SOME EFFECTS OF CYTOLYTIC ANTIBODY

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ABSTRACT

SOME EFFECTS OF CYTOLYTIC ANTIBODY

A rabbit antiserum has been produced against guinea pig peritoneal exudate cells, and its effects on the cells investigated. The investigation has been divided into a) the effects demonstrable *in vitro*, b) the effects demonstrable *in vivo*.

Immune cytolysis of tumor cells *in vitro* is characterized by swelling of the cells with an accompanying loss of nucleic acid. Peritoneal exudate cells react in an identical fashion when treated with antiserum and complement. A spectrophotometric method has been devised which measures cytolysis as the absorbance due to nucleic acid lost from the cells. This method has been used to titrate antiserum and complement, and the titrations compared with the more conventional dye exclusion method.

Attempts were made to demonstrate immune cytolysis *in vivo* by injecting sensitized cells intradermally into normal guinea pigs and studying sections. The results were inconclusive.

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INTRODUCTION

Immune hemolysis is markedly affected by the presence of an antibody against the gamma globulin of which hemolysin is composed. Onysko (1962), using a system consisting of sheep red blood cells, rabbit hemolysin (sensitizer) and guinea pig anti-rabbit serum (antisensitizer) showed that antisensitizer protected sensitized cells from lysis by complement.

The complementary lysis of normal and neoplastic tissue cells sensitized with cytolytic antibody appears to depend on the same mechanisms as the complementary lysis of sensitized erythrocytes. The present work forms part of a project designed to study the effects of antisensitizer on the immune lysis of tissue cells *in vivo* and *in vitro*, and to compare these with its effects on immune hemolysis *in vitro*.

The subject of this thesis is the measurement of immune cytolysis of guinea pig peritoneal exudate cells *in vitro*, and its demonstration *in vivo*. It is hoped that the results presented here may form the basis of a further study of the effects of antisensitizer on such a system.

TERMINOLOGY

The term "cytotoxin" for the agent in immune serum which damages cells was proposed by Metchnikoff in 1901. He further suggested that the action of a cytotoxin on cells was analogous to the action of hemolysin on red blood cells.

"Cytotoxic" is an appropriate adjective for anti-cell sera because they produce, in the presence of complement, damage and lysis of cells in vitro (Stetson and Jensen, 1960).

Cytotoxic antibody requires complement for lysis, and has been shown, in the case of tumor cells, to produce the same swelling and permeability disorders as those seen in the lysis of red blood cells by hemolysin (Green et al., 1959). Since, in this investigation, lysis of cells has been the criterion used for assessing the effect of the antisera, the term "cytolytic antibody" seems more specific and will be used on many occasions. No sharp distinction is intended between this and "cytotoxic antibody".

CHAPTER I

REVIEW OF THE LITERATURE

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REVIEW OF THE LITERATURE

The review of the literature includes selected references dealing with the effects of cytolytic antiserum and complement on cells other than red blood cells. The discussion has been divided as follows:

I. *In vivo* Effects

II. *In vitro* Effects

III. The Mechanism of Cytolysis.

I. IN VIVO EFFECTS

This discussion of the *in vivo* effects of cytolytic antibody has been divided into three sections: effects on normal cells, effects on tumor cells, and enhancement.

Effects on Normal Cells

One of the first demonstrations of the specific cytotoxic effect of heterologous antiserum *in vivo* was reported by Delezenne in 1900. He immunized ducks with preparations of dog cerebellum, medulla, and spinal cord. This "neurotoxic" antiserum was fatal to dogs when injected into the brain, the swiftness of death being proportional to the amount of serum injected, and the symptoms being those of severe brain damage.

In another early demonstration of the cytotoxicity of antiserum *in vivo*, Ledingham (1914) induced fatal purpura in guinea pigs by the injection of a potent rabbit-produced antiserum against guinea pig platelets.

Pressman (1949) labelled rabbit-produced anti-rat kidney sera with I^{131}, and recovered the radioactive label preferentially from the kidneys of rats into which the labelled antiserum had been injected. This was the first direct evidence that cytotoxic antisera combine specifically *in vivo* with the cell types used as antigens in preparing the sera. Specific binding *in vivo* of anti-leucocyte serum to circulating leucocytes and to bone marrow cells was demonstrated by Kuroyanagi *et al.* (1963), who used radioisotopic and fluorescent antibody techniques.

Specific types of circulating cells may be selectively destroyed *in vivo* by the administration of cell-specific heterologous antisera. Lymphocytes (Chew and Lawrence, 1937; Waksman *et al.*, 1961), neutrophils (Finch, 1955), and platelets (Kumar and Saraya, 1962) may be specifically destroyed *in vivo*, resulting in decreased numbers in peripheral blood. These decreased levels may be prolonged by repeated injections of antisera.

When normal circulating cells are transferred to an animal which has been previously immunized against them, evidence for their survival or destruction in the recipient is necessarily indirect, because of the impossibility of identifying such cells directly on a morphological basis. Harris *et al.* (1957) demonstrated

that when rabbit lymphocytes were injected into rabbits that had previously received immunizing injections of donor leucocytes, the injected lymphocytes were destroyed *in vivo*. These lymphocytes were "labelled" by the capacity to form antibodies to a filtrate of *Shigella dysenteriae*, and the lack of a rise of antibodies to *Shigella dysenteriae* in the recipient's serum was then evidence that the injected lymphocytes had been destroyed.

Antibodies formed during homograft rejection destroy donor spleen and lymph node cells subsequently injected into the recipient. Warwick *et al.* (1962) used delayed-type hypersensitivity to streptokinase-streptodornase as a label for donor cells. Cytotoxic antibody in the recipient rabbit's serum prevented the passive transfer of delayed-type hypersensitivity by donor cells. This blockage of transfer is evidence for destruction of the cells by the recipient.

Effects on Tumor Cells

Russell in 1908 first demonstrated that rats in which a mouse tumor had been transplanted and subsequently regressed showed an increased resistance to subsequent inoculation of the tumor. He suggested that the accelerated degeneration seen in the second tumor was caused by cytotoxic antibody.

Chickens are resistant to Krebs-2 mouse ascites tumor, although chick embryos are susceptible. Green and Lorincz (1957) correlated the development of resistance to the tumor with the appearance of a natural antibody in the serum. This antibody

appears by the sixteenth day, and at this time tumor cells disappear from the circulation and organs of the infected embryo.

The hope of developing antisera for cancer therapy has stimulated research into the specificity and effectiveness *in vivo* of antisera against tumor cells of different types. The first successful attempt to use heterologous anti-tumor serum therapeutically was reported in 1945 by Nettleship. Injection of rabbit-produced antiserum against Murphy lymphosarcoma into rats bearing the tumor caused a fifty-four per cent reduction in mortality, and retarded the rate of tumor growth in animals which succumbed.

The specific localization of antibody against Ehrlich tumor cells *in vivo* has been shown using I^{131} - labelled antibody (Wissler *et al.*, 1956). These authors could not demonstrate effective localization of antibody in Flexner-Jobling or Jensen sarcoma rat tumors, although the localization of I^{131} - labelled antibody in Wagner-osteogenic sarcoma (Pressman and Korngold, 1953) and in Murphy lymphosarcoma (Korngold and Pressman, 1954) rat tumor has been reported.

Injection of rabbit antibody against Ehrlich tumor cells into mice bearing the tumor prolongs the survival of the mice (Flax, 1956). Flax also found that the simultaneous injection of guinea pig complement further prolonged survival of the mice. The antiserum and complement *in vivo* produced a progressive loss

of nucleic acid from the cytoplasm and nuclei of the tumor cells, as judged by smears of cells harvested twelve, thirty-six, and seventy-two hours after the injection of the antiserum and complement. This loss of nucleic acid was not as evident when antiserum alone was injected. No therapeutic value of antiserum against Ehrlich cells could be demonstrated by Horn (1956), nor could Thompson (1955) affect the proliferation of mouse lymphatic leukemia cells by the administration of rabbit antiserum.

Enhancement

Enhancement has been defined by Kaliss (1958) as applying specifically to the successful establishment and progressive growth of a tumor homograft as a result of its contact with specific humoral antibody in the host. Carcinomata and sarcomata show enhancement, while tumors of lymphoid and hematopoietic origin are inhibited by the presence of antibody (Möller and Möller, 1962). The term "enhancement" has also been used to describe prolonged life of a homograft in an animal pretreated with normal tissues of the donor (Kaliss, 1958).

Kaliss (1958), in a review of enhancement, listed many characteristics of enhancing and cytolytic antibody. Paradoxically, the procedures for obtaining both types of antibody are identical, and there is a positive correlation between hemagglutinating activity and enhancing activity of an immune serum. Both antibodies are gamma globulin, but it is unknown whether they are identical or not. Enhancing antibody, like cytolytic antibody, may be produced by

1) homograft rejection, and 2) active immunization with tumor cells or extracts, and may be demonstrated by 1) passive transfer of hetero- or iso-antisera, or 2) treatment of tumor cells with antiserum *in vitro* before injecting them into the host.

Enhancement has been demonstrated with only a limited number of tumor-host combinations, and apparently represents only one of the possible reactions. Gorer (1942) demonstrated both enhancement and cytotoxicity in the same tumor antiserum-host combination. Low doses of antiserum produced enhancement of the tumor while higher doses were cytotoxic. Möller and Möller (1962) demonstrated that antisera containing antibodies against *all* isoantigens that differentiated tumor from host were capable of inducing strong enhancement, while antisera containing only antibodies against *some* of these caused less enhancement and, occasionally, inhibition. They explained this as indicating that the antibodies covered isoantigens on the cells, thus preventing them from stimulating rejection mechanisms in the host. Different host-tumor combinations could be divided into three categories: 1) those in which enhancement was induced by all antiserum doses used, 2) those in which small doses caused enhancement, and larger doses inhibition, and 3) those in which the degree of enhancement decreased as the dose of antiserum decreased.

II. IN VITRO EFFECTS

Cytotoxicity of antibody and complement has been demonstrated *in vitro* using many different types of cells and a variety of

methods. A rough, but not mutually exclusive division of these methods has been made, and this discussion will be presented under the following headings: a) tumor transplantation techniques to test viability, b) dye exclusion (the capacity of viable cells to exclude dyes such as trypan blue), c) biochemical activity, and d) morphological changes.

Tumor Transplantation

In vitro exposure of tumor cells to cytolytic antibody causes sensitization of the cells which results in inhibited growth of the tumor in a susceptible host. Presumably, this inhibition is a result of lysis of the sensitized cells by host complement. Thus, effects of cytolytic antibody on tumor cells may be shown by the complete absence of any visible tumor (Gorer, 1942), a prolonged time interval before the appearance of tumor (Landy et al., 1960), or prolonged survival of infected animals (Flax, 1956).

Heterologous antisera have been shown to be effective against a variety of tumors. Rabbit antisera were inhibitory for transplantable mouse leukemia cells (Dulaney and Arneson, 1949; Werder et al., 1952), mouse lymphosarcoma 6C3HED (Nungester and Fisher, 1954) Ehrlich mouse ascites tumor cells (Flax, 1956; Horn, 1956) and mouse mammary carcinoma (Imagawa et al., 1954). Natural cytotoxic antibodies in normal human serum destroy Ehrlich, Krebs, and Sarcoma 37 mouse tumor cells (Landy et al., 1960), and natural antibodies in normal chicken serum destroy Krebs cells (Green and Lorincz, 1957).

Homologous antisera are equally effective against tumor cells *in vitro*. Using sera from animals which had recovered from a tumor Gorer (1942) destroyed mouse leukemic cells, Burmester (1947) destroyed avian lymphoid tumor cells, and Kalfayan and Kidd (1953) destroyed rabbit Brown-Pearce carcinoma.

Dye Exclusion

The ability of cells to exclude certain dyes is a sensitive and reliable indication of viability (Hanks and Wallace, 1958). Loss of this property of dye exclusion affords a direct and immediate means of demonstrating cytotoxicity. Cytolytic antibody and complement alter the permeability properties of the cell membrane so that affected cells can no longer exclude dyes such as neutral red (Niven, 1929), trypan blue (Kapitchnikov *et al.*, 1962), azure B (Flax, 1956), and eosin (Hanks and Wallace, 1958).

Cytolysis of Ehrlich mouse tumor cells (Flax, 1956), mouse lymphocytes (Stetson and Jensen, 1960), Krebs and Sarcoma mouse tumors (Landy *et al.*, 1960) and guinea pig kidney cells (Bassett, 1962) has been demonstrated using this technique.

Reif (1962) used as criteria of damage both deformation of cells and staining with a mixture of trypan blue and eosin. The immune cytolysis of mouse Ehrlich, Sarcoma IA, and leukemia tumor cells was studied. He performed titrations of antibody and complement by this method and showed that the curves so obtained were very similar to titration curves of antibody and complement using the conventional red cell-hemolysin system. He also noted that deformed

cells in the early stage of cytolysis do not stain, but with optimal conditions for cytolysis, all deformed cells ultimately became permeable to the stain.

Biochemical Activity

Treatment with cytotoxic antiserum and complement results in inhibition of vital metabolic processes, as well as loss of the ability to exclude dyes or proliferate *in vivo*. Incubation of Ehrlich tumor cells with antiserum and complement results in loss of the capacity to respire in the presence of glucose (Flax, 1956; Bickis *et al.*, 1959). Glycolysis of Krebs and Sarcoma 37 tumor cells is also inhibited (Landy *et al.*, 1960). Flax reported that although glycolysis was inhibited, respiration in the presence of succinate was not affected. It was later suggested by Green and Goldberg (1960) that the soluble glycolytic enzymes of the cytoplasm were lost from cells undergoing cytolysis more readily than were the particulate respiratory enzymes of the mitochondria, and that this is the explanation of the difference noted. Bassett (1962) reported that Forssman antiserum and complement inhibited the oxygen uptake of guinea pig kidney cells.

The incorporation of glycine-2-C^{14} into protein by Ehrlich and 6C3HED mouse tumors was inhibited by cytolytic antibody (Colter *et al.*, 1957). Bickis *et al.*, (1959) reported that the uptake of glycine from the medium was also inhibited.

This inhibition of enzymatic activity appears to be dependent on the presence of complement (Bickis *et al.*, 1959).

Nowinski (1948) could not demonstrate an inhibitory effect on oxygen uptake by rat spleen and brain. He used antiserum alone, and did not add complement to his system.

Morphological Changes

Morphological changes occurring as a result of treatment with cytolytic antibody and complement have been described in a wide variety of cells, both in tissue culture and in suspension. Tissue culture techniques were first introduced by Lambert and Hanes in 1911. With culture techniques, the effects of cytolytic antibody on the proliferation of cells can be shown, as well as its effects on morphology. Lambert and Hanes showed that the plasma of rats which had been previously injected with a mouse sarcoma was unsuitable for growth of the sarcoma *in vitro*.

Lumsden (1925) cultured mouse and rat tumors in plasma clot cultures in the presence of fresh rat and rabbit antiserum respectively. He confirmed Lambert and Hanes's observations of the inhibitory effect of antiserum on the proliferation of cells, and further demonstrated that, upon the addition of antiserum, healthy cells growing in normal serum underwent clumping, degeneration, and lysis.

A description of the morphological changes occurring in immune cytolysis was given by Niven in 1929. Rabbit antiserum was added to cultures of normal mouse fibroblasts and of mouse mammary carcinoma M63. Fresh antiserum was lethal to the cells within thirty minutes. Death was judged by gross morphological changes,

as well as by staining with neutral red. The earliest morphological changes were an "indistinct, ragged, and irregular" cell outline, and a more distinct and opaque appearance of the nucleus. The cytoplasm became very granular in appearance and bleb-like processes extruded from the surface of some cells. These "blisters" coalesced so that the whole cell became swollen. The mitochondria disappeared. Twelve hours later, no recognizable cell remains could be found. These changes were dependent on the presence of complement. In its absence, the cells became rounded and vacuolation of the cytoplasm occurred. However, in this case the cells did not stain with neutral red, and therefore were considered viable.

The same kind of cell deterioration as a result of treatment with fresh cytolytic antiserum, or with cytolytic antiserum and added complement, has since been described, with only minor variations, for many different kinds of cells, both in tissue culture and as single cell suspensions. Some of these are outlined below. The characteristic features of cytolysis, summarized by Ross (1957), include rounding and swelling of the cells with retraction of cell processes, followed by increasing cytoplasmic granulation, mitochondrial vesiculation, and increasingly severe nuclear damage.

Immune cytolysis of cultured normal and leukemic human and chicken leucocytes was described by Hueper and Russell (1932). The same type of damage has been reported in the cytolysis of

cultured mouse mammary cancer cells (Imagawa et al., 1954), chick, rat and mouse spleen and bone marrow cells (Pomerat, 1946), chick embryo brain and spinal cord (Grunwalt, 1949) and dog myocardium (Craciun and Sorescu, 1931). The same pattern was reported by Lumsden (1958) for cytolysis of plasma clot cultures of chicken fibroblasts, adult human muscle and human embryo cerebellum. The action of cytotoxic antibody on HeLa cells in fluid and plasma - clot cultures has been extensively described (Habel et al., 1957; McAllister et al., 1958; Goldstein and Myrvik, 1958).

Terasaki et al. (1960) described cytolytic changes in suspensions of normal chicken lymphocytes treated with fresh homologous antiserum. The changes began with a decrease in the motility of the cells, and this was followed by a series of degenerative changes which are very much like those described in the cytolysis of cultured cells.

Kalfayan and Kidd (1953) described the morphological changes during immune cytolysis of Brown - Pearce carcinoma cells in suspension. In Bagg rat tumor cells, rupture of the nucleus precedes rupture of the cell membrane (Schrek and Preston, 1956). This has not been described in the cytolysis of other cells. Initial blistering of the cell membrane prior to swelling of the cell has been a prominent feature in other descriptions of cytolysis, but was not seen in either Brown-Pearce or Bagg tumor cells.

Phase contrast and electron microscope studies of the cytolysis of chick embryo heartfibroblasts were described by

Latta (1958, 1959). Antiserum and complement added to plasma clot cultures caused an immediate cytotoxic effect which was apparent within fifteen minutes. Under phase contrast, the changes seen were identical to those described for other cells.

Electron micrographs showed that the plasma membrane disrupted following the action of antibody and complement, although this feature differs from other descriptions. Mitochondria and profiles of endoplasmic reticulum become vesicular. Lipid inclusions showed internal irregularity and stained more lightly. With nuclear shrinkage, the granules of the nucleoplasm piled up at the nuclear membrane. The outer nuclear membrane separated from the inner to form large "perinuclear vesicles".

The descriptions of immune cytolysis given by Goldberg and Green (1959, 1960), and by Easton, Goldberg and Green (1962) for the Krebs cell, and by Bickis (1959) for the Ehrlich cell are very similar. Both differ from Latta only in that there appears to be no rupture of the cell membrane.

III. THE MECHANISM OF CYTOLYSIS

Immune Hemolysis

The currently-accepted explanation of the mechanism of immune hemolysis (Kabat and Mayer, 1961) assumes the presence of an unknown number of receptor sites on the surface of the erythrocyte. Two antibody molecules attach to adjacent receptor sites. They call this a bimolecular complex. Complement binds reversibly

to this antibody-cell union. Lysis of the cell is a result of the production of a permeability defect in the cell membrane by the enzymatic activity of complement (Mayer, 1962).

The Site of Antibody Fixation

Indirect evidence for the existence of receptor sites on the surface of viable cells is afforded by the observation that heat-inactivated antiserum agglutinates cells (Horn, 1956; Schrek and Preston, 1956). After treatment with sub-agglutinating doses of heat-inactivated cytolytic antiserum, HeLa cells resisted infection with poliovirus (Quersin-Thiry, 1958). Presumably, antibody had blocked the cell surface virus receptor sites.

The indirect fluorescent antibody technique was used to show that cytolytic antibody fixes at the surface of viable HeLa cells by Hiramoto et al. in 1960. In viable cells treated with heat-inactivated antiserum, fluorescence was seen only at the border of the cell, but if the cells were killed by fixation in cold acetone before they were treated with antiserum, intracellular fluorescence was seen. When viable cells were treated with antiserum and complement, characteristic blister formation occurred, and antibody was identified on the surface of the blister.

When Krebs cells are incubated in heat-inactivated cytolytic antibody, the only structural change seen is in the cell membrane (Goldberg and Green, 1959). In electron micrographs, the membrane exhibits a complex folding, resulting in many elongated processes,

and cleft-like channels which extend into the interior of the cell. This is thought to result from the binding of divalent antibody to the cell membrane. The authors suggest that normal plasma membrane movements successively bring different areas into close enough proximity to be bound together by divalent antibody.

In electron micrographs of Krebs cells incubated with a ferritin-labelled antibody, sites of antibody fixation can be identified by the presence of the electron-dense ferritin molecule (Easton, Goldberg and Green, 1962). Ferritin was identified in characteristic array along the surface of the cell membrane. In the interior of the cell it was always found within membrane-bounded vesicles, which were considered to be elements of smooth endoplasmic reticulum, probably formed by pinocytosis. The label was never found in the cytoplasmic matrix, or adhering to rough endoplasmic reticulum, or mitochondria.

In other experiments, Easton et al. (1962), added rabbit complement to this system. Characteristic swelling of the cell and of all membrane-bounded compartments within it occurred, and in electron micrographs the ferritin label could be identified in the cytoplasmic matrix and in the nucleoplasm as well as along the cell membrane. Specific binding of antibody to mitochondria or rough endoplasmic reticulum was not seen.

Colloid Osmotic Lysis

Endosmosis as the force responsible for the swelling of cells undergoing immune cytolysis was first suggested by Lumsden et al. in 1934. Ellem (1958) renewed the idea of osmotic lysis, and further suggested that the swelling characteristic of cytolysis was the result of specific permeability damage to the cell membrane. He called the process "colloid osmotic lysis", since it is due to intracellular colloid osmotic pressure, and the loss of intracellular material is analogous to that described as occurring in hemolysis by Ponder (1948).

Lee et al. (1960) reported experiments in which Ehrlich cells were treated with cytolytic antibody and complement. The cells became swollen, increasing in area over two hundred per cent, and at the same time lost large amounts of intracellular material, equal to almost half their dry mass. Ellem (1958) reported that large amounts of acid-soluble organic phosphate were lost from the cells. Using cytochemical techniques, Flax (1956) demonstrated that ribonucleic acid (RNA) was lost from Ehrlich cells undergoing immune cytolysis and Colter et al. (1956) showed that protein was lost. The loss of RNA from Brown-Pearce carcinoma cells (Kalfayan and Kidd, 1953) and chick fibroblasts (Latta, 1959) has also been shown by staining techniques.

Changes seen in the Krebs cell by phase contrast and electron microscopy were correlated with quantitative changes in the distri-

bution of small molecules and macromolecules between the cells and the medium by Green et al. (1959). In the initial stages of the reaction, before swelling has become pronounced, ninety per cent of the intracellular potassium, and a large proportion of the free amino acids and ribonucleotides are lost. Sodium enters the cell freely. After the cell has swollen, large molecules, such as protein and nucleoprotein are lost. In this way, the cells lose thirty-five per cent of their free amino acids, from thirty to sixty per cent of their protein, and up to seventy-five per cent of their cytoplasmic RNA. RNA is lost mainly as intact ribosomes. These losses correspond to the clearing of the cytoplasmic matrix seen in electron micrographs of the cells.

Swelling of the tumor cells and the loss of macromolecules can be prevented by placing the cells in a medium of very high protein concentration (130mg/ml of albumin) (Green and Silverblatt, 1960). Such a high protein concentration balances the intracellular colloid osmotic pressure. In such a medium, swelling and lysis of red blood cells is also prevented, although the loss of small molecules still occurs in both types of cells (Green et al., 1959).

On the basis of these results, Green and Goldberg (1959) postulate that immune cytolysis occurs in the following way:

Because of the high concentration of colloids within a cell, its concentration of small ions is normally slightly lower than that of an isotonic salt solution. This prevents the development of

an osmotic pressure gradient across the cell membrane. As a result of the action of cytolytic antibody and complement, a permeability defect, which may be thought of as the formation of functional "holes" is produced in the cell membrane. Small molecules pass freely into the cell as a result of this permeability defect, until their concentration is equal on both sides of the membrane. The osmotic pressure of the cell is therefore increased, and its excess over the osmotic pressure outside the cell is solely due to the osmotic pressure exerted by the intracellular colloids Water flows in, causing the cell to swell. As the cell swells, the "holes" are stretched so that macromolecules and ribosomes leak out. This loss constitutes "colloid osmotic lysis".

INTRODUCTION TO EXPERIMENTAL WORK

INTRODUCTION TO EXPERIMENTAL WORK

It is clear from this review of the literature that cytolytic antibody is toxic to cells, both *in vitro* in the presence of complement, and *in vivo*. Many methods for demonstrating this cytotoxicity have been described.

The ultimate purpose of the project of which this work forms a part is an investigation of the effects of antisensitizer (antibody against sensitizer) on immune cytolysis *in vivo*. As a preliminary to such a study, it was necessary to select methods for demonstrating cytolytic effects, and this thesis covers this preliminary work. Only after these effects can be adequately demonstrated on a quantitative basis *in vitro* can the effects of cytolytic antibody *in vivo* be evaluated. Accordingly, the work will be presented under the following headings:

I. *In vitro* Cytolysis

II. *In vivo* Cytolysis.

In each case the following will be described: 1) Materials, 2) Methods (this will include experiments performed in devising the methods), 3) Observations, and 4) Discussion.

CHAPTER II

IN VITRO CYTOLYSIS

CHAPTER II

IN VITRO CYTOLYSIS

I. MATERIALS

Cells

Guinea pig peritoneal exudate cells. Peritoneal exudate cells (PEC) consisting primarily of monocytes were obtained from guinea pigs eighteen to twenty-four hours after the intra - peritoneal injection of 10.0 ml. sterile physiological saline containing 0.001 per cent glycogen. The animals were bled out by cardiac puncture under chloroform, and 20.0 ml. Tyrode's solution[1] containing 0.01 per cent heparin injected intraperitoneally. The peritoneal cavity was then opened aseptically and the cell-rich exudate collected with a wide bore pasteur pipette. Bloody exudates were not used because of the observation that they contained a very high proportion of neutrophils. The cell suspensions were centrifuged in an angle-head centrifuge for five minutes at 1500 rpm. Any red blood cells present were lysed by resuspending the cell pellet in 8.0 ml. distilled water. Isotonicity was restored by the immediate addition of 2.0 ml. five times concentrated Tyrode's solution, and the pH adjusted to about 7.4 by adding 0.12 ml. 1.4 per cent sodium bicarbonate.

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[1] see appendix

PEC were not affected by this treatment. The cells were washed twice, centrifuging for three minutes at 1500 rpm, and resuspended. Tyrode's solution was used as diluent for all procedures.

A drop of the cell suspension was smeared on a coverslip and stained with a May-Grünwald Giemsa technique[2] for differential counts. From eighty to ninety per cent of the cells were mononuclear leucocytes and the rest were neutrophils. In order to identify the mononuclear cells, smears were made on slides and stained with the Sato-Sekiya peroxidase stain[3]. From fifty to sixty per cent of the cells contained peroxidase granules, which is usually taken to indicate monocytic origin.

The concentration of cells in suspension was measured by adding 0.1 ml. cell suspension to 2.9 ml. 0.1 N sodium hydroxide. The tubes were mixed in a Vortex Junior mixer to hasten digestion of the cells. The absorbance of this solution was read at a wavelength of two hundred and sixty mmu in a Beckman DU Spectrophotometer. The blank used to zero the machine contained 0.1 ml. Tyrode's solution and 2.9 ml. 0.1 N sodium hydroxide. The absorbance of such a solution increased directly as the concentration of cells determined by counting. An absorbance of 0.650 corresponded to a count of 60,000 cells per cubic mm., and 0.325 to a count of 30,000 cells per cubic mm. Cell concentrations were expressed as the number of cells per cubic mm. This method was used in preference to direct counting because it was more rapid and reproducible. An average harvest of PEC from one guinea pig

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[2] see appendix [3] see appendix

yielded 18.0 ml. with a count of 5000 cells per cubic mm. Because of this small yield, all procedures were designed to use as few cells as possible.

Preparation of PEC suspensions was always accomplished as rapidly as possible, and suspensions were always used immediately. Speed was important, because PEC tend to settle out on glass if they are left standing, and because Tyrode's solution may be toxic to cells after thirty minutes (Parker, 1961). Chilling of the cells was avoided, where possible, because of the observation of Green and Goldberg (1960) that osmotic control is impaired at lower temperatures.

Red blood cells. Guinea pig blood was collected in Alsever's solution[4] and stored in the refrigerator at least four days before it was used. The cells were washed three times in Tyrode's solution, centrifuging for five minutes at 1500 rpm for the first two washings, and for ten minutes at 1500 rpm for the third. A "one per cent" suspension was made in Tyrode's solution so that 1.0 ml. of the suspension added to 9.0 ml. distilled water gave an optical density of 0.225 at wavelength five hundred and fifty mmu in a Bausch and Lomb Spectronic Twenty. The blank used to zero the machine consisted of 1.0 ml. Tyrode's solution added to 9.0 ml. distilled water. Suspensions were kept in the refrigerator, and used within an hour of preparation.

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[4] see appendix

Antisera

Antiseraagainst guinea pig PEC and red blood cells were prepared in rabbits. PEC were prepared for injection by suspending washed cells in Tyrode's solution so that 1.0 ml. contained approximately 16 x 10^7 cells. 0.5 ml. of this suspension was injected intraperitoneally.

Guinea pig red blood cells were prepared for injection by washing three times in Tyrode's solution. 1.0 ml. of washed packed cells was given intraperitoneally at each injection.

Immunizing injections were given semi-weekly for three weeks. The rabbits were bled by cardiac puncture on the seventh and eighth days after the last injection. Normal rabbit serum for control purposes was pooled from four normal donors. After the serum was collected it was inactivated by heating at 56°C. for thirty minutes, and stored in the deep freeze at -20°C. Before use, it was thawed at room temperature and re-inactivated by heating for ten minutes at 56°C.

Four antisera against PEC were prepared. One, antiserum 16, produced strong agglutination of PEC, and was chosen for investigation. Control serum did not agglutinate PEC.

Complement

Blood collected from PEC donors by cardiac puncture was allowed to clot, and the fresh serum used as the source of complement.

In order to estimate the activity of guinea pig complement against guinea pig PEC, it was titrated using guinea pig red cells

and rabbit-produced hemolysin. This system was chosen because of the observation that complement is less active against homologous cells than it is against cells of a different species (Boyd, 1956). Accordingly, the titre determined by using guinea pig red cells seemed likely to be a better measure of probable activity against guinea pig PEC than one obtained using heterologous cells.

The methods used for the titration of rabbit anti-guinea pig hemolysin and for the titration of complement using this system were adapted from those of Onysko (1962). These were four-volume tests, designed by her for the study of the effects of antisensitizer on complementary lysis of red cells.

Titration of hemolysin. Doubling dilutions of antiserum were made in 1.0 ml. volumes of Tyrode's solution. 1.0 ml. of washed one per cent guinea pig red cells was added to each tube. A control tube containing no antiserum was included. The tubes were stoppered and incubated for twenty minutes in a waterbath at 37°C. They were removed from the waterbath, and 1.0 ml. Tyrode's solution and 1.0 ml. 1:4 complement added to each tube. The tubes were again incubated for thirty minutes at 37°C., then centrifuged in the cold. The supernatants were decanted and their optical density measured at five hundred and forty mmu using the control tube containing no antiserum as the blank. The last tube showing one hundred per cent lysis was taken as the endpoint. This tube contained an initial hemolysin concentration of 1:512, and this was the dilution chosen to sensitize cells for complement titration.

Preparation of sensitized red cells. Equal volumes of hemolysin (1:512) and one per cent cells were incubated for twenty minutes at 37°C. Normal cells for a control series were incubated with equal volumes of Tyrode's solution. The cells were then centrifuged for five minutes at 1500 rpm, washed once in Tyrode's solution, and re-suspended to the original volume. They were kept refrigerated until they were used.

Titration of complement. Doubling dilutions of complement, starting with a 1:2 dilution, were made in 1.0 ml. volumes of Tyrode's solution. Duplicate rows of tubes were set up. 2.0 ml. Tyrode's solution was added to each tube. 1.0 ml. sensitized cells was added to each tube of one row, and 1.0 ml. normal cells to each tube of the second row. The tubes were stoppered, and incubated for thirty minutes in a waterbath at 37°C. They were then chilled, centrifuged in the cold for ten minutes at 1500 rpm, and the supernatants decanted. The optical density of the supernatants of the first row was measured at five hundred and forty mmu, using the corresponding tube of the second row as a blank. A standard tube containing 3.0 ml. distilled water and 1.0 ml. sensitized cells was read against a blank containing 3.0 ml. distilled water and 1.0 ml. Tyrode's solution, and this value was taken as representing one hundred per cent lysis. The percentage lysis in each tube was calculated using the formula:

$$\text{percentage lysis} = \frac{\text{Optical density of tube}}{\text{Optical density of cells lyzed in distilled water}} \times 100.$$

Log probability paper was used to plot the results, with the volume of undiluted complement as abscissae, and the percentage

lysis as ordinates. The best straight line was drawn, and the dilution of complement causing fifty per cent lysis determined from the graph. This dilution of complement contained one fifty per cent hemolytic dose (one HD_{50}). In titrations of eight samples of complement, one HD_{50} was contained in dilutions of complement from 1:23 to 1:38. Dilutions of complement varying from 1:4 to 1:10 were therefore used in assessing the effect of cytolytic antibody on PEC, on the assumption that this dilution contained between 2 HD_{50} and 10 HD_{50} complement.

II. METHODS

Two methods were used to demonstrate immune cytolysis in vitro. The first part of this section describes an original spectrophotometric method, and the experiments performed in developing it. The second part describes a method selected from the literature, the dye exclusion technique.

Spectrophotometric Measurement of Lysis

Theory. Immune cytolysis and immune hemolysis are similar in that both involve loss of large amounts of intracellular material (Green and Goldberg, 1959; Kabat and Mayer, 1961). When sensitized red cells are exposed to complement, hemoglobin, among other substances, is released into the medium, and hemolysis is apparent. Thus, the amount of lysis may be measured by the increase in the optical density of the medium, using a spectrophotometer.

When other cells undergo immune cytolysis, material lost from cells includes ribonucleoprotein (as ribosomes) and cytoplasmic ribonucleic acid. A method has been devised which measures cytolysis by measuring the imcrease in absorbance of the medium due to nucleic acid lost from the cells.

After sensitized cells have been incubated in complement and cytolysis has occurred, the cells are separated from the medium by centrifugation. The supernatant, consisting of complement and material lost from the cells, is removed to fresh tubes. Perchloric acid is added, and protein and ribonucleoprotein precipitate. The mixture is then heated in a waterbath to allow the perchloric acid to hydrolyze ribonucleoprotein and release acid-soluble nucleic acid and nucleotides. Absorbance of the acid-soluble material is then measured in a Beckman DU Spectrophotometer at two hundred and sixty mmu, the wavelength of maximal absorbance of nucleic acid. Microcuvettes holding 0.5 ml. are used to hold the sample.

Perchloric acid was chosen for precipitation and hydrolysis because it absorbs very little at two hundred and sixty mmu, and its absorbance does not change with heating (Ogur and Rosen, 1950).

Tyrode's solution was chosen for this procedure, and used in all other procedures, because its absorbance at two hundred and sixty mmu is negligible, in contrast to that of Veronal buffer. The concentrations of magnesium and calcium, are, respectively, 0.1 grams and 0.2 grams per liter of Tyrode's solution, which are adequate

for complementary lysis.

No attempt has been made to identify chemically the material responsible for increasing the absorbance of the supernatant. For this reason, although it is believed to be mainly nucleic acid, it will be called "acid-soluble cellular material" (ASCM).

Accordance with Beer's Law. Such a method is practicable only if the hydrolysis of ASCM is unaffected by the unavoidable presence of serum. According to Beer's Law, the absorbance contributed by the complement and ASCM comprising the supernatant of lyzed cells should be equal to the sum of their separate contributions. To demonstrate that this is true, and that any increase in absorbance is directly due to ASCM lost from the cells, the following procedure was adopted: Two tubes, each containing 0.6 ml. washed PEC at a concentration of 20,000 cells per cubic mm. were centrifuged for three minutes at 1500 rpm. The cell sediment in one tube was resuspended in 1.8 ml. distilled water in order to obtain "cell lysate", and the cells of the second tube in 1.8 ml. Tyrode's solution as "control". The tubes were allowed to stand at room temperature with occasional vigorous shaking. After twenty minutes they were centrifuged for five minutes at 1500 rpm, and the supernatants decanted. A series of four tubes was set up as shown in table I. Hydrolysis was allowed to proceed for thirty minutes at 70°C. The precipitate was removed by centrifuging the tubes for thirty minutes at 2500 rpm, and the absorbance of each super-

natant measured at two hundred and sixty mmu. The blank used to zero the machine contained distilled water. The results are shown in table I.

Due to the presence of complement, tubes 2 and 4 contained seventy-five per cent of the amount of lysate or control, respectively, which was contained in tubes 1 and 3. Correction for this has been made in the calculations. The absorbance due to ASCM in the presence of complement is 0.217 and this is not considered to be significantly different from 0.205, its absorbance in the absence of complement. These results show that the absorbance of a mixture of complement and ASCM approaches the sum of their separate absorbancies, and thus the hydrolysis of ASCM is not affected by the presence of complement.

Optimal experimental conditions. The following series of experiments were performed in order to define the optimal conditions for obtaining the maximum amount of ASCM. The procedure followed in these experiments is first described in detail, followed by a separate account of each experiment.

1. Procedure.

Sensitization. High concentrations of antiserum were chosen for sensitization of PEC in these experiments because, initially, its strength was not known. Dilutions of 1:2 or 1:4 were used. Equal volumes of washed PEC, with concentrations from 20,000 cells per cubic mm. to 60,000 cells per cubic mm. and of antiserum were incubated in 11 x 80 mm. serology tubes for twenty minutes at 37°C.

TABLE I

THE ADDITIVE EFFECT OF THE ABSORBANCIES OF COMPLEMENT AND ASCM

Tube No.	1	2	3	4
3.5 N perchloric acid	0.2	0.2	0.2	0.2
* Lysate	0.5	-	-	-
*** Complement 1:4 in lysate	-	0.5	-	-
** Control	-	-	0.5	-
*** Complement 1:4 in control	-	-	-	0.5
Absorbance at 260 mmu	0.345	0.615	0.070	0.450

Absorbance due to 1:4 complement = (absorbance of tube 4, complement 1:4 in control) minus 0.75 (absorbance of tube 3, control)
= 0.450 - 0.75 (0.070) = 0.398

Absorbance due to ASCM in the presence of complement
= (absorbance of tube 2, complement 1:4 in lysate) minus (calculated absorbance due to 1:4 complement)
= 0.615 - 0.398 = 0.217

Absorbance due to ASCM in the absence of complement
= 0.75 (absorbance of tube 1, lysate) minus 0.75 (absorbance of tube 3, control)
= 0.75 (0.345) - 0.75 (0.070) = 0.258 - 0.53
= 0.205.

Hence, absorbance due to ASCM in the presence of complement is essentially the same as in its absence.

* lysate - supernatant of PEC lysed in distilled water
** control - supernatant of PEC suspended in Tyrode's solution
*** This means that 1 part of complement was added to 3 parts of lysate (tube 2) or control (tube 4). Thus, 0.75 of the volume of tubes 2 and 4 consists of lysate or control, respectively.

A control tube containing Tyrode's solution in place of antiserum was always included. After incubation, the tubes were chilled briefly, washed once in Tyrode's solution, centrifuging for three minutes at 1500 rpm, and the supernatant removed.

Cytolysis. A volume of complement equal to three times the original volume of washed PEC suspension was added to the cell sediment, and the cells resuspended by shaking. Dilutions of complement ranged from 1:4 to 1:10 in individual experiments. The tubes were re-incubated at 37°C. for thirty minutes to allow cytolysis to occur, then chilled briefly and centrifuged for five minutes at 1500 rpm. The supernatants were decanted to fresh tubes. The supernatant of sensitized cells was termed the "test lysate", and the supernatant of unsensitized cells the "control". Care was taken to ensure that no contamination of the supernatants by cells occurred, since such contamination would result in an abnormally high absorbance.

A drop of the cell sediment left in each tube was placed on a slide and examined under a phase microscope for the presence of deformed cells. The presence of such cells was a visual means of confirming that cytolysis had occurred (see Observations).

Hydrolysis. A row of 11 x 80 mm. serology tubes containing 0.2 ml. perchloric acid was set up, and 0.5 ml. supernatant added to each. For greater accuracy, micropipettes holding 0.2 ml. and 0.5 ml. were used to measure these volumes. A precipitate formed immediately. The tubes were heated in a waterbath for

hydrolysis, then centrifuged for thirty minutes at 2500 rpm. The clear supernatants were removed to fresh tubes.

Measurement of cytolysis. The absorbance of each supernatant was measured against a distilled water blank. The absorbance due to ASCM was expressed as the difference in absorbance between "test lysate" and "control" tubes.

2. Concentration of perchloric acid. Duplicate rows of tubes were set up, containing 0.2 ml. volumes of 0.7, 1.75, and 3.5 N perchloric acid*. One row received 0.5 ml. "test lysate", and the other received 0.5 ml. "control". The final concentrations of perchloric acid were then 0.2, 0.5, and 1.0 N. Hydrolysis was allowed to proceed for sixty minutes at 60°C.

The absorbance of both "test lysate" and "control" are presented graphically in figure 1. On the basis of these results, a final concentration of 1 N perchloric acid was chosen for hydrolysis, since it did not affect the control reading, but increased the hydrolysis of ASCM.

3. The effect of time and temperature on hydrolysis. In order to determine the optimum time and temperature for hydrolysis, the following procedure was devised. Three sets of duplicate rows of tubes each received 0.2 ml. 3.5 N perchloric acid. One row of each set received 0.5 ml. "test lysate", and the other 0.5 ml. "control". One set of tubes was then placed in a waterbath at 60°C., one at 70°C., and one at 80°C. At intervals of fifteen, thirty,

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* Stock perchloric acid, B.D.H. "Analar" 60% was taken as 10 N.

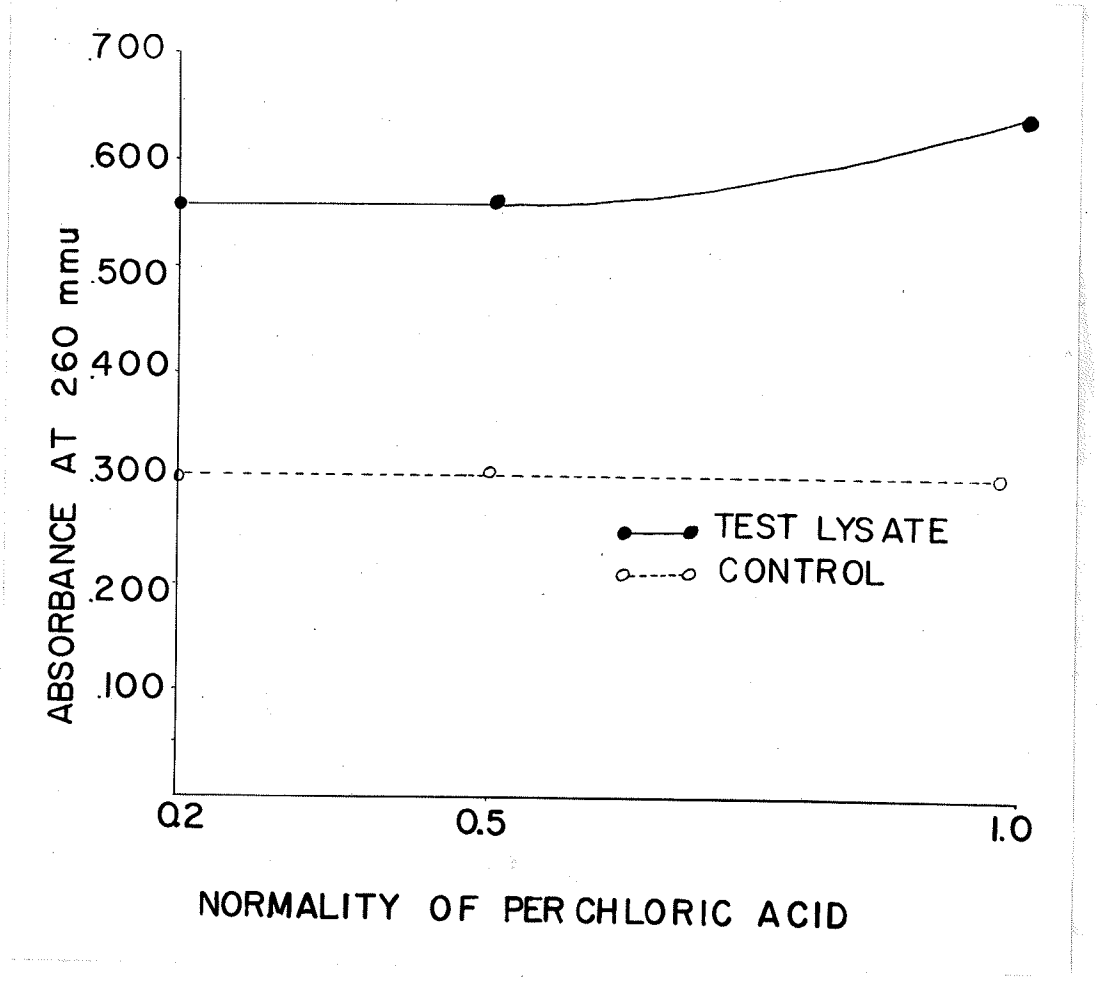

Figure 1. Effect of perchloric acid concentration on the absorbance of ASCM.

forty-five, and sixty minutes, one pair of tubes was removed from each waterbath.

The absorbance due to ASCM was determined by subtracting "control" from "test lysate". The results are presented in figure 2. Since the absorbance of ASCM is maximal after thirty minutes at 60°C., this time and temperature were chosen for hydrolysis.

4. The effect of cell concentration. Doubling dilutions of PEC, starting with a concentration of 80,000 cells per cubic mm. were made in 0.2 ml. volumes in duplicate rows of tubes. An equal volume of antiserum was added to each tube of the row which was to provide "test lysate", and equal volume of Tyrode's solution to the second row, which was to provide "control". After sensitization, cytolysis, and hydrolysis, the absorbancies due to ASCM were calculated by subtracting "control" from "test lysate".

The results are presented graphically in figure 3. There is a linear relationship between the number of cells and the amount of ASCM in the supernatant when the amounts of antiserum and complement remain constant, and in excess.

5. The effect of time allowed for cytolysis. Sensitized PEC were resuspended in 1:6 complement and incubated in a waterbath at 37°C. A duplicate series of unsensitized PEC were treated in an identical fashion. At intervals of ten, twenty, thirty, and sixty minutes, a tube containing "test lysate" and one containing "control" were removed for hydrolysis.

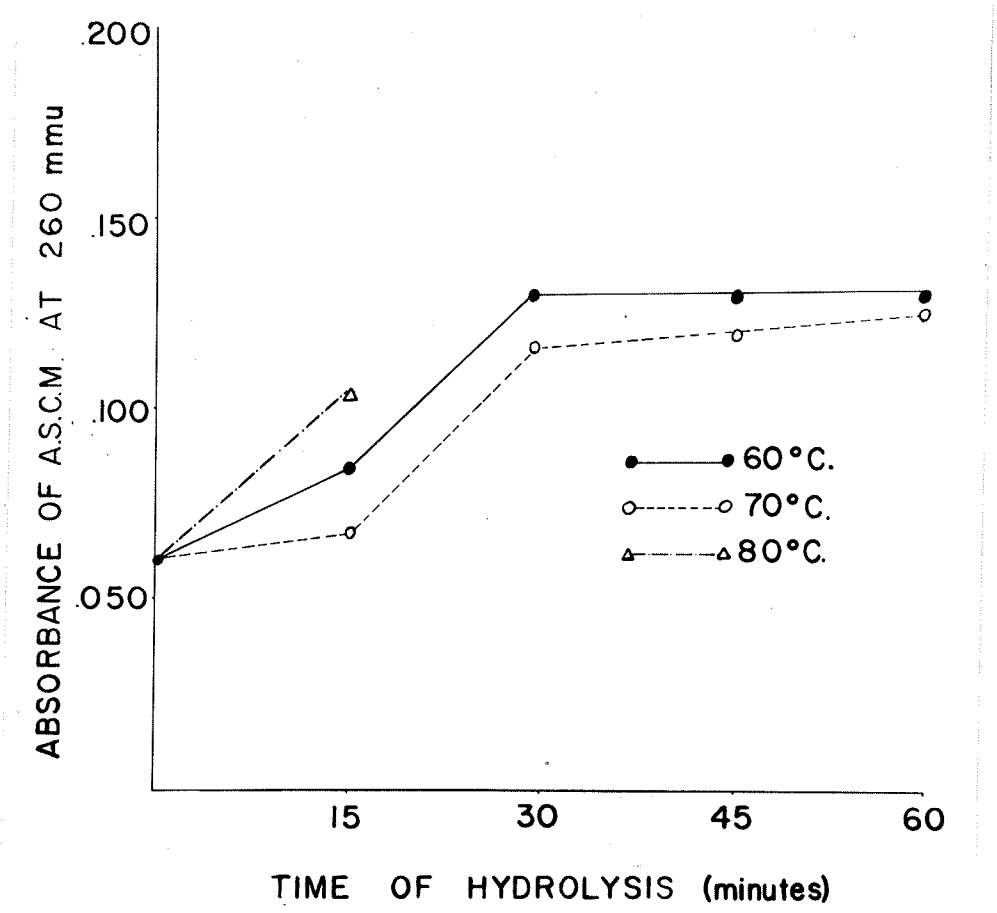

Figure 2. Effect of time and temperature of hydrolysis on the absorbance of ASCM.

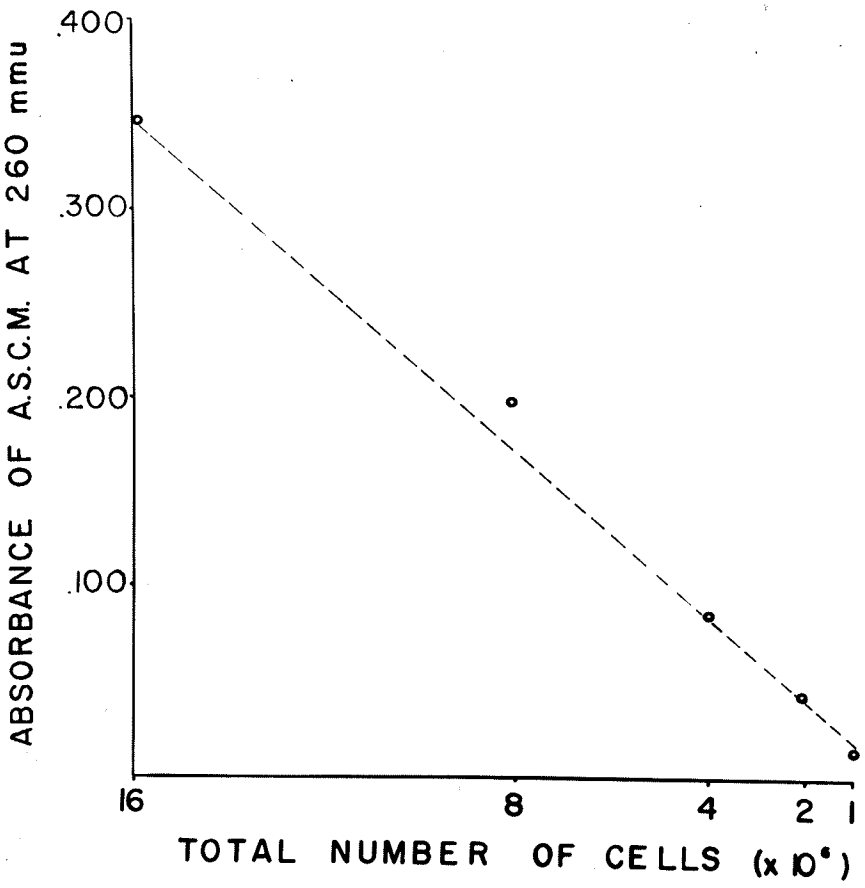

Figure 3. Effect of PEC concentration on the absorbance of ASCM.

The results are presented graphically in figure 4. Thirty minutes was adequate for cytolysis, and was the time chosen for incubation in complement.

Precision of the method. This experiment was performed in order to estimate the accuracy of the method. Five aliquots of a suspension of PEC at a concentration of 60,000 cells per cubic mm. were sensitized, washed, and incubated in 1:6 complement for thirty minutes at 37°C. A 0.5 ml. aliquot of each supernatant was hydrolyzed with 0.5 ml. 2.0 N perchloric acid, and the absorbance of each recorded. The volume of perchloric acid was increased to 0.5 ml. here and for the titration of antibody and complement so that more material was available for measurement. The final concentration of acid remained one normal.

The results are presented in table II. Error due to the machine is less than three per cent in this range of the scale (Hiskey, 1955). Presumably the error is due to technical limitations and is unavoidable with such small volumes.

Dye Exclusion

The cell sediment remaining in each tube after the supernatant had been removed for perchloric acid hydrolysis was examined for cytolysis in the following manner. Each tube received 0.15 ml. of cytolysis stain[5], containing a mixture of eosin and trypan blue. After mixing with a pasteur pipette, a drop of each suspension was placed in a hemocytometer. Differential counts of

[5] see appendix

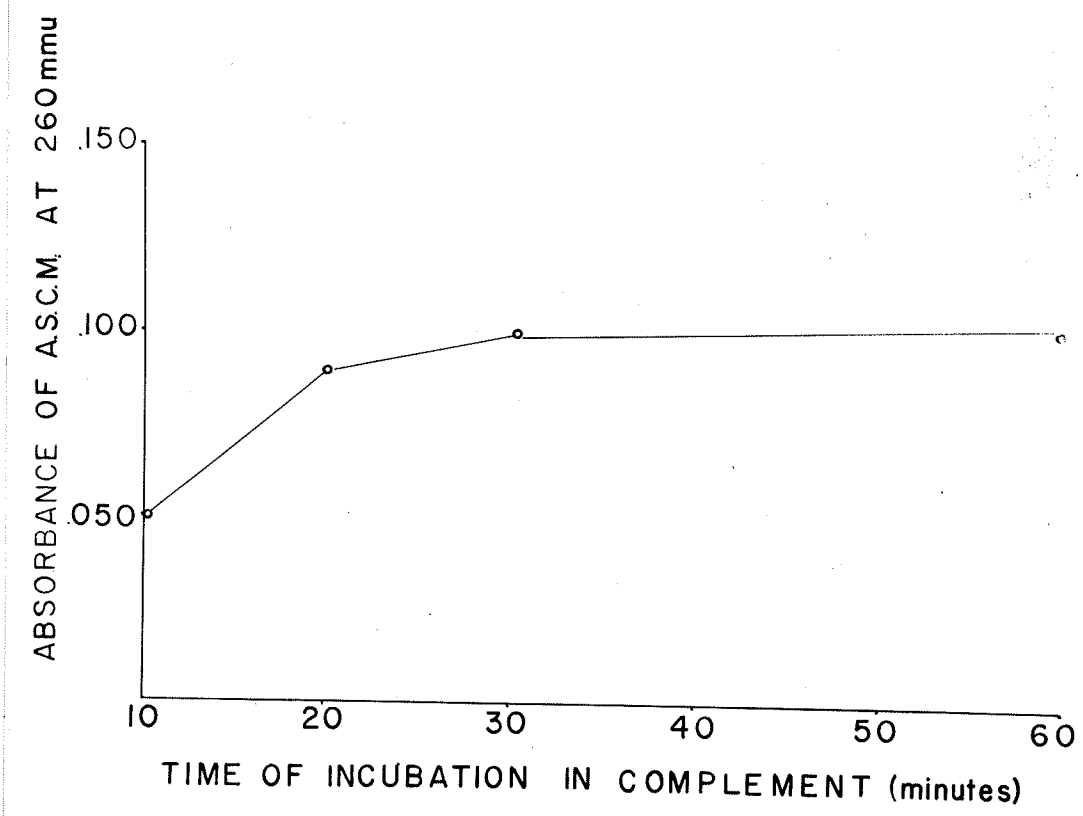

Figure 4. Effect of time allowed for cytolysis on absorbance of ASCM.

TABLE II

PRECISION OF THE SPECTROPHOTOMETRIC METHOD FOR MEASUREMENT OF LYSIS

Sample number	Absorbance at 260 mmu
1	0.290
2	0.325
3	0.325
4	0.390
5	0.350

mean = 0.336

standard deviation = 0.037

stained cells, unstained deformed cells, and normal cells were performed on two hundred cells from each tube. Clumps of more than six cells were not counted. Since very few cells which were deformed were not stained, both stained cells and unstained deformed cells were classified as damaged. The amount of cytolysis was expressed as the per cent of the suspension which was damaged.

III. OBSERVATIONS

Morphological Changes

Sensitized PEC were suspended in complement, and a drop of the suspension placed on a slide and covered with a coverslip. The resulting morphological changes during cytolysis were followed by phase microscopy.

The first change seen is the formation of cytoplasmic blisters or blebs on the cell membrane, which rapidly enlarge so that the whole cell becomes swollen. The cytoplasm becomes hyaline in appearance, and the cell membrane more sharply defined. The normally indistinct nucleus becomes well-defined, and large cytoplasmic granules cluster around it. A photomicrograph of PEC undergoing immune cytolysis is shown in figure 5. These changes are well-advanced within fifteen minutes after the addition of complement. No further alteration occurred, even after prolonged incubation. Rupture of the cell membrane or of the nuclear membrane was never seen, nor was complete lysis and disappearance of the cells. A

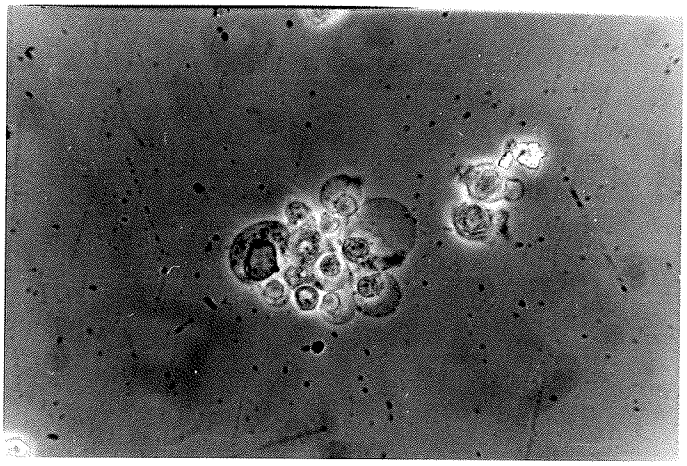

Figure 5. Sensitized PEC fifteen minutes after the addition of complement. Phase contrast, 900 x.

few cells in every suspension remained unaffected, even in the highest concentrations of antibody and complement.

Control suspensions examined at various times included unsensitized PEC in complement or in Tyrode's solution, sensitized PEC in heat-inactivated complement, and PEC which had been incubated in normal rabbit serum and suspended in complement. In all control preparations the cells retained their normal morphology. A photomicrograph of sensitized PEC resuspended in heat-inactivated complement is presented in figure 6. The cells are round, with indistinct cell outlines and no well-defined nuclear border. Sensitized cells were often clumped due to agglutination, but in the absence of complement no morphological changes were evident.

Titration of Cytolytic Antiserum

Doubling dilutions of antiserum were made in 0.2 ml. volumes of Tyrode's solution. A control tube containing only Tyrode's solution was included. Each tube received 0.2 ml. of a washed PEC suspension containing 60,000 cells per cubic mm. After sensitization and washing, the cell sediment in each tube was resuspended in 0.6 ml. 1:6 complement. For hydrolysis of the ASCM-containing supernatants, 0.5 ml. of each supernatant was added to 0.5 ml. 2 N perchloric acid.

The results of a titration of antiserum 16 are presented graphically in figure 7. Antiserum dilutions are expressed as the initial dilution before PEC were added.

The cell sediments left in the tubes after removal of the

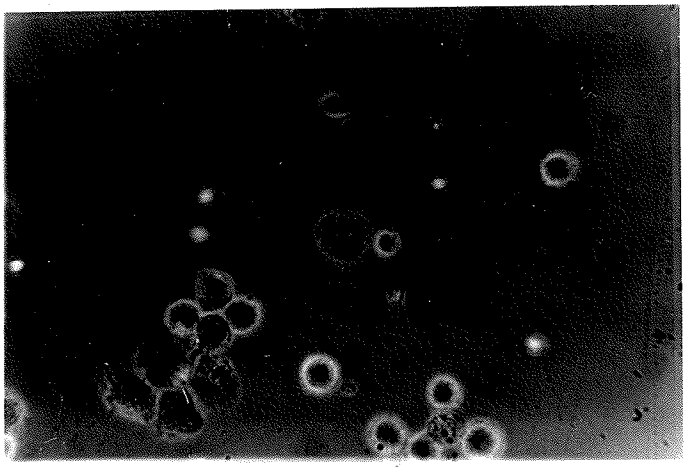

Figure 6. Sensitized PEC fifteen minutes after the addition of heat-inactivated complement. Phase contrast, 900 x.

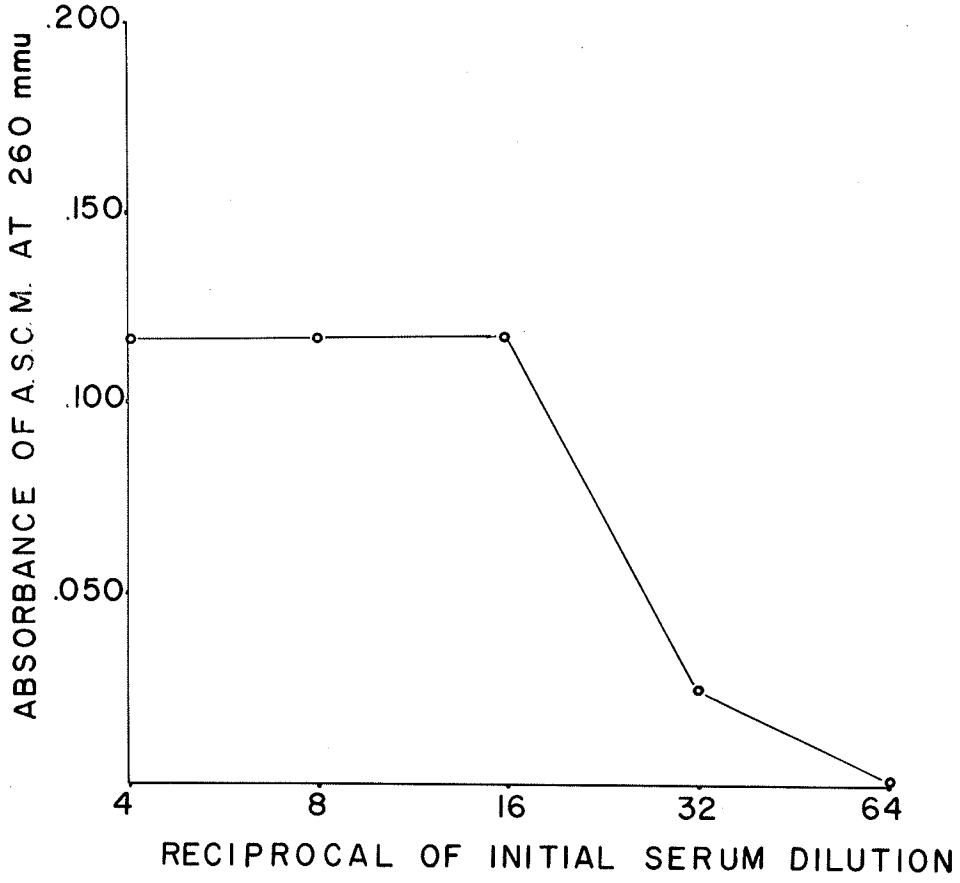

Figure 7. Spectrophotometric titration of antiserum 16 against PEC.

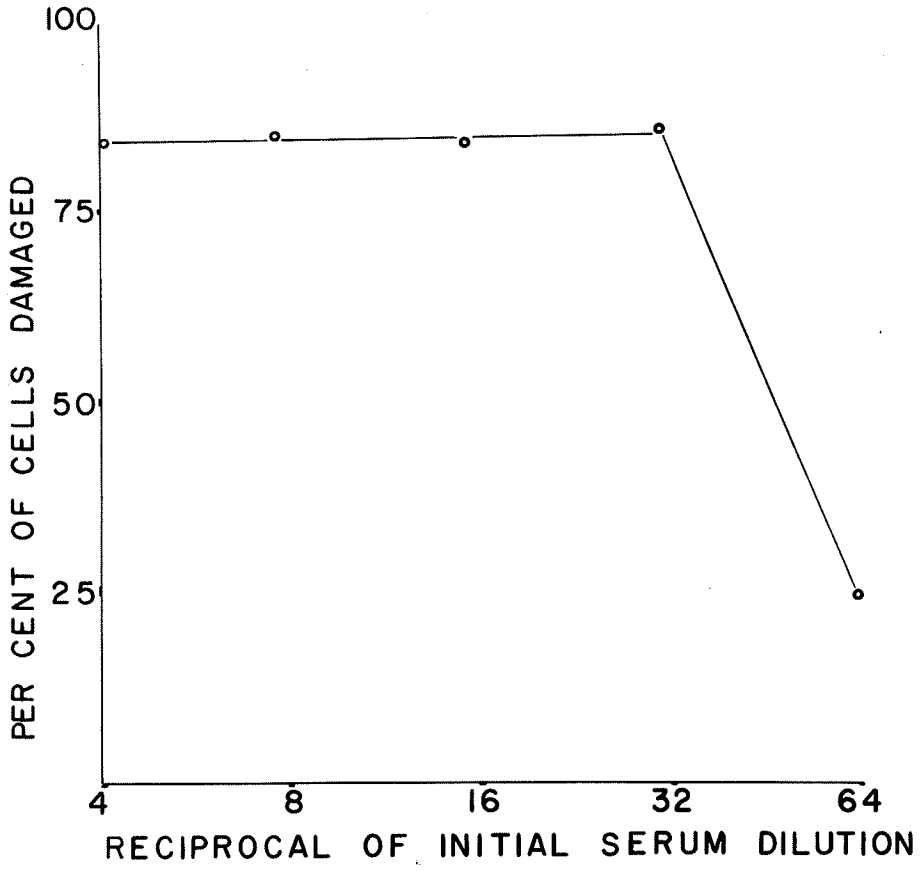

Figure 8. Titration of antiserum 16 against PEC by the dye exclusion technique.

supernatants for hydrolysis were mixed with 0.15 ml. cytolysis stain for titration by the dye exclusion technique. The results of this titration are presented graphically in figure 8.

From the results of the spectrophometric titration of antiserum 16, a 1:16 dilution was considered to contain one maximum cytolytic dose (CD_{max}) for an equal volume of PEC at a concentration of 60,000 cells per cubic mm. For sensitization of PEC for the titration of complement, 2 CD_{max} or a 1:8 dilution was used.

Titration of Complement

Doubling dilutions of complement, starting with a 1:4 dilution, were made in 0.4 ml. volumes of Tyrode's solution. Duplicate rows were set up. 0.2 ml. sensitized PEC was added to each tube of one row, to provide "test lysate", and 0.2 ml. normal PEC to the second row, to provide "control". The dilution of complement in the first tube of each row was now 1:6. After cytolysis and hydrolysis, the absorbance of each tube was measured against a distilled water blank. For purposes of comparison, these results are presented in figure 9a. To obtain the absorbance due to ASCM, each "control" value was subtracted from the corresponding "test lysate". The resulting curve is presented in figure 9b. The characteristic sigmoid shape of a complement titration curve was obtained.

For titration of complement by the dye exclusion technique, 0.15 ml. cytolysis stain was added to the cell sediment of

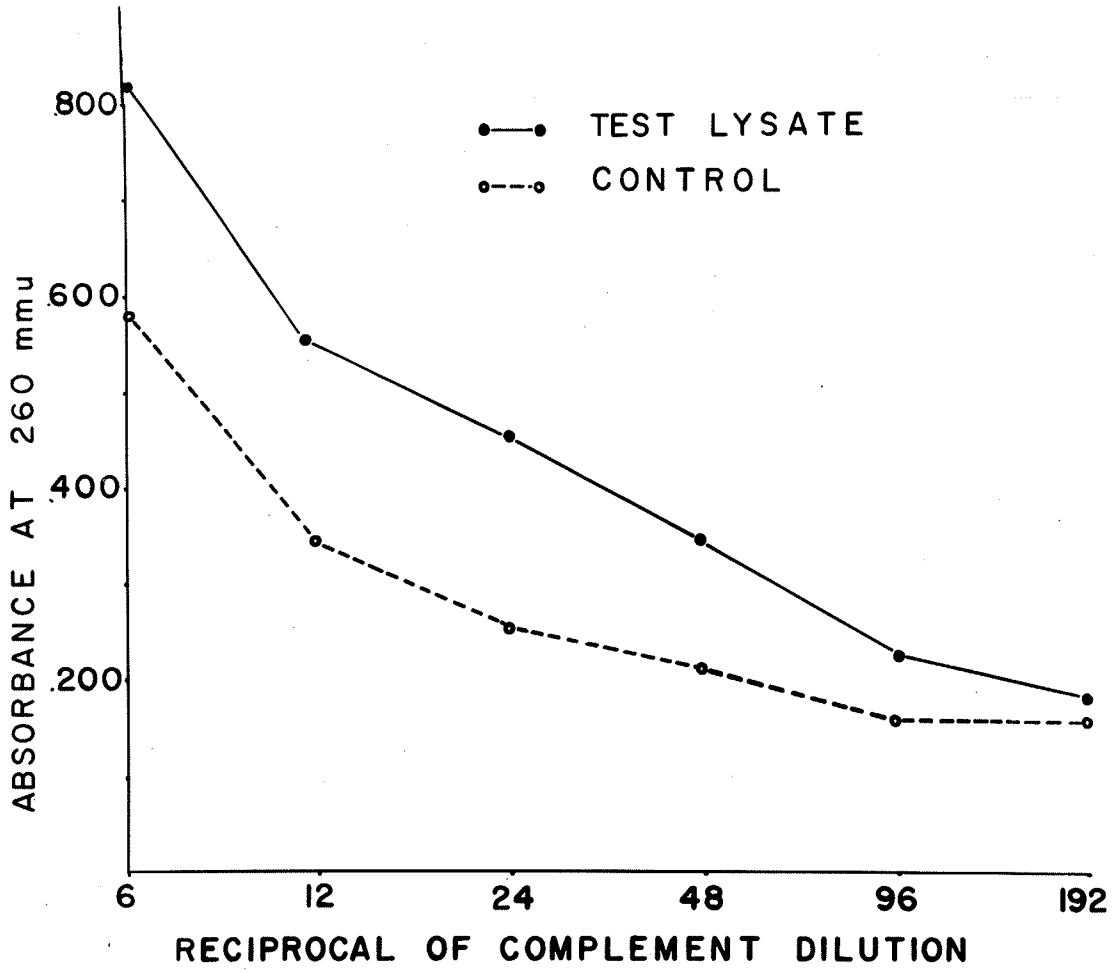

Figure 9a. Spectrophotometric titration of complement using sensitized PEC: absorbancies of "test lysate" and "control" read against distilled water.

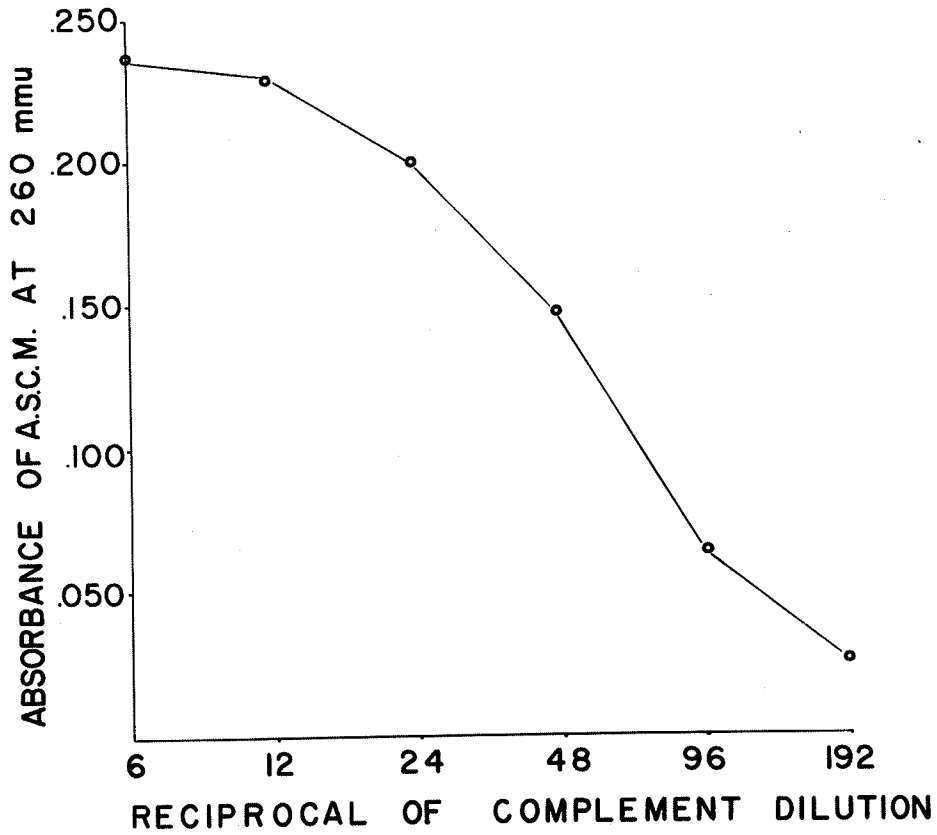

Figure 9b. Spectrophotometric titration of complement using sensitized PEC: absorbance due to ASCM.

each tube after the supernatant was removed. The per cent of damged cells was calculated. The results are presented in figure 10.

The value for each tube in figure 9b was expressed as per cent of maximum lysis, taking the absorbance due to ASCM in the first tube as representing maximum lysis. Log proability paper was used to plot these values, using per cent of maximum lysis as ordinates and ml. of undiluted complement per ml. as abscissae. The results are shown in figure 11.

A parallel titration was performed on the same sample of complement using sensitized guinea pig red cells and following the technique outlined under "Materials". The results of this titration are included in figure 11. In this case, maximum lysis is equal to one hundred per cent lysis.

In the guinea pig red cell system one HD_{50} is contained in 0.027 ml. undiluted complement per ml. reaction mixture, or a dilution of 1:37. This system contains approximately 25×10^6 cells per ml.

In the system using PEC, a fifty per cent cytolytic dose is contained in 0.018 ml. undiluted complement per ml. reaction mixture, or a dilution of 1:55. The cell concentration here is 20×10^6 cells per ml.

IV. DISCUSSION

The purpose of this part of the work was to demonstrate immune cytolysis of PEC <u>in vitro</u> on a quantitative basis, so that

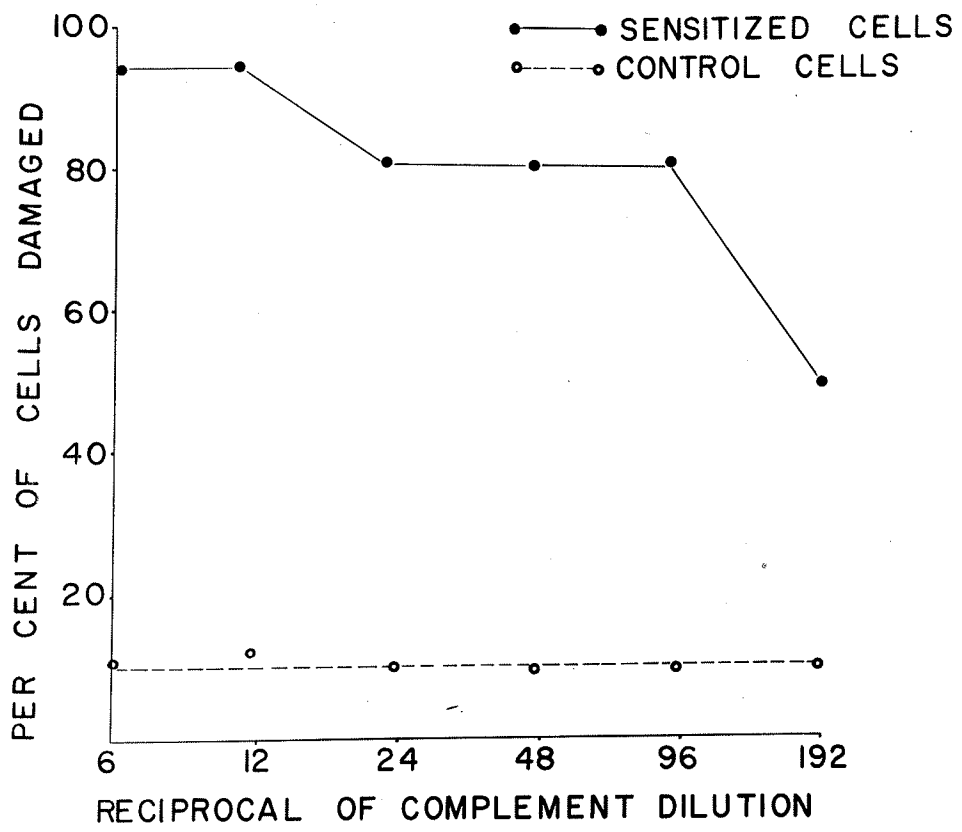

Figure 10. Titration of complement by the dye exclusion technique.

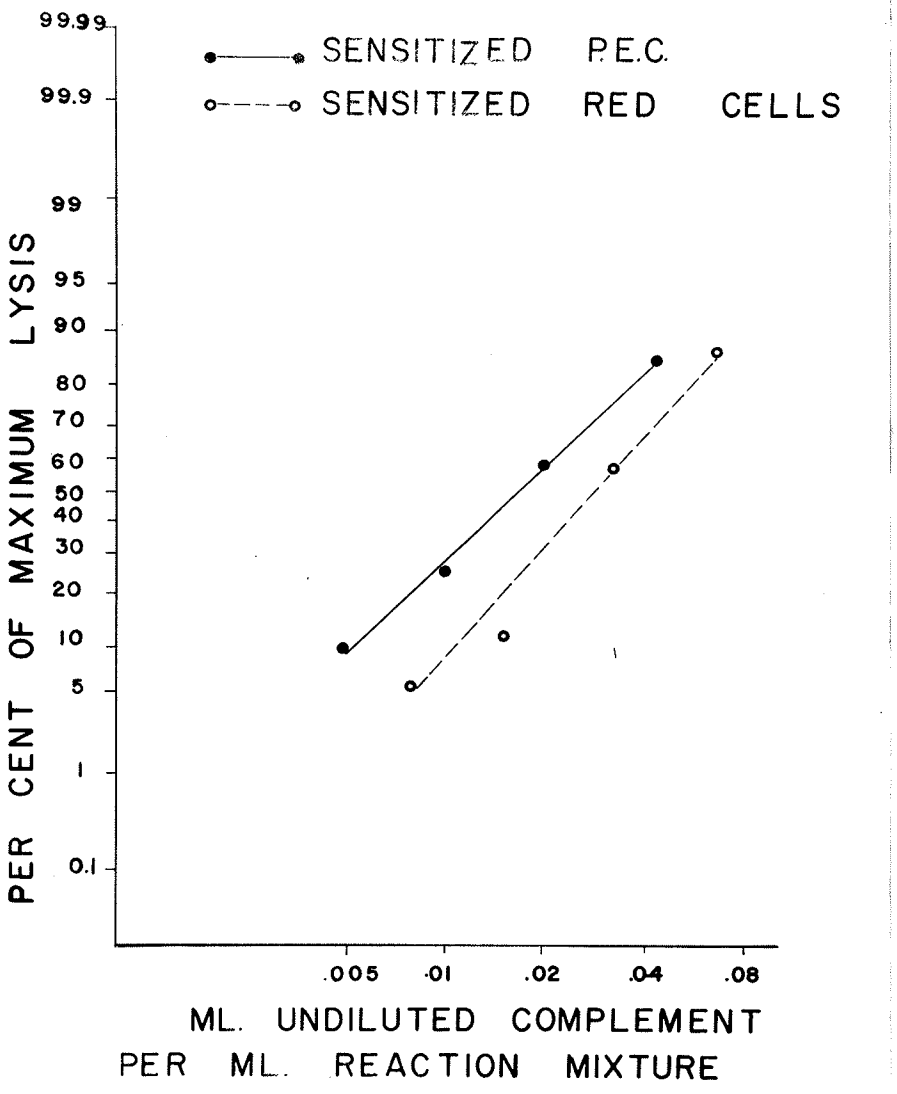

Figure 11. Titrations of complement with a) sensitized PEC and b) sensitized guinea pig red cells plotted on log probability paper.

it could be investigated in vivo. A method was devised to accomplish this, and compared with an existing method.

Immune cytolysis is a form of colloid osmotic lysis (Green and Goldberg, 1959). It is characterized by degenerative changes in the cells, including swelling of the cell and shrinkage of the nucleus, with accompanying loss of intracellular protein and nucleic acid. PEC undergoing immune cytolysis exhibit the same morphological degeneration described in the literature for other cells. The loss of intracellular nucleic acid has formed the basis of a spectrophotometric method of measuring cytolysis. The supernatant of cells which have undergone cytolysis is decanted. After precipitation of protein and hydrolysis with perchloric acid, ASCM (acid-soluble cellular material, presumed to be nucleic acid) is released. Cytolysis is measured as the absorbance of ASCM at two hundred and sixty mmu. It has been shown that the absorbance of a mixture of complement and ASCM is equal to the sum of their separate absorbancies, and therefore such a method is theoretically sound.

Titrations of antibody and complement have been performed with this method, and compared with the dye exclusion technique, an established method for demonstrating cytolysis on grounds of morphology and permeability to stains. The two methods differed slightly in the results obtained. The spectrophotometric method has the theoretical advantage that it measures only cell degeneration occurring due to incubation in complement, that is, only the

results of immune cytolysis, since any ASCM released by mechanically - damaged cells is washed away before complement is added. Cells damaged mechanically are included in estimates of immune cytolysis by the dye exclusion method.

Even in the highest concentrations of antibody and complement, from five to fifteen per cent of the cells retained their normal morphology and remained impermeable to stain. The reason for this is unknown, but two suggestions are offered. First, they may be antigenically different, and therefore insusceptible. Second, not enough time may have been allowed for complete cytolysis.

Because of this, it was assumed that the maximum absorbance due to ASCM did not represent one hundred per cent lysis, and accordingly the term "maximum cytolysis dose" (CD_{max}) was adopted for expressing doses of antibody and complement.

Complement was titrated with sensitized PEC, and with a guinea pig red cell-hemolysin system. Both systems gave similar results.

The spectrophotometric method makes it possible to study cytolysis of tissue cells on the same well-defined quantitative basis as the study of hemolysis. The time required to perform a titration is only a little longer than that required to perform titrations using a red-cell hemolysin system, and it is a less time-consuming method than one based on morphological degeneration or staining characteristics, and potentially more accurate.

CHAPTER III

IN VIVO CYTOLYSIS

CHAPTER III

IN VIVO CYTOLYSIS

I. MATERIALS

The preparation of PEC and of cytolytic and control antisera have been described under in vitro cytolysis in the section on "Materials". Cell suspensions were washed in exactly the same way, and the concentration adjusted to 60,000 cells per cubic mm.

II. METHODS

The intradermal route was chosen for injection because the cells then remained in a well-defined area, producing a local reaction, similar to a tuberculin reaction, which could be studied macroscopically as well as microscopically in stained sections of the skin.

All injections were given on the shaven abdomen of normal guinea pigs. Recipient guinea pigs were selected and shaven the day before an experiment.

For histologic examination of the skin, animals were first sacrificed by bleeding from the heart under chloroform. Specimens of skin were fixed in Carnoy's fluid[6] for six hours. The sections were stained with hematoxylin and eosin[7].

The following experiments were performed in developing the

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[6] see appendix [7] see appendix

method used to demonstrate cytolysis *in vivo*. Only brief descriptions will be given of each. In each experiment where sensitized PEC were used, PEC were divided into two parts. One part was incubated in 1:2 antiserum, and the other in 1:2 normal serum, for twenty minutes at 37°C. The volumes of serum used for sensitization varied. This account is divided into two sections: identification of injected cells, and the effects of antiserum *in vivo*.

Identification of injected PEC. In order to determine whether injected PEC could be found in sections of skin, a guinea pig received four intradermal injections of washed PEC. 1×10^6 cells were given in a volume of 0.1 ml. Very little reaction was observable microscopically. No trace of injected PEC was visible in stained sections of skin removed by biopsy one, two, three, and four days after injection.

It was therefore decided to attempt to mark the area into which the PEC were injected, so that it could be identified for sectioning. This could be accomplished by suspending the PEC for injection in a suitable dilution of India ink. An experiment to determine a suitable dilution was performed. Doubling dilutions of India ink, from 1:256 to 1:2048, were made in saline, and 0.1 ml. of each injected intradermally into each of three guinea pigs. India ink, diluted 1:2048 was still evident in sections after one week.

An attempt was made to use this marking method for the demonstration of cytolysis *in vivo*. 0.1 ml. of a suspension

containing 1 x 10^6 washed sensitized or control PEC suspended in 1:2048 India ink was injected intradermally into a normal guinea pig. The areas containing India ink could be identified in sections at forty-eight hours, but no collections of cells were seen.

Twelve guinea pigs were injected with varying numbers of sensitized and control PEC to determine how many cells had to be injected in order to find collections of cells microscopically. The total number of cells injected varied from 1 x 10^6 to 3 x 10^7. It was found that when 10 x 10^6 cells were injected, collections of cells could readily be found, even six days after injection, and marking with India ink was therefore unnecessary.

The effects of antiserum. When antiserum alone was injected, a severe inflammatory reaction was elicited. It was noticed that in experiments in which PEC were left suspended in antiserum or normal serum for injection, the reactions due to sensitized cells were larger than when the sensitized cells were washed. This was caused by the presence of excess antiserum, and henceforth all PEC were washed free of serum before they were injected.

No evidence of immune cytolysis could be found in any of these experiments. It was decided, therefore, that the most important problem was to learn how to sensitize PEC with a measured amount of antibody, so that it could be shown that they were lyzed by host complement in vitro. Only after sensitization and cytolysis could be quantitatively measured in vitro would it be practical to proceed with in vivo experiments. Methods for solving this problem

have been described in the previous section. Further attempts to demonstrate cytolysis *in vivo* were based on results obtained by the spectrophotometric method.

III. OBSERVATIONS

Washed PEC at a concentration of 60,000 cells per cubic mm. were sensitized with 2 CD_{max} antiserum, as described in the section on *in vitro* cytolysis, or with an equal amount of normal serum. After sensitization, the cells were washed once and re-suspended to one-half their original volume in Tyrode's solution. 12×10^6 cells were now contained in 0.1 ml., the amount given at each injection.

Recipient guinea pigs were bled the day before the experiment, and the serum collected and stored in the deep freeze. Before the PEC were injected, a drop of each cell suspension was mixed with a drop of cytolysis stain on a slide, and the number of damaged cells counted. At the same time, the serum of each recipient guinea pig was tested for its complementary activity on the injected cells in the following way: 0.1 ml. of each suspension was added to 0.5 ml. of a 1:5 dilution of recipient serum. The tubes were incubated for thirty minutes at $37^{\circ}C.$, and the cytolysis estimated by the spectrophotometric and dye exclusion techniques described under "Methods" of *In vitro* Cytolysis.

Each of four guinea pigs received 0.1 ml. sensitized PEC on the right side of the shaven area, and 0.1 ml. control PEC on

the left side. 0.1 ml. Tyrode's solution was injected in the centre. At various time intervals, the macroscopic appearance of the reactions was measured, and one animal sacrificed for microscopic examination of the reaction. In the first experiment, time intervals of one, two, four, and twenty-four hours were chosen; in the other two, time intervals of one, two, four, and eight hours. In all three, the results were identical.

Twenty-five per cent of the sensitized PEC took up cytolysis stain before they were injected, as compared with ten per cent of the control PEC. The cells were resuspended for injection by aspiration with the syringe used, and this vigorous treatment is assumed to be responsible for damaging the cells. It seems possible that sensitized cells are more fragile than normal, as suggested by Ellem (1958). However, because of this result, none of the evidence of cell degeneration described below can be considered as the result of immune cytolysis *in vivo*.

Sensitized PEC in all cases were lyzed by host complement *in vitro*, and normal cells were not affected. An average of eighty-seven per cent of the sensitized PEC were damaged as estimated by the dye exclusion technique, and the absorbance due to ASCM, calculated by subtracting the absorbance of control cell supernatant from the absorbance of sensitized cell lysate, had an average value of 0.205.

The macroscopic appearance of the reactions is summarized in table III. In each case, sensitized cells induced a slightly greater reaction than did control cells. No visible reaction was

TABLE III

SIZE OF REACTIONS PRODUCED IN NORMAL GUINEA PIGS BY THE INJECTION OF SENSITIZED AND OF NORMAL PEC

Guinea Pig Number	1		2		3		4	
Time After Injection (Hours)	Sens.	Norm.	Sens.	Norm.	Sens.	Norm.	Sens.	Norm.
1	12x14	12x10	13x14	6x9	8x8	6x6	8x8	6x10
2			12x16	7x7	8x10	6x8	8x10	6x8
3					8x10	6x8	8x8	5x6
4							5x5	4x4

produced by the injection of Tyrode's solution.

In all sections, even at the earliest time interval studied, a mass of basophilic stringy material was present. It is presumed that this material is composed of nuclei of degenerating cells. If this is so, the majority of injected PEC are destroyed almost immediately in vivo, whether or not they are sensitized.

At the edge of this mass, it is possible to identify mononuclear and polymorphonuclear leucocytes. Since the cell suspensions injected contained a low percentage of neutrophils, it is likely that most of the neutrophils are host cells. Presumably, most of the mononuclear cells were injected PEC, although some at least would be host cells.

In sections containing sensitized PEC, damage to these cells surrounding the basophilic mass was evident after one hour, and was maximal in two hours. This damage was shown by the presence of many pyknotic and karyorrhectic nuclei. It was not seen to the same degree in control sections. Figure 12 is a micrograph of an area in which sensitized PEC are undergoing karyorrhexis. Figures 13a and 13b are micrographs of an area containing control PEC. Very little karyorrhexis is present. These sections are from guinea pig 2 (see table III), and were taken two hours after the injection of PEC.

Twenty-four hours after injection, this evidence of damage had disappeared. Sections containing sensitized PEC were indistinguishable from those containing control PEC. Both contained healthy - looking mononuclear cells in the area of the basophilic mass.

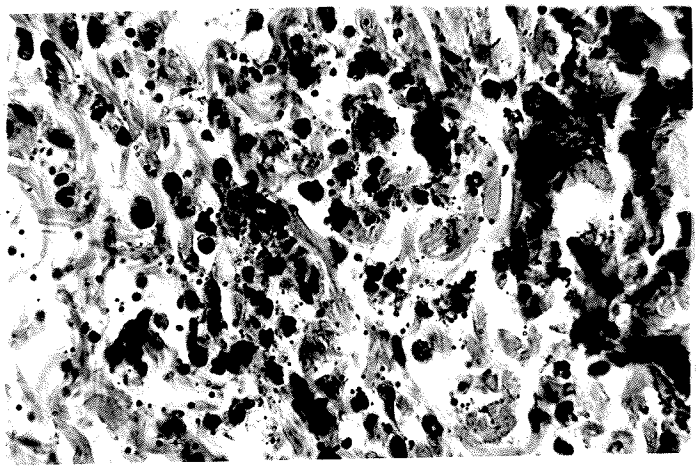

Figure 12. Sensitized PEC two hours after intradermal injection into a normal guinea pig. Hematoxylin and eosin 900 x.

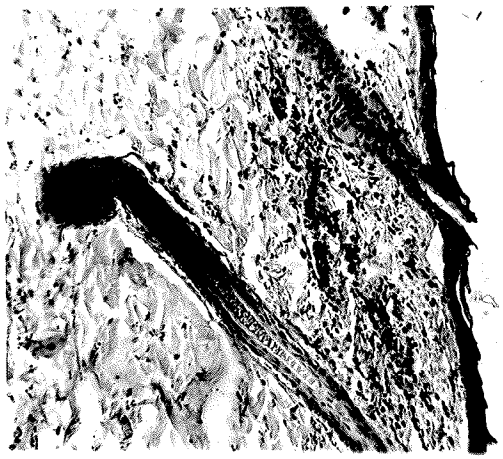

Figure 13. Normal PEC two hours after intradermal injection into a normal guinea pig. Hematoxylin and eosin 80 x.

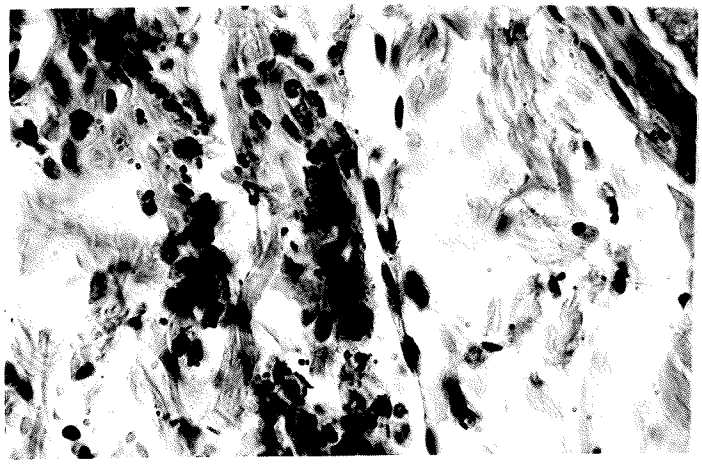

Figure 13b. Normal PEC two hours after intradermal injection into a normal guinea pig. Hematoxylin and eosin 900 x.

It is clear that when such large numbers of PEC are injected intradermally a large number of them die, whether or not they were sensitized. This is evident from the large mass of nuclear stringy material. Damage to the cells surrounding this material occurred only when sensitized PEC were injected. It is tempting to suggest that this destruction of sensitized PEC is due to the action of host complement *in vivo*, but in view of the results of the *in vitro* dye exclusion test, it is impossible to draw this conclusion.

IV. DISCUSSION

The purpose of this part of the work was to demonstrate immune cytolysis of PEC *in vivo*. Many descriptions of acute morphological changes caused by antibody and complement acting on different types of cells *in vitro* have been recorded, but a search of the literature reveals only one attempt (Flax, 1956) to demonstrate morphological changes seen in tumor cells undergoing cytolysis *in vivo*.

The problem, then was to show whether or not sensitized PEC in a homologous host disintegrate due to the action of host complement the same way as they do *in vitro*.

Techniques for injecting sensitized PEC and following their degeneration in the host were developed only after many attempts. It was found that large numbers of cells had to be injected in order to find them in sections, but the dense stringy basophilic mass seen in all sections, whether containing sensitized or control PEC,

suggested that when such large numbers are injected, a considerable percentage are destroyed, presumably due to unphysiological conditions in the injected area. For this reason it was very difficult to decide whether sensitized cells behaved differently from normal cells *in vivo*.

Only three experiments were adequately controlled to ensure that host complement destroyed optimally sensitized cells *in vitro*. In these experiments, specific destruction of sensitized cells was evident. This was seen as karyorrhexis surrounding a dense area of injected material.

No conclusions can be drawn as to whether this destruction is evidence of immune cytolysis of PEC *in vivo*. First of all, some twenty-five per cent of the sensitized PEC were damaged before the cells were injected, and karyorrhectic nuclei seen in sections may be derived from these cells. Secondly, there is no way of identifying the damaged cells as PEC instead of host cells.

It was noticed that injection of the same suspension of PEC into different guinea pigs produced reactions of different sizes and intensities. Joysey (1959) reported that well-defined blood groups do not exist in guinea pigs, but it is possible that differences in isoantigens could have been responsible for this observation.

Because of these drawbacks, the results obtained from these morphological studies are difficult to interpret. Accordingly, an experiment was performed in which loss of function was the cri-

terion for cytolysis. Attempts were made to passively transfer local cutaneous hypersensitivity by the technique of Metaxas and Metaxas-Bühler (1948), and to block this transfer by sensitizing the hypersensitive PEC *in vitro* before transfer, as was done in rabbits by Warwick *et al.* (1962). Immune cytolysis of PEC *in vivo* might then prevent the local reaction to tuberculin. However, a positive transfer was not achieved with control cells, and therefore the results have not been included.

It is suggested that for a detailed study of this phenomenon another type of cell be used. If tumor cells are chosen, their immune cytolysis would result in inhibition of tumor formation, and the fate of injected cells could be easily established. A tumor such as the Ehrlich ascites mouse tumor is suggested, since the cells may be studied in smears, and large numbers of mice can be used. Information gained from such a study could be applied to a further investigation of the immune cytolysis of normal cells *in vivo*.

CHAPTER IV

SUMMARY

CHAPTER IV

SUMMARY

The effects of cytolytic antibody and complement on guinea pig peritoneal exudate cells have been investigated.

The morphological changes produced in PEC undergoing immune cytolysis are identical with those described for other types of cells. Swelling of the cells is a prominent feature, with an accompanying loss of cytoplasmic density.

Cells undergoing cytolysis become permeable to a staining solution containing eosin and trypan blue. This has been used as a basis for one method of titrating antiserum and complement.

An original method for measuring cytolysis has been devised, based on the observation that cells lose nucleic acid to the medium during immune cytolysis. The absorbance due to nucleic acid gained by the medium is measured after precipitation of the protein and hydrolysis with perchloric acid. Optimum conditions have been defined for this method. Antibody and complement have been titrated, and the results compared with the results obtained by the dye exclusion technique. The curve obtained for the titration of complement is very close to that obtained when complement is titrated using a guinea pig red cell-hemolysin system.

Attempts have been made to demonstrate immune cytolysis *in vivo*. The results of these experiments were equivocal.

CHAPTER V

BIBLIOGRAPHY

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CHAPTER VI

APPENDIX

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1. Tyrode's Solution (mod. from Parker, 1961)

NaCl	80.0 gms.
KCl	2.0 gms.
$NaH_2PO_4 \cdot H_2O$	0.5 gms.
Glucose	10.0 gms.
$CaCl_2$	2.0 gms.
$MgCl_2 \cdot 6H_2O$	1.0 gms.

Dissolve the last two components in 100.0 ml. distilled water, add to the solution of other components and make up to 2 l. Sterilize by autoclaving. For use each day, add 20.0 ml. to 80.0 ml. distilled water and adjust the pH to 7.35 by the addition of 1.4% $NaHCO_3$.

2. May-Grünwald Giemsa Stain (Khairat, personal communication)

1. Fix dry coverslip smears five minutes in methyl alcohol. Dry.
2. Stain ten to fifteen minutes in a coverslip staining jar containing:

1.0 ml. May-Grünwald

2.0 ml. Giemsa

22.0 ml. 0.1 M phosphate buffer pH 7.2

3. Sato and Sekiya Peroxidase Stain (Bessis, 1956)

1. Apply 0.5% $CuSO_4$ to a fresh dry smear one minute. Pour off.

2. Apply saturated benzidene and H_2O_2 two minutes. Gently wash off.

3. Counterstain two minutes with 1% safranin.

4. Alsever's Solution (mod. from Osler et al., 1952)

dextrose	10.25	gms.
Na citrate	4.0	gms.
NaCl	2.1	gms.
citric acid	0.275	gms.
distilled water	500.0	ml.

Adjust to pH 6.1.

Sterilize by Seitz filtration.

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

Dissolve 500 mg. in trypan blue in 100.0 ml. hot Tyrode's solution. Add 150 mg. eosin, with stirring. Store at $4^{\circ}C$.

6. Carnoy's Fluid (Jones, 1950)

absolute alcohol	60.0 ml.
chloroform	30.0 ml.
glacial acetic acid	10.0 ml.

7. Routine Hematoxylin andEosin Stain (Armed Forces Inst. Path, 1957)

Solutions

1. Harris's Hematoxylin

hematoxylin crystals	5.0 gms.
alcohol, 95%	50.0 ml.

ammonium or potassium alum	100.0 gms.
distilled water	1000.0 ml.
mercuric oxide	2.5 gms.

Dissolve the hematoxylin in the alcohol, the alum in the water by the aid of heat. Mix the two solutions. Bring the mixture to a boil as rapidly as possible and then remove from the heat and add the mercuric oxide. Reheat the solution until it becomes a dark purple, about one minute, and promptly remove the container from the flame and plunge it into a basin of cold water. The solution is ready to use when cool. Add 2.0 to 4.0 ml. of glacial acetic acid to 100.0 ml. of solution if desired.

2. Acid Alcohol

70% alcohol	1000.0 ml.
concentrated hydrochloric acid	10.0 ml.

3. Ammonia Water

tap water	1000.0 ml.
strong ammonia water	2 to 3 ml.

4. Saturated Lithium Carbonate

lithium carbonate	1.0 gm.
distilled water	100.0 ml.

5. Alcoholic Eosin Solution

Eosin Y, water soluble	2.0 gms.
distilled water	160.0 ml.
95% alcohol	640.0 ml.

Dissolve eosin Y in the distilled water, then add the 95%

alcohol. If a deeper shade is desired, add a drop of acetic acid to each 100.0 ml. of solution.

6. Lugol's Iodine (Weigert's Modification)

potassium iodide	2.0 gms.
iodine crystal	1.0 gm.
distilled water	100.0 ml.

7. Alcohol Iodine Solution

iodine crystals	1.0 gm.
95% alcohol	100.0 ml.

8. Sodium Thiosulfate Solution (Hypo)

sodium thiosulfate	5.0 gms.
distilled water	100.0 ml.

Staining Procedure

1. Xylene, absolute alcohol, 95% alcohol.
2. Tap water.
3. Harris's hematoxylin for fifteen minutes.
4. Rinse in tap water.
5. Differentiate in acid alcohol.
6. Wash briefly in tap water.
7. Dip in ammonia water or saturated lithium carbonate until sections are bright blue.
8. Wash in running tap water ten minutes.
9. Stain with eosin one minute.
10. 95% alcohol.
11. Absolute alcohol - at least two changes.

12. Xylene - two changes.

13. Mount in permount.