with C3H/HeJ spleen cells and L-M target cells. Initial cell concentrations were $9.3 \times 10^4$ L-M cells/ml. and $6.5 \times 10^5$ lymphocytes/ml.
Figure 10--Cytotoxicity in the absence of fetal calf serum. Syngeneic C3H/HeJ--L-M system. Viability values plotted represent differences between normal and sensitized cultures.
Cytotoxicity as Measured by O₂ Consumption

Considering the small mass and low metabolic activity of the mouse lymphocyte in comparison to the target cells used in this investigation, it seemed plausible that measurements of O₂ utilization by cell suspensions consisting of target cells and lymphoid cells should reflect target cell destruction. It was hoped that the technique might even reveal injury to target cells not discernible by dye uptake. Figure 11 shows two curves depicting viability and O₂ utilization differences between cultures containing normal and sensitized lymphocytes. Both the O₂ consumption and the viability data were collected on the same tube cultures. It appears that a difference in O₂ utilization only became apparent some time after cultures were already showing a loss in viability by trypan blue. This probably reflects a difference in sensitivity which might be altered by increasing the number of target cells in the tube cultures. However, even the cell concentrations used were approaching the maximum allowable if nonspecific cell death of control and normal cultures due to overcrowding nutrient depletion, etc. were to be avoided.

Cytotoxicity as Measured by Chromium Retention

When 5 × 10⁷ L-M cells were exposed to 40 microcuries of Cr-51 as outlined earlier in Materials and Methods, they
Figure 11--Cytotoxicity as measured by O₂ consumption. O₂ consumption is difference between normal and sensitized cultures in microliters O₂/10 mins./3 ml. culture fluid. Percentage viability is difference between normal and sensitized cultures.
retained 25% of the label. Sixty-nine percent of the label was recovered in the washes and first supernate and 6% was lost in transfers and on the surface of glassware. With the 4198 and 4198V cells, only 5% of the label was retained by the cells, 89% was recovered in washes, and 6% again lost in transfers. Four percent of the original label was recovered in the first wash, 1% each in the second and third washes, and only about 1/2% in the fourth wash. Since some cells were lost with each wash and only about 1/2% of the original label was being recovered in the fourth wash, this number of washes was judged sufficient and was adopted as routine for the labeling procedure. The greater portion of the label (62-82%) remained in the supernate after the 30-minute incubation for labeling.

For testing with the L-M cell, 1 X 10^6 viable cells representing 4000 CPM under existing experimental conditions were placed into each tube. By the following day when TCF was removed and replaced with lymphoid cell suspensions, 3000 CPM remained with the cells and 1000 CPM was not cell-associated and, therefore, was discarded with the TCF. Of the 3000 CPM remaining on the cells only 2000 CPM were released into the supernate by three freeze-thaw cycles. The other 1000 CPM were retained by the dead lysed cells and cell debris.
In both the 4198 and 4198V systems approximately 3500 CPM were placed into each tube with \(1 \times 10^6\) cells, 2000 CPM were in the TCF by the time of lymphocyte addition, 1000 CPM were released by the cells on freeze-thawing, and 500 CPM were retained on the dead cells and cell debris.

Figure 12 summarizes data gathered from two runs using L-M target cells with L-M sensitized C57BL/6J lymphoid cells and 4198V target cells with 4198V sensitized C\(^3\)H/HeJ lymphoid cells. The C\(^3\)H/HeJ-4198V tubes were scored by both Cr-51 retention and trypan blue dye exclusion, and both curves are presented for comparison. The dye exclusion curve represents, as in previous experiments, the difference in viability between normal and sensitized cultures. Values for the Cr-51 retention curves represent percentage differences in Cr-51 retention between normal and sensitized, where:

\[
\text{Percent Cr-51} = \left(1 - \frac{\text{CPM Test Supernate}}{\text{CPM Freeze-Thaw Supernate}}\right) \times 100
\]

As in earlier experiments with trypan blue, the allogeneic C57BL/6J Cr-51 response is greater than the syngeneic C\(^3\)H/HeJ response. C\(^3\)H/HeJ tube samples at 24 hours revealed a 13% difference in the trypan blue procedure as compared to a 5% difference with the Cr-51 procedure. Peak differences were seen at 24 hours and a negative slope
Figure 12—Cytotoxicity as measured by Cr-51 retention. Values represent differences between normal and sensitized cultures for Cr-51 retention or dye exclusion.
is apparent after 24 hours. This early decline in response is a result of cell death in normal tube cultures seeded initially with high cell numbers (1 X 10^6 cells/tube). The high initial cell inocula were necessary to insure sufficient label for counting.
Discussion

In the initial phases of this study some difficulties were encountered obtaining polyoma virus preparations with high enough virus content to induce tumors in neonatal mice. These low titer stocks were concentrated by ultracentrifugation so they were suitable for injection purposes. Most certainly these low titers were a result of the harvest technique used in early work. The standard procedure was to centrifuge the crude virus preparation at 4°C to remove cells and cell debris leaving the virus in the supernate. Since polyoma virus is strongly cell-associated at 4°C, however, much of the virus was lost in the cell button. Centrifugation at warmer temperatures or after pretreatment of the crude cell-virus preparation with receptor destroying enzyme (Crawford, 1969) would have increased free virus in the TCF.

Other procedures which are known to give higher virus yields are a low temperature of adsorption (Helgeland, et al., 1963) and high concentrations of sodium bicarbonate in the TCF (Amako, 1964).

The appearance of tumors in DBA/2 mice with few or no tumors in C57BL/6J mice injected with polyoma virus was
expected (Jahkola, 1965). No information is available in the literature concerning the relative resistance of the C₃H/He mouse strain to polyoma oncogenesis, but judging from results observed here, this strain of mouse is also at least somewhat resistant to tumor development.

In vitro immunocompetence of mouse lymphoid cells toward polyoma tumor cells was observed throughout this study, and on at least one occasion in vivo immunocompetence was also observed. In this instance four of six animals which had received subthreshold doses of 4198 tumor cells failed to develop tumors on subsequent challenge with sufficient numbers of cells to induce tumors in all of the animals.

In the 4198 and 4198V systems, high passage tumors proved to be unsatisfactory sources of tumor cells from two standpoints: 1) single cell suspensions could not be harvested by trypsinization without encountering problems with a mucinous-like material that entrapped the cells, and 2) the higher passage cells grew into tumors so rapidly that syngeneic mice injected with these cells did not live through the 12-day immunization schedule used to sensitize test animals. Mitomycin C or a similar agent could have been used to prevent outgrowth of the tumor cells, but tumor formation proved no problem within the 12-day period if monolayer tumor cells were used for immunization.
Another less obvious advantage to using monolayer cells rather than cells from tumor tissue is that cells grown in monolayer tend to have antigenic sites exposed that are covered by a sialomucinuous coating of the same cells grown in vivo. A number of workers have found recently (Simmons and Rios, 1971) that treatment of tumor cells with Vibrio cholerae neuraminidase to remove the sialic acid moiety of this coating enhances antigenicity of tumor cells and even leads to tumor regression under proper conditions. It would be interesting if this same sialomucinuous coating were increasing the growth capacity of the 4198 or 4198V late passage cells in the mice and also causing problems with cell harvest due to the formation of the mucinous material encountered on cell harvest.

The numerous instances that have come to light in recent years of contamination of one cell line with another or mistaken identity of cell lines (see Herrick, et al., 1970) indicate the value of karyological or serological testing to substantiate the identity of a line under study, especially if the cell line has passed through a number of different hands.

Karyological data collected on the nonpolyoma cell line used in this study indicated that it was indeed the L-M cell. The modal counts of 15 biarms and 42 telocentrics, and the appearance of the marker chromosome
compare favorably with data collected by Hsu and Merchant (1961) in which the L cell was found to have 60-75 total chromosomes, 10-20 of which were biarmed to have a marker (D) similar to the one observed in this study. In another study Giles, et al. (1966) found 62-70 total chromosomes and 12-17 biarms with an E or F marker chromosome. His results also compare favorably with our own.

The two procedures used for lymphocyte isolation were comparable in most respects, yielding equivalent numbers of equally viable lymphoid cells. Lymphoid cell preparations obtained by either method were competent in target cell destruction, however, since no definitive studies comparing the cytotoxicity of the two types of lymphoid cell preparations were undertaken, quantitative differences might exist. The Isopaque-ficoll technique is a single step technique with less opportunity for contamination and it avoids the harsh hypotonic lysis step used in the differential centrifugation procedure. It does require, however, that one purchase two rather expensive reagents, whereas the differential technique involves no such expense.

For freeze-storage of splenic lymphoid cells or mouse tumor cell lines methylsulfoxide (DMSO) at a 10% concentration proved quite satisfactory. Since its introduction, DMSO has been used with equal success as
glycerol in freezing many animal cells, the only exception being human spermatozoa for which glycerol seems to be superior (Sherman, 1964). This substance is more readily dissolved than glycerol in aqueous preparations, appears to be less toxic to living tissues, and can be washed out of cells with less difficulty than glycerol. No viability loss was observed with lymphoid cells during the wash procedure with DMSO, whereas a rapid wash with glycerol proved fatal to a majority of lymphoid cells in a preparation.

There was significant splenic enlargement in animals sensitized with tumor cells (over and above control animals injected with TCF only), but the amount of splenomegaly did not reflect differences in magnitude of the immune response detectable by the many in vitro test procedures. Spleens from animals sensitized with allogeneic tumor cells were no larger than spleens from animals sensitized with syngeneic tumor cells. Larger lymphoid cell yields from enlarged spleens and histological sections of the spleens both suggested that the splenomegaly observed was a result of an increase in numbers of lymphoid cells as well as an increase in intercellular fluid.

Each in vitro cytotoxicity test procedure used had its disadvantages. The trypan blue cytotoxicity test required numerous cell counts at assay time, thus limiting
the number of tests that might be conducted simultaneously. For oxygen consumption and chromium retention procedures it was necessary to use such high initial target cell numbers that nonspecific cell death due to target cell overcrowding became a problem prior to the time at which maximal target cell destruction should have been observed. The colony inhibition test procedure did not detect tumor specific transplantation antigens under the conditions used in this work unless large numbers of lymphoid cells were used in the test plates, but such large numbers of lymphoid cells led to background problems which made colony counting difficult.

No test procedure could compete with the trypan blue cytotoxicity test for potential information available from one assay. Since there was no difficulty encountered in differentiating between lymphocytes and any of the tumor cells used in this study, data on target cell viability, lymphoid cell viability, and total cell numbers could all be collected on the same tube samples.

The total cell count might be important in some cell systems where inhibition of cell division occurs in the absence of cell death. Proliferation inhibitory factor, or PIF, is the descriptive name for a soluble factor which exercises such an effect on target cells (Green, et al., 1970). Target cells which will not undergo cell division
in the presence of this factor will exclude trypan blue, will reattach to glass and are capable of cell division if placed into fresh TCF. Green demonstrated that a number of cells including Hela, HEP 2, and KB cells were all sensitive to this factor, whereas the L cell was relatively insensitive. It is interesting to note that where cytotoxin is concerned, Granger and Williams (1968) found the L cell to be one of the most sensitive cells and other cells including the Hela cell to be relatively insensitive.

Others do not share this predilection for the dye exclusion test. Wigzell (1965) points out that the dye exclusion technique can be very time consuming and that the whole test must be completed at sampling time. He also feels that isotope labeling provides a more objective test procedure and notes that test samples do not have to be quantitated immediately, but may be stored for a more suitable occasion.

Cytotoxicity by soluble factor(s) was considered in one experiment in this study. In that instance cytotoxic activity was seen in 12 and 24-hour supernates from L-M sensitized C57BL/6J lymphocytes incubated in the presence of L-M target cells, but was not found in 48 and 72-hour supernates from the same cultures. As already pointed out, this would imply degradation or at least neutralization of the cytotoxic factor at 37°C in the presence of target and
lymphoid cells. A simple follow-up experiment would be to incubate some of this soluble factor at 37°C in the absence of any cells to see if the observed inactivation results from instability of the factor(s) at 37°C. Experience of other workers with cytotoxin has indicated that it is heat stable at temperatures up to 70°C for 1/2 hour or even 100°C for 20 minutes, but no one has characterized long-term stability at lower temperatures.

Lymphotoxin does not bind strongly to cell membranes because the toxicity of a cytotoxic fluid is not decreased by adsorption with high concentrations of target cells (Williams and Granger, 1969). Williams and Granger also note that cytolysis is reversible by washing for up to 25 hours after cells are initially exposed to cytotoxin. The site of action of cytotoxin is the cell membrane. Cytotoxin weakens the membrane until it ruptures at one point leading to a characteristic blebbing phenomenon which was quite evident under the microscope during cell counts.

In vitro target cell destruction is a function of the antigenic disparity between target and lymphoid cells (assuming that the lymphoid cells are from target-cell-sensitized animals), and on cell concentrations used in the assay tubes or plates. Target cell destruction was earlier in onset and attained a greater level when major
histocompatibility lines were crossed and/or when cell concentrations were increased in the mixed cultures.

Since the same or about the same tumor cell to lymphocyte ratio of 1:7 was used throughout this study, it is not possible to say for certain whether the concentrations of one or both cells are important in controlling the onset and ultimate magnitude of the cell to cell interaction. It is likely, however, that a certain minimum number of target cells is required to provide sufficient antigenic stimulus to all competent lymphocytes, and that increasing the target cell number above this would not increase the reaction response. Since lymphocytes are motile in culture, a small number of target cells might be sufficient to stimulate this maximal response. However, the short in vitro lifespan of lymphocytes and the necessity of their being metabolically active to participate in target cell destruction (produce cytotoxin) would necessitate a reasonable number of target cells if all the competent lymphocytes were to be active participants.

In contrast to target cell concentration, lymphocyte concentration would most likely be directly related to cytotoxin concentration and thus to the response pattern, for target cell destruction is proportional to lymphotoxin concentration in assay cultures (Williams and Granger, 1969).
Lymphocyte viability of mouse splenic lymphocytes in vitro did not remain high for any length of time under conditions used in these assay procedures. In control assay tubes containing lymphocytes only, the viability had dropped to 30% by 24 hours. Viability remained higher in assay tubes containing target cells. By 24 hours the viability of normal lymphocytes in cultures with target cells was at 60% and the viability in tubes containing sensitized lymphocytes was at 50%. The lower viability of sensitized lymphocytes is probably a result of the activity of cytotoxin on the lymphoid cells, for cytotoxin is non-specific in its action to the extent that it will not only destroy any number of target cells but the lymphocytes which produce it as well. In support of this argument, it was noted that the difference in viability between normal and sensitized lymphoid cells increased during the course of the reaction paralleling patterns seen with target cell destruction.

Improved survival of lymphocytes in the presence of target cells possibly resulted from nutrients supplied by the target cells or from the higher number of cells/surface area in tubes with both cell types. The effect of the target cells on the pH of the tissues culture fluid may also have had some bearing on the viability differences noted. Even though open systems were used in CO₂ incubators
(the Morton Stainless steel caps do not provide a gas-tight seal), the pH in tubes containing only lymphocytes was usually significantly higher than the pH in tubes containing lymphoid cells plus target cells.

Nutrient supplementation or higher cell density might also explain the more rapid increase in target cell numbers in assay tubes containing normal lymphocytes than in control assay tubes containing target cells only.

Cytotoxicity in the absence of FCS with the L-M target cell provided conclusive proof that the response seen in tube cultures was not a response of FCS sensitized lymphoid cells to FCS in the assay TCF. Many workers routinely run target cell-lymphocyte reactions in FCS-free TCF to alleviate variations encountered from test to test with different lots of FCS. Tests were not run routinely in such a manner in this investigation because neither the target cells or the lymphocytes under study maintained viability over any length of time in the absence of serum. Well defined additives such as proteins, peptones, or insulin, or some of the newer chemically defined TCF's might prove useful in this respect.

The tumor cell systems under consideration in this study could prove very useful models for additional research into the mechanisms underlying target cell-lymphocyte interactions. The L-M cell, due to its sensitivity to the
cytotoxic factor, could be a useful detection system for lymphocyte-target cell interactions. To carry out this indirect test it would be necessary to harvest TCF from reaction tubes containing lymphoid and target cells and to test it for cytotoxicity against sensitive L cells by any of the previously discussed assay procedures. Many problems encountered with direct assays would cease to be problems in such an indirect test. TCF samples could be freeze-stored for more convenient testing in large lots. Automatic cell counting units would be more useful if only one cell type were present in the assay tubes. Finally, problems with the chromium retention and oxygen consumption assays which resulted from minimum numbers of cells needed to run the assay procedures could be more readily circumvented.

The 4198-4198V system would be an excellent system for both additional in vivo and in vitro studies. Both the quantitative differences in the amount of tumor-specific antigen found on the cell surfaces (V cell contains 8.8X the amount of polyoma specific antigen found on the 4198 cell) and the availability of the parent 4094 cell which has never been in contact with polyoma virus (and therefore does not carry the tumor specific antigen) would present innumerable possibilities for further investigation.
Summary

Lymphoid cells isolated from the spleens of tumor-cell sensitized mice were shown to be immunocompetent against the sensitizing tumor cell when tested in vitro by such cytotoxicity methods as dye exclusion, oxygen consumption, Cr-51 retention, colony inhibition and spot formation.

Immunocompetence directed against the polyoma transplant antigen was observed with C3H/HeJ splenic lymphoid cells from animals sensitized with the syngeneic polyoma 4198 or 4198V tumor cells. Specificity of the response was shown by demonstrating that splenic cells from DBA/2J animals sensitized with the DBA/2J polyoma-antigen-carrying POD cell were competent against the 4198V cell but not against the L-M cell.

The polyoma-specific immunocompetence was detectible using dye exclusion and Cr-51 retention assays, but not with the colony inhibition procedure. Colony inhibition was observed with the allogeneic and the L-M syngeneic systems.

Immunocompetence against the major histocompatibility antigens was of a greater magnitude than that against the
tumor-specific transplant antigens. For example, splenic cells from C57BL/6J mice immunized with the allogeneic L-M cell were more strongly immunocompetent when tested in vitro against the sensitizing cell than were sensitized syngeneic C3H/HeJ splenic cells tested against the same target cell.
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