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Cell Cycle Kinetics And Antibody Diversity: The Effect Of A Specific Dextran Antigen And Monospecific Anti-Immunoglobulin On Cell Proliferation In The Mopc 104E (Mineral Oil - Induced Plasmacytoma 104, Transplant Subline E) Murine Plasmacytoma.

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CELL CYCLE KINETICS AND ANTIBODY DIVERSITY: THE EFFECT OF A SPECIFIC DEXTRAN ANTIGEN AND MONOSPECIFIC ANTI-IMMUNOGLOBULIN ON CELL PROLIFERATION IN THE MOPC 104E MURINE PLASMACYTOMA

by

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LIST OF ABBREVIATIONS

B cell	'Bursa derived' or bone marrow lymphocyte
^{14}C	Carbon (14)
$CHCl_3$	Chloroform
CPM	Counts per minute
DNA	Deoxyribonucleic acid
Fab	Fragment, antigen binding
Fc	Crystallizable fragment of antibody molecule
FCS	Fetal calf serum
FLM	Fraction labeled mitoses
G1	Gap 1 phase of cell cycle
G2	Gap 2 phase of cell cycle
GF	Growth fraction
Go	Resting phase (non-cycling)
^{3}H	Tritium
$^{3}HTdR$	Tritiated thymidine
HI	Heat inactivated
HOAc	Acetic acid
^{125}I	Iodine 125
Ig	Immunoglobulin
L_I	Hypothetical labeling index

List of Abbreviations (continued)

M.....Mitosis

MEM.....Medium

M_I.....Mitotic index

MOPC.....Mineral oil induced plasmacytoma

Pen-strept.....Penicillin Streptomycin mixture (5,000 units Penicillin and 5,000 mcg Streptomycin/ml)

PHA.....Phytohemagglutinin

RBC.....Red blood cells

S.....S-phase

SRBC.....Sheep red blood cells

T_D.....Doubling time

λ.....Light chain of antibody molecule

μ.....Heavy chain of antibody molecule

I. INTRODUCTION

Statement of the Problem

The study of cell proliferation has emerged from basic studies on bacteria and other lower life forms to a study of animal cells (68, 77, 85). Investigations of control of cell division in bacteria have provided considerable insight into the problems of cell proliferation in general; however, there are apparently more complex levels of control which demand attention in higher animals. For example, the study of lymphocyte proliferation in the immune response has been utilized previously as a model for studying the cell response to certain stimuli in mammals.

The general purpose of this dissertation is to study the control of cell proliferation in the immune response and specifically to investigate the origin of antibody diversity and how antigen, antibody and antigen-antibody complexes exert control over proliferation of lymphoid cells.

The kinetics of the mineral oil-induced plasmacytoma 104, transplant subline E (MOPC 104E plasmacytoma), were investigated since this system represents not only a neoplastic cell line but also synthesizes IgM antibody of a homogenous specificity. Moreover, Leon et al (57) have found that certain dextran linkages are specific for this IgM. Consequently, this system provides an ideal

model to study simultaneously the relationship between antibody synthesis and the cell cycle phases, and antibody/antigen influence on cell proliferation. The cell cycle kinetics, therefore, were essential to interpret these results and to serve as a basis for further studies on this system, i.e., antigen/antibody studies, drug regulation and antisera effects on DNA synthesis and other cell cycle parameters.

T and B Cell Immunoglobulin Surface Receptors

The current immunological concept of antigen recognition is that plasma cell precursors exhibit cell surface immunoglobulins which serve as specific receptor sites for antigen recognition. This idea was proposed in Burnet's theory of clonal selection (12) in 1959 and considerable evidence has been obtained since that time to substantiate it (1, 38, 60, 73, 103).

Most immunologists agree that both thymus lymphocytes (T cells) and bone marrow-derived lymphocytes (B cells) possess immunologic specificity; however, some doubt exists as to whether or not T cells exhibit cell surface Ig. Various techniques have been employed to demonstrate the presence of cell surface immunoglobulin: Sell and Gell (90) showed that anti-Ig could induce lymphocyte transformation; Segal et al (89) and Plotz (78) showed that the ability of cells to reconstitute irradiated recipients was inhibited by affinity labeling; Pernis et al (74), Greaves (38), and Raff et al (82) used I^{125} or fluorescent labeled antibody to Ig on the surface

of bone marrow-derived cells; and Wigzell (103) absorbed antibody-forming-cells onto a column of antigen coated beads. Other attempts at demonstrating cell surface Ig have involved use of radioactive antigens to induce radioactive suicide of the antibody-forming-cells (1, 6).

There is doubt, however, not that T cells lack specificity and that this specificity is mediated through some sort of Ig receptor, but what the nature of the receptor is and how it is expressed on the cell surface. Reports exist in the literature that T cells have no detectable Ig on their surface (101) while others indicate the converse (3, 61, 70).

T cells are thought to migrate directly from the bone marrow into the thymus whereas B cells arise in the bone marrow and migrate to peripheral lymphoid tissues. The B cells are now known to be the precursors of antibody-secreting cells (14, 22, 66) and T cells are responsible for cell mediated immunity, i.e., graft vs host reactions and delayed hypersensitivity.

Most antigens require the presence of both T and B cells for the elicitation of an antibody response and are appropriately referred to as 'thymus-independent' since they are capable of eliciting an antibody response in the absence of T cells (23, 43, 45).

Regulation of the Immune Response

The immune system has a number of ways to exert control over

proliferation of a clone of cells. The first of these is T cell control of B cell response. Evidence of T and B cell interaction was confirmed by Claman et al (14) in 1966. Since then it has been observed that the manner in which T cells respond influences B cells with respect to the class of immunoglobulin synthesized; they can regulate pressure by antigen on B cells and can regulate B cells through T cell soluble mediators. Speculation also exists that T cells possess receptors which bind with low affinity, thus implicating a role for tolerance in T cell control of B cell response.

The following terms are defined in order to provide better understanding of regulation of the immune response.

Immunological Tolerance

Tolerance was defined in 1960 by Medawar (62) as: "an antigen-induced state of specific unresponsiveness where no detectable sign of immune reaction (immune cells or antibodies) to the tolerated antigen is found. It is usually considered as a form of induced immunological non-reactivity... (specific consequence of) exposure to antigen".

Immunological Memory

In contrast to tolerance, immunological 'memory' is the ability of a cell to respond to a secondary challenge by antigen, mediated presumably through immunoglobulin cell surface receptors.

Antigenic Competition

While memory enables cells to respond to a subsequent antigenic challenge, antigenic competition causes suppression of the antibody response whenever more than one antigen is administered simultaneously.

Antigenic Modulation

Antigenic modulation, a phenomenon which may be closely related to tolerance, involves the specific disappearance of a surface antigen induced by antibody directed against the antigen (9, 71, 97). Antigenic modulation was first demonstrated by Old et al (71), who studied modulation of thymus-leukemic (TL) antigens. Since cell surface antibody can be considered as antigen, similar modulation of cell surface Ig might be possible using anti-immunoglobulin sera. Indeed, Takahashi et al (98) and Hutteroth et al (46) studied the modulation of membrane associated immunoglobulins by specific antibodies from lymphoid cells in suspension cultures from normal individuals. Incubation with anti-κ antiserum led to disappearance of both κ and μ antigen; exposure to anti-μ antiserum induced complete disappearance of antigen, whereas κ antigens were only partially affected. These authors suggested that multivalent binding of antigens to cell surface receptors might represent a step in the induction of immunity and tolerance.

Macrophage Influence on the Immune Response

In addition to the T and B cell, the macrophage is the

remaining cell type normally required to elicit an optimal immune response. Macrophages do not synthesize antibody but rather interact with antigen and catabolize and destroy it. They also demonstrate a helper function in presenting the antigen to B cells in a highly immunogenic fashion.

It was once thought that macrophages were responsible for both antigen uptake and synthesis of antibody (87). Only as late as the early 1960's did Fishman (31) and Fishman and Adler (32) show that macrophages were necessary to elicit an optimal immune response.

Cell Surface Antibody-Antigen Reactions

The immune response is dependent not only on the different cell types involved but depends also on the nature of events occurring on individual cell surfaces. For example, lymphocytes respond to mitogens and other antigens by undergoing lymphocyte transformation, a process characterized by synthesis of new DNA and subsequent cell division. There is growing evidence implicating antigen-antibody complexes as a possible trigger for DNA synthesis in the immune response. Oppenheim found that antigen-antibody complexes (Ab-Ag) either stimulated or suppressed lymphocyte transformation depending on whether antigen or antibody was present in excess (72). Banks (4) demonstrated in 1973 that the cellular response of ovalbumin (OA) sensitive lymphocytes was consistently depressed when OA was mixed with anti-OA antisera at concentrations equal to equivalence and 2 times antigen excess with one antiserum and 50 times antibody excess with another antiserum. Other

concentrations varying from antigen excess to antibody excess varied from suppression to augmentation of lymphocyte transformation. The implications for potential application of these phenomena are obvious since a tumor immunologist might desire to enhance the immune response and a transplantation immunologist would desire to inhibit the graft vs host during transplantation therapy.

Antibody, therefore, is capable of inhibiting the immune response, either through a peripheral mechanism (action of antibody is on potentially immunogenic sites of antigen) or through a central effect (the effect of antibody on antibody-forming cells or their precursors) (88).

Antiglobulins, Suppression and Stimulation

In addition to antibody control over the immune response, antiglobulins can also exert control. Sell and Gell (90) demonstrated that antiglobulin reagents trigger lymphocyte transformation and Fanger et al (28) provided evidence that cross-linkage was required in the stimulation of transformation in rabbit peripheral lymphocytes by antiglobulin reagents. Fab^1 fragments of goat anti-rabbit fragment Fab and anti-rabbit fragment Fc were ineffective in the stimulation of transformation but $F(ab^1)_2$ fragments had stimulatory activity comparable to that of the intact antibody. This work supports that of Woodruff et al (107) who provided evidence that univalent fragments do not cause transformation of human lymphocytes.

Recently, Theis (100) demonstrated suppression of delayed hypersensitivity reactions in chickens by passive administration of anti-IgG and anti-IgM and Herschowitz (40) showed that anti-IgG serum suppressed the anamnestic response of rabbit lymph node cells *in vitro*. In these studies anti-IgM treatment did not interfere with antibody formation. The author suggested two mechanisms by which anti-IgG might act to suppress the anamnestic response (1). It is possible that the anti-IgG combines with the receptors and blocks interaction with specific antigen and the subsequent initiation of antibody formation or (2) anti-IgG could act on a cell other than the lymphocyte, such as a macrophage (which has cytophilic antibody on its surface) and thereby prevent antigen processing.

Suppression of DNA Synthesis by Antigen

Zatz *et al* (109) demonstrated depression of DNA synthesis in mouse spleen after intravenous administration of sheep erythrocytes, *Salmonella typhi H*, or keyhold limpet hemocyanin. Depression of DNA synthesis was observed as early as three hours. Three mechanisms were considered by the authors which might account for suppression: (1) Thymus-dependent emigration of DNA-synthesizing cells out of spleen, or immigration of non-DNA-synthesizing cell into spleen in the time interval studies; (2) specific depression of a T-cell population in response to antigen; (3) nonspecific inhibition of DNA-synthesizing cells in response to the antigen administered.

Cap Formation

Antiglobulins can also induce 'cap formation', an event which might play a role in control of immune response. When lymphocytes were labeled in suspension with fluorescein-conjugated anti-Ig antibody three different labeling patterns could be observed depending on conditions of the experiment (39):

(1) Diffuse surface labeling

(2) Patch labeling

(3) Cap labeling.

Cap labeling (accumulation of the stain over one pole of the cell) occurs in suspension at temperatures above 15°C and was the main pattern seen when multivalent anti-Ig antibody was used. Cap formation was almost always accompanied by pinocytosis of labeled membrane (81); however, the fact that pinocytosis of labeled membrane could occur to some extent in the absence of capping (and in the presence of monovalent Fab-anti-Ig) suggested that the stimuli for capping and pinocytosis were different (24). Although capping could occur within minutes it was usually several hours after addition of antiglobulin before most cells lost their surface Ig (81). Attempts to relabel them were unsuccessful until after 12-24 hours. These authors also suggested that immunological tolerance could be related to cap formation and/or pinocytosis of Ig receptors, since it is possible that antigen could induce disappearance of receptors without activating the lymphocyte, and thus cause a state of tolerance. It is interesting to note that Allison (2) observed

a block in lymphocyte transformation (to phytohemagglutinin or concanavalin A) induced by cytochalasin B, a drug which inhibits endocytosis.

The Cell Cycle and Autoradiography

A knowledge of cell cycle kinetics is necessary to interpret the effect of antigen and antiglobulin on tumor cell proliferation. The cell cycle of the MOPC 104E tumor is also important from the standpoint of gaining a better understanding about the growth of neoplastic cell lines even in the absence of antigen and antiglobulin. The cell cycle was discovered originally by Howard and Pelc (44) in 1953, and is used to estimate the amount of cell proliferation in a specific tissue. Figure 1 shows the flow of cells as they acquire the radioactive label and progress through the cycle. (This figure is useful for visualizing the use of the labeled mitoses curve, Figure 4).

Cell cycle kinetics have been examined for a spectrum of cell types. During the past decade cell cycle research has been concentrated primarily on elucidating tumor cell kinetics. Since autoradiography was used to obtain the cell cycle kinetics certain features about radioisotopes must be understood fully.

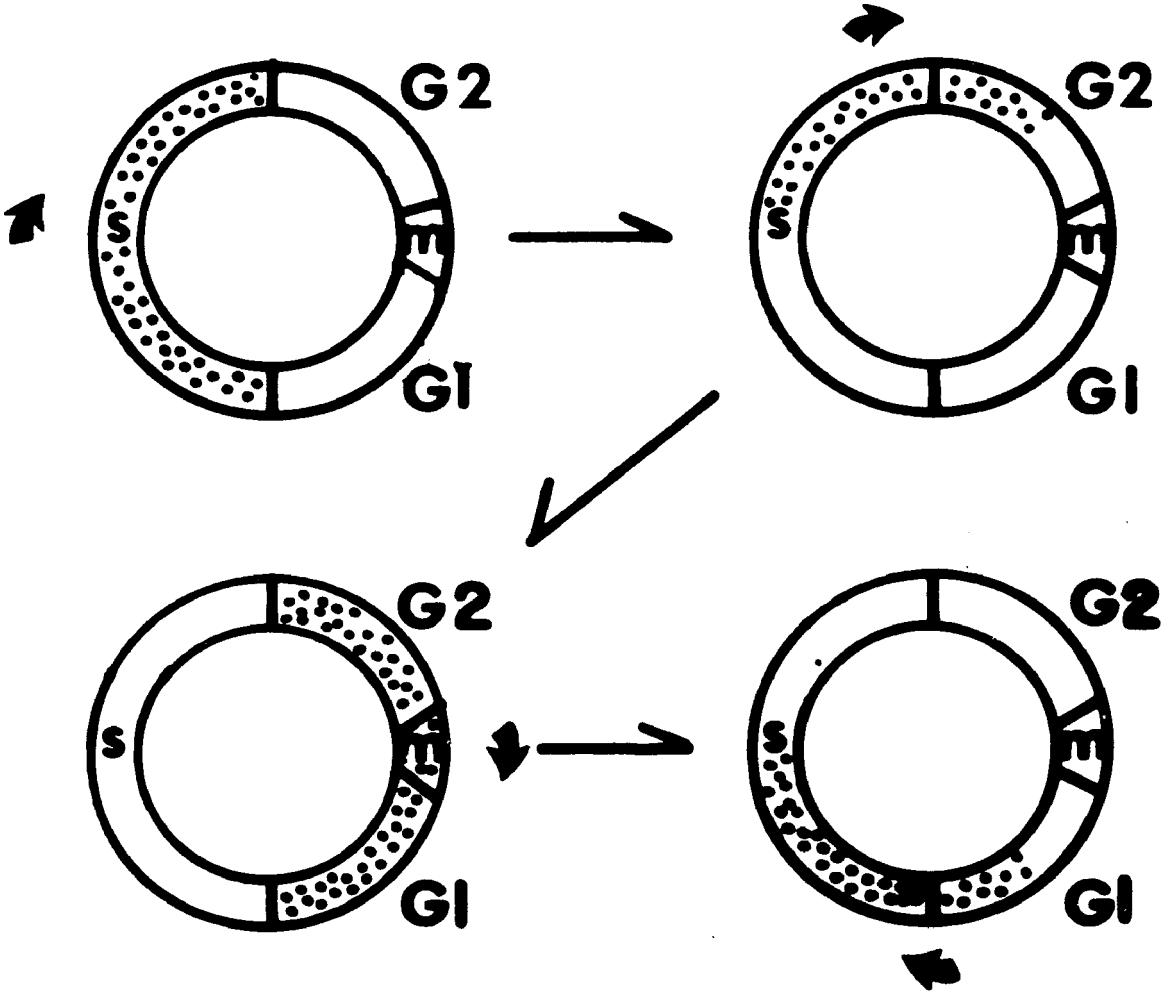
Half-Life

The half-life of a radioisotope is the time required for the disintegration of one-half the atoms. Information on the half-life

Figure 1

Diagram of the cell cycle. Top left, the shaded area represents those cells labeled during a pulse label of tritiated thymidine. Top right, the labeled population of cells progress into G2. Lower left, some cells have gone through mitosis and all mitotic figures are labeled. Lower right, all cells previously labeled have divided and any mitotic figures are unlabeled.

S.....DNA synthesis phase
G2.....Gap 2 or period just prior to mitosis
M.....Mitosis
G1.....Gap 1 or period just prior to DNA synthesis


G2
S
G1
G2
S
G1
G2
S
G1
G2
S
G1

is necessary to determine the length of time an autoradiograph should be exposed and how long a radioactive tissue can be stored before application of the emulsion. For example, the half-life of ^{3}H is twelve years, therefore, only 0.5% of the radioactivity is lost each month so that autoradiographs could be stored for up to several years before applying the emulsion.

Energy

The energy determines the range of particles in the tissues and emulsions. Energy is usually expressed in MEV (million electron volts). Beta particles from a given source vary in energy, while alpha particles are constant in energy. Alpha particles are also less penetrating than beta particles.

Chemical Properties

^{3}H is more often used in autoradiography than ^{14}C because beta particles emitted by ^{3}H are of lower energy and, therefore, have a shorter path and result in better autoradiographic resolution. ^{3}H-labeled compounds are also less expensive and have a much higher specific activity. When working with animals *in vivo*, one should take consideration of the dose of radiation the animal can withstand as well as the route of injection.

Particle Range

The range of ionizing radiation should be considered in making autoradiographs. The thickness of tissue sections and

emulsion, as well as the grain size of photographic emulsion should be coordinated simultaneously with particle range. For example, ^{3}H has a short range and, therefore, one would not want to use sections or emulsion too thick for the particles to penetrate.

Some Previous Autoradiographic Studies Pertaining to the Immune System

The kinetics of plasma cell proliferation were studied in 1961 by Nossal and Makela (69), using autoradiography, which led these authors to hypothesize that immunological memory depended on the persistence, following primary stimulation, of a continuously dividing stem line of premature lymphocytes, reactive at all times to further antigenic stimulation.

The relationship of the cell cycle to tumor growth and control of cell division has been reviewed adequately by Baserga (5). Killman et al (52) and Ponti et al (80) studied the uptake of $^{3}HTdR$ and other radioactive precursors in human multiple myeloma cells.

Continuous cell cultures of mouse myeloma have provided advantageous models for investigating malignant changes of these cells as well as the regulation of globulin synthesis and control of cell proliferation. Pettengill and Sorenson (76) investigated the growth characteristics of the MOPC 21 myeloma tumor and found the myeloma cells to be considerably heterogeneous with respect to the cell cycle, with particular variation in S-phase. Other

characteristics showing change were the amount of Ig synthesized as well as morphological changes in chromosomes.

Sorenson and Pettengill (94) investigated the morphology and functional characteristics of murine myeloma cells proliferating in culture for up to five years. They found that the rate and quantity of globulin synthesis tended to decrease with time in vitro and this was inversely related to growth rate. The specificity of globulin synthesized remained the same over a five year period.

Unlike myeloma cells which synthesize antibody of homogeneous specificity, antibody synthesized by normal cells is heterogeneous in specificity. How this heterogeneity or antibody diversity is arrived at is of considerable importance in the study of the immune system.

Antibody Diversity

The success of the immune system is truly remarkable in providing appropriate macromolecules for combination with the innumerable agents encountered in the lifetime of an individual. In addition to this tremendous versatility of host defense, the immune response involves all aspects of regulatory biology and can provide insight into environment dictated biologic responses in the forms of macromolecular specificity, memory and differentiation, as well as a variety of diseases including cancer.

Several basic theories have been proposed previously to account for antibody diversity and specificity (26, 56, 93, 96). The key question related to diversity is whether antibody production represents a continuing adaptive or differentiation process occurring during the lifetime of an individual organism, or whether it simply represents the preservation from generation to generation of the integrity of the gene pool.

The germ line theory proposes that gene diversity arose during evolution, and lymphoid cells carry a sufficiently large number of variable genes to provide ample diversity for response to all antigens encountered throughout the life span of the organism (96). In this theory the primary problems are those of gene preservation and appropriate regulation of gene expression, since little or no additional diversity can be generated during the life of a single individual. This does not, however, preclude the occurrence of some somatic mutations as the result of DNA replication. Differentiation of the immune response according to this model, then, consists of establishing the expression of genes carried in the germ line to provide cells which produce all classes of antibodies as well as the fine diversity of specific antibodies. The number of required genes is rather large, of course, as well as the demand for precise and complete differentiation. For example, Levin et al (58) has estimated at least sixty different antibody populations could be raised to a single simple hapten if it is placed on an oligolysine backbone of variable size. Given the hundreds of

haptens available, the minimum number of antibodies could easily reach 10^4 to 10^5. This estimate combined with other possible synthetic antigens and the abundance of natural antigens in the environment, would require a very large number of germ line genes to provide the necessary diversity under the germ line theory. Previous estimates on the number of germ line antibody variable genes have been of the order of 10^5. Moreover, as Levin suggested, the high specificity of antibody observed in his experiments with hapten-carrier molecules demands a system capable of recognizing subtle changes in antigen. The major disadvantages for this model are that germ line mutations are the only means of adaption to a changing environment, and both differentiation and gene preservation must be accomplished in the absence of selective pressures.

In contrast to the germ line idea, the somatic theory postulates that the organism is provided through the germ line with a finite number of ancestral genes, and diversification and contemporary adaptation are provided by amplifying this pool through mutations and recombination during somatic differentiation (56, 93). Thus, quite efficient means must be provided for mutations and scrambling of these basic germ lines. Certain features of antibody structure coupled with the extreme diversity required have led to the contention that somatic mutations and/or recombination are too restrictive in potential to account for the observed facts, particularly since it has been implied that each mutation could arise only through probability of mistakes during cell division. It has also

been difficult to rationalize the tremendous stability of certain gene loci in the face of such hypermutability.

The involvement of DNA repair enzymes in creating antibody diversity was suggested as a possibility by Dreyer and Bennett (26) and a model based on this mechanism was proposed in 1966 by Brenner and Milstein (10). However, prior to the present discussion, no comprehensive theory has been proposed using this idea to generate somatic mutations for production of antibody diversity. The Brenner and Milstein model seems no less restrictive than replication errors in providing antibody diversity since in their model a round of cell division is required following each base sequence error that occurs during repair.

DNA Repair

DNA repair functions as a homeostatic mechanism capable of correcting any distortions in the DNA molecule caused by physical or chemical means. Bacteria have been shown to undergo spontaneous mutations (104) apparently due to faulty DNA repair. Xeroderma pigmentosum is an abnormal state in humans characterized by a defect in one component of the DNA repair apparatus (the excision enzyme). Several types of DNA repair have been described including: (1) photoreactivation (50, 91); (2) post-replicative repair or recombinational repair (92); and (3) excision repair (8, 39). The major form of repair reported in mammals is of the excision variety. The

first step involves recognition of damage and endonuclease incision into the phosphodiester backbone of the DNA molecule. An exonuclease excises the damaged bases as well as several adjacent bases on each side of the damaged portion. The gap (5 or 6 bases long) is filled in by a repair polymerase with new bases complementary to the opposing chain. A ligase then seals the gap to complete the repair process.

In view of vast immunological developments in the past several years and the increase in knowledge relative to DNA repair, it is desirable to re-examine the possibility that non-conservative DNA synthesis may provide for antibody diversity with the idea in mind that its role is that of complementing gene diversity provided through the germ line.

In the present model (presented in discussion) somatic mutations are not limited to replication errors but depend in a major way on mistakes made during such non-conservative (segmental) DNA synthesis. In this way mutations can occur repeatedly between cell divisions and in resting cells. In this model selective pressures on antibody structure operate on the contemporary time scale as well as during phylogeny.

II. MATERIALS AND METHODS

Antigen

Dextran, fraction S, from Leuconostoc mesenteroides NRRL B-1355 was provided by Dr. R. N. Hiramoto.

Antibody

IgM provided by Dr. R. N. Hiramoto was obtained from peritoneal cultures of MOPC 104E tumors maintained by serial passage of 10^6 cells in six week old female Balb/c mice and was purified as follows. MOPC 104E ascites fluid was passed through a column of B-1355 dextran conjugated to Sepharose 2B. The IgM which reacts with the dextran was subsequently removed from the column by 0.1 M glycine - HCl buffer pH 2.8. The column was washed also with 0.1 N HCl to remove any strongly bound IgM. This technique is possible because the IgM is directed toward certain linkages of bacterial dextran and, therefore, reacts specifically with bacterial dextran B-1355.

The Tumor

The MOPC 104E plasmacytoma was obtained through the courtesy of Dr. R. N. Hiramoto from Dr. Michael Potter of the National

Institutes of Health. The ascites form of the IgM producing tumor has since been maintained by serial passage of 10^6 tumor cells into the peritoneal cavity of normal, six week old, female Balb/c mice (Laboratory Supply Company, 5010 Mooresville Road, Indianapolis, Indiana).

Antisera Preparation

All antisera were provided through the courtesy of Dr. R. N. Hiramoto of this institution. Preparation of monospecific goat anti-μ was by the method of Hiramoto et al (41), described as follows. An immuno-adsorbent column of purified IgM was prepared by coupling to CNBr-activated Sepharose 2B. Goat anti-mouse IgM serum was passed through this column and washed free of unbound proteins with 0.1 M borate-saline buffer pH 8.6. Adsorbed antibody was then eluted from the IgM column with 0.1 M glycine-HCl pH 2.8. The eluates were then passed through a λ-light chain column to resolve the anti-μ and anti-λ antibodies.

Tumor Cell Cultures

Tumor cells were withdrawn by means of a 2½ cc plastic syringe from the peritoneal cavity of six week old female Balb/c mice bearing ten day ascitic MOPC 104E plasmacytomas. The ascites fluid was centrifuged at 300 x g for five minutes in an International Clinical Centrifuge. The supernatant was removed by aspiration and

the cells were resuspended in 1 ml of Spinner Modified Medium (BBL), 20% fetal calf serum (Gibco) and 0.1% penicillin-streptomycin. This cell suspension was diluted 1/10 and 1/100 so that viability and cell counts could be made simultaneously by adding 0.1 ml of the 1/100 dilution to 0.1 ml of 0.33% trypan blue. The cell suspension was then adjusted to 10^6 tumor cells/ml to serve as stock cell suspension. Samples were then prepared by adding the desired amount of stock cell suspension to 16 x 125 mm plastic incubation tubes with screw caps (Falcon plastics) to obtain a final cell concentration of 10^5 cells/cc. After addition of antigen or antiserum to the appropriate concentration, the samples were mixed well and allowed to stand in ice for 1/2 hour. Subsequent to adding medium to attain the final concentration desired, tritiated thymidine (New England Nuclear, sp. act. 6.7 mCi/mM) was added to each culture to a final concentration of 2.68 μCi/ml medium. A zero time assay for tritiated thymidine was taken and then the samples were placed on a multi-purpose rotator (Scientific Industries, Inc., Model 150V) all under incubation at 37°C. Assays were made for uptake of ^{3}HTdR every two hours for a total of 10 hours. Viability checks were made at each interval by the trypan blue exclusion technique.

Differential Counts

Cytocentrifuge differential counts were made in order to determine the relative number of tumor cells, macrophages and other

nucleated cells in the stock cell suspension. Cell dilutions were made to a final concentration of 10^4 nucleated cells/ml (0.3 ml) and sedimented on microscope slides in a cytocentrifuge. The slides were stained with Wright's stain and the percent tumor cells counted. Based on the total number of nucleated cells as counted with a hemacytometer and the percent tumor cells, the actual number of tumor cells was calculated.

Nucleopore Filter Technique for Uptake of Tritiated Thymidine

These experiments were based on the nucleopore technique of Evans and Normal (27) with a modification by Gaudin (34) which utilizes a counting solution to lyse the cells prior to filtering and washing. Incorporation of ^{3}HTdR was measured by liquid scintillation in counts per minute (CPM).

Radial Hemolysis Technique for the Detection of Complement (36)

Radial hemolysis in gel plates was performed to compare the complement lysing effect of dextran-IgM complex in the presence of normal fetal calf serum and guinea pig complement respectively. Essentially, this method involved oxidation of the dextran (fraction S Leuconostoc mesenteroides NRRL B-1355) (47) with periodate and coupling this reaction product to SRBC. Pooled ascites fluid from the MOPC 104E tumor (20 µl dil 1/64) was added to each well of the plate holding the dextran conjugated SRBC in agarose. The plate

was then placed in the refrigerator overnight to allow the IgM to diffuse out of the well and coat the SRBC by binding the dextran. The plate was removed from the cold and 20 µl guinea pig complement or other medium components were added to each well. The plates were placed in an incubator at 37°C for two hours and then photographed to record evidence of cell lysis.

The Growth Curve

Sixty, six week old female Balb/c mice were inoculated with 3×10^6 tumor cells by injection into the peritoneal cavity and distributed at random, six per cage. The growth curve was established by counting total tumor cells every two days, through the eighteenth day, after injection. Five mice were sacrificed at each time interval and total cell counts were made with a hemacytometer after washing the peritoneal cavity four times with cold saline.

The appropriate dilutions of 10^4 to 10^5 nucleated cells/ 0.3 ml were made and the cells were sedimented by centrifuging on slides in a cytocentrifuge. The slides were then stained with Wright's stain and the percent tumor cells was determined on the basis of cell morphology and intensity of staining, under an ordinary light microscope. Cell viability was monitored by the tryphan blue exclusion technique.

The Labeled Mitoses Curve

The fraction of labeled mitoses (FLM) was studied 10 days after injection of 10^6 tumor cells. Twenty microcuries (μCi) of tritiated thymidine, ^{3}HTdR (New England Nuclear, specific activity 6.7 ci/mM) were injected intraperitoneally into each mouse at zero time. Of the four groups of mice which were used, two groups were assayed for the 0-8 hours period, one group 10-20 hours and the last group 22-36 hours. This was done in order to circumvent any possible changes in cell cycle time due to the withdrawal of too much ascites fluid from an individual mouse over an extended period of time.

Ascites fluid (0.05 ml) was withdrawn from each mouse at each time interval after injection of ^{3}HTdR. The cells were washed twice with cold saline, centrifuged and the supernatant discarded. The cells were then fixed for 4 minutes with cold Carnoy II solution (6 parts EtOH, 1 part glacial HOAc, 3 parts $CHCl_3$). The cells were centrifuged, the supernatant discarded and fresh medium added. Several drops of this solution were spread uniformly on separate slides with a Pasteur pipette and autoradiography was performed with Ilford K-2 liquid emulsion. The slides were then placed in black Bakelite boxes, wrapped with black tape, covered with aluminum foil, and stored at 4°C for three weeks before developing. At least 50-100 labeled mitoses were counted for each mouse at each point in time. Background grains were estimated by counting the

number of grains over a given area, not above cells. Cold slides were also prepared by dipcoating slides prepared from cells not incubated with tritiated thymidine. Mitotic figures with three or more grains were considered labeled. At least three mice were used to measure the percentage labeled mitoses at each point in time. Figure 2 shows autoradiographic silver grains on a tumor cell during mitosis.

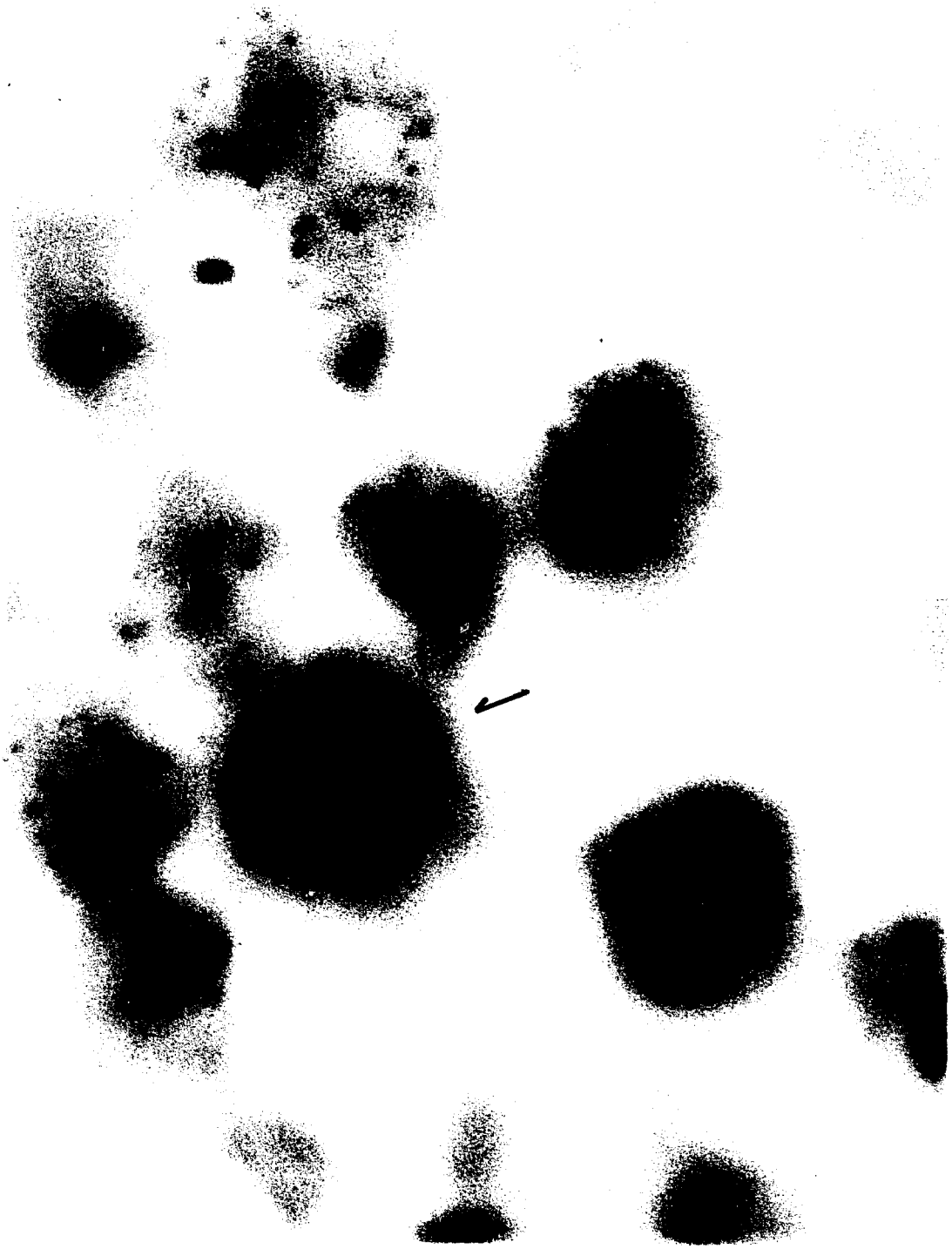
Identification of Labeled Mitotic Figures

It is essential to identify properly the cell population on which autoradiographic analysis is to be applied. In surveying more than 100 reports of biochemical and pharmacological studies using ascites tumors, Stewart et al (95) found only three which mentioned the presence of other cell types in the ascitic tumor suspension. "Most reports showed no evidence that heterogeneity of the cellular composition had been considered in interpretation of the experimental data."

A typical example of a heterogeneous ascites tumor is the MOPC 104E investigated by Stewart et al (95) which contains polymorphonucleocytes, red blood cells, small and large lymphocytes, and tumor cells as well as macrophages, etc. MOPC 104E tumor cells, however, can be easily identified on the basis of cytologic examination. Furthermore, when scoring cells as labeled or unlabeled mitotic figures, the higher frequency of mitoses of tumor cells vs other

Figure 2

Photograph of a tumor cell in metaphase during mitosis. The cells are slightly out of focus in order to show clearly the photographic grains which are in the plane above the cell.



cell types further reduces any already small error in cell identification. For example, small lymphocytes are end cells and do not divide except upon appropriate stimulation by antigen and mouse macrophages exhibit a mitotic rate of about 0.03% (50 x less than the tumor cell population) (7). Macrophages normally divide in the bone marrow and infiltrate to the site of inflammation. Macrophages from the peritoneal cavity of MOPC 104E containing Balb/c mice show very few labeled macrophages and, of course, labeled macrophages in mitosis would represent an even smaller fraction of the cell population. Consequently, identification of tumor cells in this system based on cell morphology, i.e., a large basophilic nucleus and large cell diameter, combined with the much higher tumor cell mitotic frequency eliminate any serious error due to miscounting labeled mitotic figures.

Labeling Index (Potential or Calculated)

The potential labeling index (L_I) was determined by the method of Cleaver (15) expressed mathematically as follows:

$$L_I = e\left[\frac{\ln 2\ (T_{G2} + T_m)}{T_c}\right] e\left[\frac{\ln 2\ T_s}{T_c} - 1\right]$$

Where T_c = cell cycle time

T_m = mitotic time

T_s = time of DNA synthesis

T_{G2} = time of G2 phase

Proliferative (Growth) Fraction

The fraction of cells actively proliferating was termed the growth fraction by Mendelsohn (63) and may be ascertained from the following equation.

$$\text{Proliferative Fraction} = \frac{T_I}{L_I} = \frac{\text{cells in S phase / (proliferative + non-proliferative cells)}}{\text{cells in S phase / proliferative cells}}$$

$$\frac{T_I}{L_I} = \frac{\text{proliferative cells}}{\text{proliferative + non-proliferative cells}}$$

Where T_I = thymidine labeling index

$$T_I = \frac{\text{labeled cells}}{\text{labeled + unlabeled cells}}$$

T_I is determined by counting the number of cells showing label after a one-half hour pulse of tritiated thymidine. The calculated labeling index (L_I) is based on the method of Cleaver (15) as described under labeling index.

The growth fraction (GF) can also be estimated from cell cycle time (T_c) and the 'observed' doubling time (T_D).

$$GF = \frac{T_c}{T_D}$$

The 'potential' doubling time (T_D) for a population with a growth fraction less than one can be obtained likewise from a knowledge of T_c and GF.

$$T_D = \frac{T_c}{GF}$$

DNA Repair in T Cells

Five (5) cc of peripheral blood were withdrawn from a sixteen year old male agammaglobulinemic by means of syringe with needle and put into a glass test tube containing five glass beads. The blood was defibrinated by rotation for 15 minutes in a 37° incubator and then decanted into another test tube containing approximately 250 mg of carbonyl iron powder and 1.3 ml of 1% methyl cellulose in phosphate buffered saline. The tube was allowed to sediment in an upright position for 1-15 minutes in the incubator. The clear lymphocyte-rich supernatant was carefully removed with a Pasteur pipette, transferred to a plastic test tube and centrifuged in a Serofuge at 3,000 RPM for 5 minutes. The supernatant was discarded leaving a RBC-lymphocyte pellet. Either anti-Rh or anti-A, B was added to the cell pellet depending on blood type:

Type O^+:	4-5 drops of anti-Rh
Type A, B or AB:	4-5 drops of anti-A, plus
	1 drop of anti-Rh

The tube was shaken until RBC agglutination was noted. The entire contents were transferred to a plastic Spinco test tube and centrifuged for 3 seconds in a microfuge (RBCs agglutinate). The lymphocyte-rich supernatant was transferred to another Spinco test tube. Cells were centrifuged again, for 10 seconds and the lymphocytes formed a cell pellet. The supernatant was discarded and the

pellet was resuspended in Hank's solution. The cells were serofuged for 3 seconds, the lymphocyte rich supernatant was removed to agglutinate the remaining RBCs and the cell count adjusted with Hank's solution based on counts made with a hemacytometer. After the cells were divided equally into two separate culture tubes, 20 µl hydroxyurea were added to a final concentration of 10^{-3} M to inhibit any semi-conservative DNA synthesis and the tubes were centrifuged at 500 x g for 5 minutes in an International Clinical Centrifuge. The supernatant was withdrawn and 2 ml of phosphate buffered saline added to each tube. The contents of tube #2 were poured into a 50 ml beaker and irradiated from a distance of 15 cm with 254 nm light for 10 seconds (dose rate 9 ergs/mm^2). Both tubes were centrifuged and the supernatant removed. Tritiated thymidine (10 µCi in 0.1 cc) was also added to each tube to a final concentration of 5 µCi/ml. The tubes were then put on a rotator for 2 hours while incubating at 37°C. The tubes were removed from the rotator and cell counts were taken with a hemacytometer. One (1) cc of each culture was added to separate 8 inch test tubes. Tritiated thymidine incorporation was assayed by the method of Norman and Evans (27) as modified by Gaudin et al (34). Liquid scintillation counting was used to measure the incorporation of ^{3}HTdR (a measure of DNA repair in lymphocytes). Aquasol (New England Nuclear) was used as the scintillation cocktail.

Developing and Staining of Autoradiographs

Developing

Slides were developed for 5 minutes in D-19 developer with occasional agitation, rinsed for 10 seconds in water and then put into a tray containing Kodafix solution for 8 minutes. The slides were air dried after rinsing in two changes of distilled water 5 minutes each. All staining and developing was carried out at 18°C.

Staining

Slides were hydrated for 5 minutes and then stained in toluidine blue for 2-5 minutes. After rinsing in water the slides were dehydrated by rinsing 15 seconds each in successive baths of 70%; 95% and 100% ethanol. Slides were cleared twice in xylene 5 minutes each and then mounted with Permount.

TABLE I

Experimental Materials

Spinner Modified Medium	Baltimore Biological Laboratories (BBL)
Fetal Calf Serum	Grand Island Biological Company (GIBCO)
Penicillin-Streptomycin Mixture (5,000 Units Penicillin and 5,000 mcg Streptomycin/ml)	Grand Island Biological Company
Tritiated Thymidine	New England Nuclear
Multipurpose Rotator	Scientific Industries, Inc.
Ilford K-2 Liquid Emulsion	Ilford, LTD
D-19 Developer	Kodak
Kodafix Solution	Kodak
Bakelite Boxes	Sargent-Welch Scientific Company
Permount	Fischer Scientific Company
Slides, Frosted End	Corning
Coverslips	Corning
Aluminum Foil	Viking
Trichloroacetic Acid	Sargent-Welch Scientific Company
Sodium Pyrophosphate	J. T. Baker
Glacial Acetic Acid	DuPont
Culture Tubes, w/Screw Caps 16 x 125 mm	Falcon Plastics
Sodium Cetrimide Solution (500 mg/10 cc distilled water)	Sigma Chemical Company

TABLE I (continued)

Scintillation Vials	New England Nuclear
Nucleopore Filters (0.8 μ)	Nucleopore Membrane Filters
Balb/c Mice	Lab Supply Company
Sodium Phosphate	J. T. Baker Chemical Company
Counting solution was prepared by the addition of 5 ml glacial acetic acid to 10 ml buffered formalin per liter distilled water.	
Aquasol Liquid Scintillation Cocktail	New England Nuclear
Trypan Blue Stain	Allied Chemical
Toluidine Blue 0.3% in 1% Borax	Allied Chemical
One (1) cc Plastic Syringes	Becton-Dickinson
Sodium Borate	J. T. Baker Chemical Company
Hydroxyurea	Sigma Chemical Company
Absolute Ethanol	U. S. Industrial Chemicals
Xylene, for Histological Purposes	Sargent-Welch Scientific Company
Chloroform, Reagent Grade	Merck
Sodium Chloride	J. T. Baker Chemical Company
Millipore Filter Apparatus	Millipore Company
Instruments	
Beckman DPM-100 Liquid Scintillation Counter	Beckman Instruments
Incubator	Blue M Electric Company

TABLE I (continued)

Hemacytometer	American Optical
Microscope (Phase Contrast)	American Optical
International Clinical Centrifuge	International Equipment Company
Cytocentrifuge	Shandon Elliott Company
UV-Lamp (8 Watts)	Arthur H. Thomas Company
Mettler Balance	Mettler Instrument Corporation

III. RESULTS

The Cell Cycle Kinetics

The cell cycle kinetics have been elucidated previously for a variety of animal tumors (5). The kinetics of tumor growth provide the necessary data for construction of models for *in vivo* and *in vitro* proliferation of these tumors. Such models, of course, may be found useful in planning tumor therapy.

Mouse myelomas make particularly interesting models for tumor growth since they not only represent a cell line which has lost control of cell proliferation, but also synthesize antibody of a homogenous specificity.

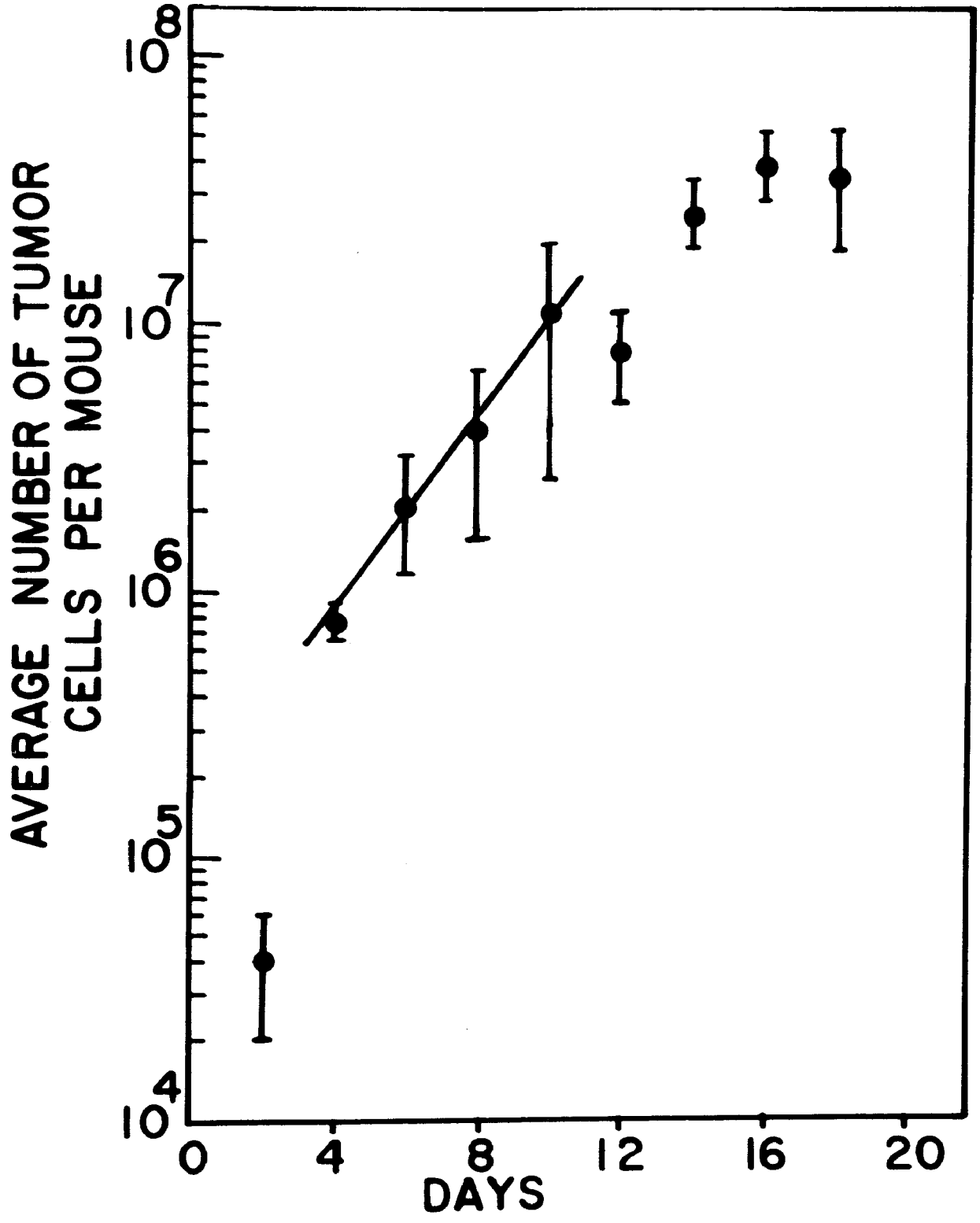
It was necessary to determine the cell cycle kinetics of the MOPC 104E plasmacytoma in order to interpret more fully the effect of antigen and monospecific antisera on cell proliferation.

The Growth Curve Experiment

First, the growth curve was established to gain an estimate of tumor cell doubling time. A least squares plot (Figure 3) of this tumor cell population vs time (days) revealed exponential kinetics from day 4 through day 10 after which time tumor growth slowed considerably until 18 days when the animals expired. The

Figure 3

Semi-log plot of average number of tumor cells per mouse vs time in days. Vertical lines represent ± S.E. of the mean. Each point in time represents the mean of five mice. This line represents a least squares plot of the data from 4 through 10 days after inoculation.


AVERAGE NUMBER OF TUMOR CELLS PER MOUSE
10^8
10^7
10^6
10^5
10^4
0
4
8
12
16
20
DAYS

exponential doubling time (T_D) was estimated at 38 hours from this curve.

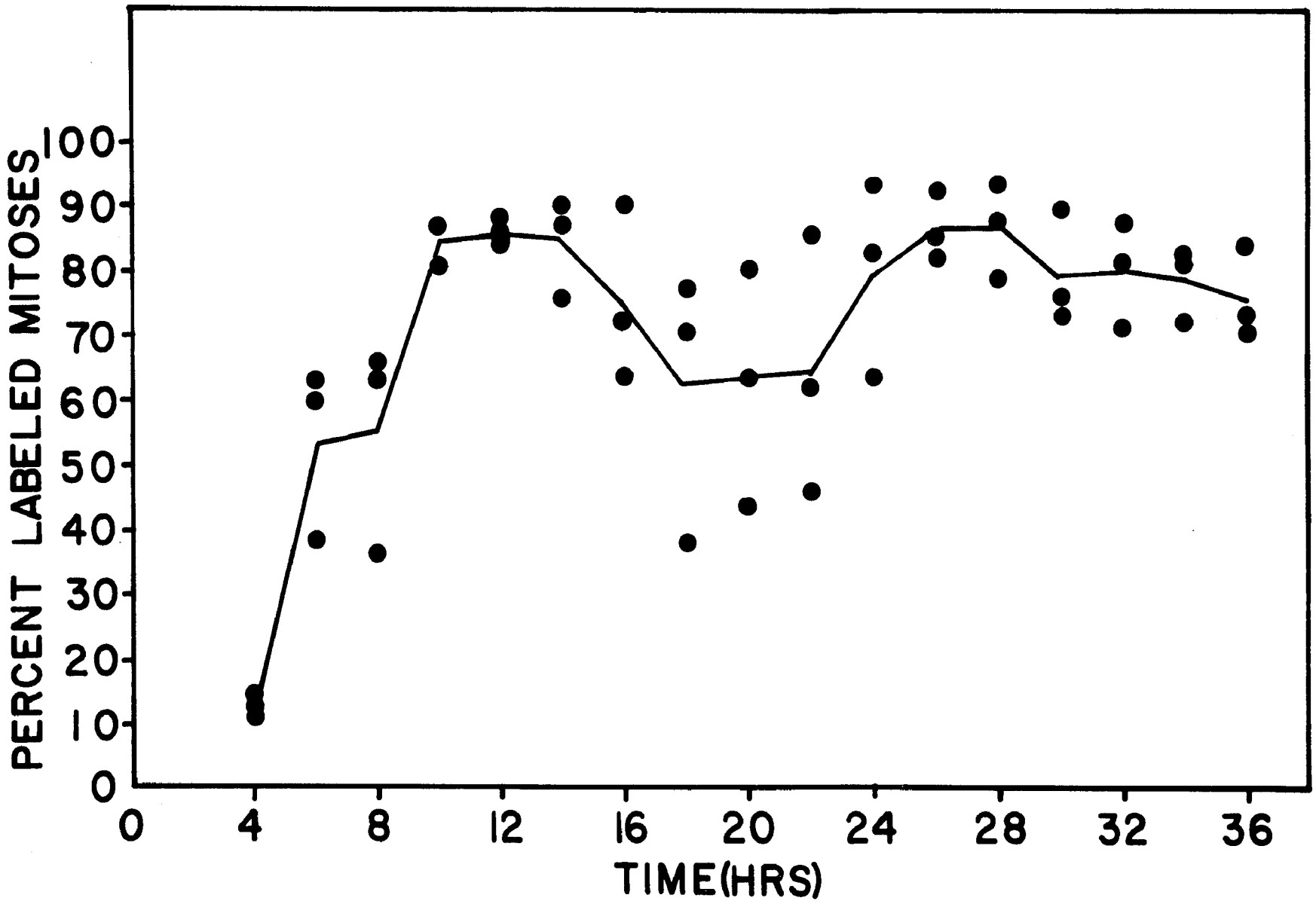
The Labeled Mitoses Experiment

The cell cycle parameters were obtained from the percent labeled mitoses curve (Figure 4). The 10 day old tumor showed 86% labeled tumor cell mitotic figures 12 hours after the injection of ^{3}HTdR. A plot of the points for individual mice revealed that the trough of each separate mouse occurred at 16, 18 and 20 hours respectively. This indicates that although there was considerable variability of the points in the 14-22 hour range, the trough does indeed lie somewhere between 16 and 20 hours. The trough of the line drawn through the mean of each set of points occurred at 18 hours. A second peak occurred at 27 hours where again 86% of the mitoses were labeled. The subsequent course of the curve is not well defined.

The duration of the cell cycle as measured by the half-height method (64) was 17.1 hours. The time of DNA synthesis (T_S) was measured as that time period between the half-height of the ascending arm of the first wave of labeled mitoses and the half-height of the descending arm of the first wave. The cell cycle time (T_C) was taken as the time period between the half-height of the ascending arm of the first wave of labeled mitoses, and the half-height of the ascending arm of the second wave. $T_G + \frac{1}{2} T_m$ was measured as that time interval between injection of ^{3}HTdR and the

Figure 4

The percent labeled mitoses curve for the MOPC 104E ascites tumor (percent labeled mitotic figures vs time in hours). Each circle represents one mouse at each point in time. Total curve represents pooled data from four separate experiments. The line is drawn through the mean of three mice at each point in time.


PERCENT LABELED MITOSES
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
28
32
36
TIME(HRS)

half-height of the ascending arm of the first curve. T_m was estimated at 1 hour and T_{G1} was assumed to be equal to $T_C - (T_S + T_{G2} + T_m)$.

Table II shows the data gained from the percent labeled mitoses curve by the half-height method. The doubling time (38 hours) was approximately twice the cell cycle time (T_C = 17.1 hours). T_S was 10.5 hours and therefore comprised most of the cycle. T_{G1} was 0.6 hours. Table III compares the observed kinetic data with some of the corresponding potential parameters (calculated). It is obvious that a considerable fraction of tumor cells was not actively proliferating since the growth fraction was considerably less than 1. This can be attributed to cell death or to cells in Go (non-cycling). Comparison of the doubling times (calculated vs observed) indicates that much of this discrepancy must be attributed to cell death. The labeling index was calculated according to the method of Cleaver (15) and the growth fraction by Mendelsohn (63). The labeling index (L_I) indicates that 68% of the cells which were actively cycling were in S phase at any given time. In contrast, the thymidine labeling index (T_I) indicates that 41% of cells (both cycling and non-cycling) were in S-phase at any given time. T_I and L_I were used to calculate the growth fraction which was 60%. This indicates that only 60% of the tumor cell population were actively proliferating in the 10 day tumors. These kinetic data were useful for interpreting the dextran and antiserum effect on the <u>in vitro</u> uptake of tritiated thymidine.

TABLE II

The Cell Cycle Parameters of the MOPC 104E Ascites Tumor

	Time (hours)
T_c	17.1
T_s	10.5
T_{G1}	0.6
T_{G2}	5.0
T_m*	1.0
$T_{G2} + \frac{1}{2} T_m$	5.5
Mitotic Index†	1.5%

* Arbitrary time.
† Based on counts of 1,000 tumor cells.

TABLE III

A Comparison of the Observed Kinetic Data of the MOPC 104E Plasmacytoma with Potential (Calculated) Parameters.

Parameter	Calculated	Actual
Cell Cycle Time		17.1 hrs
Doubling Time	28.5 hrs	38.0 hrs
Growth Fraction	60%	
Labeling Index	68%	
Thymidine Labeling Index		41%

Effect of B-1355 Dextran Antigen and Monospecific Goat Anti-μ Antiserum on Tritiated Thymidine Incorporation in MOPC 104E Cell Suspensions

The B-1355 dextran was shown to inhibit uptake of tritiated thymidine (^{3}HTdR) from cell suspensions of MOPC 104E tumor cells. Monospecific goat anti-μ antiserum showed a similar type of inhibition in the same concentration range. Tumor cell suspensions (10^5 cells/cc) were preincubated on ice with dextran antigen or anti-μ respectively for one-half hour. An assay was made, and then the tubes were rotated in an incubator at 37°C for 8-10 hours. Antigen (or antisera) concentrations varied from 10^{-7} to 10^{-1} mg/ml (excess antigen to excess antibody). Inhibition effects were observed within the range of 10^{-3} to 10^{-1} mg/ml for both antigen and antiserum. Figure 5 shows dextran inhibition of ^{3}HTdR uptake by the MOPC 104E cell cultures. The inhibitory effect becomes obvious after 2 hours of incubation and there was little further uptake of ^{3}HTdR in the antigen containing samples (10^{-1} to 10^{-3} mg/ml) subsequent to that time. At 10^{-4} mg/ml the inhibitory effect was not demonstrated. This decrease in ^{3}HTdR incorporation apparently was relative to the degree of homogeneity of the tumor cell preparations as well as the concentration of antigen. The inhibition was reproducible, however, even with a relatively low fraction of tumor cells (12-17%), although the effect was more obvious in more homogenous tumor preparations (80%).

Figure 5

Inhibition of tritiated thymidine incorporation by B-1355 dextran antigen in 80% MOPC 104E tumor cells suspensions (CPM vs time in hours).

O.....10^{-1} mg/ml dextran B-512 (non-specific antigen).
X.....10^{-1} mg/ml dextran B-1355.
●.....10^{-3} mg/ml dextran B-1355.
Δ.....10^{-4} mg/ml dextran B-1355.

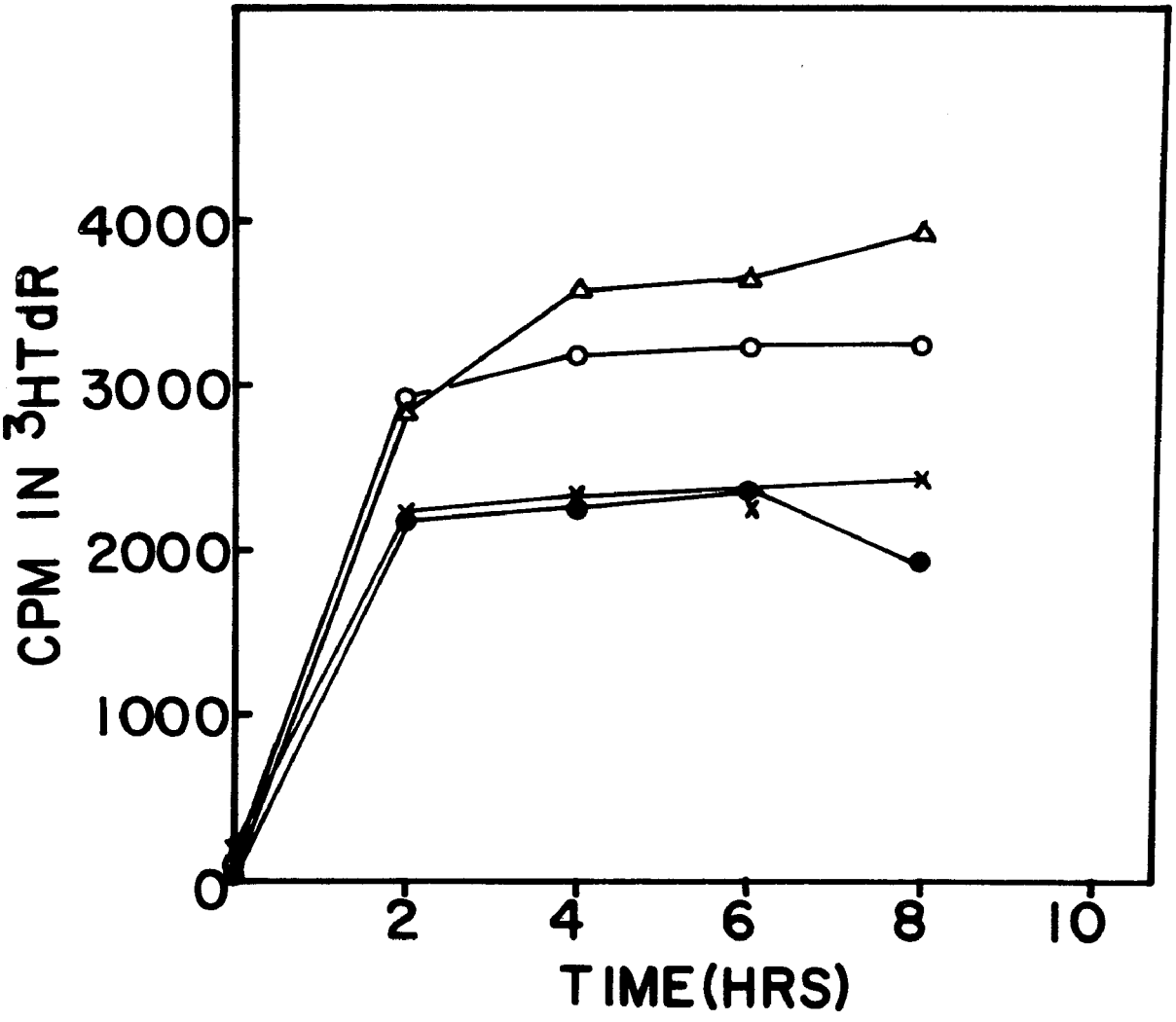

CPM IN ^{3}HTdR
4000
3000
2000
1000
0
2
4
6
8
10
TIME(HRS)

Figure 6 shows similar inhibitory effects due to antigen, the primary difference being that in this case the cell preparation was only 12% tumor cells (based on the total number of nucleated cells). The obvious difference in total ^{3}HTdR uptake was probably also dependent on the age of the tumor cell suspensions, since the growth fraction in most ascites tumors has been shown to vary with the tumor age (33). (The growth fraction is that fraction of the tumor cell population which is actively proliferating.)

Similar to the previous dextran experiment, inhibition was obvious at 10^{-1} to 10^{-3} mg/ml. The inhibitory effect was obviously less when the percent tumor cells in the suspensions was lower, since the dextran would inhibit uptake of ^{3}HTdR only in the cells demonstrating IgM on their cell surfaces. Dextran/IgM complexes have been shown previously to fix complement in this tumor system (57). If complement were present in the fetal calf serum, the possibility existed that complement cytotoxicity could cause the inhibition noted with dextran. Therefore, dextran-conjugated SRBC's were utilized to examine this possibility and these results shown in Figure 7 lead to the conclusion that the fetal calf serum did not contain complement. Note that there was a small amount of lysis even in the presence of heat inactivated guinea pig complement.

The effect of anti-μ on ^{3}HTdR incorporation in MOPC 104E tumor cell suspensions is shown in Figures 8 and 9 for 80% and 17% tumor cell suspensions respectively (percent is based on total nucleated cells). Note that there was no further incorporation of

Figure 6

Inhibition of tritiated thymidine incorporation by B-1355 dextran antigen in 12% MOPC 104E tumor cell suspensions (CPM vs time in hours).

O.....control, 10^{-1} mg/ml dextran B-512.
X.....10^{-1} mg/ml dextran B-1355.
●.....10^{-2} mg/ml dextran B-1355.
Δ.....10^{-3} mg/ml dextran B-1355.
▲.....10^{-4} mg/ml dextran B-1355.

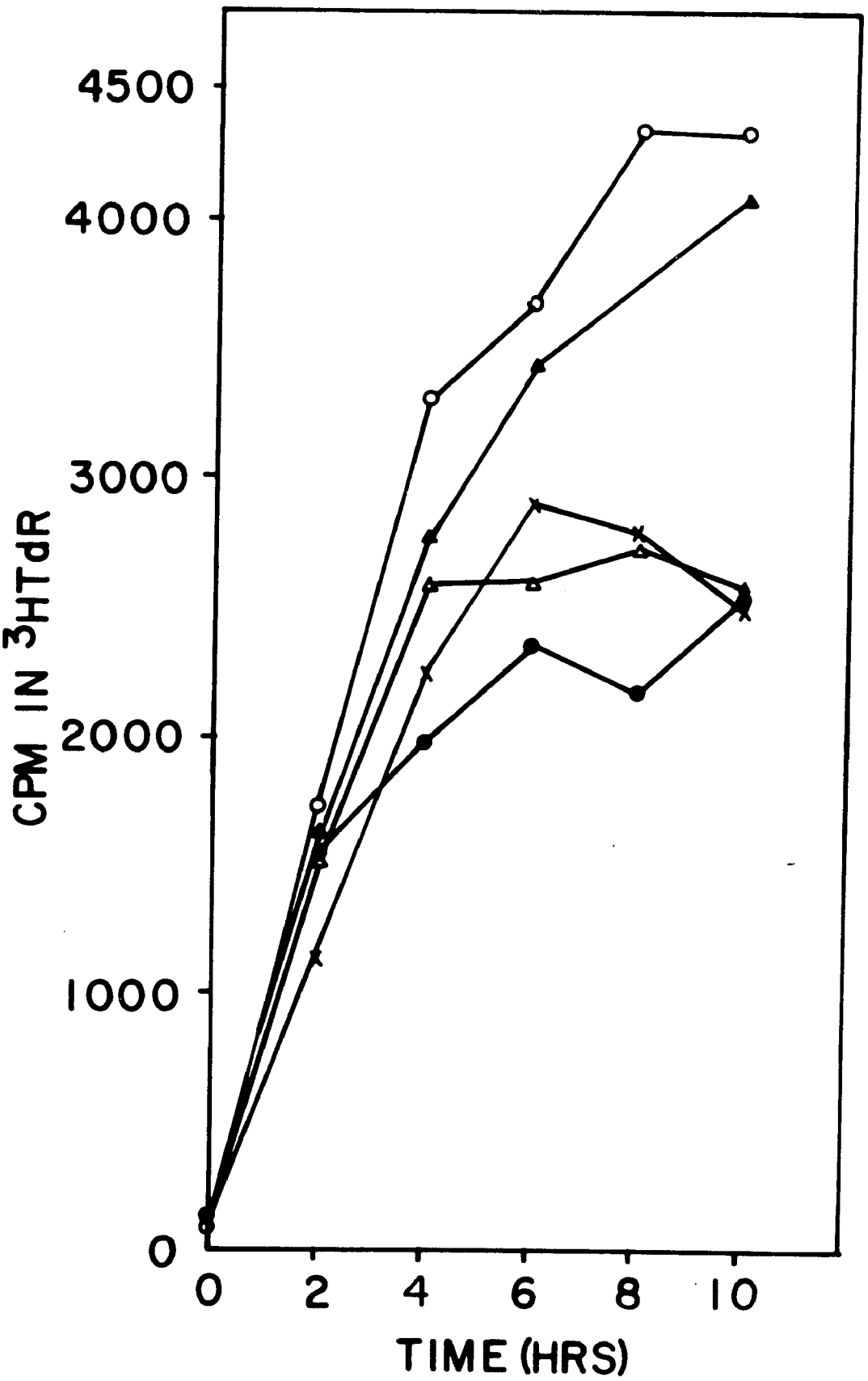

4500
4000
3000
2000
1000
0
CPM IN ^{3}HTdR
0
2
4
6
8
10
TIME (HRS)

Figure 7

Photograph of the results of the radial hemolysis method for detecting the presence of complement under conditions of the antigen and antiserum inhibition experiments. This experiment tests the ability of complexes of IgM-dextran to lyse SRBC's conjugated to the dextran under conditions of the previous experiments and in the presence of guinea pig complement (GPC). Note the large circle around wells containing GPC which represent complement lysis of SRBC. Also note small zone of lysis around even the heat inactivated (HI) GPC. No lysis was observed in the absence of the GPC, indicating complement is not present under the conditions of the previous experiments. Other abbreviations:

FCS.....Fetal calf serum.
MEM.....Medium.
HI.....Heat inactivated.
Pen-strept.....Penicillin-streptomycin (1%).
GPC.....Guinea pig complement.

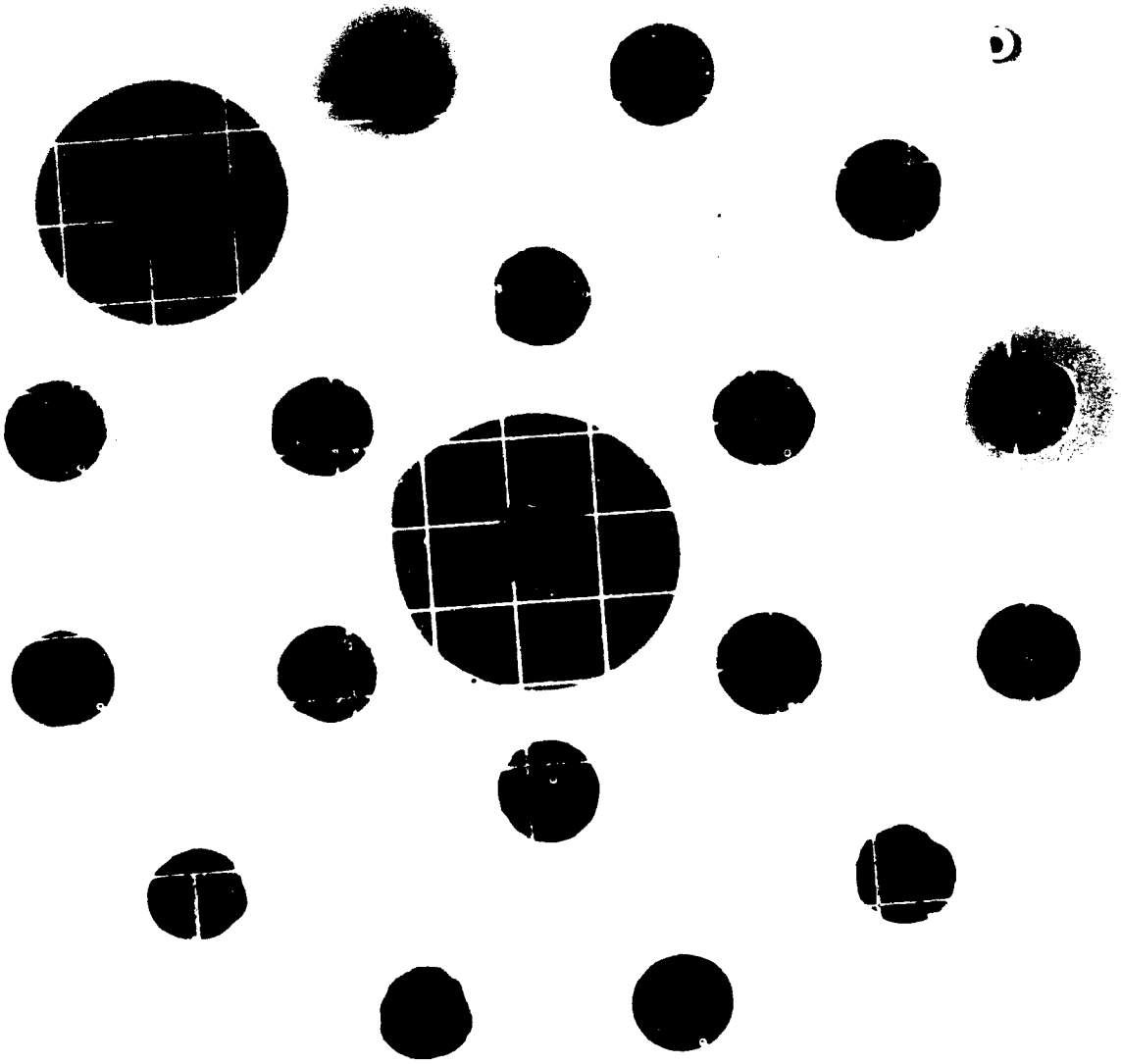
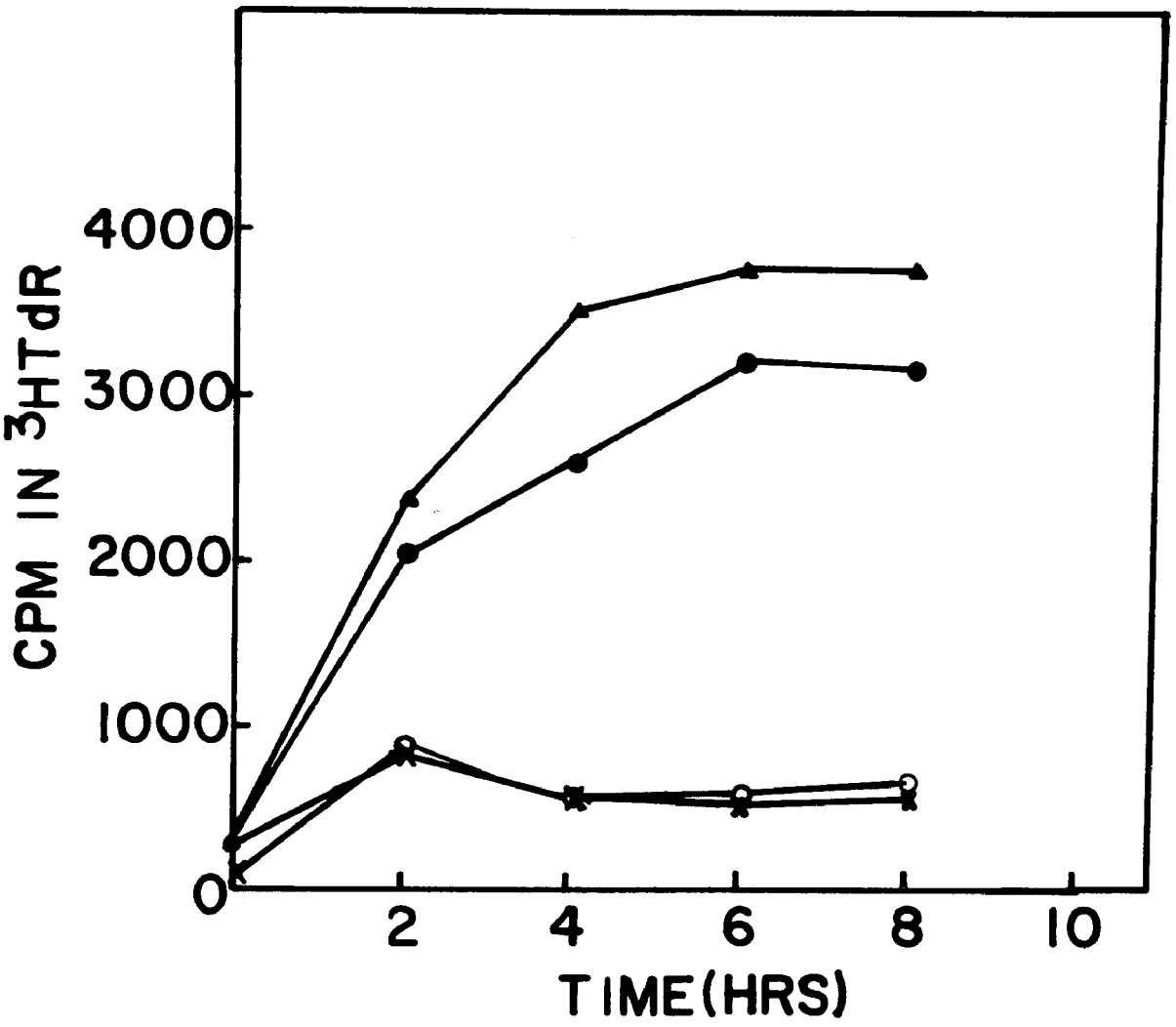


Figure 8

Inhibition of tritiated thymidine incorporation by monospecific antiserum in 80% MOPC 104E tumor cell suspensions (CPM vs time in hours).

●.....control, 0 mg/ml antiserum.
X.....10^{-1} mg/ml anti-μ antiserum.
0.....10^{-3} mg/ml anti-μ antiserum.
▲.....10^{-5} mg/ml anti-μ antiserum.


CPM IN ^{3}HTdR
4000
3000
2000
1000
0
2
4
6
8
10
TIME(HRS)

^{3}HTdR after 2 hours (Figure 8) for the 10^{-1} and 10^{-3} mg/ml samples. At a concentration of 10^{-5} mg/ml anti-μ there was no inhibition of ^{3}HTdR uptake, but rather a continued uptake of ^{3}HTdR comparable to that of the control sample. Anti-μ showed suppression of ^{3}HTdR incorporation in the concentration range of 10^{-3} to 10^{-1} mg/ml similar to the results found with the dextran antigen. Again the inhibition was less when tested on tumor preparations with low percent tumor cells; however, the effect was a similar one (Figure 9). Figure 9 shows inhibition of ^{3}HTdR uptake in the concentration range of 10^{-3} to 10^{-1} mg/ml anti-μ antiserum. Anti-μ at 10^{-5} mg/ml resulted in ^{3}HTdR uptake comparable to the control sample. The ^{3}HTdR response as percent of control in the presence of monospecific anti-μ compared with that of dextran is shown in Table IV. The inhibition by monospecific anti-μ was similar to, but more drastic than, inhibition by the B-1355 dextran.

Anti-μ was an effective inhibitor at 10^{-1} to 10^{-3} mg/ml, but as the ratio of anti-μ molecules/no. tumor cells dropped to 3.7×10^5 the inhibition was no longer observed (Table V). The average rate of release of IgM from the MOPC 104E cell surface was found by Ghanta et al to be $2.7 \pm 0.2 \times 10^5$ molecules/cell/hour (37). Therefore, after 10 hours of incubation 2.7×10^6 molecules of IgM could be released into the medium for each tumor cell present. At an antiserum concentration of 10^{-3} mg/ml or 3.7×10^7 antisera molecules/tumor cell, there would still be excess antiserum present in the medium even after 10 hours. At 10^{-5} mg/ml

Figure 9

Inhibition of tritiated thymidine incorporation by anti-μ antiserum in 17% MOPC 104E tumor cell suspensions (CPM vs time in hours).

0.....control, 0 mg/ml anti-μ.
X.....10^{-1} mg/ml anti-μ.
●.....10^{-3} mg/ml anti-μ.
▲.....10^{-5} mg/ml anti-μ.
Δ.....10^{-7} mg/ml anti-μ.

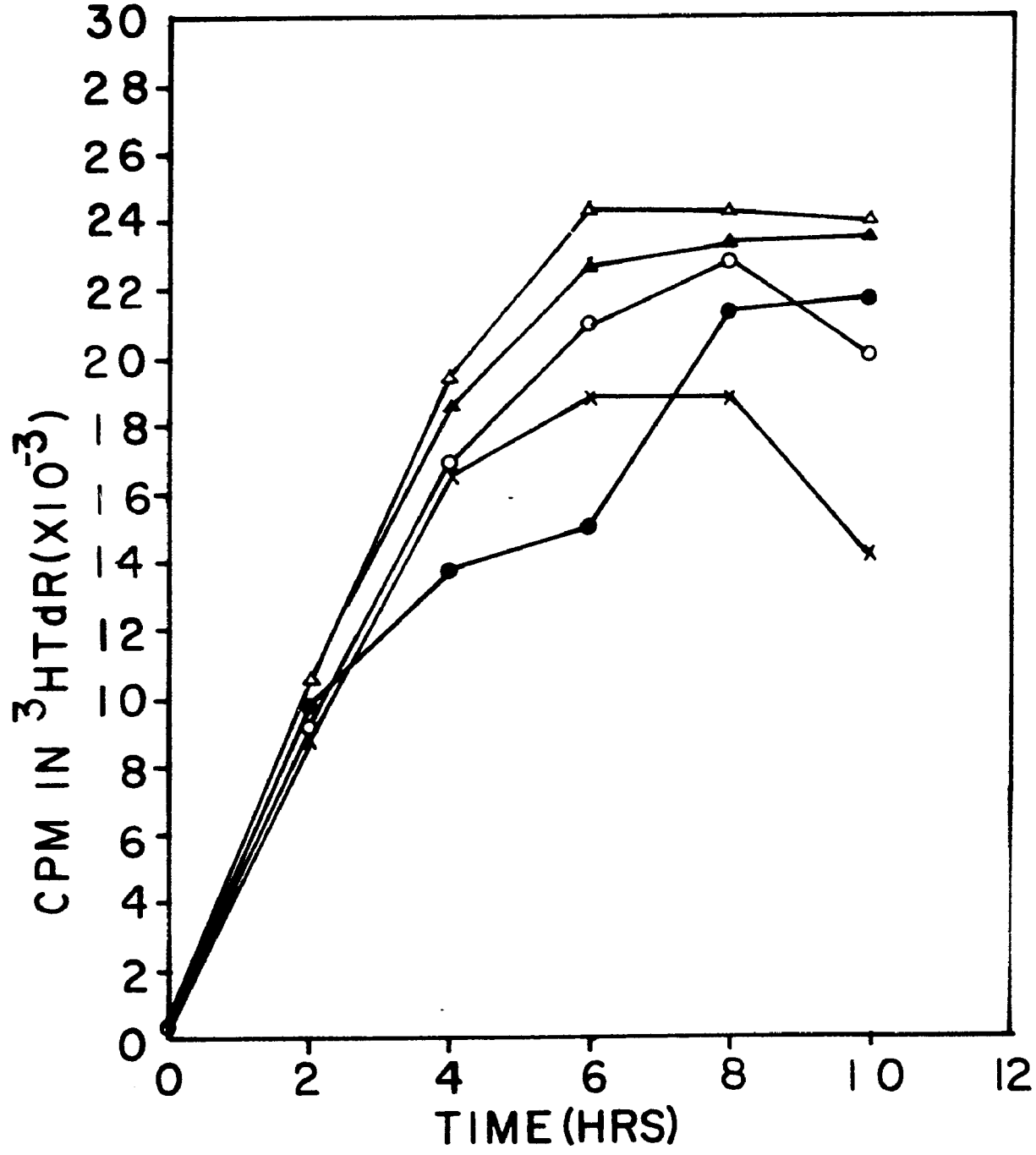

30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
CPM IN ^{3}HTdR(X10^{-3})
0
2
4
6
8
10
12
TIME (HRS)

TABLE IV

A Comparison of Tritiated Thymidine Uptake as Percent of Control in the Presence of Anti-μ Antiserum vs Uptake in the Presence of Dextran B-1355 Antigen After 4, 6 and 8 Hours Incubation in 80% MOPC 104E Tumor Cell Suspension.

Inhibitor	4 Hrs	6 Hrs	8 Hrs
Dextran (10^{-1} mg/ml)	74.3%	70.6%	75.8%
Anti-μ (10^{-1} mg/ml)	39.7%	15.7%	18.6%

TABLE V

The Number of Antisera Molecules per Tumor Cell and Antiserum Concentration Correlated with Antiserum Effect.

Conc. Anti-μ (mg/ml)	Antiserum Effect	No. Antiserum Molecules/Tumor Cell
10^{-1}	Inhibition	3.7×10^{9}
10^{-2}	Inhibition	3.7×10^{8}
10^{-3}	Inhibition	3.7×10^{7}
10^{-5}	No Inhibition	3.7×10^{5}

antiserum would not be in excess after 8-10 hours of incubation, and it was at this concentration and below that the inhibition was no longer observed. It appears, therefore, that excess antiserum was required in order for inhibition of DNA synthesis to take place.

Viability counts in these experiments ranged from 65-85% after 8-10 hours incubation. Figures 10 and 11 show the viability results throughout the experiments with dextran and anti-μ respectively (corresponds to Figures 6 and 9). These viability plots refer to the experiments represented by Figures 6 and 9 in which 17% tumor cell populations were used. Although the total number of nucleated cells scored in these particular experiments was not sufficient, it was observed in subsequent experiments using more homogenous tumors (not included) that the viability of the antigen containing sample and control paralleled very closely throughout the period of incubation.

DNA Repair in T Cells

While the MOPC 104E cell line provides a good model for studying control of cell proliferation (since these cells synthesize and secrete immunoglobulin profusely), the B cell deficient agammaglobulinemic lacks circulating antibody and thus provides a good source of T cells in which to examine for DNA repair capacity. Although DNA repair has been examined previously in humans, no one has directed attention specifically at the T cell population. For

Figure 10

Viability plot (percent viable cells vs time) with B-1355 dextran and with B-512 dextran.

B-1355.....0
B-512.....●

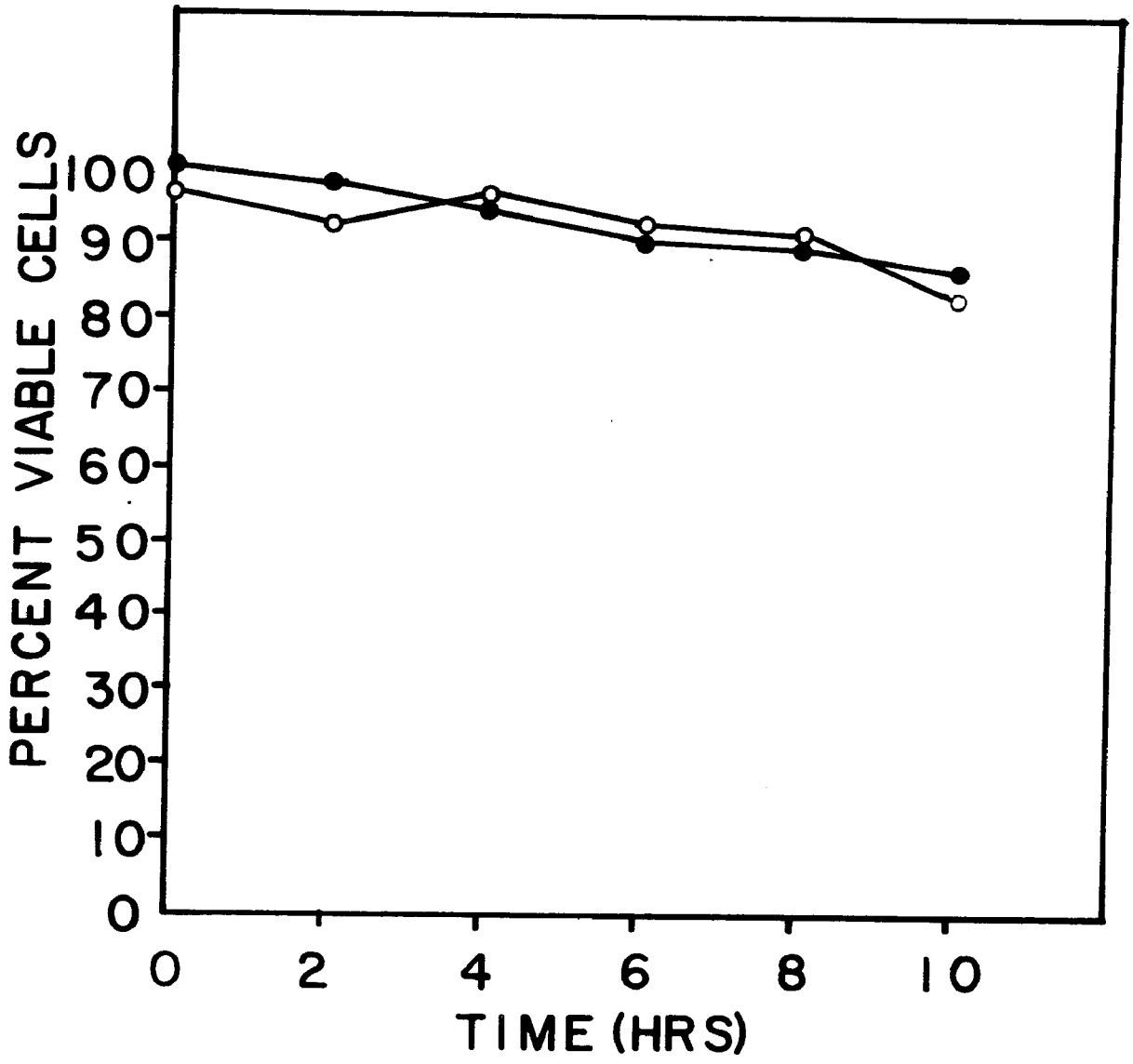
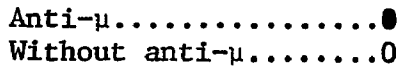
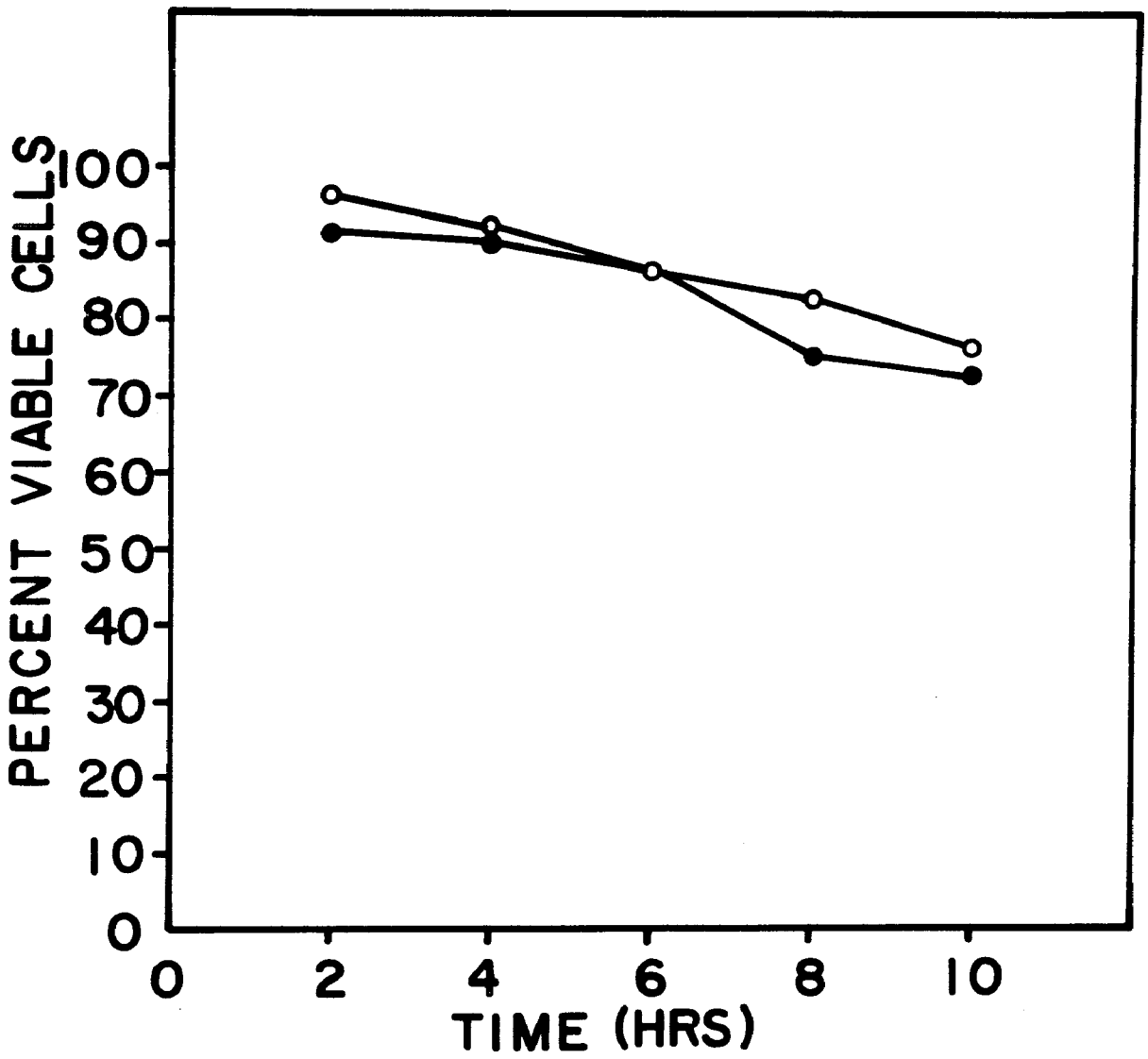

PERCENT VIABLE CELLS
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
TIME (HRS)

Figure 11

Viability plot (percent viable cells vs time) with anti-μ and without anti-μ.




PERCENT VIABLE CELLS
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
TIME (HRS)

this reason and since it is conceivable that agammaglobulinemia might represent a defect in DNA repair, the extent of ^{3}HTdR incorporation subsequent to UV-irradiation was determined. The extent of incorporation by the control sample (no UV) was 282 CPM per 1.43 x 10^{6} cells (19.7 CPM/10^{5} cells) and the irradiated sample measured 762.2 CPM per 8 x 10^{5} cells (95.28 CPM/10^{5} cells).

IV. DISCUSSION

The present studies demonstrate that both B-1355 dextran antigen and monospecific goat anti-μ produce a profound inhibition of ^{3}HTdR incorporation in suspensions of MOPC 104E tumor cells. The inhibition could not be attributed to viability differences and was shown not to be the result of complement cytotoxicity.

The cell cycle kinetics were also obtained for this tumor system and DNA repair capacity was examined in T cells from an agammaglobulinemic patient.

Inhibition of ^{3}HTdR uptake by antigen and antiserum in myeloma tumor cells is of considerable importance since an important contemporary question in immunology concerns the signals which control the initiation of DNA synthesis in the lymphocyte. For example, it has been suggested that antigen-antibody complexes might somehow trigger lymphocytes to proliferate. Furthermore, inhibition of cell proliferation by such agents might have potential in tumor therapy.

The cell cycle kinetics add to the total of our knowledge concerning tumor cell proliferation in vivo and also serve to interpret the effects of antigen and antiserum on the cell cycle.

The capacity for DNA repair in agammaglobulinemics was investigated since this condition might involve a lack of DNA repair

which, according to the model presented in this discussion, provides for antibody diversity.

Cell Cycle Kinetics

The growth fraction (GF) of a tumor cell population can be estimated from a knowledge of the thymidine labeling index (T_I, obtained experimentally) and the calculated or potential labeling index (L_I). The potential labeling index was calculated to be 68% and the GF 60%. The potential doubling time (T_D) was 28.5 hours and was obtained from the equation $T_D = \frac{T_c}{GF}$. The discrepancy between T_D and the actual doubling time, T_d, must be attributed to cell death since the fraction of non-proliferating cells has been taken into consideration in the use of the thymidine labeling index (T_I).

Estimation of T_m to be 1 hour probably does not introduce any serious error in calculating the other cell cycle parameters. The minimum T_{G2} was greater than 2 hours since no labeled mitotic figures were observed as late as 2 hours after ^{3}HTdR injection. That this tumor population is highly variable with respect to individual cell cycle residence times is demonstrated by the shallow trough.

Extensive variation in residence times would be predicted if antigen/antibody complexes on the cell surface or antibody feedback bear any relationship to control of DNA synthesis. Several researchers have shown that antibody could either depress or augment

DNA synthesis in lymphocytes depending on whether it was present in antigen or antibody excess (4, 72). In this context, it is reasonable to suggest that the MOPC 104E tumor cells vary in their potential for antibody synthesis, and therefore may demonstrate a tendency for variation in cell cycle residence times. (A definite stimulating antigen has not been established for myeloma cells, however, it is conceivable that loss of self-recognition to an endogenous antigen such as some cell wall component could serve this purpose.) A bacterial dextran from Leuconostoc mesenteriodes NRRL B-1355 (47) has been found which binds specifically to the MOPC 104E IgM (57, 108). Although no attempt was made to analyze all kinetic aspects of this tumor system, sufficient data were accumulated to determine the cell cycle parameters necessary to utilize this system as a model for future studies, i.e., drug and antigen antibody studies.

Effect of B-1355 Dextran Antigen and Monospecific Goat Anti-μ Antiserum on Tritiated Thymidine Uptake in Suspensions of MOPC 104E Tumor Cells

The results of the antigen and antisera studies indicate that both the B-1355 dextran and monospecific antiserum were capable of inducing a profound suppression of DNA synthesis of tumor cells in the MOPC 104E plasmacytoma. The specific dextran might be expected to stimulate G0 or G1 tumor cells to enter S phase more quickly or it might suppress DNA synthesis by short-circuiting the

'true antigen' (by blocking) for this tumor system. Whatever the mechanism, either effect would be interesting and would be reflected by the *in vitro* incorporation of ^{3}HTdR. The inhibitory effect was especially interesting since this system could serve as a model for control of neoplastic growth.

A similar inhibitory effect by monospecific antiserum was also noted and was equally exciting since considerable interest has been generated recently in the potential of blocking antibodies in transplantation therapy (30). The tumors used in these studies contained a wide distribution of cell types (RBC's, polymorphonuclear leucocytes, macrophages, small lymphocytes, etc., as well as tumor cells). A greater inhibitory effect was noted consistently with purer tumor preparations with respect to the percent of tumor cells. The possibility that the inhibition was due to complement toxicity was eliminated for the dextran system by showing that although dextran-IgM complexes will cause lysis of SRBC in the presence of complement, there was no complement (i.e., in the fetal calf serum) present in the system as demonstrated by the failure to lyse dextran-conjugated SRBC's. Furthermore, Takahashi *et al* (98) provided firm evidence that anti-μ was not cytotoxic to MOPC 104E tumor cells in the presence of complement. Viability checks assured that the inhibition was not due to viability differences in the control and the antigen-containing samples.

In the case of the dextran experiments, the fact that this inhibition was immunologically specific was demonstrated by the lack

of inhibition by the non-specific B-512 dextran in the control sample. Evidence that the MOPC 104E tumor cells have immunoglobulin receptors on their surface has been demonstrated previously through the efforts of Ghanta et al, 1972 (35), who used fluorescein-conjugated dextran and dextran-conjugated SRBC for the detection of these tumor cells.

The mechanism by which anti-μ and the dextran antigen (B-1355) exert their effects is not clear at this time. Whether the mechanism of inhibition by monospecific anti-μ antiserum is due to antigenic modulation, blocking effects, or some other mechanism must be left for future experiments, as must the mechanism of dextran inhibition. Antigenic modulation is a process induced by antibody by which cell surface antigen (IgM in this case) is removed specifically and selectively from the surface of the cell. This idea might provide an explanation for tolerance (71, 98).

The relationship between antigenic modulation and cap formation has not been established but it has been suggested that cap formation might provide the mechanism for the phenomenon of antigenic modulation (46). Whether cap formation occurs with these tumor cells in the presence of anti-μ antibody has not been investigated but such studies could provide some evidence linking the phenomenon of antigenic modulation with cap formation, and whether cells respond to antigen by undergoing DNA synthesis and further cell division, or simply become non-responsive to further stimulation.

It should also be interesting to ascertain where the antigen and antiserum inhibition resides with respect to the cell cycle. Is the effect directly on S phase or does it simply involve some mechanism whereby cells are prevented from making the G1/S transition? The probability that cells are inhibited in late G2 is unlikely since inhibition of ^{3}HTdR uptake relative to the control is seen early and T_{G1} + M is less than 2½ hours. Cells would not have time to progress from early G2 into S phase within this amount of time since G2 + M + G1 was found to be about 7 hours. Future experiments involving the use of a mitotic inhibitor could be utilized to determine in what phase of the cell cycle the inhibition effect is exerted.

If antigen/antibody complexes serve as the trigger for DNA synthesis in these tumor cells, then any tendency to interfere with that trigger, such as antiserum blocking, would suppress further DNA synthesis as observed in these experiments. Some such trigger is required for clonal expansion which is assumed in all respected theories of antibody diversity. The idea that antigen/antibody complexes might serve this role is incorporated into the following model and discussion on antibody diversity which provided the basis of these studies.

A Model for Antibody Diversity

Any model for antibody diversity, of course, must provide explanations for a variety of rather complex observations including:

provision of diversity on an appropriate time scale consistent with data on antibody structure, genetic patterns, and known disease states; stimulation by antigen of appropriate cell proliferation; and the existence of 'memory' following both the development of the immune state and the induction of tolerance.

The present model proposes simply that diversity of antibody genes can arise from a limited number of germ line genes through the accumulation of somatic mutations acquired during repeated events of non-conservative DNA synthesis. These mutant cells are then selected, stabilized, and regulated to provide the diversity of the immune response.

Our model proposes, therefore, that the germ line provides a stable population of 'parent' or stem immunocytes in which the ancestral immunoglobulin genes are preserved. The 'parent' cells give rise to an unstable pool of cells in which the DNA of variable genes is subject to rapid changes in base sequence information due to segmental resynthesis by non-conservative DNA synthesis. (The model does not exclude accumulation of some DNA base errors due to ordinary cell replication, although the frequency of errors due to ordinary cell replication would be much less than those induced by non-conservative synthesis.) Selection of cells from this mutant population by antigen (or some other selective device) then provides stable clones of proliferable diversified cells for the 'differentiated' immune response. These three cell compartments are summarized in Figure 12. The time scale of this differentiation

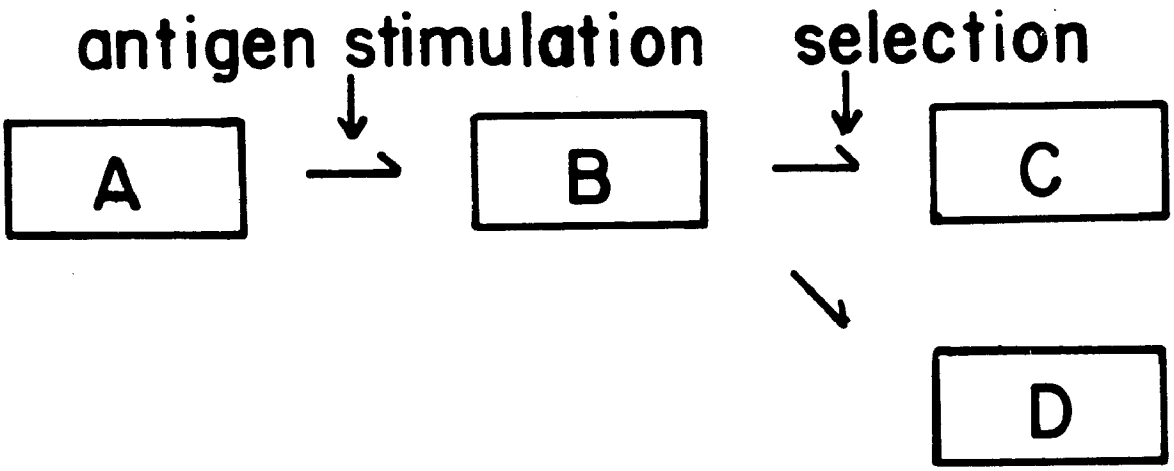
Figure 12

Generation of antibody diversity.

Compartment A = stem cell compartment.
Compartment B = unstable steady-state population.
Compartment C = stable, proliferative cells.
Compartment D = eventual cell death or interphase.

Proposed characteristics of compartment B:

1. Short-lived
2. Non-dividing or dividing
3. Mutation prone


antigen stimulation
selection
A
B
C
D

and the nature of the selective processes depend upon the details of the model.

Figures 13 and 14 present the details of two possible alternates of our model. The first allows for accumulation of base errors in antibody V-genes in the absence of antigen stimulation, and could represent either staged differentiation exclusively or could continue as a spontaneous process throughout the life of the organism. The second alternate requires antigen to trigger the hyperactive non-conservative DNA synthesis process in 'naive' immunocytes. Both alternates permit accumulation of somatic mutations in the absence of cell division, although cell division may amplify the genetic results of the mutation producing process. A distinction between these two alternates can be made only through complete knowledge of the nature of the selective pressures, for example, whether an 'internal' selective device (such as that proposed by Jerne, 48) could operate to fix diversified cells in the absence of external antigens.

This diversification probably occurs in the primary lymphoid organs (bursa of Fabricius or its equivalent and thymus), which provide for the extensive immunocyte regeneration and turnover consistent with our theory (19). For example, the thymus renews its lymphocyte population about every fourth day (65).

Jerne has postulated recently a somatic mutation process operating during cell proliferation in the primary lymphoid organ

Figure 13

The model, alternate 1: spontaneous somatic mutation.

Lymphoid cells pass through an unstable phase during differentiation in which non-conservative synthesis is hyperactive causing mutations as the result of errors made in the variable region of a limited number of ancestral (germ line) genes. Other sites on the genome are protected from the mutation by a stabilizing process such as DNA modification or protein binding. A small amount of immunoglobulin is continuously synthesized and is transported to cell surface where it serves as specific receptor. Ig which cannot combine with the stimulating antigen undergoes normal turnover from cell surface (5-6) hours. Specific Ig binds antigen and is stabilized on the cell surface. Antigen-antibody complex formation serves as the signal to turn on semi-conservative DNA synthesis and to turn off non-conservative synthesis and thus further mutations. This specific cell line is expanded by clonal proliferation as shown in figure.

Vg = germ line variable gene.
V_1, V_2 = mutated variable genes.

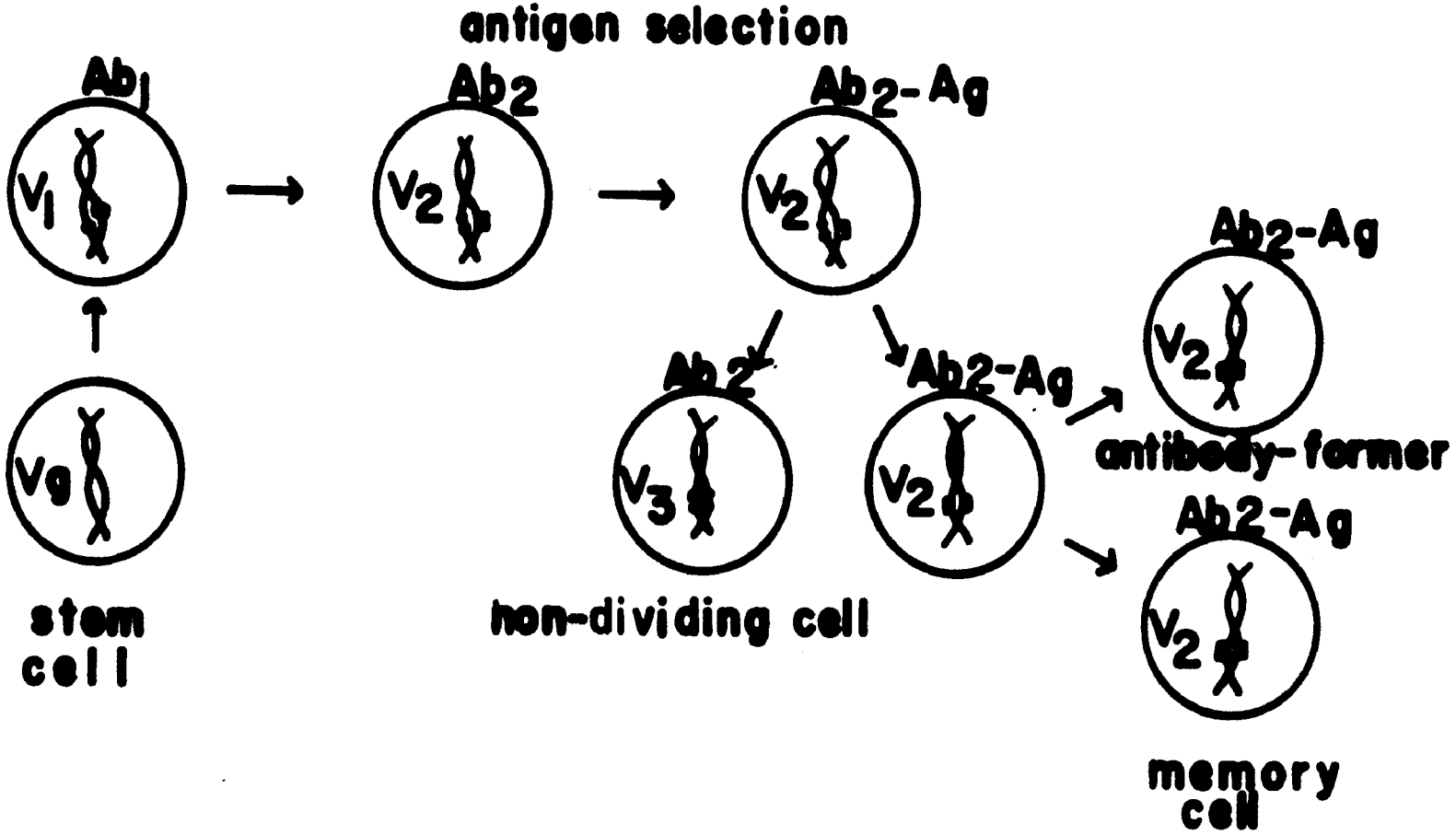
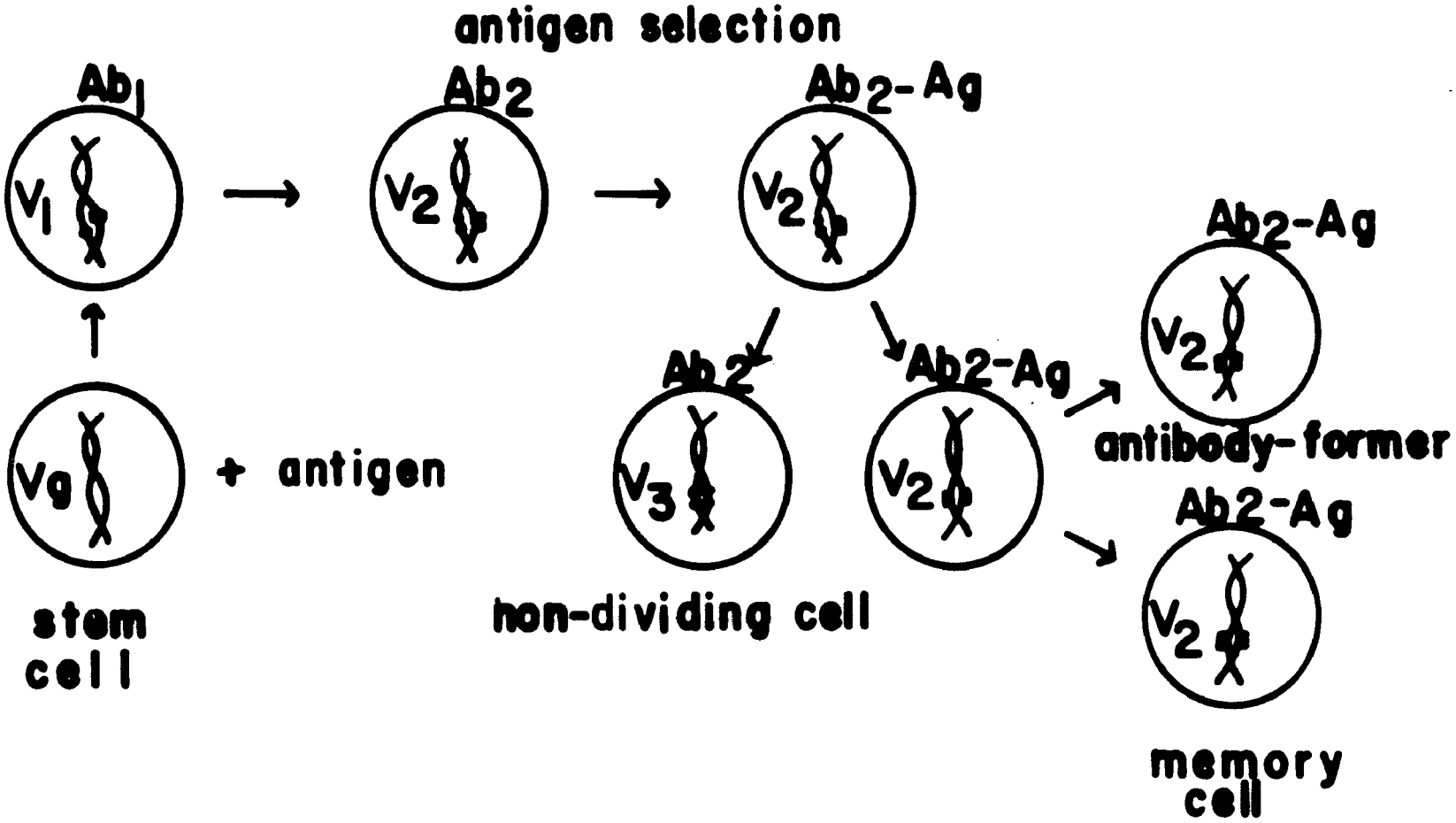

antigen selection
Ab1
Ab2
Ab2-Ag
V1
V2
V2
Ab2-Ag
Ab2
Ab2-Ag
V2
V3
V2
antibody-former
Ab2-Ag
Vg
V2
stem cell
non-dividing cell
memory cell

Figure 14

The model, alternate 2: antigen activation of non-conservative synthesis errors.

Presence of antigen serves to activate non-conservative synthesis through non-specific interaction with lymphocyte cell surface or possibly through secondary effect such as lysosome activation or macrophage - T or B cell membrane interaction. Limited regions of variable germ line genes accumulate errors in DNA base sequence introduced by non-conservative synthesis. During this phase antibody is continuously synthesized and migrates to cell surface where it serves as antigen receptor. Antibody not specific for the stimulating antigen is released from the cell surface after several (5-6) hours, while antigen-antibody complex is stable on cell surface. The complex formation serves as the trigger to turn on proliferation of a clone of similar cells by turning on semi-conservative DNA synthesis. (Non-conservative synthesis is turned off and genetic information on V-gene is locked in.)

Vg = germ line variable gene.
V_1, V_2 = mutated variable genes.


antigen selection
Ab1
Ab2
Ab2-Ag
V1
V2
V2
Ab2-Ag
Ab2
Ab2-Ag
V2
V3
V2
antibody-former
Vg
+ antigen
Ab2-Ag
V2
stem cell
non-dividing cell
memory cell

based on recognition of self antigen as the stimulating factor giving rise to mutations and cell proliferation (48).

Alternate 2 provides a more active role for antigen in provoking non-conservative DNA synthesis (and errors) in an otherwise stable population of germ line specified cells. This could result from a non-specific interaction of antigen with the cell membrane, although there is no evidence of lymphocyte-antigen binding other than through immunoglobulin receptor sites. The triggering effect of antigen could also be through some secondary mechanism (such as lysosomal activation). For example, lysosomes contain hydrolytic enzymes which are released during induction of the immune response and although there is yet no supporting evidence, it could be that these enzymes could activate non-conservative DNA synthesis through limited digestion of the genome. In this context it is fairly well established that macrophages are required in order to elicit an immune response to most types of antigen. Lysosomes are also clearly involved early in the course of lymphocyte transformation subsequent to PHA stimulation. Brittinger et al (11) and Hirschhorn et al (42) established that lysosomal hydrolases become redistributed from granular to less sedimentable fraction of cell homogenates within 2 to 4 hours after lymphocyte stimulation. Enhanced permeability of lymphocyte membranes to substances such as neutral red was also observed. Other studies have demonstrated such changes in nuclear properties of transformed cells as acetylation of

histones (79) and enhanced binding of actinomycin D (11) or acridine orange (51).

Alternates 1 and 2 differ, therefore, in the initiating role for antigen. Alternate 2 seems more efficient since DNA turnover and synthesis would be substantial only in the presence of an antigen which stimulates non-conservative synthesis and subsequent cell proliferation. For either model, however, the presence of antigen is essential for selection and proliferation of the appropriate cell lines. There is, of course, precedent for considering membrane interaction as means of controlling cell proliferation in view of the effects of cell contact and such agents as phytohemagglutinin.

Studies on DNA repair mechanism serve to illustrate the various types of 'non-conservative' DNA synthesis that might be employed in generating antibody diversity. At least three types of repair appear to operate in animal cells. These include excision repair (repair synthesis) (20, 83, 84); post-replicative repair through recombination or localized synthesis (16) of single strand defects which persist after replication; and a special error correction process occurring during replication.

The presence in cells of such enzymes as those responsible for these various processes, coupled with the chemical instability of the DNA polymer illustrate the extensive potential for localized changes in DNA, particularly since it has been recognized that both 'error-prone' and 'error-free' types of repair can be studied in

individual bacterial cell lines (104). A mutational role for such non-conservative events is suggested by such findings as mutations in stationary phase bacterial cells, inhibited by agents which also inhibit repair synthesis (86). Certain repair inhibitors have also been reported to be antimutagenic in animal cell lines (49). The properties of modification and restriction enzymes in bacteria and phase in relation to DNA stability are particularly interesting (59), since some such selective mechanisms might operate to localize the instability and high frequency mutations of the gamma globulin genes to the V-regions.

The present theory proposes that antibody diversity can arise through somatic mutations occurring in the course of non-conservative DNA synthesis or DNA turnover in a naive cell population. In contrast to the germ line theory which demands a full set of very stable pre-established specific genes for immunologic success, our theory provides a mechanism for contemporary adaption of the organism during life based on a much smaller genetic load. Table VI summarizes the principal differences between these models in reference to specific problems. Although somatic mutations have often been discussed as a diversification mechanism, the feature of selective mutations in the absence of cell division with the additional potential for control by antigen adds strength to the idea.

In addition to the idea that somatic mutations complement the germ line in providing for antibody diversity we make the following postulates:

TABLE VI

A Comparison of Germ Line and Somatic Mutation Theories

	Germ Line	Somatic Mutations
1.	Both class and all gene specificity in germ line. (All of genes stable).	1. Class specificity in germ
2.	Genes must be preserved rigorously throughout life (because selective pressures do not operate in each generation).	2. Genes are preserved after selection process (or based on selection) otherwise gene pool (with respect to V genes) may be rather fluid.
3.	Differentiation involves expression of one (or a few) genes in each of a large number of individual cell lines to provide both classes and fine diversity.	3. Differentiation results in classes of antibody producing cells which can diversify.
4.	Primary antibody production based simply on gene (and therefore single cell line) activation in large number of stable cell lines.	4. Primary antibody production based on selection from adapting population of cells. Note that at time of challenge the number of cell lines may be as large as with germ line theory but the added capacity to adapt adds another dimension of potential.
5.	Memory represents switched on cell line.	5. Memory represents adapted cell line stabilized by antigen exposure.

TABLE VI (continued)

PROBLEMS	
1. Large number of genes required.	1. Mechanism for appropriate control of mutation process.
2. Adaptation only in germ line.	2. Functioning genes (in terms of producing appropriate antibody) must be preserved.
3. Absolute preservation of genes with no selective pressures.	3. How to preserve constant gene region.

1. The immunocyte population consists of three cell groups:
   a. Stable stem cells in which germ lines are preserved.
   b. An unstable subpopulation of cells in which non-conservative DNA synthesis is active with resultant base sequence errors.
   c. A stable committed population which is capable of proliferating.
2. This process of mutation occurs in the primary lymphoid organs.
3. There exists a limited number of mutable germ line genes (as few as 10-20 would suffice). We do not feel compelled to accept a large number of V-genes based on sequencing of myeloma proteins, particularly since myeloma may well involve selection by antigen of a pool of lymphoid cells containing V-genes which could have arisen either through the germ line exclusively, through somatic mutations, or a combination of both. Selection could be by exogenous antigen (virus, etc.) or it could represent loss of self-recognition to some cell component.
4. The total number of lymphoid cells in an individual is approximately 10^{11} - 10^{12} but even if only 10% or 10^{10} - 10^{11} of such cells were actively engaged in the non-conservative synthesis and mutation inducing process, that would be sufficient to provide an ample response.

Based on the foregoing assumptions, an estimate may be made with respect to the amount of antibody diversity which can be generated in a specific amount of time by assuming a rate and fidelity of non-conservative DNA synthesis. Immediate transcription following an error would allow a small amount of antibody to be synthesized and expressed immediately as cell surface receptors for interaction with antigen if an appropriate one is available. If the correct amino acid sequence is achieved, the mutation process would be turned off by the antigen-antibody interaction and cell proliferation would be triggered.

Assuming that the rate of non-conservative DNA synthesis is such that one mistake/10^3 base pairs can be made in 1 hour, then we would expect one mistake per 10 bases in 100 hours. This would result in 33 mistakes per variable gene. We have assumed 10 genes/cell but mutations would occur in only one V-gene at any one time so that multiple specificities would not be expressed on the cell surface at the same time. The process of non-conservative synthesis could act on the variable genes in a sequential manner. The large number of lymphoid cells capable of undergoing this mutation process (10^{10}) would, therefore, increase the number of genetic combinations to 3.3×10^{11} neglecting any back mutations. If the turnover rate of antibody receptors on the lymphoid cell surface is assumed to be about 5-6 hours, the theory could predict a maximum mutation rate for each cell. Vitetta and Uhr (102) have demonstrated a 50% loss of IgA from lymphoid cell surfaces in 2 to

4 hours *in vivo*. A new population of antibodies could be synthesized sequentially from the same mutated germ line gene if at least one mistake could be made in that gene every 5 hours, but transcription would have to occur in such a way that the antibody cell surface receptors would reflect faithfully the information encoded in the genome. (Turnover of receptors on the cell surface must be fast as compared with the time interval between transcription of newly altered DNA and arrival of 'new' immunoglobulin moieties on the cell surface. A relatively unstable m-RNA that could not be used repeatedly would also be appropriate so that immunoglobulins of different specificities would not be likely to reside concurrently on the cell surface. If a cell sustained additional mutations before fixing the information, its progeny would not persist since they could not produce the appropriate antibody.) Consequently, to get a rough idea of the maximum rate of mutations that could be sustained by this model we simply accept one mistake per 5 hours in the variable gene (or 20 mistakes/gene/100 hours). This process is not postulated to be completely random in that certain regions of the V-gene would be more susceptible to errors than would others. (The susceptibility to mutation would be determined by the base sequences and the quantitative and qualitative nature of proteins binding DNA in those regions). Table VII shows the relationship between error rate and the number of mistakes and different genetic combinations for two given time spans after primary antigenic stimulation. One can readily see from this table that adequate genetic diversity can

TABLE VII

Number of Ig Populations Possible under Non-conservative Synthesis Theory 100 and 1000 Hours after Primary Antigen Stimulation

100 Hours after Primary Stimulation

Error rate/base pair/hour	No. mistakes/ 10^{10} cells	Maximum No. possible Ig populations
10^{-6}	3.3×10^{8}	3.3×10^{8}
10^{-5}	3.3×10^{9}	3.3×10^{9}
10^{-4}	3.3×10^{10}	3.3×10^{10}
10^{-3}	3.3×10^{11}	3.3×10^{11}

1000 Hours after Primary Stimulation

Error rate/base pair/hour	No. mistakes/ 10^{10} cells	Maximum No. possible Ig populations
10^{-8}	3.3×10^{7}	3.3×10^{7}
10^{-7}	3.3×10^{8}	3.3×10^{8}
10^{-6}	3.3×10^{9}	3.3×10^{9}
10^{-5}	3.3×10^{10}	3.3×10^{10}

be acquired in a relatively short time span even in the absence of cell division, and that this demonstration is applicable to both alternate 1 and 2. The theory involves little 'waste' of DNA since mutations result from limited DNA turnover and are concentrated in limited regions of the variable genes. This contrasts with somatic theories based on mutation as the result of errors made during semi-conservative synthesis. In these theories the entire genome is replicated only to initiate a small number of mistakes in one (or a few) small V-genes. The steady-state population of cells in compartment B of Figure 12 simply continues to undergo non-conservative synthesis spontaneously (alternate 1) or as long as antigen concentration is optimal (alternate 2) and 'successful' mutant cells are selected by antigen and thus are released into the proliferative compartment. Binding by antigen also serves to turn off the mutation process and turn on semi-conservative DNA synthesis and, therefore, cell proliferation.

Alternate 2 which requires antigen stimulation of diversification is challenged by evidence that apparently 'naive' or 'virgin' lymphocytes bind certain antigens without having previous exposure to those antigens. However, it may be that: (1) self-antigen could have stimulated this population to accumulate mutations in the absence of exogenous antigen with resulting diversity; (2) there is initial, low affinity binding due to cross-reaction to account for binding in absence of previous exposure. The specificities included in the initial binding would also be

reflected as an integral part of the diversity of the progeny cells.

Melvin Cohn has stressed frequently the idea of antigen selection of somatic mutations in the immune system (18). In the immune system, antigen selection provides an additional driving force to allow expression of appropriate mutations as they arise.

It is not possible at this time to decide with certainty between the germ line and the somatic mutation theory. A number of facts, however, are difficult to rationalize on the basis of stable germ line gene theory and are consistent with the adaptive nature of the somatic mutation idea. The structural basis of allotype specificity in rabbit immunoglobulin G has been studied by R. R. Porter, who among others has found that sixteen positions in the variable region of the heavy chain show a correlation between the amino acid residue present and the allotype of the protein as identified by anti-allotype sera (54, 67, 105, 106). Multigene theories of diversity would predict scrambling of these allotypes during mating. However, mating and population studies reveal that the allotypes are inherited in a simple Mendelian fashion, i.e., the α_1, α_2, and α_3 serological markers behave as alleles at a single locus. Our theory is consistent with the existence of rabbit allotypes since we postulate that certain base pairs are selectively protected from mutation through protein stabilization. The rabbit allotypes are apparently protected by some such process. The V_H gene rabbit allotypes also show a

hypervariable region at amino acid positions 95-115, two less variable regions at 30-35 and a region at positions 50-60 with even fewer changes (67). Mole also has stated that, in some positions, residue changes (for rabbit allotypes) require a double nucleotide base change in the codon, implying that mutations were occurring frequently during the divergence of allotypes, but the allotypes are now stable. This is also difficult to accept in terms of germ line theory. The nature of the double base mutation would be explained more easily through mutations during non-conservative DNA synthesis or some other somatic mutation mechanism.

It is extremely interesting that certain immunosuppressive drugs are also potent DNA repair inhibitors. (Acridines apparently inhibit DNA repair through binding to the DNA molecule). Diamino-acridines and acriflavin have been shown by Farr et al (29) to inhibit the primary immune response but similar doses fail to inhibit a secondary response. In terms of our second alternate, especially, one would predict that inhibition of DNA repair (a type of non-conservative synthesis) would suppress the immune response. Apparently, lower doses of 9-aminoacridine will inhibit DNA repair but still allow replicative DNA synthesis as shown by Lankford and Yielding (55). Secondary response would be allowed under both alternate 1 and 2 because this would simply involve response of the memory cells for which diversity is already established.

Nature often provides alternate pathways for many biological processes, e.g., the pentose phosphate shunt. Therefore, it is highly reasonable to postulate that a backup system has been provided for creation of antibody diversity and possibly a process of continuous immune adaptation. Indeed, Coffin and Scharff (17) have provided evidence for a higher rate of somatic mutations in mouse myeloma cells by fluctuation analysis than has been demonstrated for other somatic cell markers by the same method of analysis, i.e., the rate of conversion of heavy plus light chain producing cells to light chain producers was 1.1×10^{-3} per cell per generation and Chinese hamster lung cells develop resistance to 8-azaguanine at a rate of 1.5×10^{-8} per cell generation (13).

DNA Repair in T Cells from a Patient with B Cell Deficient Agammaglobulinemia

An attempt was made to examine the extent of DNA repair both in T cell deficient and B cell deficient agammaglobulinemic patients. The individual examined was deficient in B cells (Bruton type agammaglobulinemia). The rationale of this test was that agammaglobulinemia might represent a defect in DNA repair, which according to this model provides for antibody diversity.

Comparison of the DNA repair values in CPM noted for the agammaglobulinemic patient with those averages of normal individuals indicated DNA repair in T lymphocytes was well within the range of

values established by Gaudin and Yielding (unpublished data from this laboratory).

Sufficient data were not accumulated to make a statistical comparison of DNA repair in agammaglobulinemia due to limited access to the patient and his abnormally low lymphocyte blood count. Moreoever, data from this limited study did not indicate further experiments of this nature on B cell deficient agammaglobulinemics. Nevertheless, examination of B cell DNA repair in T cell deficient patients might show a completely different picture since it is the B lymphocyte which is the precursor of the antibody-secreting-cell. (T cells do not secrete antibody.) No T cell deficient patients have been available to pursue this aspect any further.

V. CONCLUSIONS

The following conclusions were derived from data in this dissertation:

1. Both dextran B-1355 antigen and monospecific goat anti-μ antiserum suppressed specifically the incorporation of ^{3}HTdR in MOPC 104E tumor cell suspensions.

2. Complement was not cytotoxic in the presence of the anti-μ antiserum, and was shown to be absent under the conditions of the dextran inhibition experiments; therefore, it was not likely to exert an inhibitory effect on the uptake of ^{3}HTdR.

3. The *in vitro* uptake of ^{3}HTdR subsequent to UV-irradiation (a measure of DNA repair) was comparable to averages established from normal healthy individuals.

4. The cell cycle kinetics were elucidated for the MOPC 104E tumor system.

5. A new model for antibody diversity based on non-conservative DNA synthesis errors was proposed.

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