IN VITRO ANTIBODY PRODUCTION

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ABSTRACT

A system was developed in which fragments of guinea-pig spleen obtained from immunized animals, produced detectable quantities of antibody *in vitro*.

Using this method of culture, an attempt was made to stimulate the production of antibody in cultures of spleen fragments, obtained from normal animals, by exposure of tissue fragments to various dilutions of a rabbit-produced antiserum against guinea-pig spleen (cytolytic antibody). Control cultures were treated with a normal rabbit serum. In one of seven experiments, a substance was produced which behaved like antibody: it caused agglutination of sheep erythrocytes sensitized with a rabbit-produced haemolysin. This activity appeared in cultures treated with either anti-spleen serum, or normal rabbit serum. Storage of such culture fluids for a period of approximately five months, resulted in almost complete loss of activity. Antibody production could not be demonstrated in any of six other experiments.

Since repeated attempts to reproduce the results of the former experiment have failed, no significant conclusions can yet be drawn concerning the ability of cytolytic antibody to stimulate antibody production *in vitro*.

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INTRODUCTION

INTRODUCTION

The production of antibody by an animal following active immunization is a well-recognized phenomenon, and the presence of antibody in the circulation of the host may readily be demonstrated by routine serological methods. Rigourous physio-chemical techniques have now made it possible to isolate γ-globulin from the serum in a relatively pure state, and to ascertain many of its physical and chemical characteristics. More recently, Porter (1963) and Edelman and Gally (1964), as a result of exhaustive analyses of γ- globulin, have suggested fairly detailed structural models of the antibody molecule.

However, in spite of all the facts which have been compiled concerning the nature and the production of the antibody molecule, the exact mechanism whereby cells are stimulated by exposure to a foreign antigen, to produce specific antibody, is still unknown.

Work on this subject has proceeded in several different directions. In order to simplify the highly complex environment afforded by the body of a live host, a number of research workers have attempted to demonstrate

the entire process of antibody production in vitro. Since the in vitro situation provides a more precise means of controlling the experimental conditions, it seems logical that this procedure might reveal valuable information about the mechanism of antibody production.

Several investigators have independently attempted to stimulate normal, immunologically competent cells to produce antibody in tissue culture following an in vitro exposure to antigen. In this instance, "normal" cells refer to those cells obtained from an animal which has not previously been immunized in vivo. The majority of such attempts have met with failure. Fishman (1961, 1963) however, has reported success in the production of a virus-neutralizing antibody against T_2 coli-phage by first pretreating the viral antigen with a suspension of macrophages, and subsequently using an RNA-extract obtained from the macrophages, to stimulate a culture of normal, homologous lymph node cells to produce virus-neutralizing substance. However, there still exists some doubt as to whether the transfer of RNA from one cell type to another, is a necessary feature of antibody production. Askonas and Rhodes (1965) have produced evidence which suggests that RNA might act merely as a carrier of small amounts of residual antigenic material in this process, and that the combination of RNA plus antigen acts as a more potent

stimulant to cells than the antigen alone.

It has been suggested in this laboratory however, that in addition to antigen, and possibly a specialized type of RNA, a third factor may be necessary for the production of antibody. The third factor is a "cytolytic antibody", that is, an antibody which is directed specifically against a cell, and which will cause lysis of that cell, when complement is present.

The hypothesis states that antibody against a host's own cells is normally present, and that such cytolytic antibody, when directed specifically against an immunologically competent cell, stimulates the cell to produce an anti-antibody, which is then detected in the serum as normal γ-globulin, or "non-immune" globulin (Fig. 1.). It has further been suggested that the production of the so-called "immune" globulins is a modification of this process. It is postulated that the cytolytic antibody molecule acts as a carrier of foreign antigenic material. The combination of cytolytic antibody plus foreign antigen, stimulates immunologically competent cells to produce a modified anti-antibody against this complex, which will then combine specifically with the foreign antigenic material alone, as well as with the whole complex. The foreign antigen has in effect, changed the antigenic character of the cytolytic antibody molecule, but has left the antibody

FIG. 1 PRODUCTION OF NORMAL γ-GLOBULIN

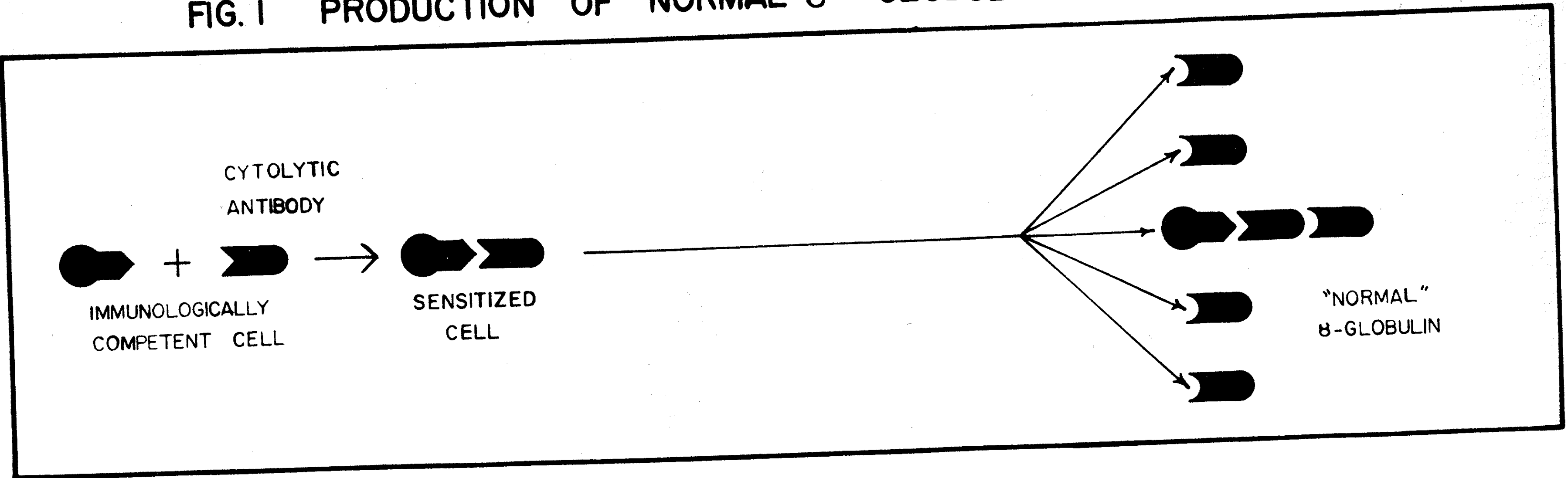
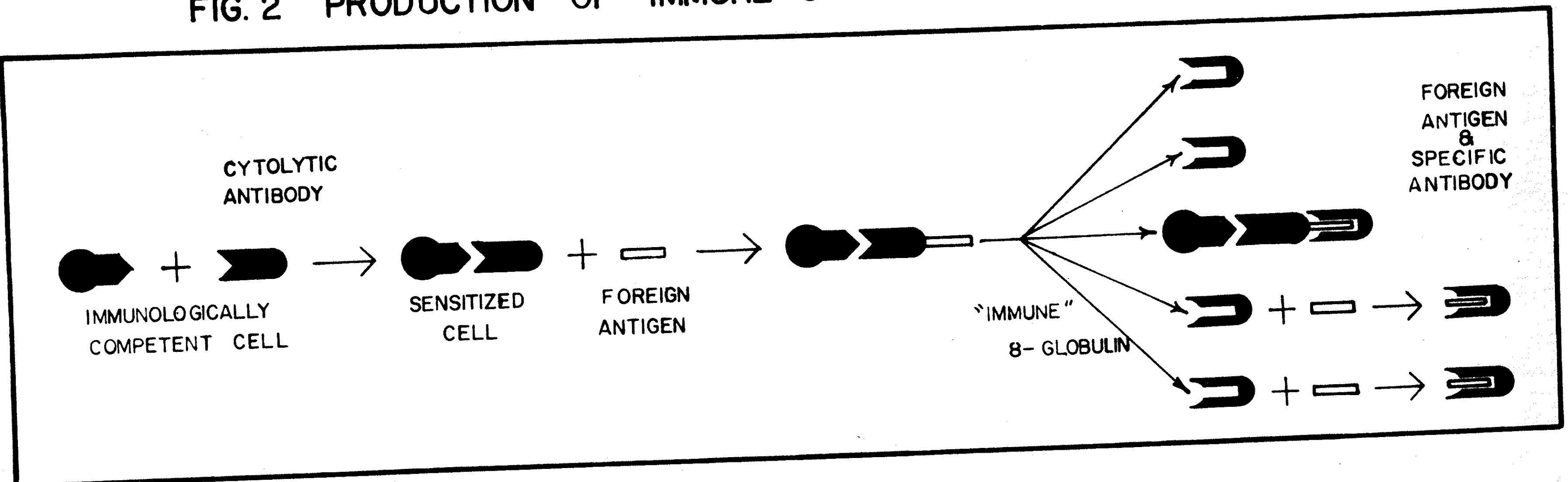


FIG. 2 PRODUCTION OF IMMUNE γ-GLOBULIN



receptor site free to act upon the cells against which it is directed (Fig. 2.).

The primary aim of the present experimental work was to stimulate normal, immunologically competent cells with a cytolytic antibody, to produce an antibody _in vitro_. Preliminary experiments were first conducted in order to establish a tissue culture system of immunologically competent cells which would produce detectable quantities of antibody _in vitro_ when cells were obtained from animals previously immunized _in vivo_. For this purpose, spleen, lymph node, and peritoneal exudate cells, taken from immunized guinea pigs, were incubated under various conditions, and subsequently, assays were done on the tissue culture fluids for their content of antibody. The second group of experiments was designed to test the ability of the same type of cells, obtained in this case however, from normal animals, to produce an antibody when stimulated by exposure to a heterologous cytolytic antibody _in vitro_. In the latter experiments, fragments of guinea-pig spleen were first incubated with a rabbit-produced, anti-spleen serum (which is itself composed of rabbit γ-globulin), then washed and cultured in fresh medium. Following an incubation period, the culture fluids were removed and titrated for the presence of antibody against rabbit γ- globulin.

CHAPTER I

REVIEW OF THE LITERATURE

CHAPTER I

REVIEW OF THE LITERATURE

References have been selected from the literature concerning the production of antibody *in vitro*, and are reviewed in the following chapter. Brief mention is also made of some of the *in vitro* effects of cytolytic antibody, as they pertain to the experimental work. The literature is presented under the following headings:

A. *In Vitro* Antibody Production

B. Some *In Vitro* Effects of Cytolytic Antibody

A. IN VITRO ANTIBODY PRODUCTION

1. *General*

The production of antibody *in vitro* has been demonstrated by many investigators. One of the earliest reports of success was published by Parker in 1937 (Parker, R.C., 1937). Rabbits were immunized with intravenous injections of guinea-pig erythrocytes, and following a rest period, a booster dose was given by the same route. Two or three days after the last injection, spleens were excised, and about seventy-five fragments of tissue (100 mg.) were cultured in 2 ml. of medium in Carrel flasks. The

cultures were incubated in an atmosphere of 80% O_2, 8% CO_2, and 12% N_2, for four days at 37°C. The medium consisted of a mixture of: (1) one part 1.4% sodium bicarbonate, (2) two parts Tyrode's solution with added glucose and phenol red, (3) three parts normal rabbit serum. At the end of the incubation period, culture fluids were titrated for the presence of antibody against guinea-pig erythrocytes using a direct hemagglutination test. Removal of spleens within forty-eight hours of the last injection of antigen, resulted in failure of cultures to produce antibody, whereas antibody could be detected in those cultures in which spleens were obtained after longer intervals of time following immunization. Controls, consisting of (1) culture medium incubated without tissue, (2) cultures incubated at 4°C, failed to produce antibody during the same period of time. Parker concluded that antibody had been produced _in vitro_ by fragments of spleen, and that a factor present in the body of the live animal, was necessary during the first forty-eight hours following immunization for the production of antibody to occur.

Parker's experiments have since been confirmed by a number of investigators. Fagraeus (1948) achieved similar results when she cultured fragments of spleen obtained from rabbits following secondary immunization with intravenous injections of killed _Salmonella typhosa_. In this case

however, tissues were incubated for only five to forty-eight hours before culture fluids were withdrawn and titrated for agglutinins against the "H" and "O" antigens of *Salmonella typhosa*. In order to determine whether antibody was actually produced in culture, or was merely released by the cells during the incubation period, extracts were made of tissue prior to the culture period, and these were titrated for their content of antibody. The titres of culture fluids were compared with those of extracts made of the same amount of tissue, and a higher titre in the culture fluids than in the extracts was interpreted as evidence that antibody production had occurred during the incubation period.

The methods of Parker and Fagraeus have since been modified to include a great variety of tissues, antigens, immunization procedures, culture media, methods of tissue culture, and antibody assay techniques. A number of these modifications are reviewed in the following sections.

2. Tissues and Cells

Investigations of the mechanism involved in the production of antibody have necessarily been intimately concerned with the sites of antibody formation. A variety of tissues have been tested for their ability to produce

antibody *in vitro* following immunization *in vivo*. Fagraeus (1948) determined the relative amounts of antibody formed by fragments of red and white splenic pulp, lymph nodes, bone marrow, thymus and liver following *in vivo*, secondary, intravenous immunization with killed *Salmonella typhosa*. These tissues were chosen because they all contained large numbers of reticulo-endothelial cells. The titres of culture fluids were compared with those of extracts made of the same amount of tissue. It was found that maximal antibody production occurred in cultures of the red pulp of spleen. Lymph node, bone marrow, and white splenic pulp produced lesser amounts of antibody, but still in excess of that found in tissue extracts. Cultures of thymus and liver apparently failed to produce antibody during the culture period, although antibody could be detected in extracts of these same tissues.

Thorbecke and Keuning (1953) conducted a similar study in which antibody production was compared in fragment cultures of red and white splenic pulp, popliteal lymph nodes, bone marrow, thymus and liver. Tissues were obtained from rabbits following primary immunization with paratyphoid B vaccine, given either intravenously, or subcutaneously into the right, hind footpad. The results corresponded roughly with those of Fagraeus. Splenic red pulp was the most active tissue following either intravenous or subcutaneous immunization. Bone marrow, lymph node and splenic white pulp were

active, but to a lesser extent, while thymus and liver appeared to be completely inactive under these conditions. Following subcutaneous immunization, cultures of the injected lymph node produced moderate amounts of antibody, while little or none was produced by the contralateral lymph node.

Stavitsky (1955) has also demonstrated that localization of antibody production may be dependent upon the route of immunization to a certain degree. After intravenous immunization of rabbits, he found greatest production of antibody against diphtheria of tetanus toxoids occurred in cultures of spleen, while small amounts of antibody were detected in culture fluids of liver. Cultures of popliteal lymph node apparently failed to produce antibody under these conditions. However, after subcutaneous immunization via the hind footpads, maximal antibody production occurred in cultures of the homolateral, popliteal lymph node, spleen, and bone marrow, while relatively small amounts of antibody could be detected in culture fluids of the contralateral node, kidney and liver.

Askonas and White (1956) assayed the ability of various tissues to incorporate radioactively labelled amino acids into specific antibody following a single, subcutaneous injection of antigen into the footpads of guinea-pigs. Both the homolateral, and contralateral flank nodes, as well as

the cervical nodes and bone marrow, synthesized large amounts of specific γ-globulin, whereas cultures of lung, spleen, lumbar nodes and granulomatous tissue produced lesser, but still detectable amounts of antibody.

In a later study, Askonas and Humphrey (1958[a]) found that after intramuscular or intraperitoneal immunization, cultures of bronchial, cervical, axillary and inguinal nodes, as well as bone marrow and granulomatous tissue, synthesized antibody in amounts which were two to six times as great as that produced by spleen under the same conditions. However, if similar animals were boosted with two intravenous doses of antigen, there was a marked increase in the production of antibody by cultures of spleen. Following hyperimmunization via the intravenous route, maximal synthesis of specific antibody occurred in cultures of spleen and bronchial lymph nodes.

Askonas and Humphrey distinguished between the amount of antibody synthesized per unit weight of tissue, and the amount of antibody synthesized by an entire organ. According to the latter method of calibration, the greatest production of antibody, after intramuscular or intraperitoneal immunization, occurred in the bone marrow, and the granuloma formed at the site of injection; following intravenous immunization, greatest production took place in the bone marrow, the lung and the spleen.

Askonas and Humphrey (1958[b]) have confirmed the ability of lung to produce large amounts of antibody in response to repeated intravenous injections of antigen. They have reported that in cultures of perfused rabbit lung, synthesis of antibody occurred at a rate of 7.0 mg. per hour.

It has been reported by Walker, Thomson and Gray (1960) that fragments of omentum, when cultured *in vitro* following intraperitoneal immunization, produce relatively large quantities of antibody. This observation was correlated with the accumulation of large numbers of plasma cells in the omentum following immunization, many of which appeared to contain antibody when stained using a fluorescent antibody technique.

Roberts, Adams, and White (1949) have reported that following repeated intraperitoneal injections of antigen, preparations of minced mesenteric lymph node produced detectable quantities of antibody when incubated for a twelve hour period *in vitro*. Production of antibody was markedly inhibited by incubation of tissues in an anaerobic atmosphere. Little or no production of antibody could be detected in preparations of mesenteric nodes following intravenous immunization, although splenic tissue was active under these conditions.

McKenna and Stevens (1960) have also claimed production

of specific antibody by cultures of peritoneal exudate cells following intraperitoneal injections of antigen and mineral oil.

On the basis of experimental evidence reported in the literature, it appears that:

(1) Following intravenous immunization, greatest antibody synthesis *in vitro*, per unit weight of tissue, is usually associated with the spleen, while various degrees of activity may be found in cultures of lymph node, bone marrow, and lung. Particularly after hyperimmunization, the lung assumes a role of major importance in the total quantity of antibody formed by individual organs.

(2) Following intramuscular or subcutaneous immunization, greatest synthetic activity is usually associated with the regional lymph nodes, while varying amounts of antibody are formed in the spleen, bone marrow, granuloma, and other groups of lymph nodes. However, when the total amount of antibody produced by an entire organ is calculated, it appears that granulomatous tissue and bone marrow are the major contributors of antibody.

(3) Following intraperitoneal immunization, large quantities of antibody may be produced in cultures of mesenteric lymph nodes, omentum, or peritoneal exudate cells induced by the injection of various adjuvant mixtures.

Although many tissues are thought to produce anti-

body both in vitro and in vivo, a great deal of confusion still exists concerning the morphological identification of the individual cells concerned in antibody production. Comprehensive reviews of this subject have been published by Fagraeus (1948), Coons, Leduc, and Connolly (1955), and Stavitsky (1961). Nossal's experiments (Nossal, G.J.V., 1959), in which he assayed the ability of single cells obtained from the lymph nodes of immunized donors, to produce antibody during an incubation period, have supplied overwhelming evidence in support of the plasma cell being the cell most intimately concerned with the production of antibody. A closely related problem has been that of the genesis of plasma cells. Speculation concerning the nature of the precursors of plasma cells, has revolved around a number of cells, including macrophages, reticular or histiocytic cells, and various cells of the lymphocyte series. Thorough reviews of the interrelationships of these, and other cells of the reticulo-endothelial system, have been made by Rebuck and LoGrippo (1961), and by Nossal (1962).

3. Immunization

The type of immunization employed in the production of antibody in vitro has been a significant feature of investigation in this field. Experiments may be placed into one of three categories on the following basis:

(1) Primary, secondary or hyperimmunization *in vivo*, followed by antibody production *in vitro*.

(2) Primary immunization *in vivo*, secondary immunization *in vitro*, followed by antibody production *in vitro*.

(3) Primary immunization *in vitro*, followed by antibody production *in vitro*.

(a) *Primary, Secondary, or Hyperimmunization In Vivo*

The production of antibody *in vitro* by tissues obtained from animals following *primary* (Askonas, B.A. and White, R.G., 1956), (Jerne, W.K. and Nordin, A.A., 1963), (Mountain, I.M., 1955), (Svehag, S.E., 1964), (LaVia, M.F., *et al*, 1960), *secondary* (Steiner, D.F. and Anker, J.S., 1956), (Vas, S.J. and Medzon, E.L., 1964), (Fagraeus, A., 1948), and *hyperimmunization in vivo* (Vaughan, J.H. *et al*, 1960), (Askonas, B.A., and Humphrey, J.H., 1958[a]), is now a well recognized phenomenon.

Using such methods, it has been shown that maximal antibody production *in vitro* occurs when tissues are removed from the live host three days after the last injection of antigen (Stavitsky, A.B., and Wolf, B., 1958[a]). It has also been established that actual production of antibody occurs, as opposed to the secretion or excretion of preformed, intracellular material, through the incorporation of radioactively labelled amino acids into tissue culture media (Stavitsky, A.B., 1958), (Askonas, A.B. and Humphrey, J.H., 1958[a]). The

appearance of labelled antibody in the medium of such cultures has been detected after an initial "lag" period of thirty minutes of incubation, in cultures of spleen fragments (Askonas, A.B., and Humphrey, J.H., 1958[a]), or lymph node cells (Helmreich, E., *et al*, 1960). Askonas and Humphrey have shown that during the "lag" phase, intracellular incorporation of radioactive material into antibody occurs, which is later followed by the secretion of radioactively labelled antibody into medium. They have estimated that secretion of the total amount of intracellular antibody occurs approximately every two hours in cultures of rabbit spleen slices.

It has also been demonstrated that production of antibody *in vitro* is a process requiring intact cells. Slight damage to cells, as by teasing cells out from a lymph node, may almost completely inhibit the response, while more severe treatment of cells, as by homogenization (Askonas, A.B., and Humphrey, J.H., 1958[a]), heating to 63°C. for twenty-five minutes (Stavitsky, A.B., 1955), or repeated freezing and thawing (Askonas, A.B., and Humphrey, J.H., 1958[a]), results in total inhibition of antibody production.

It also appears that oxygen is an essential requirement in the *in vitro* production of antibody, and incubation of tissues in an anaerobic atmosphere such as nitrogen or helium, results in partial or total abolishment of the antibody response (Stavitsky, A.B., 1955). Similarly, respiratory

poisons, such as 0.006 M cyanide, and 0.001 M dinitrophenol, when added to culture media, completely inhibit the *in vitro* formation of antibody (Stavitsky, A.B., 1955).

Although there are few mitotic figures observed in cultures of antibody-producing cells (Nossal, G.J.V., 1959), (Bauer, D.C., and Stavitsky, A.B., 1960), it appears that turnover of RNA and DNA is involved in the process of antibody production since various inhibitors of nucleic acid metabolism may cause partial or complete inhibition of the immune response *in vitro* (Dutton, R.W., *et al*, 1960).

(b) *Secondary Immunization In Vitro*

In more recent years, the initiation of a secondary immune response *in vitro* has been reported. Michaelides and Coons (1963) have succeeded in stimulating a secondary antibody response against diphtheria toxoid or bovine serum albumin in cultures of popliteal lymph node. Tissues were obtained from rabbits immunized one or more months previously with a single injection of antigen into the footpads. Fragments of lymph node were incubated in medium containing antigen for two hours, then washed free of residual antigen and cultured in homologous plasma clots in medium without antigen. Antibody could be detected in culture fluids approximately two days after exposure to antigen. Peak production of antibody occurred on the sixth or seventh day after stimulation, and continued for a period of four weeks or more.

The ability to respond simultaneously, but independently, to two different antigens was also demonstrated in this system. The presence of peak antibody titres in culture fluids coincided roughly with the appearance of large numbers of antibody-containing cells within tissue fragments, demonstrated by fluorescent antibody techniques.

Elves et al (1963[a]) have demonstrated the development of as many as 30% blast cells in cultures of human lymphocytes obtained from previously immunized blood donors, when cells were exposed to antigen in vitro. The presence of antibody within many of these cells was demonstrated using a fluorescent staining technique. Antibody was specific for the antigen which elicited the response. Similarly, using a fluorescein-conjugated anti-human γ-globulin serum, the presence of human γ-globulin was demonstrated within these same cells (Elves, M.W., et al, 1963[b]).

Tao (1964) has also succeeded in the stimulation of a secondary response in cultures of lymph node fragments obtained from animals three to six months following primary immunization in vivo with bovine serum albumin or human chorionic gonadotropin. He found that the anamnestic response could be initiated by incubation of cells with phytohemagglutinin, as well as with specific antigen. Phytohemagglutinin is a substance known to have a mitogenic effect upon cultures of human leukocytes (Nowell, P.C., 1960[a], 1960[b]).

This would seem to suggest that the magnitude of the secondary response is partially due to the rapid multiplication of previously "sensitized" cells, and that the presence of specific antigen is not a necessary prerequisite for this phenomenon to occur.

(c) Primary Immunization In Vitro

Antibody production *in vitro* has been reported in cultures of lymphoid tissue following primary, or secondary immunization *in vivo*, or following a second exposure of cultures to antigen *in vitro*. Attempts to initiate the primary immune response *in vitro*, in cultures of normal tissue, have met with less success.

In 1912, Carrel and Ingebrigsten reported the production of hemolysin, against goat red blood cells, in cultures of normal guinea-pig bone marrow and lymph glands (Carrel, A. and Ingebrigsten, R.J., 1912). Since that time, other investigators have tried unsuccessfully to initiate a primary immune response *in vitro*. Salle and McOmie (1937) attempted to produce antibody in cultures of chick embryonic tissue in Tyrode's solution. Their failure may be attributed to the lack of a mature immune mechanism in the animals from which tissues were obtained.

Michaelides and Coons (1963) attempted to stimulate a primary immune response in cultures of normal lymph node fragments by the incubation of tissue in antigen-containing

medium. In spite of the fact that they had been able to initiate a secondary antibody response in this manner, their attempts to stimulate normal tissues to produce antibody *in vitro*, failed.

Stevens and McKenna (1958) have reported the production of antibody against bovine γ-globulin in cultures of rabbit spleen fragments, and peritoneal exudate cells (McKenna, J.M., and Stevens, K.M., 1960). Low titres of antibody were detected in culture fluids using a passive hemagglutination test. They reported that a concentration of 0.1 to 10.0 mg./ml. of bovine γ-globulin in medium was optimal in the initiation of the immune response *in vitro*. Hemagglutination-inhibition tests, performed by adding bovine γ-globulin to the diluent, resulted in diminished titres, while addition of casein to the diluent had little or no effect upon the titres of antibody. The response was inhibited by the addition of prednisolone phosphate, 100 μg./ml., to culture media. Stavitsky (1961) reports that he has attempted to confirm the results of these experiments without success. He detected a substance which was capable of agglutinating sensitized cells, but which was not inhibited by the presence of homologous antigen in the diluent. Moreover, such activity could be absorbed out by an heterologous antigen-antibody precipitate.

Fishman (1961) has reported the production of antibody against T_2 coli-phage in cultures of rat lymph node cells.

The antigen, T_2 coli-phage, was pretreated by incubation with a suspension of rat macrophages. Subsequently, an homogenate of macrophages was filtered and the filtrate added to cultures of homologous lymph node cells. Culture fluids were removed after various periods of incubation, and γ-globulin precipitated and concentrated ten times by ammonium sulfate precipitation. Tests done on the γ-globulin concentrate revealed the presence of neutralizing activity for T_2 coli-phage. This activity was specific for T_2 coli-phage, and did not cross-react with T_1, or T_5 phage. The active principle was identified as γ-globulin by precipitation with a rabbit-produced anti-rat γ-globulin serum. Studies of the macrophage filtrates revealed that the activity of the transfer factor, capable of stimulating antibody production, was destroyed by ribonuclease. In later experiments (Fishman, M., and Adler, F.L., 1963), it was demonstrated that RNA extracts of macrophage filtrates were equally capable of stimulating virus-neutralizing antibody in cultures of homologous lymph node cells.

It is interesting to note in this regard, that Schoenberg _et al_ (1964) have produced electronmicrographs showing what appeared to be bridges of cytoplasm extending between macrophages and clusters of surrounding lymphocytic cells, in sections of tissue obtained from immunized rabbits. In some sections, they report having seen RNA granules about

the size of ribosomes, within the corridor of cytoplasm. Mannick (1962) has reported that the transfer of RNA extracts of lymph node cells, obtained from immunized donors, to normal recipients resulted in manifestation of an immune response in the recipient animals.

However, Askonas and Rhodes (1965) have shown the injection of antigen, complexed with RNA, into normal animals results in a greater antibody response to antigen than the injection of antigen alone. This would seem to suggest that a second possible explanation of Fishman's experiments might be that transfer of small amounts of antigenic substance, complexed with RNA, had occurred, which subsequently stimulated a primary immune response *in vitro*. The RNA has in effect, acted like an adjuvant, in the initiation of antibody production.

4. Culture Techniques

(a) Media

It has been demonstrated that amino acids are essential components of media in the production of antibody *in vitro* (Stavitsky, A.B., and Wolf, B., 1958[b]). Cultures of immunized spleen and lymph nodes fragments produced detectable quantities of antibody when cultured in media containing amino acids in buffered saline, but failed to do so when cultured in saline alone. It has also been shown that increasing the concentra-

tion of amino acids within culture media results in proportional increases in the amounts of antibody formed by tissues up to a certain point, beyond which, further increases in the concentration of amino acids fail to stimulate greater antibody production (Stavitsky, A. B., and Wolf, B., 1958[b]), (Vaughan, J. H., et al, 1960). Stavitsky and Wolf also reported that cultures of spleen and lymph node fragments failed to incorporate preformed polypeptides, obtained from a yeast protein hydrolysate, into specific antibody.

Serum is frequently added to culture media in an attempt to promote antibody production *in vitro*. Stavitsky and Wolf (1958[b]) have reported that undialyzed homologous serum caused only slight enhancement of antibody production by cultures of spleen and lymph node fragments, while dialyzed serum had no effect, or caused slight inhibition of the immune response. Heterologous serum generally had no observable effect upon the antibody-forming capacity of such cultures.

However, McKenna and Stevens (1960) have reported increased production of antibody by cultures of peritoneal exudate cells when 25% homologous serum was added to synthetic media. It was also noted that autologous serum produced still greater enhancement of the immune response *in vitro*, while both of the above were more effective than heterologous serum.

Similarly, Ambrose (1964) has found that the addition of 25% homologous serum to cultures of lymph node fragments

resulted in a very marked rise in the amount of antibody produced. The active principle appeared to be in the dialysable fraction of serum. Steiner and Anker (1956) have also reported that the addition of homologous serum to culture media stimulated increased production of antibody, in cultures of isolated spleen cells. In comparing the results of the latter three investigators with those of Stavitsky, it must be noted that Stavitsky's cultures were only maintained for periods of twenty-four hours, while those of the latter three were incubated for periods of one to four weeks. It would therefore appear that the beneficial effects of serum may be most marked when cultures are incubated over extended periods of time.

The effects of purines and pyrimidines upon antibody production *in vitro* have been assayed. Mountain (1955), Stavitsky and Wolf (1958[b]), Michaelides and Coons (1963) and Vaughan *et al* (1960) have uniformly reported failure of these substances to enhance antibody production.

Embryo extract has likewise been found to have no enhancing effect upon the ability of cultures to produce antibody (Stavitsky, A.B., and Wolf, B., 1958[b]), (Mountain, I.M., 1955). Several vitamins and hormones have been tested in an effort to determine whether such compounds stimulate the *in vitro* production of antibody. Stavitsky and Wolf (1958[b]) found that para-amino benzoic acid, and thiamin, failed to increase production of antibody in cultures of spleen and lymph node frag-

ments. Mountain (1955) has also reported that riboflavin, thiamin, and nicotinamide had little or no effect upon antibody production.

Michaelides and Coons (1963) have found that a mixture of vitamins A, C, E, K, B_{12}, inositol, thioctic acid, linoleic and linolenic acids, resulted in noticeable enhancement of the secondary response in vitro. Such an effect was manifested in some cultures while not in others. These authors have also reported that the addition of cortisone, insulin, and vitamin B_{12} to culture media, increased production of antibody by such cultures when serum was omitted from the media. Of particular interest, are the reports of Ambrose (1964) that cortisone, at concentrations of 0.1 to 10.0 μg./ml. of media, stimulated production of antibody in amounts equal to, or greater than that of similar media containing 25% homologous serum. This is in contrast with the commonly held view that cortisone results in a marked reduction of lymphoid tissue and depression of the immune response in vivo (Berglund, K., 1956[a], 1956[b]). Mountain (1955) however, has reported that 320 μg./ml. or more, of cortisone in media, resulted in a reduction of antibody production in cultures of spleen fragments.

In this respect, it is interesting to note that Weissmann and Thomas (1962) have reported the ability of cortisone to stabilize the lysosomal membranes of phagocytic cells. Since it is known that these cell organelles contain numerous

autolytic enzymes, a possible role of cortisone in such cultures might be the protection of cells from autolysis by preventing activation of lysosomes.

(b) Culture Apparatus

At the present time, a great variety of culture techniques have been employed in the production of antibody by cells cultured *in vitro*.

The earliest methods consisted of cultivating fragments of tissue in glass vessels, in fluid media (Fagraeus, A., 1948), (Parker, R.C., 1937), (Stavitsky, A.B., 1955).

Since that time, the fragment culture technique has suffered many innovations. Michaelides and Coons (1963) have cultured fragments of lymph node by embedding the tissue in homologous plasma clots in Roller tubes, and incubating the cultures in a roller drum.

Other investigators have grown fragments of tissue on the surface of wire gauze (LaVia, M.F., *et al*, 1960) supported just at the surface of fluid medium.

Egdahl *et al* (1962) have developed a technique in which fragments of immunized lymph node were layered over the surface of a layer of neutral agar in a small volume of medium. Beneath the neutral agar was a second layer of agar containing specific antigen. Antibody which was produced in culture, and antigen already present, diffused into the neutral layer of agar, where a specific immune precipitate was formed.

Although fragment culture techniques are plagued by the fact that cells in the centre of a fragment frequently become necrotic, antibody production in this type of system has been demonstrated for at least three days in cultures of spleen (LaVia, M.F., *et al*, 1960), and for as long as four weeks in cultures of lymph node (Michaelides, M.C., and Coons, A.H., 1963). It must be observed however, that cultures of spleen appear to undergo rapid loss of lymphoid elements under these conditions, followed by proliferation of fibroblasts (Trowell, O. A., 1958). Cultures of lymph node fragments retain their lymphoid character for longer periods of time (Michaelides, M.C., and Coons, A. H., 1963).

Other investigators have tackled the problem of tissue culture in a slightly different manner, in which cells were first removed from tissues by proteolytic enzymes (McKenna, J. M., and Stevens, K. M., 1960), teasing (Fishman, M., 1961), or scraping of tissue across a wire mesh grid (Vas, S. I., and Medzon, E. L., 1964). The cells so collected, have been cultured by various methods in order to demonstrate antibody production *in vitro*.

McKenna and Stevens (1960) have utilized the monolayer technique in the culture of peritoneal exudate cells with a considerable degree of success. Under these conditions, they found that it was mainly the mononuclear cells of an exudate, which adhered to the glass, while lymphocytes were lost to the

culture following the first medium change.

Attempts to culture spleen cells in suspension in fluid medium for the purpose of demonstrating antibody production, have met with variable degrees of success. Vas and Medzon (1964) cultured suspensions of spleen cells in Erhlenmeyer flasks, on rotary shakers, and subsequently detected antibody in the culture fluids. However, Steiner and Anker (1956) reported that similar cultures of spleen cells failed to produce antibody. Glucose utilization of such cells dropped to low or negligible levels áfter forty-eight hours of incubation.

Steiner and Anker (1956) devised a different type of apparatus, in an attempt to maintain spleen cells in a viable state for longer periods of time. Their method consisted of growing cells in a thin layer of medium on the surface of a cellophane membrane, which in turn was supported at the surface of a reservoir of medium. Cells were aerated by a continuous flow of 95% O_2 : 5% CO_2. Using such an apparatus, reticular cells, large lymphoid and myeloid cells, and motile lymphocytes remained viable after three days in culture, while after five days the presence of mononuclears, and multinucleated giant cells were observed. No fibroblast formation was seen, and only a few mitoses were evident. Such cultures produced detectable quantities of antibody. Ainis (1962) has designed a similar apparatus in which suspended cells were

cultured on the surface of a dialyzing membrane, over the surface of a reservoir of medium. Antibody continued to be produced for nine or ten days in these cultures.

A technique has recently been developed by Jerne and Nordin (1963) in which spleen cells, taken from mice immunized with sheep erythrocytes, were suspended in medium containing 0.7% agar, and sheep red cells. The medium was allowed to solidify, and after an incubation period, complement was layered over the surface of the culture. After further incubation, the production of hemolysin could be demonstrated as "plaques of lysis" surrounding single or multiple spleen cells. Such a technique was of use in the detection and enumeration of antibody-producing cells within a culture, and for the detection of small amounts of antibody. Ingraham and Bussard (1964) have further modified this technique for the detection of antibody against soluble protein antigens.

Richardson and Dutton (1964) have succeeded in combining the use of Jerne's technique for detection of antibody-producing cells, with a method of culturing cells in suspension. Spleen cells, obtained from animals previously immunized *in vivo* with sheep erythrocytes, were cultured in suspension in the presence of sheep erythrocytes. Subsequently, antibody-producing cells were detected by "plating out" of the cell suspensions in a manner similar to that of Jerne and Nordin (1963).

Although a variety of culture techniques have been utilized in the demonstration of antibody production *in vitro*, methods of tissue culture still require improvement. A satisfactory method for the long-term culture of isolated lymphoid cells has yet to be developed.

Of particular interest in this regard are the reports of Auerbach (1963). He has claimed that a type of synergism exists between embryonic spleen and thymus tissue. When rudiments of both tissues are cultured together, development of lymphoid cells within the fragments was observed. However, when either tissue was cultured alone, tissues failed to develop beyond the fibroblast stage. It would be of interest to know whether such cultures would be capable of producing antibody when exposed to antigen for the first time *in vitro*. The author has reported that such experiments are currently in progress.

5. Antibody Assay Techniques

There are an ever-increasing number of techniques available for the detection of antibody produced *in vitro*. A few of the more commonly used methods, together with some recently developed methods which are particularly suited to this purpose, are mentioned in the following section. One of the most frequently used techniques is the passive hemagglutination test, as developed by Boyden (1951). This method entails the absorption of protein antigens onto the surface of erythro-

cytes which have been pre-treated with tannic acid. Such cells will undergo agglutination when mixed with sera containing specific antibody. Stavitsky (1961) has reported the production of non-specific substances in culture, which may cause agglutination of cells, giving rise to falsely positive results. He stresses the need for adequate controls using this system of detection. The method may be used to detect as little as 0.006 μg./ml. of antibody nitrogen (Marrack, J. R., 1963).

Neutralization tests have been used successfully in the detection of anti-viral antibodies (Fishman, M., 1961), and anti-toxin antibodies (Vas, S. I., and Medzon, E. L., 1964) such as the lecithinase toxin produced by _Clostridium perfringens_. The extreme sensitivity of these tests, combined with the highly antigenic nature of such antigens, make this a highly effective system for the study of _in vitro_ antibody production.

Small amounts of precipitating antibody produced _in vitro_ may be detected using gel diffusion techniques developed by Ouchterlony (1948), with the advantage that only small amounts of fluid are required for the test. However, this system is less sensitive than passive hemagglutination methods (Marrack, J. R., 1963). Egdahl _et al_ (1962) have utilized this technique in the detection of antibody produced in cultures of lymph node fragments by growing the tissues directly

on the surface of agar, below which was a second layer of agar containing antigen, so that a precipitation reaction occurred within the culture medium itself. Jerne and Nordin's (1963) method of detecting antibody-producing cells has made similar use of the ability of antibody to diffuse through agar-containing medium.

Coons et al (1955) have developed fluorescent antibody techniques which may be utilized in the detection of antibody within cells. Such a method may be manipulated to establish the specificity of antibody contained within cells, as well as to demonstrate the γ-globulin nature of the antibody present (Elves, M. W., et al, 1963[b]), (Michaelides, M. C., and Coons, A. H., 1963).

One of the most sensitive, and useful, techniques in current use for the detection of very small amounts of antibody, was originally developed by Ranney and London (1951), and Keston and Katchen (1956). Radioactively labelled amino acids were incorporated into tissue culture media. Subsequently, culture fluids were harvested, and specific antibody precipitated by the addition of homologous antigen, or antigen-antibody aggregates. Such co-precipitation was shown to be a more sensitive technique than direct precipitation. The amounts of radioactively labelled antibody were measured using a radioactive counter, and the values obtained were considered to represent antibody, newly formed in vitro. An estimate of

the amount of non-specific reactivity was obtained by the addition of heterologous antigen-antibody complexes to a second aliquot of the same culture fluid. Any radioactivity detected in the precipitate formed, was considered to be non-specific in nature.

Each of the techniques described, offer disadvantages, as well as advantages, in the detection of antibody produced *in vitro*. A combination of several tests have often supplied the most useful information.

B. SOME IN VITRO EFFECTS OF CYTOLYTIC ANTIBODY

Our interest in the effects of cytolytic antibody upon cells cultured *in vitro*, have centred principally around those cytotoxic, and mitogenic effects which have been reported. Comprehensive reviews of the cytotoxic effects of cytolytic antibody *in vitro* are already available (Rigby, C., 1963), (Bitensky, L., 1963).

More recently, it has been reported that treatment of rat fibroblasts with specific cytolytic antiserum, resulted in loss of lysosomal enzymes coupled with autolysis of cells. This was correlated with swelling of cells, and loss of their ability to exclude vital dyes. Such observations may offer a possible explanation of the cytotoxic effects of cytolytic antisera.

A second notable effect of cytolytic antisera upon

cells, has been the ability of such sera to stimulate mitosis of human leukocytes. Grasbeck and Nordman (1963) observed the presence of 10% or more mitotic figures in cultures of human peripheral blood leukocytes incubated for three or four days in media containing rabbit-produced cytolytic antibody. This was in contrast with less than 0.5% mitoses observed in control cultures which had been incubated with media containing saline in place of antiserum. Pearlmain et al (1963) have observed similar effects in cultures of human leukocytes when previously "sensitized" cells, obtained from tuberculin-positive donors, were incubated with specific antigenic substance (Purified Protein Derivative).

The similarity in the mitogenic activity of cytolytic antibody upon normal cells, and specific antigen upon sensitized cells, might suggest a function of cytolytic antibody in immunological phenomena such as was described in the Introduction.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

1. Animals

Adult guinea-pigs of both sexes were maintained on a diet of Purina alfalfa pellets, oats, lettuce and cabbage leaves, and water.

Adult, male albino rabbits were fed a diet consisting of Purina alfalfa pellets, lettuce and cabbage leaves, and water.

2. Sheep's Blood

Sheep's blood was originally obtained by bleeding from the jugular vein into a vessel containing an equal volume of Alsever's solution.[1] Blood was routinely stored in this form at 0 to 4°C for a period of three weeks, at which time it was discarded and a fresh lot obtained in its place. Newly obtained blood was allowed to stand at 0 to 4°C for at least two days before it was used. In later experiments, blood was collected and stored in a Bacto Transfuso-vac bottle containing dextrose-sodium citrate solution.[2]

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[1]see appendix [2]see appendix

3. Antigens

Guinea-pigs were immunized with four different antigens throughout the experimental work: (1) sheep erythrocytes, (2) human erythrocytes, (3) rabbit γ-globulin, and (4) guinea-pig erythrocytes sensitized with rabbit-produced haemolysin. Immunization procedures varied from one experiment to another, and will therefore be described under the section, Procedures and Results.

(a) Sheep erythrocytes. Sheep's blood, suspended in Alsever's solution, was centrifuged in a sterile graduated centrifuge tube at 1500 r.p.m. for five minutes. The supernatant fluid was discarded, and the cells washed three times with 0.85% sodium chloride, centrifuging at 1500 r.p.m. for five minutes and discarding the supernate each time. The final packed cell volume was recorded, and cells resuspended in a volume of saline equal to nine times the volume of packed cells. The final suspension obtained by this method consisted of 10% sheep cells (by volume) in saline. This suspension was used to immunize guinea-pigs against sheep erythrocytes.

(b) Human Erythrocytes. Human type "O" blood, stored in Alsever's solution, was treated in an analagous fashion to sheep's blood, to yield a suspension of 10% human erythrocytes (by volume) in saline.

(c) Rabbit γ-Globulin. Rabbit γ-globulin (RGG) was obtained from Manor Research Laboratories in an amorphous state. It was dissolved in sterile saline so as to yield a final concentration of 1.0% RGG. This solution was stored at -18°C, and thawed at room temperature before it was injected into guinea-pigs.

(d) Guinea-pig Erythrocytes Sensitized with Rabbit-produced Haemolysin. Washed, packed erythrocytes were prepared in the manner described previously, and the cells resuspended in Dulbecco's Balanced Salt Solution (DBSS).[3] The cells were sensitized by incubation for one to two hours, with approximately two-thirds of a hemagglutinating dose of rabbit-produced amboceptor.[4] Sensitized cells were centrifuged, the supernate discarded, and the cell pellet washed once and resuspended in DBSS to give a final concentration of 50% sensitized guinea-pig erythrocytes in DBSS.

4. Media

(a) Basic Media. Medium 199, without $NaHCO_3$, obtained from Microbiological Associates, Bethesda, Maryland, and Eagle's Minimum Essential Medium (Eagle's MEM)[5] were utilized throughout all experiments. To these media, supplements were added,

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[3] see appendix

[4] Amboceptor was obtained from Markham Laboratories, Chicago 20, Ill.

[5] see appendix

including: (1) normal guinea-pig serum, (2) antibiotics, and (3) cortisone derivatives. The initial pH of the medium was adjusted to 7.2 to 7.4 with 4.4% $NaHCO_3$. Thereafter, the pH of the medium in each culture was maintained at this level by adding 1.4% $NaHCO_3$ whenever it was required.

(b) Supplements

(i) Serum. In a number of experiments, guinea-pig serum was added to basic media at concentrations of 20 to 25%. Serum was obtained by cardiac puncture of normal guinea-pigs. The blood was allowed to clot, and the serum removed. In some cases, the serum was stored in a frozen state at -18°C, while in other instances serum was stored at 0 to 4°C for no longer than forty-eight hours before it was incorporated into medium and added to cultures. These will be referred to as frozen NGPS, and fresh NGPS, respectively. In one experiment, lyophilized guinea-pig serum, obtained from the Department of National Health and Welfare, Ottawa, Ontario, was reconstituted with glass distilled water and used for this purpose.

(ii) Antibiotics. A combination of three antibiotics was added to all media in the following concentrations, to prevent contamination by bacteria or fungii:

1. Penicillin, 200 units/ml.
2. Streptomycin, 100 μg./ml.
3. Mycostatin, 200 units/ml.

(iii) Cortisone Derivatives. In several experiments, sodium hydrocortisone hemisuccinate,[6] and cortisone acetate,[7] were added to media in the place of serum, at concentrations of 0.1, and 1.0 μg./ml. of medium.

(c) Transport Media. Tissues were collected, transported, and prepared for culture in a variety of fluids which have been labelled collectively as "transport media". They included:

1. DBSS
2. 1.5% gelatin in DBSS
3. Hank's balanced salt solution (HBSS), pH7.2-7.4[8]
4. Medium 199

(d) Plasma. Guinea-pig plasma was required for the embedding of fragments of guinea-pig spleen. For this purpose, guinea-pigs were bled by cardiac puncture, and 9.5 ml. of blood transferred immediately to a tube containing 0.5 ml. of 0.02% heparin in saline, and inverted to mix. The final concentration of heparin, 0.0010 mg/ml. of blood was sufficient to prevent immediate clotting of whole blood, while not to prevent clotting of the plasma when it was later mixed with

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[6]Obtained from The Upjohn Co. of Can., Don Mills, Ont.

[7]Obtained from Merck, Sharp and Dohme of Can. Ltd., Mont.

[8]see appendix

tissue fragments. The heparinized blood was centrifuged at 1500 r.p.m. for fifteen minutes, and the supernatant plasma withdrawn and stored in a sterile container at 0 to 4°C for not longer than twenty-four hours before being used.

5. Cell Suspensions

Suspensions of guinea-pig spleen, lymph node, and peritoneal exudate cells were required for three purposes:

1. Immunization of rabbits in the production of anti-spleen sera.
2. Titration of rabbit-produced anti-guinea-pig spleen sera.
3. Culture of isolated spleen cells in monolayers.

The same techniques were used to obtain cell suspensions for all three purposes.

(a) Peritoneal Exudate Cells. Guinea-pigs were injected intraperitoneally with 10.0 ml. of 0.001% glycogen in saline one to three days prior to sacrifice, to induce a peritoneal exudate. On the day of sacrifice, animals were first injected with 20 - 25 ml. of 0.1% heparin in DBSS by the intraperitoneal route. The injection of DBSS served to increase the fluid content of the exudate making the cells more easily obtainable, while the heparin was necessary to prevent the formation of a clot.

Animals were anaesthetized with chloroform, and

killed by cardiac exsanguination. The abdomen was opened using aseptic techniques, and the peritoneal exudate withdrawn using a sterile Pasteur pipette. If the exudate was bloody, it was discarded and a fresh one obtained in its place.

(b) Spleen and Lymph Node Cell Suspensions. Guinea-pigs were anaesthetized and killed by cardiac exsanguination. The abdominal cavity was opened using sterile precautions, the spleen, and/or mesenteric and flank lymph nodes removed and placed in a vessel containing chilled transport medium. The remainder of the procedure was carried out in an ultra-violet cabinet. Tissues and fluid were transferred to a Petri dish where as much as possible of the fat and surrounding connective tissue were removed. The tissues were cut into fragments using sterile scalpel blades, and the fragments placed on a brass 60-mesh wire grid. The fragments of tissue were combed across the grid using a piece of 60-mesh copper screen, and the cells isolated by this procedure were washed through the grid with transport fluid. The cell suspension was collected in a dish below. Further treatment of the suspension was carried out according to the requirements of a particular experiment.

6. Tissue Fragments

Fragments of guinea-pig spleen were cultivated in plasma clots. Spleens were obtained for this purpose in the

same manner as described in the preceding paragraph. After removal of fat and connective tissue, the spleen was first cut into thin slices using sterile scissors, and then into smaller fragments, about 1.0 mm^3 in size using sterile scalpel blades. A great deal of care was exercised during the cutting procedure to ensure a clean-cut edge on each fragment of tissue, and to avoid tearing of the spleen wherever possible. A new scalpel blade was used for each spleen.

B. METHODS

1. Tissue Culture

Two different techniques were applied to the culture of cell suspensions and tissue fragments. Isolated cells were suspended in medium, seeded in tubes, and incubated horizontally. Under these conditions, cells settled to the lower side of the tube, adhered to the glass, and formed a type of monolayer. In contrast, fragments of splenic tissue were embedded within tubes in a layer of homologous plasma, covered with medium, and incubated in a roller drum. During the incubation period, cells migrated outward from the fragment, into the surrounding layer of plasma. The details of these techniques are as follows:

(a) Monolayers. Lymph node and peritoneal exudate cell suspensions were centrifuged at 1000 r.p.m. for five minutes. The supernates were discarded, and cell pellets

resuspended in media by pipetting.

Spleen cell suspensions were subjected to a differential centrifugation procedure at this point, to remove as many red cells as possible from the original suspension. In this process, a suspension was centrifuged, the supernate decanted, fresh transport medium added, and cells resuspended by pipetting. The process was repeated three or four times, centrifuging at 1000 r.p.m., 800 r.p.m., and 200 - 300 r.p.m., repeating the last centrifugation procedure when it was deemed necessary. The cell pellet, which consisted mainly of white cells, was resuspended in a small volume of medium.

Aliquots of each cell suspension were mixed with equal volumes of Reif cytolysis stain,[9] and a drop of each mixture placed in a hemocytometer. The number of viable cells, and the total number of cells were estimated, assuming that stained cells were dead or dying cells, and unstained cells were viable cells. From these figures, the total number of viable cells in the original suspension was calculated and the suspension was adjusted accordingly by dilution in medium, to the desired cell concentration. Cells were dispensed to tubes in 1.0 ml. volumes, and incubated horizontally at 37°C. Both Leighton tubes containing flying coverslips, and roller tubes, were used for this purpose. Some cultures were incubated in air, while others were stoppered with cotton plugs

[9] see appendix

and incubated in a mixture of 95% O_2 : 5% CO_2.

At various intervals, old medium was removed and stored at -18°C, and fresh medium added to cultures in its place.

(b) Fragment Cultures. Fragments of spleen were obtained by the method described previously. Parker's technique (Parker, R.C., 1961) was adopted for the embedding of fragments of tissue in plasma clots. A few drops of homologous plasma were placed in a roller tube and spread over the bottom half of one wall of the tube. Ten fragments of tissue, bathed in HBSS, were placed just inside the mouth of the tube, and the excess fluid removed with a pipette. The fragments were arranged in a linear fashion in the layer of plasma at the lower end of the tube. The excess plasma was allowed to drain to the bottom, and mixed with five or six drops of medium. The mixture of plasma and medium was then spread over the fragments, and the tubes placed horizontally until the clot adhered to the side of the tube. Cultures each received 1.0 ml. of medium, and were incubated at 37°C in a roller drum revolving at 1/5 r.p.m. Periodically, medium was replaced, and old medium stored in sterile containers at -18°C.

2. Titration of Culture Fluids and Sera

Culture fluids and sera were titrated for the presence of antibody using either a direct hemagglutination test for detection of antibody against erythrocytes, or an indirect

hemagglutination test for detection of antibody against rabbit γ-globulin. The following description applies to both tests except where otherwise noted:

(a) Culture Fluids. All culture fluids were stored at -18°C. Before titrating, they were thawed and inactivated at 56 - 58°C for thirty minutes to destroy any residual complement activity which might remain in the serum fraction of media. Culture fluids were diluted 1/5 or 1/10 in DBSS prior to the titration procedure, since in most cases there was less than 1.0 ml. of fluid. The process of dilution also served to neutralize the pH of fluids, which in some cases was low.

(b) Standardized Erythrocyte Suspension. Human or sheep's blood, suspended in anticoagulant solution, was centrifuged at 2000 r.p.m. for five minutes, the supernate discarded, and the cell pellet resuspended and washed three times with DBSS, centrifuging at 2000 r.p.m. and discarding the supernate each time. A measured volume of washed, packed erythrocytes was mixed with DBSS to give a suspension of approximately 1.2% cells. An aliquot of suspension was diluted 1/10 in distilled water. Red cells were lysed in such a hypotonic solution, and the haemoglobin released imparted an orange colour to the solution, the optical density of which was proportional to the concentration of haemoglobin, and therefore to the number of erythrocytes present in the original suspension.

The optical density of this solution was determined in a Bausch and Lomb spectrophotometer at 550 mμ, reading against a blank consisting of DBSS diluted 1/10 in distilled water. The optical density was recorded, and a calculation made to determine the dilution necessary to obtain a standard suspension of optical density, 0.1125, which is approximately equal to 0.5% erythrocytes:

$$\frac{\text{optical density}}{0.1125} = \text{dilution factor}$$

The standard suspension, known as 0.1125 cells, was obtained by diluting the original suspension thus:

$$\frac{1}{\text{dilution factor}} = 0.1125 \text{ suspension}$$

For example, had the dilution factor been 2.5, the original suspension of 1.2% cells would be diluted 1/2.5 to obtain a suspension of 0.1125 cells.

The 0.1125 cell suspension was utilized directly in the titration of culture fluids by the direct hemagglutination method. For the indirect hemagglutination test, further treatment of the cells was required as follows:

(c) <u>Sensitization of 0.1125 Cells</u>. The presence of antibody in culture fluids or sera against rabbit γ-globulin was detected using an indirect type of hemagglutination test. Essentially, sheep erythrocytes were "sensitized" with a "subagglutinating" dose of rabbit-produced haemolysin. The

concentration of haemolysin was sufficient to coat the cells with antibody, but not to cause agglutination. These are referred to as "sensitized" cells. Such cells are agglutinated by antibody against rabbit γ-globulin.

Cells were sensitized in 25.0 ml. volumes. Tubes were labelled "sensitized" cells, and "normal" cells. To the former, 0.1125 cells, DBSS, and haemolysin were added, while 0.1125 cells and DBSS were mixed in the latter, in the amounts indicated in Table I.

TABLE I

SENSITIZATION OF SHEEP ERYTHROCYTES

Material	Sensitized Cells	Normal Cells
DBSS	23.75 ml.	25.00 ml.
0.1125 cells	25.00 ml.	25.00 ml.
1/1000 haemolysin	1.25 ml.	0.00 ml.

The haemolysin added to the first tube was diluted to a final concentration of 1/40,000. This constituted one-third of a normal 100% agglutinating dose of haemolysin.

Tubes were capped with parafilm, inverted to mix, and incubated in a 37°C water bath for twenty minutes, inverting the tubes every five minutes to mix. Tubes were then centrifuged in the cold at 2000 r.p.m. for five minutes. The

supernates were decanted and cell pellets resuspended and washed with 25.0 ml. of DBSS. The suspensions were centrifuged again at 2000 r.p.m. for five minutes, and supernates discarded. The remaining cell pellets were resuspended by pipetting, in 24.9 ml. of DBSS to obtain a suspension of 0.1125 sensitized cells, or 0.1125 normal cells.

(d) The Titration. Titrations were performed in Perspex plates, in 0.5 ml. volumes. A Perspex plate consists of a clear plastic plate, with rows of shallow wells indented over the surface, which will hold 1.0 to 2.0 ml. of fluid.

Culture fluids, or sera, were diluted serially in DBSS in 0.25 ml. volumes, beginning with a 1/5, 1/10, or 1/20 dilution in the first cup.

(i) Direct Hemagglutination Test. In the direct test, 0.25 ml. of normal 0.1125 cells was added to each cup in a series. A control cup was prepared by mixing 0.25 ml. of DBSS with 0.25 ml. of 0.1125 cells.

(ii) Indirect Hemagglutination Test. In the indirect test, 0.25 ml. of sensitized 0.1125 cells was added to each cup in a series, while 0.25 ml. of normal cells was added to a control cup containing 0.25 ml. of the most concentrated dilution of fluid. A second control consisted of 0.25 ml. of sensitized cells mixed with 0.25 ml. of DBSS.

In both tests, a known positive serum, and a known negative serum, were titrated with each group of cells prepared.

Plates were covered with aluminum foil, and placed on a rotary shaker at 190 r.p.m., at 37°C for twenty minutes. At the end of this time, the plates were removed and allowed to stand overnight at room temperature before reading. The titre of a culture fluid, or serum, was expressed as the reciprocal of the highest dilution of fluid at which agglutination could be observed.

3. Preparation of Cytolytic Antibody

Antisera against guinea-pig spleen was prepared in rabbits. Suspensions of guinea-pig spleen cells were centrifuged at 1500 r.p.m. for five minutes, the supernates discarded, and the cell pellets resuspended in a small volume of saline or DBSS, to obtain a turbid suspension cells. Cells prepared in this manner, were either injected immediately into rabbits, or were stored at -18°C and then thawed and injected into rabbits at a later time.

Immunization of rabbits was accomplished by injecting 0.5 ml. of cell suspension into the marginal ear vein of each of three rabbits. Each animal was given two courses of injections. Rabbits were then bled from the marginal ear vein, and the blood collected in sterile containers and allowed to clot at 4°C. The sera were removed, pooled, sterilized by Millipore filtration, and then stored at -18°C.

4. Titration of Cytolytic Antibody Sera

The pooled cytolytic antibody-containing sera were titrated for their lytic activity using a system consisting of: (1) guinea-pig spleen cells, (2) rabbit-produced anti-spleen serum, (3) guinea-pig complement, and (4) Reif cytolysis stain as an indicator of immune cytolysis.

(a) Spleen Cell Suspensions. Spleens were removed from normal guinea-pigs, and a suspension of cells obtained by scraping fragments of tissue across a wire mesh grid as described previously. Cells were collected in 1.5% gelatin in DBSS, and were subjected to a differential centrifugation procedure to remove as many of the red cells as possible. The cell pellet obtained, consisting mainly of white cells, was resuspended in medium 199, pH 7.2 - 7.4, to a final concentration of 1.0 x 10^6 cells/ml. of medium.

(b) Complement. Lyophilized guinea-pig serum, obtained from the Department of National Health and Welfare, Ottawa, Ontario, and reconstituted with glass distilled water, was used as a source of complement. It was diluted 1/30 in DBSS, and then divided into two equal portions. One portion was inactivated in a water bath at 56°C for thirty minutes, while the second portion was kept in an ice water bath.

(c) Anti-spleen Serum. Rabbit-produced antiserum against guinea-pig spleen cells were removed from the freezer, and inactivated at 56°C for thirty minutes. Dilutions of serum were prepared in DBSS, and included dilutions of:

1/50, 1/100, 1/500, 1/1000, 1/5000, and 1/10,000.

(d) The Titration. Two series, of six tubes each, were arranged and labelled series "A" and series "B". To one tube in each series, was added 1.0 ml. of each dilution of serum. Subsequently, 0.25 ml. of cell suspension at a concentration of 1.0×10^6 cells/ml., was added to each tube, and all tubes were then shaken to obtain an even suspension of cells in serum. Both series of tubes were placed in an ice water bath for one hour to allow the cells time to become coated with antibody. At the end of this time, 1.0 ml. of a 1/30 dilution of active complement was added to each tube in series A, while 1.0 ml. of 1/30 inactivated complement was added to each tube in series B. Both sets of tubes were incubated in a water bath at 37°C for one hour to allow complementary lysis to occur. At the end of the incubation period, tubes were plunged into an ice water bath to stop the reaction.

In order to measure the amount of lysis which had occurred, an aliquot of cell suspension was added to an equal volume of Reif cytolysis stain, mixed by pipetting, and a drop of suspension placed on a clean dry slide. A coverslip was laid over the drop, and the slide observed under the microscope. At least ten fields were examined, and the number of stained and unstained cells recorded. The number of lysed (stained) cells was then expressed as a percentage of the total number of cells counted:

$$\frac{\text{number of stained cells}}{\text{total number of cells counted}} \times 100 = \%\ \text{lysed cells}$$

The results of a titration are seen in Table II.

TABLE II

PERCENTAGE OF LYSED SPLEEN CELLS FOLLOWING INCUBATION WITH CYTOLYTIC ANTIBODY AND COMPLEMENT

Dilution of Serum	Series A Active C	Series B Inactive C
1/50	57%	20%
1/100	46%	41%
1/500	25%	19%
1/1000	26%	17%
1/5000	29%	22%
1/10,000	21%	15%
Average		22.3%

Under conditions of the titration, serum dilutions of 1/500 or greater, caused little if any detectable lysis of cells in the presence of complement (series A) when compared with cells which had been treated with inactivated complement (series B). The titre of the cytolytic antibody-containing serum therefore appeared to lie between dilutions of 1/100

and 1/500. Since this method of titration was not considered a very sensitive one, a more accurate determination of the serum titre, as could possibly be achieved by using a finer series of dilutions, was not attempted.

5. Gel Diffusion

The method of gel diffusion adopted for the detection of precipitating antibody, was basically that developed by Ouchterlony (1948). To a solution of barbital-sodium barbital buffer,[10] pH 8-6, Special Noble Agar was added to a final concentration of 1.0%. The mixture was heated to dissolve the agar, and 3.0 ml. of solution pipetted onto the surface of a glass slide. When the agar had solidified, small circular wells were cut in the agar. Eight wells (about 1.0 mm. in diameter) were arranged symmetrically about the periphery of a single central well (about 2.0 mm. in diameter). Culture fluids or sera to be tested, were placed undiluted in the centre well, while different antigens were added to the peripheral wells. Slides were allowed to stand overnight at room temperature, and for another day at 4°C in a moist atmosphere, to allow diffusion and precipitation of antigen and antibody in the agar. Slides were then observed for lines of precipitation.

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[10]Obtained from Buchler Instruments, Fort Lee, New Jersey.

CHAPTER III

PROCEDURES AND RESULTS

CHAPTER III

PROCEDURES AND RESULTS

A. Introduction to the Experimental Work

The ultimate aim of this project was to treat cultures of normal, immunologically competent cells with a cytolytic antibody, in an attempt to stimulate the production of antibody *in vitro*. However, it was felt that a necessary preliminary objective should be the development of a tissue culture system in which cells obtained from animals previously immunized *in vivo*, would produce detectable quantities of antibody *in vitro*. The following chapter therefore contains a description of preliminary experiments, utilizing tissues or cells obtained from previously immunized animals, followed by an account of experiments in which normal cells were treated *in vitro* with cytolytic antibody, in an attempt to stimulate anti-antibody production.

Since the experimental work required frequent modifications of technique, the description of each experiment, or group of experiments, has been arranged under the following headings: (1) Theory, (2) Procedure, and (3) Results.

B. Attempts to Produce Antibody in Monolayer Cultures of Lymph Node, Spleen, and Peritoneal Exudate Cells

THEORY

Initial attempts to produce antibody *in vitro* were made using a modification of the procedure of McKenna and Stevens (1960). These authors had reported the production of antibody *in vitro*, by cultures of guinea-pig peritoneal exudate cells following either *in vivo*, or *in vitro* antigenic stimulation. The method was adapted to the culture of spleen and lymph node cells as well as peritoneal exudate cells, as is described in *Materials and Methods*.

The technique, which entailed the culture of dispersed cells in monolayers in tubes, was chosen because it allowed rapid uniform access of medium to cells, as well as providing a relatively accurate means of enumeration of cells within a culture.

The first group of experiments were designed to test the ability of cells cultured by this method, to produce antibody against sheep or human erythrocytes, when obtained from previously immunized guinea-pigs.

PROCEDURE

Guinea-pigs were immunized with five or six intraperitoneal injections of 0.5 ml. of 10% erythrocytes, of either sheep or human origin. Following a rest period of at least one month, all animals received one or more booster doses of the same antigen, by the intraperitoneal route. Twenty-four hours pervious to sacrifice, 10.0 ml. of 0.001% glycogen was adminis-

tered by the same route. Two days following the last injection of antigen, guinea-pigs were sacrificed with chloroform, or by cardiac exsanguination. The spleen, mesenteric lymph nodes, and peritoneal exudate cells were removed and prepared for culture according to the technique outlined in Materials and Methods for monolayer cultures. Cells were seeded in Leighton tubes in medium 199 with 25% NGPS (frozen), and incubated at 37°C. At various intervals, old medium was withdrawn, and fresh medium added in its place. Culture fluids were then titrated for antibody against sheep or human erythrocytes using the direct hemagglutination test. An aliquot of each lot of fresh culture medium was retained and titrated separately for any natural hemagglutinating activity which might reside in the serum fraction of the medium.

RESULTS

The results of three such experiments are outlined in Table III. No antibody was detected in any of the culture fluids of any of these experiments. This failure occurred in spite of the fact that titres of 2048, >256, and >65,536, were obtained in titrations of the post-immunization sera of the same animals from which the cells were obtained, 218, 220, and 216 respectively. It therefore seemed apparent that the failure of cultures to produce detectable quantities of antibody, could not be attributed to a failure of the guinea-pigs to respond to immunization.

TABLE III

HA TITRES IN CULTURE FLUIDS OF SPLEEN, LYMPH NODE AND PERITONEAL EXUDATE CELLS FROM IMMUNIZED GUINEA-PIGS

Exp.	Tissue	Cell conc./ml.	Days in culture		
			1 - 5	5 - 9	9 - 14
218	Spleen	$6.0x10^6$	< 2 < 2 < 2 < 2 < 2 < 2 < 2	< 2 < 2 < 2 < 2 < 2 < 2 < 2	< 2 < 2 < 2 < 2 < 2 < 2 < 2
	Lymph node	$1.8x10^6$	< 2 < 2 < 2	< 2 < 2 < 2	< 2 < 2 < 2
	PEC*	$2.7x10^6$	< 2 < 2 < 2	< 2 < 2 < 2	< 2 < 2 < 2
			6 - 9	9 - 13	
220	Spleen	$8.0x10^6$	< 2 < 2 < 2 < 2 < 2 < 2	< 2 < 2 < 2 < 2 < 2 < 2	
	Lymph node	$5.0x10^6$	< 2 < 2	< 2 < 2	
	PEC	$3.3x10^6$	< 2	< 2	
			1 - 4	4 - 7	7 - 10
216	Spleen	$4.0x10^6$	< 2 < 2 < 2	< 2 < 2 < 2	< 2 < 2 < 2
	Lymph node	$8.4x10^6$	< 2 < 2	< 2 < 2	< 2 < 2

*peritoneal exudate cells

C. Attempts to Stimulate Antibody Production by Increasing the Oxygen Tension

THEORY

Since the above described failure could not be explained as a failure of the animals to respond to immunization, it seemed likely that the defect might lie in the culture system itself. Steiner and Anker (1956) had suggested that individual cells, suspended in medium, required a high oxygen tension to support optimal cell metabolism and antibody production. Accordingly, an experiment was designed to test the effect of increased oxygen content in the atmosphere, upon the ability of our cultures to produce antibody. For this purpose, a number of cultures were incubated in an atmosphere of 95% O_2 : 5% CO_2, while the remainder were incubated as usual in air.

PROCEDURE

Guinea-pig 219 was immunized with six injections of 0.5 ml. of 10% human erythrocytes. Four months later, two more intraperitoneal injections of the same amount were given as booster doses, spaced two days apart. Three days after the last injection, the animal was killed, the spleen removed, and prepared for culture in the usual manner. Spleen cells were seeded in Leighton or roller tubes, in medium 199 with 25% NGPS (frozen) at a concentration of 3.0×10^6 cells/ml.

Control cultures consisted of cells which were killed

by heating at 56°C for thirty minutes, and then incubated along with other cultures at 37°C. Five cultures were incubated in 95% O_2 : 5% CO_2, while an equal number were incubated in air. Medium was replaced after five, ten, and sixteen days, and culture fluids titrated for antibody against human erythrocytes using the method of direct hemagglutination.

RESULTS

The results of titrations may be seen in Table IV. No antibody was detected in culture fluids obtained after the first ten days of incubation. However, media from days eleven to seventeen, caused a type of agglutination of erythrocytes at dilutions of 1/1600 or greater. The same type of agglutination, at comparable dilutions, was also observed in culture fluids of heat-killed cells. A second notable feature of these titrations was the fact that the type of agglutination observed differed from that which was normally seen in similar titrations of antibody containing sera: agglutinated cells rapidly underwent complete dispersal upon gentle shaking of the plates. Unfortunately, there was not sufficient culture fluid left to permit further investigation of the hemagglutinating activity detected. In view of the factors mentioned, it was concluded that production of specific antibody *in vitro* had not occurred. As a result, it was difficult to assess the effect of increased oxygen tension upon cells,

TABLE IV

HA TITRES IN CULTURE FLUIDS OF SPLEEN CELLS AFTER INCUBATION IN $95\%O_2 : 5\%CO_2$

Tubes	Atmosphere	Days in Culture		
		1 - 6	6 - 11	11 - 17
Leighton	$95\%O_2 : 5\%CO_2$	< 2 < 2 < 2	< 2 < 2 < 2	> 256 > 1600 > 800
Leighton	Air	< 2 < 2 < 2	< 2 < 2 < 2	64 > 256 > 256
Roller	$95\%O_2 : 5\%CO_2$	< 2 < 2	< 2 < 2	> 256 > 256
Roller	Air	< 2 < 2	< 2 < 2	64 128
Culture media		< 2	< 2	< 2
Heated controls: Leighton	$95\%O_2 : 5\%CO_2$	< 2 < 2	< 2 < 2	> 1600 > 256

other than to conclude that this at least was not the only factor needed to promote antibody production in these cultures. Nevertheless, the procedure of incubating in $95\%O_2 : 5\%CO_2$, was adopted in future experiments.

D. Attempts to Stimulate Antibody Production *IN VITRO* by the Addition of Cortisone Derivatives to Media

THEORY

Throughout the experimental work so far, normal guinea-pig serum had been added at a concentration of 25% to medium 199. Such serum, obtained by cardiac puncture of normal guinea-pigs, had been stored in a freezer at -18°C for periods of time extending up to several months. However, Ambrose (1964) has reported that certain cortisone derivatives, in "physiological amounts", were capable of replacing serum as a supplement to basic media for the purpose of supporting antibody production *in vitro*. Since cortisone and its derivatives are relatively labile substances, it was thought that these hormones, normally present in small amounts in freshly drawn serum, might be destroyed by the procedure adopted for the storage of serum. If these hormones were a necessary factor in the production of antibody, their absence in serum might possibly account for the failure of our cultures to produce antibody. The following experiments were designed to test this hypothesis.

For this purpose, hydrocortisone sodium hemisuccinate, and cortisone acetate were incorporated into medium 199 at concentrations of 0.1, and 1.0μg./ml. Both of these substances had been utilized successfully by Ambrose, and the concentrations

selected were within the range at which maximal enhancement of antibody production had been reported. A third group of cultures were incubated in medium containing 25% NGPS in order that the effects of cortisone and serum might be compared.

PROCEDURE

Guinea-pigs 211 and 212 were given a series of six injections of 0.5 ml. of 10% sheep erythrocytes suspended in saline. Five months later, booster doses of 0.1% erythrocytes were given each animal, 0.5 ml. by the intraperitoneal route, and 0.2 ml. by the intravenous route. Twenty-four hours later, spleen and mesenteric lymph nodes were removed and prepared for culture. Cell suspensions obtained from both animals were pooled in media containing one of the following:

(1) 25% NGPS (lyophilized)

(2) hydrocortisone sodium hemisuccinate, 0.1μg./ml.

(3) hydrocortisone sodium hemisuccinate, 1.0μg./ml.

(4) cortisone acetate, 0.1μg./ml.

(5) cortisone acetate, 1.0μg./ml.

Cells were seeded in Leighton tubes, at concentrations of 4.0×10^6, and 6.0×10^6 cells/ml. for spleen, and lymph node cells respectively. Tubes were incubated at 37°C in $95\%O_2 : 5\%CO_2$. Medium was removed after four, nine, and nineteen days of incubation, and titrated for antibody against

sheep erythrocytes using the direct hemagglutination test.

Control cultures, which consisted of cultures heated to 63°C for thirty minutes, were incubated at the same time, and the media of these cultures titrated by the same method.

RESULTS

The results of titrations may be seen in Table V. No antibody was detected in any of the culture fluids of either the experimental group, or of the control group (not shown in Table V). However, sera obtained from the same animals just prior to sacrifice showed antibody titres of 80 and 320. Apparently, the failure of cultures was not due to lack of an immune response by the animals.

In two other similar experiments, cultures also had failed to produce antibody, but in these instances, antibody was not detected in sera obtained from the donor animals. Since it appeared that the animals had not responded to immunization, the details of these experiments have not been reported.

E. Viability Studies of Cells in Culture

The reason for the failure of cultures to produce antibody was not apparent. However, it began to appear that the difficulties encountered stemmed from a more basic defect in the culture technique, than a simple deficiency in the culture medium, or an insufficient supply of oxygen. Attention

TABLE V

HA TITRES OF CULTURE FLUIDS OF SPLEEN AND LYMPH NODE CELLS CULTURES IN MEDIA CONTAINING CORTISONE DERIVATIVES

Tissue	Supplement	Conc.	No. of tubes	Days in Cultures		
				1 - 5	5 - 10	10 - 19
Spleen	Na hydrocort. hemisuccinate	0.1 μg/ml.	4	< 5	< 20	< 20
		1.0 μg/ml.	4	< 5	< 20	< 20
	Cortisone acetate	0.1 μg/ml.	4	< 5	< 20	< 20
		1.0 μg/ml.	4	< 5	< 20	< 20
	NGPS	25%	4	< 5	< 20	< 20
Lymph node	Na hydrocort. hemisuccinate	0.1 μg/ml.	2	< 5	< 20	< 20
		1.0 μg/ml.	2	< 5	< 20	< 20
	Cortisone acetate	0.1 μg/ml.	2	< 5	< 20	< 20
		1.0 μg/ml.	2	< 5	< 20	< 20
	NGPS	25%	2	< 5	< 20	< 20

was therefore drawn to the cultures themselves, for a possible solution to the problem.

It had been observed that during the culture period, cells began sticking to the glass after two or three hours of incubation, and continued doing so, to form a type of monolayer on the inside of the glass tube. Many of the cells continued to adhere to glass throughout the entire culture period. Observations of cells under the light microscope revealed what appeared to be normal, healthy cells with clearly defined margins. After forty-eight hours, spleen cells became less sharply defined, and many of the cells appeared to undergo a process of fragmentation and disintegration. Lymph node cells however, appeared to remain in good condition for several days, and continued to do so for as long as twenty days.

It was also observed that when cells were cultured in media supplemented by cortisone derivatives, they adhered to the glass in greater numbers, and appeared to remain healthier than those cells cultured in serum-containing media.

The following experiments were therefore designed in an attempt to gain further information concerning the health and viability of cells during the culture period. Spleen and lymph node cells, obtained from normal animals, were cultured in the usual fashion, and after various time intervals, cells were removed and examined using Reif cytolysis stain as an

indicator of cell viability.

The use of peritoneal exudates was discontinued because of the frequent occurrence of bloody exudates, and the limited number of cells which could be obtained from this source.

PROCEDURE

Normal guinea-pigs were sacrificed, and the spleens and/or mesenteric lymph nodes removed and cultured in the usual manner. Media, either 199, or Eagle's MEM, contained hydrocortisone sodium hemisuccinate or cortisone acetate at a concentration of 1.0µg./ml. or 25% NGPS (inactivated at 56°C for thirty minutes). After various intervals of incubation, aliquots of cell suspension were removed from each culture, mixed with an equal volume of Reif Cytolysis stain, and examined under the microscope. The number of stained and unstained cells was recorded, and from these figures, the percentage of viable (unstained) cells was calculated.

RESULTS

The results of three such experiments are seen in Table VI. In all cases, the percentage of viable cells had dropped considerably after forty-eight hours of incubation to low, and in many instances, negligible numbers. In spite of the fact that lymph node cells stuck to the glass in greater numbers, and in all cases appeared to be in a healthier state than similar cultures of spleen cells, the

TABLE VI

% VIABILITY OF SPLEEN AND LYMPH NODE CELLS CULTIVATED IN MONOLAYERS

Medium	Tissue	Cell conc./ml.	Incubation time in hrs.		
			0	24	48
199 + Na hydrocort. succinate, 1.0 µg./ml.	Spleen	1.0×10^7	78	-	16
		1.0×10^7	78	-	14
	Lymph node	5.0×10^6	72	-	0-5
199 + Cortisone acetate, 1.0 µg./ml.	Spleen	1.0×10^7	78	-	25
		1.0×10^7	78	-	28
		1.0×10^7	71	59	0-5
		1.0×10^7	71	36	0-5
	Lymph node	5.3×10^6	72	-	0-5
199 + 25% NGPS (inactivated)	Spleen	1.0×10^7	78	-	34
		1.0×10^7	78	-	30
	Lymph node	6.0×10^6	72	-	14
Eagle's MEM + Cortisone acetate, 1.0 µg./ml.	Lymph node	1.0×10^7	56	5	0-5
		1.0×10^7	56	5	0-5
		1.0×10^7	56	5	0-5

latter cells continued to remain viable for longer periods of time in all media tested.

Similarly, although media containing cortisone derivatives appeared to promote greater sticking of cells to glass than similar serum-containing media, the latter appeared to maintain a greater number of cells in a viable state than the former. In two cultures containing serum-supplemented media, as many as 30% of the spleen cells remained viable after forty-eight hours of incubation, in contrast to the consistently lower values obtained in cultures in which cortisone derivatives were present.

However, since in most cases the number of healthy cells in a culture after forty-eight hours of incubation had fallen below 25%, it appeared that the technique was inadequate for the maintenance of cells in a functional state over extended periods of time. Consequently, this method of cell culture was discarded and future efforts directed towards the establishment of a more suitable method of cell culture in which antibody production might be demonstrated.

F. Attempts to Grow Spleen Cells on Cellophane Membranes

THEORY

Steiner and Anker (1956) had developed a culture method which they reported was capable of maintaining spleen

cells in a viable state for at least six days, and which would support detectable production of antibody by cells obtained from animals previously immunized *in vivo*. Spleen cells were cultivated in a thin layer of medium, about 1.0 mm. deep, on the surface of a cellophane membrane which was stretched tightly over the surface of a reservoir of medium. The medium in the reservoir was kept in a state of constant motion by a rotating magnet placed in the bottom of the vessel. Fluid could readily be withdrawn from either the lower compartment containing medium alone, or from the upper compartment containing cells on the surface of the membrane. The upper chamber was aerated by a continuous flow of 95% O_2 : 5% CO_2.

The cellophane membrane allowed passage of small molecular weight compounds such as amino acids, from one compartment to the other, while larger molecules such as γ-globulin were excluded.

The success of this method was attributed by these authors, to the large supplies of nutrient substances and oxygen which were available to cells, the rapid removal (by dilution) of waste products, and the protection of cells from agitation which might lead to cell damage.

It therefore seemed desirable to culture spleen cells in a manner analogous to the one just described. In the following experiments, an attempt was made to incorporate

some of these principles into a simpler type of culture apparatus.

PROCEDURE

A make-shift apparatus was devised as pictured in Fig. 3. It consisted of a cellophane membrane held taughtly between two concentric, stainless steel water bath rings, which was placed over the surface of a small Petri dish filled with medium 199. The medium contained 1.0 µg. ml. of cortisone acetate, and 0.7% Difco Bacto-agar. The addition of agar was necessary to impart a degree of solidity to the medium, such that it could support the membrane in a completely horizontal position just touching the medium at the surface of the dish.

Spleen cells were suspended in medium 199 with or without 25% NOPS (fresh), and 2.0 ml. of suspension pipetted onto the surface of the membrane. The culture was placed within a larger Petri dish which contained a moistened ball of cotton wool, and the entire apparatus incubated in 95% O_2 : 5% CO_2 at 37°C.

In one culture, the cellophane membrane was omitted and cells layered directly onto the surface of the agar-medium mixture, while in another culture the agar-medium mixture and the membrane were both omitted, and cells pipetted into the bottom of a glass Petri dish.

After various intervals, cells were withdrawn and the

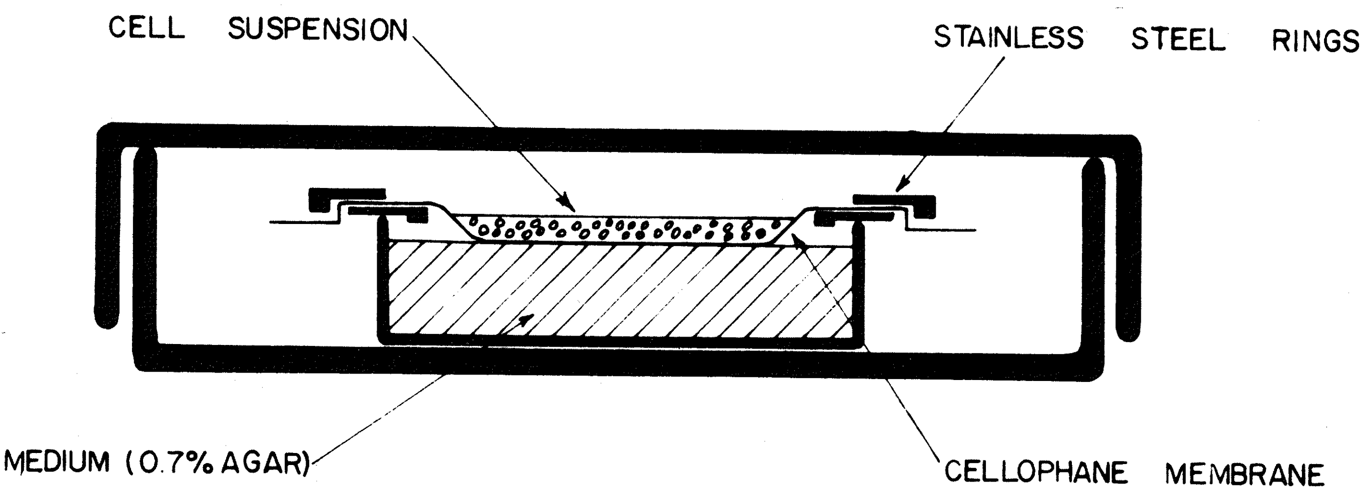

FIG. 3 CELLOPHANE MEMBRANE CULTURE

percentage of viable cells estimated using the Reif dye-exclusion technique as a measure of cell viability.

RESULTS

The results of these experiments are seen in Table VII. The number of viable cells fell to 10% or less in all cultures after forty-eight hours of incubation. The inclusion of fresh serum in the medium appeared to have very little, if any, effect upon prolonging the life span of cells cultured in such an apparatus. From the results of these experiments, it appeared that this apparatus was not an adequate substitute for the one used by Steiner and Anker. Therefore, since this method afforded no obvious advantages, its use was discontinued.

G. Attempts to Produce Antibody Against Sheep Erythrocytes in Cultures of Spleen Fragments Obtained from Immunized Animals

THEORY

Both the monolayer, and cellophane membrane techniques had proved to be of little value in the culture of spleen and lymph node cells, except for periods of short duration. The next experiments were therefore designed to test a third method of culture in which fragments of tissue were embedded in homologoud plasma clots, covered with medium, and incubated in a roller drum. Michaelides and Coons (1963) had used this method successfully in the production of antibody *in vitro* by fragments of

TABLE VII

PERCENTAGE VIABILITY OF SPLEEN CELLS GROWN ON A CELLOPHANE MEMBRANE

Agar-medium mixture	Cellophane membrane	Cell conc./ml.	Medium Suppl.	Hours of incubation					
				0	1	6	24	48	72
+	+	4.4×10^6	25% NGPS	65	-	-	30	10	0-5
+	+	4.4×10^6	25% NGPS	65	-	-	40	4	0-5
+	+	1.1×10^7	-	65	66	-	44	0-5	
+	+	1.1×10^7	-	65	-	-	-	0-5	
+	-	1.1×10^7	-	65	-	44	50	0-5	
-	-	1.1×10^7	-	65	42	-	-	0-5	

rabbit lymph node.

The details of this method have been outlined in *Materials and Methods*. Because of the very small size of guinea-pig lymph nodes, it was deemed impracticable to attempt culturing this tissue by the fragment technique. Consequently, the remainder of the experimental work has been done using fragments of guinea-pig spleen. Initial attempts to produce antibody in cultures of spleen fragments were made using tissue obtained from animals previously immunized with sheep erythrocytes *in vivo*.

PROCEDURE

Guinea-pigs 271, 273, and 274 were given a single injection of 0.5 ml. of 10% sheep erythrocytes by the intraperitoneal route. Approximately three months later, each animal received a booster injection by the method described above. Three days after the last injection of antigen, animals were sacrificed and their spleens removed. Tissues were cut into fragments and embedded in homologous plasma clots as described in *Materials and Methods*. Each culture received 1.0 ml. of Eagle's MEM with 20% NGPS (fresh). All tubes were incubated in a roller drum (1/5 r.p.m.) at 37°C. After six, nine and twelve days, medium was withdrawn and titrated for antibody against sheep erythrocytes by the direct hemagglutination method.

In each of these three experiments, a control culture,

consisting of heat-killed cells (56°C for thirty minutes), was incubated along with other cultures at 37°C.

RESULTS

The results of titrations are shown in Table VIII. In one experiment (273), culture fluids showed agglutinating activity against sheep erythrocytes at dilutions of 1/20 or 1/40. This activity was detected in five out of five cultures, while it was not found in titrations of the culture fluids of heat-killed cells, or of culture medium which had not been incubated with tissue. Activity was detected in culture fluids after the first six days of incubation, but was not observed after that time. It appeared therefore, that small amounts of hemagglutinin had been produced *in vitro* by fragments of immunized spleen, and that its production was dependent upon the presence of intact cells in the culture.

In two other experiments (271 and 274), cultures failed to produce appreciable amounts of antibody. This was correlated with antibody titres of 5, and 10, in sera obtained from the same animals. Although serum obtained from guinea-pig 273 contained antibody only to a titre of 80, it appeared that even this seemingly small amount of antibody in the circulation was indicative of significantly greater immune response by this animal than by either of the other two, since, in the case of guinea-pig 273, a serum titre of 80 was associated with production of antibody by fragments of spleen cultured *in vitro*.

TABLE VIII

HA TITRES OF CULTURE FLUIDS OF SPLEEN FRAGMENTS
OBTAINED FROM ANIMALS IMMUNIZED
WITH SHEEP ERYTHROCYTES

Expt. number	Tube number	Days in Culture		
		1 - 6	6 - 9	9 - 12
271	1	5	< 5	< 5
	2	5	< 5	< 5
	3	5	< 5	< 5
	4	< 5	< 5	< 5
	5	< 5	< 5	< 5
	Heated control	< 5	< 5	< 5
Post-immun. serum titre: 5	Medium alone	< 5	< 5	< 5
273	1	40	< 10	< 10
	2	40	< 10	< 10
	3	40	< 10	< 10
	4	20	< 10	< 10
	5	40	< 10	< 10
	Heated control	< 10	< 10	< 10
Post-immun. serum titre: 80	Medium alone	< 10	< 10	< 10
		1 - 6	6 - 12	12 - 16
274	1	< 10	< 10	< 10
	2	< 10	< 10	< 10
	3	< 10	< 10	< 10
	4	< 10	< 10	< 10
	5	< 10	< 10	< 10
	Heated control	< 10	< 10	< 10
Post-immun. serum titre: 10	Medium alone	< 10	< 10	< 10

H. Attempts to Produce Antibody Against RGG in Cultures of Spleen Fragments Obtained from Immunized Animals

THEORY

In the previous experiment, production of an antibody-like substance had been demonstrated in cultures of spleen fragments obtained from animals immunized by the intraperitoneal route. The titres of antibody detected in culture fluids were low. However, it is believed that maximal production of antibody by the spleen occurs following intravenous immunization, as has already been discussed in the Review of the Literature. Therefore, in the next group of experiments, animals were immunized by the intravenous route in an attempt to increase the amounts of antibody produced by fragments of spleen cultivated *in vitro*.

PROCEDURE

Guinea-pigs 360, 361, and 365 were given five biweekly injections of 0.5 ml. of 1.0% RGG in the marginal ear vein. After a rest period of approximately one month, each animal received three booster doses of the same amount of antigen by the intravenous route. Three days after the last injection, animals were killed, and the spleens removed. Fragments of tissue were embedded in homologous plasma clots in Eagle's MEM with 20% NGPS (fresh). Culture fluids, removed after various intervals, were titrated for antibody against rabbit γ-globulin using the indirect hemagglutination technique

described in Materials and Methods.

RESULTS

Antibody could not be detected in the culture fluids in any of these experiments. Similarly, titration of post-immunization sera obtained from these animals failed to reveal the presence of antibody. Failure of cultures to produce antibody was therefore attributed to a lack of response by the animals to immunization.

I. Attempts to Produce Antibody Against RGG in Cultures of Spleen Fragments Obtained from Animals Immunized with Sensitized Cells

THEORY

Since the intravenous injection of rabbit γ-globulin had failed to stimulate the production of detectable antibody either *in vivo* or *in vitro*, the next experiments utilized animals which had already been immunized with guinea-pig red cells sensitized with a rabbit-produced haemolysin. Since the haemolysin is itself composed of rabbit γ-globulin, such sensitized cells will stimulate the production of antibody against rabbit γ-globulin. By injecting the antigen (RGG) on the surface of particulate matter (guinea-pig red cells), it was hoped that a greater immune response might be achieved than by the injection of rabbit γ-globulin alone.

PROCEDURE

Guinea-pigs 372 and 373 had previously been given two courses of intraperitoneal injections of guinea-pig erythrocytes sensitized with rabbit-produced haemolysin. One week later, two booster doses of the same antigen were given each animal intraperitoneally. Three days after the last injection of antigen, spleens were removed and cultured in the usual fashion. One tube containing heat-killed cells (56°C. for thirty minutes), and one tube containing plasma and medium without tissue fragments, were included in both experiments. Culture fluids removed after five, eleven, and fifteen days, were titrated for antibody against rabbit γ-globulin using the indirect hemagglutination technique.

RESULTS

The results of these two experiments are shown in Table IX. A substance was detected in culture fluids in both experiments which caused agglutination of erythrocytes sensitized with rabbit-produced haemolysin. This substance could be detected at dilutions as high as 1/80, in two cases. Its presence was detected in culture fluids obtained after the first eleven days of incubation, and after that time was absent. Production of an agglutinating substance could not be demonstrated in the culture fluids of either the heat-killed cells, or plasma and medium incubated without tissue fragments. Similarly, titration of each lot of culture medium failed to reveal the presence of antibody. It was therefore

TABLE IX

HA TITRES OF CULTURE FLUIDS OF SPLEEN FRAGMENTS
OBTAINED FROM ANIMALS IMMUNIZED
WITH SENSITIZED CELLS

Expt. number	Tube number	Days in Culture		
		1 - 5	5 - 11	11 - 15
373	1	10	10	< 10
	2	20	10	< 10
	3	20	< 10	< 10
	4	10	< 10	< 10
	5	10	10	< 10
	Plasma + medium	< 10	< 10	< 10
	Medium alone	< 10	< 10	< 10
Post-immun. serum titre: 40,960	Heated control	< 10	< 10	< 10
372	1	80	20	< 10
	2	80	10	< 10
	3	20	10	< 10
	4	40	10	< 10
	5	40	10	< 10
	Plasma + medium	< 10	< 10	< 10
	Medium alone	< 10	< 10	< 10
Post-immun. serum titre: 81,920	Heated control	< 10	< 10	< 10

concluded that the appearance of an antibody-like substance had occurred during the incubation period, and that its appearance in culture fluids was dependent upon the presence of viable cells in the culture.

In both experiments, examination of post-immunization sera revealed the presence of antibody in titres of 40,000 or greater. The reason for the apparent inconsistency between the titres of sera and culture fluids was not obvious. It may be partially explained by the fact that the antigen had been administered by the intraperitoneal route in these experiments. Under these conditions, one might expect the major sites of antibody formation to be in the omentum or in various groups of lymph nodes draining the peritoneal cavity.

In order to test the specificity of the substance which had been detected, two culture fluids were selected from each experiment, and titrated using a hemagglutination-inhibition test.

PROCEDURE

Culture fluids from tubes "1" and "2" in experiment 372, and tubes "2" and "3" in experiment 373, were selected for hemagglutination-inhibition tests since these culture fluids had shown greatest agglutination activity against sensitized cells in each of these two experiments.

The titrations were performed in triplicate in essentially the same manner as the indirect hemagglutination

tests, with the following modifications: rabbit γ-globulin, or human γ-globulin, were first dissolved in the standard diluent (DBSS)to a final concentration of 0.5 mg./ml. Culture fluids were diluted serially in each of the above, and incubated at 37°C. for ten minutes on a rotary shaker at 190 r.p.m., then removed from the shaker and incubated an additional fifty minutes at 37°C. A third group of titrations were performed in the same manner using DBSS alone as the diluent. Thereafter, sensitized cells were added to each dilution of culture fluid, and the test carried out in exactly the same fashion as the indirect hemagglutination test described in Materials and Methods.

RESULTS

The results of inhibition tests may be seen in Table X. Agglutination of sensitized cells was inhibited by the incorporation of the homologous antigen (rabbit γ-globulin) in the diluent, but not by the presence of an heterologous antigen (human γ-globulin). This suggested that the substance present in culture fluids was a specific antibody against rabbit γ-globulin.

In order to gain further information regarding the nature of this substance, an attempt was made to demonstrate its presence using a gel diffusion technique.

PROCEDURE

The details of the gel diffusion technique are

described in Materials and Methods. Culture fluids were placed in the centre well, while the following antigens were added to the peripheral wells: (1) 1.0% rabbit γ-globulin, (2) 1.0% human γ-globulin, (3) undiluted horse serum, (4) undiluted, rabbit-produced, anti-guinea-pig spleen serum, (5) undiluted guinea-pig serum.

TABLE X

HA TITRES OF HEMAGGLUTINATION - INHIBITION TESTS OF CULTURE FLUIDS CONTAINING ANTIBODY AGAINST RGG

Expt. number	Tube number	Diluent		
		DBSS	RGG	HGG*
372	1	40	<10	20
	2	20	<10	40
373	2	40	<10	80
	3	20	<10	40

*human γ-globulin

Culture fluids from tubes "1" and "2" in experiment 372, and tubes "2" and "3" in experiment 373, were assayed by this method. A known positive serum (373) was included in the test. This serum had a titre of 81,920, when tested by the indirect hemagglutination test. In order to determine whether the gel diffusion technique was sensitive enough to

detect antibody in amounts comparable to that present in the culture fluids to be tested, the positive control serum was also tested at a dilution of 1/1000. At this dilution, the serum contained approximately the same amount of agglutinating activity as the most strongly positive of the culture fluids to be assayed.

RESULTS

None of the four culture fluids tested precipitated with any of the five antigens. The undiluted positive control serum (373) produced a single line of precipitation with 1.0% rabbit γ-globulin, and two precipitation lines with normal rabbit serum and rabbit-produced anti-guinea-pig spleen serum. However, when the same serum was diluted 1/1000, no precipitation could be detected with any of the antigens tested. Therefore, if the agglutinating activity of a serum or culture fluid can be considered an indicator of the precipitating activity of the same fluid, it may be concluded that the gel diffusion method was not sensitive enough to detect any precipitating antibody which might have been present in those culture fluids tested.

J. Attempts to Initiate a Primary Immune Response IN VITRO using Cytolytic Antibody as a Stimulus

THEORY

It had been demonstrated in the previous experiments

that the fragment culture technique could support small amounts of antibody production by fragments of guinea-pig spleen taken from immunized animals. The next undertaking was to determine whether or not a rabbit-produced anti-serum against spleen cells, when added to fragments of normal spleen, would stimulate production of antibody against rabbit γ-globulin *in vitro*. For this purpose, one group of cultures was exposed to different dilutions of cytolytic antibody-containing serum, while a second group of cultures was exposed to the same dilutions of a normal rabbit serum.

PROCEDURE

Normal guinea-pigs were killed by cardiac exsanguination, their spleens removed, and fragments of tissue prepared for culture in the usual fashion. Fragments from one or more spleens were pooled, and embedded in homologous plasma clots.

Rabbit-produced, anti-spleen serum (cytolytic antibody), and normal rabbit serum, were diluted in HBSS to final concentrations of: 1/25, 1/50, 1/100, 1/500, and 1/1000.

Two tubes of spleen fragments embedded in plasma, were treated with each dilution of the cytolytic antibody-containing serum, while control cultures were treated with the same dilutions of normal rabbit serum (NRS). Each culture was incubated with 1.0 ml. of the appropriate dilution of serum at 37° C. for two hours, to allow time for exposure of all cells with-

in a fragment, to serum. Fluids were then withdrawn, and cultures washed once with fresh HBSS to remove residual serum. All tubes then received 1.0 ml. of Eagle's MEM with 20% NGPS (fresh) and were incubated in a roller drum (1/5 r.p.m.) at 37°C.

Culture fluids, removed after various intervals of incubation, were titrated for antibody against rabbit γ-globulin using the indirect hemagglutination test.

RESULTS

The results of one of three such experiments are shown in Table XI. In this particular experiment, a substance was detected in culture fluids which caused agglutination of sensitized cells, at dilutions as high as 1/320, or 1/640. This substance was present in cultures which had been treated with either the rabbit-produced anti-spleen serum, or with normal rabbit serum. It also appeared that a 1/100 dilution of serum was the maximal concentration of serum which would stimulate production of this substance, since treatment of cultures with more concentrated sera, failed to elicit a responce. Peak titres of agglutination were observed in culture fluids after the first four days of incubation, after which time, activity rapidly diminished to low or negligible levels. Titrations of culture media failed to demonstrate the presence of natural agglutinating substances within the serum fraction of media, which might account for the agglutination titres

TABLE XI

HA TITRES OF CULTURE FLUIDS OF NORMAL SPLEEN FRAGMENTS TREATED WITH CYTOLYTIC ANTIBODY OR NRS IN VITRO

Cytolytic Antibody						NRS					
Tube No.	Recip. of Serum dil'n	Days in Culture				Tube No.	Recip. of Serum dil'n	Days in Culture			
		1-4	4-7	7-12	11-14			1-4	4-7	7-12	11-14
F-1	25	<20	<20	<20	20	F-11	25	<20	<20	20	20
F-2	25	<20	<20	<20	20	F-12	25	<20	<20	20	20
F-3	50	20	<20	40	20	F-13	50	<20	<20	<20	20
F-4	50	<20	<20	40	20	F-14	50	<20	<20	<20	20
F-5	100	160	40	20	20	F-15	100	320	40	20	20
F-6	100	160	40	20	20	F-16	100	80	40	<20	<20
F-7	500	320	80	40	20	F-17	500	320	40	20	20
F-8	500	320	80	40	20	F-18	500	160	80	20	20
F-9	1000	320	80	20	20	F-19	1000	320	80	40	20
F-10	1000	320	-	-	-	F-20	1000	640	80	20	20

observed in positive culture fluids. However, when culture fluids were again titrated following approximately five months storage at -18°C, the activity previously observed had almost completely disappeared. In the earlier titration, culture fluids had been stored for a period of approximately two weeks prior to titration.

In two similar experiments (not shown in the table), no antibody could be detected in culture fluids of either the cytolytic antibody, or the normal rabbit serum group.

K. Further Attempts to Stimulate Antibody Production, with Cytolytic Antibody, in Cultures of Normal Spleen Fragments

THEORY

In one instance out of three in the previous group of experiments, a substance had been produced in cultures of normal spleen fragments treated with either cytolytic antibody or normal rabbit serum, which caused agglutination of sensitized cells. The following experiments were therefore performed in an attempt to reproduce the results of this last experiment. It was also hoped that an optimal concentration of serum necessary to elicit such a response, might be established. Accordingly the range of dilutions of cytolytic antibody-containing serum was extended beyond that of the previous experiments. Control cultures, consisting of fragments of spleen treated with HBSS alone, and heat-killed cells treated with each

dilution of serum, were included in an attempt to establish further, the nature of the stimulant which had been observed in the previous experiment. In the following experiment, fragments of spleen were incubated with serum prior to the embedding of tissue in plasma. It was hoped that such a procedure might allow greater penetration of serum into fragments.

PROCEDURE

Normal guinea-pigs were sacrificed by cardiac exsanguination, and their spleens removed and cut into fragments. Fragments from one or more spleens were pooled, and incubated in either the cytolytic antibody serum, or the normal rabbit serum, at dilutions of 1/25, 1/100, 1/800, 1/5600, in HBSS. In two experiments, fragments of tissue were placed in roller tubes containing 2.0 ml. of one of the above fluids, and incubated in a roller drum (1/5 r.p.m.) for two hours at 37°C. In two other experiments, fragments were placed in about 10.0 ml. of fluid in a glass Petri dish, and incubated at 37°C for two hours. Following the incubation period, fragments were washed once with fresh HBSS, and then embedded in homologous plasma clots, covered with 1.0 ml. of Eagle's MEM with 20% NGPS (fresh), and incubated in a roller drum at 37° C. Control cultures, included in each group, were heated at 56°C for thirty minutes and then incubated along with other cultures at 37° C. Culture fluids were withdrawn after various periods of incubation and titrated in the same manner as before.

RESULTS

Table XII illustrates the results of one of four such experiments. Antibody could not be detected in the culture fluids of any of four experiments. Such culture fluids were stored at -18°C for a period of one or two months before they were titrated. It is not known whether this procedure might result in the destruction of agglutinating activity in such culture fluids. However, storage of antibody-containing sera for similar or longer periods of time, did not result in appreciable reduction of anti-antibody titres (McIllmurray, H., 1965).

L. Histology of Spleen Fragments Cultured in Homologous Plasma Clots

THEORY

Observations of spleen fragments cultured in plasma clots, revealed the migration of cells outwards from fragments, into the surrounding layer of plasma. This was coupled with the gradual digestion of the fibrin clot surrounding a fragment. After approximately six days of incubation, many cells appeared to have developed into fibroblasts. In some cultures, many cells resembling giant-cells could be observed.

In order to gain more information about the nature of cells within fragments, studies were made of sections of tissue after various periods of incubation.

TABLE XII

HA TITRES OF CULTURE FLUIDS OF NORMAL SPLEEN FRAGMENTS TREATED WITH CYTOLYTIC ANTIBODY OR WITH NRS IN VITRO

Serum	Tube No.	Recip. of serum dilution	Heated (56°C, 30 mins.)	Days in Culture	
				1 - 5	5 - 9
Cytolytic antibody	P-1	25	+	< 10	< 10
	P-2	25	-	< 10	< 10
	P-3	25	-	< 10	< 10
	P-4	100	+	< 10	< 10
	P-5	100	-	< 10	< 10
	P-6	100	-	< 10	< 10
	P-7	800	+	< 10	< 10
	P-8	800	-	< 10	< 10
	P-9	800	-	< 10	< 10
	P-10	5600	+	< 10	< 10
	P-11	5600	-	< 10	< 10
	P-12	5600	-	< 10	< 10
NRS	P-13	25	+	< 10	< 10
	P-14	25	-	< 10	< 10
	P-15	25	-	< 10	< 10
	P-16	100	+	< 10	< 10
	P-17	100	-	< 10	< 10
	P-18	100	-	< 10	< 10
	P-19	800	+	< 10	< 10
	P-20	800	-	< 10	< 10
	P-21	800	-	< 10	< 10
	P-22	5600	+	< 10	< 10
	P-23	5600	-	< 10	< 10
	P-24	5600	-	< 10	< 10
HBSS	P-25	-	+	< 10	< 10
	P-26	-	-	< 10	< 10
Plasma alone	P-27	-	-	< 10	< 10
Culture medium				< 10	< 10

PROCEDURE

Spleen fragments, obtained from normal or immunized guinea-pigs, were cultured in homologous plasma clots in the usual fashion. After zero, three, six, and fourteen days of incubation, fragments of tissue were removed from culture, and fixed in 10.0% formalin. Tissues were sectioned and stained with haemotoxylin and eosin.

RESULTS

Sections of spleen fragment cultures may be seen in Figs. 4 - 9. At zero time, fragments of tissue show the appearance typical of normal splenic tissue. Areas of white pulp, containing many lymphocytes, alternated with areas of red pulp, in which a meshwork of reticulo-endothelial cells intermingled with red cells could be observed (Figs. 4 and 5).

By day three, organization of tissue was beginning to disappear, and the presence of lymphoid follicles could no longer be distinguished. Many of the lymphocytes had become pyknotic, while others were undergoing the process of karyorrhexis (Figs. 6 and 7).

On day six, the majority of lymphocytes had disappeared leaving a network of reticular cells, many of which appeared to have developed into fibroblasts (Fig. 8). By day fourteen, lymphocytes had vanished from the scene entirely, and there was marked proliferation of fibroblasts throughout the whole

fragment (Fig. 9).

It may be remembered that antibody was detected in similar cultures only during the first few days of incubation, after which antibody ceased to be produced. The loss of antibody-forming capacity coincided roughly with the disappearance of cells of the lymphocyte series from cultures.

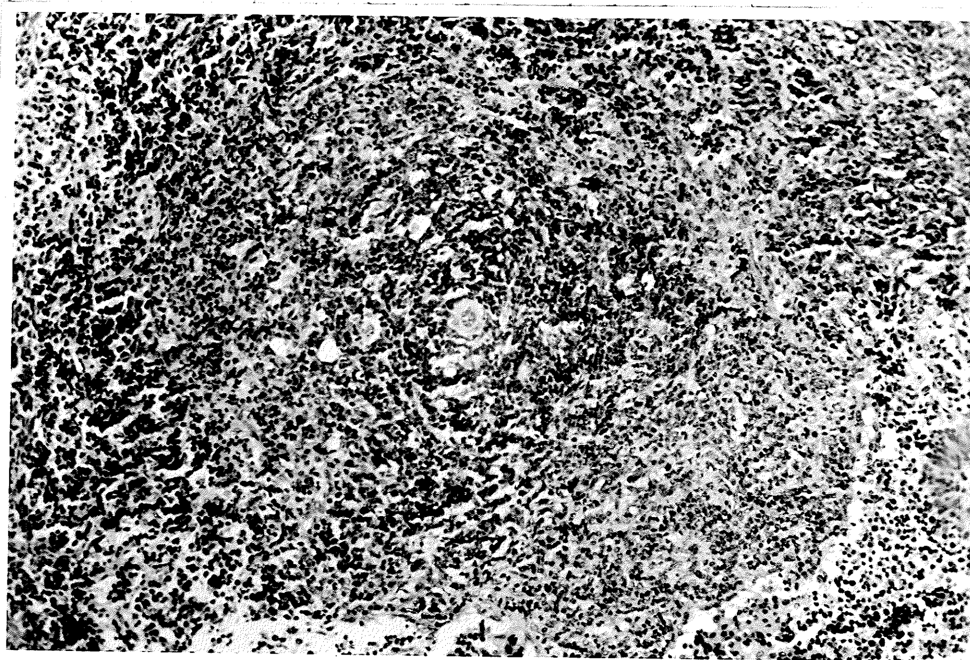

Fig 4. Freshly obtained spleen fragments (photographed at magnification x 100).

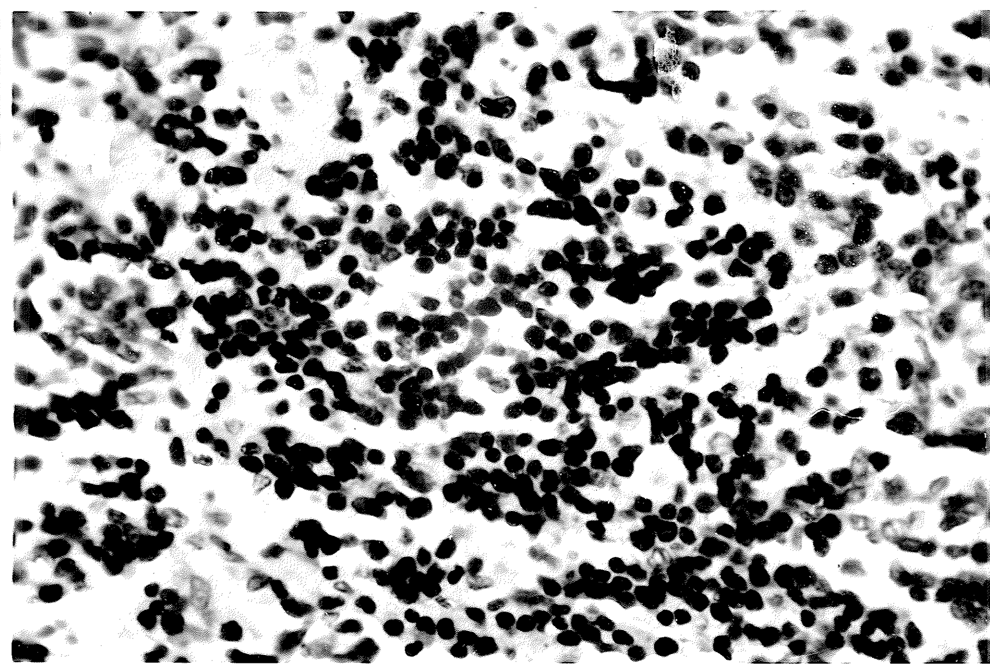

Fig. 5. Freshly obtained spleen fragments (photographed at magnification x 400).

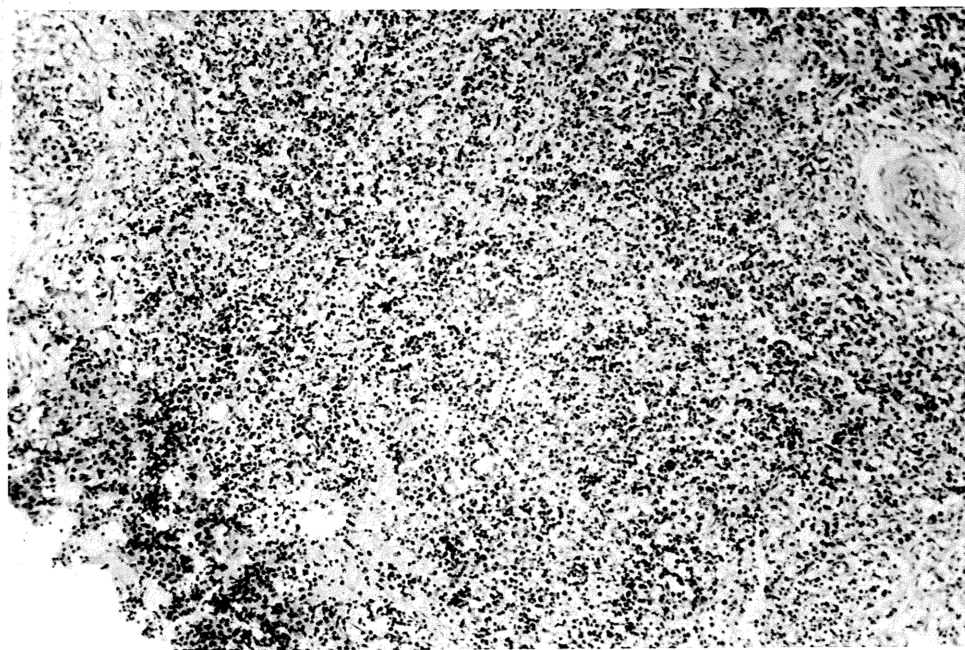

Fig. 6. Spleen fragments after three days of incubation (x 100).

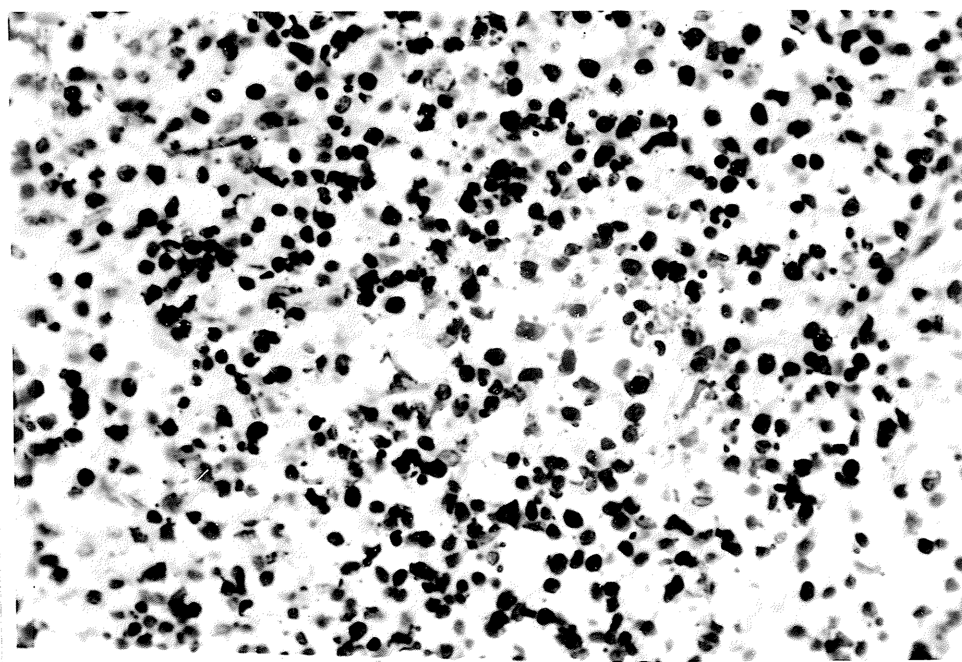

Fig. 7. Spleen fragments after three days of incubation (x 400).

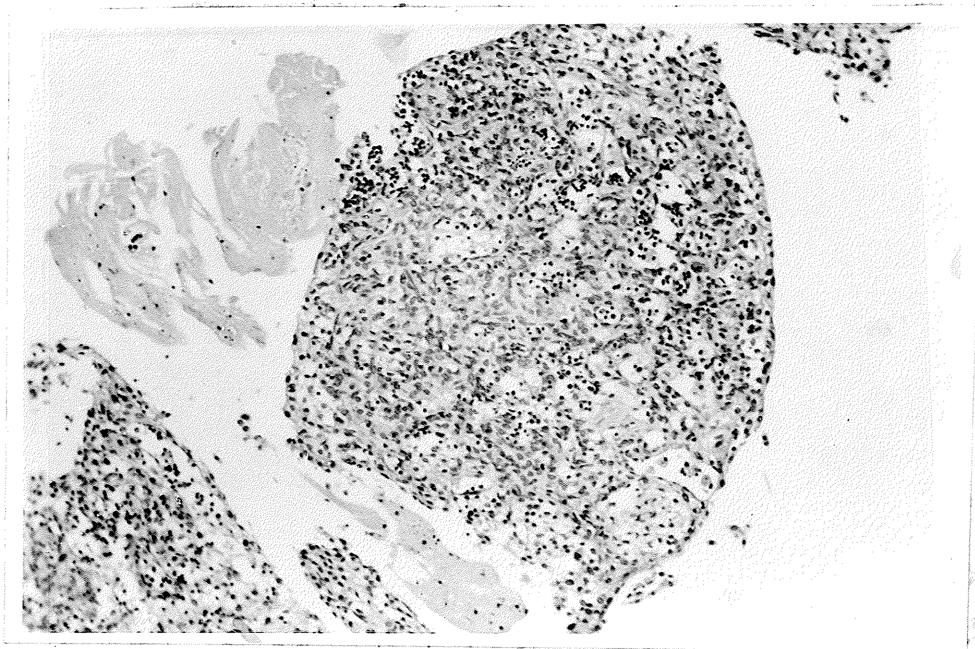

Fig. 8. Spleen fragments after six days of incubation (x 100).

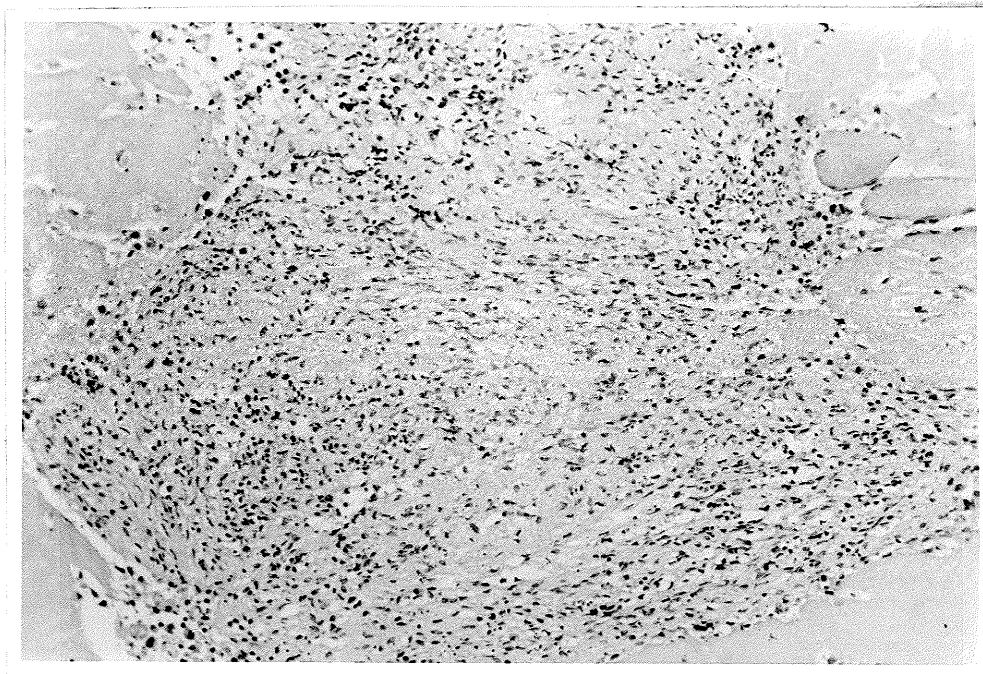

Fig. 9. Spleen fragments after fourteen days of incubation (x 100).

CHAPTER IV

DISCUSSION OF RESULTS

CHAPTER IV

DISCUSSION OF RESULTS

The preliminary experiments were designed to establish a method of tissue culture in which cells obtained from animals previously immunized *in vivo*, would produce detectable quantities of antibody *in vitro*.

Initial attempts to achieve this objective were made using spleen, lymph node and peritoneal exudate cells, obtained from guinea-pigs immunized with sheep or human erythrocytes. Cells were cultured in monolayers in medium 199 containing either homologous serum, or cortisone derivatives. Antibody production could not be demonstrated in either of these media, or when cultures were incubated in an atmosphere of 95% O_2 : 5% CO_2.

The reasons for the failure of such cultures are likely two-fold. Using a dye-exclusion technique, it was shown that the majority of cells in such cultures were damaged, or dead, after forty-eight hours of incubation. Damage to cells may possibly be the result of the procedures used in isolating cells from the individual organs. Askonas and Humphrey (1958[b]) have shown that even slight damage to cells caused by teasing out from a lymph node, resulted in almost total loss of the antibody-forming capacity of tissue.

These observations are also consistent with those of

Steiner and Anker (1956) who have reported that spleen cells, suspended in fluid medium, rapidly lost their ability to metabolize glucose. Antibody production could not be demonstrated in such cultures using a radioactive labelling technique for detection of antibody.

A second cause of failure may lie in the relative insensitivity of the direct hemagglutination test, for the detection of small amounts of antibody. Vas and Medzon (1964) have reported the production of antibody by suspensions of spleen cells. However, such antibody was detected by radioactive methods, or using an intracutaneous toxin-neutralization test. Both of these methods appear to be more sensitive than the direct hemagglutination technique (Stavitsky, A.B., 1955), (Stavitsky, A.B., 1958), (Marrack, J.R., 1963). In addition, Vas and Medzon found that a concentration of 2.5×10^7 cells/ml. of medium, was optimal for demonstration of antibody production, while below a concentration of 1.2×10^7 cells/ml., no antibody could be detected. These numbers are in excess of the number of cells present in our cultures.

Using a suspended cell system, it appears that large numbers of cells, combined with the use of a very sensitive assay technique, are required for the demonstration of antibody production *in vitro*.

In contrast, cultures of spleen fragments, obtained

from guinea-pigs previously immunized with sensitized cells (guinea-pig erythrocytes sensitized with rabbit-produced haemolysin), produced what appeared to be specific antibody against rabbit γ-globulin. Such cultures continued to produce antibody for about five days of incubation. Loss of antibody-forming capacity occurred at approximately the same time as the disappearance of lymphoid cells from culture fragments. This would suggest that these cells are responsible for the antibody production which was observed.

It was not demonstrated that the substance present within culture fluids, which caused agglutination of sensitized cells, was in fact, guinea-pig γ-globulin. However, the fact that its activity was inhibited by the presence of homologous antigen, but not by heterologous antigen, is suggestive evidence that it was indeed specific antibody.

Similarly, it was not shown conclusively, that antibody was actually produced during the incubation period. However, since this material was not detected in the culture fluids of heat-killed cells, it seems likely that actual synthesis of antibody _in vitro_, did occur. Adequate proof could best be achieved by the incorporation of radioactively labelled amino acids into culture media, followed by the demonstration of specific radioactively labelled antibody in the culture fluids.

In the last two groups of experiments, an attempt

was made to stimulate production of antibody *in vitro* in cultures of normal spleen fragments, by exposure of fragments to a rabbit-produced antiserum against guinea-pig spleen cells (cytolytic antibody). Following incubation of fragments with cytolytic antibody, tissues were washed and cultured in fresh medium without cytolytic antibody.

The results of these experiments are inconclusive. A substance was detected in the culture fluids of one experiment, which was capable of agglutinating sensitized cells, but its activity appeared to be destroyed by storage at -18°C for approximately five months. However, when sera, containing antibody against rabbit γ-globulin, were stored in a similar fashion, their activity was not destroyed (McIllmurray, M., 1965).

Repeated attempts to reproduce this material in similar cultures of spleen fragments, have failed. It is not possible to draw any definite conclusions from these experiments until these results have been confirmed, and the active substance subjected to more detailed examination.

It is notable however, that exposure to normal rabbit serum, as well as to cytolytic antibody-containing sera, resulted in production of this material. This would tend to disprove, rather than support, the theory that cytolytic antibody is an essential factor in the production of antibody.

It has been noted, that after six days of incubation, the majority of cells within cultures of spleen fragments, were of the fibroblast type; lymphocytic cells were almost completely absent. Trowell (1959) has reported similar findings. The explanation for the failure of lymphoid cells to develop under these conditions, is unknown. Auerbach (1963) suggests the possibility that thymus may play an important role in the development of lymphoid elements in the spleen. However, Ioachim (1965) has disputed the necessity of a thymic factor, and has in fact reported the continued production of plasma cells in cultures of spleen cells, for as long as seven months. Such cells were obtained by mincing of splenic tissue from various species, and were cultured in Puck's medium containing 15% fetal bovine serum, in the bottom of glass Petri dishes.

Accordingly, it is suggested in future attempts to demonstrate antibody production by isolated spleen cells, an effort be made to utilize Ioachim's technique for the culture of such cells. Since this method appears to offer a system of replicating cells which continue to differentiate into plasma cells, it would seem to be particularly suited to studies of antibody production <u>in vitro</u>.

An alternative method suggested for such studies is that of Michaelides and Coons (1963) in which fragments of rabbit lymph node were cultured in homologous plasma clots.

Such a system also appears to supply the necessary elements for the development of mature plasma cells.

CHAPTER V

SUMMARY

CHAPTER V

SUMMARY

Attempts were made to stimulate antibody production *in vitro* in cultures or immunologically competent tissue obtained from normal guinea-pigs. Preliminary experiments were done in an effort to establish a culture system in which cells obtained from immunized animals, would produce detectable quantities of antibody *in vitro*.

It was found that :

1. Guinea-pig spleen, lymph node, and peritoneal exudate cells, obtained from guinea-pigs immunized against sheep or human erythrocytes, failed to produce detectable quantities of antibody when cultured in monolayers, in medium 199 with 25% NGPS (frozen). The replacement of serum in media, by cortisone acetate, or sodium hydrocortisone hemisuccinate, failed to promote antibody production in these cultures.

2. After forty-eight hours incubation, the majority of cells cultivated in monolayers were no longer viable.

3. Cultivation of spleen cells on the surface of cellophane membranes, failed to increase the number of survivors in a culture.

4. Fragments of spleen, obtained from guinea-pigs

immunized with guinea-pig red cells sensitized with a rabbit-produced haemolysin, produced detectable amounts of what appeared to be specific antibody against rabbit γ-globulin. This material caused agglutination of sensitized erythrocytes. Incorporation of specific antigen (rabbit γ-globulin) in the diluent inhibited this reaction, while the presence of heterologous antigen (human γ-globulin) did not. This substance was not produced in cultures of heat-killed cells.

5. Attempts to initiate antibody production _in vitro_ using cytolytic antibody as the stimulus, are inconclusive. A substance was detected in the culture fluids of one experiment, which caused agglutination of sensitized cells. It was produced in those cultures treated with normal rabbit serum as well as cytolytic antibody. The activity of this material disappeared after storage at -18° for approximately five months. Efforts to reproduce these results have met with failure in six similar experiments.

CHAPTER VI

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CHAPTER VI

BIBLIOGRAPHY

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CHAPTER VII

APPENDIX

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1. <u>Alsever's Solution</u> (mod. from Osler *et al*, 1952)

dextrose	10.25	gm.
Na citrate	4.00	gm.
NaCl	2.10	gm.
citric acid	0.275	gm.
distilled water	500.00	ml.

Adjust to pH 6.1.

Sterilize by Millipore filtration.

2. <u>Dextrose-sodium Citrate Solution</u> (Dept. of Biochem., McGill University)

2.3 gm. dextrose

1.7 gm. Na citrate

Dissolved in 100 ml. distilled water.

Combine 70 ml. of solution with 180 ml. of blood.

3. <u>Dulbecco's Balanced Salt Solution</u> (DBSS) (mod. from Merchant *et al*, 1961, p.162)

Unit #1 In each of two 2 liter flasks:

80.0	gm.	NaCl
2.0	gm.	KCl
11.5	gm.	Na_2HPO_4
2.0	gm.	KH_2PO_4
1400.0	ml.	distilled water

Unit #2 In a 500 ml. flask:

10.0 gm. dextrose

400.0 ml. distilled water

Unit #3 In a 500 ml. flask:

0.34 gm. $CaCl_2$

400.0 ml. distilled water

Unit #4 In a 500 ml. flask:

2.0 gm. $M_4Cl_2 \cdot 6H_2O$

400.0 ml. distilled water

Autoclave all units separately at 10 lbs. for thirty minutes. To each of the 2 liter flasks (#1) add:

200 ml. #2

200 ml. #3

200 ml. #4

4. Reif Cytolysis Stain (mod. from Reif, 1962)

Dissolve 50.0 mg. of trypan blue in hot DBSS. Cool, and add 150 mg. of eosin with stirring. Store at 4°C.

5. Hank's Balanced Salt Solution (HBSS) (mod. from Merchant *et al*, 1961)

A. 10 X Solution

Unit #1 3.5 gm. $NaHCO_3$

250.0 ml. distilled water

Unit #2	80.0	gm.	NaCl
	4.0	gm.	KCl
	2.0	gm.	$MgSO_4 \cdot 7H_2O$
	0.6	gm.	$Na_2HPO_4 \cdot 2H_2O$
	10.0	gm.	glucose
	0.6	gm.	KH_2PO_4
	800.0	ml.	distilled water
Unit #3	1.4	gm.	$CaCl_2$
	100.0	ml.	distilled water
Unit #4	0.4	gm.	phenol red
	150.0	ml.	distilled water

Adjust to pH 7.0

Make up to final volume of 200 ml.

Add 100 ml. of Unit #4 to Unit #2, and then add Unit #3 to make 1000 ml. Store at 4°C.

B. Working Solution

Dilute 10 X stock 1:10 with distilled water. Autoclave at 10 lbs. for 20 minutes. Adjust pH to 7.2 - 7.4 with sterile 4.4% $NaHCO_3$.

6. Eagle's Minimum Essential Medium (MEM) (mod. from Eagle's)

A. Stock Solution

Unit #1 Hank's balanced salt solution.

Unit #2 (X100)	1.05	gm.	L - arginine
	0.31	gm.	L - histidine
	0.52	gm.	L - isoleucine
	0.52	gm.	L - leucine
	0.58	gm.	L - lysine
	0.15	gm.	L - methionine
	0.32	gm.	L - phenylalanine
	0.48	gm.	L - threonine
	0.10	gm.	L - tryptophane
	0.46	gm.	L - valine

Dissolve in 100 ml. of #1 by heating to 80°C.

Unit #3 (X100)	0.24	gm.	L - cystine
	0.36	gm.	L - tyrosine

Dissolve in 100 ml. of 0.1N HCl.

Unit #4 (X1000)	0.10	gm.	choline chloride
	0.20	gm.	I - inositol
	0.10	gm.	nicotinamide
	0.10	gm.	calcium pantothenate
	0.10	gm.	pyridoxal
	0.01	gm.	riboflavin
	0.10	gm.	thiamine

Dissolve in 100 ml. of #1.

Unit #5 (X100) 0.01 gm. folic acid

0.01 gm. biotin

Dissolve in 100 ml. of #1 by the addition of a few drops of 0.5N NaOH.

Unit #6 (X100) 2.92 gm. glutamine

Dissolve in 100 ml. of #1. Store at -18°C.

B. Working Stock

Combine:

10.0	ml.	#2
10.0	ml.	#3
1.0	ml.	#4
10.0	ml.	#5
10.0	ml.	#6
20.0	ml.	10% glucose

Add 10.0 ml. of a mixture of penicillin (at 20,000 units/ml.) and streptomycin (at 10,000 μg./ml.), and 10.0 ml. of mycostatin (at 20,000 units/ml.). Store at 4°C for not longer than one week.

For use, combine:

8.1	ml.	Working Stock
20.0	ml.	NGPS (fresh)
71.9	ml.	HBSS

Adjust to pH 7.2 - 7.4 with 4.4% $NaHCO_3$.