An Investigation of the Changes in the Cell Surface Isoantigens of the Hamster Pouch Epithelium Induced by Malignant Change

Robert F. Chavez

Loyola University Chicago

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AN INVESTIGATION OF THE CHANGES IN THE CELL SURFACE ISOANTIGENS OF THE HAMSTER POUCH EPITHELUM INDUCED BY MALIGNANT CHANGE

BY

ROBERT F. CHAVEZ

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 1968
AUTOBIOGRAPHY

Robert F. Chavez was born in Chicago, Illinois on September 6, 1937.

He was graduated from Crane Technical High School, Chicago, Illinois in June, 1956.

In September, 1956 he entered the University of Illinois, College of Engineering, Chicago, Illinois. After two years he changed his curriculum to pre-dentistry, and he pursued the required courses in the evening at various colleges and universities in Chicago, Illinois.

In September, 1962 he entered Loyola University, School of Dentistry, Chicago, Illinois. He received the degree of Doctor of Dental Surgery in June, 1966.

One week following his graduation from the School of Dentistry, he began graduate studies in the Department of Oral Biology of the same University.
ACKNOWLEDGEMENTS

My sincere thanks to Patrick D. Toto, D.D.S., M.S., Professor of Oral Pathology, Loyola University, my teacher and thesis director for his guidance in the preparation of this thesis. His comments on the interpretation of histologic data gave clarity to a difficult portion of my work.

To John Madonia, D.D.S., Ph.D., Assistant Professor of Microbiology and Pathology, Loyola University, for his advice on testing the immune serum.

To Richard Prendergast, D.D.S., M.S., for his helpful comments on immunofluorescent staining and purification of the conjugated globulin.
DEDICATION

To my wife, Eleanor
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CHAPTER I

INTRODUCTION AND STATEMENT OF THE PROBLEM

A. Introductory Remarks:

Cancer is a word which can fill one with a sense of complete hopelessness; a hopelessness born of the certainty of its irreversible course once it has progressed beyond a certain point. This progression has an almost insignificant origin: A normal cell experiences a change in its basic structure. An understanding of the exact nature and degree of that change still eludes us today.

Once the basic structure of a cell has been altered, the cell starts on a course of malignant growth which is well documented in both its chemical and physical aspects. Investigations of these chemical and physical aspects have yielded a wealth of basic information which is pointing the way and will ultimately enable us to understand the subtle mechanism of malignant change.

Without question, the most promising avenue of cancer research today is immunology because of the highly specific nature of antigen-antibody systems. Good progress on the immunologic aspects of cancer has prompted researchers to optimistically predict a cure for cancer.
in the near future. This is a noble goal, and all work which hastens
the day of its realization is a worthy endeavor. It is in that spirit
that this investigation was undertaken.

B. Statement of the Problem:

It is known that normal cells produce a surface which is chiefly
mucopolysaccharide. The native antigens (species, blood group, etc.)
for any species are known to be found chiefly in this mucopolysaccharide
surface; they are inherently a part of this surface.

This investigation will attempt to show qualitatively what changes
occur, if any, in the native antigens when hamster pouch epithelium
undergoes malignant change.
A. Fluorescent Antibody Technique:

Prior to the advent of immunofluorescence in 1942, investigators had been searching for a method which would graphically show the distribution of soluble antigens in tissues. Up to that point, two principal methods of determining the content and distribution of soluble antigens in a tissue sample were available to investigators. One method involved the chemical analysis of a tissue sample to determine its approximate content of soluble antigens. The second method involved the uptake of radioactive trace elements and the use of tissue autoradiograms to determine the distribution of soluble antigens. Both methods have merit, but at that point they did not have the graphic qualities which researchers were interested in.

Coons and his co-workers Creech, Jones, and Berliner (1942) at about the beginning of World War II were experimenting with the conjugation of fluorescent dyes to protein molecules. They theorized that it might be possible to attach a fluorescent tracer to an antibody
molecule without altering the immunologic specificity of the antibody. In this investigation they prepared an antiserum which contained antibodies against a specific type of pneumococcus. A fluorescent dye, fluorescein isocyanate, was then conjugated to the antibody molecules. This conjugated antibody was then applied to previously prepared sections of mouse liver heavily infected with the same type of pneumococcus. These sections were then viewed under a microscope equipped with an ultraviolet light source. The distribution of the soluble pneumococcal antigens in the mouse liver were clearly revealed. Thus a new technique with great promise was created.

Technical difficulties were associated with the various aspects of this new method. At the end of World War II, Coons and Kaplan began to work on improvements for this new technique. In 1950, their results were published. The most notable improvements which they made were an improved technique for preparing tissue sections with less destruction of its antigenic activity and methods for reducing non-specific staining. Non-specific staining occurred in two ways. The first type of non-specific staining was due to unconjugated fluorescent material which remained in the immune serum. They separated the conjugates from
the unbound fluorescein by dialysis and precipitations with half saturated solutions of ammonium sulfate. These procedures did not remove all of the unconjugated fluorescein, but they were an improvement. The second type of non-specific staining occurred because of the presence of extraneous antibodies in the immune serum. These two workers discovered empirically that this type of non-specific staining could be removed by shaking the dialyzed conjugates with a washed and dried suspension of ground liver.

Despite the improvements introduced by Coons and Kaplan, non-specific staining remained a problem for many using this new technique. In 1951, Coons published another article which mentioned this problem specifically. He pointed out that it was becoming clear to him that much of the non-specific staining encountered was due to what he termed natural antibody. He speculated that natural antibody was the result of the possession of common antigens by animals of different species and bacteria which often infect the serum donors. Much of this non-specific staining, he pointed out, could be avoided by absorbing the conjugated antisera with normal tissues from the animals being studied. This method is successful only when appreciable amounts
of the antigen belonging to the interfering antigen-antibody system are present.

The paraffin embedding method of preparing tissues for histologic study provides a minimum of morphologic disturbance in the tissue sections. This method, however, is not suitable for use with the fluorescent antibody technique because it involves the use of heat and chemical agents which denature tissue antigens. Sainte-Marie (1962) worked out a method by which tissues intended for study by immunofluorescence could be prepared by a modification of the usual paraffin embedding method. In his modification 95% ethanol cooled to 4°C is used as a fixative, and heat is kept to a controlled minimum. He claims that this modified paraffin embedding method is an improvement over frozen sections because it gives brighter staining and more precise delineation between stained and unstained areas. This modification will be used in our study.

Another problem which prevented the widespread use of the fluorescent antibody technique was the inability of the researcher, in many situations, to obtain a high titer antiserum against one or several antigens. In the process of conjugation and removal of non-specific
materials enough protein of the required type would be removed from the weak antiserum that it became useless. Weller and Coons (1954) introduced an improvement which permitted this technique to be employed effectively with a weak antiserum. This improvement they termed sandwich staining. A weak antiserum was not conjugated with the fluorescent tracer; it was only exposed to tissue preparations which would remove non-specific antibodies. A high titer antiglobulin against the species in which the weak antiserum was produced was conjugated with the fluorescent tracer. Since the antiglobulin was obtained in high titer the antibody loss caused by conjugation and purification became insignificant. With this improvement more researchers, especially in the field of cancer, began to apply immunofluorescence to their problems.

Tanaka and Leduc (1956) found the Forssman antigen to be common to many species of animals commonly used in research. Their work supports the earlier assumption by Coons, that there are antigens common to many species, and these common antigens in turn are responsible for some of the non-specific staining encountered in the fluorescent antibody technique.
B. Antigenic Change In Neoplasia:

An interesting hypothesis was put forth by Green (1954). He hypothesized that cells derive their individual identity from one or more substances which are probably protein complexes or their related enzyme systems. In malignant change these identity substances are altered and thus behave as isoantigens. The cells involved, because of the loss of some of their identity substances, become more antigenically neutral. The immunologically regulated disposal mechanisms no longer affect them to the same degree, and their growth becomes less restricted.

Hiramoto and Pressman (1957) performed an interesting experiment. They were interested in determining whether the malignant melanoma arose from skin or underlying dermis. They prepared two sera; one against skin and one against a malignant melanoma. Using the fluorescent antibody technique they found that both sera stained melanoma cells, neurilemma, connective tissues, and the papillary region of skin. The sera differed only in that the anti-epidermis serum stained only epidermis, hair follicles, and several types of carcinomas. They explained this behavior by the existence of common or cross-reacting antigens in the papillary layer of normal skin and the cytoplasm of
melanoma cells. Since none of the several antimelanoma sera prepared reacted with epidermis they concluded that the melanoma does not arise from the epidermis.

Nairn, Richmond, Mc Entegart, and Fothergill (1960) used the fluorescent antibody technique to test for organ-specific antigens in three types of normal and tumor tissues. They used rat liver and hepatoma induced by aminoazo-dye feeding; hamster kidney and renal carcinoma induced by estrogen implant; human skin and various skin tumors occurring spontaneously in man. The antisera against these tissues were rendered organ-specific by absorptions with appropriate tissue preparations. In liver and kidney tissue they were able to demonstrate an organ-specific antigen in normal tissue. They used complement fixation and gel diffusion as well as fluorescent antibodies to demonstrate the organ-specific antigens. These same antigens were absent from or present only in small amounts in the corresponding malignant tumor. In malignancies of human skin they found a depletion of specific antigen. The carcinomas showed greatly diminished fluorescent staining. They found that the specific antigen appears to be concentrated in the cell membrane and in the perinuclear zone. This they consider a significant
finding, and they cite the work of Green (1954), in which he writes of "identity proteins" and their relationship to the immunologically regulated disposal mechanisms. They were thus able to demonstrate a loss of antigens in various malignant tumors.

Carruthers and Baumler (1965) did a very cogent study with the fluorescent antibody technique in which they tested for loss of epithelium-specific antigen and gain of new antigenicity by malignant cells. Using the mouse as their test animal, they tested epithelium under the following conditions: normal, undergoing normal changes induced by the hair growth cycle (hair was removed), hyperplastic, tissues undergoing abnormal changes produced by topical application of methylcholanthrene, and squamous cell carcinoma induced by a carcinogen. The antisera against these three tissue states were prepared in rabbit and rendered epithelium-specific by absorption with appropriate tissue preparations (liver, lung, and kidney tissue sediments). Their tests suggested that normal and hyperplastic epidermis contain antigens not found in squamous cell carcinoma, and squamous cell carcinoma contains antigens not found in normal and hyperplastic epidermis.

In the same year another investigator, Brandtzaeg, used indirect
immunofluorescence to show the presence of substances A and B on the
surface of cells in human gingiva. His study also showed that these
antigens are resistant to water extraction, and they are not affected by
alcohol fixation. He used the method developed by Sainte-Marie, to
prepare his tissue sections.

The disclosure by Brandtzaeg, of blood-group substances A and B
in human gingiva prompted another investigator, Prendergast (1968), to
carry out a study in which he wished to determine whether or not these
antigens are lost when human oral mucous membrane undergoes
malignant change. His tissue samples consisted of eight specimens of
epidermoid carcinoma and fifteen specimens of clinically normal
gingiva. Each specimen was obtained from a different individual. His
findings indicate that the blood group substances A and B are lost in
malignization of human oral mucosa.

Our study was carried out with the oral mucous membrane
of the hamster. Prendergast was not able to obtain specimens of
carcinoma and normal tissue from the same individual. Using the
hamster as the test animal allowed me to obtain carcinoma from one
pouch and use the other pouch as a normal control in the same animal.
C. Chemical Carcinogenesis:

Polycyclic hydrocarbons capable of carcinogenic activity were first discovered by Kennaway and Heiger, in the early 1930's. The first carcinogen isolated in 1930 was 1, 2, 5, 6, -dibenzanthracene. Soon after 3, 4-benzpyrene and 20-methylcholanthrene were isolated. Many studies were carried out using these chemicals to induce malignancies of different types. These chemicals have ultimately become known as universal carcinogens because they produced malignant change by topical application, intramuscular and intravenous injection, or simply by incorporating them into the diet.

Salley (1954) undertook an extensive study to determine which of these three carcinogens was most effective in producing carcinoma of the hamster cheek pouch. He used a half percent solution of carcinogen in each of two solvents, acetone and benzene. There were two groups of animals. One group received the acetone solution and the other received the benzene solution. The right pouch of each animal was painted three times per week for sixteen weeks. The results in both groups showed conclusively that the 9, 10-dimethyl-1, 2-benzanthracene (DMBA) is the chemical of choice for producing carcinomas in the hamster pouch cheek.
Salley (1955) carried out another investigation in which he attempted to shorten the time necessary to obtain malignant tumors of the hamster cheek pouch. In this study he used mineral oil as a solvent for the DMBA. He found that the induction of carcinoma in the hamster was shortened by as much as seven weeks. It was reasoned that the DMBA must be kept in solution to be readily available to the tissues. Since acetone is volatile it evaporates quickly leaving a residue of DMBA crystals. Mineral oil is able to keep the DMBA in solution. Also, the mineral oil did not produce the tissue inflammation and ulceration seen when volatile solvents were used.

It has been demonstrated in clinical epidemiologic studies that chronic alcoholism enhances the formation of oral carcinoma. Henefer (1966) carried out an investigation in which he attempted to determine whether or not the ingestion of alcohol increased the incidence of carcinoma in the hamster cheek pouch. He divided sixty hamsters into four equal groups. Two of the groups received only 30% ethanol for fluid intake. The other two groups received water. In the 30% ethanol group the pouches of half of the animals were coated with DMBA three times per week for seven weeks. The pouches of the other
animals in the ethanol group were coated with mineral oil to serve as controls. The animals in the group receiving plain water had their pouches coated in a similar manner. The results, though not conclusive for the number of animals used, indicated that chronic ingestion of ethyl alcohol increases the incidence of carcinoma in the hamster cheek pouch. Since I was interested in obtaining the highest incidence of carcinogenesis in the test animals their fluid intake was limited to 30% ethanol.
CHAPTER III
MATERIALS AND METHODS

A. Animals:

The Syrian Golden Hamster was selected for this study because it is generally recognized that a closely inbred group of animals will yield more consistent experimental findings than a heterogeneous group. Since its introduction to the United States in 1938, the Syrian Golden Hamster has proven its value as a laboratory animal in many epidemiological studies.

The normal hamster pouch wall consists of four distinct layers. The surface is stratified squamous epithelium two to four cell layers thick. The second layer is dense fibrous connective tissue containing no accessory structures or glands. The third layer is a thin band of striated longitudinal muscle fibers. The fourth layer is loose areolar connective tissue.

Twenty-four male Syrian Golden Hamsters were purchased for this study. When the animals were received they were only three weeks old. Reiskin (1968) has extensive experience in chemically induced carcinoma of the hamster pouch. He observed that neoplasia occurs most
rapidly in young hamsters.

Twelve hamsters were removed from the shipping case; each was placed in a separate cage which was provided with abundant laboratory food. The fluid intake of these animals was limited to 30% ethanol. This was done in the hope that it would increase the incidence of neoplasia in this group of hamsters.

Six large male rabbits were also purchased. Each weighed at least five pounds. These animals were used to produce an antiserum against normal hamster pouch.

B. Induction of Neoplasia in the Hamster Cheek Pouch:

The carcinogen chosen for this study is 9,10-dimethyl-1,2-benzanthracene (DMBA) because it has been shown to be the most potent in producing neoplasia of the epidermis. Previous researchers have established that only one type of malignant change, squamous cell carcinoma, is produced by DMBA in the hamster cheek pouch. Ethylene glycol was chosen as the vehicle because it is miscible with water as well as a solvent for DMBA. It seems reasonable that a water soluble vehicle will facilitate the absorption of the DMBA into the tissues because while the vehicle holds the carcinogen in solution it in
turn, because it is water soluble and does not stain the tissues. A saturated solution of DMBA was prepared in glycol which will dissolve

Frequent daily cleaning was required for this experiment. Detecting the early signs of the tumors in the pouches was very important. We were able to detect them quickly across the animal's back. The usual

The usual method of holding the animal is by brushing my thesis director. He suggested that a small round rubber pellet saturated with a carcinogen could be placed in the pouch. When the animal attempts to remove it, he will, in effect, paint his own pouch. A small straight hemostat was used to grasp the rubber pellet and place it in the deepest part of the cage. This was done only in the right pouches; the left pouches served as controls.

**FIGURE I**

**WIRE MESH CAGE WITH FOOD AND 30% ETHANOL**
turn, because it is water soluble, will be slowly absorbed into the tissues. A saturated solution of DMBA was prepared because ethylene glycol will dissolve DMBA only to the extent of 0.5 to 1.0 per cent.

Frequent direct visualization of the hamster pouches is not required for this study because we are not primarily interested in detecting the earliest clinical change in the mucosa of the hamster pouches. Therefore, apparatus to immobilize the hamster was not used. Application of the carcinogen only requires that we be able to hold the animal in the hand. Usually, the hamster will quickly accept being held in the hand. If biting had been a problem the animal could have been held with a gloved hand.

The usual method for introduction of carcinogen to the hamster pouch is by brush application. A different method was suggested by my thesis director. He feels that a small cotton pellet saturated with carcinogen could be placed in the pouch. When the animal attempts to remove it he will, in effect, paint his own pouch. A small straight hemostat was used to grasp the saturated pellet and place it in the deepest part of the hamster pouch. The carcinogen was placed only in the right pouches; the left pouches served as controls. The
application of carcinogen continued until the appearance of evidence of neoplasia.

In the laboratory the pellet method for introducing the carcinogen proved to be both rapid and effective. After the second week, the animals were held without the need for a protective glove. The pellet was consisted of diethyl ether in the hamsters with the addition of methanol to the ethylene glycol. Hamsters were cut and the pouches were cut and the diethyl ether and the tissues were reassembled. The pouches were cut and food debris were placed in the freezer until the pouch material was frozen solid. A single edge safety razor blade was used to shred the frozen pouch material. The shredded pouch material was harvested from the ice. The yield was 12.3 grams. Forty-eight milliliters of normal saline were added to the weighed pouch material.

A motor driven mortar and pestle was set up. The mortar is a

FIGURE 2
CARCINOGEN SOLUTION AND ITS COMPONENTS
application of carcinogen continued until the appearance of clinical evidence of neoplasia.

In the laboratory the pellet method for introducing the carcinogen proved to be both rapid and effective. After the second week, the animals were held without the need for a protective glove. The pellet was consistently placed in the depth of each pouch, and in general the hamsters allowed the pellet to remain there unmolested.

C. Preparation of Anti-Hamster Serum in Rabbit:

Twelve hamsters were sacrificed by prolonged exposure to diethyl ether in a covered glass jar. When vital signs had ceased the hamsters were removed and their pouches were everted. The pouches were cut away from the hamsters, rinsed in normal saline to remove food debris, and placed on the surface of a tray of ice. The trays were placed in the freezer until the pouch material was frozen solid. A single edge safety razor blade was used to shred the frozen pouch material. The shredded pouch material was harvested from the ice. The yield was 12.3 grams. Forty-eight milliliters of normal saline were added to the weighed pouch material.

A motor driven mortar and pestle was set up. The mortar is a
FIGURE 3

MATERIALS REQUIRED TO INTRODUCE THE CARCINOGEN

FIGURE 4

PELLET SATURATED WITH THE CARCINOGEN SOLUTION IS INTRODUCED TO THE DEPTH OF THE POUCH.
FIGURE 4

A HAMSTER WELL ACCLIMATED TO BEING HELD IN THE HAND

FIGURE 5

PELLET SATURATED WITH THE CARCINOGEN SOLUTION IS INTRODUCED TO THE DEPTH OF THE POUCH
FIGURE 6
MATERIALS REQUIRED FOR HAMSTER SACRIFICE

FIGURE 7
GLASS COVER REMOVED AT CONCLUSION OF HAMSTER SACRIFICE
glass tube which resembles an ordinary test tube. The pestle is made of teflon, and it fits closely to the inner dimension of the mortar. The mortar was held in an ice water bath while the pestle was revolving to prevent heat denaturation of protein material.

Ten to fifteen milliliter increments of the saline-pouch mixture were added to the mortar. This material was acted upon by moving the pestle up and down in the mortar while the pestle revolved. The resulting mash was strained through two layers of cotton gauze. The yield was 50 milliliters of saline-tissue homogenate. The homogenate was placed in a cool centrifuge for thirty minutes at 100 g. The supernate was stored in a glass bottle at -15°C.

The supernate was prepared for injection in two forms. The first form consisted of 10 milliliters of gross supernate mixed with an equal volume of Freund's adjuvant. The second form was an attempt to purify the mucopolysaccharide portion of the supernate. Antonopoulos et al (1964) showed that mucopolysaccharides can be quantitatively precipitated from a tissue sample with cetylpyridinium chloride.* Since the isoantigens with which we are concerned in this study are part of the mucopolysaccharide cell surface, concentration of this

*Purchased from Eastman Organic Chemicals, Rochester 3, New York
material should eliminate much extraneous protein material. Accordingly, 16 milliliters of 5% cetylpyridinium chloride (CPC) were mixed with 4 milliliters of the supernate. This formed a 1% solution of CPC with the supernate. This mixture was allowed to stand overnight in the refrigerator at 4°C. The next day a white precipitate had formed and settled to the bottom of the container. The precipitate was separated by centrifugation in a cold centrifuge. Equal portions of precipitated mucopolysaccharide and Freund's adjuvant were combined. The precipitate became resuspended in Freund's adjuvant.

Each form of antigen material was injected into one rabbit once per week for four weeks. This was a pilot study intended to determine whether or not we could create an antiserum against normal hamster pouch. Five days after the last injection the two rabbits were bled from the ear veins, and the antibody titer was determined. The tests showed a weak but acceptable titer in the rabbit which had received the CPC purified antigen. The second rabbit which had received the gross form of antigen showed no detectable titer with the qualitative precipitin test.

The remaining four rabbits were separated into two groups.
Each group received one type of antigen as in the pilot study. At the end of the immunization period the rabbits were test-bled, and the serum titer was determined. All rabbits were then exsanguinated by cardiac puncture. The serum was separated from the pooled blood of each group of two rabbits. The separated serum was further clarified by centrifugation. Non-specific protein in the antisera was reduced by two absorptions with liver powder. The two antisera were mechanically agitated for one hour with 100 milligrams of liver powder per milliliter of antiserum.

D. Preparation of Tissues for Study:

When carcinoma was found in the right hamster pouches the animals were sacrificed. Specimens of cancer and normal tissue were obtained from the right and left pouches respectively of the same animal and processed according to the method of Sainte-Marie.

Sandwich staining, as outlined previously, was used to stain the sections of normal and cancer tissue. The first layer was the antiserum obtained from the rabbit. The second layer was goat anti-rabbit gamma globulin conjugated with fluorescein isothiocyanate.* The unconjugated fluorescent material was removed by passing the

*Purchased from Hyland Laboratories, Los Angeles, California
FIGURE 8
RABBIT HELD IN SUPINE POSITION IN PREPARATION FOR CARDIAC PUNCTURE

FIGURE 9
PROBING FOR THE HEART
FIGURE 10

A SUCCESSFUL CARDIAC PUNCTURE

TWENTY ML OF BLOOD IS BEING WITHDRAWN FROM THE RABBIT
FIGURE 11

TWENTY MILLILITER SYRINGE FILLED WITH BLOOD IS BEING WITHDRAWN FROM THE RABBIT
conjugated globulin through a Sephadex column. The elution was carried out with phosphate buffered saline (pH 7.2). The eluant was concentrated by evaporation in a chemical dessicator at 4°C.

Two blocking reactions were used to determine staining specificity. In the first test normal rabbit serum was substituted for immune rabbit serum in the first layer. In the second test the first layer of rabbit serum was eliminated; the conjugated globulin was used with no intermediate layer.

A Richert Zetopan fluorescence microscope with an HBO high pressure mercury vapor lamp was used to examine the slide sections. Magnifications of 100 and 400 diameters were used.

Photomicrographs of the stained sections were taken using outdoor highspeed Ectachrome film with an ASA of 160. The exposure times varied from 90 to 120 seconds.
CHAPTER IV

FINDINGS

A. Incidence of Neoplasia:

The hamster is one of the most interesting animals to work with in the laboratory because of its extreme curiosity. We found the Syrian Golden required a minimum of twelve hours of observation per day. We divided the group of twelve hampers into three groups of four hampers each. The remaining two hampers were used as a control group. The remaining hampers were observed daily without further incidence. The hamsters became accustomed to the cage and the method of observation. The experiment was conducted without any complications.

The experiment entered its second phase where we observed tumor growth by external palpation. The right and left pouches of each animal were palpated once per week. The first evidence of tumor growth was noted in the tenth week. Three hampers developed small palpable tumors at the base of the pouch. These tumors were removed by evertment. A papillary tumor approximately one millimeter in diameter was found in one of these hampers. At this point, application of the carcinogen was discontinued.

FIGURE 12

REICHERT ZETOPAN RESEARCH MICROSCOPE
CHAPTER IV
FINDINGS

A. Incidence of Neoplasia:

The hamster is one of the most interesting animals to work with in the laboratory because of its extreme curiosity. We found the Syrian Golden Hamster to be a hardy laboratory animal which required a minimum of care. By the end of the fifth week, our original group of twelve hamsters had dwindled to nine because of faulty cage latches. The remaining nine hamsters continued through the experiment without further incident. As I mentioned previously, the animals quickly became acclimatized to the reception of the carcinogen by the method which we described earlier. As the period of carcinogen application entered its sixth week we began to check each animal for tumor growth by external palpation. The right and left pouches of each animal were palpated once per week. The first evidence of tumor growth was noted in the tenth week. Three hamsters had developed small palpable tumors at the base of their right pouches. When the hamster pouches were everted a papillary tumor approximately one millimeter in diameter was seen. At this point application of the carcinogen was discontinued.
FIGURE 13

EVERTED NORMAL HAMSTER POUCH
FIGURE 14

LIVING HAMSTER WITH A TUMOR IN THE RIGHT POUCH

FIGURE 15

EXCISED TUMOR
FIGURE 15

LARGE TUMOR IN THE RIGHT POUCH OF A SACRIFICED HAMSTER

FIGURE 16

EXCISED TUMOR
according to plan, but their fluid intake was still restricted to 30% ethanol. At the end of the thirteenth week tumors were present in all but one of the remaining group of nine hamsters. One hamster was sacrificed at this point to test the techniques for preparing and examining tissue specimens. In the fourteenth week one hamster died. It had a large tumor in its right pouch. At the end of the fifteenth week all remaining hamsters were sacrificed.

The rate of tumor growth was fairly rapid. The increase in diameter averaged one-half millimeter per week. Sections of each tumor were stained with H&E and examined microscopically for evidence of malignant change. Histologically, each tumor showed the characteristics of well differentiated squamous cell carcinoma. Eight of the remaining nine hamsters developed squamous cell carcinoma. This is an incidence of eighty-nine percent.

B. Immune Serum:

The gross and mucopolysaccharide forms of the normal hamster pouch material provoked significantly different immune responses in rabbit. The same two forms of normal hamster pouch material had been prepared two months earlier; each form had been injected into a single
rabbit in order to determine if the material could provoke a significant immune response. The immune response in the early group of test rabbits was qualitatively the same as in the later, larger group.

The gross form of hamster pouch material provoked a weak immune response in both the early and the late groups of rabbits. The titer of the immune serum was so low that the qualitative precipitin test showed a negative reaction. Subsequently, however, this immune serum did show a weak reaction line when gel diffusion tests were carried out on agar plates.

The mucopolysaccharide form of hamster pouch material provoked a significantly greater immune response. The qualitative precipitin test showed an extinction point of one part antiserum in thirty-two parts of buffered saline. Although this is still a low titer, it can be used in our investigation because we are using the sandwich (two layer) staining technique. This is the technique which was developed expressly for the purpose of making effective use of immune serums of low titer. The gel diffusion test showed a strong line of reaction for this serum. This antiserum was used in all staining reactions; the gross form of immune serum was discarded.
C. Staining in Normal Tissue:

All specimens of normal hamster pouch showed positive staining in the epithelium. We also saw a variable amount of non-specific staining in the sub-epithelial tissue layers. The techniques which we employed to reduce unconjugated fluorescent material and non-specific antibodies enabled us to reduce the non-specific staining to an acceptable level. I was never able to see pure tissue autofluorescence in the sub-epithelial layers. The staining reaction in the epithelium was unmistakably bright positive.

Previously, I described two types of blocking tests which were employed in this investigation. The sections of normal tissue subjected to these tests showed an almost complete extinction of all staining. Even in blocking tests we were not able to see pure tissue autofluorescence. The low level diffuse background staining was probably due to unconjugated fluorescent material.

D. Staining in Neoplastic Tissue:

This investigation was designed to permit me to compare the intensity of immunofluorescent staining in normal and neoplastic tissues from the same test animal. Accordingly, the staining reactions in the
FIGURE 17

HISTOLOGIC SECTION OF NORMAL POUCH SHOWING FOUR DISTINCT TISSUE LAYERS: EPITHELIUM, DENSE CONNECTIVE TISSUE, STRIATED MUSCLE, AND LOOSE CONNECTIVE TISSUE (H&E 100X)

FIGURE 18

SQUAMOUS CELL CARCINOMA OF THE HAMSTER POUCH SHOWING PEARL FORMATION (H&E 100X)
FIGURE 19
NORMAL HAMSTER POUCH SHOWING BRIGHT POSITIVE STAINING OF THE EPITHELIUM (400X)

FIGURE 20
NORMAL HAMSTER POUCH SHOWING POSITIVE STAINING OF THE EPITHELIUM (100X)
corresponding sections of normal and neoplastic tissue were compared. A consistent finding in the sections of malignant tissue is a generalized decrease in the intensity of positive staining. The intensity of staining in normal sections was definitely bright, but the positive staining in the malignant tissue was dull by comparison. The intercellular areas in the tumor sections were stained, but the positive staining was diminished. The blocking tests in malignant tumor sections, as seen in the blocking tests in normal sections (Fig. 20), were negative.

The blocking reaction was performed before the conjugated fluorescent antibody was added. In subsequent stained sections the background staining was greatly reduced. The background staining, however, could still be seen in tissue sections subjected to the blocking tests. When the conjugated antihuman antibody was filtered through the Sephadex column a second time the background staining could still be observed. The conjugated antihuman antiserum was obtained from the same patient from whom the tumor tissue had been obtained, and the conjugated antihuman antiserum had been used in the blocking tests. The conjugated antihuman antiserum was also blocking the positive staining in the normal sections.

FIGURE 21
NORMAL HAMSTER POUCH SHOWING AN ABSENCE OF POSITIVE STAINING BECAUSE OF A BLOCKING REACTION (100X)
corresponding sections of normal and neoplastic tissue were compared. A consistent finding in the sections of malignant tissue is a generalized decrease in the intensity of positive staining. The intensity of staining in normal sections was definitely bright, but the positive staining in the malignant tissue was dull by comparison. The intercellular areas in the tumor sections were the primary area of positive staining. Some staining was seen intracellularly in the perineuclear area. The blocking tests in malignant tissues produced the same lack of staining seen in sections of normal tissue.

E. Non-Specific Staining:

The background staining which can be attributed to unconjugated fluorescent material was quite intense in tissue sections stained before the conjugated antiglobulin was filtered through the Sephadex column. In subsequent stained sections the background staining was greatly reduced. The background staining, however, could still be seen in tissue sections subjected to the blocking tests. When the conjugated antiglobulin was filtered through the Sephadex column a second time the background staining was reduced to a very low level.
FIGURE 22
SQUAMOUS CELL CARCINOMA SHOWING DIMINISHED POSITIVE STAINING (100X)

FIGURE 23
SQUAMOUS CELL CARCINOMA SHOWING AN ABSENCE OF POSITIVE STAINING BECAUSE OF A BLOCKING REACTION (100X)
The objective of this investigation was to observe any changes in the intensity of immunofluorescent staining between normal and carcinogen-induced neoplasia of hamster pouch epithelium. The findings consistently show that the intensity of staining in squamous cell carcinoma is decreased to a significant degree. Such a finding suggests that the mucopolysaccharide cell surface of epithelium in the hamster pouch contains an antigen or antigens which are lost or depleted when the cells become malignant.

In this study normal and malignant tissues from the same animal were compared. This is a significant contribution to the uniformity of the findings because it eliminates some of the variation introduced when samples are taken from different individuals of even a closely inbred group. Further, it was noted that every animal which developed a malignant tumor showed the same type and grade of malignancy. A well differentiated squamous cell carcinoma developed in each case. This finding was expected because other researchers have pointed out that the only type of malignant tumor which develops in the hamster
pouch as a result of a carcinogen is a well differentiated squamous cell carcinoma. An additional degree of uniformity is added by this finding. Thus, the ingredients for consistent results were inherent in the experimental plan, and they expressed themselves in the findings as a qualitative change of consistent degree in the intensity of immunofluorescent staining.

The positive staining reaction which I observed in normal tissue was confined primarily to the cells of the epithelium. I cannot say, however, that the immune serum was rendered tissue-specific for epithelium because only liver powder was used to remove non-specific protein. Had I absorbed the immune serum with preparations of hamster liver, lung, and kidney I would have been able to say that the findings were tissue-specific for epithelium. I do feel that the use of liver powder alone reduced the non-specific staining in non-epithelial tissues to a level acceptable in a qualitative study. The success of the two types of blocking tests employed support the specificity of the findings obtained with the immune serum.

The non-specific staining seen in connective tissue and muscle could not be completely abolished. The elimination of unconjugated
fluorescent material from the conjugated antiglobulin was fairly complete and easily accomplished with a Sephadex column. However, the elimination of non-specific antibodies from the immune serum was a more difficult task. While I am convinced that repeated absorptions with tissue homogenates of several hamster organs would eliminate most of the non-specific antibodies from the immune serum, it must be remembered that the titer of the immune serum was too low to permit me to carry out this procedure without seriously depleting the desired antibodies as well.

If we interpret the loss or depletion of cell surface antigens to be a consistent finding in malignant change, we must ask ourselves what bearing this finding has on the behavior of such cells. We know that malignant cells exhibit a general lack of cohesiveness, and the greater the degree of malignancy the less cohesive or social such cells become. As Green (1959) suggests, it well may be that there are proteins which identify the cell and keep its growth under the control of immunologic regulators. It is also possible that the behavior of neoplastic cells is in part a purely surface phenomenon. The mucopolysaccharide cell surface contains various antigens as an inherent
part of its structure. When these antigens are lost or altered in malignant change the cell must suffer a loss or alteration of surface structure. The cell becomes less and less able to hold itself in close association with its neighbor cells. Freed from the close confinement of its neighbors its growth becomes accelerated. Further investigation must determine the exact nature, degree and location of the antigenic loss or alteration produced by malignant change.

The securing of an immune serum of sufficient titer against normal hamster pouch was a crucial part of this investigation. As I pointed out in the findings the gross homogenate of hamster pouch combined with Freund's adjuvant was almost totally ineffective in provoking an immune response in both groups of rabbits. However, when the mucopolysaccharide portion of the gross homogenate was extracted as a complex with cetylpyridinium chloride it proved to be a much more effective antigen. It was a surprise to see the greater antigenic potency of the mucopolysaccharide preparation because, in general, mucopolysaccharides are poor antigens. The complex of mucopolysaccharide and cetylpyridinium chloride probably enhanced the antigenicity of the mucopolysaccharides. Also, the extraction of the
mucopolysaccharide portion of the gross pouch homogenate eliminated much extraneous protein. The total immunologic capability of the rabbit was devoted to a smaller group of antigens.

The hamsters which received triweekly applications of carcinogen (DMBA) in their right pouches experienced a high incidence of carcinoma (89%). I feel that this high incidence was due in part to the systemic effect of the 30% ethanol which they consumed as their sole fluid intake. Statistical studies on human populations have suggested that ethanol has a systemic effect in facilitating malignant transformation. However, I could not evaluate the effect of ethanol because the test population was too small and I did not institute controls for comparison.
A. Summary:

An alteration in cell surface antigens associated with malignant change has been investigated with the fluorescent antibody technique. Normal hamster pouch epithelium and squamous cell carcinoma (induced by topical DMBA) from the same animal were compared. The findings consistently show that the intensity of staining is diminished in squamous cell carcinoma.

Immune serum against normal hamster pouch was produced in rabbit. The hamster pouch material used to immunize the rabbits was prepared in two forms. One form consisted of the crude supernate of the homogenized and centrifuged pouch material combined with an equal part of Freund's adjuvant. In the second form, the mucopolysaccharides were precipitated from the supernate with cetylpyridinium chloride. The precipitate was combined with an equal part of Freund's adjuvant. Only the second form of antigen material provoked a titer high enough to be used in our study.

Non-specific protein in the immune serum was reduced by two
absorptions with liver powder. Unconjugated fluorescent material in the conjugated globulin was reduced to a low level by gel-filtration with Sephadex. The specificity of the staining reaction was demonstrated by two types of blocking tests.

As an additional measure, the fluid intake of the hamsters receiving carcinogen was limited to 30% ethanol in an effort to insure a high incidence of carcinoma. The animals did experience a high incidence of squamous cell carcinoma (89%), but there is no way of determining the effect of ethanol on such a small population.

B. Conclusions:

1. The hamster pouch has tissue antigens specific for epithelium.
2. The epithelium-specific antigens of the hamster pouch are lost or altered in malignant change.
3. The epithelium-specific antigens of the hamster pouch were effective in producing an immune serum in rabbit.
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The thesis submitted by Dr. Robert F. Chavez has been read and approved by three 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 3, 1968

Signature of Director