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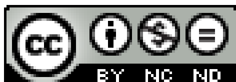

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ANTIGENICITY OF BUCCAL HAMSTER CARCINOMA

A PRELIMINARY STUDY

By

Arnold S. Morof

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

1971

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AUTOBIOGRAPHY

Arold S. Morof was born in Detroit, Michigan on December 2, 1939.

He was graduated from Humford High School, Detroit, Michigan on January 17, 1959.

In January 1959 he entered the University of Detroit, Detroit, Michigan, pre-dental curriculum. After one year, he changed Universities, continuing his education at Wayne State University, Detroit, Michigan and Loyola University, Chicago, Illinois.

In September, 1963 he entered Loyola University, School of Dentistry, Chicago, Illinois. He received the Degree of Doctor of Dental Surgery in June, 1967.

In July 1967, he entered the Graduate School of Loyola University to begin a two year program toward a Master of Science Degree in Oral Biology.

On 1 July 1968, he started a two year residency at Cook County Hospital, completing the residency on 31 June 1970.

1 July 1970, he returned to Loyola University Graduate School to complete the research, thesis and curriculum for his Masters Degree.

ACKNOWLEDGMENTS

My sincere thanks to Patrick D. Toto, D.D., M.S., Professor of Oral Pathology, Loyola University, who provided valuable suggestions, comments, direction and interpretation of histologic data, that helped me during the difficult portion of my work.

To Dale Birdsell, Ph.D., Department of Microbiology, Loyola University, for his advice on testing the immume serum.

To the entire Staff of the Department of Oral Pathology, Loyola University School of Dentistry, for their Technical Assistance in the preparation of the tissues sections and staining.

DEDICATION

To my wife Phyllis

and sons

Benjamin M.

Richard A.

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CHAPTER I

"PREFACE"

Webster's Definition: "Cancer: a malignant growth of tissue, usually ulcerating, tending to spread by local invasion and also through the lymph and blood stream, associated with general ill health, and progressive emaciation." A normal cell changes its function and physical appearance and develops into a malignant cell. With this change, it radically alters its function and physical appearance. Research of these biochemical, functional and physical appearances has been well documented. This knowledge will one day enable us to understand the total mechanism of malignant change and permit us to prevent the progressive destruction it causes.

Immunology, because of the highly specific nature, shows the most promising avenue of cancer research. Immunologic aspects of cancer treatment has made researchers optimistic in developing a "cure" for cancer.

After diagnosing patients with oral cancer, treating them by surgical, radiological and biochemical methods, and

following the "accepted route of treatment and having no survivors over 18 months, other means of treatment must be developed.

Based on the difficulties I encountered treating these patients, I chose to do this research project.

CHAPTER II

Statement of the Problem:

Investigations have revealed that tissue antigens specific for stratified squamous epithelium are found in the hamster cheek pouch. The epithelium specific antigens of the hamster cheek pouch are either lost or they change when malignancy occurs.

The purpose of this research project was to determine whether or not tumor antigens can be found in the buccal epithelium of the hamster carcinoma produced by 9,10,-dimethyl 1,2, benzanthracene.

CHAPTER III

REVIEW OF THE LITERATURE

A. Chemical Carcinogenesis:

Polycyclic hydrocarbons have aroused great interest because of their ability to produce malignant tumors in experimental animals.

Shean (1941) in his experiments with benzanthracene derivatives, found that these compounds were useful tools in various types of studies in experimental cancer.

Berenblum (1945) noted that it had been established that for mouse skin the most potent of the known agents was 9,10, dimethyl 1,2, benzanthracene. He therefore tested this compound on the rabbit's skin and compared the results with those obtained with tar and benzpyrene. Histological examination of the lesions confirmed the malignant nature of the growth, as evidenced by their anaplastic character. There was deep penetration down to the cartilage and in several cases, through the cartilage to the other side, with one case leading to development of a large tumor on the other side of the ear which ulcerated to the surface. Metastases was not

found in any of the animals.

Levy, et al (1950) implanted crystals of 9,10, dimethyl -1,2, Benzanthracene (DMBA) in the food pouch, under the masseter muscle, on the fundus of the mandible. He also implanted crystals of DMBA under the gingival margin of the lower mandibular incisors. Tumors (sarcomas) were produced in 80 per cent of the hamsters treated with DMBA. These tumors spread by invasion along the periodontal membrane.

Levy and Ring (1950) studied the mucous membrane and skin surfaces of the lower lip of mice, age two months, after they were painted with a 0.6 per cent solution of DMBA in benzene. Animals were sacrificed at intervals following the applications of DMBA. Changes in the mucous membrane that he noted were increased amounts of surface keratin and increase in the size and number of granules in the stratum granulosum. Some of these granules extended into the stratum spinosum. There was a slight hyperplasia of the epithelial cells, the proliferation being most marked in the stratum germimativum. Hydropic changes occurred in the cells of the stratum spinosum and stratum granulosum. The lamina propria becomes thickened by edema and a fine fibrinous precipitate.

Salley (1953) used several carcinogenic chemicals on the hamster cheek pouch. He noted that the hamster pouch, because of its ready adaptability in invivo microscopic observations, served as a useful tool in studying initiation, development and metabolism of oral carcinoma.

Salley (1954) studied the carcinogenic activity of three carcinogens, 3,4, Benzpyrene, 20 Methylcholanthrene, and 9,10, Dimethyl 1,2, benzanthracene, dissolved in acetone and benzene. He noted that an acetone solution of DMBA proved to be the best combination, though the other chemical carcinogens gave positive results, but to a lesser degree.

Salley (1955) investigated a non-volatile solvent (mineral oil, U.S.P. heavy), for DMBA that would decrease tumor induction time from 6 to 7 weeks (acetone solvent) to 4½ weeks. In hamster 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 24 hours after application. In 48 to 72 hours these droplets were emulsified into small particles which were distributed in the intercellular spaces of the basal epithelial cell layer. It was noted 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 the epithelial cells. Less tissue deformity was also observed when mineral oil was the solvent than when volatile substances were used.

Salley (1957) described the early changes associated with DMBA carcinogenesis in the hamster cheek pouch observed by conventional histologic methods. The pouch appeared to pass through four distinct histologic gradations before the appearance of definite neoplasms. These are inflammation, degeneration, regeneration, and hyperplasia.

Morris (1961) noted that tissues of the cheek pouch of old hamsters are more resistant to carcinogenic stimuli than those of young hamsters. Five weeks appears to be the ideal age for hamsters to be used in experimental carcinogenesis from the standpoint of ease of manipulation and tumor production. DMBA concentration of 0.5 per cent produces maximum tumor response with minimum latent period, and with no loss of animals due to toxicity. A shorter latent period is required for tumor development in animals exposed to a carcinogen three times per week than in those receiving

the carcinogen twice weekly. Conditions of caging had no apparent effect on the experimental results.

Renstrup, et al (1962) demonstrated that chronic mechanical irritation by use of producing ulceration in the cheek pouch of the hamster increases the onset of cancer induced by DMBA.

Morris and Reiskin (1965) showed that when the hamster cheek pouch was exposed to tri-weekly applications of DMBA for four weeks, all animals developed tumors in a minimum amount of time. Painting less frequently than three times a week, failed to produce tumors in all animals in twenty-one weeks.

Elzay (1966) studied hamster pouches painted with DMBA in alcohol and noted that clinically they developed epithelial tumors earlier and larger than those painted with DMBA in mineral oil. All pouches painted with DMBA in alcohol and mineral oil developed tumors.

Reiskin and Berey (1968) showed that carcinomas induced in the pouches of eight to twelve week old hamsters by DMBA 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.

Duncan (1969) studied the relation of certain hydrocarbons to metabolism and binding to cellular macromolecules. He found that the potent carcinogens were to bind DNA and RNA ten times more readily than non-carcinogens.

B. Carcinogenic Immunology

Hiramoto and Pressman (1957) were determining whether the malignant melanoma arose from skin or underlying dermis. They prepared two sera: one against skin and one against melanoma. Using the fluorescent antibody technique they found that both sera stained melanoma cells, neurilemma, connective tissues, and papillary region of the 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 existance of common or cross-reacting antigens in the papillary layer of normal skin and the cytoplasm of melanoma cells. Since none of the several anti-melanoma sera prepared reacted with epidermis they concluded that the melanoma does not arise from the epidermis.

Carruthers and Baumler (1965) using fluorescein-labeled antibody as an immunochemical stain, demonstrated differences between the antigenic composition of the mouse epidermis; normal, normal growth changes induced by the hair growth cycle, hyperplastic, abnormal growth changes produced by the topical application of methylcholanthrene and carcinogen-induced squamous cell carcinomas. Antisera against epidermis,

early and late hyperplastic epidermis, and highly differentiated squamous cell carcinoma were used. 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 test concluded 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.

Nairm, et al (1960) noted that immunofluorescent staining anti-skin serum revealed loss of skin-specific antigen in squamous cell carcinomas, basal cell carcinomas, naevocarcinomas and sweat gland carcinoma, whereas benign skin tumors stained as brilliantly as normal epidermis.

Hillemann's (1962) demonstrated that human carcinoma of the cervix could be stained by antiserum prepared against such tumors while the normal cervical epithelium failed to stain.

Graffi and Horn (1966) showed that cancinomas induced by carcinogenic hydrocarbons reveal a specific antigenicity. This tumor specific antigen is a characteristic of sarcomas and carcinomas.

Maisin (1966) stated that the majority of experimental tumors of chemical or viral origin have lost at least some of their tissue-specific antigens while they produce an antigen specific to themselves. These specific tumor antigens are not necessarily the same, though they may be, in tumors of the same origin induced in animals belonging to an isologous strain.

Southam (1966) stated that it is now generally conceded that cancer specific antigens do exist in experimental animal tumors.

Carruthers and Baumler (1966) concluded a study with the use of fluorescein-labeled antibodies of the antisera prepared against normal and hyperplastic epidermis and squamous cell carcinomas of mice, the normal epidermis and hyperplastic epidermis were found to have antigens not detected in the carcinomas. In addition, the carcinomas had antigens not apparent in the normal and hyperplastic epidermis.

Elves (1966) stated that injections in antigen either by subcutaneous or intravenous routes, leads to the appearance in the serum of the animal of antibodies directed against the foreign protein. On first encounter with the

antigen there may be a long delay before the appearance of antibody, and the level of the antibody is relatively low. A second injection of the same antigen some time after the primary stimulation leads to a more rapid rate of antibody production and the serum levels achieved are considerable higher. Further administration of the antigen after the secondary response will result in still higher serum levels of antibody until the animal becomes hyperimmunized and no further increase in antibody levels can occur for some time.

Toto (1967) stated that physical changes in the cancer cell probably are the basis for antigenic changes in the cell surfaces.

Mellors (1968) stated that there is evidence, including observations by the immunofluorescence method, that tumor cells may either lose antigens found in the normal cells of origin, gain new ones, or possibly revert to the production of antigens typical of earlier stages of development.

Carruthers (1970) prepared antisera in rabbits against urea-extractable proteins of normal mouse epidermis, papillomas and squamous cell carcinomas. He showed that the epidermis has urea-extractable antigens which are present

in much smaller amounts in the urea extracts of papillomas and squamous cell carcinomas. Papillomas and carcinomas have several urea-extractable antigens in common, and both the benign and malignant growths each have at least on antigen present in greater amount in one tissue than in the other. The papilloma and carcinoma antigens are present, but in small amounts in normal epidermis.

C. Fluorescent Antibody Technique:

Marrack (1934) demonstrated that without impairing the capacity of the antibody to react specifically with the antigen which stimulated its synthesis dye molecules can be chemically linked to antibody molecules.

Coons, et al (1942) used fluorescent antibody technique utilizing fluorescein isocyanate to detect the presence of antigenic material attributable to pneumococcal organism.

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 emulsifier like Falba.

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 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 procedured are unimportant.

Fishel, et al (1952) noted that the addition of killed tubercle bacilli to Freud's adjuvant mixture elicited increased and prolonged antibody levels to selected antigens

and increased antibody formation was correlated with local granuloma formation and hyperplasia in the regional lymph nodes and spleen of animals receiving the emulsion containing killed tubercle bacilli.

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; and non-living antigens, the use of adjuvants will increase the titer.

Coons (1956) stated that the use of an intermediate layer of antiserum followed by the conjugated immune globulin is ten times more sensitive than the single layer staining technique. Gains 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.

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

Riggs, et al (1958) developed fluorescein isothiocyanate as a labelling agent. The isothiocyanates are an

improvement over the isocyanates in that they are more easily prepared, stable and less toxic.

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

Chavez (1968) using the fluorescent antibody technique that showed the fluorescent of tumor cells was diminished as compared to normal cells.

CHAPTER IV

MATERIALS AND METHODS

A. Animals:

Fifteen 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. Four female New Zealand white rabbits, each weighing about four pounds, were utilized in the production of the antiserum to hamster tissue carcinoma.

B. Induction of the neoplasium:

The carcinogen chosen for this study is 9,10, Dimethyl 1,2, Benzanthracene (DMBA), which has been shown to be the most potent in producing neoplasia of the epithelium. The normal hamster pouch wall consists of four distinct layers. The surface is stratified squamous epithelium two to four layers thick, the second layer being dense fibrous connective tissue, the third layer is a thin band of longitudinal muscle fibers and the fourth layer is loose areolar connective tissue. Studies have shown that only one type of malignant

Change occurs, squamous cell carcinoma produced by DMBA in the hamster cheek pouch. Mineral oil was chosen as the vehicle because it is non-volatile, causes less tissue deformity, is distributed in the intercellular spaces of the basal epithelial cell layer, and is a solvent for DMBA. Induction of the tumors occurred in the left cheek pouch of fourteen eight week old dark-eared Syrian hamsters, using 9,10, Dimethyl 1,2, benzathrancene (DMBA, Eastman Organic Chemical, 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 the animal was painted with DMBA solution three times a week, using a No. 7 camel-hair brush. The right cheek pouch went untreated so that it could be utilized as a control. All hamsters were kept on a diet of Purina rat food and tap water. Painting of the pouches continued for six weeks and was discontinued at a time when tumor formation was both seen and palpated externally.

C. Immunization of the Rabbits:

Fourteen dark-eared Syrian hamsters were sacrified after twelve weeks, by prolonged exposure to diethy ether

in a covered glass jar. The right and left cheek pouch of all the hamsters were removed, rinsed in normal saline and placed on the surface of a tray of ice. After the tissue became firm on the ice, the tumors were removed from the normal appearing tissue by sharp dissection. The tumors were then cut in half, half being imbedded in paraffin and the other half was then shredded using a surgical blade wiped free of any surface contaminents. The shredded pouches were collected and placed in a tissue homogenizer (Sorvall Omni-Mixer Homogenizer, Ivan Sorvall, Inc., Inc., Newton, Conn., Model OM-1150), which had been precooled to 0°C. All materials were kept in the frozen state, except when homogenization was done. During homogenization fifty milliters of sterile physiologic saline was added to the tumor cheek pouches. When homogenizing the outside of the tissue, homogenizer was packed with ice chips in water in an attempt to minimize heat denaturation of the tissue proteins. (International Equipment Co., Model HN 6300 RPM) 3,000 rpm frequency for ten minutes, alternating one minute on one minute off, then thirty minute stop period with the homogenate being cooled in ice water. This was done to prevent any heat generation and any protein degeneration of the gross homogenate.

After six hours of this procedure was completed, approximately 30 minutes of total homogenation, fifty milliliter gross homogenate solution was produced. Tumor gross homogenate was kept in a frozen state until injection into the rabbits.

Five sealed ten milliliter vials of Freund's Adjuvant (Difco Laboratories, Detroit, Michigan) were pooled. Three milliliters of Freund's Adjuvant were added to three milliliters of gross homogenate and mixed until a uniform white creamy consistency appeared prior to injecting the rabbits.

Three female New Zealand white rabbits, each weighing about four pounds, were inoculated with 1 ml. of gross homogenate and 1 ml. of Freund's Adjuvant mixed together, using a disposable syringe with a 20 gauge needle. The rabbits were then allowed to rest for a period of two days. This procedure was repeated for five weeks, three injections per week. One week after last injection the first challenge injection was administered six weeks after the first injection. One week later, a second challenge was given. One week after the second challenge injection, nine weeks after the first 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. 1780 in Catalog) to facilitate bleeding. Thirty milliliters of blood was collected from each rabbit, permitted to clot for 24 hours, and centrifuged to separate the serum from the blood cells. All the serum collected from the rabbits were pooled, equal amounts of serum coming from each of the three rabbits. Pooled serum was cleared with normal hamster pouch gross homogenate. The normal tissue was then cut in half, half being imbedded in paraffin and the other half was then shredded using a surgical blade wiped free of any surface contaminents. The shredded pouches were collected and placed in a tissue homogenizer (Sorvall Omni-Mixer Homogenizer, Ivan Sorvall, Inc., Newton, Conn., Model OM-1150), which had been precooled to $0^{o}C$. All materials were kept in a frozen state, except when homogenization was done. During homogenization fifty milliters of sterile physiologic saline was added to the normal tissue cheek pouches. When homogenizing the outside of the tissue, homogenizer was packed with ice chips in water in an attempt to minimize heat denaturation of the tissue proteins. (International Equipment Co., Model HN 6300 RPM) R.P.R. blood tube precipitin test, using .5cc of cleared pooled serum and .5cc of clear gross

homogenate gave a positive precipitation reaction. Control serum from normal untreated rabbit was also used with no precipitation noted.

D. Preparation of the Tissues.

Half of the tumor in the left cheek pouch was immediately placed in 95 per cent ethanol. The tissue specimens were then embedded in paraffin and prepared according to the method of Sainte-Marie (1962). Normal tissue of the hamster cheek pouch of a control animal was also prepared, in a like manner.

The paraffinized section of the tumor and normal tissue were stained with hematoxylin and eosin. These sections were utilized for identifying and localizing tumor cells. They were both viewed under low and high magnification and photomicrographs were taken. Ektachrome High Speed film, ASA 160, was used for all photomicrograph pictures.

Other sections of the paraffinized section of the tumor and normal tissue were stained with the fluorescent dye, fluorescein isothiocyanate, using the "sandwich" technique to visualize antigen-antibody reaction in the tissues. This technique consists of first deparaffinizing the section

embedded in paraffin by exposing them to three consecutive washes of one minute each using xylol, fresh amounts of solvent being used for each washing. The tissue sections were then washed for one minute each in three consecutive physiologic saline baths. Application of the intermediate antiserum, (first antibody antigen reaction), was completed on sections of both normal and tumor tissues for thirty minutes in a high humidity environment "wet chamber" and placed in an incubator at 37°C. Each section was then washed in buffered saline for three minutes. The sections were then reacted with Sheep Anti-Rabbit Globulins, (second antibody antigen reaction), (Hyland Laboratories, Los Angeles, California), conjugated with fluorescein isothiocyanate for thirty minutes in the "wet chamber" within the incubator. Fixation was accomplished using 95 per cent ethanol for twenty minutes, followed by a three minute wash in buffered saline. Glycerol was used to mount the coverslip. The slides were immediately viewed with a Reichert-Zetopan Fluorescent Microscope (Reichert, Austria) illuminated with a mercury vapor lamp. (Lamp #1273WL and Filters KG2B612 and VG1UV). The following tests were used to verify the results

of the immunofluorescence reaction using rabbit immune serum against carcinoma cells.

1. Elimination of first layer of the rabbit immune serum or the immunofluorescence technique.
2. Replacing rabbit immune serum with normal serum.
3. Using rabbit serum cleared in squamous cell carcinoma gross homogenate.

Photomicrographs were taken immediately upon preparation of the sections which were exposed to the fluorescent preparations. Exposure times of 90 to 120 seconds were used on the Ektachrome High Speed film.

CHAPTER V

RESULTS

A. Neoplastic Growth:

Two weeks after the applications of 0.5 per cent of DMBA to the left cheek pouch of the hamsters, the oral mucosa of the pouch appeared reddened, ulcerative and a desquamative reaction occurred. The third week of DMBA application brought significant changes in the disposition as well as the pouches of the hamsters. The hamsters were more irritable than at any previous time. Definite nodular areas were seen in the treated cheek pouches during the applications of the carcinogen. The beginning of the fourth week brought with it a generalized change in the coat of the hamster under treatment. It was at this time that the animals began to loose some hair and matting of the remaining hair was noted. By the fifth week, small tumors (1mm-2mm) were both palpated and viewed in the base of all cheek pouches being treated with DMBA. In the eighth week all tumors were readily palpated externally and all of the animals exhibited loss of external hair over the tumor area. Three animals showed ulceration of the external epithelium

over the base of the pouch. All of the animals had well defined multiple masses in the base of the left cheek pouches. These tumors had a well defined fibrous base and were firmly attached. (Fig. #9) All animals treated were killed at the end of the twelfth week, and tumor specimens from the left cheek pouch were removed. Half of each of the specimens were collected and utilized in the making of the gross homogenate and the other halves were fixed in C 95 per cent ethanol at 4°C.

None of the animals showed any type of reaction in the untreated pouches.

B. Evaluation of the Serum:

Using R.P.R. blood tubes (Rapid Plasma Reaction Capillary Tubes, Hynson, Westcott and Dunning Inc., Baltimore, Maryland), a precipitin test was run on the test serums of the three rabbits six weeks after the first injection and after the second challenge. Half of the R.P.R. tube was filled with cleared immune serum and half with clear gross homogenate. All R.P.R. blood tubes tested showed a positive reaction eight weeks after injection with homogenate, characterized by the formation of a creamy colored precipi-

tation formed between the pooled rabbits serum and the centrifuged hamster pouch gross homogenate supernate. The pooled rabbit serum obtained after nine weeks were again tested for precipitation. Serum from the untreated normal rabbit was also tested. Positive precipitation occurred only when the treated rabbit serum was used against the supernate from the gross homogenate of the hamster pouch tumors. No precipitation reaction occurred when normal rabbit serum was used.

C. Normal and Neoplastic Tissue Staining:

Sections of the normal hamster cheek pouch stained with hematoxylin and eosin showed that the epithelium consisted of stratum corneum, stratum spinosum and stratum germinativum. The thickness of the stratified squamous epithelium was in most instances four to six layers. The lamina propria, a dense connective tissue, contained no accessory glands or structures. The thin striated muscle appeared longitudinal in same section or transverse, depending on the plane of the sectioning. The loose connective tissue contained blood vessels. (Fig. 1 and 2)

Sections of the tumor stained with hematoxylin and

eosin were composed of surface epithelium which was acanthotic and which proliferated downward into the supporting tissue in an irregular manner. There were variable areas of hyperkeratosis. The basal layer was not intact. A relatively marked, predominantly lympocytic inflammatory reaction was seen in the lamina propria. The epithelial tumor cells themselves showed degrees of differentiation from anaplastic epithelium with many mitotic figures to a relatively well differentiated squamous epithelium containing numerous epithelial pearl formations and occasionally individual keratinization. (Fig 3-3A)

D. Immunofluorescent Staining:

Positive staining appeared as an apple green fluoresence. The intensity of the fluorescent staining in malignant tissue sections was bright, while positive, the fluoresence in the normal tissues were dull. The intercellular area found on the periphery of the cells demonstrated in the tumor sections the brightest fluoresence. The staining seen intracellularly in the cytoplasm around the nucleus was less intense. The nucleus did not stain and appeared black. (Fig. 4) A consistant finding in the section of

normal tissue was a lower intensity of positive staining. (Fig.5) The staining of sqaumous cell carcinoma using the normal rabbit serum while positive, the flouresence was dull. The staining of squamous cell carcinoma using the rabbit immune serum cleared in squamous cell carcinoma gross homogenate, while positive, the flouresence was dull. It was apparent that the squamous cell carcinoma stained better than the normal cells when the antisera against carcinoma was used.

CHAPTER VI

DISCUSSION

It was noted that all fourteen DEA Syrian hamsters developed multiple squamous cell carcinomas and papillomas of various size in the treated pouch. The induction of these tumors occurred in the fifth week when using 0.5 per cent DMBA in mineral oil. This finding corroborates those of Levy (1950) and Salley (1953, 1954), who in their research studies showed the same results. In all cases some of the tumors produced in the pouches were squamous cell carcinoma.

The cleared gross homogenate supernate which was prepared from squamous cell carcinoma of the buccal hamster cheekpouch produced an antibody in the rabbits after nine weeks. The gross homogenate was centrifuged to remove any tissue fragments that would give a false precipitation reaction. When the cleared gross homogenate reacted with the immune rabbit serum in an R.P.R. blood tube a positive precipitation occurred. These results concur with Chavez (1968) and Rossa (1970). Southam (1966) has found similar results when using mouse epidermis carcinoma. Carruthers (1970) found using urea extractable technique, antigens that were

present in mouse squamous cell carcinoma.

However, the cleared gross homogenate and the untreated rabbit serum failed to react, supporting the observation of specificity of the treated rabbit serum.

The fluorescence of the cells noted in malignant tissue was a bright apple green (Fig. 4). This indicated an antibody-antigen reaction between the immune serum produced in the rabbit, and the squamous cell carcinoma of the hamster cheek pouch. A blue fluorescent color only (Fig. 6 and Fig. 6A) was noted when the immune serum was omitted from the squamous cell carcinoma indicating the absence of an antibody-antigen reaction.

A dull fluorescent color was noted (Fig. 7-7A) when normal rabbit serum was used on the squamous cell carcinoma indicating some cross reactivity.

It was also noted that a dull blue fluorescent color was present (Fig. 8-8A) when immune rabbit serum cleared with squamous cell carcinoma indicating some cross reactivity.

Chavez (1968) and Rossa (1970) had similar findings. This fluorescence antibody technique is a useful tool to detect the antibody-antigen reaction in cancer cells. Hiramoto and Pressman (1957) and Carruthers and Baumler (1965)

used similar techniques in detecting antibody reactions in cancer cells. They also noted that there was a weak fluorescense in normal tissue, the existance of common or cross-reacting antigens explained this response.

CHAPTER VII

SUMMARY

Fourteen male dark-eared partial albino Syrian hamsters were used in the induction of squamous cell carcinoma. The left cheek pouch of these hamsters were painted twice a week with 0.5 per cent solution of DMBA in mineral oil for eight weeks, until tumors were both palpated and seen directly in the cheek pouch. The right cheek pouch was left untreated to serve as a control. Antibodies were prepared in the rabbit using hamster tumor gross homogenate. Non-specific proteins in the immune serum were removed by absorption with gross homogenate normal hamster material. The gross homogenate antigenic material was centrifuged and the supernate was reacted with immune serum and showed a positive percipitation reaction. Antigens associated with squamous cell carcinoma were manifested employing the fluorescent antibody technique. Normal hamster pouch epithelium and squamous cell carcinoma from the same animal were compared. The findings consistently showed that the fluorescence intensity of the staining is brighter in the squamous cell carcinoma.

CHAPTER VIII

CONCLUSION

This research study concluded that:

1. Squamous cell carcinoma is produced using 0.5 per cent DMBA in buccal pouches of DEA hamsters.
2. Gross homogenate of the hamster cheek pouch carcinoma with the addition of Freund's Adjuvant (complete) can effectively elicit an antibody response in the rabbit.
3. The fluorescent staining indicated that there was an antibody produced against squamous cell carcinoma and to a lesser extent against normal tissue (squamous cells).

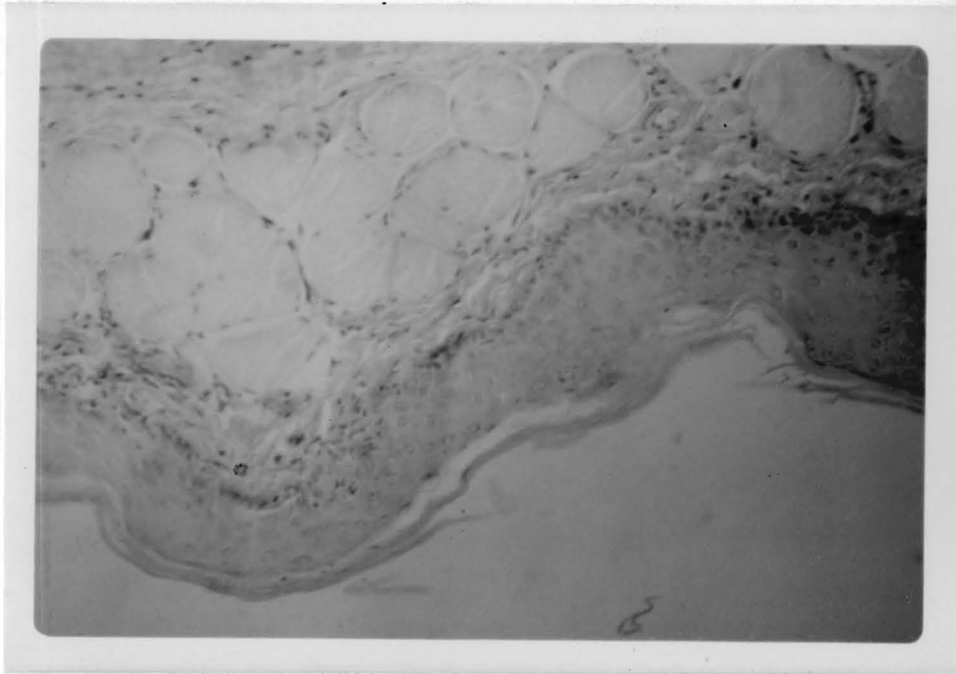

Figure 1

This photomicrograph of normal hamster cheek pouch is stained with hematoxylin and eosin. Four distinct layers are shown. Epithelium, dense connective tissue, striated muscle. The magnification of this section is 100X.

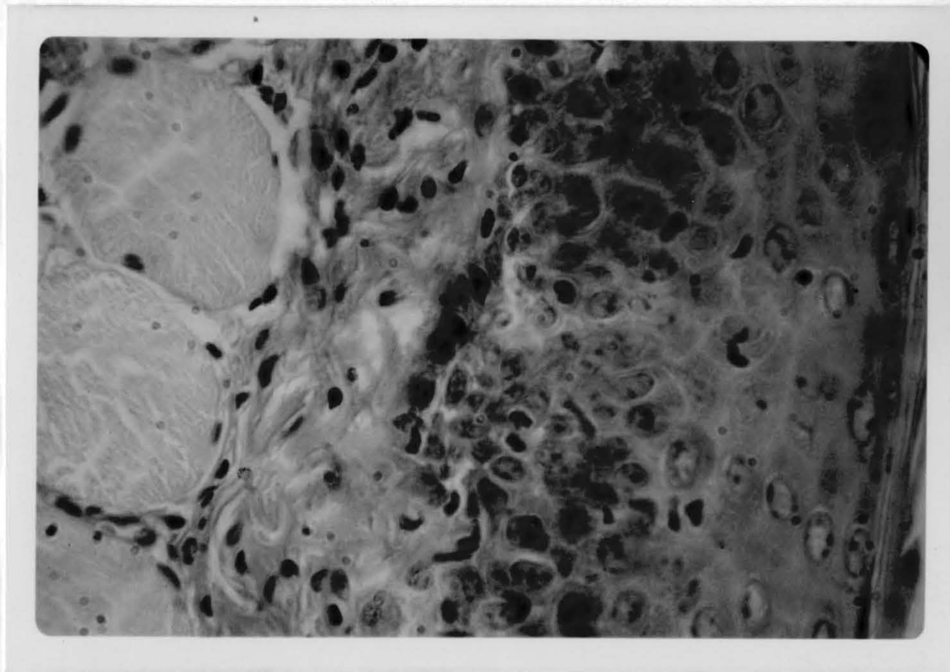

Figure 2

This photomicrograph of normal hamster cheek pouch is stained with hematoxylin and eosin. Appearing in the photomicrograph is the stratum corneum, stratus spinosum, stratum germinativum, lamina propria, and muscle layer. The magnification of this section is 250X.

Figure 3

This photomicrograph of squamous cell carcinoma of the hamster cheek pouch is stained with hematoxylin and eosin. The magnification is 100X. Note the dyskeratosis of the squamous epithelial cells in a stroma of connective tissue.

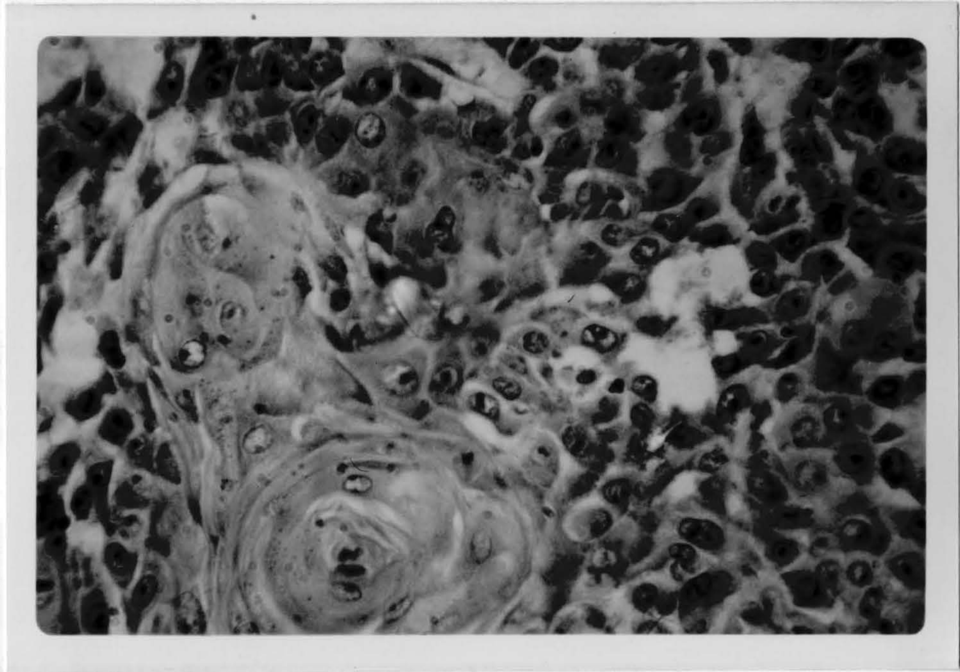

Figure 3A

This photomicrograph of squamous cell carcinoma of the hamster cheek pouch is stained with hematoxylin and eosin. The magnification is 250X. Note the dyskeratosis of the squamous epithelial cells in a stroma of connective tissue.

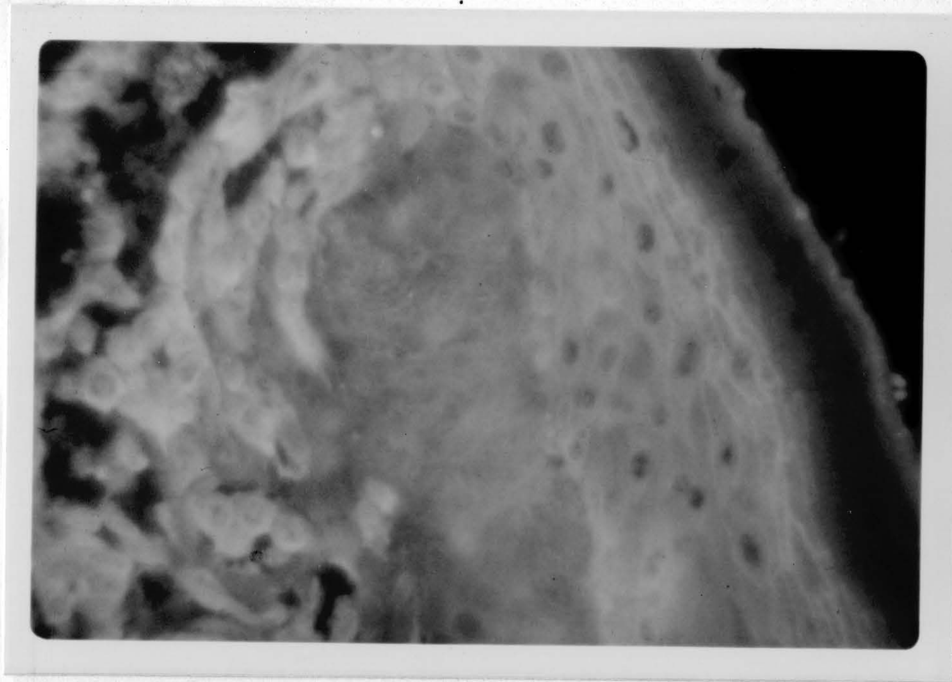

Figure 4

This photomicrograph of squamous cell carcinoma of the hamster cheek pouch stained apple green, using the gross homogenate induced rabbit anti-hamster squamous cell carcinoma serum as an intermediate layer prior to the application of the sheep anti-rabbit immune globulin conjugated to fluorescein isothiocyanate. The magnification of this photomicrograph is 250X, and the exposure was 90 seconds. This photomicrograph shows the proliferation of squamous cell carcinoma into the loose connective tissue layer.

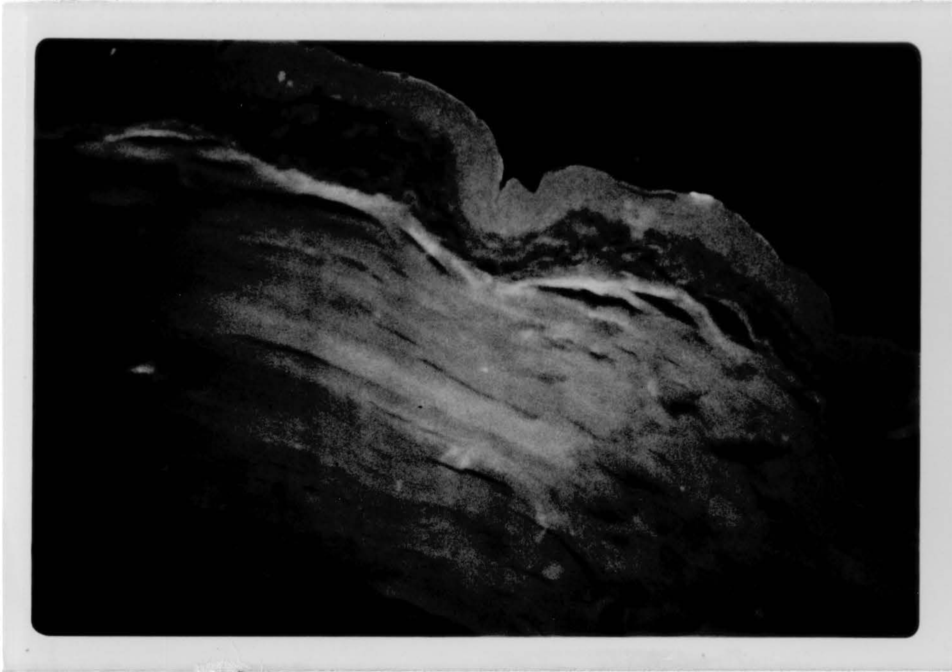

Figure 5

This photomicrograph of normal hamster cheek pouch was stained pale-aqua using the gross homogenated induced rabbit anti-hamster carcinoma serum as an intermediate layer. Magnification is 100X.

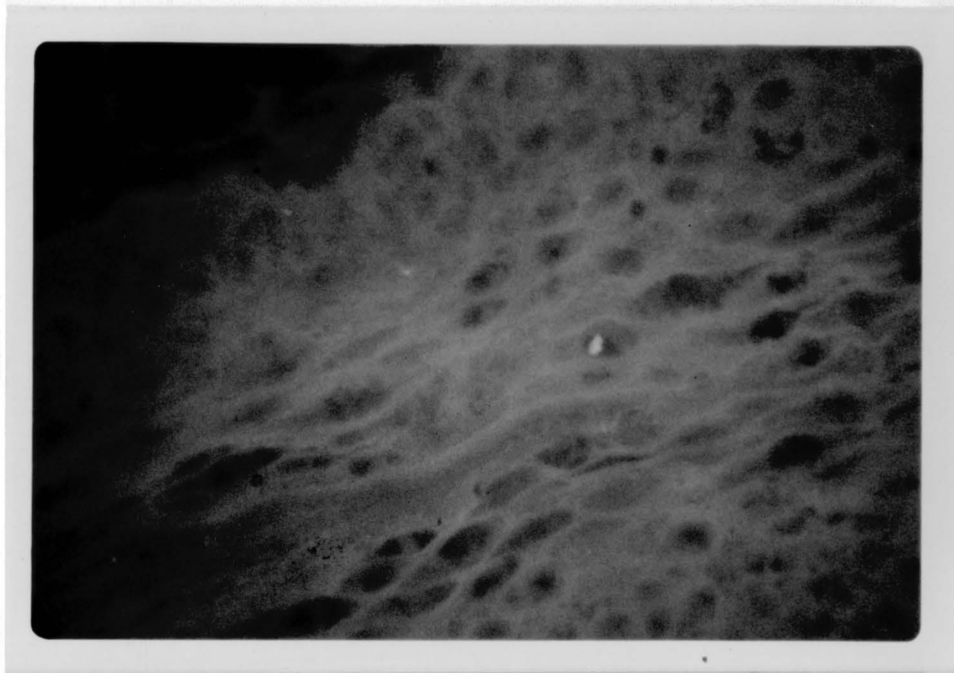

Figure 5A

This photomocrograph of normal hamster cheek pouch was stained pale-aqua using the gross homogenated induced rabbit anti-hamster carcinoma serum as an intermediate layer. Magnification is 250X.

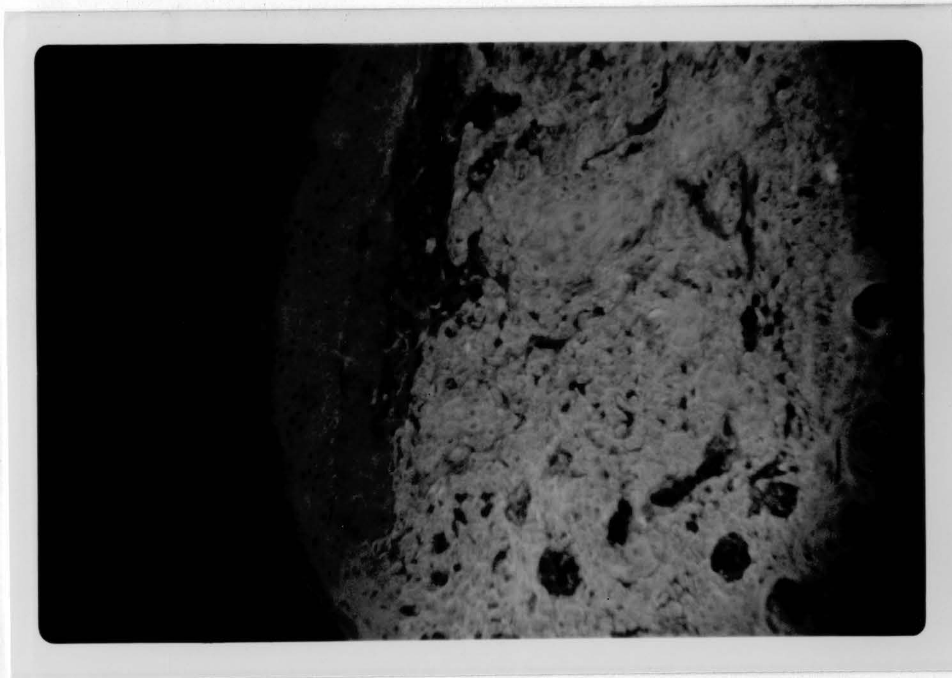

Figure 6

This photomicrograph of squamous cell carcinoma was stained blue when we did not use the gross homogenate induced rabbit anti-hamster squamous cell carcinoma serum as an intermediate layer prior to the application of sheep anti-rabbit immune globulin conjugated to fluorescein isothiocyanate. Magnification of this photomicrograph is 100X.

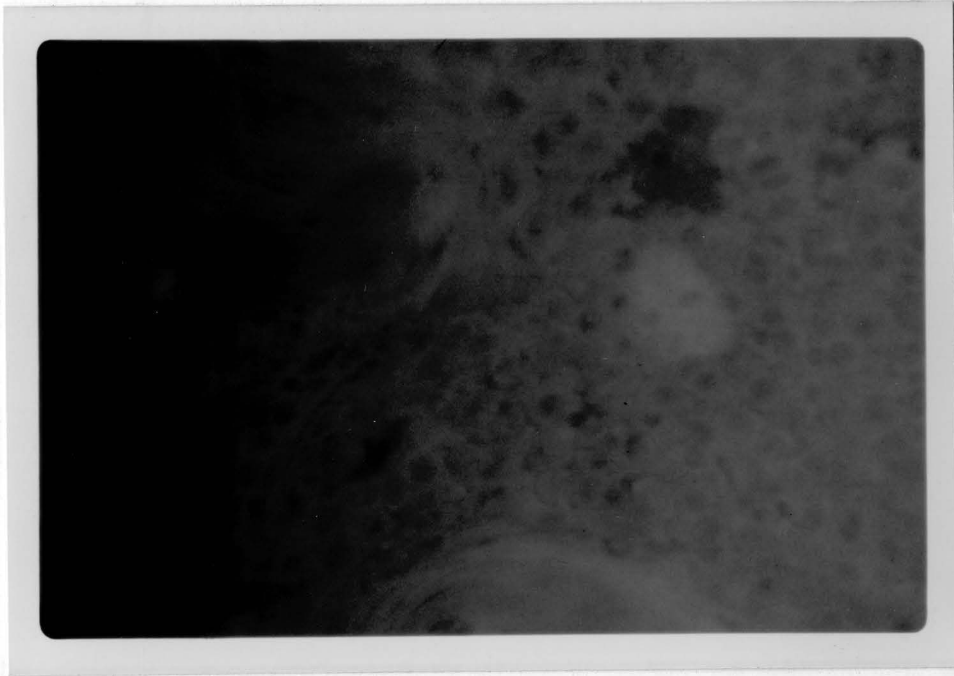

Figure 6A

This photomicrograph of squamous cell carcinoma was stained blue when we did not use the gross homogenate induced rabbit anti-hamster squamous cell carcinoma serum as an intermediate layer prior to the application of sheep anti-rabbit immune globulin conjugated to fluorescein isothiocyanate. Magnification of this photomicrograph is 250X.

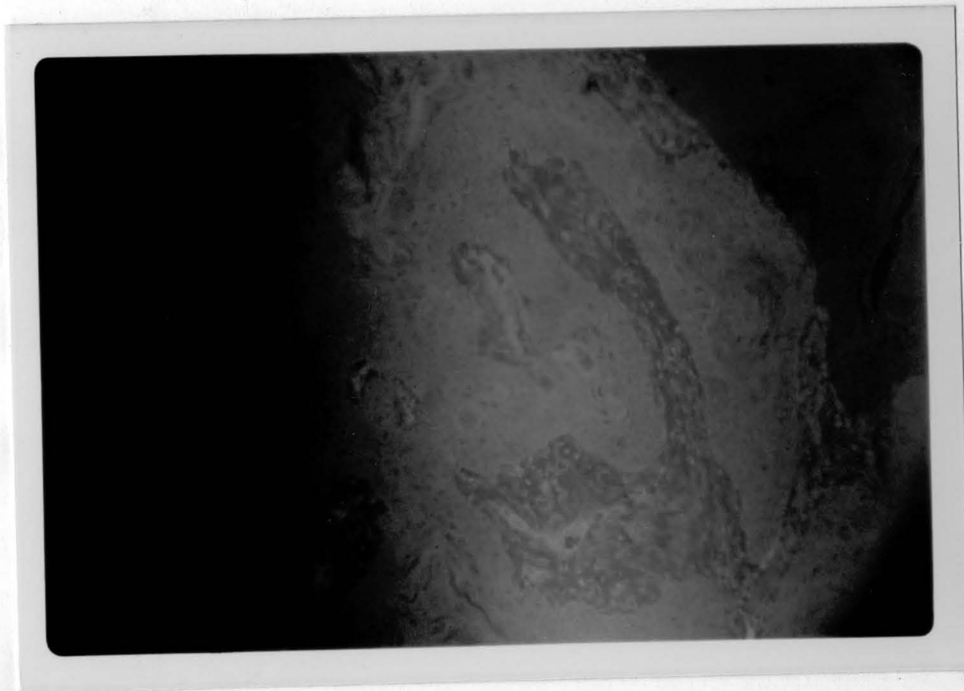

Figure 7

This photomicrograph of squamous cell carcinoma was stained blue when we used normal rabbit serum prior to the application of sheep anti-rabbit immune globulin conjugated to fluorescein isothiocyanate. Magnification of this photomicrograph is 100X.

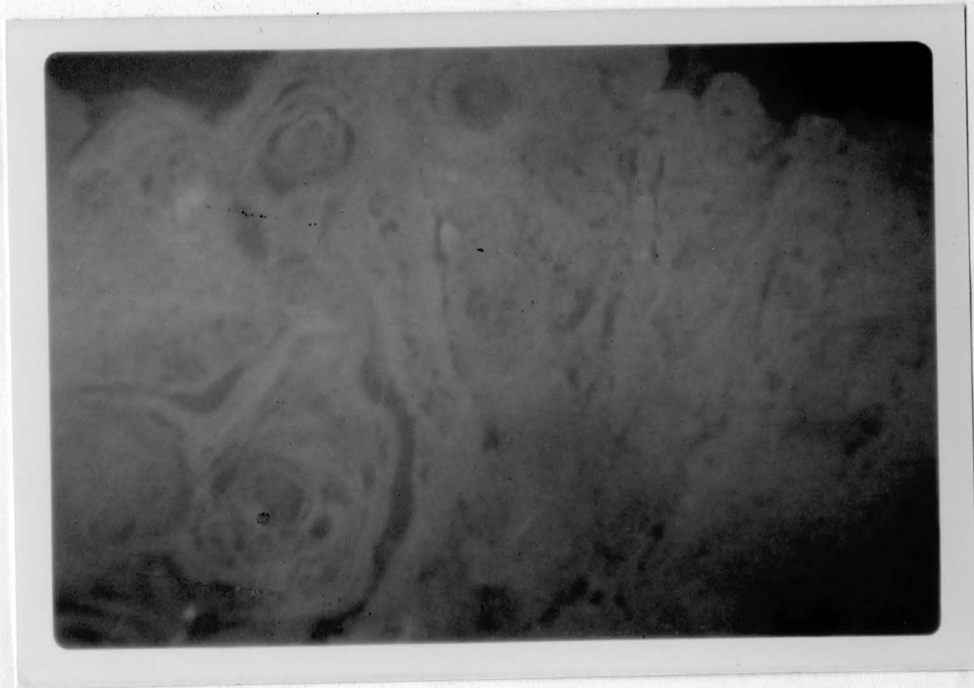

Figure 7A

This photomicrograph of squamous cell carcinoma was stained blue when we used normal rabbit serum prior to the application of sheep anti-rabbit immune globulin conjugated to fluorescein isothiocyanate. Magnification of this photomicrograph is 250X.

Figure 8

This photomicrograph of squamous cell carcinoma was stained blue when we used rabbit immune serum cleared in squamous cell carcinoma homogenate, prior to the application of sheep anti-rabbit immune globulin conjugated to fluorescein isothiocyanate. Magnification of this photomicrograph is 100X

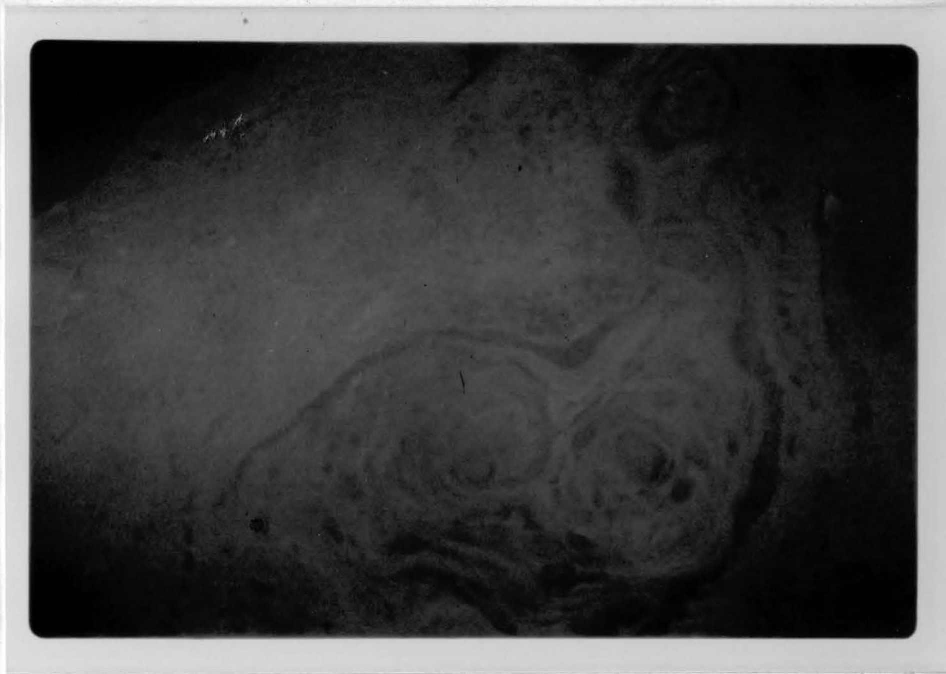

Figure 8A

This photomicrograph of squamous cell carcinoma was stained blue when we used rabbit immune serum cleared in squamous cell carcinoma homogenate, prior to the application of sheep anti-rabbit immune globulin conjugated to fluorescein isothiocyanate. Magnification of this photomicrograph is 250X.

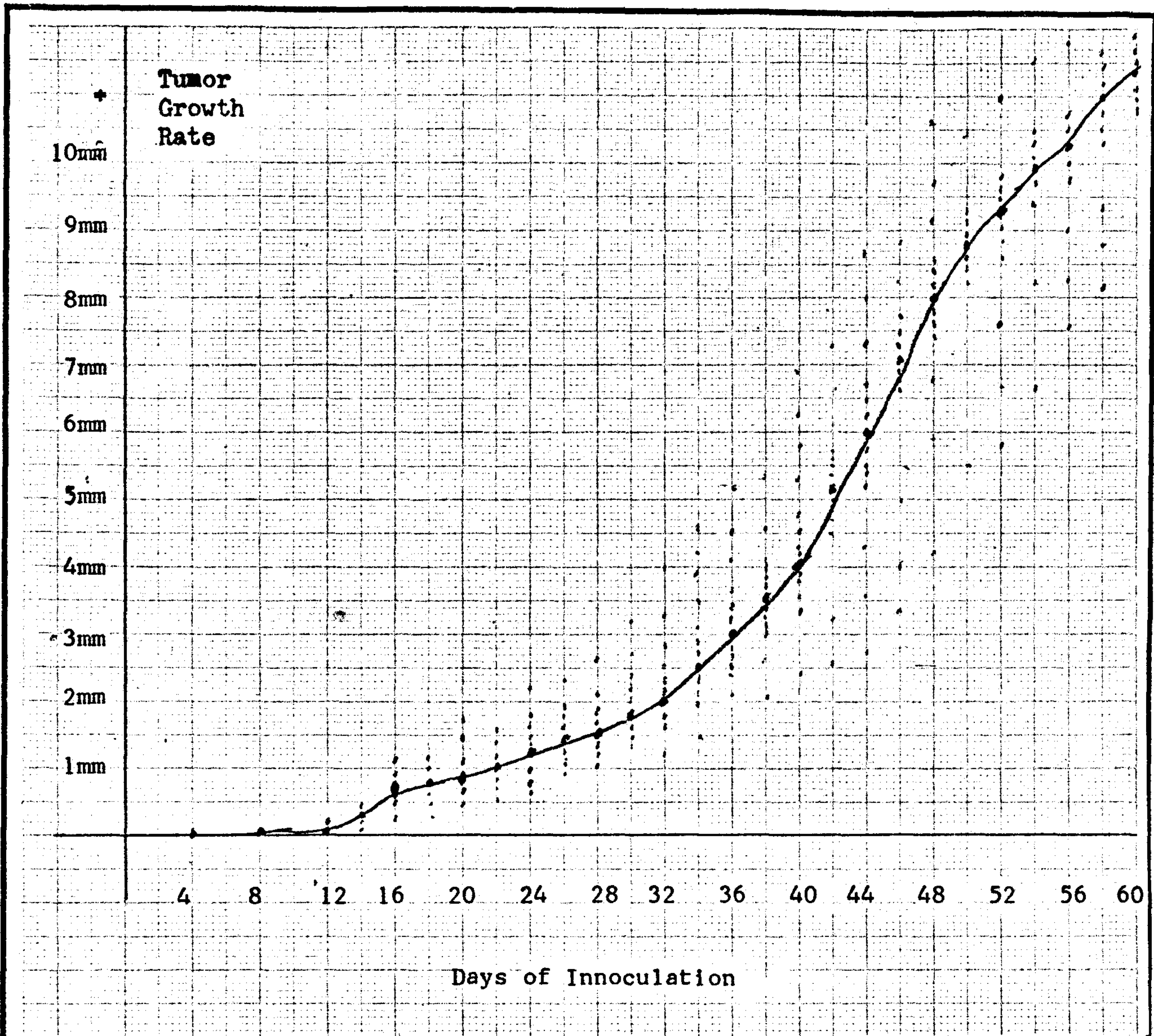

Figure 9

Graft of Tumor Induction.

This graph indicates the rate of tumor growth vs time. In this study I used 15 male Dark eared Partial albino(DEA) Syrian hamsters. To the left cheek pouch of the hamsters I applied DMBA and examined each hamster at two day intervals. All growths were measured with a calipers and recorded.

	1:1	1:2	1:5	1:10	1:20	1:50	1:100	1:200	1:500	1:1000
Treated Rabbit Serum	+	+	+	+	-	-	-	-	-	-
Normal Rabbit Serum	-	-	-	-	-	-	-	-	-	-
Antigen	-	-	-	-	-	-	-	-	-	-

+ aglutination occured (greater than)
(.1mm)

- aglutination did not occure (less than)
(.1mm)

Figure 10

Table of serum titers.

This table indicates: A. Dilution reactivity of the treated rabbit serum

B. Dilution reactivity of Normal rabbit serum

C. Dilution reactivity of antigen

CHAPTER IX

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APPROVAL SHEET

The thesis submitted by Dr. Arnold S. Morof, 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.

October 30, 1971 — Date

Patrick D. Toto — Signature of Director