IN VITRO IMMUNE RESPONSE OF LYMPHOID ORGANS TO ADENOVIRAL ANTIGENS

by

Rhoda Anne Reddick

Submitted to the Faculty of the School of Graduate Studies

of the Medical College of Georgia in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

June

1968

IN VITRO IMMUNE RESPONSE OF LYMPHOID

ORGANS TO ADENOVIRAL ANTIGENS

This dissertation submitted by Rhoda Anne Reddick has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

The signatures which appear below verify the fact that all required changes have been incorporated and that the dissertation has received final approval with reference to content, form, and accuracy of presentation.

This dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[redacted]

Advisor

JUN - 8 1968

Date

[redacted]

Dean, School of Graduate Studies

ACKNOWLEDGMENTS

Sincere appreciation is expressed to my advisor, Dr. Stanley S. Lefkowitz for his invaluable guidance and assistance throughout the course of this study and the preparation of this dissertation.

Appreciation is also expressed to Dr. Robert B. Dienst, Chairman, to the members of the faculty and staff of the Department of Medical Microbiology, and to the members of my advisory committee for their help and interest.

The technical assistance of Miss Nancy Wood with certain aspects of this project is gratefully acknowledged. Appreciation is also expressed to Mrs. Georganne Boudet for typing the manuscript.

TABLE OF CONTENTS

TABLE OF CONTENTS (Cont.)

LIST OF FIGURES

LIST OF FIGURES (Cont.)

LIST OF TABLES

LIST OF TABLES (Cont.)

Introduction

I. Statement of the problem

Immune responses to infectious agents have been studied for many years. Recently considerable interest has been directed toward studies of the immune response *in vitro*. This approach provides a system in which the antibody producing potential of an individual animal can be studied under various conditions which could not be simulated in the intact animal. Many reports have been published which describe in detail the secondary immune responses of antibody producing organs, tissues and cells of various animal species. The antigens used in these studies have been quite varied, ranging from relatively simple proteins such as egg albumin, human or bovine serum albumin to more complex materials such as bacterial products, viruses or erythrocytes. More recently there have been reports of primary immune responses initiated entirely *in vitro*. These responses have been induced by the antigen alone and by nucleoprotein-antigen complexes extracted from cells exposed to the antigen. Improvements in methodology, refinement of existing techniques and development of extremely sensitive assays have made possible a greater understanding of the immune response.

Advances have been made in elucidating the structure and composition of antigens which evoke immune responses. This is

particularly evident in the case of viruses. Knowledge of the structure and localization of the antigenic components of the virus particle have opened the way to new approaches to the study of the possible roles played by viral proteins in the course of infection and in the host response.

Interest in the immune responses of animals to adenoviruses is based on the fact that these viruses are important in human respiratory diseases. Since the discovery of the oncogenic properties of certain adenovirus types, much emphasis has been placed on the study of immune responses to the viral antigens during the process of oncogenesis. One approach is through an *in vitro* study of antibody synthesis to viral antigens. The antigenic structure of this group of viruses has been defined sufficiently to make this approach feasible.

The purpose of this investigation was to study *in vitro* the immune responses of animal lymphoid tissues to adenovirus antigens. There have been no published reports of a study in which the adenoviruses were used as antigenic stimuli for the demonstration of *in vitro* antibody responses. In many of the previous studies of the immune response *in vitro*, the agents used to evoke the responses have been non-infectious or when infectious, the agent was not thought to produce disease in the host. The adenoviruses used in the present investigation are infectious and produce either a persistent, asymptomatic infection or overt disease in the hosts selected for study.

This study was divided into two parts. Part I was concerned with a system in which a latent infection without disease is

established in the experimental animal. Part II was concerned with a system in which the infection leads to overt disease, i.e. carcinogenesis.

Part I of this study was concerned with the antibody response of the rabbit to adenovirus type 5. Adenovirus type 5 was selected: 1) because the virus can be produced and purified with relative ease and 2) because the structure of the virus is well established. The rabbit was selected because it has been demonstrated that rabbits injected intravenously with adenovirus type 5 develop a latent infection (Pereira and Kelly, 1957). The virus may persist in the spleen for at least eight weeks without overt expression of disease.

The purposes of Part I were: 1) an attempt to demonstrate in vitro immune responses in lymphoid tissues removed from rabbits immunized in vivo with adenovirus type 5, and 2) to study some of the factors or variables which might affect the ability of these tissues to respond to antigenic stimulation in vitro.

Lymph node, spleen and thymus tissues were studied with regard to their ability to synthesize viral antibodies following challenge in vitro with the specific viral antigens. The response was studied with regard to the time of appearance, duration of synthesis and specificity of the antibody produced in vitro. Attempts were made to ascertain the cell types which may be found in the cultures of the antibody-producing tissues.

For the elucidation of the factors or variables which effect the ability of the tissues to respond in vitro, the following were studied:

1) the effect of various time intervals between the initial injection of the animal with viral antigens and the subsequent re-exposure *in vitro* of their lymphoid tissues to the viral antigens;

2) the effect of various preparations used as antigens to stimulate antibody synthesis *in vitro*, i.e. partially purified homotypic intact virus in various concentrations and/or their highly purified structural subunits, and heterotypic adenoviruses;

3) the effect of exposure of the tissue to compounds reported to enhance antibody production, specifically hydrocortisone-succinate (Ambrose, 1964) and phytohemagglutinin (Elves, Roath and Taylor, 1963; Tao, 1964); and

4) the effect of compounds reported to inhibit antibody or protein synthesis, specifically chloramphenicol (Ambrose, 1963, 1966; Svehag, 1964) and puromycin (Darken, 1964).

Part II of this study was concerned with a system in which viral infection leads to carcinogenesis. When newborn hamsters are injected with adenovirus type 12, tumors develop at the site of injection thirty to ninety days later. The adenovirus type 12-tumor "system" in hamsters provides a model for studying the immune responses of the host during viral oncogenesis. It has been shown that once the virus induces tumor formation or malignant transformation, the virus can no longer be recovered from the tissues of the animal or from the transformed cell (Landau *et al.*, 1966;

Larson, Gosnell and Hilleman, 1966). Certain viral structural antigens may, however, persist in the tumor cell (Huebner, et al., 1963, 1964). This indicates that some portions of the viral genome may replicate in tumor cells even though infectious virions are not produced. It has also been shown that antibody to various antigens of the virions can be detected in serum from certain tumor bearing animals. The antibody is detectable in serum from many of the animals, but not all (Huebner, Rowe and Lane, 1962; Huebner, et al., 1963). One possible explanation is that the serum antibody levels are too low to be detected even by the most sensitive techniques. However, if it could be demonstrated through in vitro stimulation of "primed" lymphoid tissue that synthesis of antibody to the viral antigen(s) does occur, then another approach to methods of searching for hidden viral genomes would be available.

The purposes of Part II were: 1) to demonstrate in vitro synthesis of antibody to adenovirus type 12 antigens by spleen and lymph node tissues from hamsters bearing adenovirus type 12-induced tumors, and 2) to determine if there was a relationship between the capacity of the lymphoid tissues to respond to challenge in vitro and the presence of serum antibodies and tumors.

II. Review of related literature

A. Antibody synthesis in vitro

The study of antibody synthesis in vitro began with the development of techniques and methods for maintenance of functional tissues outside the intact host. The earliest report of antibody response in vitro was that of Carrel and Ingebristen (1912), who

claimed to have shown antibody production in tissue cultures of lymph nodes and bone marrow cells from normal guinea pigs after in vitro exposure to goat erythrocytes. Subsequent investigators, however, failed to reproduce these findings.

Since the early reports, extensive investigations have been undertaken to study both the primary and secondary responses in vitro. Two recent reviews (Stavitsky, 1961; Nossal, 1966) have covered in detail the developments of work in these areas. The following is a brief summary of the highlights of these developments.

Coons and associates undertook rather detailed studies of secondary immune responses in vitro and of certain factors effecting these responses. Michaelides and Coons (1963) reported that rabbit lymph node fragments could be maintained and stimulated to produce antibody in vitro for periods up to three weeks. They found that fragments were superior to cell suspensions for prolonged studies. O'Brien, Michaelides and Coons (1963) showed that the anamnestic response in vitro closely resembles the response in vivo in sensitivity to antigen, in time course of antibody production, in sequence of appearance and morphology of the antibody producing cells. They found as little as 0.001μg/ml of bovine serum albumin would stimulate an antibody response and as much as 0.5 mg/ml would not inhibit the response. In these studies they consistently observed a lag phase between in vitro stimulation and appearance of detectable antibody. Once synthesis began the antibody titers rose abruptly, reached a plateau and declined slowly. The active cells in the early periods (3-4 days) were classified as immature plasma cells; those found to be the active cells in later periods

(8-10 days) were classified as mature plasma cells.

Further studies by O'Brien and Coons (1963), Ambrose and Coons (1963), and Ambrose (1966) on the effect of metabolic inhibitors such as 5-bromodeoxyuridine, chloramphenicol, salicylate, and puromycin indicated that the *in vitro* response was the result of *de novo* synthesis of antibody rather than passive release of preformed antibody. It was found in the above studies that the concentration, the time of addition and the duration of exposure to the inhibitor all affected the response. These studies suggest that the secondary *in vitro* response is biphasic, characterized by an inductive phase followed by a productive phase (Ambrose, 1966).

Many workers have confirmed and extended the early reports of Coons and co-workers. The antibody producing tissues of various animals (mice, chickens, dogs, and guinea pigs) have been utilized in studies of the secondary immune response *in vitro*. Patterson *et al*. (1963 a, b; 1966), Dutton and Eady (1964) and Svehag (1964) have shown that spleen fragments from immunized animals can be stimulated *in vitro* to produce antibody. Their findings are in general similar to those of workers studying lymph node cultures, i.e., 1) the antibody response is antigen specific; 2) the quantity and duration of synthesis depends upon the conditions of culture; 3) the appearance of the antibody follows a lag phase after exposure of the tissue to the antigen; and 4) the response is sensitive to metabolic inhibitors.

Certain differences in the response of lymph node and spleen fragments have been reported. Stavitsky (1961) reported that per

unit weight of tissue, rabbit lymph nodes synthesized more antibody than the spleen. In a study of the antibody synthesizing capacity of various tissues of several strains of mice, Friedman (1964) found that the spleen cells were more active than lymph node cells; however, the capacity of the spleen cell was also found to vary according to the strain of mouse. The response of the lymph node and spleen has been shown to vary according to the time interval between in vivo injection of antigen and removal of the tissue for in vitro study (Askonas and Humphrey, 1958; Dutton and Vaughn, 1960; and Grabar and Corvazier, 1960). Grabar and Corvazier (1960) observed that the architecture of the lymph fragments is maintained in culture better than that of spleen fragments. This may account for some of the differences in responses reported by other investigators.

Studies with thymic explants have been much less successful than those with lymph node and spleen. In contrast with other lymphoid organs, there is little or no antibody formation and no plasma cell proliferation or formation of germinal centers in the thymus after systemic administration of antigen (Fagreus, 1948; Askonas and White, 1961). Direct injection of antigens into this organ, however, induces formation of germinal centers and plasma cells as well as the production of antibodies (Marshall and White, 1961). Therefore, it would appear that the immunological competence of lymphoid cells within the thymus in vivo is less than that of the lymphoid cells in other organs, although this competence may be influenced by the age of the animal.

It has been reported that cultures of thymus from various animal species produce small amounts of immunoglobulin (Askonas

and White, 1956; Thorbecke, 1960; Freidman, 1964). Van Furth et al. (1966) found during in vitro studies of normal and pathological human thymic tissue that normal adult thymus synthesizes immunoglobulins A and G but no M. Tissues from patients with myasthenia gravis and systemic lupus erythematous were found to synthesize immunoglobulin M as well as A and G. The specific antibody activity of these immunoglubulins was not studied.

Ortiz-Muniz and Sigel (1967) reported synthesis of specific antibody in fragments of thymus from rabbits immunized with bovine serum albumin. The fragments were found to elaborate antibody only after secondary stimulation in vitro; there was no evidence of continued synthesis as a result of primary in vivo stimulation. Although their findings were not construed as proof of the antibody forming capacity of the thymocytes, they are suggestive and are interesting in view of the work of Claman (1966) and Winkelstein and Craddock (1967). These workers have shown that a dual population of cells exists in the thymus. The major population has autonomous proliferative ability, actively synthesizes DNA for a short period of time, but does not respond to antigenic stimulation or phytohemagglutinin. The minor component is the "resting" population of small cells (lymphocytes) which proliferate and undergo blastoid transformation, not unlike that seen in lymphocytes of the peripheral blood.

In view of the heterogenous nature of lymphoid cells, many attempts have been made to determine the cell type responsible

for antibody production. In her now classical study of the spleen, Fagreus (1948) demonstrated that the red pulp of the spleen consists primarily of plasma cells and is more active *in vitro* in antibody production than the white pulp which consists mainly of lymphocytes.

Two groups of workers have attempted to extend knowledge of antibody production by studying individual lymph node cells isolated from immunized animals. Nossal and co-workers (1966) have concluded that: a) most antibody producing cells belong to a specialized family of cells, the plasma cell series, characterized by RNA-rich cytoplasm and an eccentric nucleus. b) The chief difference between the primary and secondary response is a greater proportion and absolute number of antibody producing plasma cells proliferating in the lymph nodes after the secondary stimulation. c) The plasma cells are highly specialized in that, with rare exceptions, each cell forms one type of antibody. d) Many plasma cells or cell clones go through a sequence whereby each forms 19S and 7S antibody with identical or very similar combining sites. In contrast, Attardi *et al.* (1959) found in their system that antibody production is by no means confined to the plasma cell family. These workers maintained that the lymphocyte forms approximately as much antibody as each plasma cell and the numbers of cells capable of producing two different types of antibody are greater than the work of Nossal's group indicates.

The studies of Gowans (1959, 1962) with thoracic duct lymphocytes and the demonstration by Hungerford *et al.* (1959) that peripheral lymphocytes exposed to phytohemagglutinin are capable of blastoid transformation have created new interest in the role of the small lymphocyte in antibody production. Elves *et al.* (1963) reported

that peripheral blood lymphocytes from individuals previously immunized with tetanus or diphtheria toxoid undergo a blastoid transformation either in the presence of the specific antigen or in the presence of phytohemagglutinin. These cells produce specific antibody. They postulated that the small lymphocyte has the intrinsic ability to "remember" a previous experience with an antigen. Tao (1964) similarly reported a specific anamnestic response was produced when lymph node fragments from rabbits hyperimmunized with bovine serum albumin were exposed to phytohemagglutinin in vitro.

Although many investigators have successfully demonstrated antibody synthesis and anamnestic responses in vitro, the demonstration of a primary response initiated in vitro has been more difficult to achieve. Stevens and McKenna (1958) and McKenna and Stevens (1960) claimed to have produced a primary antibody response in vitro. They added bovine γ-globulin to splenic fragments and peritoneal exudates obtained from rabbits which had been non-specifically primed with endotoxin 24 hours prior to sacrifice. They reported the detection of agglutinins for erythrocytes coated with bovine γ-globulin. The material they detected was found to be heat labile at 56°C and lost activity after storage at -20°C. These properties are not shared by most antibodies which have been described and, therefore, it is doubtful that the material they detected was classical antibody.

Globerson and Auerbach (1965) reported the initiation of a primary antibody response following the exposure of spleen explants from mice to sheep erythrocytes. In the initial experiments they had pretreated their animals *in vivo* with either phytohemagglutinin or Freund's adjuvant. In a later report (Globerson and Auerbach, 1966), they repeated their results without pretreatment of the animals *in vivo*. Tao and Uhr (1966) demonstrated the presence of 19S antibody followed by appearance of 7S antibody in organ cultures of lymph nodes from non-immunized rabbits which were exposed *in vitro* to bacteriophage Ø x 174. They concluded this to be suggestive of a primary response which had been initiated entirely *in vitro*. Saunders and King (1966) reported the induction of a primary response when paired spleen and thymus explants from non-immune mice were incubated *in vitro* with coliphage R-17. The animals used in both of the above experiments received no pretreatment *in vivo*, nor was it found necessary to subject the antigen to any preliminary treatment.

In the previous experiments, fragments or explants had been studied. Under these conditions the architecture of the tissue is more or less maintained. Mishell and Dutton (1966) exposed dissociated spleen cells from non-immune mice to heterologous erythrocytes and reported the initiation of a primary response. They claimed to have shown a response *in vitro* comparable in magnitude to that seen *in vivo*.

Fishman (1959, 1961, 1963) and associates have also reported the induction of a primary response *in vitro*. These workers

employed a two-step system in which the antigen (hemocyanin or T2 bacteriophage) was incubated with macrophages from peritoneal exudates obtained from non-immunized rabbits. The macrophages were then disrupted and the cell free extracts were incubated with "normal" lymphocytes. Their experiments indicate that during the incubation of the antigen with the macrophages a macromolecule, presumably RNA, is produced which is capable of converting non-immune lymphoid cells into antibody producing cells. The antibody response has been shown to be biphasic, consisting of an early wave of 19S antibody and a second wave composed mainly of 7S antibody.

Other workers (Askonas and Rhodes, 1965; Friedman et al., 1965) using the "Fishman system" have found that small amounts of antigen remain in the cell free extracts and that the antigen complexes with the RNA. Therefore, these workers have suggested that the macromolecule acts as an adjuvant. However Adler, Fishman and Dray (1966) used rabbits chosen so that the peritoneal exudate cells and lymph node cells were from animals of different allotypes. With this procedure they showed that the IgM antibody produced by the lymph node cells contains antigenic markers characteristic of the donor of the peritoneal cells and the IgG antibody has the allotypic specificity of the donor of the lymph node cells. These studies indicate that the RNA acts in some fashion other than as an adjuvant.

From studies on the conversion of non-immune spleen cells to antibody producing cells by RNA extracts, Cohen (1967) has

postulated the presence of specific recognition sites on the competent recipient cells. The presence of specific binding sites on recipient cells is predictable from a model of antibody formation which requires the cooperative efforts of two cell types for antibody formation. This model postulates that macrophages transfer an activating or informative material to a small number of competent lymphocytes which possess recognition sites for the product of the macrophages.

The increase in information obtained from studies of antibody synthesis *in vitro* has been possible because of the development of very sensitive assays capable of detecting small amounts of antibody.

The technique of tanned cell hemagglutination was described by Boyden (1951) and modified by Stavitsky (1954). This technique is suitable for detection of antibody to almost any soluble protein antigen. The assay is extremely sensitive. When combined with hemagglutination inhibition reactions and carefully controlled, the assay is reported capable of detecting less than 0.01μg of specific antibody (Stavitsky, 1961; 1963).

Ranney and London (1951) described a technique which is comparable to the tanned cell hemagglutination method in sensitivity and can differentiate passive antibody release from active synthesis. This technique makes use of radioactively labelled amino acids which are incorporated into newly synthesized antibody and can be detected by precipitation with the specific antigen or by co-precipitation with homologous antigen-antibody complexes.

Jerne and Nordin (1963) introduced a hemolytic plaque assay requiring complement which makes possible the detection of hemolysins produced by single cells or clones of cells. In the original assay the antibody producing cells and antigens (erythrocytes) were incorporated in an agar matrix. Ingraham and Bussard (1964) described a similar technique in which carboxymethyl cellulose replaced the agar matrix. Subsequent modifications of the technique by Sterzl and Riha (1965), and Dresser and Wortis (1965) have improved the method to the point that cells producing 19S and 7S immunoglobulins can be differentiated. Pick and Feldman (1967) described a further modification in which they combined autoradiographic methods with the plaque technique and were able to detect antibody in isolated lymph node cells to soluble protein antigens.

B. Antigenic composition of adenoviruses

The human adenoviruses were discovered by Rowe et al. (1953). This group of viruses is associated with a variety of respiratory diseases (Huebner et al., 1954; Parrott et al., 1954) as well as a variety of other disease syndromes in man (Soheui, et al., 1965; Goffe and Maassab, 1967; Boniuk et al., 1965).

Separation of these viruses into specific groups and types within a group has become possible as a result of intensive studies of their morphology and antigenic properties. A separation of the human adenoviruses into subgroups and types within a group was proposed by Rosen (1960). This method is based on their ability to agglutinate monkey and rat erythrocytes.

The morphology of the adenoviruses was first elucidated by Horne et al. (1959). These workers were able to show by

electron microscopy that the viral capsid is an icosahedron constructed of 252 morphological units. Subsequent studies on the architecture of the adenovirus capsid have revealed the presence of 240 non-vertex capsomeres, 12 vertex capsomeres plus projections with terminal knobs (Smith, 1965; Smith et al., 1965; Valentine and Pereira, 1965; Norrby, 1966 a; Norrby et al., 1967). Multiplication of these viruses in infected cells has been shown to be associated with the synthesis of three distinct soluble antigens (Pereira, 1959) which are subsequently incorporated into the viral capsid (Wilcox and Ginsberg, 1963 b; Valentine and Pereira, 1965; Norrby, 1966 a, b). Recently a non-structural antigen has been demonstrated in preparations of certain adenovirus types (Hoggan et al., 1965; Russell and Knight, 1967).

The antigens or morphologic components can be separated from each other and from the infectious virions by a variety of methods including immunoelectrophoresis (Pereira, Allison and Farthing, 1959), chromatography on DEAE-cellulose columns (Klemperer and Pereira, 1959; Wilcox and Ginsberg, 1961; Haruna et al., 1961; Gelderblom et al., 1965; Huebner et al., 1965), density gradient centrifugation (Allison et al., 1960; Wilcox and Ginsberg, 1963 b; Smith, 1965) and polyacrylamide gel electrophoresis (Maizel, 1966; Petterson et al., 1967).

The three antigens associated with multiplication of the adenoviruses were originally designated as antigen A, B, C (Pereira et al., 1959). Antigen A is a group-specific antigen

shared by most adenoviruses serotypes of human and animal origin; antigen C is largely type-specific. Antigen B corresponds to the "cell detaching factor" of Rowe et al. (1958) or the "toxin-like" factor of Everett and Ginsberg (1958). Antigens A and C have been shown to be antigenically distinct, whereas antigens B and C are related. Pereira (1960) demonstrated that antigen B is a complex formed by antigen C in combination with an additional component. This was subsequently confirmed by electron microscopy (Valentine and Pereira, 1965).

These investigations were able to show that antigen A is analogous to the non-vertex caposomeres; antigen B is composed of the vertex capsomeres plus the projections and antigen C is analogous to the projections on the vertex capsomeres. The projections on different serotypes have been shown to vary in length (Wilcox et al., 1963; Valentine and Pereira, 1965; Norrby, 1966 a; Norrby et al., 1967; Peterson et al., 1967). To standardize terminology Ginsberg et al. (1966) proposed that the name "hexon" be used for antigen A and "penton" for antigen B to denote that each unit of these antigens has six or five nearest neighbors respectively in the viral capsid. Antigen C is now designated as the "fiber".

The components of the virus which are responsible for the direct hemagglutination properties of different serotypes reflect certain differences in the adenoviruses types. These components have been described as the complete hemagglutinin and the

incomplete hemagglutinin, the latter of which requires the presence of heterotypic antiserum for demonstration. The two hemagglutinins present in adenovirus types 1, 2, 4, 5 (Rosen's group III) have been identified as the penton and the fiber antigen (Pereira and de Fiqueiredo, 1962). The penton causes complete hemagglutination; the fiber causes incomplete hemagglutination and requires the presence of heterotypic antiserum. The complete hemagglutinin of types 3 and 7 (Rosen's group I) and type 9 (Rosen's group II) consist of an aggregate of 12 penton units; the isolated pentons are the incomplete hemagglutinins (Norrby, 1966 a, b; Norrby and Skaaret, 1967; Norrby et al., 1967). In other serotypes, hemagglutination is associated with both the virus particle and the soluble antigens (Wigand and Wunn, 1966).

The elution patterns of the soluble antigens separated on DEAE also indicate differences in the viral subunits of the members of the four sub-groups as well as of the types within a sub-group. The elution profile of Group I, II and IV is such that with increasing sodium chloride concentration, the antigens separate in the order hexon, penton, fiber (Haruna et al., 1961; Gelderblom, 1965; Huebner et al., 1964). The order in which the subunits of types 2 and 5 in Group III elute is fiber, penton, hexon; however, components of type 4, also in Group II, elute in the order of Groups I, II and III (Klemperer and Pereira, 1959; Wilcox and Ginsberg, 1961).

The viral subunits are proteins which differ in composition between related and unrelated serotypes. However, relatively

little is known about the size or number of polypeptide chains composing the substructures. Polyacrylamide gel electrophoresis of adenoviruses disrupted by treatment with sodium docecyl sulfate and 2-mercaptoethanol resulted in the separation of ten different bands (Maizel, 1966). Laver et al. (1967) have demonstrated the presence of two N-terminal amino acids (alanine and glycine) in certain adenoviruses. Both of these amino acids were found in protein associated with the DNA of the virus and were thought to be associated with an internal protein rather than with components of the capsid.

A variety of serologic methods have been used to demonstrate antibodies to the various components of the adenoviruses. These include neutralization of infectivity, complement fixation, hemagglutination inhibition and indirect hemagglutination of tanned erythrocytes coated with the antigenic components. The nature of the viral antigens responsible for these serologic reactions has been the subject of several investigations. The neutralization of adenoviruses is a type specific reaction and was suggested by Wilcox and Ginsberg (1963 b) to be associated with both the fiber and the hexon. However, recent studies by Petterson (1967) indicate that purified hexon is not capable of stimulating neutralizing antibodies in rabbits. All the major components can be demonstrated by complement fixation (Wilcox and Ginsberg, 1961). The antigens involved in the passive hemagglutination reactions were reported by Friedman and Bennett

(1957) to be associated with both type and group specific antigens. Further study of this reaction by Ross and Ginsberg (1958) indicated that the reaction was associated with soluble components rather than the intact virion. They showed that the reaction could be made type specific by absorption of the antisera with a pool of heterotypic adenoviruses. Pereira and de Figueiredo (1962) suggested the reaction may be due primarily to the group specific or hexon antigen. However, recent studies by Lefkowitz (1967, 1968) with purified components of adenovirus types 5 and 12 indicate that the principal antigen involved is the fiber. Some activity is associated with the penton, but the hexon could not be assayed by this method and, therefore, apparently does not attach to the tanned erythrocytes.

C. Adenovirus 12-tumor system

Since the demonstration by Trentin and co-workers (1962) of the oncogenic effects of adenovirus type 12 in newborn hamsters, the adenovirus tumor system has been widely studied from the standpoint of humoral antibody response to the structural virion and tumor or neoantigens.

Huebner, Rowe and Lane (1962) demonstrated antibodies in the serum of tumor bearing hamsters which would fix complement in the presence of adenovirus 12 antigens. These antibodies were shown to be essentially type specific and were found only in sera from tumor bearing animals. The antibodies were not found in sera from animals which did not develop tumors although the animals had been injected with adenovirus 12 at birth. In further studies

Huebner and associates (1963) found that virus specific complement fixing antibody responses developed in hamsters in which transplants of adenovirus tumors grew progressively and the degree of response could be correlated with the tumor size. The tumor transplants were capable of inducing complement-fixing antibody to adenovirus type 12 even when the animals bearing the primary tumor from which the transplant was made failed to develop the specific complement-fixing antibody. In view of the reported failures to recover infectious virus from the tumor cells, these findings suggested that although complete infectious virus was not being synthesized, a specific antigen or component of the virus was persisting and perhaps replicating with the tumor cells. However, the complement fixing antigen which had been utilized in these early studies was quite crude and the reaction was caused in part by the presence of tumor antigen which is also present in cells infected with adenovirus type 12.

Subsequently, Huebner et al. (1963, 1964) demonstrated the presence of specific adenovirus complement-fixing antigen present in virus free cells from hamster and rat tumors. The evidence presented indicated that the antigen present was the structural type-specific C or fiber antigen rather than the structural group-specific A or hexon antigen. The presence of the structural fiber antigen was not confirmed by Pope and Rowe (1964) with immunofluorescent techniques or by Berman and Rowe (1965) with immunodiffusion techniques.

Specific non-virion antigens have been demonstrated in adenovirus 12 tumor or cells transformed by adenovirus 12. These antigens (neoantigens or tumor antigens) have been demonstrated by complement fixation reactions (Huebner et al., 1964; Gilead and Ginsberg, 1965), and with immunofluorescent techniques (Pope and Rowe, 1964; Levinthal et al., 1966; Shimojo et al., 1967). The results of the latter studies indicate that the tumor antigens are localized predominately within the nucleus and are probably distinct from the transplantation antigens. The transplantation antigens are thought to be present at the cell surface (Winn, 1960; Moller and Moller, 1962; Berman, 1967).

Berman and Rowe (1965) using agar gel diffusion techniques, found three types of antibodies to "new antigens" in the sera of tumor bearing hamsters. Only one of these antigens was demonstrable in the tumor. This antigen is also found during early stages of the infectious cycle in tissue culture cells; it is usually referred to as the "T" or "tumor" antigen. Other new antigens may be present in the tumor; however, these are demonstrable only indirectly through the presence of specific serum antibodies or through transplantation resistance. Antibodies to the type-specific C or fiber antigen and a new antigen, termed D, may be found in the sera of hamsters bearing adenovirus type 12 tumors.

The structural antigens of adenovirus type 12 have been fairly well characterized. However, until recently, much less was known about the nature of the tumor or "T" antigen induced by adenovirus

type 12. Gilead and Ginsberg (1965, 1968 a, b) have presented evidence that the "T" antigen is a relatively small (S_{20w} 2.58), heat labile, acidic protein of a single molecular species. The protein is synthesized early in the course of infection before the production of viral DNA and capsid proteins. Its production is dependent upon de novo protein synthesis, but it may be synthesized in the absence of DNA synthesis. The role of the tumor antigen remains to be defined. Although the evidence suggests that it is probably an "early protein" or enzyme necessary for synthesis of viral DNA, the possibility remains that it has no role in either virus replication or viral oncogenesis and merely serves as a marker of a functioning portion of the viral genome in the tumor or virus-infected cells.

With regard to the antibody response in the tumor bearing hamster, it has been shown that the antibody to both the tumor or "T" antigen and the structural virion antigen are found exclusively in the 7S fraction of the gamma globulin (Hollinshead et al., 1966; Fugmann and Sigel, 1967; Van Hoosier et al., 1968). Fugmann and Sigel (1967) found that the hamster 7S antibodies to the "T" antigen could be subdivided by DEAE chromatography into two fractions, γ-1 and γ-2.

The role of the antibodies in relation to the process of tumorigenesis has not been established. Several recent studies have indicated that the antibodies to the structural components of the virus afford some protection to the host. Eddy and coworkers (1964) found an inhibition of tumor production in hamsters repeatedly

injected with live adenovirus type 12 during the period of tumor formation. Peries et al. (1966) demonstrated inhibition of the formation of adenovirus 12 induced tumors in hamsters inoculated subcutaneously with live adenovirus type 5. No inhibition occurred when killed type 5 was used. Schild et al. (1967) reported production of tumor inhibition when hamsters were immunized with both live and killed adenovirus type 5, live types 2, 7 and 12. In their study, tumor inhibition was found to be greater in animals immunized with the heterotypic viruses than with the homologous type 12. Larson et al. (1967) demonstrated the prevention of virus tumorigenesis in newborn hamsters when the mothers had been immunized with virus prior to conception. This study was done with the SV-40 system and no report of a similar study with adenovirus type 12 has been published to date.

While much is known about the in vivo immune responses in tumor bearing hamsters, relatively little has been reported concerning studies of the in vitro immune responses of tissues or cells of these animals.

Hellstrom and Sjorgren (1967) demonstrated by colony inhibition tests that lymphocytes from animals sensitized to the specific tumor antigen of adenovirus type 12 tumor cells are capable of inhibiting the establishment of the tumor cells when the two cell types are planted together. This inhibition was mediated in the absence of complement. Evidence presented in this report suggests that the response of the sensitized lymphocytes was

directed against the transplantation antigen rather than CF antigens present in the tumor cells.

MATERIALS AND METHODS

I. Cell cultures

HeLa, KB, and human embryonic kidney (HEK) cells were grown as monolayers in 8 or 32 ounce prescription bottles. Adenovirus type 12-transformed hamsters cells were grown in 8 ounce prescription bottles for "tumor" antigen preparations. Growth medium consisted of Eagle's mimimum essential medium (MEM) supplemented with 10 per cent heat inactivated fetal calf serum (FCS) for HeLa and KB cells and 20 per cent FCS for HEK and adenovirus type 12-tumor cells. The following antibiotics were added per ml of growth medium: 100 units penicillin-G, 100 μg dihydro-streptomycin, 2.5 μg tetracycline, and 30 units nystatin. Following inoculation of virus, the cultures were maintained with the same medium containing 2 per cent FCS.

II. Antigen preparation

A. Virus production

Adenovirus type 5 (adenoid 75), type 2 (adenoid 6), type 7a (S 1058), and type 12 (Huie) were grown in HeLa or KB cell monolayers. The cells were harvested when 75 to 90 per cent of the cell sheet exhibited cytopathic effects. At the time of harvest the medium was decanted and the infected cell sheet was washed twice with phosphate buffered saline (PBS), pH 7.2. The amount of PBS used was equal to the original media volume. The cells

were scraped from the glass surface into a 2 to 3 ml of PBS and were centrifuged at 700 x G for 10 minutes. The cells were then resuspended in PBS to a final volume equivalent to 10 per cent of the original volume of the tissue culture fluid. This suspension was subjected to four to six cycles of freezing (-20°C) and thawing (37°C) to release intracellular virus. The lysed cells were centrifuged at 1800 x G for 20 minutes to remove the cell debris. Such preparations will be referred to as "crude" virus.

B. Virus purification

Virus purification and separation of the structural viral components accomplished with methods of freon extraction, CsCl density gradient centrifugation and diethylaminoethyl (DEAE) cellulose column chromatography. The methods employed were similar to those described by Klemperer and Pereira (1959) and Wilcox and Ginsberg (1963).

1. Freon extraction

One volume of crude virus in PBS (pH 7.2) and two volumes of cold (4°C) freon (Genesolv-D) were homogenized for one minute in a Virtis "45" homogenizer at a setting of 30. The virus-freon mixture was centrifuged for 20 minutes at 1800 x G at 4°C in a Servall RC-2 centrifuge. The resulting aqueous layer containing virus was harvested and re-extracted with two volumes of freon. The aqueous layer from the second extraction was collected, aliquoted, sterility checked and stored at -20°C until used. These preparations were titrated in HeLa cell cultures for toxicity and infectivity. Virus preparations were analyzed further by passive hemagglutination

(PHA) and complement fixation (CF) for antigenic activity against various reference sera. All adenovirus types listed previously were subjected to the freon extraction procedure. Preparations of uninfected KB cells were also subjected to this extraction procedure; these preparations were used as antigen controls in antibody assays. Adenovirus types 5 and 12 were purified further.

2. CsCl density gradient centrifugation

Preparative ultracentrifugation of freon extracted virus was carried out similar to methods described by Wilcox and Ginsberg (1963). Freon extracted virus preparations of adenovirus types 5 and 12 were dialyzed overnight at 4°C against 0.01 M Tris (hydroxy-methyl) amino methane buffer (pH 8.1). The virus solution was added to 2.27 gm of CsCl to give a final density of 1.34. This mixture was placed in a 1/2 x 2 inch cellulose nitrate centrifuge tube. Equilibrium centrifugation was carried out in a Beckman Model L-2 preparative centrifuge, employing the SW-50 swinging bucket rotor. The centrifugation period was 24 hours at 165,000 x G.

After centrifugation the tubes were placed in a Buchler puncturing device and consecutive twenty drop fractions (0.25 ml) were collected from the tubes by the bottom puncture method. Each fraction was diluted to a final volume of 1.5 ml with PBS (pH 7.2) and was assayed as described for freon extracted virus. The active fractions were pooled and in some cases rebanded in CsCl according to the above procedure to achieve further concentration and purification. The active fractions were dialyzed against multiple changes of 0.01 M

phosphate buffer (pH 7.0) and were chromatographed on DEAE cellulose columns to achieve further purification and separation of the structural components.

3. Diethylaminoethyl cellulose column chromatography

The procedure for DEAE column chromatography was similar to that reported by Klemperer and Pereira (1959) with minor modifications. DEAE (Cellex-D, Bio-Rad Laboratories, exchanger capacity 0.98 meq/gm) was treated with several liters of 95 per cent ethyl alcohol and equilibrated with 0.01 M phosphate buffer (pH 7.0). Glass columns 300 mm in length and 8 mm inside diameter were used. The equilibrated DEAE slurry was added and allowed to pack by gravity to a height of 150 mm. The amount of DEAE required to prepare the columns was approximately 0.7 gm (dry weight).

Samples applied to the columns were freon extracted virus, freon extracted KB cell extracts or pools of antigenic viral material separated by CsCl density gradient centrifugation. Prior to application on the column the samples were dialyzed overnight at 4°C against 0.01 M phosphate buffer (pH 7.0). Protein determinations were made on the dialyzed samples and an amount not exceeding 2.5 mg was applied to the columns. The samples were added to the top of the DEAE and stirred into the upper 2 cm. The DEAE was allowed to resettle before addition of the eluting solutions.

The antigens were eluted from the columns by the stepwise addition of solutions of increasing NaCl concentration. These solutions were prepared in 0.01 M phosphate buffer (pH 7.0). The NaCl

concentrations were increased by 0.025 M or 0.05 M increments and covered the range from 0.00 M to 0.55 M. Twenty to 30 ml of each solution was added to the column. Ten ml fractions were collected and assayed for antigenic activity using reference sera and culture fluids. The assay methods were passive hemagglutination and complement fixation.

Fractions with low initial activity were concentrated five-fold by dialysis against polyethylene glycol 20,000 (Carbowax). In certain experiments the active fractions from adenovirus type 5 virus preparations, which eluted with 0.05 to 0.075 M and 0.20 M NaCl were pooled separately, concentrated and rechromatographed to achieve greater purification of the viral antigens. Column chromatography was carried at room temperature.

C. Viral assays

1. Infectivity titrations

Virus preparations were titrated in HeLa or KB cell tube cultures. Serial tenfold dilutions were made in Hank's balanced salt solution (BSS); 0.1 ml of each dilution was inoculated into each of four tubes. The tubes were incubated for one hour at 25°C to allow for virus adsorption and were fed with 0.9 ml of MEM containing 2 per cent FCS. After 7 days incubation at 37°C, the amount of virus necessary to infect 50 per cent of the cultures ($TCID_{50}$) was calculated by the formula of Reed and Muench (1938) and recorded as the initial dilution of virus inoculated.

2. Toxin titrations

Twofold serial dilutions of virus preparations were made in MEM containing 2 per cent FCS. Duplicate tube cultures of

HeLa cells were inoculated with 0.1 ml of each dilution. After one hour at 25°C each culture was fed with 0.9 ml of the maintenance medium and incubation was continued at 37°C. Readings for cytopathic effects were made after 4 and 24 hours incubation. Fifty per cent endpoints were calculated after 24 hours incubation.

Toxin and infectivity titrations were done on each virus preparation before and after freon extraction. Active fractions from the CsCl density gradients and from the DEAE cellulose columns were also assayed for toxin and infectivity.

3. Protein determinations

The protein content of the virus material applied to DEAE cellulose columns and of the preparations used to expose tissue fragments was determined by measuring the absorbance at 280 mu in a Bausch and Lomb Precision spectrophotometer. The method of Kalchar (1947) was used to correct for nucleic acid absorbance at 260 mu.

D. Tumor antigen preparation

Adenovirus type 12- transformed hamster cells were used as a source of tumor antigen. The cells were removed from the surface of the culture bottles and were washed with two volumes of PBS (pH 7.2). The cells were resuspended in PBS to contain 2×10^7 cells/ml and the suspension was sonified with a Branson sonifier. The sonified preparations were centrifuged at 700 x G for 10 minutes to remove the cellular debris. The resulting supernate was used as the tumor antigen.

E. Antigen titrations

Each virus preparation and tumor antigen preparation used in the antibody assays were titrated in checkerboard titrations against

various reference sera. The optimal dilutions were determined for passive hemagglutination and complement fixation tests.

III. Animals

A. Rabbits

Female New Zealand white rabbits (5 to 7 lbs) were injected intravenously (IV) with one 0.5 ml dose of freon extracted adenovirus type 5. This was the equivalent of $5 \times 10^{4.5}$ (HeLa) $TCID_{50}$. Certain animals were injected with adenovirus type 5 structural subunits prepared by DEAE cellulose column chromatography. The latter animals were injected three times (IV) with 1.5 ml per injection. Serum antibody titers were determined periodically and at the time of sacrifice of the animals. Various intervals of time elapsed following the primary exposure of the rabbits to the antigens. The animals injected with freon extracted virus were sacrificed 3, 6, 9, and 12 months after the initial injection. The animals injected with the viral subunits were sacrificed 9 to 12 months after the third injection. All animals were sacrificed by exsanguination and their sera were saved for antibody assays. The lymph nodes, spleen and thymus of the animals were removed for maintenance in vitro.

B. Hamsters

Random bred pregnant female Syrian hamsters were obtained from Lakeview Farms, Vineland, New Jersey. Newborn hamsters from these animals were used for primary tumor induction. Weanling hamsters from the same supplier were used as recipients of the tumor transplants.

1. Primary tumor induction

Newborn hamsters were injected subcutaneously on the dorsal flank with 0.1 ml (10^3 $TCID_{50}$) of adenovirus type 12 in order to induce tumors. The animals were injected within 24 hours after birth. When tumors became one to two inches in diameter, the animals were sacrificed by exsanguination. The serum was saved for antibody assays. The lymph nodes and spleen of the animals were removed for maintenance in vitro and the tumors from certain animals were removed for transplantation.

2. Tumor transplantation

Serial transplants of the viral induced tumors were made to weanling (30 to 50 gm) male and female hamsters. The tumor tissue from the donor animals was removed aseptically and non-necrotic portions were washed three to five times in Hank's BSS containing twice the standard concentration of antibiotics used for cell cultures. The tissue fragments were finely minced, diluted with a small amount of BSS, and 0.2 to 0.3 ml of the mince was injected subcutaneously into the dorsal flank of the recipient animals. Animals bearing transplanted tumors were studied in a manner identical to the animals bearing virus induced tumors. In this study two serial transplants of four viral induced tumors were made.

IV. Organ culture methods

A. Tissue preparation

The tissues used in these experiments were lymph nodes (popliteal and inguinal), spleen and thymus from rabbits and lymph nodes

(inguinal and mesenteric) and spleen from hamsters. All tissues were processed according to the following procedure.

The tissues were removed aspetically, washed twice in BSS containing double concentrations of antibiotics. The lymph nodes were trimmed of fat and the capsule was removed from the spleens. The tissues were minced into fragments approximately 1 to 2 mm in their longest dimension and were subjected to two additional washes in BSS. The fragments were divided into two groups: (1) a control group which was exposed only to BSS and (2) a group which was exposed to appropriate dilutions of viral antigens in BSS. Dilution of the virus preparation was based either on infectivity determined as $TCID_{50}$ in HeLa or KB cell cultures or on protein content when non-infectious subunit preparations were used. All dilutions were made in BSS without antibiotics. The hamster tissues were exposed to 100 $TCID_{50}$ of freon extracted adenovirus type 12. The rabbit tissues were exposed to 1000 $TCID_{50}$ of freon extracted adenovirus types 2, 5, 7a or 12 depending upon the design of the experiment or to approximately 10 ug of protein in the viral subunit preparations. In certain experiments the dilution of adenovirus type 5 was varied in order to determine the effect of various concentrations of the antigens on antibody responses of the tissues. The diluted antigen preparations and tissue fragments were placed in one dram vials and incubated at 37° C for 2 hours in an atmosphere of 5 per cent CO_2 and 95 per cent air. After incubation the fragments were washed three times with BSS to remove unabsorbed antigens, then were placed in culture.

B. Organ cultures

Methods of organ culture were similar to those of Ortiz-Muniz and Sigel (1967). The tissue fragments were placed on Noble agar discs in 60 mm petri dishes. The agar discs consisted of a bottom layer of 1 per cent agar and a top layer of 0.5 per cent agar prepared in BSS adjusted to pH 7.0 with 0.3 N NaOH. Discs 2.5 cm in diameter and 2-3 mm in thickness were cut with a Bellco tube closure. Six to eight tissue fragments were placed around the periphery of the discs and two discs were placed in each culture dish. The organ culture system is depicted in Figure 1.

The basic culture medium consisted of Eagle's MEM supplemented with 20 per cent heat inactivated FCS and antibiotics in the concentrations used for cell cultures with the omission of tetracycline. Two ml of medium was used per culture. Additional ingredients were added to the basic medium depending upon the objectives of the experiment. Hydrocortisone-succinate (Solu-Cortef, Upjohn) and phytohemagglutinin-P (Difco) were used in studies of the effect of compounds which have been reported to produce enhancement of the antibody response. In studies with hydrocortisone succinate, the composition of the medium was varied with respect to serum and the steroid. The composition of the medium was: a) Eagle's MEM containing 20 per cent FCS, b) Eagle's MEM containing 20 per cent serum and hydrocortisone succinate (1 μg/ml), and c) Eagle's MEM containing only hydrocortisone succinate. Phytohemagglutinin-P was added to the basic medium in a concentration of 250 μg/ml. Some studies were done in which the cultures were exposed to medium containing phytohemagglutinin. In

Figure 1. Organ culture system for maintenance of tissue fragments *in vitro*. Mag. 1/3X

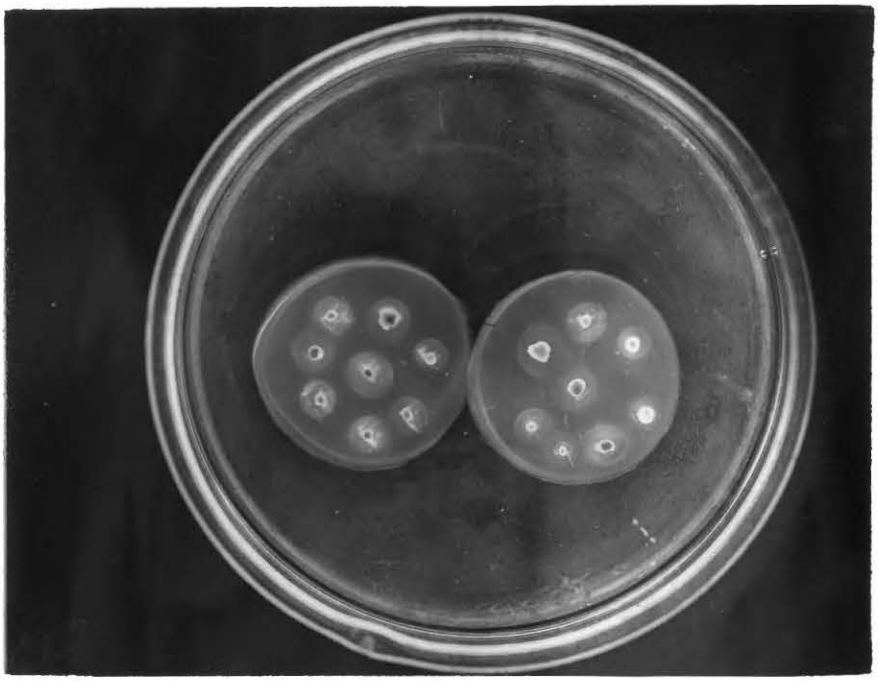


Figure 1

these studies, the cultures had been maintained 30 days prior to the exposure to phytohemagglutinin. These cultures were exposed to phytohemagglutinin for 12 hours, then the medium containing the phytohemagglutinin was removed and the culture dishes were rinsed three times with balanced salt solution to remove the residual phytohemagglutinin. New medium without phytohemagglutinin was added and the cultures were re-incubated. In other studies, fragments of tissue were exposed to 250 μg/ml phytohemagglutinin for 2 hours before they were placed in culture.

Chloramphenicol and puromycin were added to the basic culture medium in the studies of the effects of protein synthesis inhibition. Chloramphenicol was used at a concentration of 0.15 M (50 μg/ml) and puromycin was used at two concentrations (1 μM and 3 μM). In these studies the tissues were cultured in medium containing the inhibitors for various periods of time.

In routine maintenance of all cultures, the entire medium was changed every third day and the fluid harvests were assayed for antibody activity immediately or stored frozen at -20°C. Fluids from cultures of rabbit tissues were assayed for antibody activity without further processing; however, early experiments indicated that fluids from cultures of hamster tissues required concentration prior to antibody assay. Pools were made of the culture fluids harvested over a nine to twelve day period. Each pool represented three serial samples. Pool I contained samples 2, 3, and 4; pool II

contained samples 5, 6, and 7; pool III contained samples 8, 9, and 10. Sample 1 was not included to minimize the possibility of interference by any residual antigen in this sample. The pooled fluids were placed in 8/32 inch dialysis tubing attached to 500 ml glass bulbs and were subjected to pressure (12 psi) dialysis at 4°C. This resulted in a four to fivefold concentration of the fluids. Samples of complete medium were also subjected to this procedure to serve as controls and were assayed along with concentrated culture fluids.

The production of antibody was taken as one index of the viability of the cultures; however, other criteria were used. The cultures were examined microscopically for evidence of cellular proliferation around the explants. The viability of the cells was determined by the trypan blue exclusion technique (Pappenheimer, 1917). Cells growing from the explant on the surface of the agar and, where possible, the tissue fragments were examined after staining with 1.25 per cent trypan blue in normal saline. The cultures were stained at the termination of the experiment.

Fragments from certain cultures were harvested periodically and histological sections and impression smears were made for cytological studies. The fragments were fixed for 30 to 60 minutes in Zenker-formal fixative (9:1). The tissue sections were prepared and stained by the histology laboratory of the Department of Pathology. Certain sections were stained with hematoxylin and eosin; others were stained with periodic acid Schiff. Impression smears were also made of of the cells surrounding the explants. These preparations were air-dried,

fixed in absolute methanol and stained with Wright-Giemsa.

V. Antibody assays

A. Complement fixation

Complement fixation tests were done by the micro-technique described by Lefkowitz et al. (1966). Sera were inactivated at 56°C for 30 minutes prior to assay; culture fluids were not inactivated. Twofold serial dilutions of sera or culture fluids were made in veronal buffered saline (VBS). Dilutions were made in tubes or directly in microtiter U plates (Linbro Plastics) with a 0.025 ml calibrated loop (Cooke Engineering Co.). Exactly 0.025 ml of each dilution was mixed with two units of antigen contained in 0.025 ml and two full units of guinea pig complement contained in 0.05 ml were added to each dilution of the antigen-antibody mixture. The mixture was shaken and incubated overnight at 4°C, after which time the indicator system was added. This system was prepared by mixing equal volumes of amboceptor, 1:2000 in VBS, and 2 per cent sheep red blood cell suspension. After 10 minutes incubation at 25°C, 0.05 ml of the sensitized cell suspension was added to each well of the microtiter plate. The plates were incubated at 37°C for one hour and then were read for hemolysis. Endpoints were determined as the highest dilutions of serum or culture fluid which produced two plus fixation of complement. Each test contained a complement titration and controls for the antigen, serum or culture fluid and sensitized cells. All tests routinely contained positive serum controls to assess the effectiveness of the assay.

B. Neutralization tests

Neutralization tests were carried out with rabbit sera and culture fluids from the rabbit explants. These tests were done in human embryonic kidney (HEK) cell cultures. Adenovirus type 5 was diluted in MEM without serum to contain 50-100 $TCID_{50}$/ 0.1 ml. Twofold serial dilutions of serum and culture fluids were made in the same diluent. Equal volumes of virus and either serum or culture fluid dilutions were mixed and held at 25°C for one hour. Two-tenths ml of each mixture was added to triplicate cultures of HEK cells. The serum-virus mixtures were allowed to remain on the monolayer for one hour at 25°C before the addition of 0.8 ml maintenance medium. The cultures were then incubated at 37°C.

The cultures were read daily for cytopathic effects (CPE). Final readings were made when the virus control exhibited three to four plus CPE. Neutralization endpoints were taken as the highest dilution of serum or culture fluid which produced complete inhibition of CPE in all of the triplicate cultures. Virus titrations were included with each test.

Pre-and post-immunization sera were titrated in the same test. All serum samples were heat inactivated at 56°C for 30 minutes prior to assay. In assays of culture fluids, samples from cultures which were negative for antibody by other assays were included as controls on non-specific factors in the fluids which might cause virus neutralization.

C. Passive hemagglutination

The procedure of Stavitsky (1954) as modified by Lefkowitz (1967) was used. Sheep red blood cells (SRBC) in Alsever's solution were obtained from Baltimore Biological Laboratories. The cells were washed three times in PBS (pH 7.2) and resuspended as a 2.5 per cent suspension. Equal volumes of the SRBC suspension and tannic acid, 1:20,000 in PBS (pH 7.2), were incubated at 37°C for 10 minutes. The tanned cells were centrifuged at 700 x G for 10 minutes, washed once in an equal volume of PBS and were resuspended to the original RBC volume (2.5 per cent) in PBS (pH 6.4).

One volume of the appropriate antigen dilution in PBS (pH 6.4), four volumes of PBS (pH 6.4) and one volume of tanned SRBC were incubated at 25°C for 10 minutes. The antigen coated cells were centrifuged at 700 x G for 10 minutes, washed with two volumes of one per cent normal rabbit serum-saline (NRS) buffered at pH 7.2, and were resuspended in four volumes of NRS. The final dilution of the antigen coated cells yielded a 0.625 per cent suspension. Tanned SRBC were treated in the same manner except an additional volume of the buffer was substituted for the antigen dilution. Such preparations were used as tanned cell-saline controls and were included with each assay. Five one-hundredths ml of the final suspension was added to 0.1 ml serum or culture fluid dilutions contained in the microtiter plates. The mixtures were shaken and all allowed to settle for 2 to 3 hours at room temperature. Titration endpoints were determined

as the reciprocal of the highest serum or culture fluid dilution which produced a three plus agglutination pattern.

The optimum dilution of each virus preparation or cell extract used to sensitize tanned SRBC was determined by checkerboard titrations against reference antisera. The antigen dilution which gave maximum sensitivity without non-specific agglutination of the SRBC was used to coat the tanned cells.

Sera were heat inactivated at 56°C for 30 minutes and were tested for SRBC agglutinins prior to assay for virus specific antibody. Two or fourfold dilutions of sera or culture fluids were made either in tubes or in microtiter plates with loops calibrated to deliver 0.025 or 0.05 ml. The diluent was NRS. When antibody dilutions were made with loops, appropriate volumes of NRS were added to bring the final volume to 0.1 ml prior to the addition of sensitized SRBC.

In addition to the tanned cell-saline controls, the following test controls were used: (1) untanned and unsensitized SRBC, (2) tanned SRBC sensitized with freon extracted preparations of uninfected KB or HeLa cells, (3) tanned SRBC sensitized with fetal calf serum, and (4) tanned SRBC coated with freon extracted preparations of adenovirus types 2, 5, 7a, or 12.

Passive hemagglutination inhibition (HI) tests were done on the culture fluids in order to assess further the specificity of this reaction. Homotypic and heterotypic adenovirus preparations, KB cell extracts and fetal calf serum were used as the inhibiting or

blocking antigens in the inhibition tests. The dilution of the blocking antigen used was twice the concentration which was found to produce a fourfold decrease in antibody titer when the specific antigen-sensitized SRBC were reacted with the homologous antiserum. An equal volume of the blocking antigen was added to each dilution of serum or culture fluid. The mixtures were incubated at 25°C for one hour and then were tested for hemagglutinating activity for tanned SRBC sensitized with specific adenovirus test antigen. The test antigen was adenovirus type 5 in the studies on the rabbit culture fluids; adenovirus type 12 in the studies on the hamster culture fluids. After addition of the antigen coated cells, the mixtures were allowed to settle and agglutination patterns were read as previously described.

In HI tests with serum and culture fluids from the rabbit experiments, the blocking antigens used were adenovirus type 5, structural subunits of type 5, adenovirus types 2, 7a, and 12, KB cell extracts and fetal calf serum. The test antigen was adenovirus type 5. In the experiments with hamsters, the blocking antigens were adenovirus type 12 or the structural subunits of type 12 in addition to the same ones used in the rabbit experiments. The test antigens were adenovirus type 12 or the structural subunits.

D. Determination of the molecular species of antibody

Sucrose density gradient centrifugation (Kunkel, 1960) of fluids from rabbit lymph node and spleen cultures was carried out to determine the molecular species of antibody produced in vitro.

A Buchler gradient forming device was used to prepare linear gradients from 10 per cent (w/v) to 37 per cent (w/v) sucrose. Sufficient quantities of the two solutions were added to the mixing chambers to prepare three 5 ml gradients simultaneously. Gradients were formed in cellulose nitrate centrifuge tubes (1/2 x 2 inch). Serum or culture fluids were diluted 1:1 with isotonic (0.15 M) saline and a 0.2 ml sample was layered on the gradient. The tubes were placed in the SW-50 swinging bucket rotor and were centrifuged in a Beckman Model L-2 preparative ultracentrifuge at 130,000 x G for 16 hours.

After centrifugation the tubes were placed in a Buchler puncturing device at 4°C. Consecutive twenty drop fractions (0.25 ml) were collected from a puncture in the bottom of the tube. The first fractions collected had the greatest density. Each fraction was brought to a final volume of 0.5 ml with PBS (pH 7.2). Subsequent twofold dilutions were made in one per cent normal rabbit serum-saline for assay by passive hemagglutination or in veronal buffered saline for assay by complement fixation.

Portions of each fraction were mixed with an equal volume of 0.2 M 2-mercaptoethanol (2-ME) to test for the presence of 2-ME sensitive antibody (Deutsch and Morton, 1957). These mixtures were incubated at 37°C for one hour and at 4°C overnight before assay by passive hemagglutination. Some fluids were treated with 0.2 M 2-ME prior to centrifugation. Comparable untreated samples were centrifuged at the same time for comparison. Fractions were not dialyzed

prior to assay since it was shown (Lefkowitz et al., 1966) that neither sucrose nor mercaptoethanol interferred with the assay procedures utilized here.

VI. Demonstration of adenovirus type 5 in spleen and lymph node fragments from rabbits

Portions of the spleen and lymph nodes from rabbits originally injected with either freon extracted adenovirus type 5 or the structural subunits were suspended in 2 ml BSS. Each sample was macerated in a Ten-Broeck tissue grinder. The cell debris was removed by centrifugation at 700 x G for 10 minutes. Two-tenths ml of the supernate was inoculated into triplicate tube cultures of human embryonic kidney (HEK) cells. After inoculation, the cultures were incubated at 25°C for one hour and were fed with 0.8 ml MEM containing 3 per cent FCS. Incubation was then continued at 37°C. The cultures were read daily for cytopathic changes characteristic of adenovirus infection. Blind passages of negative cultures were made after 10 days. The cells were scraped from the surface of the tubes. The media and cells from the triplicate cultures were pooled and centrifuged at 800 x G for 10 minutes. The supernate was removed and the cell pellet was resuspended in 0.6 ml of medium. This cell suspension was frozen and thawed three times and recentrifuged. The resulting supernate was used as inoculum for further passages in HEK cells. Uninoculated control cells from the previous passages were processed identically. The passage cultures were read daily for 10 days for evidence of the presence of adenovirus.

The cytopathic agents recovered from the tissues were identified as adenovirus type 5 by neutralization of infectivity with specific antiserum.

RESULTS

PART I

A. In vivo antibody responses of rabbits to adenovirus type 5 antigens.

Nine rabbits were used in this study. Seven of the animals were injected with a freon extracted preparation of adenovirus type 5. Two animals were injected with more highly purified structural viral subunits prepared by DEAE-cellulose column chromatography. One of these animals received the type-specific C or fiber antigen; the other received the group-specific A or hexon antigen. The antibody titers of serum samples were determined by complement fixation and passive hemagglutination methods. The results are presented in Tables 1 and 2. The data presented indicate that the animals (001-009) which received a single injection of infectious virus had detectable levels of antibody for as long as twelve months after the single exposure. The animals (8 and 9) which received multiple injections of the viral structural subunits did not have antibody levels at the time of sacrifice detectable by the methods used. The antibody response of animal 9 was detectable only by complement fixation, whereas the antibody response of animal 8 was detectable both by complement fixation and passive hemagglutination. Animal 9 was injected with the hexon antigen, and it has been shown (Lefkowitz, 1967) that the hexon does not

TABLE 1

IN VIVO ANTIBODY RESPONSES OF RABBITS* INJECTED WITH ADENOVIRUS TYPE 5 ANTIGENS AS DETERMINED BY COMPLEMENT FIXATION**

Animal No.	Preimmunization	Weeks After Initial Injection							
		2	3	4	12	24	36	48	52
001	<2		64		64+				
002	<2		32			32+			
003	<2	128			256+				
005	<2	128							16+
006	<2	256					64+		
008	<2	64						8+	
009	<2	64						8+	
8	<2	8	16						<2+
9	<2	32	64				<2+		

* Animals 001-009 received a single injection of freon extracted adenovirus type 5. Animals 8 and 9 received 3 injections of DEAE-purified structural subunits of adenovirus type 5. Animal 8 received the fiber antigen; animal 9 received the hexon antigen.

** Titer expressed as reciprocal of serum dilution.

+ Titer at the time of sacrifice.

TABLE 2

IN VIVO ANTIBODY RESPONSES OF RABBITS* INJECTED WITH ADENOVIRUS TYPE 5 ANTIGENS AS DETERMINED BY PASSIVE HEMAGGLUTINATION**

Animal No.	Preimmunization	Weeks After Initial Injection							
		2	3	4	12	24	36	48	52
001	<10		2560		10,240+				
002	<10		640			10,240+			
003	<10	2,560			10,240+				
005	<10	10,240							640+
006	<10	40,960					10,240+		
008	<10	10,240						2560+	
009	<10	10,240						2560+	
8	<10	256		1024					<4+
9	<10	4		4			<4+		

* Animals 001-009 received a single injection of freon extracted adenovirus type 5. Animals 8 and 9 received 3 injections of DEAE-purified structural subunits of adenovirus type 5. Animal 8 received the fiber antigen; animal 9 received the hexon antigen.

** Titer expressed as reciprocal of serum dilution.

+ Titer at the time of sacrifice.

attach to the tanned erythrocyte. Animal 8 was injected with the fiber antigen. Antibody to this antigen can be assayed by both serologic procedures.

B. In vitro antibody responses of rabbit lymphoid tissues exposed to adenovirus type 5 antigens

1. Antibody responses of various tissues maintained in vitro

Lymph node, spleen and thymus fragments were exposed in vitro to freon extracted adenovirus type 5. In some experiments lymph node and spleen fragments were exposed to DEAE-purified structural subunits of the virus. Fragments of each type of tissue were also maintained without exposure in vitro to the virus preparations.

The results of the antibody responses obtained are given in Table 3. These data are presented according to the tissue type studied, the antigen preparation used, and the time interval between in vivo exposure of the animal and exposure of the tissues to the viral antigens in vitro. The lymph node exhibited the most consistent antibody response. Eighty per cent (57 of 71) of the lymph node cultures established from animals (001-009) injected with the freon extracted virus synthesized antibody following exposure of the tissue to the viral antigens in vitro. The ability of this tissue to respond was not affected by prolonged intervals between the in vivo and in vitro exposures to the antigens. The lymph node cultures established from animals (8 and 9) hyperimmunized with the fiber or hexon antigen failed to respond to exposure to any of the antigenic preparations.

TABLE 3

ANTIBODY RESPONSES OF RABBIT LYMPHOID TISSUES FOLLOWING IN VITRO EXPOSURE TO ADENOVIRUS TYPE 5 (Ad. 5) ANTIGENS

Animal No.	Interval Between Injection and Sacrifice (Weeks)	Antigenic Exposure Primary (In Vivo)	Antigenic Exposure Secondary (In Vitro)	Cultures Synthesizing Antibody Total Cultures Exposed In Vitro: Lymph Node	Spleen	Thymus
001	12	Ad. 5*	Ad. 5	4/6	2/3	0/4
003	12	Ad. 5	Ad. 5	4/4	2/4	0/4
002	24	Ad. 5	Ad. 5	6/6	2/2	0/4
006	36	Ad. 5	Ad. 5	12/12	0/6	NC
009	48	Ad. 5	Ad. 5	8/12	0/8	NC
008	48	Ad. 5	Ad. 5	12/16	0/8	NC
005	52	Ad. 5	Ad. 5 Hexon* Fiber*	11/15	0/7	NC
9	36	Hexon	Ad. 5 Hexon Fiber	0/8	0/8	NC
8	52	Fiber	Ad. 5 Hexon Fiber	0/14	0/9	NC

* Ad. 5 - freon extracted preparation; hexon and fiber--DEAE-purified structural subunits.
NC - no cultures.

Each of these animals was sacrificed as a time interval comparable to the sacrifice time of the animals injected with the freon extracted virus.

The splenic tissue was much less active than the lymph node in synthesizing antibodies in vitro. Levels of antibody from cultures of spleen fragments which did respond were lower than the levels from comparable lymph node cultures. Only the spleen cultures established from animals sacrificed within twelve to twenty-four weeks after the in vivo injection could be stimulated to produce antibody in vitro. This indicated that the time interval between in vivo and in vitro exposure to the antigens may effect the response which can be evoked from splenic tissue in vitro.

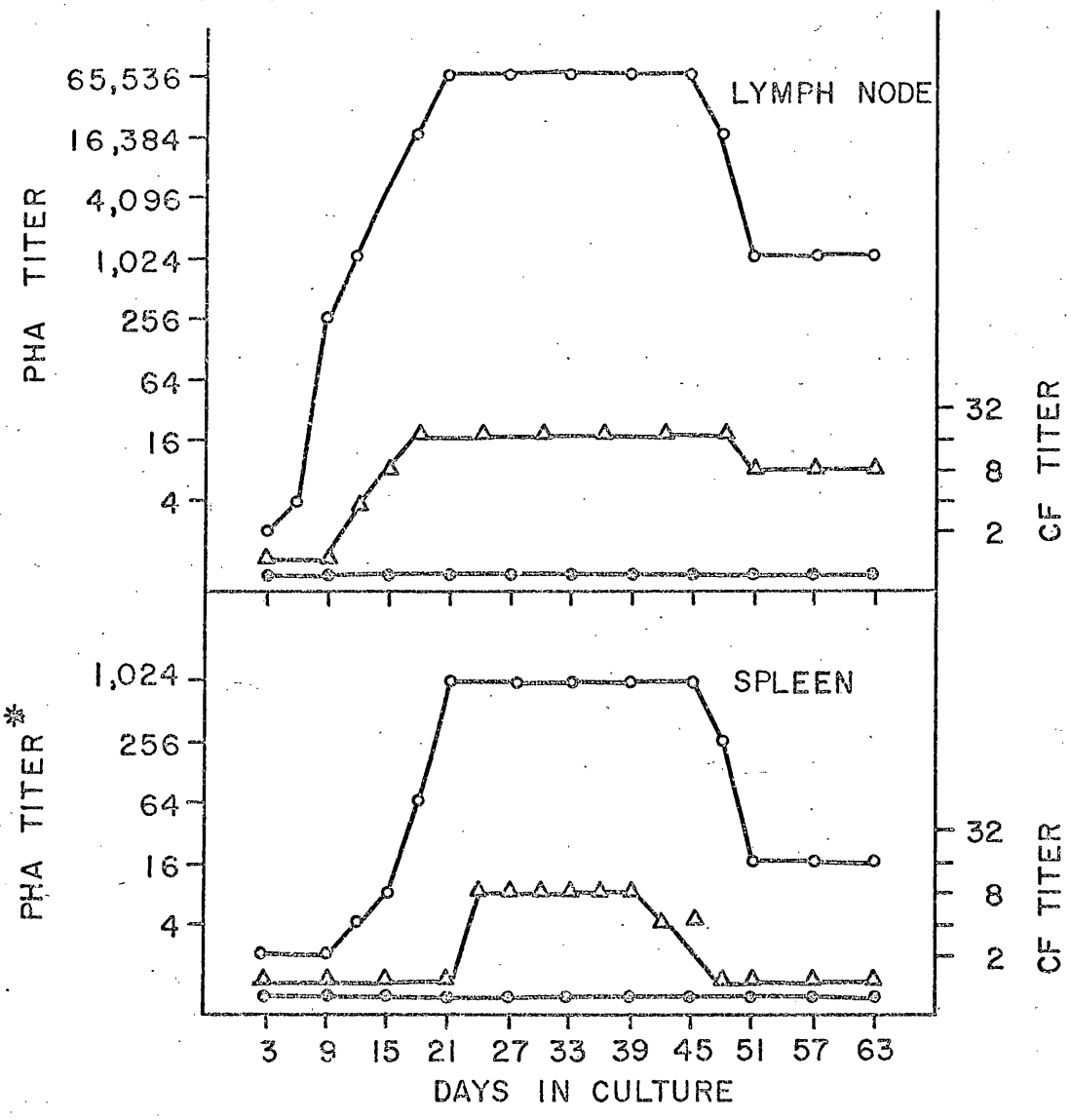
No antibody was detected in cultures of thymic tissue. In one experiment fragments of spleen and thymus, or fragments of lymph node and thymus were cultured together. Low levels of antibody were detectable in the fluids from the cultures of thymus and lymph node; however, the response was probably the result of antibody synthesis by the lymph node tissue. Antibody was not detected in culture fluids from the thymus and spleen fragments.

In all antibody-producing cultures the tissue fragments had been exposed to the viral antigens in vitro. Cultures of fragments maintained without in vitro exposure did not produce antibody at detectable levels.

2. Time course of antibody responses in vitro

Figure 2 represents a typical response of the lymph node and spleen fragments which synthesized antibody in vitro. This response

Figure 2. Antibody response of lymph node and spleen fragments *in vitro*.



ANTIBODY RESPONSES OF LYMPH NODE AND SPLEEN FRAGMENTS IN VITRO

o—o Passive hemagglutination (PHA) titer; tissue exposed to adenovirus type 5 in vitro

Δ—Δ Complement fixation (CF) titer; tissue exposed to adenovirus type 5 in vitro

o—o Tissue not exposed to adenovirus type 5 in vitro

* Expressed as reciprocal of dilution

Figure 2

is expressed in terms of time of appearance of antibody, levels of antibody attained and duration of synthesis of antibody.

As indicated in the figure, the antibody was detectable both by complement fixation and passive hemagglutination methods. However, the passive hemagglutination method was the more sensitive of the two. Antibody was not detected until the tissues had been in culture for six to nine days. This lag phase was a consistent finding in all experiments. Once antibody became detectable, there was a rapid rise in titer within the following two or three sampling periods. Peak titers were generally reached within twelve to fifteen days after initiation of the culture. The levels of antibody remained fairly stationary for periods up to thirty to forty-five days, then began a gradual decline. Most of the experiments were terminated within sixty to eighty days after initiation; however, one experiment was continued for 120 days. At the termination of this experiment low levels (1:32) of antibody were still detectable by passive hemagglutination. The antibody titers of fluids from the spleen cultures were always lower than the titers of samples from the lymph node cultures.

Replicate cultures established from the tissues of individual animals were, in general, comparable in antibody levels. Greater differences were noted, however, between cultures established from tissues of different animals. Table 4 is a composite of data from replicate lymph node cultures established from animals 001-009. All of the cultures were treated in an identical manner as to the amount of virus used for <u>in vitro</u> stimulation of the tissue and the medium

TABLE 4

ANTIBODY TITERS FROM REPLICATE CULTURES OF LYMPH NODE TISSUE FROM RABBITS INJECTED WITH ADENOVIRUS TYPE 5 ANTIGENS*

Animal No.	Culture No.	Days in Culture							
		3	9	15	21	33	51	66	120
001	1	<4	256	16,384	16,384	4,096	64	64	ns
	2	<4	64	4,096	4,096	4,096	64	ns	ns
002	1	<4	1024	16,384	65,536	65,536	4,096	4,096	ns
	2	<4	256	16,384	65,536	65,536	4,096	4,096	ns
	3	<4	1024	16,384	65,536	65,536	4,096	1,024	ns
003	1	<4	256	4,096	4,096	4,096	1,024	256	32
	2	<4	64	4,096	4,096	4,096	256	64	32
	3	<4	64	1,024	1,024	1,024	64	64	16
005	7	<4	256	65,536	65,536	65,536	16,386	ns	ns
	8	<4	256	65,536	65,536	65,536	4,096	ns	ns
	9	<4	1024	262,144	262,144	262,144	65,536	ns	ns
006	6	<4	4	16,386	16,384	16,384	ns	ns	ns
	7	4	16	16,386	65,536	65,536	16,384	ns	ns
	8	4	4	16,386	4,096	16,384	ns	ns	ns
008	17	<4	16	512	4,096	4,096	ns	ns	ns
	20	<4	64	1,024	1,024	4,096	ns	ns	ns
009	3	<4	4	1,024	4,096	4,096	ns	ns	ns
	7	<4	16	512	1,024	1,024	ns	ns	ns
	24	<4	64	4,096	4,096	4,096	ns	ns	ns

* Passive hemagglutination titers expressed as reciprocal of dilution.

ns - no sample

used for maintenance of the cultures. The antibody was assayed by passive hemagglutination. The data presented in the table were obtained from a test in which all the samples were assayed at the same time in order to have the test conditions standard.

Five samples from each of three lymph node cultures were assayed for the presence and persistence of neutralizing antibodies. Two of the cultures selected were from the same animal (005) and the third from a different animal (002). All of the cultures were producing antibodies detectable by the other assay methods (PHA and CF). The samples of all cultures tested were harvested 6, 12, 18, 24, and 45 days after initiation of the cultures. Samples from non-antibody producing lymph node cultures from both animals were included in the tests as control on non-specific neutralization of the test virus. The results are given in Table 5. The complement fixation and passive hemagglutination titers are included for comparison. Neutralizing antibodies were detected in the three cultures tested. This activity was quite low; the maximum titers obtained were 1:8. The presence of neutralizing antibodies could be correlated in time with the appearance of antibodies detectable by the other tests. There was, however, a more rapid decline of antibodies detectable by neutralization tests than by complement fixation or passive hemagglutination.

3. Effect of various concentrations of viral antigens on antibody responses in vitro

A standard amount of virus (1000 $TCID_{50}$) was used in most experiments. One experiment was designed to measure the limits

TABLE 5

ANTIBODY RESPONSES OF LYMPHOID TISSUES EXPOSED IN VITRO TO ADENOVIRUS TYPE 5 ANTIGENS AS DETERMINED BY NEUTRALIZATION, COMPLEMENT FIXATION AND PASSIVE HEMAGGLUTINATION TESTS*

Animal No.	Culture No.	Assay**	Days in Culture				
			6	12	18	24	45
002	19	NT	2	2	8	2	<2
		CF	<2	4	8	8	8
		PHA	4	1,024	16,384	65,536	65,536
005	8	NT	4	4	4	4	<2
		CF	2	32	32	16	16
		PHA	64	16,384	65,536	65,536	65,536
005	10	NT	2	8	8	4	<2
		CF	2	32	64	32	16
		PHA	64	65,536	262,144	65,536	65,536

*Titer expressed as reciprocal of dilution

**NT - neutralization test

CF - complement fixation

PHA - passive hemagglutination

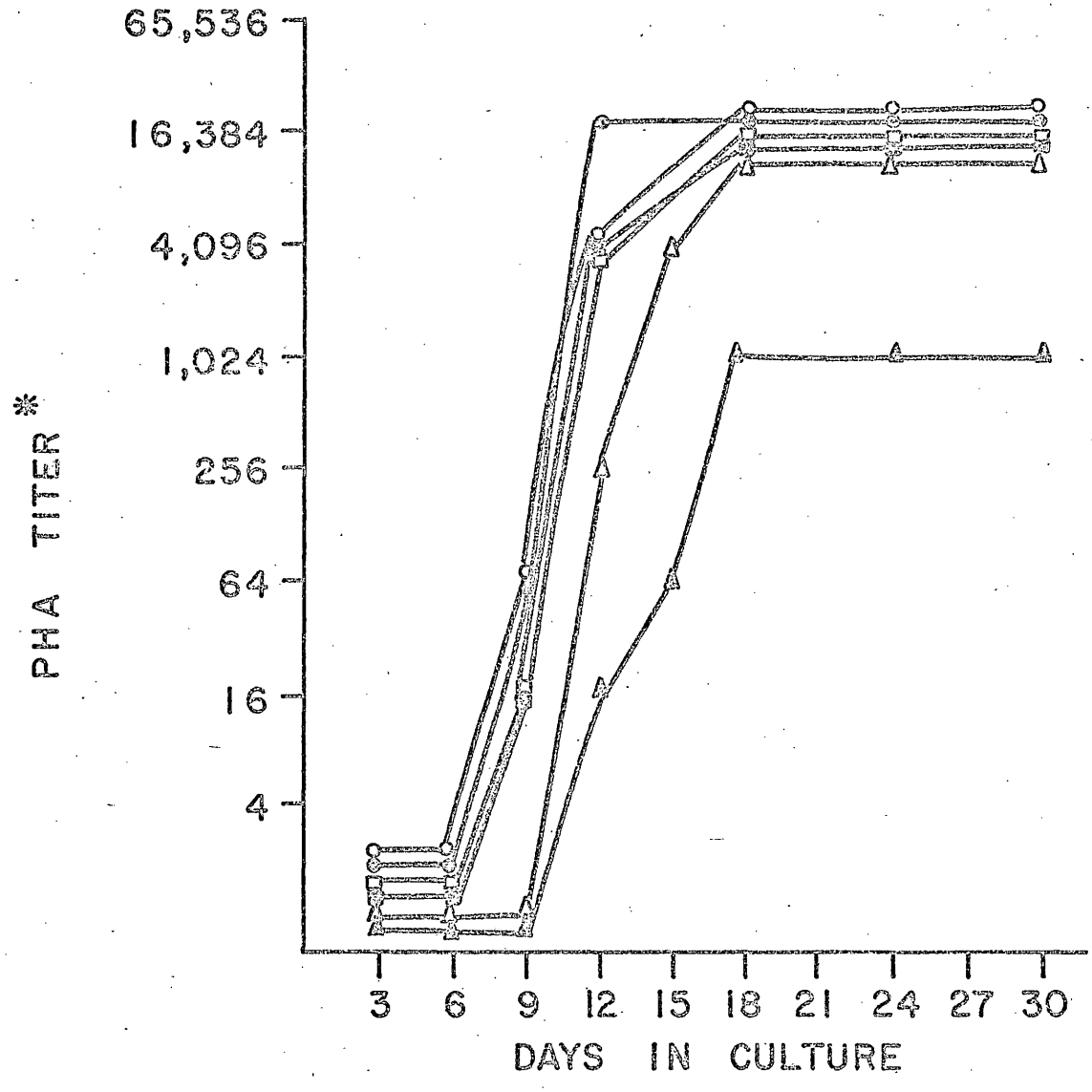
of sensitivity of the antibody response. The freon extracted virus preparation was prepared in dilutions to contain infectious units from 10 $TCID_{50}$ to 10^6 $TCID_{50}$. The results of this experiment are presented in Figure 3. The response of the lymph node fragments from this animal (006) to dilutions of virus containing from 10^2 to 10^6 $TCID_{50}$ was essentially the same. There was no apparent inhibition of the response when the high concentration (10^6 $TCID_{50}$) was used. At the lowest concentration (10 $TCID_{50}$) some decrease in the response was noted, and there appeared to be a slower rise in the titer. The maximum levels attained by these cultures were not as great as the levels attained by the fragments exposed to higher concentrations of virus.

Protein determinations were made on each of the various dilutions used for the in vitro stimulation of the tissue. Based on the absorbance at 280 mμ, the protein content of the preparations ranged from 50 μg to 3 μg/ml.

3a. Antibody responses following re-exposure to antigens after initiation of the cultures

Several attempts were made to evoke additional antibody responses after the tissues had been in culture for several weeks. When antibody production appeared to have reached a stationary level, the cultures were exposed again to the viral antigens. The fragments were not removed from the surface of the agar blocks when the re-exposure was carried out. The virus dilution was added to the culture dishes and allowed to remain for two hours. The culture dish was washed with several changes of balanced salt solution before new

Figure 3. Antibody responses of lymph node fragments exposed to various concentrations of adenovirus type 5.



ANTIBODY RESPONSE OF LYMPH NODE FRAGMENTS EXPOSED TO VARIOUS CONCENTRATIONS OF ADENOVIRUS TYPE 5

10^6 $TCID_{50}$ — 10^3 $TCID_{50}$

10^5 " — 10^2 "

10^4 " — 10^1 "

* Expressed as reciprocal of dilution

Figure 3

medium was added and the culture was re-incubated.

All attempts to induce additional antibody responses were unsuccessful. The fluids harvested at the first sampling period after the cultures were re-exposed to the viral antigens generally had lower titers than the fluids harvested just prior to re-exposure. Titers of the fluids from subsequent samples usually rose to approximately the same level as the titers of the fluids taken just prior to re-exposure, but never exceeded these titers.

4. Specificity of the antibodies synthesized in vitro

Three tests were used to determine the specificity of the antibodies synthesized in vitro. These were: 1) passive hemagglutination tests in which antigens other than adenovirus type 5 were used to sensitize the erythrocytes, 2) passive hemagglutination inhibition tests, and 3) complement fixation tests. The antigens used in the above tests were: fetal calf serum, freon extracted preparations of KB cells and freon extracted preparations of adenovirus types 2, 5, 7a, and 12. Included in some tests were DEAE-purified structural subunits of adenovirus type 5. The results of typical experiments are given in Tables 6, 7 and 8. The same samples are recorded in all the tables for comparative purposes.

The results shown in Table 6 were obtained with passive hemagglutination tests on the culture fluids. The erythrocytes were sensitized with the various antigens listed in the table. Untanned and tanned-unsensitized erythrocytes were included as controls. There was no agglutination of the control erythrocytes which indicated that there were no non-specific factors in the fluids which

TABLE 6

SPECIFICITY OF ANTIBODY SYNTHESIZED IN VITRO AS DETERMINED BY PASSIVE HEMAGGLUTINATION*

Animal No.	Culture No.	Antigens on RBC								
		Ad. 5 Hexon	Ad. 5 Fiber	Ad. 5	Ad. 2	Ad. 7a	Ad. 12	FCS	KB	SRBC
001	7	2	8	2048	4	2	2	2	2	2
002	1	2	512	16,384	8	2	2	2	2	2
	19	2	256	16,384	16	2	2	2	2	2
003	3	2	16	512	2	2	2	2	2	2
	4	2	32	1024	4	2	2	2	2	2
005	7	2	1024	16,384	64	2	2	2	2	2
	8	2	1024	16,384	16	2	2	2	2	2
006	7	2	64	16,384	16	2	2	2	2	2
	8	2	16	16,384	8	2	2	2	2	2
008	17	2	64	2048	4	2	2	2	2	2
	20	2	16	1024	4	2	2	2	2	2
009	3	2	32	4096	4	2	2	2	2	2
	24	2	64	8092	4	2	2	2	2	2

* Titer expressed as reciprocal of dilution.
Ad. - adenovirus, freon extracted preparations, numeral indicates type
Ad. 5 Hexon - DEAE-purified fraction eluted with 0.20 M NaCl
Ad. 5 Fiber - DEAE-purified fraction eluted with 0.05-0.075 M NaCl
FCS - fetal calf serum
KB - freon extracted preparation of KB cells
SRBC - sheep red blood cells, untanned and tanned, unsensitized-saline controls

would cause agglutination of the sensitized cells used in the test. There was no reaction of the fluids with the cells sensitized with either KB cell extracts or fetal calf serum. Antibody activity was detected only with cells sensitized with adenoviral antigens. Higher titers were detected when the fluids were tested with cells sensitized with freon extracted adenovirus type 5. Significant titers were also obtained with cells sensitized with the DEAE-purified fiber or C antigen. No agglutination was obtained with cells treated with the DEAE-purified hexon or A antigen. This was not surprising since, as previously mentioned, the hexon apparently does not attach to the erythrocytes. Some cross-reactivity was observed with cells sensitized with adenovirus type 2 antigens. There was no agglutination of cells sensitized with adenovirus types 7a or 12.

The results of the complement fixation tests are presented in Table 7. There was no complement-fixing activity observed when the fluids were reacted with KB cell antigens or fetal calf serum. The activity was present only when the viral preparations were used as antigens. The highest titers were obtained with the homotypic adenovirus type 5 antigens. When both the DEAE-purified hexon and fiber antigens were used, positive reactions were also obtained. There was more evidence of cross-reactions with the antigens of the heterotypic adenovirus types 2, 7a, and 12 than was noted in the passive hemagglutination test. This was attributed to the fact that complement fixation measures antibody to the hexon antigen as well as antibody to the fiber and penton antigens.

TABLE 7

SPECIFICITY OF ANTIBODY SYNTHESIZED IN VITRO AS DETERMINED BY COMPLEMENT FIXATION*

Animal No.	Culture No.	Antigen							
		Ad. 5 Hexon	Ad. 5 Fiber	Ad. 5	Ad. 2	Ad. 7a	Ad. 12	FCS	KB
001	7	2	2	8	8	<2	2	<2	<2
002	1	2	2	16	8	2	2	<2	<2
	19	2	2	8	4	2	2	<2	<2
003	3	<2	<2	2	<2	<2	<2	<2	<2
	4	2	2	8	4	<2	2	<2	<2
005	7	8	4	64	16	4	4	<2	<2
	8	16	4	32	4	4	4	<2	<2
006	7	2	2	16	4	2	2	<2	<2
	8	2	2	16	4	2	2	<2	<2
008	17	2	2	8	2	2	2	<2	<2
	20	4	2	8	2	2	2	<2	<2
009	3	2	2	4	2	<2	2	<2	<2
	24	2	2	8	4	2	2	<2	<2

* Titer expressed as reciprocal of dilution.

Ad. - adenovirus, freon extracted preparations, numeral indicates type
Ad. 5 Hexon - DEAE-purified fraction eluted with 0.20 M NaCl
Ad. 5 Fiber - DEAE-purified fraction eluted with 0.05-0.075 M NaCl
FCS - fetal calf serum
KB - freon extracted preparation of KB cells

The results obtained with the passive hemagglutination inhibition tests (Table 8) further substantiated the conclusion that the antibody detected in the culture fluids was specifically antibody to the viral antigens. There was no inhibition of the agglutination of erythrocytes sensitized with adenovirus type 5 antigens when the heterologous antigens (KB or fetal calf serum) were used as the "blocking" antigens. When homotypic adenovirus type 5 (freon extracted preparation) and the fiber antigen of adenovirus type 5 were used as the "blocking" antigens, there was a marked reduction in the titers obtained. When the hexon antigen was used as the "blocking" antigen, there was no inhibition of the reaction. This further substantiated the conclusions that the passive hemagglutination test measures antibodies to the penton and fiber antigens rather than antibodies to the hexon antigen. When the heterotypic adenovirus types 2, 7a, and 12 were the "blocking" antigens, only type 2 caused any inhibition of agglutination of the cells sensitized with antigens of adenovirus type 5. This inhibition was less than a fourfold reduction in the titer. Adenovirus types 7a and 12 did not inhibit the reaction.

5. Antibody responses of lymphoid tissues exposed *in vitro* to antigens of homotypic and heterotypic adenoviral antigens

An experiment was designed to compare the response of lymph node tissue exposed *in vitro* to antigens of homotypic adenovirus type 5 with the response of tissue exposed to antigens of heterotypic adenoviruses. The animals (005) from which the tissue was taken

TABLE 8

SPECIFICITY OF ANTIBODY SYNTHESIZED IN VITRO AS DETERMINED BY PASSIVE HEMAGGLUTINATION INHIBITION*

Antigen** on RBC	Blocking Antigen	Animal No./Culture No. 001/7	002/1	002/19	003/3	003/4	005/7	006/7	008/17	009/24
Ad. 5	-	2048	16,384	16,384	512	1024	>16,384	16,384	2048	8092
Hexon	-	<2	< 2	< 2	<2	<2	< 2	< 2	<2	<2
Fiber	-	8	512	256	16	32	1024	64	64	64
Ad. 5	Ad. 5	<2	4	4	< 2	< 2	16	4	< 2	< 2
"	Hexon	2048	16,384	16,384	512	1024	>16,384	8092	1024	8092
"	Fiber	4	64	64	8	16	64	64	16	64
"	Ad. 2	2048	8092	8092	512	1024	>16,384	4096	512	4096
"	Ad. 7a	2048	16,384	16,384	512	1024	>16,384	8092	1024	8092
"	Ad. 12	2048	16,384	16,384	512	1024	>16,384	8092	1024	8092
"	KB	2048	16,384	16,384	512	1024	>16,384	8092	1024	8092
"	FCS	2048	16,384	16,384	512	1024	>16,384	8092	1024	8092

* Titer expressed as reciprocal of dilution.

** Ad. - adenovirus, freon extracted preparation; numeral indicates type
Hexon-DEAE- purified fraction eluted with 0.2 M NaCl
Fiber-DEAE- purified fraction eluted with 0.05-0.075 M NaCl
KB - freon extracted preparation of KB cells
FCS - fetal calf serum

was injected with freon extracted adenovirus type 5. Tissue fragments were exposed to the antigens listed in Tables 9 and 10. The results of the complement fixation tests on the culture fluids are given in Table 9. Fragments exposed to the hexon antigen of adenovirus type 5 produced antibody which reacted with all the viral test antigens except the fiber antigen of type 5. Fragments exposed to the fiber antigen produced antibody reactive with the hexon, fiber and freon extracted preparations of types 2 and 5. However, no reaction was observed when types 7a and 12 were the test antigens. These results indicated that the antibody cross-reacting with the heterotypic adenovirus types was antibody to the hexon antigen which is common to all the adenoviruses. Fragments exposed to freon extracted adenovirus types 2 and 5 produced antibody reactive with all viral antigens used in the test. In general, the higher titers were obtained with fluids from tissues exposed to type 5. The freon extracted preparation contained the hexon, fiber and penton antigens; therefore, the antibodies produced in vitro would be expected to react with both the fiber and the hexon antigens. The antigen in the preparations of types 7a and 12 which stimulated antibody production was more than likely the hexon antigen. This is indicated by the cross reactivity which was found with all test antigens except with the fiber of type 5. These reactions simulated those observed when the purified hexon of type 5 was the antigen used to stimulate the fragments in vitro. No reactions were observed in culture fluids from fragments exposed to the KB cell antigens.

TABLE 9

ANTIBODY RESPONSES OF LYMPH NODE TISSUE EXPOSED IN VITRO TO HOMOTYPIC AND HETEROTYPIC ADENOVIRUS ANTIGENS AS DETERMINED BY COMPLEMENT FIXATION*

Culture No.	Antigenic Exposure In Vitro	Test Antigens						
		Hexon	Fiber	Ad. 5	Ad. 2	Ad. 7a	Ad. 12	KB
1	-	<2	<2	<2	<2	<2	<2	<2
2	-	<2	<2	<2	<2	<2	<2	<2
3	Hexon**	4	<2	4	2	2	2	<2
4	"	4	<2	4	2	2	2	<2
5	Fiber	8	16	32	4	<2	<2	<2
6	"	8	32	32	4	<2	<2	<2
7	Ad. 5***	8	4	64	16	4	4	<2
8	"	16	4	32	4	4	4	<2
9	Ad. 2	8	2	32	8	2	4	<2
10	"	8	4	32	16	4	4	<2
11	Ad. 7a	2	<2	4	2	2	2	<2
12	"	4	<2	8	8	4	4	<2
13	Ad. 12	2	<2	4	2	2	2	<2
14	"	4	<2	8	4	2	2	<2
15	KB****	<2	<2	<2	<2	<2	<2	<2
16	"	<2	<2	<2	<2	<2	<2	<2

* Titer expressed as reciprocal of dilution

** Hexon - DEAE-purified fraction eluted with 0.2 M NaCl
Fiber - DEAE-purified fraction eluted with 0.05-0.075 M NaCl

*** Ad. - adenovirus, freon extracted preparations, numerals indicate virus types

**** KB - freon extracted preparation of KB cells

The results of the passive hemagglutination tests are shown in Table 10. The tissues exposed in vitro to the hexon antigen produced low levels of antibody detected only with cells sensitized with the fiber antigen or the freon extracted adenovirus type 5 preparation. No reactions occurred with the other test antigens. Tissue exposed to the fiber antigen, likewise, produced antibody detected only with cells sensitized with the homotypic antigens, and antibody levels in these cultures were higher. Fluids from tissue fragments exposed to freon extracted adenovirus type 5 had the highest antibody titers when assayed with cells sensitized with the homotypic antigens, although some cross reactivity with adenovirus type 2 occurred. The tissue exposed in vitro to adenovirus type 7a produced antibody detected only when type 7a was the antigen coated on the erythrocytes. The tissues exposed to adenovirus type 12 did not produce antibody detectable by passive hemagglutination.

6. Molecular species of antibody synthesized in vitro

To determine the molecular species of antibody synthesized in vitro, culture fluid samples were subjected to sucrose density gradient centrifugation. Fluids from cultures of lymph nodes of seven different animals and a spleen culture were analyzed. The results of a representative experiment are given in Figure 4. The greatest activity of all the samples tested was found in the 7S region of the gradient. The antibody detected in this region was resistant to treatment with 2-mercaptoethanol. Antibody activity was also found in the 19S region of the gradient in culture fluids from four of the seven animals. The activity in this region was sensitive to treatment

TABLE 10

ANTIBODY RESPONSES OF LYMPH NODE TISSUE EXPOSED IN VITRO TO HOMOTYPIC AND HETEROTYPIC ADENOVIRUS ANTIGENS AS DETERMINED BY PASSIVE HEMAGGLUTINATION*

Culture No.	Antigenic Exposure In Vitro	Test Antigen on RBC					
		Hexon	Fiber	Ad. 5	Ad. 2	Ad. 7a	Ad. 12
1	-	<2	<2	<2	<2	<2	<2
2	-	<2	<2	<2	<2	<2	<2
3	Hexon**	<2	2	32	<2	<2	<2
4	"	<2	2	32	<2	<2	<2
5	Fiber	<2	4096	16,384	<2	<2	<2
6	"	<2	1024	4096	<2	<2	<2
7	Ad. 5***	<2	1024	>16,384	64	<2	<2
8	"	<2	1024	>16,384	16	<2	<2
9	Ad. 2	<2	16	64	64	<2	<2
10	"	<2	16	128	32	<2	<2
11	Ad. 7a	<2	<2	<2	<2	4	<2
12	"	<2	<2	<2	<2	8	<2
13	Ad. 12	<2	<2	<2	<2	<2	<2
14	"	<2	<2	<2	<2	<2	<2
15	KB****	<2	<2	<2	<2	<2	<2
16	"	<2	<2	<2	<2	<2	<2

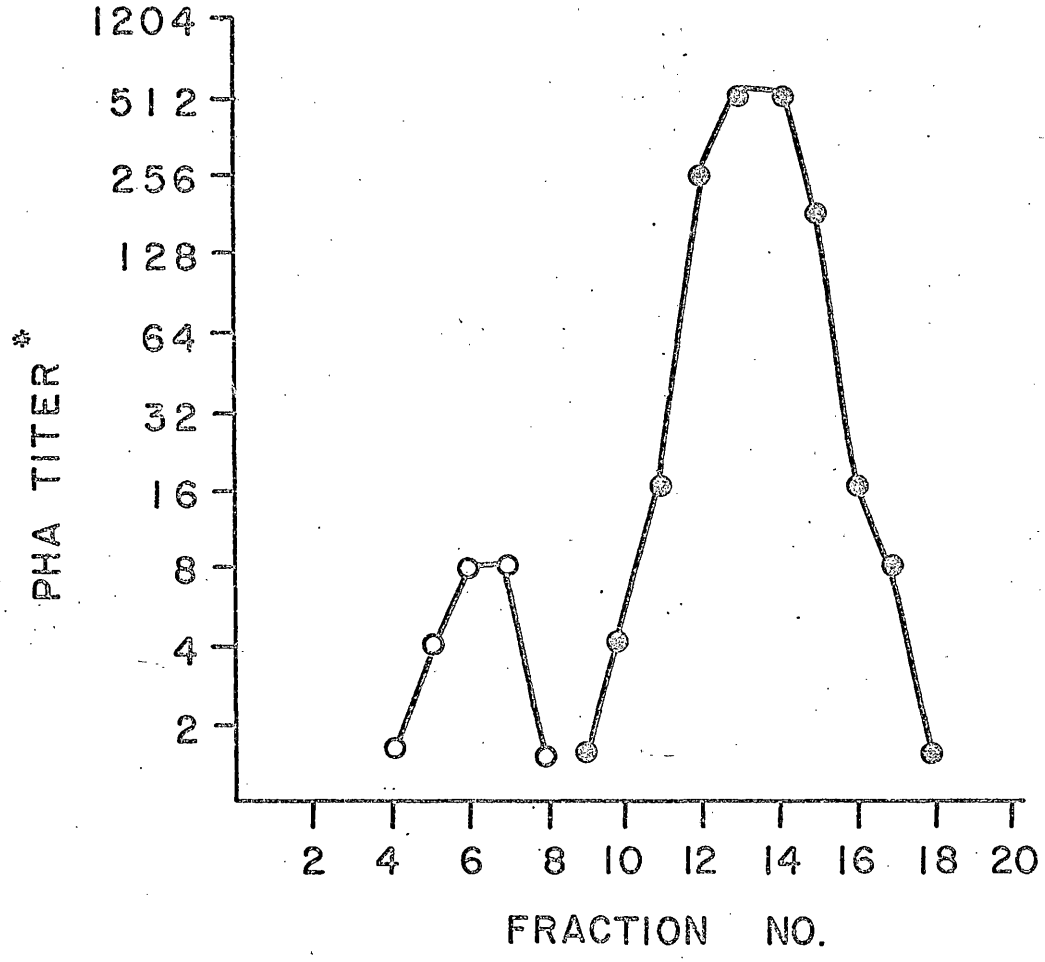
* Titer expressed as reciprocal of dilution

** Hexon - DEAE-purified fraction eluted with 0.20 M NaCl
Fiber - DEAE-purified fraction eluted wiht 0.05-0.075 M NaCl

*** Ad. - adenovirus, freon extracted preparation, numerals indicate virus types

**** KB - freon extracted preparation of KB cells

Figure 4. Sucrose gradient centrifugation of antibody synthesized by lymph node fragments *in vitro*.



SUCROSE GRADIENT CENTRIFUGATION OF ANTIBODY SYTHESIZED BY LYMPH NODE FRAGMENTS IN VITRO

o——o 2-Mercaptoethanol sensitive

●——● 2-Mercaptoethanol resistant

* Expressed as reciprocal of dilution

Figure 4

with 2-mercaptoethanol. This provided further evidence that the antibody was a 19S macroglobulin.

The results of the assays of serial samples from the same lymph node culture are given in Figure 5. Low titered antibody activity in the 7S region was present in the 9 day sample. The activity in this region increased in the subsequent samples. The antibody in the 19S region became detectable in later samples. The antibody may have been present in the 9 day sample, but at a level too low to be detected even with a method as sensitive as passive hemagglutination.

Antibody activity was detectable in both the 19S and 7S regions in the sample from the spleen culture tested. The lymph node cultures from this animal also synthesized 19S antibody.

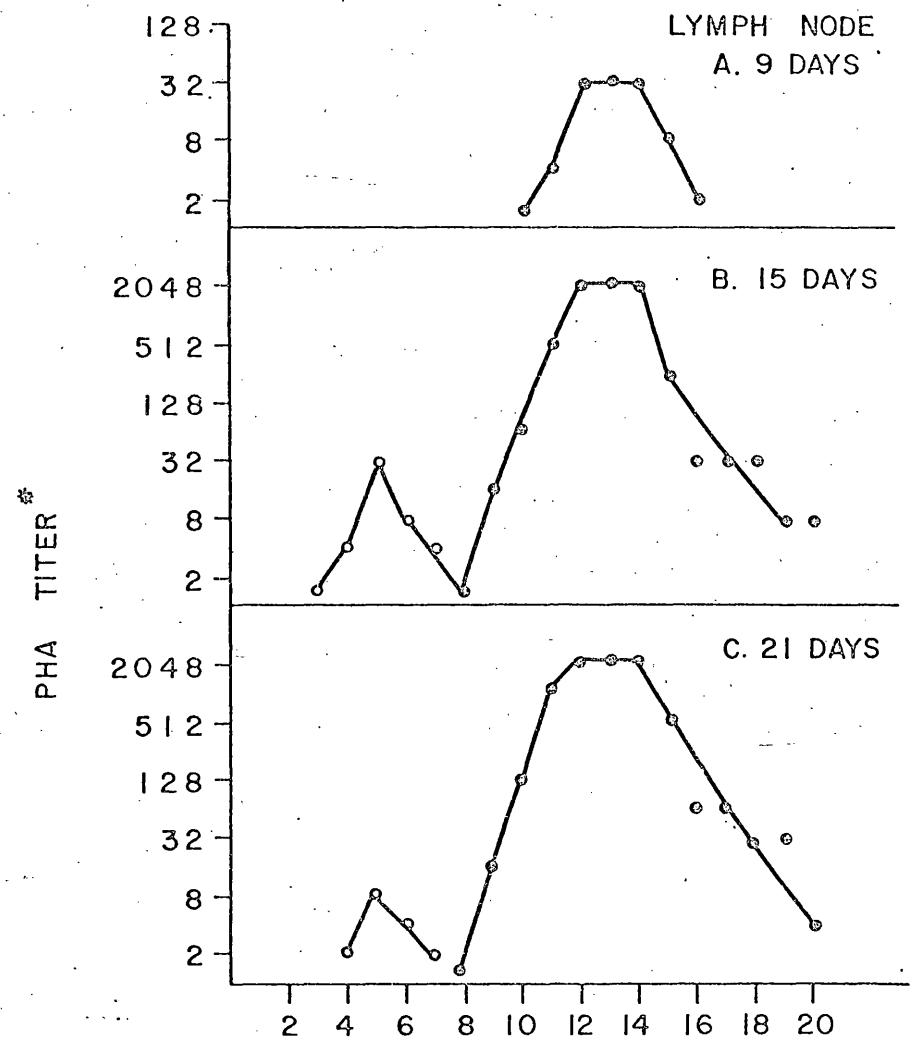
Activity in the 19S region was detectable by passive hemagglutination only. Complement-fixing antibody was detectable in the 7S region, but only in the samples which had titers of 1:64 or greater when measured by passive hemagglutination.

7. Effect of various compounds on antibody synthesis *in vitro*

a. Effect of hydrocortisone-succinate

Several investigators (Ambrose, 1964, 1966; Ortiz-Muniz and Sigel, 1967) have reported that antibody synthesis *in vitro* could be enhanced by the addition of hydrocortisone-succinate (Solu-Cortef) to the culture medium. These investigators have found that the compound could eliminate the requirement for serum in the culture medium. To assess the effect of addition of the compound to

Figure 5. Antibody distribution after sucrose density gradient centrifugation of sequential samples from a lymph node culture.



ANTIBODY DISTRIBUTION AFTER SUCROSE DENSITY GRADIENT CENTRIFUGATION OF SEQUENTIAL SAMPLES FROM A LYMPH NODE CULTURE

o—o 2- Mercaptoethanol sensitive
o—o 2- " resistant
* Expressed as reciprocal of dilution

Figure 5

the culture medium used in this study, several cultures were set up in which the composition of the medium was varied with respect to serum and hydrocortisone-succinate.

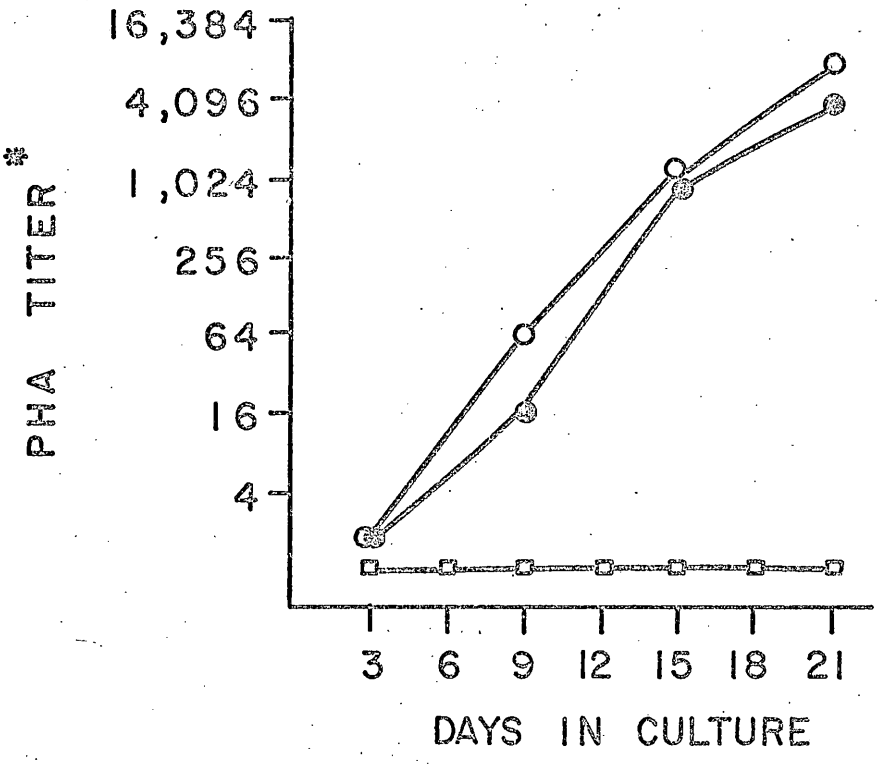
The results of the experiment are shown in Figure 6. No difference in the antibody synthesis could be detected between cultures maintained in the medium containing serum and hydrocortisone-succinate and the cultures maintained in the medium containing serum only. There was no detectable antibody in the fluids from cultures maintained on the medium which contained hydrocortisone-succinate without serum. The requirement for serum could not be eliminated by the addition of the steroid.

Some fragments which were not exposed to the viral antigens in vitro were also maintained on the different media. No antibody was detected in the fluids from these cultures. Therefore the addition of hydrocortisone-succinate to the culture medium used in this study did not produce any effect either in terms of enhancing the antibody production or as a substitute for serum.

b. Effect of phytohemagglutinin-P

Phytohemagglutinin has been reported to evoke specific anamnestic responses when "primed" or sensitized tissues are exposed to the compound (Tao, 1964). Therefore, experiments were designed to study the effect of phytohemagglutinin on antibody synthesis in lymphoid tissues from the rabbits sensitized to adenovirus type 5.

Figure 6. Effect of hydrocortisone-succinate on antibody synthesis by lymph node fragments *in vitro*.



EFFECT OF HYDROCORTISONE-SUCCINATE ON ANTIBODY SYNTHESIS IN VITRO

o—o Eagle's medium, 20% fetal calf serum

●—● " " , " " " " + hydrocortisone—succinate (1 µg/ml)

□—□ Eagle's medium, without serum + hydrocortisone-succinate (1 µg/ml)

* Expressed as reciprocal of dilution

Figure 6

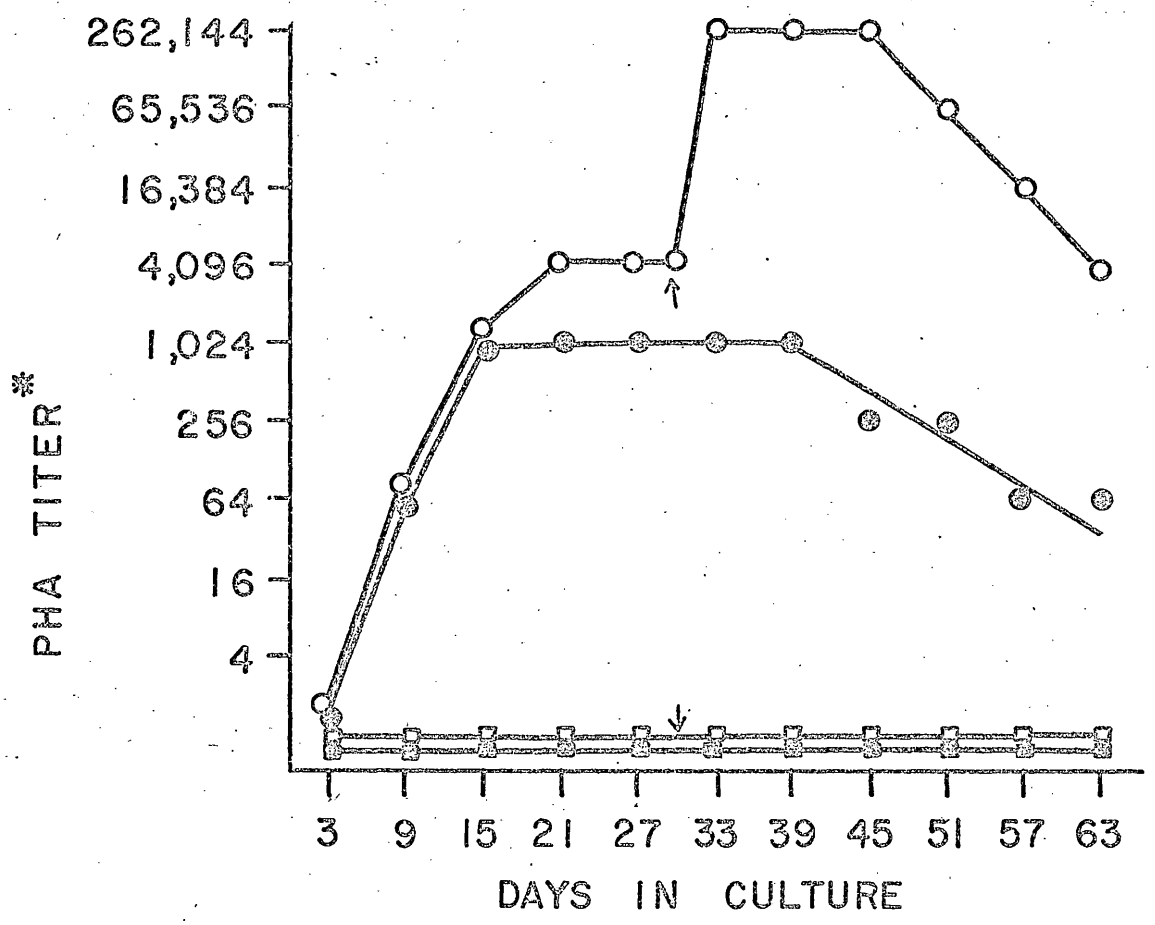
In the first experiment, tissues which had been maintained in culture for 30 days were exposed to phytohemagglutinin-P.

Figure 7 depicts the results of this experiment. Cultures exposed to phytohemagglutinin on day 30 exhibited a definite increase in antibody synthesis as compared with the cultures which were maintained without exposure to phytohemagglutinin. The antibody levels were maintained approximately two weeks and then there was a gradual decline. Although a decline was observed, the treated cultures were still producing antibody at higher levels than the untreated cultures at the termination of the experiment. The response was specific in terms of the antibody produced. This was determined by tests designed to show that the antibody detected was specifically viral antibody. These tests were described in a previous section.

Although phytohemagglutinin can cause agglutination of red blood cells, the increase in antibody titer detected by passive hemagglutination was not the result of non-specific agglutination of the antigen-coated erythrocytes. There was no agglutination of the saline-control tanned cells, nor did the fluids from cultures which were not producing antibody prior to addition of phytohemagglutinin cause agglutination of the antigen coated cells.

As a result of the findings in the first experiment, a second experiment was designed to study the effect of exposure of lymph node fragments to phytohemagglutinin prior to their being placed in culture. Some fragments were exposed <u>in vitro</u> only to phytohemagglutinin; some were exposed only to the viral antigens; and some were exposed to the viral antigens and phytohemagglutinin together.

Figure 7. Antibody responses of lymph node fragments exposed to phytohemagglutinin 30 days after initiation of cultures; → indicates time of addition (day 30) of phytohemagglutinin.



ANTIBODY RESPONSES OF LYMPH NODE FRAGMENTS EXPOSED TO PHYTOHEMAGGLUTININ 30 DAYS AFTER INITIATION OF CULTURES

Fragments exposed to adenovirus type 5 in vitro

o—o Phytohemagglutinin added

◉—◉ Without phytohemagglutinin

Fragments not exposed to adenovirus type 5 in vitro

□—□ Phytohemagglutinin added

▣—▣ Without phytohemagglutinin

* Expressed as reciprocal of dilution

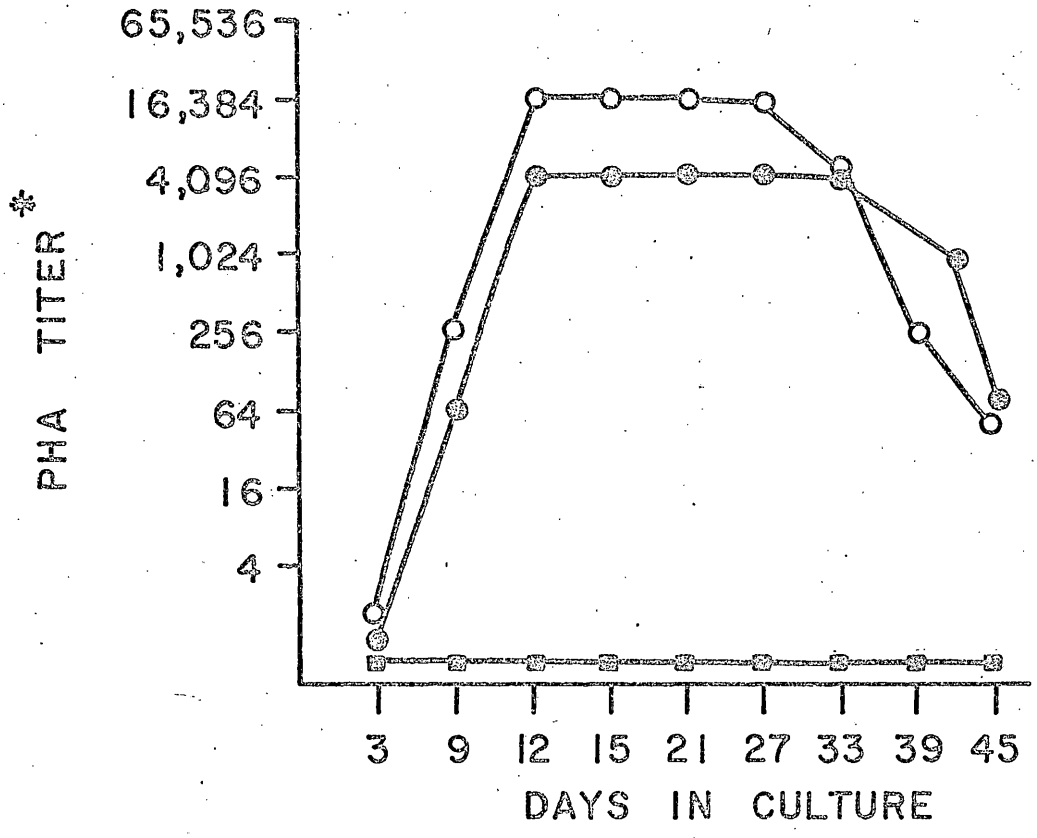
Figure 7

The results of this experiment are given in Figure 8. From the data it was concluded that phytohemagglutinin did not produce either enhancement or inhibition of the antibody response of the lymph node fragments. There was no appreciable difference in the response of the fragments exposed only to the viral antigens and those exposed both viral antigens and phytohemagglutinin. Phytohemagglutinin did not evoke a response in the tissue which was not exposed to the viral antigens *in vitro*. The difference in the findings of the first and second experiments may be the result of the difference in the length of time the fragments were exposed to phytohemagglutinin.

c. Effect of chloramphenicol

Experiments were designed to determine if the antibody detected *in vitro* was the result of *de novo* synthesis. In the first experiment lymph node and spleen fragments were exposed to the viral antigens as described previously. Certain of the cultures were maintained on medium which contained chloramphenicol (50 µg/ml). Comparable cultures were maintained on inhibitor-free medium. The antibody titers of the culture fluids were compared. These results are given in Figure 9. When chloramphenicol was incorporated into the medium at the initiation of the culture (time 0), no antibody could be detected in the fluids from the spleen cultures. The tissues which were exposed to virus and maintained on inhibitor-free medium produced antibody. Although the inhibitory effect of chloramphenicol was not as marked in the cultures established from the lymph node

Figure 8. Effect of phytohemagglutinin on antibody responses of lymph node fragments.



EFFECT OF PHYTOHEMAGGLUTININ ON ANTIBODY RESPONSES OF LYMPH NODE FRAGMENTS

O——O Exposed to virus

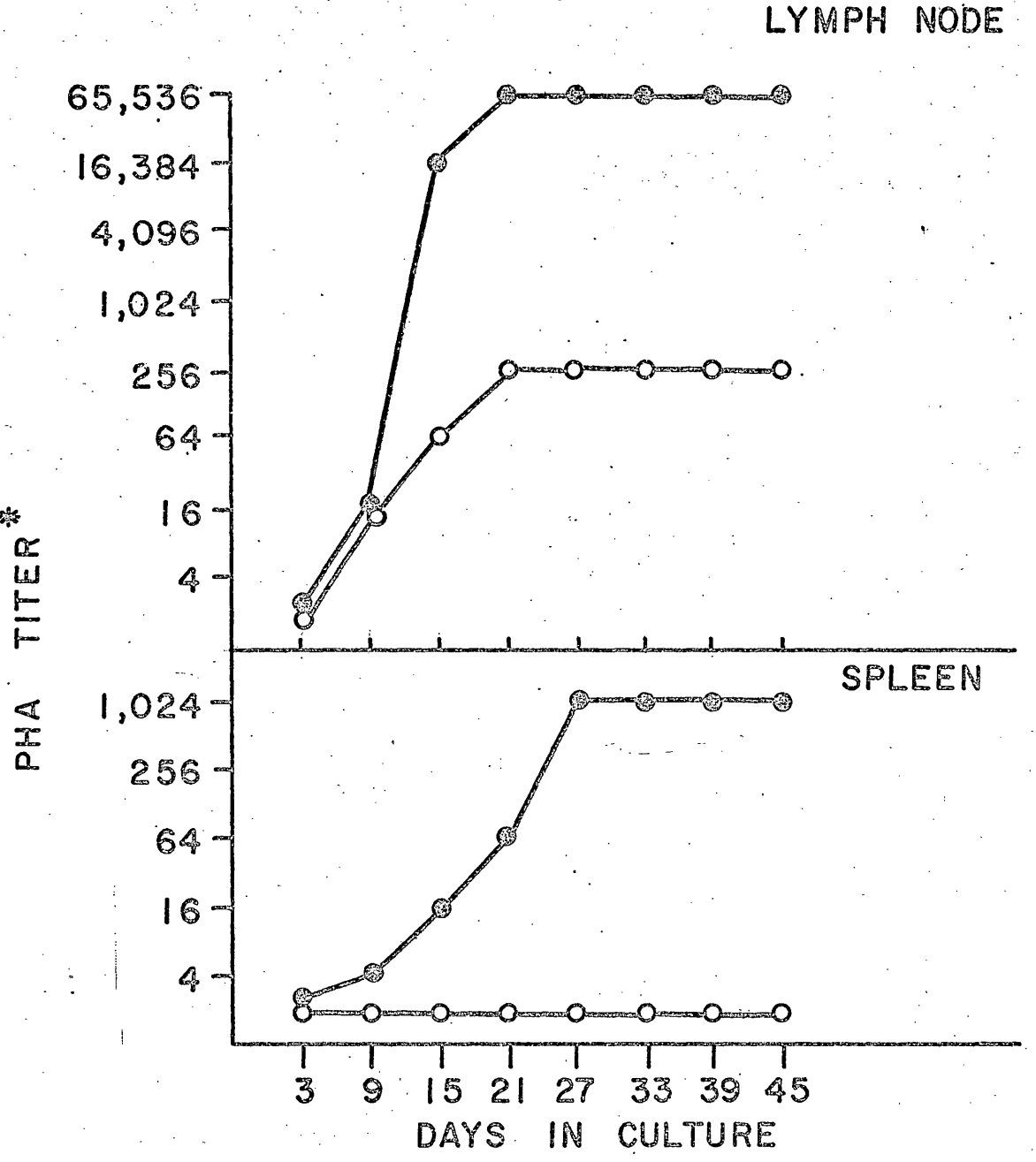
●——● " " " + phytohemagglutinin (250 µg)

■——■ " " phytohemagglutinin (250 µg)

* Expressed as reciprocal of dilution

Figure 8

Figure 9. Effect of chloramphenicol on antibody responses of lymph node and spleen fragments.



EFFECT OF CHLORAMPHENICOL ON ANTIBODY RESPONSES OF LYMPH NODE AND SPLEEN FRAGMENTS

●—● Exposed to virus

○—○ " " " + chloramphenicol (50 µg/ml)

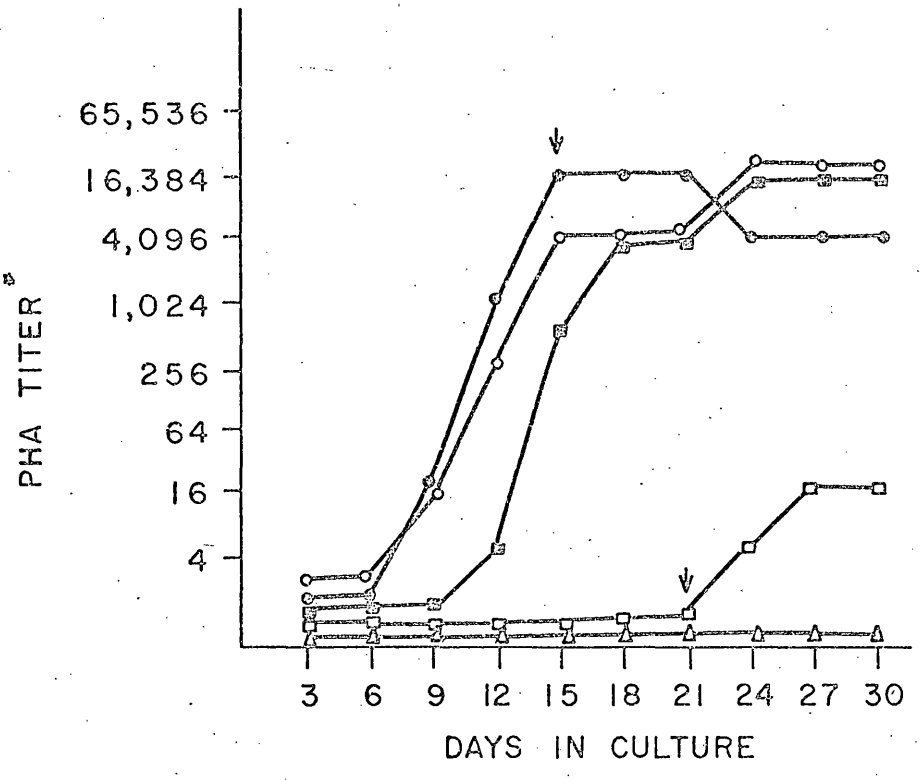
* Expressed as reciprocal of dilution

Figure 9

fragments, lower levels of antibody were detected in the cultures maintained on the medium containing chloramphenicol than in the cultures maintained on the inhibitor-free medium.

A second experiment was designed to determine the effect of the presence of chloramphenicol for different periods of time. Lymph node fragments were used in the experiment. The results of the experiment, given in Figure 10, indicate that the inhibitory effect of chloramphenicol depends upon the time of addition of the inhibitor. When the fragments were exposed to the viral antigens and chloramphenicol for a short period of time (2 hours), the effect was negligible. There was a slight delay (3 days) in the appearance of detectable antibody; however, the fragments responded by synthesizing antibody at levels comparable to fragments which were exposed only to the viral antigens. The continued presence of chloramphenicol appeared to be necessary for maximum inhibitory effects to be produced. This is indicated by the results from cultures maintained on chloramphenicol from time 0. It is interesting that cultures which had been exposed to the inhibitor from time 0 did show slight antibody production when the inhibitor was removed on day 21. Once antibody production had begun the tissue appeared to be refractory to the effect of the inhibitor as indicated by the cultures which were maintained on inhibitor-free medium through day 15. Addition of chloramphenicol on day 15 had little inhibitory effect. From these data, it appeared that the effect of chloramphenicol is most pronounced during the early or inductive phase of antibody production rather than at a later stage when active synthesis is in progress.

Figure 10. Effect of chloramphenicol on antibody responses of lymph node fragments, → indicates addition (day 15) or removal (day 21) of chloramphenicol.



EFFECT OF CHLORAMPHENICOL ON ANTIBODY RESPONSES OF LYMPH NODE FRAGMENTS

o—o Exposed to virus

o—o " + chloramphenicol (50µg/ml) DAY 15-30

▣—▣ " + " " " 0-2 hrs.

□—□ " + " " " 0-21

△—△ " + " " " 0-30

* Expressed as reciprocal of dilution

Figure 10

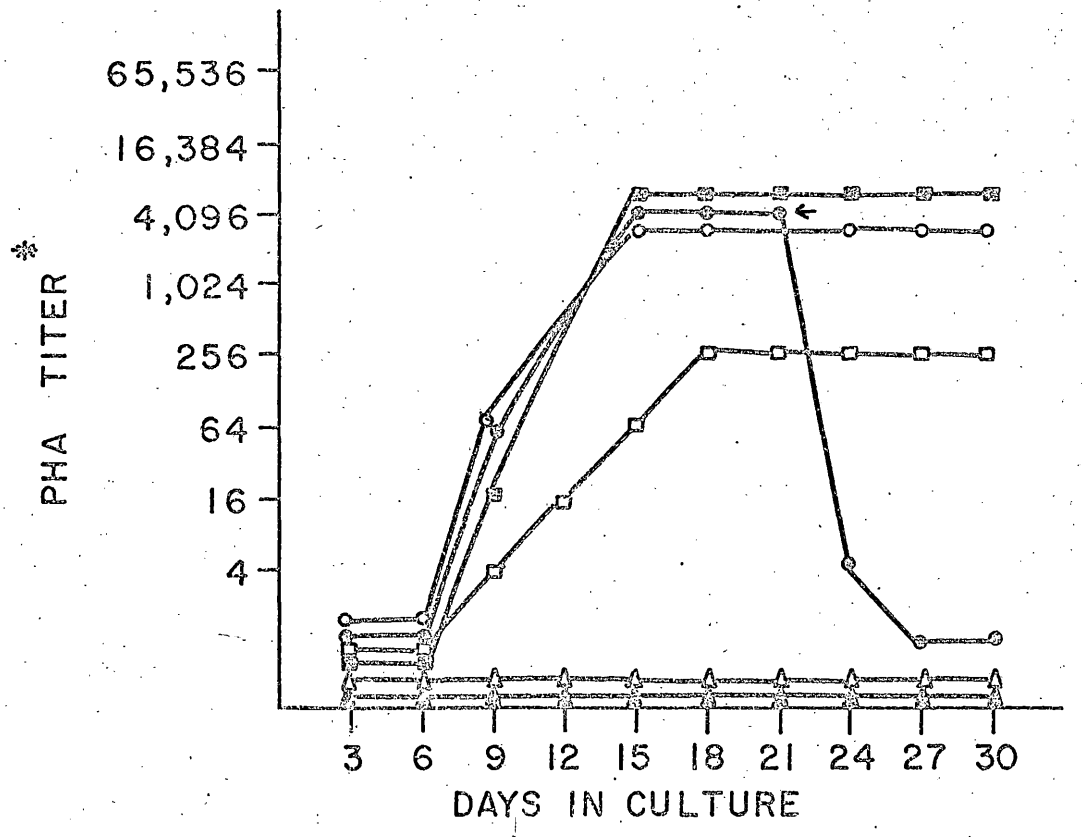
d. Effect of puromycin

The effect of puromycin on antibody synthesis by lymph node fragments was studied as a part of the experiments designed to determine whether de novo antibody synthesis was occurring.

The design of this experiment was similar to the design of the second experiment with chloramphenicol. The tissue fragments were exposed to the viral antigens and to the inhibitor for varying periods of time.

The results (Figure 11) indicated that the antibody response was inhibited by puromycin and that this inhibition was concentration dependent. Fragments exposed to 3 μM puromycin for a period of only 2 hours were completely inhibited. In contrast, fragments exposed to 1 μM from day 0 through day 30 were only partially inhibited. The appearance of detectable levels of antibody was delayed in these latter cultures and the levels did not reach those of the cultures which were not exposed to the inhibitor. The inhibition of the cultures exposed to the higher concentration of puromycin was not the result of toxicity of this concentration. These cultures remained viable as determined by trypan blue exclusion tests. In contrast to chloramphenicol, puromycin was found to exert an inhibitory effect on antibody production when the inhibitor was added on day 21. A marked decrease in detectable levels of antibody was observed within the next sampling period after the addition of the inhibitor. These data indicate that antibody synthesis is sensitive to puromycin in both the early and later stages of antibody production.

Figure 11. Effect of puromycin on antibody responses of lymph node fragments, ⟶ indicates addition (day 21) or 3 μM puromycin.



EFFECT OF PUROMYCIN ON ANTIBODY RESPONSES OF LYMPH NODE FRAGMENTS

o—o Exposed to virus

o—o " + puromycin (3μM) DAY 21–30

□—□ " + " (1μM) " 0–30

▣—▣ " + " " " 0–2 hrs. only

Δ—Δ " + " (3μM) " 0—30

▲—▲ " + " " " 0—2 hrs. only

* Expressed as reciprocal of dilution

Figure 11

8. Recovery of infectious virus from rabbits immunized with adenovirus type 5

Attempts were made to recover infectious adenovirus type 5 from the spleen and lymph nodes of eight of the nine rabbits used in this study. Tissue homogenates were inoculated into cell cultures of human embryonic kidney (HEK). This cell line was used because of its sensitivity to small quantities of adenovirus. The results of this study are presented in Table 11. Agents producing the characteristic cytopathology induced by adenovirus infection were recovered from tissues of three of the animals. The agents were recovered in the second passage of the samples in the HEK cell cultures. The isolations were made from spleens of animals which had been injected with the freon extracted adenovirus type 5 preparation. The isolates were made from animals which had been injected three, ten and twelve months prior to sacrifice. Two isolates were also obtained from the lymph nodes. One of the animals was injected three months prior to sacrifice; the other was injected ten months prior to sacrifice. The negative cultures were passaged a third time, but these remained negative. The isolates were identified as adenovirus type 5 by neutralization of the infectivity with specific antiserum. These results were taken as evidence that adenovirus type 5 produces an infection in the rabbit which becomes persistent although the infection produces no outward symptoms of disease. The animals remained healthy throughout the period of the study.

Because adenoviruses were originally discovered in lymphoid tissues maintained in culture for periods of time, an attempt was

TABLE 11

RECOVERY OF INFECTIOUS ADENOVIRUS TYPE 5 FROM LYMPHOID TISSUES OF RABBITS INJECTED WITH VIRAL ANTIGENIC PREPARATIONS*

Animal No.	Weeks After Injection	Virus Isolations			
		Tissue Homogenates		Cultured Fragments**	
		Spleen	Lymph Node	Spleen	Lymph Node
001	12	+	+	+	+
002	24	-	-	NT	+
006	36	-	-	+	-
008	48	+	+	NT	NT
009	48	-	-	-	-
005	52	+	NT	NT	NT
8	52	-	-	-	-
9	36	-	-	-	-

* Animals 001-009 injected intravenously with freon extracted adenovirus type 5.
Animals 8 and 9 injected intravenously with DEAE-purified subunits of adenovirus type 5.

** Tissue fragments tested after periods of time in organ culture.

NT - not tested.

made to recover virus from some of the fragments which were maintained in culture without re-exposure *in vitro* to the virus. The cultures from which the fragments were taken were not producing detectable levels of antibody. All fragments tested were from cultures which had been maintained at least 30 days. The results are included in Table 11. With this procedure virus isolations were made from tissue of two animals (002 and 006) which had not yielded virus directly in the tissue homogenates.

Rabbits 8 and 9 were the animals which had been injected with the structural subunits of adenovirus type 5 rather than infectious virus. Virus recovery was neither expected nor obtained from the tissues of these animals.

PART II

A. Adenovirus type 12 tumor induction in Syrian hamsters

The animals used in Part II of this study were Syrian hamsters bearing adenovirus type 12 tumors. Newborn hamsters from a total of seven litters were used to obtain the viral induced tumors. Tumors which developed in the newborn hamsters were used as a source of material for serial transplantation to weanling hamsters. Two serial transplants of four separate viral induced tumors were made.

The results of tumor induction and transplantation are given in Table 12. Tumors generally appeared within 30 to 60 days after the injection of virus into the animals. It can be seen that the incidence of tumor induction was quite variable among the seven litters.

The tumors used for transplantation were taken from animals in litters 2, 3, 5, and 7. The percentage of successful transplants was somewhat higher in the first passage of the tumor than in the second passage. Palpable tumors were detectable within nine to fifteen days following transplantation.

When the tumors reached 1 to 2 inches in diameter, the animals were sacrificed. The sera were assayed for antibody to the viral antigens of adenovirus type 12 and for antibody to the tumor or "T" antigen. The spleen and lymph nodes were removed for in vitro

TABLE 12

INCIDENCE OF ADENOVIRUS TYPE 12 TUMORS IN SYRIAN HAMSTERS

A. Incidence of tumors in newborn hamsters injected with 2×10^3 $TCID_{50}$ adenovirus type 12

Litter No.	No. Injected	No. Tumors	Per Cent Positive
1	6	0	0
2	8	3	38
3	9	6	67
4	9	0	0
5	9	7	78
6	9	1	11
7	6	1	17

B. Incidence of tumors in weanling hamsters injected with 0.2 ml tumor mince

Primary Transplant	No. Injected	No. Tumors	Per Cent Positive
HA2	9	5	56
HA3	8	5	63
HA5	14	9	64
HA7	12	4	33
Secondary Transplant	**No. Injected**	**No. Tumors**	**Per Cent Positive**
HB2	22	3	14
HB3	21	9	43
HB5	26	8	30
HB7	12	6	50

stimulation with antigens and for culture. Table 13 is a composite of the data on the animals whose lymphoid organs were used for culturing. The following data are presented: 1) method of tumor induction (viral induced or transplanted), 2) tumor size (mm), and 3) serum antibody titers to both the viral and tumor antigens.

Antibody to the viral antigens could be detected in serum from animals bearing viral induced tumors both by complement fixation and by passive hemagglutination tests. This antibody was detected in eight of the twelve animals. Antibody to the tumor antigen was detected by complement fixation in serum from all the animals bearing viral induced tumors. Antibody to the tumor antigen could not be detected when passive hemagglutination was the method of assay. Apparently the tumor antigen does not attach to the erythrocytes. No antibodies to either viral or tumor antigens were found in the serum from animals 33 and 34 which were injected as newborns but did not develop tumors.

Antibody to the viral antigens was detected by complement fixation or passive hemagglutination in serum from only four of the twelve animals bearing transplanted tumors. Antibody to the tumor antigen was found in the sera of ten of the twelve animals. Animals 35 and 36 were injected with tumor mince but did not develop tumors.

B. Antibody responses <u>in vitro</u> of lymphoid tissues from hamsters bearing adenovirus type 12 tumors

The design of the experiments in which the lymphoid tissues of the tumor bearing hamsters were studied was similar to the design

TABLE 13

SERUM ANTIBODY TO VIRAL AND TUMOR ANTIGENS OF HAMSTERS BEARING ADENOVIRUS TYPE 12 TUMORS

Animal No.	Tumor Size (mm)	Serum Antibody Titer+			
		Viral Antigens		Tumor Antigen	
		CF++	PHA+++	CF	PHA
HO 1*	40	16	640	4	<4
HO 2*	41	4	<4	4	<4
HO 3*	51	<4	<4	64	<4
HO 4*	50	<4	<4	4	<4
HO 5*	40	<4	<4	4	<4
HO 6*	40	64	2560	4	<4
HO 7*	30	4	40	32	<4
HO 8*	32	>256	2560	16	<4
HO 9*	42	4	160	64	<4
HO 10*	38	<4	<4	16	<4
HO 29*	40	64	640	>256	<4
HO 30*	30	64	40	128	<4
HO 33*	--a	<4	<4	<4	<4
HO 34*	--a	<4	<4	<4	<4
HA2-13**	45	64	2560	>256	<4
HA7-37**	50	<4	<4	>256	<4
HB3-14**	49	<4	<4	4	<4
HB5-15**	47	4	<4	16	<4
HB5-16**	45	4	40	16	<4
HB2-31**	65	<4	40	<4	<4
HB7-46**	58	<4	<4	32	<4
HB7-47**	60	<4	<4	16	<4
HB7-48**	50	<4	<4	16	<4
HB7-49**	38	<4	<4	<4	<4
HB7-51**	60	<4	<4	16	<4
HB7-52**	35	<4	<4	16	<4
HB5-35**	--a	<4	<4	<4	<4
HB5-36**	--a	<4	<4	<4	<4

\+ Titer expressed as reciprocal of dilution
++ Complement fixation
+++ Passive hemagglutination
* Viral induced tumors
** Transplanted tumors
a No tumor

of the experiments previously described in Part I. Fragments of spleen, mesenteric and inguinal lymph nodes were exposed *in vitro* to viral or tumor antigens.

1. Antibody responses of lymphoid tissue exposed *in vitro* to viral antigens

The results are recorded in Table 14. Only the fragments which were exposed *in vitro* to the viral antigens responded by production of antibody. No antibody was detected in the culture fluids from the fragments which were not exposed to the antigens *in vitro*. Only the lymph node fragments could be stimulated to respond. The spleen fragments from all the animals did not synthesize detectable amounts of antibody. As can be seen from this table, the titers obtained with cells sensitized with freon extracted preparation of adenovirus type 12 or with the DEAE-purified fiber antigen of adenovirus type 12 were comparable. This was taken as evidence that the antibody which was measured was antibody to the fiber or C antigen. Antibody was detected more frequently in the first and second pools of the culture fluids than in the third. The antibody could be detected longer in the culture fluids from animals bearing the viral induced tumors as evidenced by more positive Pool II samples from this group of animals.

Complement-fixing antibody was detected in pools of culture fluids from only one animal, number 31. Antibody was found only in the second and third pool of the samples, and the titer was low (1:4). It will be noted that both pools had titers of 1:32 when measured by passive hemagglutination.

TABLE 14

ANTIBODY RESPONSES OF LYMPH NODE FRAGMENTS FROM TUMOR BEARING HAMSTERS EXPOSED IN VITRO TO ADENOVIRUS TYPE 12 ANTIGENS

Animal No.	Antibody Measured by PHA[+]					
	Pool I		Pool II		Pool III	
	Ad. 12[++]	Fiber[+++]	Ad. 12	Fiber	Ad. 12	Fiber
HO 1*	8	16	<4	<4	<4	<4
HO 2	8	8	32	32	<4	<4
HO 3	8	8	16	32	<4	<4
HO 4	8	4	16	8	4	4
HO 5	<4	<4	16	16	<4	<4
HO 6	16	16	16	32	<4	<4
HO 8	<4	<4	16	32	<4	<4
HO 9	<4	<4	8	nd	<4	<4
HO 10	8	8	16	16	8	16
HO 30	16	16	<4	<4	ns	ns
HA2 13**	8	8	16	16	<4	<4
HA7 37	8	8	16	16	<4	<4
HB3 14	<4	<4	<4	<4	<4	<4
HB2 31	16	16	16	32	32	16
HB7 46	4	4	<4	<4	<4	<4
HB7 47	16	16	<4	<4	ns	ns
HB7 48	8	8	<4	<4	<4	<4
HB7 49	4	4	<4	<4	<4	<4
HB7 51	8	8	<4	<4	<4	<4
HB7 52	8	8	<4	<4	<4	<4

[+] Passive hemagglutination, titer expressed as reciprocal of dilution

[++] Ad. 12 - freon extracted preparation of adenovirus type 12

[+++] Fiber - DEAE-purified fraction eluted with 0.20 M NaCl

* Viral induced tumors

** Transplanted tumors

nd Not done

ns No sample

2. Antibody responses of lymphoid tissue exposed *in vitro* to tumor antigen

Attempts were made to stimulate the lymphoid tissues to produce antibody to the tumor antigen. Tissues taken from one animal bearing a viral induced tumor and from two animals bearing transplanted tumors were exposed *in vitro* to the tumor antigen. Table 15 gives the results of this experiment. It can be seen that the sera of these animals did have complement-fixing antibody to the tumor antigen. However, antibody could not be detected to the tumor antigen, nor could antibody to the viral antigen be detected in the fluids by passive hemagglutination.

3. Antibody responses of lymph node tissue from non-tumor bearing hamsters exposed *in vitro* to antigens of adenovirus type 12

Four non-tumor bearing animals were used in this experiment. Two of the animals were injected as newborns with virus, and two were injected as weanlings with tumor mince. None of these animals developed tumors nor had serum antibody to either the viral or tumor antigens. The lymph node tissues were exposed to the viral antigens *in vitro* in an attempt to stimulate antibody synthesis. Antibody could not be detected in the culture fluids from any of the cultures from these animals.

4. Specificity of antibody synthesized *in vitro*

The specificity of the antibody synthesized *in vitro* was determined by testing the culture fluids with erythrocytes

TABLE 15

RESPONSE OF LYMPH NODE FRAGMENTS FROM ADENOVIRUS TYPE 12 TUMOR BEARING HAMSTERS EXPOSED TO TUMOR ANTIGEN IN VITRO

Animal No.	Serum Antibody Titer[+]		Culture Fluid Titer	
	Tumor Ag. CF[++]	Viral Ag. PHA[+++]	Tumor Ag. CF	Viral Ag. PHA
HO 29*	>256	2560	<4	<4
HB5-15**	16	<4	<4	<4
HB5-16**	16	16	<4	<4

[+] Titer expressed as reciprocal of dilution
[++] Complement fixation
[+++] Passive hemagglutination

* Viral induced tumor
** Transplanted tumor

sensitized with heterologous antigens and by passive hemagglutination inhibition tests. Table 16 gives the results of representative tests in which various antigens were coated on the red blood cells. It can be seen that the reaction was positive only when adenovirus type 12 antigens were the antigens on the red cells.

Table 17 gives the results of the passive hemagglutination inhibition tests. Only adenovirus type 12 viral antigens caused a fourfold or greater decrease in the antibody titer. Little or no inhibition was seen when adenovirus type 5, tumor antigen, KB cells or fetal calf serum were used as the "blocking" antigens.

5. Effect of chloramphenicol on antibody synthesis *in vitro*

Synthesis of antibody could be inhibited by incorporation of chloramphenicol (50 µg/ml) in the culture media. An experiment was set up in which the tissue fragments were divided into three groups. One group was cultured without exposure to the viral antigens; the second group was exposed to the viral antigens and cultured in medium containing chloramphenicol; the third group was exposed to the viral antigens and cultured in medium without chloramphenicol. Antibody was detected only in the fluids from the latter group which indicated that the antibody was the result of *de novo* synthesis.

Table 18 is a summary of the findings in this study. Of the hamsters bearing viral induced tumors, 50 to 60 per cent had antibody to the viral antigen in their serum. All the lymph node cultures from these animals produced antibody after stimulation *in vitro* with

TABLE 16

SPECIFICITY OF ANTIBODY SYNTHESIZED IN VITRO AS DETERMINED BY PASSIVE HEMAGGLUTINATION*

Animal No.	Antigen on RBC**				
	Ad. 12	Ad. 5	FCS	KB	SRBC
HO 1	16	<4	<4	<4	<4
HO 2	32	<4	<4	<4	<4
HO 3	32	<4	<4	<4	<4
HO 4	16	<4	<4	<4	<4
HO 5	16	<4	<4	<4	<4
HO 6	32	<4	<4	<4	<4
HO 8	32	<4	<4	<4	<4
HO 9	16	<4	<4	<4	<4
HO 10	16	<4	<4	<4	<4
HO 30	16	<4	<4	<4	<4
HA2 13	16	<4	<4	<4	<4
HA7 37	16	<4	<4	<4	<4
HB3 14	<4	<4	<4	<4	<4
HB2 31	32	<4	<4	<4	<4
HB7 46	4	<4	<4	<4	<4
HB7 47	16	<4	<4	<4	<4
HB7 48	8	<4	<4	<4	<4
HB7 49	4	<4	<4	<4	<4
HB7 51	8	<4	<4	<4	<4
HB7 52	8	<4	<4	<4	<4

* Titer expressed as reciprocal of dilution

** Ad. 12 - Adenovirus type 12, DEAE-purified fraction eluted with 0.20 M NaCl

Ad. 5 - Adenovirus type 5, freon extracted

FCS - fetal calf serum

KB - KB cells, freon extracted

SRBC - sheep red blood cells, tanned, unsensitized-saline control

TABLE 17

SPECIFICITY OF ANTIBODY SYNTHESIZED IN VITRO DETERMINED BY PASSIVE HEMAGGLUTINATION INHIBITION*

Antigen on RBC	Blocking Antigen	Animal No.									
		HO-3	HO-4	HO-5	HO-6	HO-8	HA2-13	HA7-37	HB2-31	HB7-47	HB7-48
Ad. 12	-	32	8	16	32	32	16	16	32	16	8
Ad. 12	Ad. 12	<4	<4	<4	4	<4	<4	<4	<4	<4	<4
Ad. 12	Ad. 5	32	8	16	32	32	16	16	32	16	8
Ad. 12	"T" ag.	32	8	16	32	32	16	16	32	16	8
Ad. 12	KB cells	32	8	16	32	32	16	16	32	16	8
Ad. 12	FCS	32	8	16	32	32	16	16	32	16	8

* Titer expressed as reciprocal of dilution
Ad. 12 - DEAE-purified fraction eluted with 0.20 M NaCl
Ad. 5 - freon extracted preparation
"T" ag. - adenovirus type 12 hamster tumor cells, 2×10^7 cells/ml, sonified preparation
KB cells - crude cell preparation
FCS - fetal calf serum

TABLE 18

SUMMARY OF IN VIVO AND IN VITRO ANTIBODY RESPONSES OF HAMSTERS BEARING ADENOVIRUS TYPE 12 TUMORS

Tumors	Total Animals	Positive Sera		Positive Cultures
		CF*	PHA**	PHA
Viral Induced	10	6 (60%)	5 (50%)	10 (100%)
Transplanted	10	1 (10%)	2 (20%)	9 (90%)

* Complement fixation

** Passive hemagglutination

the viral antigens. Only 10 to 20 per cent of the hamsters bearing transplanted tumors had detectable serum antibodies; however, the lymph node tissues from all but one of these animals responded by production of antibody *in vitro*.

C. Histological studies of tissues maintained *in vitro*

Limited histological studies were done in an attempt to learn something of the cell type which persisted in the organ culture system used in this investigation.

Microscopic examination of the intact cultures revealed the presence of cellular proliferation around the explanted tissues on the surface of the agar blocks. Figures 12 and 13 are representative of the appearance of the fragments and cellular outgrowths around them. The explants shown are lymph node fragments from hamsters. The cultures of the spleen fragments and lymph node fragments from rabbits had the same general appearance.

Some of the fragments were fixed and histological sections were made. Figures 14 and 15 are sections from a rabbit lymph node and spleen fragment, respectively. These fragments were exposed *in vitro* to the viral antigens prior to culture. The cultures from which the fragments were taken were producing antibody. These particular fragments had been maintained in culture for eighteen days. In general the cellularity and architecture of the tissue were well maintained over the period of cultivation. However, no striking differences could be detected between sections of the tissues which were exposed

Figure 12. Explant of hamster lymph node fragment growing on surface of agar disc showing cellular proliferation. Unstained. Original mag. 45X.

Figure 13. Area of cellular proliferation around explant of hamster lymph node fragment on surface of agar disc. Unstained. Original mag. 125X.

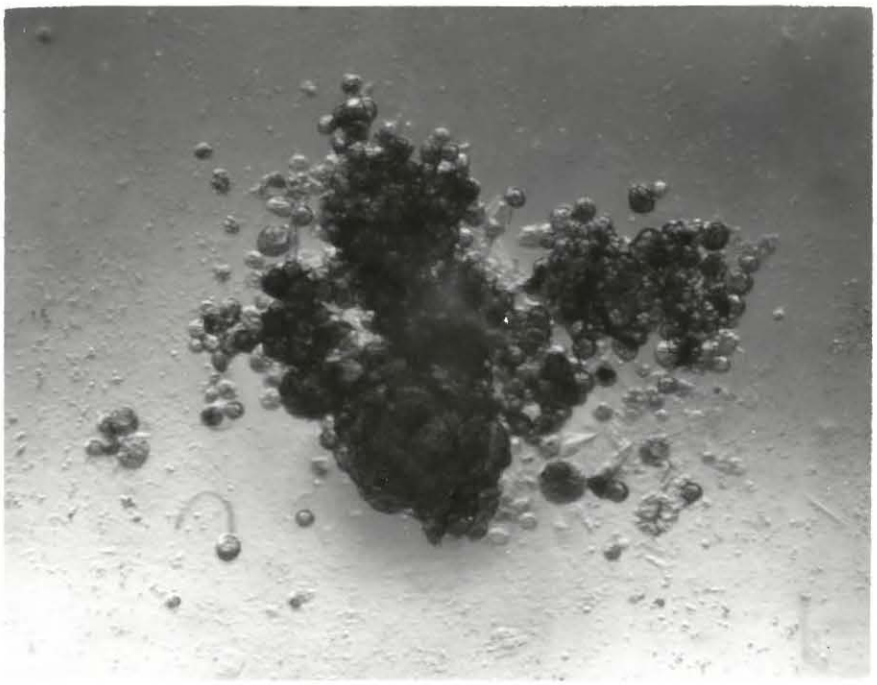


Figure 12

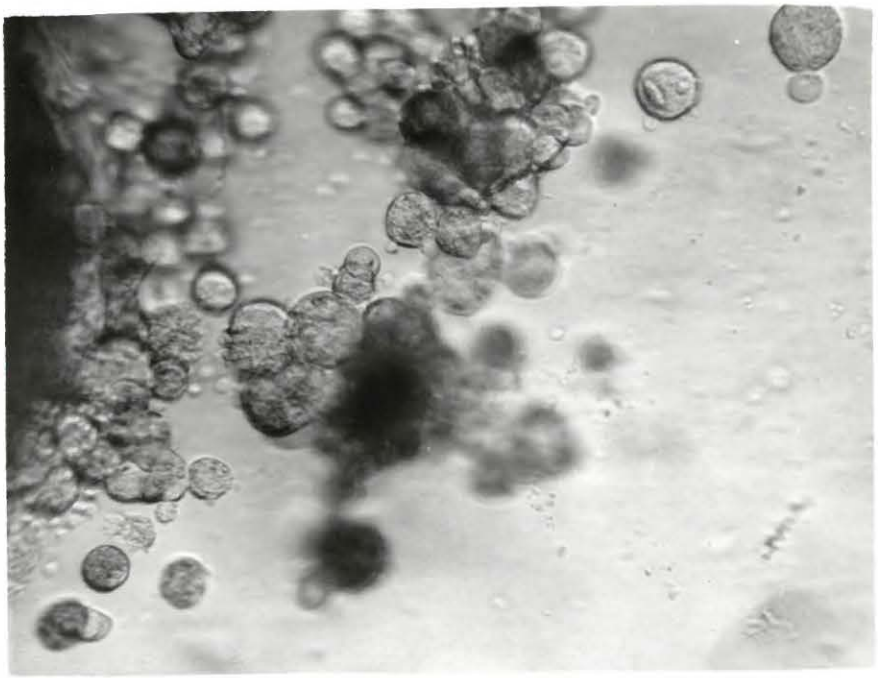


Figure 13

Figure 14. Section of rabbit lymph node fragment after 18 days in culture. Periodic acid Schiff stain. Original mag. 50x.

Figure 15. Section of rabbit spleen fragment after 18 days in culture. Periodic acid Schiff stain. Original mag. 50x.

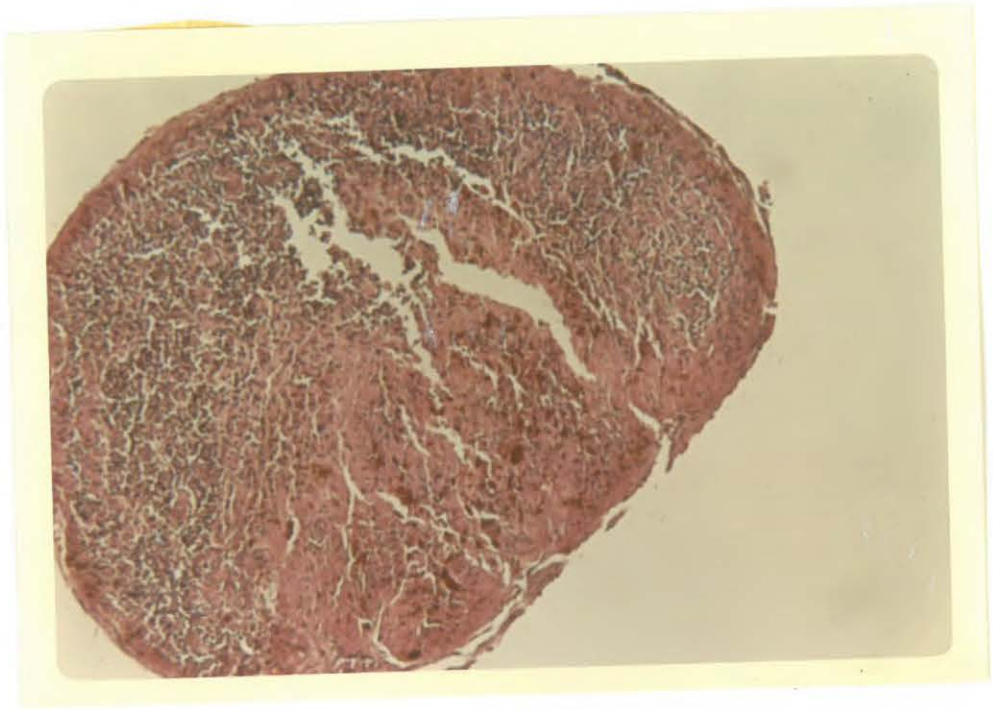


Figure 14

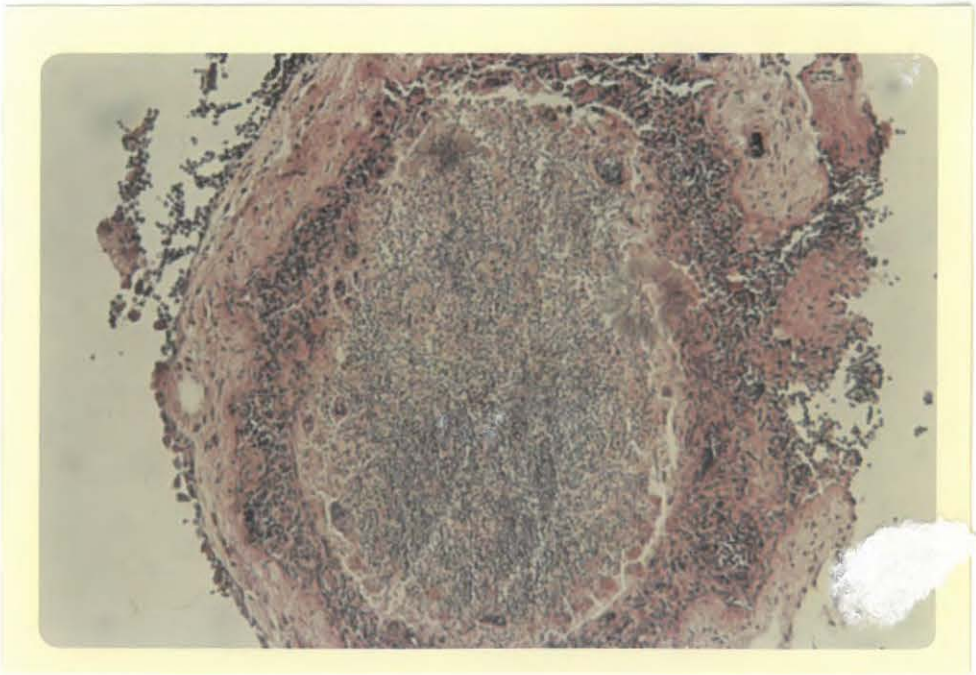


Figure 15

to the viral antigens *in vitro* and those which were maintained without *in vitro* stimulation.

In an attempt to determine the types of cells which were growing around the explants, impression smears were made of the agar blocks after the tissue fragments were removed. This type of preparation proved to be of limited value because of the difficulty in obtaining satisfactory material for study. Therefore no strict differentiation of the cells could be made. In general the cells which adhered to the slides were mononuclear cells which had characteristics of the lymphocytic series. Cells in all stages of maturation were found; however, the medium and small lymphocytes were the most readily identified. Some of the cells exhibited characteristics of mature plasma cells, i.e. an abundance of basophillic cytoplasm and a mature, eccentric nucleus.

Figures 16 through 19 are representative of the cells which were most frequently seen. The cells shown were from cultures of tissues which had been stimulated *in vitro*, and these cultures were producing antibody. No striking differences were noted, however, in cell types when the impression smears were made from the control cultures.

Figure 16. Lymphoid cells recovered by impression smear from a culture of hamster lymph node fragments after 21 days in culture. Tissue fragments were exposed *in vitro* to adenovirus type 12 antigens prior to being placed in culture. Wright-Giemsa stain. Original mag. 450X

Figure 17. Lymphoid cells recovered by impression smear from a culture of hamster lymph node fragments after 21 days in culture. Tissue fragments were exposed *in vitro* to adenovirus type 12 antigens prior to being placed in culture. Wright-Giemsa stain. Original mag. 450X

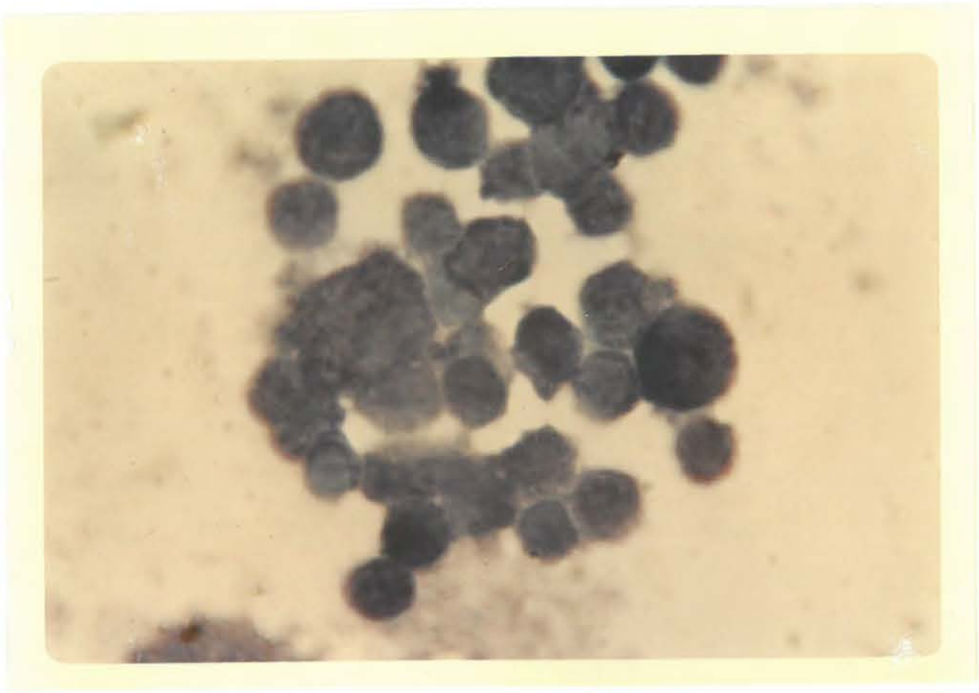


Figure 16

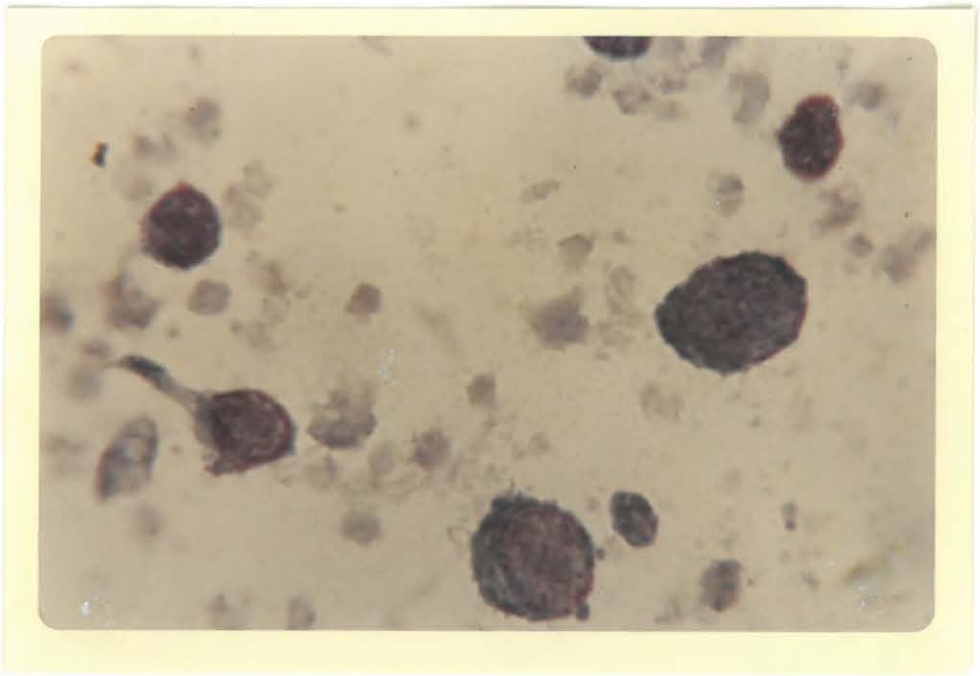


Figure 17

Figure 18. Lymphoid cells recovered by impression smear from a culture of rabbit lymph node fragments after 15 days in culture. Tissue fragments were exposed *in vitro* to adenovirus type 5 antigens prior to being placed in culture. Wright-Giemsa stain. Original mag. 450X.

Figure 19. Lymphoid cells recovered by impression smear from a culture of rabbit spleen fragments after 15 days in culture. Tissue fragments were exposed *in vitro* to adenovirus type 5 antigens prior to being placed in culture. Wright-Giemsa stain. Original mag. 200X.

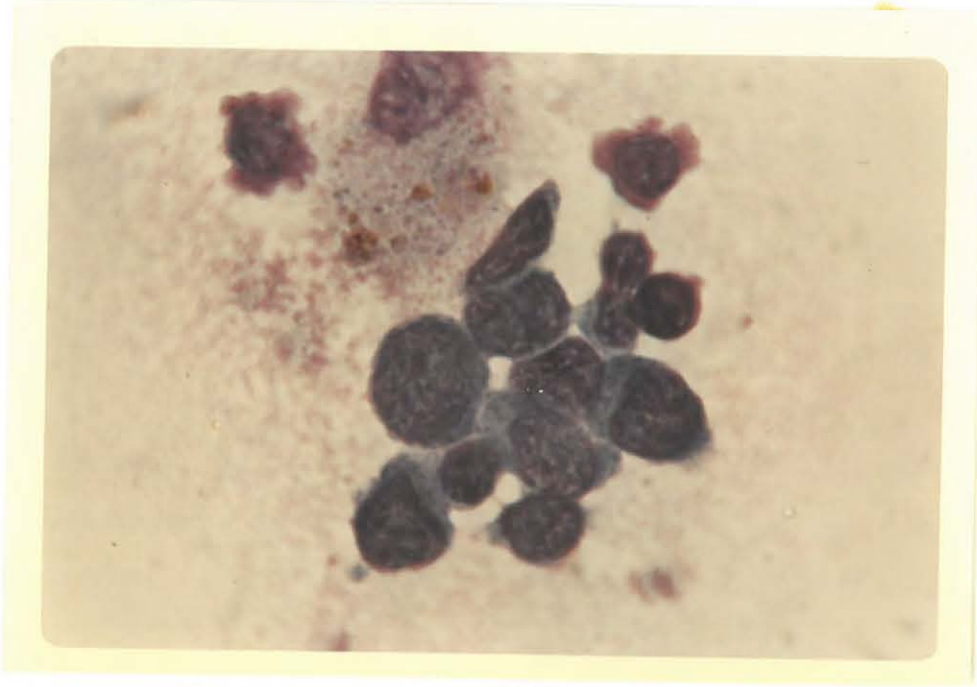


Figure 18

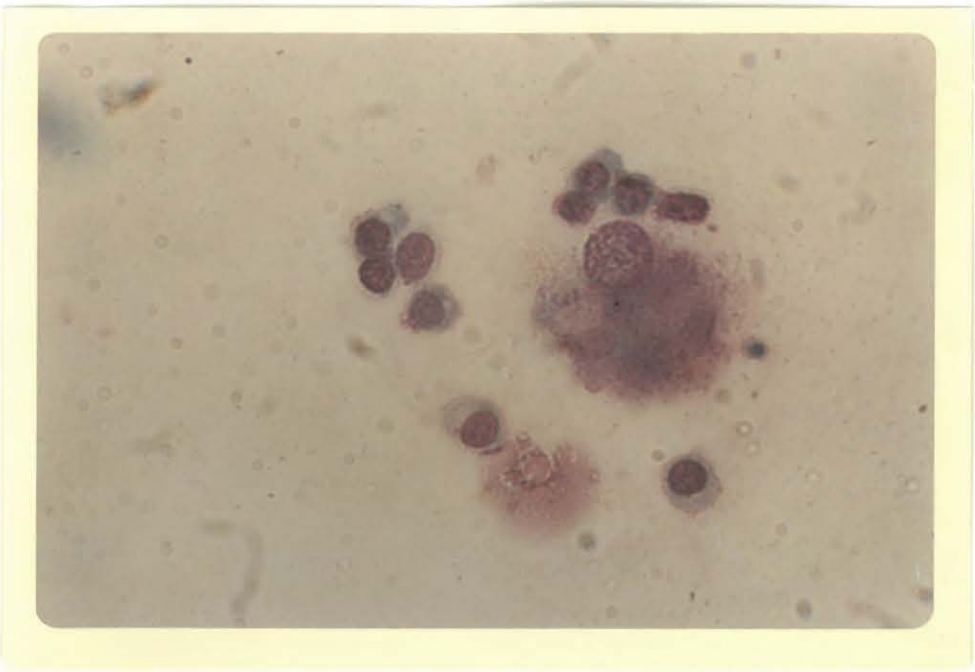


Figure 19

DISCUSSION

The results of the studies of antibody synthesis *in vitro* by fragments of rabbit lymphoid tissues were, in general, consistent with the findings reported by Michaelides and Coons (1963) and by Dutton and Eady (1964). The tissues responded to specific stimulation *in vitro* as evidenced by production of antibodies detectable by complement fixation, passive hemagglutination, and neutralization tests. The lymph node fragments could consistently be stimulated to respond to *in vitro* stimulation with the specific viral antigens regardless of the time interval between the initial exposure *in vivo* and the re-exposure *in vitro* up to the limit of the study.

When the tissues were placed in cultures there was a consistent lag period of six to nine days before the levels of antibody became detectable even with the most sensitive assay used. This lag period has been the general finding of other investigators (Michaelides and Coons, 1963; Tao, 1964; Kritzman and Harper, 1966). This interval has been attributed to: 1) the necessity for adaptation of the tissue to a changed environment; 2) the maturation and division of precursor cells to a state of active antibody synthesis; and 3) in the present study, the presence of sufficient residual antigen to combine with the earliest antibody produced. The latter point is supported by the recovery of infectious virus in the first (3 days) fluid harvests.

Once the antibody levels became detectable to the assay procedures the virus could no longer be recovered from the fluids. After antibody synthesis began, the peak titers were reached in about 12 to 15 days and remained at a constant level for 30 to 45 days followed by a gradual decline. The prolonged survival of the productive fragments was attributed to the organ culture system utilized in this study. The preservation of the architecture of the tissue fragments and the retention of the productive cells on the surface of the agar discs was considered to have extended the period of antibody production.

The concentration of viral antigens used to stimulate the fragments was not extremely critical. Lymph node fragments responded equally well to a concentration range of antigen from 10^2 to 10^6 $TCID_{50}$ of the virus preparation used for in vitro stimulation. Some decrease in effectiveness was noted at the lowest concentration (10 $TCID_{50}$). This possibly was near the limits of sensitivity of the response. Other workers have reported similar findings when wide concentration ranges were used to stimulate "primed" tissues in vitro (Michaelides and Coons, 1963; Dutton and Eady, 1964).

The attempts to induce additional antibody responses after the fragments had been in culture for various periods of time were unsuccessful. The drop in titer of the fluids which was noted immediately after re-exposure to the antigen was thought to be the result of the antigen combining with the antibody being synthesized. The titers of the fluids generally returned to the same levels as before re-exposure to the antigen. The freon extracted preparations of adenovirus type 5 which were used to re-expose the tissues contained, in addition

to other virion antigens, the penton antigen (toxin) which is associated with the "early" cytopathology caused by this virus. The action of this toxin on the exposed cells growing on the surface of the agar discs may have caused sufficient damage to the cells to prevent more active antibody production. No attempts were made to remove the fragments from the agar discs and re-expose them to the freon extracted virus or to the individual structural subunits. Several other factors may also account for this lack of increased response. All of the antigen-sensitive cells may have responded to the initial *in vitro* stimulation and the fragments could have been synthesizing antibody at their maximum capacity. Although the culture dishes were washed following the re-exposure period, sufficient virus may have remained in the agar discs to have prevented detection of any increased antibody production.

The fragments of all lymphoid tissues from the animals which had been hyperimmunized with the individual hexon or fiber antigens failed to respond to stimulation with any of the various antigen preparations. The failure of the tissues of these animals to respond to *in vitro* stimulation could be taken as evidence of the necessity of the continuing presence of antigen *in vivo*. Injection of the subunits would not establish a latent infection. Also the serum antibody titers of these animals declined considerably during the course of these experiments. The animals which were injected with the freon extracted virus were under continued stimulation by the viral antigens. This statement is substantiated by the fact that virus was recovered at the time of sacrifice from the lymph nodes and spleens of some of the animals injected with the infectious virus.

Spleen fragments did not synthesize antibodies as consistently or at levels comparable to the lymph node fragments. The spleen fragments which did respond to *in vitro* stimulation were from animals sacrificed within 12 to 24 weeks after the initial injection of the virus. This indicated that the time interval between the *in vivo* and *in vitro* exposure to the antigens may effect the response which can be evoked by splenic tissue *in vitro*. Differences in the ability of the lymph node and spleen to respond to *in vitro* stimulation have been reported by others (Stavitsky, 1961). There is apparently a correlation between the tissue weight and the synthetic capacity of the spleen. In the present study the number of fragments of lymph node and spleen placed in culture were always the same, but the tissue weights were different. The difference in the architecture of the two organs may also account for the difference in the antibody production *in vitro*. A fragment of lymph node would be expected to contain more cells capable of synthesizing antibody than an equivalent fragment of spleen.

The lack of response to *in vitro* stimulation observed with the fragments of thymus are consistent with the findings of Askonas and White (1956) and Askonas and Humphrey (1958). Ortiz-Muniz and Sigel (1967) did report antibody production by thymic fragments from animals immunized with bovine serum albumin; however, the levels were quite low and these investigators suggested that the antibody may have arisen from lymphoid cells trapped in the thymus rather than be indicative of the antibody synthesizing ability of the thymocytes.

The results of tests designed to determine the specificity of the antibody synthesized in vitro indicated that the antibody detected was specifically antiviral. The lack of reactivity of the culture fluids with KB cell antigens, fetal bovine serum and control sheep erythrocytes was taken as evidence that the antibody being measured was specific for the viral antigens used in the serologic tests. To determine the specificity of the viral antibody, the fluids were reacted in passive hemagglutination and complement fixation tests with the antigens of the homotypic adenovirus type 5 and with the antigens of the heterotypic adenovirus types 2, 7a and 12. The results obtained in the passive hemagglutination tests indicated that the antibody measured by this method was primarily antibody to the fiber and penton antigens of type 5. Highest titers were obtained with erythrocytes sensitized with the freon extracted preparations of type 5. Significant titers were also obtained with erythrocytes with the fiber antigen of type 5. The difference in titers obtained with the two antigenic preparations was attributed to the fact that the freon extracted preparations contained both the fiber and penton antigens and the DEAE column fraction contained only the fiber antigen. Also there was a quantitative difference in the amount of antigen in the two preparations. As previously mentioned, the hexon or group specific antigen apparently does not attach to the erythrocytes; therefore, passive hemagglutination does not measure antibody to this antigen and is a more type-specific assay. This is supported by the lack of reactivity of the fluids with cells sensitized with adenovirus types

7a and 12 and the slight cross-reactivity of the fluids with cells sensitized with adenovirus type 2. Adenovirus type 2 is a member of the same subgroup (III) to which type 5 belongs; adenovirus types 7a and 12 are members of subgroups I and IV, respectively. Therefore, one might expect some cross-reactivity with adenovirus type 2, particularly since both the fiber and penton antigens of type 2 were present in the preparation used to sensitize the erythrocytes. This cross-reactivity of types 2 and 5 in the passive hemagglutination reaction has been previously described (Lefkowitz, 1967).

The passive hemagglutination inhibition tests further substantiated the conclusions that the antibody synthesized in vitro was antiviral and that this assay measures antibody to the fiber and penton antigens rather than antibody to the hexon. This was demonstrated when the hexon was used as the "blocking" antigen. In these tests there was no reduction in the antibody titers obtained using erythrocytes sensitized with freon extracted type 5 as the test antigen. However, when either the fiber or the freon extracted preparation of type 5 were the "blocking" antigens, marked reduction in antibody titers were obtained.

In the complement fixation tests, antibody was detected to the purified hexon and fiber antigens of adenovirus type 5. Highest titers were obtained with the freon-extracted type 5 as the test antigen. This was expected since such a preparation contains the fiber, penton and hexon antigens. There was more cross-reactivity between the antibodies in the culture fluids and the antigens of heterotypic adenovirus types than was seen in the passive hemagglutination tests. This was attributed to the fact that complement

fixation measures antibody to the hexon antigen as well as antibody to the fiber and penton antigens. The hexon or group antigen is considered to be common to all adenovirus types and to possess a single antigenic determinant (Peterson et al., 1967).

The responses of adenovirus type 5-sensitized lymph node tissue to stimulation with homotypic and heterotypic adenoviral antigens provided some interesting observations. The purified hexon antigen of type 5 stimulated the fragments to produce anti- detectable by both complement fixation and passive hemagglutination methods. Therefore, it was assumed that antibodies to all three structural antigens were being synthesized by these fragments stimulated by the hexon antigen of type 5. However, the response of the tissue to the hexon was not as great as the response to the purified fiber antigen or to the freon extracted preparations of type 5.

The antibody detected by passive hemagglutination in the fluids of the adenovirus type 5-sensitized tissue exposed in vitro to adenovirus type 2 was considered to have resulted from the presence of some common or closely related antigenic determinant on the fiber or penton antigens of the two virus types. The purified fiber and penton antigens of type 2 were not available for testing; therefore, no conclusion would be made concerning which antigen was responsible for causing the response.

The antibody detected by complement fixation in the fluids from the adenovirus type 5-sensitized tissues exposed to types 7a and

12 was considered to have resulted from the response to stimulation by the common hexon antigen. This is supported by the lack of reactivity of these fluids with the fiber antigen of type 5. Also the lack of cross-reactivity detected by passive hemagglutination which was observed in the complement fixation tests supports the conclusion that the cross-reactivity observed in the complement fixation tests was the result of the antibody to the hexon antigen.

The major portion of antibody demonstrated in the organ culture fluids had the properties of 7S. However, tissues from four of seven animals produced antibody which had the characteristics of 19S. The titers of this antibody were low and were detectable only by passive hemagglutination after the titers of the 7S antibody reached 1:32 or greater. The fact that the 19S antibody appeared after 7S in the serial cultures is considered to reflect the extremely low levels of this antibody rather than indicate a sequence relationship in the antibody produced. No correlation could be made between the presence of 19S antibody in the culture fluids and the time interval between the primary *in vivo* exposure of the animal to the antigen and the *in vitro* re-exposure of the tissues. The presence or absence of the 19S antibody was consistent in replicate cultures from the same animal. This indicates that the variation in the response was the result of individual variation among the animals rather than variation among cultures from the same animal.

The addition of hydrocortisone-succinate to the basic medium used in this study did not enhance antibody production. In the presence of 20 per cent fetal bovine serum, antibody synthesis was

proceeding at the maximum level regardless of the presence of hydrocortisone-succinate. In contrast to the findings of Ambrose (1964, 1966) and Ortiz-Muniz and Sigel (1967), hydrocortisone-succinate did not substitute for the serum requirement. In Ambrose's studies, Eagle's MEM was used, but additives such as insulin and certain "non-essential" amino acids were also present when maximum enhancement was observed. No additions to the Eagle's MEM other than the steroid were made in the present study. In the studies of Ortiz-Muniz and Sigel, the basic medium was Medium 199 which contains ingredients in addition to those in Eagle's MEM. The difference in the composition of the medium could account for the lack of response to hydrocortisone-succinate observed in the present study.

Addition of phytohemagglutinin-P to the medium of cultures after 30 days cultivation resulted in an increase in the synthesis of specific viral antibody. This increased production was, however, noted only in cultures of fragments which had been stimulated with viral antigens prior to being placed in culture. Antibody was not synthesized in cultures of fragments which were exposed to phytohemagglutinin and maintained without re-exposure in vitro to the viral antigens. There are several possible explanations for these findings. Phytohemagglutinin has been shown to induce blastoid transformation and mitosis in rabbit lymphocytes (Fikrig, Gordon and Uhr, 1966; Sabesin, 1966); however, the highest mitotic index generally occurs four to five days after exposure to phytohemagglutination. The increased antibody production was noted in the next sampling period (3 days) after the addition of phytohemagglutination to the

cultures. Therefore the effect of the compound must have been on a metabolic process other than, or in addition to, induction of cell division. Several investigators (Muller and Le Mahieu, 1966; Cooper and Rubin, 1966) have shown that non-ribosomal RNA synthesis and protein synthesis are accelerated in lymphocytes stimulated with phytohemagglutinin. This accelerated synthesis can be seen as soon as 24 hours after addition of the compound. These findings would suggest that the rapid increase in antibody synthesis observed in the organ cultures was probably the result of accelerated synthesis of RNA and specific antibody protein. The continued production of elevated levels of antibody may have been the result of an increased number of cells producing specific antibody as a result of cell division.

The exposure of the tissue fragments to phytohemagglutinin alone or to both phytohemagglutinin and the specific viral antigens prior to cultivation produced no demonstrable effect. This is in contrast to the findings of Tao (1964) who claimed to have shown a specific anamnestic response in lymph node fragments from rabbits hyperimmunized with human chorionic gonadotropin or bovine serum albumin. This response was, however, not an all-or-none phenomenon. Tissues from only four of the seven animals he used responded to stimulation with phytohemagglutinin. He also used a higher concentration (600 μg/ml) than was used in the present study. Several factors can, therefore, account for the findings reported in the present study. The length of time (2 hours) may not have been

sufficient for phytohemagglutinin to produce an effect at the concentration used. The tissues from the animal used in this experiment may simply have been unresponsive to phytohemagglutinin. Consideration must also be given to the antigen used in this study. Montgomery et al. (1967) have recently reported that certain viruses inhibit the lymphocyte response to phytohemagglutin. Although adenoviruses were not among the viruses studied by these investigators, the observations made in the present study may be pertinent to this report.

The effect of chloramphenicol and puromycin on antibody synthesis in this system was consistent with the findings reported by Ambrose (1966) and Svehag (1964). Chloramphenicol was inhibitory to antibody synthesis at the concentration used (50 μg/ml). This effect was dependent upon the time of addition of the antibiotic and the length of the exposure. The antibiotic had to be present at all times to produce maximum inhibition. A short (2 hour) pulse exposure had little effect, and addition after 15 days produced little or no effect. These findings indicated that the antibody producing cells are most sensitive to chloramphenicol during the inductive phase of antibody synthesis and less sensitive during the productive phase. This difference in sensitivity has been suggested (Ambrose, 1966) to be the result of the presence of a longer lasting messenger RNA during the productive phase or to be the result of the formation of less-sensitive messenger RNA-ribosome complexes during the productive phase.

Puromycin was markedly inhibitory to antibody synthesis at the 3 μM concentration, but less inhibitory at the 1 μM concentration even when the latter concentration was present throughout the duration of the experiment. Addition of 3 μM puromycin after active synthesis had begun promptly terminated antibody synthesis. These studies are also in agreement with those reported by Ambrose (1966) and indicate the extreme sensitivity of the cells to an inhibitor which acts at a terminal step in protein synthesis. The results of these studies with chloramphenicol and puromycin indicated that the antibody being detected *in vitro* was the result of *de novo* synthesis rather than the result of passive release of preformed antibody.

A persistent infection was established in the rabbits. This was shown by recovery of virus from the spleens and lymph nodes of the animals injected with infectious virus preparations. These results are consistent with the findings of Pereira and Kelly (1957), who were able to demonstrate virus in the spleen of animals sacrificed three months after injection of the virus. As a result of this persistent infection the animals were under constant antigenic stimulation. This was evidenced by the levels of serum antibody in these animals at the time of sacrifice. This constant stimulation *in vivo* is thought to account in part for the successful demonstration of immune responses *in vitro* following exposure of the tissues to the viral antigens. The absence of detectable serum antibody and the lack of response *in vitro* to stimulation of the tissues taken from the animals hyperimmunized with the non-infectious structural subunits tends to support the conclusion that the persistence of virus

in vivo is necessary for maximum responses *in vitro*, at least under the conditions of this study.

The results of the studies with hamster lymphoid tissues *in vitro* were, in general, comparable with the results of the studies with the rabbit lymphoid tissues. Under the conditions of this study, only the lymph node fragments synthesized antibody *in vitro*. There was no detectable antibody synthesized by the spleen fragments *in vitro*. Only those lymph node fragments which were exposed to viral antigens *in vitro* produced antibody. There was no evidence of continued synthesis as a result of *in vivo* stimulation in fragments maintained without *in vitro* exposure to the viral antigens. These findings suggest that the *in vitro* stimulation may cause proliferation and maturation of the antibody producing cells.

Since it was necessary to pool the culture fluids because of the low antibody titers, no definite time course of antibody production could be established for the hamster tissue cultures as was possible for the rabbit cultures. However, it did appear that synthesis of antibody at detectable levels did not continue as long in the hamster cultures as in the rabbit cultures. This is indicated by the relatively few positive Pool III samples. Apparently the most active synthesis of antibody occurred within the first 2 weeks of culture. This difference in the period of active synthesis may simply reflect the difference in the productive capacity of tissues from different species. However, since adenovirus type 5 will replicate in rabbit lymphoid tissue and adenovirus type 12 does not replicate in hamster

lymphoid tissue, the difference in the length of the response may reflect responses to residual or persistent antigen in cultures of rabbit tissues which would not be present in the cultures of the hamster tissues.

Antibodies obtained from the culture fluids could be assayed readily by passive hemagglutination. Complement-fixing antibodies were detected in the pools from a single culture. The fluids which were positive in complement fixation tests had passive hemagglutination titers of 1:32. The findings that relatively few of the samples were positive by complement fixation was not surprising in view of the greater sensitivity of passive hemagglutination methods as compared to complement fixation methods. This is especially true in light of the low titers obtained by passive hemagglutination.

Although the levels of antibody synthesized in vitro were quite low, the antibody was specific for an antigen in the viral preparations. The lack of cross-reactivity of the fluids with antigens of adenovirus type 5, KB cells and fetal bovine serum supported this conclusion. The antibody could be "blocked" in the passive hemagglutination inhibition tests only with the freon extracted adenovirus type 12 or the fiber antigen of adenovirus type 12. There was no inhibition of the reaction when heterologous antigens were used as the "blocking" antigens. The failure of the tumor antigen to inhibit the reaction was taken as more evidence that the antibody detected was to a virus-specific antigen.

The absence of detectable antibody in the fluids of cultures of lymph node fragments exposed to the tumor antigen was somewhat

surprising. In view of the fact that all the animals used in this experiment had serum antibody to the tumor antigen, one might expect the lymph node to respond to *in vitro* stimulation. However, the failure to detect antibody may reflect the limits of the sensitivity of complement fixation as an assay method rather than indicate that the tissues were not producing antibody.

Consistent with the observations in the studies of the rabbit lymphoid tissues, the addition of chloramphenicol suppressed antibody synthesis in the hamster lymph node cultures. These results indicated that the antibody detected in the culture fluids was the results of *de novo* synthesis rather than release of preformed antibody.

The most significant finding in this study was that lymph node fragments from tumor-bearing hamsters which did not have detectable levels of serum antibody would synthesize antibody if stimulated *in vitro* with the specific viral antigen. This observation indicated that the lymph nodes of tumor-bearing hamsters are sensitized *in vivo* sufficiently to respond readily to stimulation *in vitro* with the viral antigen. This *in vivo* sensitization is thought to be the result of the presence of the viral type specific antigen which has been shown to persist in the tumor cells (Huebner *et al.*, 1964). The lack of response observed in tissues from animals which did not develop tumors although they were exposed to adenovirus type 12 *in vivo* indicates the necessity of the persistence of at least some antigenic component of the virus for *in vitro* responses to occur.

The results of this study of the immune responses *in vitro* in a model system in which it is established that a portion of the viral genome persists without the production of infectious virus leads one to speculate on the utility of this type of approach for the detection of the presence of inapparent infections or hidden genomes in other systems in which the viral etiology of a process is suspected but can not be demonstrated by the conventional methods of virus isolation or demonstration of serum antibody to the virus.

SUMMARY

The results of the studies of rabbit lymphoid tissues maintained *in vitro* indicate that:

1) the sensitized tissues would respond to *in vitro* stimulation with specific viral antigens in a concentration range from 10^6 $TCID_{50}$ to 10 $TCID_{50}$;

2) the lymph node fragments were more active in the synthesis of antibodies *in vitro* than were the fragments of spleen and thymus;

3) the lymphoid tissues responded to stimulation *in vitro* by production of specific antibodies which appeared in the culture fluids after a lag period, rose to peak titers in two to three weeks and declined gradually;

4) the antibodies produced *in vitro* were specific for adenovirus type 5 and could be detected by complement fixation, passive hemagglutination and neutralization tests;

5) antigens of heterotypic adenoviruses could also stimulate antibody synthesis in lymph node fragments; this response was evoked by the hexon antigen which is common to all the adenoviruses;

6) the antibodies synthesized *in vitro* were predominantly 7S; some cultures also produced 19S antibodies;

7) the addition of hydrocortisone-succinate did not enhance antibody production nor could it substitute for serum in the culture medium;

8) exposure to phytohemagglutinin elicited a specific response in tissues which had been maintained in culture for 30 days, but had no effect on fragments exposed simultaneously to virus and phytohemagglutinin or on fragments exposed only to this compound in the absence of the specific viral antigens;

9) the production of antibody could be inhibited by addition of chloramphenicol and puromycin;

10) adenovirus type 5 established a persistent infection in the rabbit and infectious virus was recovered from spleen or lymph node for periods up to 10 to 12 months after initial injection of the virus;

11) lymph node tissues from animals injected IV with infectious adenovirus type 5 responded to in vitro stimulation up to 12 months after the initial injection of virus, whereas tissues from animals hyperimmunized with non-infectious structural subunits could not be stimulated to respond in vitro 9 months after the final immunization.

The results of the studies of hamster lymphoid tissues maintained in vitro indicate that:

1) the lymph node fragments from hamsters bearing viral induced or transplanted adenovirus type 12 tumors could be stimulated in vitro to produce specific antibody to an antigen of adenovirus type 12;

2) this response could be evoked in tissues from tumor-bearing animals regardless of the presence or absence of detectable serum antibody to the viral antigen;

3) only those fragments which were exposed to antigen *in vitro* produced antibody at detectable levels;

4) the tissues from tumor-bearing hamsters exposed *in vitro* to tumor antigen did not produce antibody detectable with the assay methods used;

5) the spleen fragments stimulated *in vitro* did not produce antibody at detectable levels;

6) the synthesis of antibody could be inhibited by chloramphenicol.

REFERENCES

Adler, F. L., M. Fishman, and S. Dray, 1966. Antibody formation initiated in vitro--III. Antibody formation and allotypic specificity directed by ribonucleic acid from peritoneal exudate cells. J. Immunol. 97:554-558.

Allison, A. C., H. G. Pereira, and C. P. Farthing, 1960. Investigation of adenovirus antigen by agar gel diffusion technique. Virology 10:316-328.

Ambrose, C. T., 1964. The requirement for hydrocortisone in antibody-forming tissue cultivated in serum-free medium. J. Exp. Med. 119:1027-1049.

Ambrose, C. T., 1966. Biochemical agents affecting the inductive phase of the secondary antibody response initiated in vitro. Bact. Revs. 30:408-417.

Ambrose, C. T. and A. H. Coons, 1963. Studies on antibody production--VIII. The inhibitory effect of chloramphenicol on the synthesis of antibody in tissue culture. J. Exp. Med. 117:1075-1088.

Askonas, B. A. and J. H. Humphrey, 1958. Formation of specific antibodies and γ-globulin in vitro. Biochem. J. 68:252-261.

Askonas, B. A. and J. M. Rhodes, 1965. Immunogenicity of antigen-containing ribonucleic acid preparation from macrophages. Nature 205:470-474.

Askonas, B. A. and R. G. White, 1956. Sites of antibody production in the guinea pig. The relation between in vitro synthesis of anti-ovalbumin and γ-globulin and distribution of antibody-containing plasma cells. Brit. J. Exp. Path. 37:61-74.

Attardi, G., M. Cohn, K. Horibata and E. S. Lennox, 1959. Symposium on the biology of cells modified by viruses on antigens. II. On the analysis of antibody synthesis at the cellular level. Bact. Revs. 23:213-223.

Berman, Leonard, 1967. On the nature of transplantation immunity in the adenovirus tumor system. J. Exp. Med. 125:983-999.

Berman, L. D. and W. P. Rowe, 1965. A study of the antigens involved in adenovirus 12 tumorigenesis by immunodiffusion techniques. J. Exp. Med. 121:955-967.

Boniuk, Milton, C. A. Phillips and J. B. Friedman, 1965. Chronic adenovirus type 2 keratitis in man. New Eng. J. Med. 273: 924-925.

Boyden, S. V., 1951. The adsorption of protein on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. J. Exp. Med. 93:107-120.

Carrel, A. and R. Ingebristen, 1912. The production of antibodies by tissues living outside of the organism. J. Exp. Med. 15: 287-291.

Claman, H. N., 1966. Human thymus cell cultures--evidence for two functional populations. Proc. Soc. Exp. Biol. Med. 121: 236-240.

Cohen, E., 1967. Conversion of non-immune cells into antibody-forming cells by RNA. Nature 213:462-465.

Cooper, H. L. and A. D. Cooper, 1966. Synthesis of nonribosomal RNA by lymphocytes: a response to phytohemagglutinin treatment. Science 152:516-518.

Darken, M. A., 1964. Puromycin inhibition of protein synthesis. Pharmacol. Revs. 16:223-243.

Deutsch, H. F. and J. I. Morton, 1957. Dissociation of human serum macroglobulins. Science 123:600-601.

Dresser, D. W. and H. M. Wortis, 1965. Use of an antiglobulin serum to detect cells producing antibody with low hemolytic efficiency. Nature 208:859-861.

Dutton, R. W., A. H. Dutton and J. H. Vaughn, 1960. The effect of 5-bromouracil deoxyriboside on the synthesis of antibody *in vitro*. Biochem. J. 75:230-235.

Dutton, R. W. and J. D. Eady, 1964. An *in vitro* system for the study of the mechanism of antigenic stimulation in the secondary response. Immunology 7:40-53.

Eddy, B. E., G. E. Grubbs, and R. D. Young, 1964. Tumor Immunity in hamsters infected with adenovirus type 12 or simian's virus 40. Proc. Soc. Exp. Biol. Med. 117:575-579.

Elves, M. W., S. Roath, G. Taylor, and M. C. G. Israels, 1963. The in vitro production of antibody lymphocytes. Lancet 1:1292-1293.

Everett, S. F. and H. S. Ginsberg, 1958. A toxinlike material separable from type 5 adenovirus particles. Virology 6: 770-771.

Fagreus, Astrid, 1948. Antibody production in relation to the development of plasma cells. In vivo and in vitro experiments. Acta Med. Scand., supplement 204:5-122.

Fikrig, S., F. Gordon and J. W. Uhr, 1966. Culture of leucocytes from rabbit blood and lymph: effect of phytohemagglutinin and growth of macrophages. Proc. Soc. Exp. Biol. Med. 122: 379-382.

Fishman, M., 1959. Antibody formation in tissue culture. Nature 183:1200-1201.

Fishman, M., 1961. Antibody formation in vitro. J. Exp. Med. 114: 837-856.

Fishman, M., K. A. Hammerstrom, and V. P. Bond, 1963. In vitro transfer of macrophage RNA to lymph node cells. Nature 198:549-551.

Friedman, Herman, 1964. Distribution of antibody plaque forming cells in various tissues of several strains of mice injected with sheep erythrocytes. Proc. Soc. Exp. Biol. Med. 117:526-530.

Friedman, H. P., A. B. Stavitsky, and J. M. Solomon, 1965. Induction in vitro of antibodies to phage T_2:antigen in the RNA extract employed. Science 149:1106-1107.

Friedman, M. and C. R. Bennett, 1957. A hemagglutination test for detection of adenovirus antibodies. Proc. Soc. Exp. Biol. Med. 94:712-717.

Fugmann, R. A. and M. M. Sigel, 1967. Nature of hamster complement-fixing antibody to adenovirus 12 tumor antigen. J. Virology 1:678-683.

Gelderblom, H., R. Wigand and H. Bauer, 1965. DEAE-chromatography of adenoviruses of Rosen's group II. Nature 205:625-626.

Gilead, Z. and H. S. Ginsberg, 1965. Characterization of a tumor-like antigen in type 12 and type 18 adenovirus infected cells. J. Bacteriol. 90:120-125.

Gilead, Z. and H. S. Ginsberg, 1968a. Characterization of the tumor-like (T) antigen induced by type 12 adenovirus. I. Purification of the antigen from infected KB cells and a hamster tumor cell lines. J. Virology 2:7-14.

Gilead, Z. and H. S. Ginsberg, 1968b. Characterization of the tumor-like (T) antigen induced by type 12 adenovirus. II. Physical and chemical properties. J. Virology 2:15-20.

Ginsberg, H. S., Pereira, H. G., R. C. Valentine and W. C. Wilcox, 1966. A proposed terminology for the adenovirus antigens and virion morphological subunits. Virology 28:782-783.

Globerson, A. and R. Auerbach, 1965. Primary immune reactions in organ cultures. Science 149:991-993.

Globerson, A. and R. Auerbach, 1966. Primary antibody response in organ cultures. J. Exp. Med. 124:1001-1016.

Goffe, B. F. and H. F. Maassab, 1967. Sudden deafness associated with adenovirus infection. New Eng. J. Med. 276:1406-1409.

Gowans, J. L., 1959. The recirculation of lymphocytes from blood to lymph in the rat. J. Physiol. 146:54-69.

Gowans, J. L., D. D. McGregor and D. M. Cowen, 1962. Initiation of immune responses by small lymphocytes. Nature 196: 651-655.

Grabar, P. and P. Corvazier, 1960. In Ciba Found. Symposium on Cellular Aspects of Immunity. Eds. G. E. W. Wolstenholme and C. M. O'Connor. Churchill, London. pp. 198-206.

Haruna, I., H. Yaoi, R. Kono, and I. Watanabe, 1961. Separation of adenoviruses by chromatography on DEAE-cellulose. Virology 13:264-267.

Hellstrom, I. and H. O. Sjogren, 1967. In vitro demonstration of humoral and cell-bound immunity against common specific transplantation antigen(s) of adenovirus 12 induced mouse and hamster tumors. J. Exp. Med. 125:1105-1118.

Hoggan, M. D., W. P. Rowe, P. H. Black, and R. J. Huebner, 1965. Production of "tumor-specific" antigens by oncogenic viruses during acute cytolytic infection. Proc. Nat. Acad. Sci., U.S.A. 53:12-19.

Hollinshead, A. C., T. C. Alford, H. C. Turner, and R. J. Huebner, 1966. Adenovirus antibody and antibody to "T" antigen (neoantigen) location in gamma-globulin. Nature 211: 423-424.

Horne, R. W., S. Brenner, A. P. Waterson, and P. Wildy, 1959. The icosahedral form of an adenovirus. J. Mol. Biol. 1:84-86.

Huebner, R. J., W. P. Rowe, T. G. Ward, R. H. Parrott, and J. A. Bell, 1954. Adenoid-pharyngeal-conjunctival agents, a newly recognized group of common respiratory system viruses. New Eng. J. Med. 251:1077-1086.

Huebner, R. J., W. P. Rowe, and W. T. Lane, 1962. Oncogenic effects in hamsters of human adenovirus types 12 and 18. Proc. Nat. Acad. Sci. U.S.A. 48:2051-2058.

Huebner, R. J., W. P. Rowe, H. C. Turner, and W. T. Lane, 1963. Specific adenovirus complement-fixing antigens in virus-free hamster and rat tumors. Proc. Nat. Acad. Sci. U.S.A. 50:379-389.

Huebner, R. J., H. G. Pereira, A. C. Allison, A. C. Hollinshead, and H. C. Turner, 1964. Production of type-specific C antigen in virus-free hamster tumor cells induced by adenovirus type 12. Proc. Nat. Acad. Sci. U.S.A. 51: 432-439.

Hungerford, D. A., A. J. Donnelly, P. C. Nowell, and S. Beck, 1959. The chromosome constitution of a human phenotypic intersex. Amer. J. Human Genet. 11:215-236.

Ingraham, J. S. and A. Bussard, 1964. Application of a localization hemolysin reaction for specific detection of individual antibody-forming cells. J. Exp. Med. 119:667-682.

Jerne, N. K. and A. A. Nordin, 1963. Plaque formation in agae by single antibody producing cells. Science 140:405-406.

Kalchar, H., 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. II. Determination of adenine compounds. J. Biol. Chem. 167:445-460.

Klemperer, H. G. and H. G. Pereira, 1959. Study of adenovirus antigens fractionated by chromatography on DEAE-cellulose. Virology 9:536-545.

Kritzman, J. and A. Harper, 1966. A study of antibodies produced by spleen fragments in vitro. Proc. Soc. Exp. Biol. Med. 121:489-493.

Kunkel, H. G., 1960. Macroglobulins and high molecular weight antibodies in The Plasma Proteins. Vol. I. Ed. Frank Putnam, Academic Press, New York. p. 279-307.

Landau, B. J., V. M. Larson, G. A. Devore and M. R. Hilleman, 1966. Studies on induction of virus from adenovirus and SV_{40} hamster tumors. I. Chemical and physical agents. Proc. Soc. Exp. Biol. Med. 122:1174-1182.

Larson, V. M., P. A. Gosnell and M. R. Hilleman, 1966. Studies on induction of virus from adenovirus and SV_{40} hamster tumors. II. Helper viruses. Proc. Soc. Exp. Biol. Med. 122:1182-1191.

Larson, V. M., W. G. Raupp and M. R. Hilleman, 1967. Prevention of SV_{40} virus tumorigenesis in newborn hamsters by maternal immunization. Proc. Soc. Exp. Biol. Med. 126:674-677.

Laver, W. G., J. R. Suriano and M. Green, 1967. Adenovirus proteins. II. N-terminal amino acid analysis. J. Virology 1:723-728.

Lefkowitz, S. S., 1967. Adenovirus antigens detectable using passive hemagglutination methods. Proc. Soc. Exp. Biol. Med. 126:549-552.

Lefkowitz, S. S., 1968. Assay of viral antibodies in sera of hamsters bearing adenovirus type 12 tumors. Bact. Proc. 1968, p. 176.

Lefkowitz, S. S., J. A. Williams, B. E. Howard, and M. M. Sigel, 1966. Adenovirus antibody measured by the passive hemagglutination test. J. Bacteriol. 91:205-212.

Levinthal, J. D., C. Ahmad-Zadeh, G. Van Hoosier, and J. J. Trentin, 1966. Immunoflourescence of human adenovirus type 12 in various cell types. Proc. Soc. Exp. Biol. Med. 121:405.

McKenna, J. M. and K. M. Stevens, 1960. Studies on antibody formation by peritoneal exudate cells in vitro. J. Exp. Med. 111:573-600.

Maizel, J. V., 1965. Acrylamide-gel electrophoresis by mechanical fractionation: radioactive adenovirus proteins. Science 151: 988-990.

Michaelides, M. C. and A. H. Coons, 1963. Studies on antibody production. V. The secondary response in vitro. J. Exp. Med. 117:1035-1051.

Mishell, R. I. and R. W. Dutton, 1966. Immunization of normal mouse spleen cell suspensions in vitro. Science 153:1004-1005.

Moller, E. and G. Moller, 1962. Quantitative studies on the sensitivity of normal and neoplastic mouse cells to the cytotoxic action of isoantibodies. J. Exp. Med. 115:527.

Montgomery, J. R., M. A. South, W. E. Rawls, J. L. Melnick, G. B. Olson, P. B. Dent, and R. A. Good, 1967. Viral inhibition of lymphocyte response to phytohemagglutinin. Science 157: 1068-1070.

Muller, G. C. and M. Le Mahieu, 1966. Induction of ribonucleic acid synthesis in human leucocytes by phytohemagglutinin. Biochimica et Biophysica Acta 114:100-107.

Norrby, E., 1966a. The relationship between the soluble antigens and the virion of adenovirus type 3. I. Morphological characteristics. Virology 28:236-248.

Norrby, E., 1966b. The relationship between the soluble antigens and the virion of adenovirus type 3. II. Identification and characterization of an incomplete hemagglutinin. Virology 30:608-617.

Norrby, E., B. Nyberg, P. Skaaret, and A. Lengyel, 1967. Separation and characterization of soluble adenovirus type 5 components. J. Virology 1:1101-1108.

Norrby, E. and P. Skaaret, 1967. The relationship between soluble antigens and the virion of adenovirus type 3. III. Immunological identification of fiber antigen and isolated vertex capsomer antigen. Virology 32:489-502.

Nossal, G. J. V., 1966. Antibody production in tissue culture in Cells and Tissues in Culture. Vol. 3, Chapter 5. Ed. E. N. Willmer, Academic Press, New York.

O'Brien, T. F., M. C. Michaelides, and A. H. Coons, 1963. Studies on antibody production. VI. The course, sensitivity and histology of the secondary response in vitro. J. Exp. Med. 117:1053-1062.

O'Brien, T. F. and A. H. Coons, 1963. Studies on antibody production. VII. The effect of 5-bromodeoxyridine on the in vitro anamnestic antibody response. J. Exp. Med. 117:1063-1074.

Ortiz-Muniz, G. and M. M. Sigel, 1967. Long-term synthesis of antibody in vitro. Proc. Soc. Exp. Biol. Med. 124:1178-1182.

Pappenheimer, A. M., 1917. The reaction of lymphocytes under various experimental conditions. J. Exp. Med. 25:633-650.

Parrott, R. H. W. P. Rowe, R. J. Huebner, H. W. Bernton, and N. B. McCullough, 1954. Outbreak of a febrile pharyngitis and conjunctivitis associated with type 3 APC virus infection. New Eng. J. Med. 251:1087-1090.

Patterson, R., I. M. Suszko, and J. J. Pruzansky, 1963a. In vitro production of antibody by chicken spleen cells. I. Detection of antibody by isotope techniques. J. Immunol. 90:829-835.

Patterson, R. and I. M. Suszko, 1963b. In vitro production of antibody by chicken spleen cells. II. Immunoflourescent studies of tissue culture preparations. J. Immunol. 93: 836-842.

Patterson, R., I. M. Suszko, and M. F. Orr, 1966. Heterogencity of antibody producing cells and antibodies as a result of environmental alterations in tissue culture. J. Immunol. 96:139-148.

Pereira, H. G., 1959. Adenoviruses. Brit. Med. Bull. 15:225-234.

Pereira, H. G., 1960. Antigenic structure of non-infectious adenovirus materials. Nature, 186:571-572.

Pereira, H. G., A. C. Allison, and C. P. Farthing, 1959. Study of adenovirus antigens by immunoelectrophoresis. Nature 183:895-896.

Pereira, H. G. and M. V. T. de Figueirldo, 1962. Mechanism of hemagglutination by adenovirus types 1, 2, 4, 5, and 6. Virology 18:1-8.

Pereira, H. G. and Barbara Kelley, 1957. Latent infection of rabbits and adenovirus type 5. Nature, Lond. 180:615-616.

Peries, J., J. C. Chuat, M. Canwit, M. Boeron, 1966. Inhibition of adenovirus 12 oncogenicity in hamsters by simultaneous inoculation with adenovirus 5. Nature 209:738-739.

Petterson, U., L. Philipson and S. Hoglund, 1967. Structural proteins of adenoviruses. I. Purification and characterization of the adenovirus type 2 hexon antigen. Virology 33:575-590.

Pick, E. and J. D. Feldman, 1967. Autoradiographic plaques for detection of antibody formation to soluble proteins by single cells. Science 156:964-966.

Pope, J. H. and W. P. Rowe, 1964. Immunofluorescent studies of adenovirus 12 tumors and of cells transformed or infected by adenoviruses. J. Exp. Med. 120:577-588.

Ranney, H. M. and I. M. London, 1951. Antibody formation in surviving tissues. Fed. Proc. 10:562-563.

Reed, L. J. and H. Muench, 1938. A simple method of estimating fifty percent endpoints. Amer. J. Hyg. 27:493-497.

Rosen, Leon, 1960. A hemagglutination-inhibition technique for typing adenoviruses. Amer. J. Hyg. 71:120-128.

Ross, Eugene and H. S. Ginsberg, 1958. Hemagglutination with adenovirus. Nature of viral antigen. Proc. Soc. Exp. Biol. Med. 98:501-505.

Rowe, W. P., J. W. Hartley, B. Roizman, and H. B. Levy, 1958. Characterization of a factor formed in the course of adenovirus infection of tissue cultures causing detachment of cells from glass. J. Exp. Med. 108:713-729.

Rowe, W. P., R. J. Huebner, L. K. Gilmore, R. H. Parrott, and T. G. Ward, 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc. Soc. Exp. Biol. Med. 84:570-573.

Russell, W. C. and B. E. Knight, 1967. Evidence for a new antigen within the adenovirus capsid. J. Gen. Virol. 1:523-528.

Sabesin, S. M., 1966. Blastoid transformation of rabbit and guinea pig peripheral lymphocytes by phytohemagglutinin. Proc. Soc. Exp. Biol. Med. 122:351-355.

Saunders, G. C. and D. W. King, 1966. Antibody synthesis initiated in vitro by paired explants of spleen and thymus. Science 151:1390-1391.

Schild, G. C., C. W. Potter, J. S. Oxford, 1967. Inhibition of tumor formation with adenovirus 12 tumor cells in hamsters immunized with various adenoviruses. Nature 213:519-520.

Shimojo, H., H. Yamamoto, and C. Abe, 1967. Differentiation of adenovirus 12 antigens in cultured cells with immunofluorescent analysis. Virology 31:748-752.

Smith, K. O., 1965. Cyclic structure of adenovirus DNA. Science 198:100-102.

Smith, K. O., W. D. Gehle, and M. D. Trousdale, 1965. Architecture of the adenovirus capsid. J. Bacteriol. 90:254-261.

Stavitsky, A. B., 1954. Micromethods for the study of protein and antibodies. I. Procedure and general applications of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells. J. Immunol. 72:360-368.

Stavitsky, A. B., 1961. In vitro studies of the antibody response. Advanc. Immunol. 1:211-261.

Sterzl, J. and I. Riha, 1965. A localized haemolysis in gel method for the detection of cells producing 7S antibody. Nature 208:858-859.

Stevens, K. M. and J. M. McKenna, 1958. Studies on antibody synthesis initiated in vitro. J. Exp. Med. 107:537-559.

Sohier, R., Y. Chardonnet, and M. Prunieras, 1965. Adenoviruses: status of current knowledge. Progr. in Med. Virol. 7: 253-325.

Svehag, S. E., 1964. Antibody formation in vitro by separated spleen cells: inhibition by actinomycin and chloramphenicol. Science 146:659-661.

Tao, T. W., 1964. Phytohemagglutinin elicitation of specific anamnestic immune response in vitro. Science 146:247-248.

Tao, T. W. and J. W. Uhr, 1966. Primary-type antibody responses in vitro. Science 151:1096-1098.

Thorbecke, G. J., 1960. Gammaglobulin and antibody formation in vitro. I. Gammaglobulin formation in tissues from immature and normal adult rabbits. J. Exp. Med. 112:279-292.

Trenton, J. J., Y. Yabe, and G. Taylor, 1962. The quest for human cancer viruses. Science 137:835-841.

Valentine, R. C. and H. G. Pereira, 1965. Antigens and structure of the adenovirus. J. Mol. Biol. 13:13-20.

van Furth, R., H. R. E. Scherit, and W. Hijmans, 1966. The formation of immunoglobulins by human tissues in vitro. III. Spleen, lymph node, bone marrow and thymus. Immunology 11:19-27.

Van Hoosier, G. L., J. J. Trentin, S. S. Chenault, M. E. Byran, and K. J. McCormick, 1967. Infrequent C-F antibody response in mice with adenovirus type 12 tumors. Proc. Soc. Exp. Biol. Med. 124:1053-1055.

Van Hoosier, G. L., Jr., C. Capp, D. Baren, and J. J. Trentin, 1968. Evidence for the IgG nature of complement-fixing antibodies to adenovirus type 12 hamsters tumor neoantigens. J. Immunol. 100:128-132.

Wigand, R. and W. Wunn, 1966. Virus-associated and soluble adenovirus hemagglutinins: differences in antigenic specificity. Virology 28:484-485.

Wilcox, W. C. and H. G. Ginsberg, 1961. Purification and immunologesic characterization of types 4 and 5 adenovirus-soluble antigens. Proc. Nat. Acad. Sci. U.S.A. 47:512-526.

Wilcox, W. C. and H. S. Ginsberg, 1963a. Structure of type 5 adenovirus. I. Antigenic relationship of virus-structured proteins to virus specific soluble antigens from infected cells. J. Exp. Med. 118:295-306.

Wilcox, W. C. and H. S. Ginsberg, 1963b. Production of specific neutralizing antibody with soluble antigens of type 5 adenovirus. Proc. Soc. Exp. Biol. Med. 114:37-42.

Wilcox, W. C., H. S. Ginsberg, and T. F. Anderson, 1963. Structure of type 5 adenovirus. II. Fine structure of virus subunits. Relationship of structure subunits to virus-soluble antigens from infected cells. J. Exp. Med. 118:307-314.

Winkelstein, A. and C. G. Craddock, 1967. Comparative responses of normal human thymus and lymph node cells to phytohemagglutinin in cultures. Blood 29:594-607.

Winn, H. J., 1960. Immune mechanisms on homotransplantation. I. The Role of serum antibody and complement in the neutralization of lymphoma cells. J. Immunol. 84:530.