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THE EXTRACTION OF MUCOPOLYSACCHARIDES FROM THE
ORAL MUCOSA OF THE HAMSTER BUCCAL POUCH

by

JOSEPH WAYNE ROSSA

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF LOYOLA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE - JUNE, 1970

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LIFE

Joseph Wayne Rossa was born in Chicago, Illinois, on July 27, 1941. He graduated from Weber High School in Chicago, Illinois, in June, 1959. He attended the College of Arts and Sciences, Loyola University, Chicago, Illinois, for two years in the pre-dental curriculum, and in September, 1961, entered Loyola University School of Dentistry in Chicago, Illinois. A degree of Doctor of Dental Surgery was conferred in June, 1965.

The following year was spent in the private practice of dentistry with his father, a Loyola Dental School alumnus, Class of 1939.

In June, 1966, he was commissioned a Lieutenant in the Dental Corps of the United States Navy Reserve and served two years of active duty at Great Lakes Naval Training Center, Great Lakes, Illinois. He was released from active duty on June 30, 1968.

In September of that year, he entered the graduate school of Loyola University to begin a two year program toward a Master of Science degree in Oral Biology.

On January 10, 1970, he received an appointment to Cook County Hospital, Chicago, Illinois, for a two year residency in Oral Surgery.
To my wife
Diane Rose
and my parents
Elizabeth and Joseph
ACKNOWLEDGEMENTS

***********************

My most sincere thanks to Dr. Patrick D. Toto who provided a great measure of stimulation in the preparation of this thesis. His valuable suggestions and constructive criticism contributed much to this work.

To Dr. A. B. Reiskin who provided the initial insight into the intricacies of the carcinogenic aspect of this study and whose wisdom and judgment added much to the final content and form of this work.

To Dr. John Madonia whose helpful suggestions and advice in the immunologic aspect of this study was essential to its progress.

To Dr. B. Jarislow for his advice on immunization schedules and his interest in detail.

To the entire staff of the Department of Oral Pathology, Loyola University School of Dentistry, for their technical assistance in the preparation of tissue sections and staining.
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CHAPTER I
INTRODUCTION

In the 19th century, the cell came to be thought of as "the unit of living matter" (Herbert Spencer) or "the primary representative of life" (Claude Bernard). It was also discovered that a single cell can make up an entire organism or it can be one of a complex of many cells that are aggregated and differentiated into tissues and organs. The cell is the basic structural and functional unit of living organisms, just as is the atom in chemical structures.

Biochemical investigations have shown that the products of living matter and the living matter itself are composed of the same basic elements that compose the inorganic world. Biochemists have isolated from the complicated matter of cells not only inorganic constituents but also more complex molecules such as proteins, fats, polysaccharides, and nucleic acids.

The cell theory has clarified all the fields of biological research. It has been shown that every cell is formed by the division of another cell and that there are basic similarities in the chemical composition and metabolism of all cells. The function of the organism as a unit was also shown to be a result of the sum of the activities and relationships expressed by the individual cells.

The cell is a unit that possesses a different internal milieu than that of its environment. This difference is maintained throughout the life of the cell by the surface membrane which controls selectively the entrance and exit of molecules and ions. This surface membrane is so thin that it cannot be seen with the light microscope, but in some cells it is covered by
thicker layers of protective substances which are within the limits of observation with the light microscope.

The ability to generate new properties by the simple rearrangement of pre-existing parts is a basic characteristic of all living systems. Complex antigens illustrate this characteristic at the molecular level, and the surface membrane of cells provide and excellent example at the organelle level of cell organization. The surface membrane and the extraneous coats are intimately related to the specific association of cells to form tissues. The mechanism by which a cell can "recognize" and exist with other cells of similar kind takes place at the cell surface. The properties of the cell membrane, and in particular its adhesive characteristics, are decisive in regulating the association of cells. Whether cells take up special positions with reference to adjacent cells, remain fixed in position, or continue to migrate is of critical importance in embryonic development. Also, the capacity of malignant neoplasms to metastasize is surely dependent upon the adhesive properties of the cell membrane.

In the transformation of normal cells into metastasizing neoplasms, the cell membrane becomes less adhesive, and the neoplastic cell is enabled to dissociate from the surrounding cells and to infiltrate various organs. It is precisely this infiltration, coupled with continued proliferation, that makes such neoplasms malignant. However, the surface membrane of such cancer cells is probably very similar to the cell membranes of many kinds of embryonic cells. At least one kind of normal adult cell also has a non-adhesive surface membrane that permits it to migrate extensively throughout other tissues of the body. This is the leukocyte. If the normal migration
of leukocytes were coupled with uncontrolled cell division in the peripheral locations to which they migrate, they would certainly prove to be malignant. Thus, it is clear that cancer cell membranes, although relatively non-adhesive, are not necessarily abnormal. The malignant abnormality lies merely in coupling the production of non-adhesive membranes to continued cell division.

Immunoochemical principles are playing an important role in today's study of malignant change. The highly specific nature of the antigen-antibody reaction and the progress in the immunologic aspects of cancer has prompted researchers optimistically to predict a cure in cancer cases. Tumor-specific antigens possibly may be the key in the immunologic breakthrough in malignant change. It is for this reason that a thorough method for the investigation of antigenic profiles of normal and malignant cell surfaces must be devised. It is my belief that the extraction and treatment of antigenic components related to cell surfaces must be thoroughly studied in order to validate any further work concerning these antigens.
CHAPTER II

REVIEW OF LITERATURE

A. Chemical Carcinogenesis

1. Cellular and Tissue Response and the Effect of Extrinsic Factors

Sir Percival Pott (1775) stated that soot was the cause of cancer of the scrotum in chimney sweeps. Kennaway and Heiger (1930) were the first to describe the carcinogenic polycyclic hydrocarbons. Gye and Foulds (1939) first studied the subcutaneous carcinogenic capacity of 3,4-benzpyrene.

Levy (1950) noted the following histologic picture in the mucous membrane of mice following a single topical application of 9,10-dimethyl-1,2-benzanthracene (DMBA). The amount of surface keratin was increased and there was an increase in the size and number of granules in the stratum granulosum with some of the granules extending into the stratum spinosum. There was a slight hyperplasia of the epithelial cells, the proliferation being most marked in the stratum germinativum. There were hydropic changes in the cells of the stratum spinosum and the stratum granulosum. The lamina propria was thickened by edema and a fine, fibrous precipitate.

Shimkin (1954) stated that those substances which are capable of initiating malignant change in animals are termed "carcinogenic agents". These agents may be either chemical, physical, or living. A direct causative effect between the substance and the malignancy produced is not implied. All that is stated is that in animals injected or exposed to these agents or procedures, tumors arise in significantly higher incidence than in untreated animals.
Salley (1954) studied the carcinogenic activity of 3,4-benzpyrene, 3-methylcholanthrene, and 9,10-dimethyl-1,2-benzanthracene, dissolved in acetone and benzene respectively; he found that the most efficient combination was 9,10-dimethyl-1,2-benzanthracene in acetone, the first evidence of neoplasia being noted at seven weeks in the cheek pouch of the Syrian hamster.

Salley (1955) noted that a non-volatile solvent (mineral oil, U.S.P., heavy) for 9,10-dimethyl-1,2-benzanthracene decreased the tumor induction time from 6 to 7 weeks (acetone solvent) to $4\frac{1}{2}$ weeks. Control animals painted with mineral oil per se exhibited no neoplasia after fifty weeks of treatment and observation. In pouches treated with mineral oil solutions, the vehicle containing the carcinogen could be seen as droplets (5 to 7 microns in diameter) on the basal epithelial cells twenty-four hours after applications. In forty-eight to seventy-two hours these droplets were emulsified into small particles which were distributed in the intercellular spaces of the basal epithelial cell layer. When the same hydrocarbon was dissolved in acetone and topically applied and observed in vivo, there was rapid volatilization of the solvent, leaving a residue of crystalline DMBA adherent to the keratinized surface of the epithelium. At no time could particles be seen inside the epithelium, as was the case with mineral oil. It appears that mineral oil as a solvent acted as a co-carcinogen on oral epithelium by causing rapid dissemination of the cancer-inducing compound in and around epithelial cells. Less tissue deformity was also observed when mineral oil was the solvent than when
volatile substances were used.

Salley (1957), in a study of the reaction of the hamster cheek pouch to 9,10-dimethyl-1,2-benzanthracene, stated that the reaction of the oral mucosa may be divided into four stages before neoplastic transformation. The four stages were inflammation, degeneration (Fig. 1), regeneration, and hyperplasia.

Goldhaber (1958) found that topically applied carcinogens became localized in high concentration in mucosal ulcerations.

Rose and Gorlin (1958) said that an increased incidence in experimentally induced malignancies occurred in animals deficient in Vitamin A.

Morris (1958) noted that the basement membrane of epithelium undergoing pre-neoplastic hyperplasia exhibited increased thickness; that the basement membrane of epithelium contained in papillomatous tumors was less defined, thinner, and less intensely stained than that of normal or hyperplastic epithelium; and that in areas of cellular invasion, no basement membrane could be demonstrated. These observations were made in hamster cheek pouch treated with 9,10-dimethyl-1,2-benzanthracene and stained with toluidine blue after sulfation.

Salley (1961) stated that detectable fluorescence of the carcinogenic material was present in skin after one painting of carcinogen, and in the oral mucous membranes following three to four applications.

Morris (1961), in investigating the influence of extrinsic carcinogenic factors, found that the tissues of the cheek pouch of old hamsters are more resistant to carcinogenic stimuli than those of young
hamsters and that five weeks after birth appears to be the ideal age for hamsters used for experimental carcinogenesis from the standpoint of ease of manipulation and tumor production; that 0.5% concentration of DMBA is the optimal concentration for the rapid production of malignant tumors in the hamster cheek pouch; that a shorter latent period is required for tumor development in animals exposed to carcinogen three times a week as opposed to two times weekly; that a smaller total dose of carcinogen is required to produce tumors when it is applied twice weekly than when applied three times a week; and that the response of the hamsters to the carcinogen is not related to the sex of the animal.

Reiskin and Berry (1969) showed that squamous cell carcinomas induced in the cheek pouches of eight to twelve week-old Syrian hamsters by 7,12-dimethylbenzanthracene painting differed in behavior according to the host strain. The mean latent period in inbred dark-eared partial albino (DEA) hamsters was 7.3 weeks, compared to 10.0 to 10.75 weeks in random-bred golden or cream hamsters. The average tumor growth rate was significantly higher in DEA animals than in the other two strains.

Dipaolo, Donovan, and Nelson (1969) stated that the carcinogenic polycyclic hydrocarbons which were added to the cells of Syrian hamster embryos resulted in the formation of altered clones not seen with control conditions. The frequency of appearance of altered clones was related to the known carcinogenic potency of the compounds tested. Toxicity increased with the amount of the compound tested and was related to its potency as a carcinogen. At constant carcinogenic concentration the number of altered clones increased with the number of cells exposed. They also stated that of
the several carcinogenic polycyclic hydrocarbons which caused transformation in the embryonic Syrian hamster cell cultures, the most active compounds were 7,12-dimethylbenzanthracene (the older term denoting 9,10-dimethyl-1,2-benzanthracene) and 3-methylcholanthrene which caused 16.9% and 8.4% transformed clones at 0.1 and 5.0 micrograms per milliliter, respectively.

Duncan, Brookes, and Dipple (1969), in studying the relation of certain hydrocarbons to metabolism and binding to cellular macromolecules, found that the potent carcinogens were able to bind DNA and RNA ten times more readily than the non-carcinogens. There was no appreciable difference in binding capacity to other cellular protein between the carcinogens and non-carcinogens. There was no appreciable difference in binding capacity to other cellular protein between the carcinogens and non-carcinogens.

Levy, Taylor, and Bernick (1969) said that histologic and electron microscopic study of inflamed gingival tissue reveal changes in both the epithelium and the connective tissue. These changes appear to be morphologically related. Although precise mechanisms are not known, there is evidence that a metabolic or chemical change in one of the tissues influences the behavior or metabolism of the other.

2. Relevance to Studies on Cell Surface Antigens

Weiler (1952) described an organ-specific antigen for all healthy tissues of rat liver which was absent in hepatoma. He also correlated the loss of antigenicity with the grade of malignancy. He noted that the organ-specific antigen of normal kidney cytoplasmic particles were absent in kidney tumors using a quantitative method (1956a) and also by utilizing the fluorescent antibody technique (1956b).
Kay (1957), in studying the A and B antigens of the epithelium of the human urinary tract, noted that tumors arising from this epithelium may fail to exhibit these antigens. He attributed this to the inability of malignant cells to absorb antibody. Due to the fact that his results were variable in some instances, and that an "all-or-none-" phenomenon was not apparent, he concluded that the loss of antigen was not of genetic consequence.

Kay and Wallace (1961), in studying blood group substances in normal and malignant urinary epithelium, stated that a complete or partial loss of antigen was frequently observed. Correlation with other properties of the tumors revealed, with some exception, increased frequency of antigen loss particularly among the more pleomorphic, anaplastic, infiltrating, and rapidly fatal tumors.

Carruthers and Baumler (1965) tested mouse epithelium using fluorescein-labeled antibody as an immunochemical stain and suggested that normal and hyperplastic epidermis contained antigens not found in squamous cell carcinoma and that squamous cell carcinomas contained antigens not found in normal and hyperplastic epidermis.

Toto (1967) stated that altered cell surfaces in cancer are probably genetically determined, which may result in differences in negative charge densities, perhaps causing cells to lose their cell connection and preventing formation of the basement membrane.

Prendergast (1967) in working with both normal and cancerous human oral epithelium showed that malignant tissues as compared to normal tissues exhibit a reduced if not an absent reaction to anti-A or anti-B serum, the
antiserum used corresponding to the tissue type.

Baldwin and Moore (1968) exhibited the membrane-associated tumor-specific antigens from rat hepatomas induced by aminoaazo dye. Rat hepatoma D23 (induced by 4-dimethylaminoazobenzene) transplanted in syngeneic Wistar rats was used for the isolation of membrane fractions. The antigenicity of tumor cell membrane fractions was assayed by their capacity to absorb antibody from iso-immune or tumor-specific immune sera. Immunofluorescent staining of hepatoma D23 cells with tumor-specific antiserum was much less intense than that observed with isoantisera. Pre-absorption of the antiserum with a concentrated soluble cytoplasmic protein did not neutralize the tumor-specific antibody and it is concluded that the removal of antibody following treatment with hepatoma D23 cells or membrane is not due to non-specific absorption.

Lapp and Toto (1969) concluded that the aging process does not influence the antigenic reactivity or the cellular productivity of the blood-group substances in human stratified squamous epithelium of the oral cavity.


3. Contact Inhibition

Abercrombie and Heaysman (1953) discussed the loss of contact inhibition of sarcoma cells of fibroblasts in relation to one another or with normal fibroblasts.

Ambrose (1958) said that the contact between the cancer cells and the normal tissue cells which they invade is probably conditioned by the
surface properties of their cellular membrane.

Ambrose, James, and Lowick (1956), in comparing the electrical properties of the cell surfaces exhibited in normal and homologous mouse kidney tumor cells, noted that the tumor cells exhibited twice the average net negative charge density of the normal cells from which they were derived.

Carruthers and Suntzeff (1944) suggested that estimations of the calcium content of mouse epidermis during the process of experimental carcinogenesis revealed two distinct phases: an immediate reduction of the calcium level by as much as fifty per cent within ten days from the initiation of carcinogenesis, and a further reduction when the epithelial cells have been transformed into cancer cells. They further deduced that the reduction of calcium in the hyperplastic epidermis is an important feature in this experimentally induced pre-cancerous condition.

Coman (1944) stated that decreased adhesiveness of cells of squamous cell carcinoma was related to their decreased calcium content and that much of the phenomenon of metastasis could be directly related to this fact.

Brunschwig, Dunham, and Nichols (1946) noted that less calcium and more potassium was found in gastric carcinoma than in adjacent uninvolved gastric mucosa.

Dunham, Nichols, and Brunschwig (1946) said that less calcium and more potassium was found in carcinoma of the colon than in adjacent normal mucosa. The calcium content of normal and cancerous colonic mucosa was higher than in the normal and cancerous gastric mucosa respectively. This was to be expected since calcium is normally excreted by the mucosa of the colon.
Coman (1947) concluded that the mechanism of the invasiveness of cancer was dependent upon decreased adhesiveness of the cells due to decreased calcium content, ameboid movement of the liberated cells, and the liberation of a spreading factor (hyaluronidase) which acts upon adjacent normal tissue.

Fisher, Saffer, and Fisher (1970) studied the calcium content of twenty-five adenocarcinomas of the colon, together with normal adjacent colonic mucosa, utilizing the highly sensitive technique of atomic absorption spectrophotometry. Their results suggest that cell adhesiveness was not found to be sufficiently altered, despite a wide range of tumor calcium, to influence cell separation and consequently the incidence of metastases. The findings do not specifically refute the hypothesis relating decreased mutual adhesiveness of neoplastic cells to their decreased calcium content, or more precisely to a decrease in binding by the cell membrane. For it is possible that, despite a normal or elevated tumor calcium, there might be defective calcium binding at the cell periphery.

B. Immunological Methods and Considerations - Fluorescent Antibodies

Mayer et al. (1957) prepared monkey kidneys for immunization of rabbits by homogenizing them in a Waring blender with a quantity of physiologic saline.

Scott (1955) noted that long-chain quaternary ammonium salts such as cetylpyridinium chloride (CPC) can precipitate acidic polysaccharides from their aqueous solutions. These precipitates are soluble in salt solutions. The polysaccharides may be removed from the solution containing cetylpyridinium chloride ions and salt by precipitation with ethanol or acetone.
Neutral polysaccharides are not precipitated by the cetylpyridinium chloride, but are left in the supernate.

Antonopolous et al. (1961) stated that the minimum salt concentration required to dissolve the complex of a given polysaccharide is reproducible, well-defined, and a characteristic of the polysaccharide. This phenomenon has been used in the fractionation of polysaccharides.

Morgan and King (1943) described a method for the isolation from crude gastric mucin of a polysaccharide-amino-acid complex. The amino-acid components were then removed by extraction with 90% phenol. The polysaccharide was then recovered by alcohol precipitation.

Brown (1939) said that polysaccharides could be removed from solution by fractional precipitation with alcohol in the presence of sodium acetate and acetic acid.

Coons (1956) concluded that methods for the production of antisera are largely empirical. There are three considerations to be borne in mind: the animal must be stimulated repeatedly; the dose of the antigen must be adequate but not overwhelming; and, for non-living antigens, the use of adjuvants will increase the titer considerably.

Freund et al. (1948) stated that antibody formation was enhanced and sustained when the antigen was incorporated into a water-in-oil emulsion prepared with paraffin oil and an emulsifier like Falba. It was further said that the mode of action of the agents studied may be referable to protection of antigens against destruction, to inducing a cellular reaction favorable to immune response, or both of these factors simultaneously.

Fischel et al. (1952) noted that the addition of killed tubercle
bacilli to Freund's adjuvant mixture elicited increased and prolonged antibody levels to selected antigens and the increased antibody formation was correlated with local granuloma formation (Fig. 2) and hyperplasia in the regional lymph nodes and spleens of animals receiving the emulsion containing killed tubercle bacilli.

Tanaka and Leduc (1956) described the distribution of the Forssman antigen, as revealed by the use of specific fluorescein-labeled antibody, in tissues of the guinea pig, cat, dog, mouse, and chicken. In the species examined the principal common finding is its presence in the vascular endothelium and the perivascular connective tissue. The tissues reported to contain Forssman antigen include almost all organs of these animals.

Sainte-Marie (1962) devised a method by which tissues to be studied with immunofluorescent techniques could be prepared by a modification of the usual paraffin embedding technique. Tissues treated by the Sainte-Marie technique are fixed in 95% ethanol which has been pre-cooled to 4°C. Heat involvement of the specimens is kept to a minimum by the use of this method.

Easty and Mercer (1957) concluded that the specific precipitate formed in the precipitin reaction is formed as the result of the union between antigen and antibody molecules, followed by separation from solution of the insoluble antigen-antibody complex. This picture is supported by the electron micrographs of the ferritin-anti-ferritin precipitates.

The discovery and naming of the precipitin reaction is attributed to Krause who, in 1897, observed that a precipitate formed when cell-free filtrates of broth cultures of typhoid bacillus were mixed with anti-typhoid
Marrack (1934) first demonstrated that dye molecules can be chemically linked to antibody molecules without impairing the capacity of the antibody to react specifically with the antigen which stimulated its synthesis.

Coons, Creech, Jones, and Berliner (1942) originally used the fluorescent antibody technique, utilizing fluorescein isocyanate, to detect the presence of antigenic material attributable to the pneumococcal organism.

Coons and Kaplan (1950) described a method employing antibody labeled with fluorescein isocyanate as a histological stain to visualize the specific antigen-antibody precipitate under the fluorescent microscope.

Weller and Coons (1954) first described the "sandwich" technique for location of antigen; they treated tissue culture monolayer preparations of varicella and herpes zoster viruses with the specific human antiserum and then stained the bound human antibody globulin by means of conjugated anti-human-globulin. The antiglobulin serum can be obtained in high titer so that small antibody losses caused by conjugation and purification procedures are unimportant. Antiglobulin conjugates are valuable for locating antibody from a weak serum which may be used in the intermediate layer of the sandwich; this avoids conjugating the weak serum and reducing its antibody titer still further.

Coons (1956) states that the use of an intermediate layer of antiserum followed by the conjugated immune globulin is ten times more sensitive as the single layer technique. Presumably, the gain in sensitivity is
attributable to the additional combining sites which are made available by the antibody molecules of the middle layer acting as antigen for the fluorescent antiglobulin. The use of additional layers may be used further to increase the sensitivity.

Riggs et al. (1958) developed fluorescein isothiocyanate as a labelling agent. The isothiocyanates are an important improvement over the isocyanates in that they are more easily prepared, more stable, and less toxic.

Nairn, Richmond, McEntegart, and Fothergill (1960) used the "sandwich" technique and fluorescent staining to study the reaction of organ-specific antigen in normal and malignant tissues. The results of the study suggest that mammalian cells contain organ-specific antigen or antigens which are lost or depleted when the cells become malignant, whether the malignancy is chemically induced or spontaneous.

Nairn, Richmond, and Fothergill (1960) in an investigation of the histological staining reaction of a variety of human and animal tumors with fluorescent conjugates of non-immune rabbit globulin have shown variable results with a trend toward equal staining of normal tissue and benign tumors, and diminished staining of carcinomas. They also stated that the conjugate of non-immune rabbit globulin behaves as a histological stain in much the same way as standard acid dyes, possibly because of its net negative charge.

Chavez (1968) using the fluorescent antibody technique showed that the fluorescence of tumor cells as compared to normal cells, the source of both being the hamster buccal pouch, was diminished.
C. Immunology and Carcinogenesis

Green (1954) postulated that the "initiation" stage of chemical carcinogenesis is that at which an antigenic change in the "identity protein(s)" to an isoantigen occurs. The antibody response thus elicited either destroys the precancerous cell at some stage, or continuous hyperplasia leads to an ever-increasing immune reaction which may finally induce an adaptation in the cell. The adaptation involves the loss of "identity proteins", and the neoplastic cell emerges.

De Maeyer and De Maeyer-Guignard (1963) showed that 7,12-dimethylbenzanthracene and 20-methylcholanthrene inhibited interferon formation in tissue culture. The specific repression of the synthesis of an inhibitor of virus growth offers a first clue as to how they can stimulate the growth of tumor viruses in vivo. It is further possible that these substances not only repress the synthesis of inhibitors of viral replication, but also of cellular replication; this would explain their direct carcinogenic activity.

Prehn (1968) states that most, or perhaps all, neoplasms contain surface antigens which are not recognized as "self" by the immunologic mechanism of the animals in which they originate. Almost all of the putatively non-viral tumors are individually specific, and few, if any, cross-reactions occur among them. The antigenic titer varies greatly and is related to the latent period and the immunologic reactivity of the tumor host. The relationship is almost certainly due to immunoselection. The antigens are found in early premalignant lesions and the immune mechanism suppresses many of them before they reach macroscopic size and before they undergo progression.
to malignancy. It is hypothesized that the tumor antigens represent an alteration in a normal cell surface regulatory site or sites. Whether they are encoded but repressed in the genome of the normal cell or are the result of mutation has not been determined.

D. Cell Surface Mucopolysaccharides

Kabat (1956) states that the A-B-O antigen system of the human red cell depends on the presence on the exterior surface of the cell of a thin coating consisting in part at least of polysaccharides. These polysaccharides have molecular similarities to the mucins of gastric secretion. Immunochemical methods tell us that the red cell has a sugar coating - a covering rich in polysaccharides - or at least, that polysaccharide molecules are found on the outer surface of its plasma membrane.

Bennett (1963) stated that the polysaccharide coating is extracellular, is readily permeated by water and ions, has a low electrical capacitance, and low electrical resistance, can act as a filter in selectively screening particles of various sizes, can bind certain substances by virtue of the chemical nature of groupings available. If charged groups are available, as in acid polysaccharides, the cell coating can exhibit the properties of an ion exchange resin. It influences the environment close to the plasma membrane extracellularly by filtration and selective binding.

Toto and Grandel (1966) in studying epithelium showed that the stratum spinosum contained larger amounts of acid mucopolysaccharides than the stratum corneum, stratum granulosum, and stratum germinativum respectively. This was established by the affinity of acid mucopolysaccharides to
Alcian Blue and Dialysed Iron. The binding together of the epithelial cells (stratum spinosum) by desmosomes is mediated by the adhesive properties of the mucopolysaccharides.

Jones and Coyle (1969) in studying the interface between neoplastic epithelium and the underlying mesenchyma or connective tissue found areas of abundant loose fibroblastic tissue around the extensions of the most aggressive tumors. The histochemical reactions of this material suggest a high acid mucopolysaccharide content. The findings of this investigation indicate that the accumulation of acid mucopolysaccharides around a carcinoma is associated with its offensive capacity.

Martinez-Palomo, Braislovsky, and Bernhard (1969) showed that both the absence of tight junctions and the increase in surface mucopolysaccharide of transformed cells are likely to be related to their loss of contact inhibition.
CHAPTER III
STATEMENT OF THE PROBLEM

The object of this study is the evaluation of selected extraction procedures and complexes of mucopolysaccharides of the oral mucosa of the Syrian hamster in regard to their antigenic capabilities in antibody formation. An attempt to elicit antibody formation in rabbits against untreated hamster cheek pouch and to selected extracts which will be the result of treatment of the tissue homogenate with cetylpyridinium chloride and alcohol respectively will be carried out. An additional sample of alcohol extract of hamster buccal pouch will be further clarified using phenol prior to immunization attempts. The antisera obtained will be screened with liver powder to eliminate non-specific protein, and selected samples of antisera will be screened additionally with the supernate of the cetylpyridinium chloride treated tissue homogenate. The resultant antisera will be subjected to micro-precipitin tests to determine the magnitude of antibody response elicited by the respective antigenic complexes. These tests will be performed in the hope of clarifying which extraction procedure or complex of mucopolysaccharides of the oral mucosa could best be utilized in the study of the difference in antigenic profile in normal and malignant oral mucosa.
CHAPTER IV
MATERIALS AND METHODS

ANIMALS:

Male dark-eared partial albino (DEA) Syrian hamsters (Cricetus Auratus) which were inbred for at least fifty generations and which were identical to the day in age were used in these experiments. Female New Zealand white rabbits, each weighing about four pounds, were utilized in the production of antiserum to normal hamster tissue. All animals were kept on a diet of Purina rat chow and tap water.

PREPARATION OF ANTIGEN:

Ten dark-eared partial albino Syrian hamsters were killed by an excessive dose of diethyl ether inhalation and both the right and left cheek pouches were excised from each immediately. The excised pouches were then rinsed in sterile physiologic saline and placed on a tray of ice to impart some degree of firmness to the tissues so that shredding would be facilitated. The pouches were then shredded using a single edge razor blade which had previously been wiped free of any surface lubricant. The shredded pouches, collectively weighing 10.1 grams, were collected and placed in a tissue homogenizer (Sorvall Omni-Mixer Homogenizer, Ivan Sorvall Inc., Newton, Conn., Model OM-1150) which had been pre-cooled for twenty-four hours at 4°C. All handling of the pouch material itself or the extracts of the pouch material from this time on except for brief periods was done in the confines of a controlled environmental room (Environ-Room, Laboratory Line Instruments, Melrose Park, Illinois) where the temperature was a constant 4°C. Homogeni-
zation and storage of antigenic preparations was accomplished in the controlled environmental room.

One hundred milliliters of sterile physiologic saline was added to the harvested cheek pouch material prior to homogenization in the mechanical homogenizer. During the actual procedure of homogenization, the outside of the tissue homogenizer was additionally packed with ice chips in an attempt to prevent an excessive build-up of heat which would denature tissue components. The tissue was homogenized using the highest frequency (50,000 rpm) available on the tissue homogenizer for thirty minutes during which the homogenizer under the control of a Flexopulse timing device (Flexopulse clock, "HG" series, Bliss, Eagle Signal, Davenport, Iowa) alternately ran for ten seconds and was off for ten seconds. After thirty minutes, enough sterile physiologic saline was added to the tissue and saline homogenate to bring the total volume of gross homogenate and saline to two hundred milliliters. The gross homogenate was then divided into two separate and equal portions of one hundred milliliters each and sealed.

Two and one-half grams of cetylpyridinium chloride (Eastman Organic Chemicals, Rochester 3, New York) was placed in fifty milliliters of deionized distilled water and vibrated until a homogeneous, viscous, yellow fluid, concentration 5% cetylpyridinium chloride (CPC), was obtained. Twenty-five milliliters of this 5% CPC solution was added to one of the 100 ml. portions of gross homogenate and stirred vigorously. The result was 125 mls. of gross homogenate and 1% CPC. This mixture was allowed to stand uncovered in the controlled environmental room, until by the process of evaporation the volume of the resultant mixture reached 100 mls. This volume
was then equal to the volume of gross homogenate originally used and thus the concentration of the tissue components was the same per milliliter of saline as that of the untreated gross homogenate. This sample was then sealed to prevent further evaporation and concentration of the components.

Both the untreated gross homogenate and the CPC-treated gross homogenate were respectively agitated well immediately prior to the taking of the following samples:

Fifteen milliliters of the CPC-treated gross homogenate was bottled and labeled Number 1.

Fifteen milliliters of a 1% CPC in sterile physiologic saline solution was bottled and labeled Number 2.

Fifteen milliliters of the untreated gross homogenate was bottled and labeled Number 3.

Fifteen milliliters of the CPC-treated gross homogenate was treated further. To this portion of CPC-treated gross homogenate, 11.4 grams of magnesium chloride were added and stirred vigorously. This sample, which was then approximately 8 M magnesium chloride, was then centrifuged for ten minutes at 4000 rpm in an angle centrifuge (HN Centrifuge, International Equipment Co., Needham Heights, Mass.). The supernate was decanted and centrifuged again for an additional ten minutes. The supernate was then decanted and treated with three volumes of 100% ethanol. The following morning a white precipitate was recovered upon centrifugation and this precipitate was dried in the air of the controlled environmental room. An additional three volumes of 100% ethanol were added to the remaining supernate which had been decanted prior to the drying of the precipitate and no further precipitation occurred.
This was taken to be an indication that complete precipitation had occurred with the initial treatment with ethanol. The dried precipitate was added to 15 mls. of sterile physiologic saline and it became suspended in the salt solution. It did not dissolve in the saline. This fifteen milliliter sample was bottled and labeled Number 4.

Fifteen milliliters of untreated gross homogenate was centrifuged and the clear supernate was decanted. Three volumes of 100% ethanol were added to this supernate. The following morning a white precipitate was visible in the bottom of the container. The precipitate was recovered and dried in the air of the controlled environmental room. Three volumes of 100% ethanol were added to the remaining supernate and no further precipitation occurred. The dried precipitate was added to ten milliliters of 95% phenol and it immediately disappeared upon stirring. The phenol solution was allowed to remain undisturbed for three hours; it was then subjected to an alcohol extraction with three volumes of 100% ethanol, the result being a tan precipitate. The precipitate was recovered and placed in a 100% ethanol wash for twenty-four hours. The precipitate was again recovered and rewashed in 100% ethanol and dried by evaporation. The dried precipitate, white in color, was placed in fifteen milliliters of sterile physiologic saline and it immediately disappeared, the resulting solution being crystal clear. This sample was bottled and labeled Number 5.

Five sealed ten milliliter vials of Complete Freund's Adjuvant (Difco Laboratories, Detroit, Michigan) were opened and the contents of the individual vials were pooled. Ten milliliter quantities of the Complete Freund's Adjuvant were then added to ten milliliters of the respective
antigenic preparations (Numbers 1, 2, 3, 4, 5). All solutions upon shaking presented an identical white appearance and were creamy in consistency. They were utilized in this state for the immunizations and were stored in the controlled environmental room in sealed bottles. Five milliliters of each of the antigenic preparations prior to the addition of the Complete Freund's Adjuvant were retained for testing antibody titers and for chemical analysis. These samples were labeled with their original respective sample number.

IMMUNIZATIONS:

Fifteen female New Zealand white rabbits were divided into five groups of three rabbits each. The rabbits were identified as follows:

Group 1 - 1(1), 1(2), 1(3); Group 2 - 2(1), 2(2), 2(3); Group 3 - 3(1), 3(2), 3(3); Group 4 - 4(1), 4(2), 4(3); Group 5 - 5(1), 5(2), 5(3). Each group number corresponded to the number of the antigenic preparation which that group of rabbits received. Inoculations were in the quantity of 1 cc. per injection and were administered subcutaneously with a disposable syringe equipped with a twenty gauge needle. Initially the rabbits were injected three times over a period of a week. The rabbits were then allowed a rest period of six weeks to be followed by a challenge injection which was given six weeks after the date of the initial injection of the immunization schedule. A second challenge injection was administered one week after the first challenge. Micro-precipitin tests were run on blood samples of each rabbit two weeks after the initial injection and five days after the first challenge injection. One week after the second challenge injection, all rabbits were bled from a marginal ear vein using a suction apparatus (Bellco.
Biological Glassware and Equipment, Vineland, N.J., Rabbit Bleeding Apparatus, Item No. 1708 in catalog) to facilitate bleeding. Approximately 25 mls. of blood were collected from each rabbit and maintained during centrifugation to separate the serum from the blood cells in their respective containers. All serum collected from the rabbits immunized with a common antigenic material or preparation was pooled, equal amounts of serum coming from each of the three rabbits in the group. Serum was not collected from the rabbits injected with the CPC and saline as no precipitin reaction occurred during previous testing. The pooled serums were labeled I, III, IV, V; these Roman numerals corresponded respectively to the antigenic preparations 1, 3, 4, and 5 which were used to immunize the rabbits. Serum was also collected from an untreated rabbit to serve as a control. Micro-precipitin tests were run on all pooled serum using both the supernate from the untreated gross homogenate and the phenol-cleared solution as the antigen. All antisera when not being used were stored in the quick-frozen state.

CLARIFICATION OF ANTISERA:

Prior to use in antigen-antibody reactions utilized in the tissue comparisons, all serum samples were reacted with liver powder in the quantity of 100 mg. of powder for each milliliter of antiserum for one hour. This was done in the hope of screening out certain extraneous non-specific protein. The antiserum which showed the greatest antibody titer after the first challenge injection was also reacted with an equal amount of the supernate of the CPC-treated gross homogenate and additional amounts of this supernate until no precipitin reaction occurred.
CHEMICAL TESTING OF ANTIGENIC PREPARATION SUPERNATES:

Chemical analysis of the respective antigenic samples was done utilizing the Biuret Test, Ninhydrin Test, and a spray test specific for free carbohydrate.

CARCINOGENESIS:

Tumors were induced in the left cheek pouches of eight week-old dark-eared partial albino Syrian hamsters using 9,10-dimethyl-1,2-benzanthracene (DMBA, Eastman Organic Chemicals, Rochester, New York). A 0.5% solution of DMBA was prepared using high quality mineral oil (mineral oil, U.S.P., heavy) as the solvent. The left cheek pouch of each animal was painted with the DMBA solution twice a week using a No. 7 camel-hair brush. The right cheek pouch went untreated so that it could be utilized as a control. Painting of the pouches was discontinued at a time when tumor formation was both seen directly and palpated externally in each animal.

PREPARATION OF TISSUE SECTIONS:

When all antisera to be used were obtained and when tumor formation was advanced, the hamsters were killed by an excessive dose of diethyl ether inhalation. The tumor in the left cheek pouch as well as the normal right cheek pouch of each hamster was excised and immediately placed in 95% ethanol pre-cooled to 4°C. Specimens of both tumor and normal tissue were also immediately frozen for use in frozen sections. The tissue specimens fixed in 95% ethanol were embedded in paraffin; they were also prepared according to the method devised by Sainte-Marie (1962).

Both frozen sections and paraffinized sections of both tumor and
normal tissue were stained with hematoxylin and eosin. These sections were utilized for identifying and localizing tumor cells. They were seen both under low magnification and high magnification with photomicrographs being taken of representative sections. Kodachrome II film, ASA 25, was used.

Both the frozen and paraffinized sections of tumor and normal tissue were stained with the fluorescent dye, fluorescein isothiocyanate, using the "sandwich" technique to visualize antigen-antibody reactions in the tissues. This technique consisted of first deparaffinizing the sections embedded in paraffin by exposing them to three consecutive washes of one minute each in xylol using fresh amounts of solvent for each washing. Three fifteen second washes in 95% ethanol followed. The tissue sections were then washed for one minute each in three consecutive physiologic saline baths. Application of the intermediate serum, the antiserum produced in the rabbits, was accomplished on sections of both normal tissue and tumor for thirty minutes in a high humidity environment by placing the slide in an incubator at 37°C. on a wet piece of filter paper in the bottom of a Petri dish and covered with a Petri dish cover. Each section was then washed in 95% ethanol for twenty minutes, in buffered saline for three minutes, and dried. The dried sections were then reacted with Sheep Anti-Rabbit Globulin (Hyland Laboratories, Los Angeles, Calif.) conjugated with fluorescein isothiocyanate for thirty minutes in a high humidity environment described above. Fixation was accomplished using 95% ethanol for twenty minutes, followed by a three minute wash in buffered saline. Glycerol was used to mount the coverslips. The slides were immediately viewed with a Reichert-Zetopan Fluorescent Microscope (Reichert, Austria) illuminated with a mercury vapor lamp. Blocking reactions
were used utilizing Unconjugated Sheep Anti-Rabbit Globulin prior to the application of the Conjugated Sheep Anti-Rabbit Globulin, and also utilizing no intermediate rabbit antiserum prior to the application of the Conjugated Sheep Anti-Rabbit Globulin to the tissue specimens. Normal serum obtained from an untreated rabbit was also used in an attempt to show that antibodies other than those already in rabbit serum were elicited by the series of immunizations. Photomicrographs were taken immediately upon preparation of the sections which were exposed to the fluorescent preparations. Outdoor high speed Ektachrome film, ASA 160, was used.
CHAPTER V

RESULTS

CARCINOGENESIS:

After the initial four applications of 0.5% DMBA to the left cheek pouch of the hamsters assigned to the carcinogenesis aspect of this study, the oral mucosa of the pouch appeared reddened and raw, typical of an inflammatory and desquamative reaction. The fourth week of DMBA application brought significant changes in both the temperament and the treated pouch of the hamsters. At this time the hamsters were more irritable than at previous handlings, and definite nodular areas were seen in the cheek pouch during application of the carcinogen. The beginning of the fifth week brought with it a generalized change in the coat of the hamsters under treatment. It was at this juncture that the animals appeared to be losing some hair and clumping of the remaining hair was the rule. By the eighth week, tumors were both palpated externally and viewed in the base of the cheek pouches being treated with carcinogen. It was at this point that treatment of the pouches with carcinogen was discontinued. By the beginning of the twelfth week of the carcinogenesis phase, all tumors were readily palpable externally and all animals exhibited loss of much of their external coat in the area overlying the tumor(s) (Fig. 3). One animal even showed ulceration of the external epithelium over the base of the treated pouch. This mass externally appeared to be highly invasive as the overlying tissue appeared to be greatly indurated. All animals under treatment were killed at the end of the fourteenth week for the purpose of obtaining tumor speci-
mens from the left cheek pouches as well as for obtaining the control, or right, cheek pouches. The cheek pouches were excised immediately upon cessation of life signs in the hamsters. A portion of each specimen was utilized for frozen sections; the remainder of the individual specimens were fixed in 95% ethanol which had been pre-cooled to 4°C, for use in paraffin-embedded sections.

One-half of the animals had a well-defined, singular mass in the base of the left cheek pouch. These tumors had well-defined fibrous bases with a highly hemorrhagic crown or surface. The other one-half of the animals exhibited multiple well-defined tumor masses in the treated pouch. These masses however were much smaller than the singular mass exhibited by the former group. None of the animals at any time showed any type of reaction other than normal in the untreated pouches (Fig. 4).

**Antiserum Evaluation:**

Two weeks after the initial injections of the rabbits, blood samples were procured from each rabbit and they were separately analysed. Micro-precipitin Ring tests were performed using serial dilutions of the respective antisera tested against the supernate of the untreated gross homogenate and the phenol-clarified solution respectively. Dilutions of the antisera were done using a borate-saline buffer. The Ring tests were performed in capillary tubes layering the antigen over the antiserum. The dilutions of the antiserum were as follows: full strength; 1:4; 1:16; 1:64; 1:256. The precipitin tests on the sera collected two weeks after the initial inoculation were all negative. These were read twenty-four hours after the capillary tubes with the
layer of antigen over the antiserum were placed in an incubator at 37°C.

Five days after the first challenge injection, corresponding to about seven weeks after the initial inoculation, blood samples were again collected individually and kept as such. The first group of rabbits injected with the CPC-treated gross homogenate showed no detectable antibody titer when the serum dilutions were layered with the phenol-clarified solution, and practically no appreciable antibody titer when the serum dilutions were layered with the supernate of the untreated gross homogenate. The antiserum obtained from rabbit 1(2) showed an antibody titer to a dilution of 1:4, while the antisera from the other two rabbits, 1(1) and 1(3), showed a positive reaction only when undiluted in response to the supernate of the untreated gross homogenate.

The second group of rabbits injected with the 1% CPC in sterile physiologic saline showed no precipitin reaction at all to either the supernate of the untreated gross homogenate or to the phenol-cleared solution.

The third group of rabbits injected with the untreated gross homogenate also showed a variable response. The antisera of two of the rabbits, 3(1) and 3(2), elicited positive precipitin reactions to both the supernate of the untreated gross homogenate and the phenol-cleared solution in the dilution of 1:16. The antiserum of rabbit 3(3) elicited a precipitin reaction to the supernate of the untreated gross homogenate and to the phenol-cleared solution in the dilution of 1:256. A portion of this antiserum was later cleared with the supernate of the CPC-treated gross homogenate.

The fourth group of rabbits injected with the CPC-cleared, alcohol-precipitated gross homogenate produced a very weak response. Only one rabbit
in this group, 4(3), produced an antiserum which evoked a precipitin reaction when layered with the supernate from the untreated gross homogenate and the phenol-cleared solution respectively, and that was in a dilution of 1:4.

The fifth group of rabbits, injected with the phenol-cleared solution as the antigenic preparation, produced an antiserum uniformly which reacted with the supernate from the untreated gross homogenate in a dilution of 1:16 and with the phenol-cleared solution in a dilution of 1:64.

A portion of the antiserum produced by rabbit 3(3), which exhibited the best antibody titer after the first challenge injection, was reacted with the supernate of the CPC-treated gross homogenate. A precipitate formed and the remaining serum was cleared by centrifugation. The cleared antiserum was separated from the precipitate and reacted with: 1) the phenol-cleared solution and this resulted in a positive precipitin reaction up to a dilution of 1:32; 2) the supernate of the untreated gross homogenate and this resulted in a positive precipitin reaction up to a dilution of 1:8; 3) the supernate of the CPC-treated, alcohol-precipitated gross homogenate and this resulted in a negative precipitin reaction; and 4) with the supernate of the CPC-treated gross homogenate and this resulted in a negative reaction.

One week after the second challenge inoculation, which was administered to all rabbits except those in the second group, the rabbits were bled and the antisera of the rabbits in a given group were pooled, equal amounts of antiserum being given by each rabbit in the group to its respective pool. The results of the Micro-precipitin Ring tests on the pooled antisera were as follows.

The antiserum from the first group of rabbits, Group I, elicited a
precipitin reaction to a dilution of 1:4 to both the supernate of the untreated gross homogenate and the phenol-cleared solution respectively.

The antiserum from the third group of rabbits, Group III, elicited a precipitin reaction to a dilution of 1:64 when reacted with the supernate from the untreated gross homogenate and with the phenol-cleared solution.

The antiserum from the fourth group of rabbits, Group IV, elicited a precipitin reaction to undiluted antiserum with both the supernate of the untreated gross homogenate and the phenol-cleared solution.

The antiserum from the fifth group of rabbits, Group V, elicited a precipitin reaction to a dilution of 1:64 to both the supernate of the untreated gross homogenate and the phenol-cleared solution.

The control serum, collected from an untreated rabbit, showed no precipitin reaction when reacted with the supernate of the untreated gross homogenate and the phenol-cleared solution.

TABLE 1. FINAL SEROLOGICAL ASSAY

<table>
<thead>
<tr>
<th>ANTIGEN</th>
<th>DILUTION 0</th>
<th>1:4</th>
<th>1:16</th>
<th>1:64</th>
<th>1:256</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% CPC - Saline</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Untreated Gross Homogenate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Alcohol-Precipitated, Phenol-Cleared Gross Homogenate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CPC-Treated Gross Homogenate</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CPC-Treated, Alcohol-Precipitated Gross Homogenate</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The above chart indicates the efficiency of antigenic preparations in regard to antibody evoking potentiality demonstrated by the respective antigenic preparations. The antisera used in the dilutions were those
obtained after the second challenge injection. The antisera were tested against the supernate of the untreated gross homogenate and the phenol-cleared solution respectively using the micro-precipitin test. A grade of (+) is indicative of a positive precipitin reaction while a grade of (-) is indicative of no reaction.

SPECIFIC CHEMICAL TESTING OF ANTIGENIC SUPERNATES:

The supernate of the CFC-treated gross homogenate elicited a positive Biuret Test. The supernate of the untreated gross homogenate gave a very weak positive Biuret Test. The supernate of the CFC-treated, alcohol-precipitated gross homogenate elicited a negative Biuret Test as did the phenol-cleared solution. All supernates tested with the Ninhydrin test for protein as well as with a test specific for free carbohydrate were doubtful to negative.

MICROSCOPIC INTERPRETATION OF TISSUE SECTIONS:

Sections of normal hamster cheek pouch stained with hematoxylin and eosin demonstrated that the tissue composition of the cheek pouch from the surface inward was epithelium, dense connective tissue, striated muscle fibers, and loose areolar connective tissue (Fig. 5). Under high magnification (250X), the epithelium consisted specifically of a stratum corneum, stratum spinosum, and a stratum germinativum. The thickness of the stratified squamous epithelium was in most instances four to six cell layers. The dense connective tissue, or lamina propria, immediately underlying the epithelium contained no accessory glands or structures. The thin muscle layer appeared in both longitudinal and transverse section depending upon the
plane of the section. The loose connective tissue contained blood vessels.

Sections of the tumor stained with hematoxylin and eosin were composed of a connective tissue stroma and squamous epithelial cells (Fig. 6). The stroma was composed of dense connective tissue with areas of looser appearing connective tissue interspersed throughout. The cellular components of the tumor consisted chiefly of squamous epithelial cells in a diffuse type background. Blood vessels also were seen in the tumor sections. The histological picture of the tumor was diagnosed as being a well-defined squamous cell carcinoma.

**FLUORESCENT ANTIBODY - "SANDWICH" STAINING TECHNIQUE:**

In the utilization of the fluorescent antibody - "sandwich" staining technique, five different antisera were used in the intermediate layer. The antisera were different in the fact that their production was accomplished by differently treated antigens, or because the antisera were cleared in a different manner.

Antiserum I, produced by an antigenic mixture in which the acid mucopolysaccharides were bound by the cetylpyridinium chloride, displayed the following characteristics and specificity when used as the intermediate layer in the "sandwich" technique of fluorescent antibody staining: The stratum spinosum displayed the most intense fluorescence when this antiserum in comparison to other antisera was used. The fluorescence was more generalized and extended in some instances to the intercellular spaces in the stratum germinativum. The stratum corneum was negative, as was the muscle layer including its intercellular spaces. The lamina propria was negative with
the exception of interspersed areas of intense fluorescence.

Antiserum III, produced by the inoculation of untreated gross homogenate showed the following characteristics and specificity when used as the intermediate layer in the "sandwich" technique of fluorescent antibody staining: The stratum spinosum displayed both positive and negative areas with regard to fluorescence which was not as intense when it was present as that seen when antiserum I was used as the intermediate layer. The muscle layer was devoid of fluorescence, but the areas between the muscle bundles showed intense fluorescence. The stratum corneum as well as the lamina propria were completely negative in regard to fluorescence (Fig. 7).

Antiserum IV displayed the following characteristics and specificity when used as the intermediate layer in the "sandwich" technique of fluorescent antibody staining: The stratum spinosum as well as areas in the stratum germinativum showed a positive fluorescent reaction. The muscular layer, both muscle bundles and intermuscular spaces, was definitely negative. The stratum corneum and the lamina propria were also devoid of fluorescence.

Antiserum V displayed the following characteristics and specificity when used as the intermediate layer in the "sandwich" technique of fluorescent antibody staining: The stratum spinosum showed positive fluorescence which was more sedate than in any of the other sections using different antisera. The muscular layer, both muscle bundles and intermuscular spaces, was negative. The stratum corneum and the lamina propria were also negative although the lamina propria was speckled with scattered fluorescence.

The antiserum which was produced by rabbit 3(3), immunized against
the untreated gross homogenate and cleared by the supernate of the CPC-treated gross homogenate, displayed the following characteristics and specificity when used as the intermediate layer in the "sandwich" technique of fluorescent antibody staining: The stratum spinosum showed a positive fluorescent stain. The fluorescent stain in the stratum germinativum was greatly diminished in comparison with the stratum spinosum. The contrast between these two areas in the epithelium was excellent (Fig. 8). The muscular bundles, intermuscular areas, lamina propria, and stratum corneum were negative in regard to fluorescence.

When the serum from the untreated rabbit was used as the intermediate layer in the "sandwich" staining technique, a completely negative reaction with regard to fluorescence occurred. When no rabbit anti-hamster serum was used as an intermediate layer prior to the application of the sheep anti-rabbit immune globulin, there was no fluorescence. However, when the unconjugated sheep anti-rabbit immune globulin was applied after an intermediate layer of rabbit anti-hamster serum and prior to a layer of conjugated sheep anti-rabbit immune globulin, a generalized fluorescence was seen throughout the tissue sections.

Sections of tumor, when exposed to the fluorescent antibody technique in conjunction with "sandwich" staining, showed areas of intense fluorescence at random in a background of lessor fluorescing cells. The predominant cell seen in the tumor sections was one of diminished fluorescent staining capacity (Figs. 9 and 10).

The fluorescent characterization rendered above is a compilation of findings recorded separately by three individuals who personally viewed
coded transparencies of all tissue sections. Quantitative evaluation of fluorescence was not attempted as this would tend to be very subjective in nature. The fact that fluorescence was apparent and that some comparisons between tissue sections in regard to the areas of fluorescence were made was the end exacted by these three individuals.
The object of this investigation was to study different methods of extracting the mucopolysaccharide component of cell surfaces and to relate the extract to its ability to provoke an antibody response. Specifically, hamster cheek pouch was utilized to procure cell surface antigen, and rabbits were used in the production of antibodies to the extracted material obtained from the cells of the hamster cheek pouch. Because mucopolysaccharides are supposedly a relatively weak antigenic material, Freund's Adjuvant (Complete) was added to the inoculum for the purpose of enhancement. The study was also designed to make a comparison between the staining characteristics of normal and carcinogen-induced (9,10-dimethyl-1,2-benzanthracene) malignancy of hamster oral epithelium utilizing the fluorescent antibody technique.

The fact that one pouch of the same animal could be used for the induction of malignancy and the opposite pouch could be left untreated acted as a built-in control for tissue comparison. The fact that this control was possible is very important in that it eliminates individual variables encountered when comparing tissues of different subjects, even if the animals are closely inbred. The individual response of each animal varied in extent and degree. However, it was noted that, in this study, fifty percent of the animals developed a single well-developed tumor at the base of the pouch while the remaining fifty percent developed multiple tumors of various sizes in the treated cheek pouch.

The tissue sections were fixed and processed immediately in an
attempt to prevent the possibility of changes in cell surface antigenic characteristics. The sections utilized in the fluorescent antibody technique were examined immediately upon preparation, and initiation of photography of the area of the slide chosen for reproduction was accomplished after exposure of the section to ultraviolet light for not more than two minutes. These controls were instituted because the fluorescent reaction diminishes proportionately with the age of the preparation and with the extent of exposure to ultraviolet light.

The histologic picture of tumor specimens stained with hematoxylin and eosin was in all instances one of a well-differentiated squamous cell carcinoma. This finding is in agreement with the literature in which it is reported that the only type of malignancy which is chemically induced in the hamster cheek pouch by 9,10-dimethyl-1,2-benzanthracene is a well-differentiated squamous cell carcinoma.

CHARACTERIZATION OF ANTIGENIC PREPARATIONS AND ANTIBODY RESPONSE:

The fact that neither cetylpyridinium chloride nor sterile physiologic saline were able to evoke a precipitin reaction when the serum of the rabbits immunized with this combination would not react with the supernate of the untreated gross homogenate or the phenol-cleared solution is indicative of the fact that these materials of themselves are not directly responsible for a specific antibody response to antigenic mixtures with which they may be associated.

The finding that an appreciable antibody response was elicited when the rabbits were immunized with the untreated gross homogenate of hamster
cheek pouch was expected, for one could expect no greater spectrum of antigens
than from the homogenate of the entire tissue.

The very weak antibody response evoked by the CPC-treated gross
homogenate as compared to the relatively strong antibody response of the
untreated gross homogenate can only be attributed to a change effected by the
cetylpyridinium chloride as the addition of this substance in the concentra-
tion of 1% was the only difference between the two above mentioned antigenic
inoculums.

The low titer evoked by the use of the CPC-treated, alcohol-
precipitated gross homogenate as an inoculum could either be attributed to
the effect of the cetylpyridinium chloride on the components of the gross
homogenate, or upon the fact that the precipitate obtained was insoluble in
saline and thus probably unavailable to the tissues of the rabbit and unable
to promote anything other than a foreign body reaction.

The reason for the relatively high antibody titer evoked by the
alcohol-precipitated, phenol-clarified solution is purely speculative in
the absence of a detailed assay of the final precipitate. The fact that the
final precipitate obtained was readily soluble in sterile physiologic saline
and thus available to the immunologic system of the rabbit was definitely a
factor which enhanced its ability to provoke an antibody response in the host.
Whether an alteration in its antigenic nature by the use of phenol, or merely
the fact that it was readily available in soluble form to the tissues of the
host, was responsible for the magnitude of the antibody response is unknown.

The fact that the antiserum obtained by immunizing the rabbits with
the untreated gross homogenate and then clearing this antiserum with the
supernate of the CPC-treated gross homogenate was so successful in its specificity is a significant finding. This finding is especially noteworthy in the light of studies by Jones and Coyle (1969) and by Martinez-Palomo, Braislovsky, and Bernhard (1969) which involve acid mucopolysaccharides so heavily in the histochemistry of malignant tumors. The use of this antiserum as the intermediate layer in the "sandwich" staining method of investigation most clearly portrayed the lessor fluorescent intensity of the stratum germinativum in comparison to the intensely fluorescing stratum spinosum. It also demonstrated most clearly the relative difference in fluorescence in a random arrangement of cells in specimens of squamous cell carcinoma of the hamster cheek pouch. It is my feeling that this antiserum is specific for the acid mucopolysaccharides found on the cell surfaces of epithelial cells. It is also significant that when this antiserum was exposed to the supernate of the untreated gross homogenate that a dilution index of 1:8 was ascertained and that when exposed to the phenol-cleared solution, a dilution index of 1:32 was elicited. These indices seem to indicate that the phenol-cleared solution apparently contained more available acid mucopolysaccharide per unit volume than an equal amount of the supernate of the untreated gross homogenate. It is also significant that this antiserum established that the phenol-cleared solution contained antigens which were the same as, or similar enough to react the same as, the antigenic component(s) precipitated from the gross homogenate by the cetylpyridinium chloride. The disparity of specificity displayed between this antiserum and the antiserum obtained by immunizations with the phenol-cleared solution indicates that the latter antiserum was not screened as effectively of non-epithelial components as
was the former antiserum. It is entirely possible that if the antiserum elicited by the phenol-cleared solution were cleared with the supernate of the CPC-treated gross homogenate, we would have obtained an antiserum which was epithelial specific but with more intense fluorescence than that exhibited when the antiserum evoked by the untreated gross homogenate and cleared with the supernate of the CPC-treated gross homogenate was used in the fluorescent reaction. However, this is not entirely true as the increased degree of fluorescence exhibited by the antiserum elicited by the phenol-cleared solution might have been due to antibodies which would be screened out by the supernate of the CPC-treated gross homogenate.

In the various antisera which were cleared only by liver powder absorptions, specificity to the epithelium was not completely accomplished. The non-specific stain seen in the connective tissue and the intermuscular spaces could not be completely removed. This suggests that the non-specific staining seen in the sections was due to the method of screening extraneous antibodies rather than to unconjugated material because the staining characteristics of the antiserum screened with the supernate of the CPC-treated gross homogenate appeared to be very adequate in regard to specificity.

EVALUATION OF FLUORESCENCE IN TISSUE SECTIONS:

The relatively less fluorescence of the cells of the stratum germinativum as compared to the cells of the stratum spinosum was self-evident. The cells of the stratum germinativum fluoresced less intensely in comparison to the brightly fluorescing cells in the stratum spinosum. This
difference in fluorescent staining capacity probably represents the state of differentiation between the two cell layers.

The difference in fluorescence of cells noted in malignant tissue sections was predominantly one of intensity. However, the proportion of less intensely fluorescent cells in comparison to the more intensely fluorescent cells was predominantly in favor of the less intensely fluorescing cells. There was also a random arrangement of cells seen in malignant tissue sections which was not seen in normal tissue sections. There was no systematic relationship nor progression of less intensely fluorescing cells as related to the more intensely fluorescent areas as seen in the normal relationship of the stratum germinativum to the stratum spinosum. The existence of cellular areas with relatively different fluorescent staining capacities and its probable significance in relation to the carcinogenic mechanism is a point of interest. If the possibility exists that the malignant cells were incapable of maturing and that this factor prevented the differentiation of antigens on the cell surface, then one would not expect to see an orderly progression outward from the less intensely staining cells as is seen in the basal-cell-prickle cell relationship. This random relationship of more and less intensely fluorescing cells was the case in sections of squamous cell carcinoma of hamster cheek pouch. The possibility also exists that the loss of the ability of the affected cell to secrete the antigen is an expression of a loss of functional differentiation in neoplasia. This does not appear to be the case as frequently the area of comparatively less fluorescent cellular reactivity extends from the stratum germinativum through the stratum corneum in sections of normal tissue. Whether the lack of specific reaction in these areas is
due to an incomplete formation of antigen or lack of it, or whether it is due to a disparity in electrostatic forces on the cell surface remains unanswered. The fact that it does exist in a uniform manner progressing in a direction away from the stratum germinativum is significant.

Another possibility exists in explanation of the randomized areas of more and less intensely fluorescing islands of cells that are seen in malignant tissue sections. This possibility is that the more intensely fluorescing cells in malignancy are either islands of normal cells or malignant cells with normal antigens.
CHAPTER VII
SUMMARY AND CONCLUSIONS

The purpose of this study was to evaluate selected extraction procedures and complexes of mucopolysaccharides of the oral mucosa of the Syrian hamster as to their antigenic capabilities in antibody formation. This was done in the hope of clarifying which extraction procedure or complex of mucopolysaccharides of the oral mucosa could best be utilized to study the difference in antigenic profile between normal and malignant oral mucosa.

A number of male dark-eared partial albino Syrian hamsters were killed and their excised pouches were homogenized for the basis of antigenic preparations. Fifty percent of the homogenate was subjected to treatment with cetylpyridinium chloride and the remaining fifty percent was left untreated. Five inoculums, each containing an equal amount of Freund's Adjuvant (Complete), were tested. These inoculums consisted of the following five bases: 1) CPC 1% and sterile physiologic saline; 2) untreated gross homogenate; 3) alcohol-precipitated, phenol-clarified gross homogenate; 4) CPC-treated gross homogenate; and 5) CPC-treated, alcohol-precipitated gross homogenate. Five groups of three rabbits each were injected on a selected immunization calendar with each group being immunized with one of the five inoculums. This was done in the hope of producing antibodies specific to the cell surface antigens of the stratified squamous epithelium of the hamster cheek pouch.

Six male dark-eared partial albino Syrian hamsters were used in the production of squamous cell carcinoma. The left cheek pouch of each of these
hamsters was painted twice a week with a 0.5% solution of 9,10-dimethyl-1,2-benzanthracene in mineral oil (U.S.P., heavy) until tumors were both directly seen in the cheek pouch and palpated externally. The right cheek pouch was left untreated to serve as a control.

Upon preparation of all antisera to be tested, the hamsters used in the carcinogenesis aspect of the study were killed and specimens of both tumor and normal cheek pouch were obtained and fixed. A portion of each specimen was used in frozen sections and the remaining portion was fixed and prepared according to the method of Sainte-Marie (1962). Sections were stained with hematoxylin and eosin and also utilizing the fluorescent antibody technique. The various antisera obtained were used as the intermediate layer in the fluorescent antibody technique to demonstrate the various antigen-antibody reactions to the respective antisera in sections of normal and malignant tissue. This was done to compare the cellular fluorescent characteristics between normal hamster cheek pouch epithelium and chemically induced squamous cell carcinoma of the hamster cheek pouch.

This investigation revealed:

1. A gross homogenate of hamster cheek pouch with the addition of Freund's Adjuvant (Complete) can effectively elicit an antibody response in the rabbit.
2. Tissue antigens specific for stratified squamous epithelium are found in the hamster cheek pouch.
3. The epithelium-specific antigens of the hamster cheek pouch are either lost or changed when malignancy occurs.
4. Cetylpyridinium chloride in some way diminishes the antigenicity of the gross homogenate of the hamster cheek pouch.

5. An antiserum produced by immunization of rabbits with untreated gross homogenate of hamster cheek pouch and cleared with the supernate of the CPC-treated gross homogenate is selective to the acid mucopolysaccharides of cell surfaces.

6. The antiserum produced by immunization with untreated gross homogenate and cleared with the supernate of the CPC-treated gross homogenate and used as the intermediate layer in the fluorescent antibody technique demonstrated excellent contrast between the stratum germinativum and stratum spinosum in normal epithelium and between the more and less intensely fluorescing areas seen in the squamous cell carcinoma of the hamster cheek pouch.


Pott, Sir Percival: Chirurgical Observations Relative to the Cataract, the Polypus of the Nose, the Cancer of the Scrotum, the Different Kinds of Ruptures, and the Modification of the Toes and the Feet, London: Hower, Clark and Pollins, 1775.


Fig. 1 - This is a photomicrograph of normal hamster cheek pouch which had been exposed to 9,10-dimethyl-1,2-benzanthracene. The section was stained with hematoxylin and eosin and the original magnification was 100 X. This photomicrograph is interesting in that it shows an area of epithelium on both the right and the left sides. The epithelium on the left shows normal arrangement of cells and appears to be unaffected while the epithelium on the right shows a breakdown of the stratum corneum, an interruption of the stratum germinativum, and polymorphonuclear leukocytic invasion of the connective tissue layers. This area on the right is apparently an ulceration in the oral mucosa initiated by the application of the carcinogen.
Fig. 2 - Granulomatous area around the injection site in the rabbit. This reaction is attributed to the use of Freund's Adjuvant (Complete).
Fig. 3 - View of tumor mass intraorally and with the tumor and affected pouch everted twelve weeks after the initiation of painting the pouch with 9,10-dimethyl-1,2-benzanthracene. Note the loss of hair and the ulcerated petechiae on the neck of the hamster.
Fig. 4 - Excised specimens of normal hamster buccal pouch (left) and malignant involvement (right) of hamster buccal pouch treated with 9,10-dimethyl-1,2-benzanthracene. Both of the above specimens were taken from the same animal. The malignancy was in the left pouch and the normal specimen was taken from the control or untreated pouch which was on the hamster's right side.
Fig. 5 - This photomicrograph of normal hamster cheek pouch is stained with hematoxylin and eosin. Apparent in the photomicrograph is the stratum corneum, stratum spinosum, stratum germinativum, lamina propria, and muscular layer. The magnification of the section was originally 250 X.
Fig. 6 - This photomicrograph of cancerous hamster cheek pouch is stained with hematoxylin and eosin. The magnification was originally 250 X. Note the dyskeratosis of the squamous epithelial cells in a stroma of connective tissue.
Fig. 7 - This photomicrograph of normal hamster cheek pouch was stained using the untreated gross homogenate induced rabbit anti-hamster serum which was cleared with liver powder as an intermediate layer prior to the application of sheep anti-rabbit immune globulin conjugated to fluorescein isothiocyanate. The original magnification of the section is 500 X and the exposure to the illuminating source was 120 seconds. The stratum germinativum shows less intense fluorescence than does the stratum spinosum. Some fluorescence is also noted in the lamina propria and intermuscular spaces.
Fig. 8 - This photomicrograph of normal hamster cheek pouch was stained apple-green using the untreated gross homogenate induced rabbit anti-hamster serum which was cleared with the supernate of the CPC-treated gross homogenate as an intermediate layer prior to the application of sheep anti-rabbit immune globulin conjugated to fluorescein isothiocyanate. The original magnification of this photomicrograph is 500 X and the exposure to the illuminating source was 60 seconds. This photomicrograph illustrates specificity of the antiserum used to epithelium very well.
Fig. 9 - This photomicrograph of squamous cell carcinoma in the hamster cheek pouch was stained using the untreated gross homogenate induced rabbit anti-hamster serum which was cleared with the supernate of the CPC-cleared gross homogenate as the intermediate layer prior to the application of sheep anti-rabbit immune globulin conjugated to fluorescein isothiocyanate. The original magnification of this section was 500 X and the exposure to the illuminating source was 120 seconds. Note the presence of both intensely staining cells in some areas and less intensely fluorescing cells in other areas with no evidence of a systematic progressive arrangement as is seen in the relationship of the stratum spinosum to the stratum germinativum. Random arrangement of cells with no evidence of systematic progression is a characteristic of malignancies.
Fig. 10 - This photomicrograph of squamous cell carcinoma of hamster cheek pouch was stained using the untreated gross homogenate induced rabbit anti-hamster serum which was cleared with the supernate of the CPC-treated gross homogenate as the intermediate layer prior to the application of the sheep anti-rabbit immune globulin conjugated to fluorescein isothiocyanate. The original magnification of this section is 500 X and the exposure to the illuminating source was 120 seconds. Note the areas of intensely fluorescing cells in the lower right hand corner or in the center left area of the photomicrograph. The areas of intensely fluorescing cells as compared to the lesser fluorescing cells is characteristic of malignant tumors.
APPROVAL SHEET

The thesis submitted by Dr. Joseph W. Rossa has been read and approved by the appointed members of the Department of Oral Biology.

The final copies have been examined by the Director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 25, 1970

Date

Signature of Advisor