1984

Metastases in Lymph Nodes of Hamster Buccal Pouch Carcinomas

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by

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A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Science

May

1984

DEDICATION

To my wife Annie

To my parents

To my uncle, Bishop Iacovos of Chicago, whose love and support made this study possible.

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to Dr. Norman K. Wood, for his direction of an excellent two-year learning experience and for his advice, guidance and help during the development of this research.

I would like also to express my gratitude to Dr. Donald Doemling and Dr. Patrick Toto for their valuable suggestions, help and guidance in the completion of this study.

My deepest appreciation is extended to the following people who contributed their help in making this study possible.

To Mrs. Egle Vilutis and Mrs. Valeria Pleirys for their technical assistance in the preparation of the histologic slides.

To Mr. Jack Corliss, director of Academic Computing Services and his excellent staff, for their help and assistance.

To Dr. Marie Jacobs and Dr. James Lehnert for their help and advice.

To Dr. Michael Kiely for his friendship and valuable suggestions.

To Mrs. Sylvia Yancy, secretary of the department of Oral Diagnosis, for her help, patience and friendship during these last two years.

To Dr. Paul Kapagian and Dr. Costas Tsoumis for their love, encouragement and friendship.

Finally a very special thank you is extended to Dr. Joseph Gowgiel for his unselfish assistance, enthusiasm and help through my Graduate

Education and for providing me with the invaluable opportunity of participating in the teaching activities of his department.

VITA

Kostas Tsiklakis was born in Athens, Greece on November 1, 1953. His elementary education was obtained in Athens and he completed his secondary education in 1971 at the Moraitis High School, Psychiko, Athens, Greece.

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CHAPTER I

INTRODUCTION

Squamous cell carcinoma is the most common oral malignancy representing about 95 percent of all malignant neoplasms that occur in the mouth and the jaws. Clinically this tumor may appear as a white, red, ulcerated,or exophytic lesion. It is assumed that a greater percentage of the ulcerated oral carcinomas tend to be of the infiltrated type rather than the exophytic or verrucous types and thus they generally show a poorer prognosis (Wood and Goaz 1980). Considerable microscopic variation is presented in intraoral carcinomas from well differentiated to highly anaplastic, poorly differentiated neoplasms. The latter lesions tend to metastasize early and widely. Metastases involve mainly the regional lymph nodes and especially the submandibular, submental and deep cervical nodes.

Hamster buccal pouch carcinoma is an excellent experimental system for studying different parameters of oral mucous membrane malignancy. This tumor model which was first developed by Salley (1954) and then refined by different investigators has many major advantages, the most important of these include: (1) the early induction of tumors by topical application of DMBA; (2) the development of precancerous dysplastic lesions comparable to human oral leukoplakia; and (3) the histologic similarity of the induced carcinoma to the well differentiated type of

human squamous cell carcinoma of the oral cavity. One of the disadvantages of this model is that the tumors induced are of the exophytic type and have the tendency to proliferate on the surface rather than invade the underlying tissues and give frequent metastases as do the poorly differentiated human oral carcinomas. Metastases of the hamster buccal pouch carcinoma to the cervical lymph nodes were reported in the first study by Salley (1954) but this finding has not been confirmed by later investigators. Most of the attempts which have been made in order to induce a more anaplastic and invasive carcinoma which will constantly metastasize to the lymph nodes or distant organs, were unsuccessful.

Systemic chronic administration of cortisone has been found to exhibit a significant immunosupressive effect in both humans and experimental animals. Earlier and more anaplastic neoplasms as well as an increased metastatic rate have been observed in induced or transplanted tumors in cortisone-treated animals.

Incision through a malignant tumor may increase the local aggressiveness of the neoplasm or may mechanically encourage the movement of malignant cells towards the lymphatic vessels and thus increase the metastatic rate of the tumor.

It is the purpose of this study to try to induce a more anaplastic carcinoma of the hamster pouch, to investigate if metastases to lymph nodes will develop by extending the tumor bearing period and to study the effect of incision and cortisone on the primary tumors and on the metastatic rate.

CHAPTER II

REVIEW OF THE LITERATURE

CHEMICAL CARCINOGENESIS

MECHANISM OF ACTION OF CHEMICAL CARCINOGENS

Carcinogenesis is the transformation of normal cells into malignant or neoplastic cells and may happen in any tissue or organ of the living organism. The main characteristic of these transformed cells is the abnormal, unrestricted and uncontrolled proliferation which results in the formation of a mass or tumor. Cancer cells invade and destroy local tissues by direct extension or they can break off and leave the original mass and be carried to distant sites of the body. There they set up metastases further attacking and destroying the organs involved.

The exact cause of cancer remains undetermined but it has been found that some agents can initiate or initiate and promote the process of carcinogenesis. These agents are referred as carcinogens. Of the known carcinogens (chemicals, viruses and radiation) chemicals have been found to be the most important in the induction of human cancers. In addition chemical carcinogens and especially polycyclic hydrocarbons have been used extensively for tumor induction in experimental animals over the last seventy years (Faber 1981).

Yamagiwa and Ichikawa (1915) developed the first experimental model of chemically-induced cancer. These investigators induced skin

carcinoma in the ears of rabbits by repeated topical applications of coal tar. Tsutsui (1918) induced skin cancer in mice by topical applications of tar and Passey (1922) induced skin cancer in mice by using ether extracts of tars.

The first pure synthetic carcinogen shown to be successful in producing mouse skin carcinoma was the 1:2,5:6-dibenzanthracene (Kennaway and Hieger 1930). Later the carcinogenic hydrocarbon, benzopyrene, was isolated from coal tar (Kennaway 1955) and the carcinogenic activities of various other hydrocarbons have been studied since then.

Although we know today that polycyclic hydrocarbons and their active metabolites bind to nuclear DNA and induce mutagenesis and malignant transformation of cells, the exact molecular mechanism by which these compounds act is not yet completely understood (Janss, et al. 1972, Cavalieri, et al. 1976, Yupsa, et al. 1976, Farber 1981).

The extensive research in the area of chemical carcinogenesis has lead to some conclusions which are generally accepted today. It is now understood that polycyclic hydrocarbons are not themselves reactive substances, but they require activation to chemically reactive metabolites (Heidelberg 1973, Harvey 1982). These metabolites include substances such as epoxides, diols and phenols, but it has been established that the epoxides are the most important in causing malignant transformation of cells

Holtzman et al. (1967) reported that the metabolism of polycyclic hydrocarbons takes place principally in the microsomes of the endoplasmic reticulum and is catalyzed by the mixed-function oxidase

enzymes. These enzymes are NADPH-dependent, are present in most tissues of the mammalian species and serve to detoxify drugs and foreign compounds. These observations were supported later by other investigators (Grover, et al. 1974, Harvey, et al. 1975, Harvey 1981).

Thomson and Slaga (1976) reported that the specific microsomal enzyme complex, aryl-hydrocarbon-hydroxylase, is probably responsible for the metabolic activation of polycyclic hydrocarbons to their ultimate carcinogenic form. This enzyme complex is found in most mammalian tissues including skin and mucous membranes of mice, rats and humans.

Brooks and Lawley (1964) indicated that the metabolic activation of polycyclic hydrocarbons occurs in the cell and then the active metabolites become covalently bound to the macromolecules of the cell. Janss, et al. (1972) found that the metabolic products of the 7,12-dimethylbenz(a)anthracene bind to the DNA or proteins of cells in vivo.

Huberman and Sachs (1977) studied the metabolic activity of five polycyclic hydrocarbons, as well as the binding of their metabolites to DNA, RNA and the protein of the cell. All five carcinogens were metabolized to water-soluble products which bind to nuclear DNA but not to RNA or proteins. These results, therefore, supported the suggestion that DNA is the target of the carcinogenic hydrocarbons.

Miller (1978) reported that the metabolic derivatives of hydrocarbons have the ability to react with the genetic material and cause permanent alterations in cell phenotype. Also, early in the process of the carcinogenesis, DNA damage may be repaired by specific enzymes but later

on, the replication of the altered DNA will result in the malignant transformation of cells.

In conclusion, it is known that polycyclic hydrocarbons when applied in various tissues, are metabolized by microsomal enzymes of the cells to active metabolites. Epoxides, the most important of these metabolites, have the ability to bind to cellular DNA and induce structural alterations, mutagenesis and malignant transformation of cells. 9,10-dimethyl-1,2-benzanthracene (DMBA) is one of the most potent carcinogens and it has been used extensively for tumor-induction in various organs of the experimental animals including hamster buccal pouch.

HAMSTER BUCCAL POUCH CARCINOMA

For almost thirty years, the hamster buccal pouch carcinoma has been used by many investigators as an excellent experimental system for studing malignancy of the oral mucous membrane. The model however has received some criticism because of the unique anatomical stucture of the pouch (Kollas 1955, Stormby and Wallenius 1964).On the other hand, Shklar (1979) summarized the major advantages which include: (1) the similarity between the hamster pouch mucosa and the keratinized human oral mucosa in terms of histology, histochemistry and ultrastracture; (2) the absence of spontaneous or naturally occurring carcinomas which would confuse the data on carcinogenesis; (3) the consistent production of carcinomas with potent chemical carcinogens; (4) the development of precancerous dysplastic lesions comparable to human oral leukoplakia preceding the development of true squamous cell carcinoma; (5) the consistent time pattern of tumor development with a potent carcinogen; (6)

the susceptibility of the tumor system to systemic influences such as vitamins, hormones and various drugs; and (7) the susceptibility of the tumor system to immunologic influences.

Salley (1954) was the first one who successfully produced squamous cell carcinomas on the hamster buccal pouch. In this first report three of the most potent carcinogens were used, 9,10 dimethyl-1,2-benzantracene (DMBA), 20-methylcholathrene (20-MC) and 3,4 benzopyrene(3,4-BP). Each of the carcinogens was dissolved in both acetone and benzene in order to compare the effect of the solvent. A 0.5 per cent solution was used in each case. Using a #4 camel hair brush, each pouch was painted three times per week for sixteen weeks with an additional nine weeks of observation. This study showed that DMBA dissolved in acetone was the most potent carcinogen of the three in producing carcinomas in the hamster cheek pouch. The first exophytic papillomatous tumor was noted at seven weeks and at the end of the experiment all animals showed squamous cell carcinomas microscopically. Also cervical lymph node metastases were observed in this first study.

The organic solvent of the carcinogen plays an important role in the development of the neoplasia. DMBA alone does not produce tumors but when diluted in different solvents produces various degrees of malignancy depending on the nature of the solvent (Suss et al. 1972). In a subsequent study, Salley (1955) used mineral oil instead of acetone as a solvent for the DMBA and he found that the tumor induction time decreased from seven weeks to four and one half weeks.

Salley (1957) also studied the early histologic changes of the

hamster pouch mucosa after the application of the DMBA. Inflammatory cells appeared in the submucosal connective tissue after a single application of the carcinogen. After three applications of DMBA he observed a more intense inflammatory reaction as well as degeneration of the epithelial cells. After the fifth application regeneration of the epithelium and absence of inflammation were observed. A period of hyperplasia of the epithelial layers occurred between the sixth and the fifteenth application. Papillomas developed after fifteen applications (5 weeks) and squamous cell carcinomas developed after eight weeks of application.

Since that time Salley's technique has been used by several investigators as a model system for studing different parameters of oral carcinogenesis.

Goldhaber (1958) suggested that in order for the DMBA to enter the underlying tissues an ulceration of the mucosa should preexist. This suggestion was disproved when Salley (1961) using fluorescence microscopy observed that the DMBA penetrated into the oral tissues of the hamster through intact pouch epithelium. This was supported later by the findings of Kendrick (1964) and of Meskin and Woolfrey (1964).

Morris (1961) studied various factors which may influence hamster buccal pouch carcinogenesis. The most important conclusions of this study included the following: (1) the tissues of the cheek pouch of old hamsters are more resistant to carcinogenic stimuli than those of young hamsters; five weeks of age appeared to be ideal for experimental oral carcinogenesis; (2) 0.5 per cent concentration of DMBA is the optimal

concentration for rapid production of malignant tumors; (3) a shorter latent period is required for tumor development in animals exposed to carcinogen three times per week than those receiving the carcinogen only twice weekly; (4) the response of the hamsters to the carcinogen is not related to the sex of the animals; and (5) conditions of caging (3 or 4 animals per cage) had no apparent effect on the experimental results.

Renstrup, et al. (1962) studied the effect of chronic mechanical irritation on the hamster pouch carcinoma. Their results showed that chronic irritation alone could not produce carcinoma but it hastened significantly the onset of carcinomas produced by topical application of DMBA.

Santis and associates (1964), Shklar (1970) and MacDonald (1978), stated that another advantage of this model system is that the development of carcinomas is preceded by hyperkeratotic or dysplastic lesions comparable to human oral leukoplakia.

Levij, et al. (1968) demonstrated that malignant neoplasms did not develop when 0.5 per cent DMBA was applied for only six weeks. Reiskin and Berry (1968) found that different hamster strains showed variable latent periods of tumor induction.

Mohammad (1979), Mohammad and Micher (1976) and Marshack, et al. (1977) showed that the hamster cheek pouch is not an immunologically privileged anatomical site, as previously thought but instead it can be sensitized by a strong immunogen and a histologically measurable cell-mediated responce can be elicited.

Mock and Main (1980) studied the effect of DMBA on the hamster

pouch mucosa in cell cultures in vitro and reported that dysplastic changes were observed at 21 to 28 days but these changes were not seen at 35 to 49 days indicating repair and return to normal morphology at the early stages of the carcinogenesis.

Odukoya and Shklar (1982) described a two phase mechanism of carcinogenesis in hamster buccal pouch which agreed with the general concept that carcinogenesis is a two stage mechanism. During the initiating stage normal cells are converted to latent tumor cells, and then during the promoting phase these dormant cells are stimulated and give rise to tumors.

Finally, physical factors, drugs and chemicals have been studied by for their inhibitory or enhancing effects on the development and growth of hamster carcinomas. Inhibition of growth or delay in the appearance of the tumors have been produced by administration of supplementary dietary zinc (Poswillo and Choen 1971, Edwards 1976), by systemic administration of BCG (Giunta and Shklar 1971 , Giunta, et al. 1974), by oral administration of levamisole (Eisenberg and Shklar 1977, Cottone, et al. 1979), by systemic administration of retinoids (Shklar, et al. 1980, Tsiklakis 1982), by intramuscular injection of testosterone (Polliack, et al. 1970) and by oral administration of azathioprine (Sheehan, et al. 1971), aspirin and indomethacin (Perkins and Shklar 1982) and vitamin E (Shklar 1982). Also inhibition of growth of the tumors has been reported by topical application of: dimethyl sulfoxide (Shklar, et al. 1969), cortisone acetate (Polliack, et al. 1970), dinitrochlorobenzene (Marshack, et al. 1978), chloropromazine (Levij and

Polliack 1970) and vinblastine (Levij, et al. 1970).

On the other hand, vitamin A deficiency (Rowe and Gorlin 1959), chronic mechanical irritation (Renstrup, et al. 1962), low level x radiation (Lurie 1977), systemic administration of norethynodrel (Frensilli and Weathered 1982), alcohol in the drinking water (Freedman and Shklar 1978) and systemic or topical administration of cortisone (Sabes, et al. 1963, Shklar 1966, Shklar 1967), have been shown to have an enhancing effect on the carcinogenesis in the hamster pouch, causing earlier, more aggressive and more malignant neoplasms.

METASTASIS OF MALIGNANT NEOPLASMS

GENERAL CONSIDERATIONS

The most dreaded aspect of cancer is its ability to metastasize. Metastasis formation involves the sequential release of cells from primary tumors, their movement to other sites and their implantation and growth in these sites.

Epidemiologic studies have shown that of the million new cases of cancer expected annually, approximately two thirds or 600,000 to 700,000 people will develop and die of metastases. Pathogenesis, prevention and treatment of tumor metastases are the major topics of cancer research today and numerous experimental models have been developed.

The first important step in metastasis formation is the decreased adhesiveness of cells in the primary cancer. Such cells can break off from the primary growth and invade nearby veins or lymphatics. After such invasion the cancer cells may embolize to distant organs and develop metastases there.

The factors responsible for decreased adhesiveness of cancer cells still are poorly understood even though the phenomenon was demonstrated decades ago. In early studies Coman (1944,1953) showed that cells could be detached more easily from certain carcinomas than from corresponding normal tissues. He also thought that the reduced adhesiveness of the cancer cells was due to a decreased amount of calcium bound to the surfaces of these cells. However later on it was demonstrated that the calcium content of cancer cells does not differ significantly from that of normal cells.

The studies of Sylven and Blois (1960) among others, have shown that the intercellular regions of tumors contain a variety of enzymes. Lysosomal hydrolases are prominent among these enzymes. Release of these hydrolases has been found to promote cell detachment _in vitro_ and cell detachment and metastasis _in vivo_ (Weiss and Holyoke 1969, Sylven 1976). Studies on alteration of the malignant cell surface have shown that increased amount of sialic acid (Weiss 1973) or fucose (Turner, et al. 1980) on the surface are associated with increased discohesion of cells.

There are many ways for the cancer cells to enter the blood vessels and the lymphatics. The invasion can take place during temporary damage of the capillary or venular walls or through intercellular junctions or gaps. Cancer cells may follow lymphocytes and macrophages and enter the capillaries through intercellular junctions (Sherwin and Richters 1972). Endothelial fenestrations and "open" tumor sinusoids may lead to hemorrhage as well as to metastasis (Ward, et al. 1974).

In order to survive the circulating cells must attach to the vas-

cular endothelium and then "escape" from the blood stream. In some instances tumor cell emboli interact with the endothelial cells and a fibrin or fibrin-like material is formed. This material acts as a latticework upon which the cells can proliferate and form the thrombus which is necessary for the development of secondary tumor growths (Laki 1974). Therefore it has been suggested that prevention of blood coagulation or fibrinolysis by anticoagulant drugs will have an inhibitory effect on the development of metastases (Hilgard and Thornes 1976, Lione and Bosman 1978).

Even after the formation of tumor emboli in distant organs, metastasis is not inevitable. It is well known that only a small percentage of the large number of the circulating malignant cells can survive and give rise to metastasis. Isotope labeling studies have shown that 99 per cent of the circulating malignant cells are destroyed by the immunological mechanisms of the host (Fidler 1970). Originally it was thought that tumor cells were killed by the immune response of the sensitized lymphocytes. Fidler (1974) points to the sensitized macrophage as the killer cell.

As summary, cancer metastasis depends on the interaction of tumor cells with their host. The phenomenon is complex and is affected by multiple factors. Some of these factors include: the unique characteristic of malignant tumor cells which allows them to survive; host immune defence mechanisms; immune stimulation for inhibition of tumor growth or spread and the biological behavior of tumor cells themselves. Future studies concerning manipulation of the host defence mechanisms or tumor

properties could determine the ideal therapeutic approach towards prevention of metastatic tumor spread.

Squamous cell carcinoma represents aproximately 90 to 95 per cent of oral malignancy. The first site of metastasis of this neoplasm is usually the homolateral cervical lymph nodes. If the disease remains untreated for longer periods of time involvement of deeper or contralateral lymph nodes may be observed. Finally, fixed homolateral or bilateral lymph nodes or distant metastasis usually to the lung, liver or bones are seen in late stages.

METASTASIS IN EXPERIMENTAL ANIMALS

The pattern of metastasis has been studied extensively in experimental animals for many years. One of the earliest studies was published by Tyzzer (1913), who implanted tumors subcutaneously in Japanese Waltzing mice and studied various factors which influenced metastasis to the lungs. The results showed that the development of metastases depended on certain factors such as; the biological character of the tumor, the duration of its growth, the size of the primary mass and conditions furnished by the host tissue. However surgical operations or incisions did not increase the incidence of metastases.

Zeidman, et al. (1950) injected viable cells from mouse sarcoma 241, into the tail veins of mice and studied the tumor metastasis to the lungs. The results showed that very few tumors were formed compared to the number of tumor cells injected and this indicates high mortality of the circulating cells. On the other hand the number of metastases was directly proportional to the number of viable tumor cell emboli released

into the circulation.

The first experimental studies on the spread of cancer into the lymphatic system were published by Zeidman and Buss (1954) and by Zeidman, et al. (1955). These investigators injected tumor cells into the afferent lymphatics of the popliteal lymph nodes of the rabbit. Tumor cell emboli were arrested in the subcapsular sinus of one or more lobules of the coresponding nodes. The tumors did not spread to the next node for at least three weeks after the initial arrest. Zeidman (1955) was also able to demonstrate that tumor cells can pass directly from the thoracic duct to nearby lymph nodes without the necessity of passing through the lungs.

Fisher and Fisher (1965), studying factors influencing the development of hepatic metastases in rats, found that hypophysectomy and anticoagulants inhibited the development of metastases, where hepatic trauma resulting from manipulation of the liver, enhanced metastases.

Milas, et al. (1974) reported that the immunological response of the host deals more effectively with distant foci rather than with a single primary tumor and immunotherapy may be more effective in this situation. Jones and Castro (1976) and Fisher, et al. (1976), showed that corynebacterium parvum, a powerful immunopotentiating agent, inhibits the growth of metastases probably through macrophage activation.

Zimel, et al. (1977) studied the influence of stress and endocrine imbalance on lymph node metastases of the 256 Walker carcinosarcoma in rats and found that pain, electrical stress, cortisone and antithyroid drugs enhanced metastasis growth, where adrenalectomy, testosterone and

thyroxine inhibited metastases.

Muntzing, et al. (1976) reported that enviromental factors enhanced the growth of metastases of experimental renal tumors.

Mosley, et al. (1978) showed that limb amputation caused a significant increase in the number of the pulmonary metastases of transplated Lewis tumors in mice. It was thought that this was due to non-specific stress developed by the operation. In the same study large doses of cortisone significantly increased metastases but small doses had no effect. On the other hand Corynebacterium parvum counteracted the effects of cortisone.

Finally,much work has been directed towards treating metastases with immunotherapy in the last few years. Numerous experimental studies have been carried out in which the ability of different agents to stimulate the lymphatic system against tumor metastases was examined (Weissman 1980, Tsubura, et al. 1980, Lejuene, et al. 1980, Basic, et al. 1980).

METASTASIS OF THE HAMSTER POUCH CARCINOMA

Lymph node metastases in experimental oral carcinogenesis have been reported by Lemon and Smakula (1955), from transplatable sarcoma of the pouch and by Fujita, et al. (1973), from tumors of the tongue.

Salley (1954) in his first study on the chemical carcinogenesis of the hamster buccal pouch, reported frequent cervical lymph node metastases in animals that were painted with DMBA for 16 weeks with an additional 9 weeks of observation. The author characteristically describes the finding of metastasis: "Normal lymphoid tissue is seen bordering

masses of malignant squamous cells revealing marked keratinization. The metastasis is similar to cervical lymph node invasion sometimes seen in human squamous cell carcinoma of the oral cavity." Although this was an important finding of the oral carcinogenesis of the hamster pouch, Salley himself did not mention anything about metastases in his later publications (1955, 1957).

Morris (1961) described in his outstanding study the factors that influence the hamster pouch carcinoma. Although the metastatic spread of these tumors was not included in the study, he believed that metastasis to the submandibular lymph nodes does occur.

Renstrup, et al.(1962) were able to demonstrate that chronic mechanical irritation enhances the growth of the primary tumors but they did not mention anything about lymph node metastases.

Shklar (1966), Levij, et al. (1967), Giunta and Shklar (1971), Freefman and Shklar (1978), Eveson (1981), all reported that they never found metastases.

Rwomushana, et al. (1970) reported one case of lymph node metastasis in one hamster which was treated with DMBA and vinblastine (cytostatic drug). The authors stated that this was the only case of metastasis found out of 562 animals treated with DMBA in their laboratory during the period 1966 to 1970.

A similar statement has been made by Sheehan, et al. (1971) who reported that not a single metastasis has been observed in more than 1000 treated animals.

Shklar (1972) in a review article about chemical carcinogenesis of

the hamster buccal pouch, mentioned that these tumors have the tendency to proliferate on the mucosal surface rather than invade deeply into the underlying connective tissue. In addition he stated that metastasis does not occur to the regional lymph nodes, distant organs or bone and that an experimental oral carcinoma system that produces metastases has not yet been developed despite a number of different approaches. So the general belief is that although lymphatic drainage exists in the hamster pouch (Lindeman and Stauli 1968, Giunta, et al. 1983), chemically induced cheek pouch squamous cell carcinomas do not have the ability to metastasize to the lymph nodes.

However two recent studies (Craig 1980, Safour 1983) demonstrated that under special conditions, approximately fifty per cent of the buccal pouch carcinomas metastasized to the cervical lymph nodes. Craig (1980), in his study, extended the tumor bearing period from 16 or 18 weeks, which was used in most previous studies to more than 30 weeks. In order for the animals to survive, every exophytic tumor greater than 0.5 cm diameter was surgically excised, in some several times. At the end of the experiment 11 of the 23 animals (48%) showed histologically confirmed metastases to the ipsilateral cervical lymph nodes.

In the other study Safour (1983) found that approximately 50 per cent of the animals in which incisional biopsies were performed in clinically suspicious tumors at the 14th week of the experiment, showed definite histological cervical lymph node metastases 3 to 6 weeks after the biopsy procedure.

Confirmation of these two studies with appropriate controls will

probably prove that these tumors have the ability to metastasize and also will justify the continuing use of the hamster buccal pouch carcinoma model system in studies related to the development and spread of the oral malignancy.

FACTORS INFLUENCING METASTASIS AND TUMOR GROWTH

CORTISONE AS AN IMMUNOSUPRESSIVE DRUG

Among the side effects of the systemic chronic administration of corticosteroids is the supression of the immune system. This immunosupressive effect is dose-related and directly proportional to the anti-inflammatory degree of the corticosteroid.

The effects of corticosteroids on the immune system and on antibody production during protozoan infections of hamsters have been extensively studied by Frenkel (1960,1972) and by Frenkel and Lunde (1966). Also Frenkel and Havenhill (1963) studied the corticoid sensitivity of hamsters, rats and mice and especially the effect of dose, time and route of administration on body or organ weight and on survival rate of the animals.

Cerrili and Hattan (1974) in a review article on immunosupression and oncogenesis pointed out that with the increasing use of corticosteroids and other immunosupressive drugs, one should remain aware of the potential development of tumors.

Pomeroy (1954) reported that cortisone acetate administered subcutaneously to Swiss mice concomitant with intravenous injections of adenocarcinoma cell suspensions, developed widespread metastases. He concluded that the major mechanism involved was probably a supression of

the reticuloendothelial system resulting in failure of tissue-specific antibody production and thus it may be an immunological selection phenomenon.

Baserga and Shubik (1954) undertook a study to evaluate the action of cortisone on transplanted and induced tumors in mice.Their results showed that cortisone inhibited the primary growth of transplanted adenocarcinoma and induced skin carcinoma. On the other hand they found that cortisone favors an increase in metastatic spread of all the experimental tumors. Inhibition of the primary tumor but increased incidence of metastasis by systemic administration of cortisone has also been reported by Agosin, et al. (1952) and by Bloom, et al. (1963).

Stoker (1969a, 1969b) studied the effect of cortisone on the dissemination of cancer via the lymphatic system. Tumor cells were injected, after surgical exposure, into a popliteal afferent lymphatic vessel on the medial side of the leg of the rabbit. Cortisone was injected intramuscularly in half of the animals just before the injection of the tumor cells. In the control animals the spread of the tumor was limited to the popliteal lymph node only. In the cortisone group it was found that the popliteal nodes as well as the pelvic lymph nodes were involved.

A possible explanation of the mechanism by which cortisone induces immunosupression is given by Fisher, et al. (1976). The authors believe that corticosteroid administration is associated with a transient lymphocytopenia secondary to redistribution of the lymphoid elements. T cells are affected to a larger extent than B cells. Corticos-

teroids also may inhibit some functions of macrophages. All these result in the depression of the immunologic response mechanisms and failure to destroy the tumor cells.

The effect of corticosteroids on the depression of lymphocytes is better illustrated in experimental and clinical studies in which cortisone or dexamethasone administration inhibited or depressed lymphatic leukemias and lymphosarcomas (Shisa 1969, Huggins and Vematsu 1976).

Zimel, et al. (1977) and Cameron, et al. (1982) reported that cortisone acetate enhanced metastasis as well as primary tumor growth in rats.

Chung, et al (1973) studied the effects of both an immunosupressive drug, cortisone acetate and of an immunopotentiator agent, Mycobacterium bovis (BCG) in mice on transplanted sarcoma. BCG alone was able to depress tumor growth but when cortisone was added an enhancement of tumor growth was observed. So cortisone abrogated BCG-mediated tumor killing. Similar studies, using as immunopotentiator the Corynebacterium parvum, were performed by Mosley, et al. (1978) and by Jones, et al. (1978). Both studies showed that small doses of cortisone had no effect on the inhibitory action of the corynebacterium parvum but large doses significantly increased metastasis.

Mclaughlin, et al. (1974) stated that chronic cortisone treatment failed to alter significantly the growth of tumors induced in the kidneys of hamsters.

The effect of the systemic or topical administration of cortisone has also been studied in experimental oral carcinogenesis. Sabes, et al.

(1964) studied the effect of topical application of cortisone on carcinomas of the buccal pouch and submandibular salivary gland. In their results cortisone seemed to enhance the incidence of tumor formation in the pouch but inhibited the development of malignancy in the salivary gland.

Anbari, et al. (1965) stated that rats receiving systemic administration of cortisone developed carcinomas of the submandibular gland more rapidly and in greater number than rats in which cortisone was not administred.

Shklar (1966, 1967) observed that systemic administration of cortisone hastened the induction and development of hamster buccal pouch carcinomas. These tumors were considerably larger and also demonstrated more invasion of the underlying connective tissue and muscle. Metastasis to the regional lymph nodes was not observed in any case.

Finally Polliack, et al. (1970) reported that topical application of cortisone acetate had an inhibitory effect on the DMBA carcinogenesis of the hamster buccal pouch. The decrease of tumor formation was thought to be due to cortisone iduced depression of DNA synthesis and mitotic activity.

INCISION OF TUMORS

Incisional biopsy is the most common method used for obtaining a piece of tissue for histologic examination (Giunta, et al. 1969). The assumption that incisional biopsy or surgical trauma may contribute to the spread of tumor cells is in dispute among investigators and health professionals.

A number of clinicians and investigators emphasize that the value of the knowledge obtained by the biopsy is very important and as the complications are minimal, incisional biopsy should be developed as a routine procedure. They also mention that the possibility of hastening metastasis by local irritation from a biopsy procedure is only theoretical (Miller 1946, Bernier and Tiecke 1950, Bourgoyne 1954).

Another group of investigators believe that the spreading of the tumor cells along the lymphatic or vascular channels following a biopsy procedure is a possibility but if the procedure is done with care, the spread of tumor cells will be minimized (Tiecke 1965, Kerr, et al. 1974, Tyldesley 1978).

Some experimental studies have been performed in order to determine the effect of incision or trauma upon dissemination of tumor cells.

Tyzzer (1913) showed that incisions of the implanted tumors in mice did not increase the incidence of metastases.

Wood (1925) obtained biopsies from half of the animals in which Flexner rat carcinoma was transplanted. He found that the percentage of metastases in the lungs of the biopsied group was not significantly different from the group which was not biopsied.

Shklar (1968) used the hamster cheek pouch carcinoma in order to study the effect of incision and manipulation of the tumors. He concluded that biopsy incision together with massage and compression did not promote spread or increased rate of tumor growth.

Knox (1929) on the other hand showed that massage and surgical excision of the primary transplated tumors of the mice resulted in

increased rate of metastases to the lungs.

In addition many surgeons have observed on some occasion how surgical trauma has stimulated the formation of metastases in patients suffering from malignant disease (Skolnik, et al. 1980). Several experimental studies (Agostino and Cliffton 1965, Fisher, et al. 1967, Ivarsson 1976) have also shown that different kinds of trauma stimulate the formation of metastases after intravenous tumor cell injection. The mechanisms responsible for this effect of trauma have not yet been fully clarified. Several post-traumatic reactions have been considered, such as disturbed microcirculation, intravascular coagulation, increased microthrombus formation and damage to the vascular endothelium. Each of these mechanisms could theoretically increase the potential for entrapping circulating tumor cells.

CHAPTER III

MATERIALS AND METHODS

EXPERIMENTAL GROUPS

Sixty four male hamsters (Cricetus auratus), three months of age and weighing approximately 100 grams were obtained from Angles Lab Animals (Farmerburg, Indiana). The animals were housed four in a plastic cage with a stainless steel lid, maintained in a temperature of 65-70 F, and were fed Purina Lab chow and tap water ad libitum. After two weeks of acclimatization the animals were randomly divided into four equal groups, of sixteen animals each (Table 1).

Group A. The animals in this group received the applications of the carcinogen, 9,10,dimethyl-1,2 benzanthracene (DMBA), for thirteen weeks and remained untreated for the next seven weeks. This group served as the control group.

Group B. The animals in this group were treated with DMBA for thirteen weeks. Following the last application of the carcinogen, incisional biopsies of the developed tumors were performed. The animals received no other treatment during the next seven weeks.

Group C. In this group DMBA was applied for thirteen weeks and incisional biopsies were performed after the end of the applications as in Group B. In addition this group received cortisone acetate subcutaneously from the beginning until the end of the experiment (20 weeks).

Group D. These animals received DMBA and cortisone as in group C but incisional biopsies of the tumors were not performed.

A detailed description of the experimental procedures follows.

APPLICATION OF THE CARCINOGEN

The right pouches of all animals in all four groups were painted three times per week for thirteen weeks with a 0.5 percent solution of 9,10-Dimethyl-1,2-benzanthracene (Sigma Chemical Co., St. Louis, Mo.) in heavy mineral oil (U.S.P.). The dimethyl-benzanthracene solution (DMBA) was applied by means of a #4 camel's hair brush. The palm of the left hand was used to hold the animal steady on the working table and the thumb of the same hand was used to retract the right angle of the mouth. The brush was dipped in the DMBA solution, the excess solution was allowed to drip off and then the brush was introduced into the pouch (Figure 1). With gentle circular movements the entire surface of the pouch was painted. The left pouch of all animals remained untreated and served as control for the DMBA-treated pouches.

The right pouches of all animals were examined weekly in order to observe the surface changes and the development of tumors on the mucosa. For this purpose the animal was held steady on the working table by one person while the other person everted the pouch with the use of two blunt forceps. One forceps was used to retract the cheek and the other forceps was introduced into the pouch grasping the pouch and then everting it. Under appropriate lighting conditions detailed clinical examination of the pouch was performed and selective photographs were taken.

INCISION OF TUMORS

After thirteen weeks of DMBA application, an incisional biopsy was performed on a selected tumor in every animal of B and C groups. Half of the animals of each group, with the largest tumors were operated on the first day while the rest of the animals were operated three days later.

Under ether anesthesia the pouch was everted and the largest tumor (diameter 5 mm or more) with a red surface was selected for the incision. A sterile #15 scalpel blade was used to cut deeply into each tumor. First a tissue specimen (3 x 3 mm) was removed from the tumor and fixed in 10 percent formalin for further histological examination. Next three repeated incisions were made through the same tumor and in sufficient depth into muscularis layer of the pouch (Figure 2). The pouch then was re-inverted into the mouth and the animal was placed back into the cage, after the recovery from the anesthesia.

ADMINISTRATION OF CORTISONE

Cortisone acetate suspended in normal saline (Cortone,Merck-Sharp-Dohme, West Point, PA) was injected subcutaneously in all the animals of groups C and D, from the beginning untill the end of the experiment. Cortisone was injected twice weekly in a dose of 10 mg per Kg weight. This dose was selected based on a study by Frenkel and Havenhill (1963) who determined that doses as high as 12 mg per Kg weight administered subcutaneously twice weekly had no effect on the survival time of hamsters. On the other hand a 2-fold increase of this dose resulted in a 50 percent weight loss and death within two months.

For the cortisone injection the animal was grasped and raised with the left hand. A 0.25 ml glass tuberculin syringe with a 25 gauge short needle was used to inject the cortisone subcutaneously into the right supra-inguinal area of the animal (Figure 3).

SACRIFICE OF THE ANIMALS

Starting at seven weeks after the incision of tumors, i.e., at the twentieth week, animals were killed by inhalation of an ethyl ether overdose. The killing of the animals was completed in seven days by sacrificing equal numbers of animals from each group daily.

Immediately following death, each animal was positioned in the dissection board. A longitudinal incision was made first in the skin of the neck starting at the midline of the lower lip and terminating at the level of the superior surface of the sternum. The skin over the left and right cheeks and over the neck was reflected exposing the pouch, the submandibular and parotid glands together with the associated lymph nodes.

Both pouches of the animals were excised, opened with scissors and examined grossly. Left pouches were examined for detection of any surface changes. The right pouches were weighed and the number and size of tumors, the presence of necrotic material and any other pathologic finding were recorded for each animal.

After the pouches were excised the glandular structures of the right and left side of the neck were examined grossly. Any pathologic change, lymph node enlargement or tumor mass was recorded and photographed. The submandibular and parotid glands of each side along with

the associated lymph nodes were excised in one mass and prepared for histological study. Finally the lungs and liver from each animal were removed and examined grossly for evidence of pathosis.

The excised tissues of each animal (left and right pouches, left and right glandular structures of the neck, lungs and liver) were fixed in 10 percent formalin for at least three days, embeded in paraffin, sectioned at 6 microns, stained with Hematoxylin and Eosin (H&E) and examined under light microscopy.

HISTOLOGICAL EVALUATION

Prepared glass slides from the right and left pouches, right and left structures of the neck, lungs and liver were examined microscopically for each animal. Also microscopic examination was performed for the incisional biopsy specimens which were obtained at the thirteenth week from the animals of groups B and C. All findings were recorded on the Histopathological Evaluation Sheet (Appendix A).

First the biopsy specimens were studied in order to establish malignancy and also the degree of differentiation of the neoplasms.

For the microscopic evaluation of the right pouch two tumors were selected from each pouch. Effort was made to include in the study tumors which did not look necrotic or keratotic. The histological description of the tumors included the surface changes of the epithelial tissue, the presence of ulceration, hyperkeratosis and atypia, changes of the basement layer and the presence of invasion and inflammatory reaction.

The tumors were classified in four categories according to the degree of the differentiation (dd). Tumors which were forming large ker-

atin pearls and had neoplastic cells very well differentiated and failing to show prominent mitotic figures or hyperchromatic nuclei were classified as dd I. Tumors with large keratin pearls but with neoplastic cells showing more atypia with large and more hyperchromatic nuclei were classified as dd II. Tumors classified as dd III showed little keratin formation, hyperchromatic nuclei, prominent nucleoli and abnormal mitotic figures. Tumors classified as dd IV showed little or no keratin formation and neoplastic cells with a high degree of atypia, multipolar, hyperchromatic and large nuclei, prominent nucleoli, many and abnormal mitotic figures and deep invasion into the underlying tissues. Tumors with I or II degree of differentiation were characterized as well differentiated. Tumors with degree of differentiation III or IV were characterized as poorly differentiated.

The tumors of the right pouch were also characterized according to the degree of invasion as (a) superficial and (b) deep invading. Tumors categorized as "superficial invading" demonstrated mostly an exophytic growth pattern with minimal or no extension into the lamina propria. On the other hand deeply invading tumors were identified by the extension of the epithelial tumor masses or islands deep into the connective tissue and in some instances into the muscularis layer of the pouch.

The microscopic examination of the tissues from the left and right side of the neck included the morphology of the salivary glands, the structural alterations of the lymph nodes and the presence or absence of metastases. The stage of metastasis, the degree of differentiation of the metastatic growth and the capsular invasion of the lymph node were

also assessed.

Finally serial microscopic sections were evaluated for the presence of metastases in the lungs and liver of the animals.

TABLE I

EXPERIMENTAL GROUPS

GROUP*	DMBA	INCISIONAL BIOPSY	CORTISONE
A	+	-	-
B	+	+	-
C	+	+	+
D	+	-	+

*: Each group consisted of 16 animals
+: Procedure performed

FIGURE 1

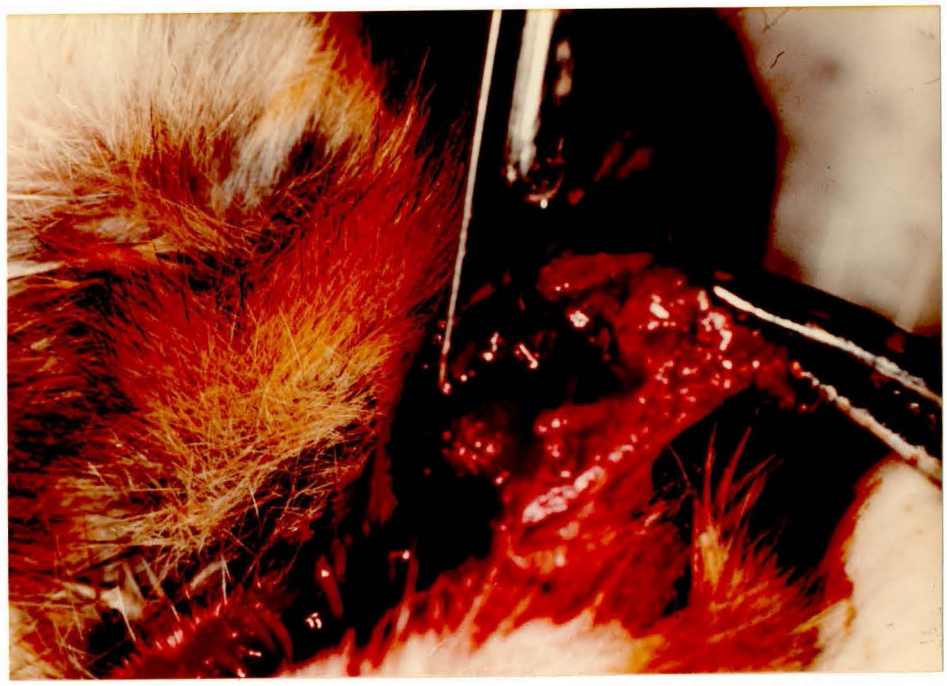
Application of DMBA to the right pouch.

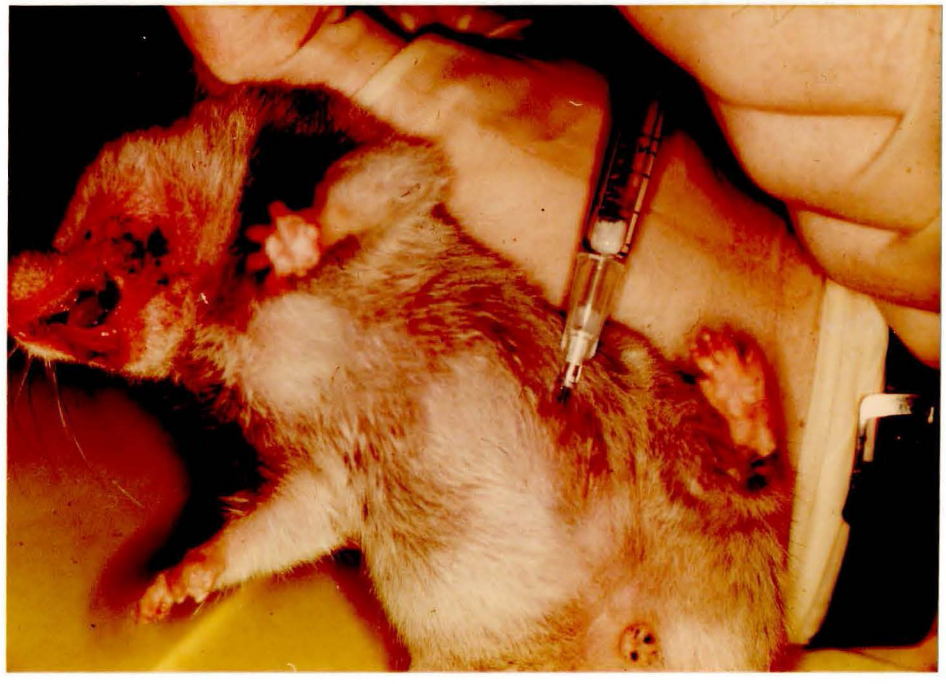
FIGURE 2

Deep incision through the tumor into the lamina propria and muscular layer after the incisional biopsy was done.

FIGURE 3

Subcutaneous injection of cortisone.





CHAPTER IV

RESULTS

CLINICAL OBSERVATIONS

An inflammatory response was initiated in the right pouches of all the animals just a few days after the initial painting with the DMBA. This was recognized clinically as edema and erythema of the mucosa which lasted approximately for two weeks.

By the end of the second week the erythema usually had subsided and the pouches appeared normal for the next two weeks. Also by this time the animals were not as aggressive as before, probably because they had become used to the painting procedure.

At the fourth week of the DMBA painting the right pouches of all the animals began to demonstrate a series of clinical changes of consistency and color. The smooth, thin and translucent mucosa of the pouch changed progressively to thick wrinkled and whitish mucosa.

By the end of the sixth week, the right pouches of all the animals became shorter, much thicker and had leukoplakic areas scattered all over the surface mucosa (Figure 4). On the other hand the mucosa of the left pouch of all the animals appeared normal (Figure 5) and much different from the altered thickened mucosa of the right pouch. At this time no clinically exophytic tumor growth was observed in any animals of groups A and B. However two animals from group C and one animal from

group D exhibited small papillomatous lesions with either red or white color in the right pouches.

By the end of eight weeks almost all animals in the four groups had papillomatous exophytic tumors in the painted pouches. Most of these tumors were measured to be 1 to 3 mm in diameter (Figure 6). These lesions were either solitary or multiple and scattered all over the pouch. They had a rough verruciform appearance with either a sesile or pedunculated base and white keratotic surface. It was interesting that some animals of the cortisone groups (C and D) had larger tumors than these in groups A and B measured 3 to 5 mm in diameter with a white or necrotic surface (Figure 7).

Examination of the right pouches at the tenth week of the experiment showed that all the animals had developed clinically detectable exophytic tumors. Some of these tumors were fixed at the base of the pouch and this made everting the pouch difficult. Most of the small papillomatous growths detected earlier were now large and had a necrotic surface. Differences in the size of the tumors between the cortisone and the non-cortisone groups which had been detected earlier were no longer apparent. The tumors of all the animals ranged from 3 to 5 mm in diameter. Some animals showed multiple smaller tumors while others had only one or two tumors but larger in size. These findings were the same for all the animals of the four groups.

From the end of the tenth week until the end of the DMBA applications (13th week) the tumors of all animals became larger, most of them were more than 5 mm in diameter and had a necrotic surface (Figure 8).

During the two weeks following incisional biopsy in B and C groups, the tumors of these animals rapidly increased in size. Some animals exhibited such large tumors that they even had difficulty in keeping their heads in an upright position. Also skin tumors developed over the painted pouches of these animals. No such a rapid increase in the size of the tumors of the animals that were not biopsied (Groups A and D) was observed. Also the skin tumors that developed over the painted pouch of the latter animals were much smaller in size.

During the four last weeks of the experiment the tumors of all animals continued growing but at a slower rate than before. Also the difference in the tumor growth rate between the cortisone and the non-cortisone groups was not as prominent as before. During the last two weeks of the experiment the animals became weaker, lost weight and had difficulty in feeding themselves because of the enormous swellings of the cancerous right pouches.

AUTOPSY RESULTS

Some animals died during the course of the experiment and so were excluded from the study. Only the animals that remained alive until the twentieth week of the experiment were included in the final results. At the end of the experiment 14 animals out of the initial 16 were evaluated in each of the A and B groups and 13 animals out of the initial 16 were included from groups C and D.

The surface mucosa of the left pouch of all animals appeared normal macroscopically, very thin, smooth and had a normal pink color. However in two cases (one animal in group A and one animal in group D), a

very small papillomatous tumor was found close to the base of the pouch. These tumors had a whitish surface and their size was less than 2 mm in diameter. Other areas of these pouches appeared entirely normal.

When the skin over the right pouch was reflected the outer fibrous connective tissue lining of the pouch appeared to be free of invading neoplastic tumors in all cases. Although the exophytic tumors had caused marked swellings of the pouches in no case had the neoplasms invaded the outer layer or directly extended into the structures of the neck or the skin. The pouch from this aspect appeared smooth and very well demarcated from the other anatomical structures of the neck (Figure 9).

When the excised pouch was opened with scissors a variation in size and appearance of the tumors was observed. Most of the animals in B and C groups were bearing large necrotic tumors which in many cases occupied the whole pouch (Figure 10). Most of the tumors in group A animals had reached a size of 12 to 15 mm and in some cases the tumors had grown all over the pouch mucosa. Group D animals generally had smaller tumors than the other groups.

Table II displays the right pouch weight in grams for all animals in all groups. In some animals the pouches were not weighed as they were found to be protruding out of the mouth at the time of sacrifice. The mean pouch weight for Group B was 4.35 g. which was higher than the control animals (Group A 3.30 g.) but the difference was not statistically significant. The mean value of pouch weight for group C was very close to the mean pouch weight of the controls. Group D showed the lowest mean pouch weight (2.55 g.) but the difference between Group D and Group A

was not statistically significant. On the other hand the difference of the mean values between Groups D and B was significant ($P<0.05$).

The submandibular salivary glands along with the associated lymph nodes were lying inferiorly to the pouch when the skin was reflected (Figure 9). The parotid glands with their associated lymph nodes were connected with the other glandular structures of the neck by fat and fascia. The cancerous pouches were found to be completely separate from the submandibular gland and lymph nodes in all animals (Figure 9). In no case was observed a direct extension of the pouch neoplasms to the lymph nodes or the salivary glands.

At the right side of the neck the parotid glands with the associated lymph nodes were normal in all cases. The submandibular area on the other hand, showed a series of pathologic macroscopic changes. Approximately half of the animals of all groups exhibited large lymph nodes, four to five times the size of the nodes on the left side. The enlarged nodes were positioned superior to the submandibular salivary glands and they had a smooth surface, brown color and rubbery consistency (Figure 9).

In five animals,(one from Group A, one from Group B and three from Group C) a large tumor mass was observed in the submandibular area. These tumors were hard in consistency, measured 12 mm or more in diameter had rough surfaces and were completely separate from the cancerous pouches (Figure 11). When these masses were cut in half a necrotic, cheesy material was found within. The lymph nodes were completely destroyed in these cases and the submandibular gland was attached to one

side of the exophytic growth.

Nothing significant was observed grossly on the left side of the neck except that some lymph nodes were slightly enlarged.

The lungs, the liver and the rest of the viscera of all the animals did not show any macroscopic, pathologic change. In one animal only (Group B), the left lung appeared enlarged, necrotic and white in color but the right lung appeared normal.

MICROSCOPIC RESULTS

The unpainted left pouch of almost all the animals did not show any microscopic changes. Figure 12 illustrates a cross section of the normal left pouch. The epithelial lining was stratified squamous type with 2 to 4 cell layers in thickness. The epithelium was keratinized and was devoid of rete ridges and epithelial accessory structures. Under the surface epithelium a thin layer of rather dense connective tissue was seen and under this a layer of longitudinal striated muscle fibers was found.

In some animals a few areas of the otherwise normal pouch exhibited a moderate degree of acanthosis or keratosis but dysplastic changes were not observed. The two exophytic tumors of the left pouch which were observed grossly and studied histologically, proved to be well differentiated squamous cell carcinomas.

All the biopsy specimens which were obtained at the thirteenth week of the experiment from B and C groups, histologically represented squamous cell carcinomas. Almost all of them were of the well differentiated type with heavy keratinization, formation of large keratin

pearls, altered nuclear-cytoplasmic ratio and loss of cell polarity. Highly anaplastic carcinomas were not observed.

Table III displays the microscopic findings of the right pouches and also the number of animals with metastases to the cervical lymph nodes in each group. No significant differences in the degree of differentiation of the tumors was observed between the groups. More than 75 percent of the tumors of all animals were characterized as well differentiated neoplasms. The neoplastic cells were forming nests, sheets and cords with otho-or parakeratin production (Figure 13). Extensive keratin production indicated low grade malignancy with cells well differentiated without prominent atypical mitotic figures. The nuclear-cytoplasmic ratio was altered but large hyperchromatic nuclei with prominent nucleoli were rare. Chronic inflammatory cells, mostly lymphocytes and plasma cells could be seen surrounding the epithelial nests. Edema and large sinusoidal spaces were observed in the connective tissue. Finally large areas of these tumors appeared necrotic consisting of epithelial cell remnants, homogenous eosinophilic material and polymorphonuclear leukocytes.

Only 14 to 23 percent of the total tumors in all groups were poorly differentiated neoplasms. The neoplastic cells of these tumors formed small nests or sheets with little or no keratinization. Sometimes isolated malignant cells were seen invading deeper structures. The cells in these tumors displayed one or more of the following: Pleomorphism, alteration of the nuclear-cytoplasmic ratio, hyperchromatism, prominent nucleoli, dissapearance of the cytoplasm in some cases and

bizzare mitotic figures (Figure 14). Large areas of necroses, intense inflammatory reaction and edema of the connective tissue were also prominent in these neoplasms.

The tumors of the right pouch were characterized also as superficially or deeply invasive. It was interesting to note that a difference in the degree of invasion was observed between the cortisone and non-cortisone groups. As it can be seen from Table III, 46 and 42 percent of the tumors of Groups C and D respectively had deeply invaded the underlying tissues, where only 18 percent of the tumors of Group A were characterized as deeply invading. The differences between Group C and Group A and between Group D and Group A were found to be statistically significant (Table III, $P<0.05$). Group B tumors also showed deeper invasion than the controls but this difference was not significant.

The epithelial nests of the invading tumors were found deep in the lamina propria and frequently had infiltrated the muscularis layer of the pouch. Figure 15 shows small epithelial nests invading deeply into the underlying lamina propria.

Metastasis to the cervical lymph nodes was found microscopically in 3 out of 14 animals in Group A (21%), in 3 out of 14 animals in Group B (21%), in 5 out of 13 animals in Group C (38%) and in 2 out of 13 animals in Group D (15%). Although Group C showed the higher incidence of metastasis (38%), the difference was not statistically significant. The five cases of grossly detectable tumors in the cervical area were histologically diagnosed as metastatic squamous cell carcinomas. All metastatic lesions were found in the ipsilateral submandibular lymph nodes

except in one case (Group B) where both ipsilateral and contralateral nodes were involved. In all animals, lymph nodes associated with the parotid gland were free of tumors.

The histology of the diagnosed metastatic lesions showed a great variation in terms of malignancy, atypia and in the degree of the lymph node involvement. Figures 16 to 21 display the most characteristic findings of the metastatic involvement of the lymph nodes.

In cases where metastasis was characterized as early, malignant cells were indetified in only a few of the serial sections studied. The malignant cells were forming single or sometimes multiple foci occupying the peripheral sinus and the outer cortex of the lymph node. Figures 16 and 17 show the characteristic appearance of the early metastatic lesion with the tumor cells invading the peripheral lymphatic sinusoids and located close to the fibrous capsule of the lymph node.

In more advanced cases the metastatic mass had replaced a large area of the node, the normal architecture of the node had been lost and lymphocytes surrounded the tumor mass (Figure 18). In other cases the tumor mass was so large that almost the whole node was replaced by the metastatic deposit (Figure 19). In the cases of grossly detected metastases, the rapid proliferation of the malignant cells resulted in extensive amount of necroses similar to the necrotic areas found in the large exophytic tumors of the right pouch. In these cases the metastatic lesion had totally replaced the lymph nodes and had extended into the surrounding tissues. It is of interest to note that the associated salivary glands, parotid and submandibular, were found normal in all

cases studied, without any evidence of malignant invasion.

All the metastatic lesions represented histologically squamous cell carcinoma which was well differentiated in some cases (Figure 20) but highly anaplastic in others showing a greater degree of atypia than the primary tumors (Figure 21).

In most of the animals that were free of metastases the submandibular lymph nodes were significantly enlarged but metastatic deposits were not detected although serial sections were studied. These nodes showed an alteration of the normal architecture with prominent reactive and hyperplastic changes. Histiocytic proliferation, a marked B cell proliferation, prominent lymphatic sinusoids and hypertrophy of the lining endothelial cells were noted in most cases.

Finally distant metastatic deposits were not found in any case in the lungs or the livers of the animals. These organs appeared normal in all cases except in one animal in which one of the lungs was found to be necrotic and had dystrophic calcification.

TABLE II

WEIGHT OF RIGHT POUCH AT THE END OF THE EXPERIMENT

GROUP A		GROUP B		GROUP C		GROUP D	
Animal	Pouch weight g.	Animal	Pouch weight g.	Animal	Pouch weight g.	Animal	Pouch weight g.
A2	2.68	B1	4.94	C1	4.37	D1	2.48
A3	1.46	B2	7.62	C2	3.28	D2	3.62
A4	2.42	B3	4.03	C4	2.11	D3	4.03
A5	2.52	B4	5.72	C5	3.80	D5	2.01
A6	2.95	B5	3.07	C6	3.32	D6	2.67
A7	2.06	B6	2.06	C7	6.43	D7	2.01
A8	5.82	B7	6.01	C8	3.40	D9	1.06
A9	4.15	B8	4.07	C9	2.51	D11	2.27
A10	3.97	B9	3.73	C10	2.00	D13	2.45
A11	3.44	B11	4.14	C11	3.25	D14	2.92
A13	3.38	B12	4.94	C12	2.13	D15	2.59
A14	4.79	B13	2.21	C13	5.05	D10	-
A15	-	B15	4.77	C14	3.13	D4	-
A1	-	B14	-				
Mean	3.30	Mean	4.35	Mean	3.44	Mean	2.55
SD	1.23	SD	1.59	SD	1.26	SD	0.81

P values (t test)

$P < 0.05$ in: D vs B.
$P < 0.1$ in: B vs A, B vs C, C vs D, D vs A.
$P > 0.1$ in: C vs A.

TABLE III

MICROSCOPIC FINDINGS

RIGHT POUCH CARCINOMAS*	GROUP A (N=14) Cases	%	GROUP B (N=14) Cases	%	GROUP C (N=13) Cases	%	GROUP D (N=13) Cases	%
Differentiation								
Well differentiated	24	86	22	78	20	77	21	80
Poorly differentiated	4	14	6	22	6	23	5	20
Invasion								
Superficially	23	82	20	71	14	54	15	58
Deeply	5	18	8	29	12	46	11	42
Metastases	3	21	3	21	5	38	2	15

P values (x^2 test)

Differentiation	$P > 0.1$ for all groups
Invasion	$P < 0.05$ in: C vs A, D vs A. $P < 0.1$ in: C vs B, D vs B. $P > 0.1$ in: A vs B, D vs C.
Metastases	$P > 0.1$ for all groups.

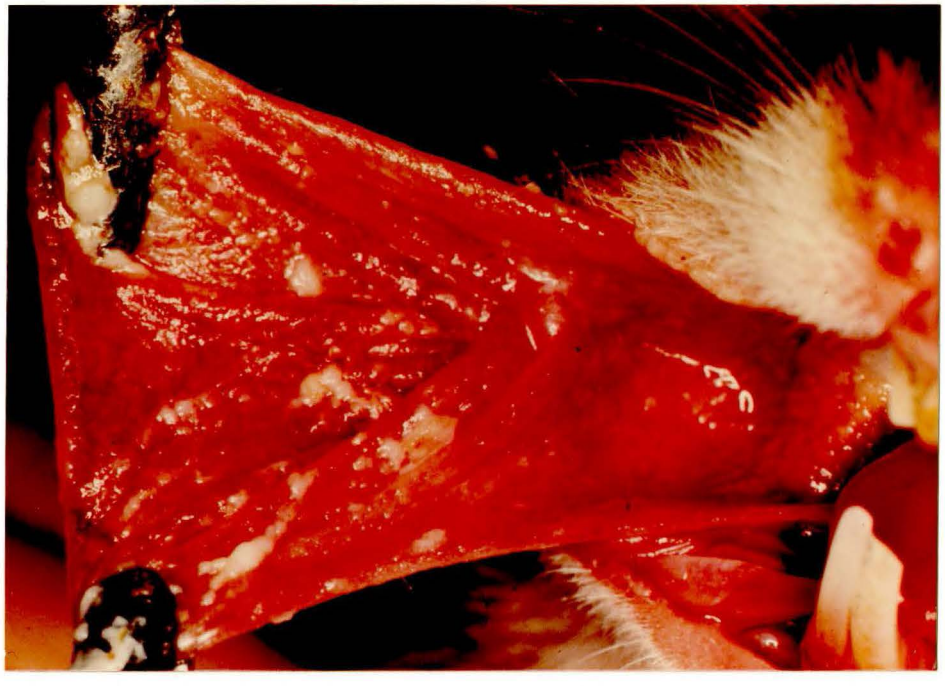
N: Number of animals in each group
*: Two tumors from each animals studied

FIGURE 4

Right treated pouch after 6 weeks of DMBA application. Note the general thickening as well as the leukoplakic areas.

FIGURE 5

Left untreated normal pouch six weeks from the beginning of the experiment.



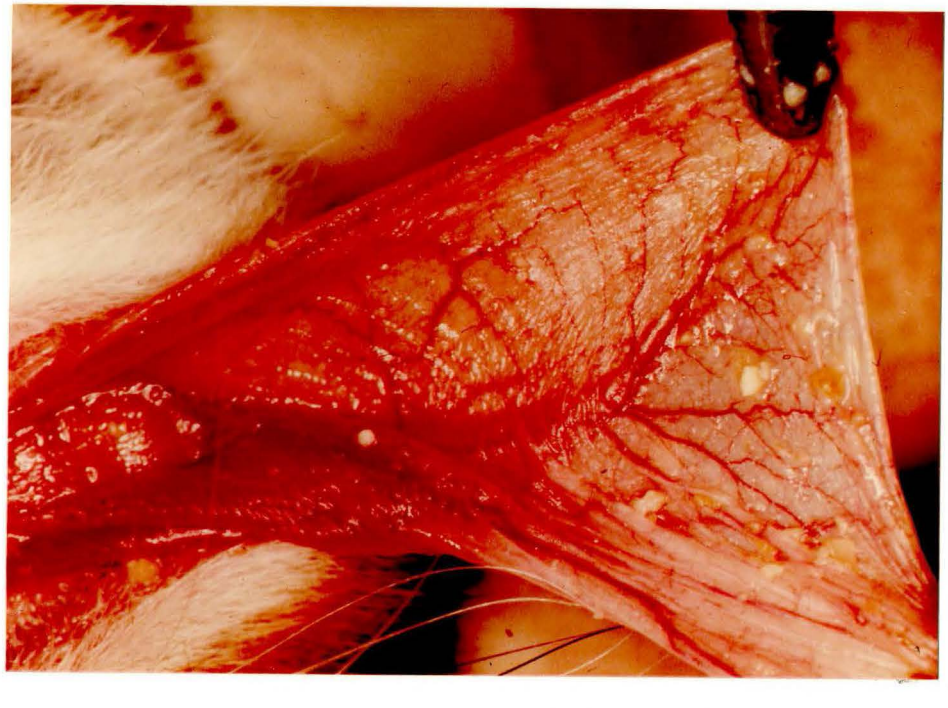
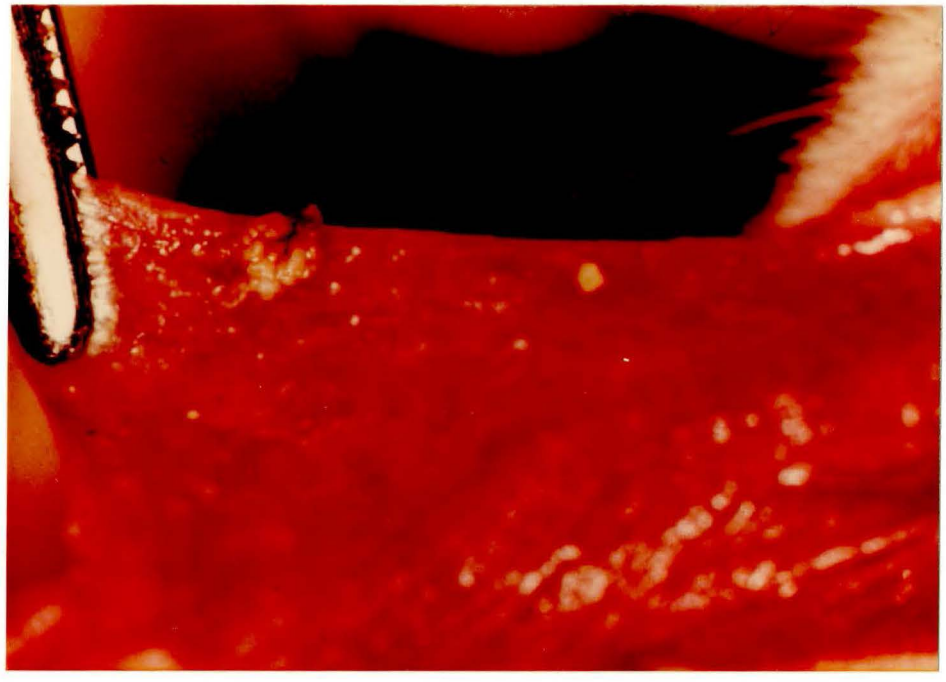


FIGURE 6

Small papillomatous tumor after 8 weeks of DMBA application to the right pouch of the control Group A.

FIGURE 7

Exophytic tumor 5 mm in diameter after 8 weeks of DMBA application to the right pouch of the cortisone Group C.



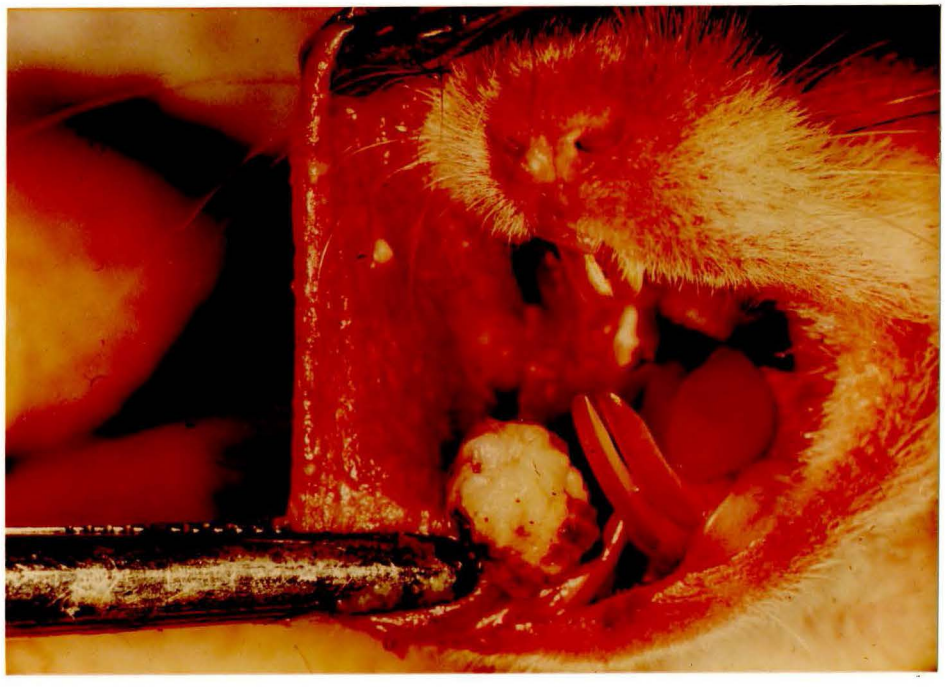
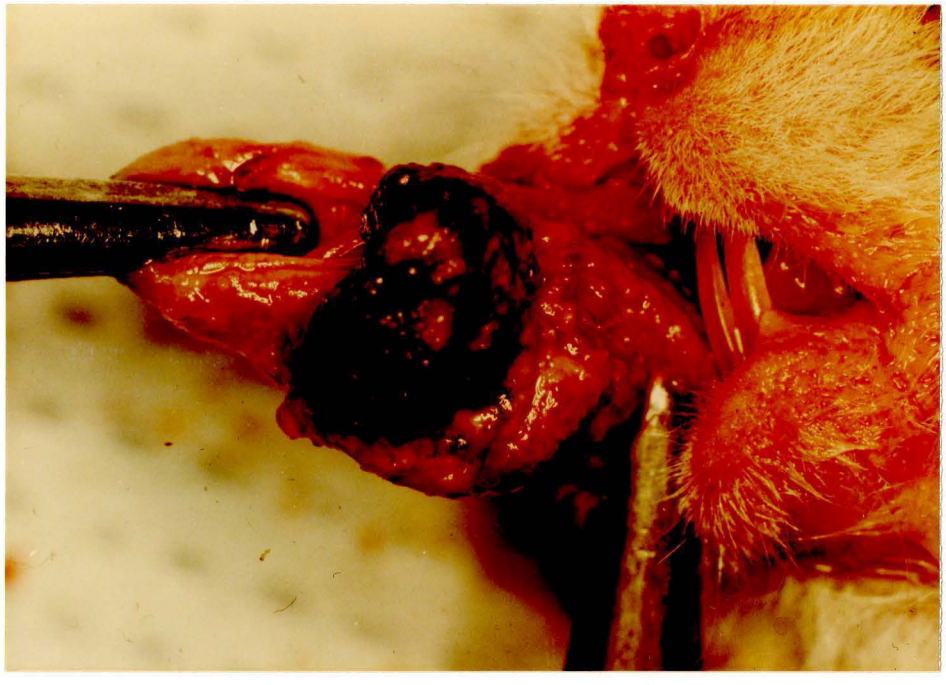


FIGURE 8

Necrotic exophytic tumor 10 mm in diameter after 13 weeks of DMBA applications.

FIGURE 9

Right side of animal with the skin reflected: The cancerous pouch (P) is clearly separated from the submandibular gland (S) and lymph nodes (LN) which are greatly enlarged.




S
LN
P

FIGURE 10

Excised right pouch showing large necrotic tumors occupying the whole surface.

FIGURE 11

Metastatic tumor mass (M) at the right submandibular area. The cancerous pouch (P) appears separate from the mass.

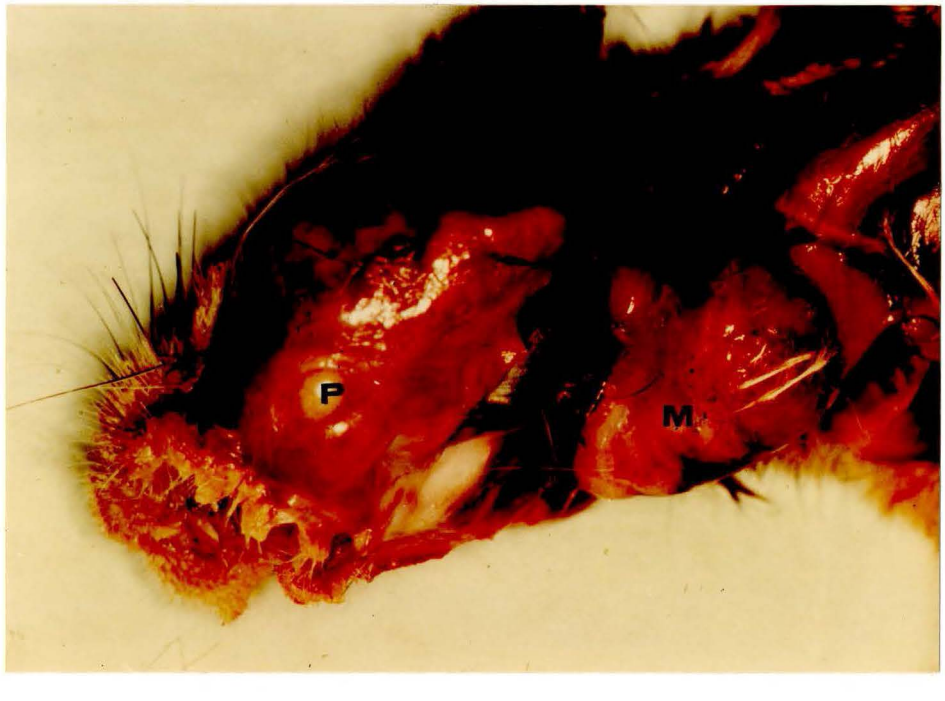

P
M

FIGURE 12

Photomicrograph of normal untreated pouch showing thin epithelium, lamina propria and muscular layer. (H & E stain--magnification X144).

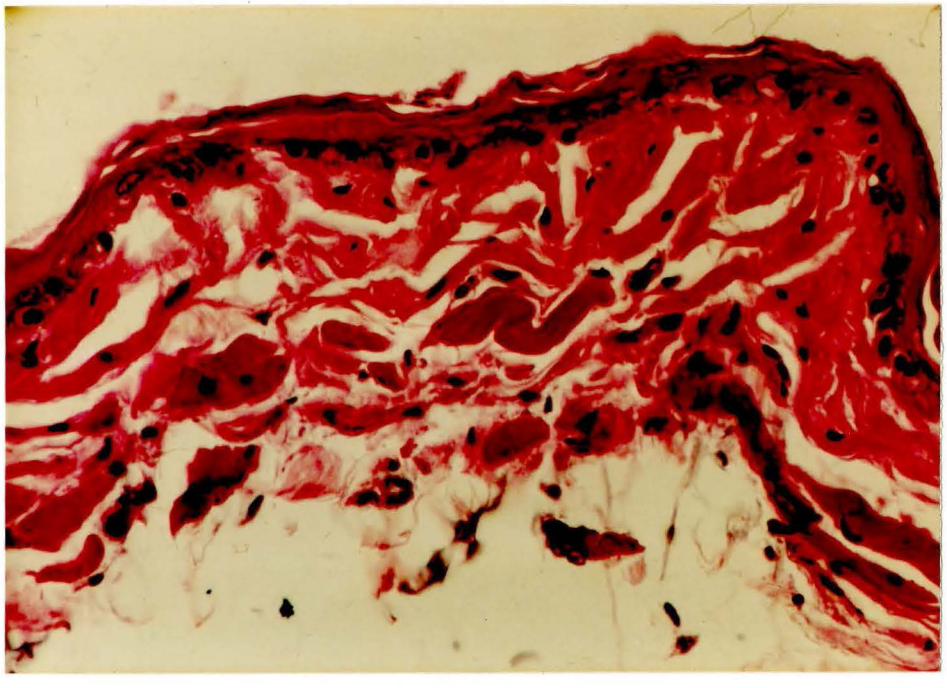
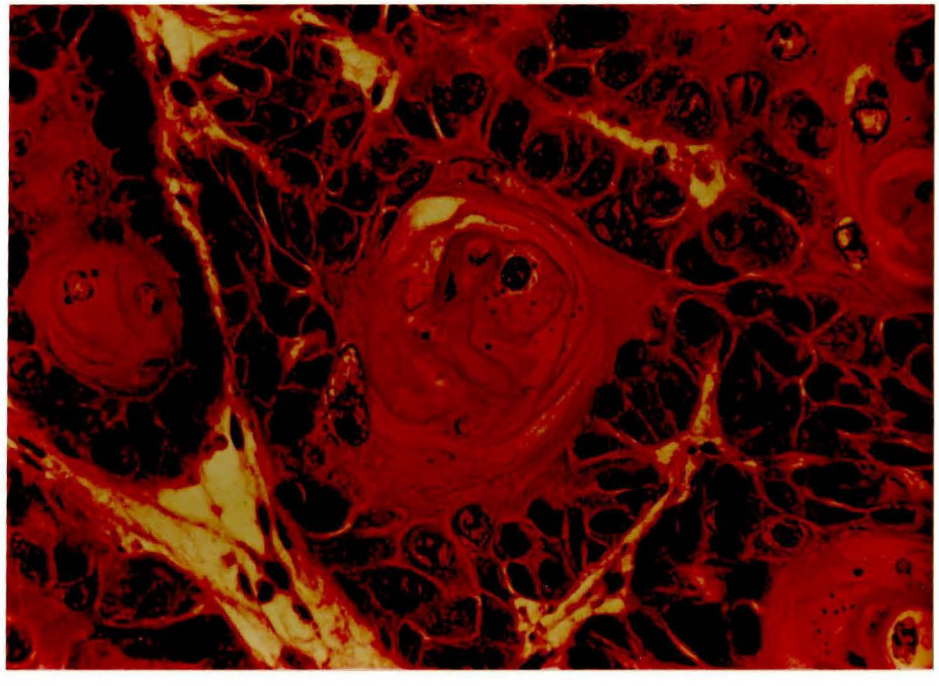


FIGURE 13

Photomicrograph of a well differentiated carcinoma of the right pouch, demonstrating epithelial nests with keratin production (H & E stain--- magnification X360).

FIGURE 14

Photomicrograph of a less differentiated carcinoma of the right pouch, demonstrating pleomorphic hyperchromatic cells with large nuclei and prominent nucleoli (H & E stain--- magnification X360).



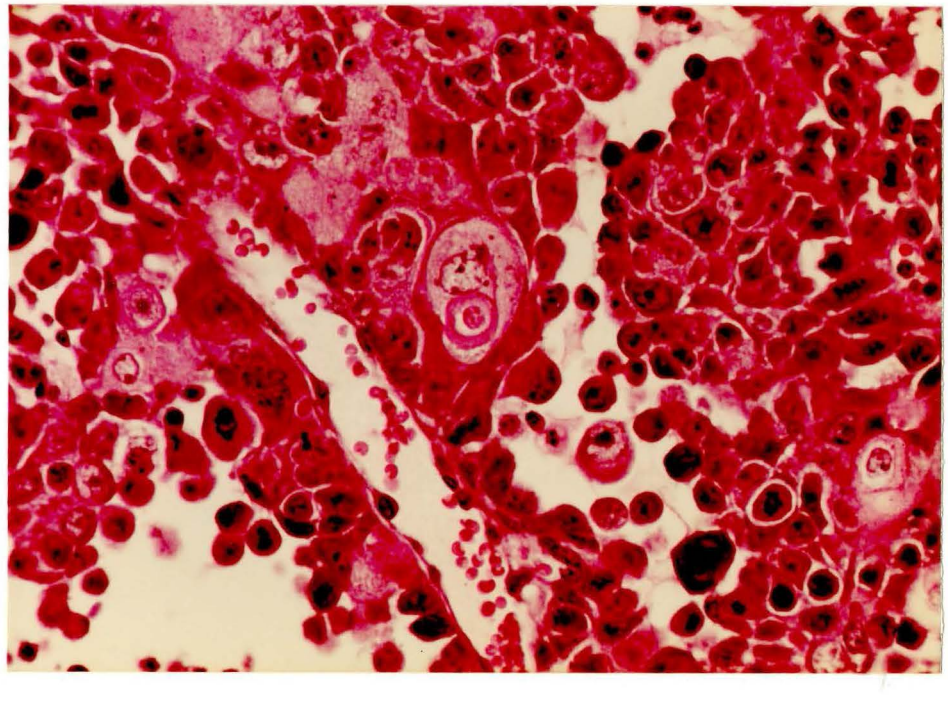


FIGURE 15

Photomicrograph of an invading carcinoma of the right pouch, demonstrating epithelial masses deeply into the underlying lamina propria (H & E stain--- magnification X58).

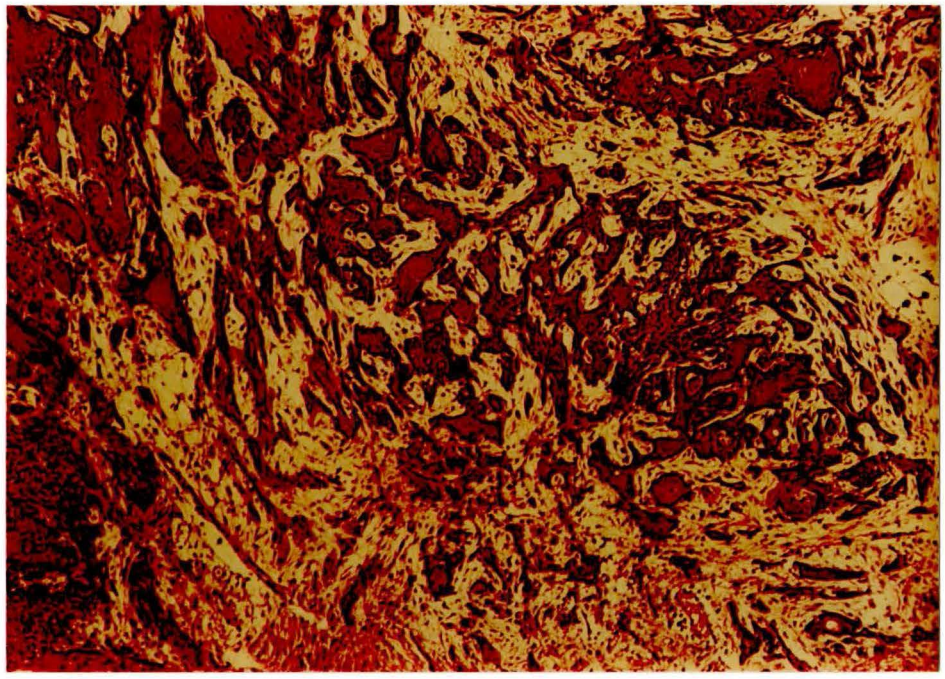
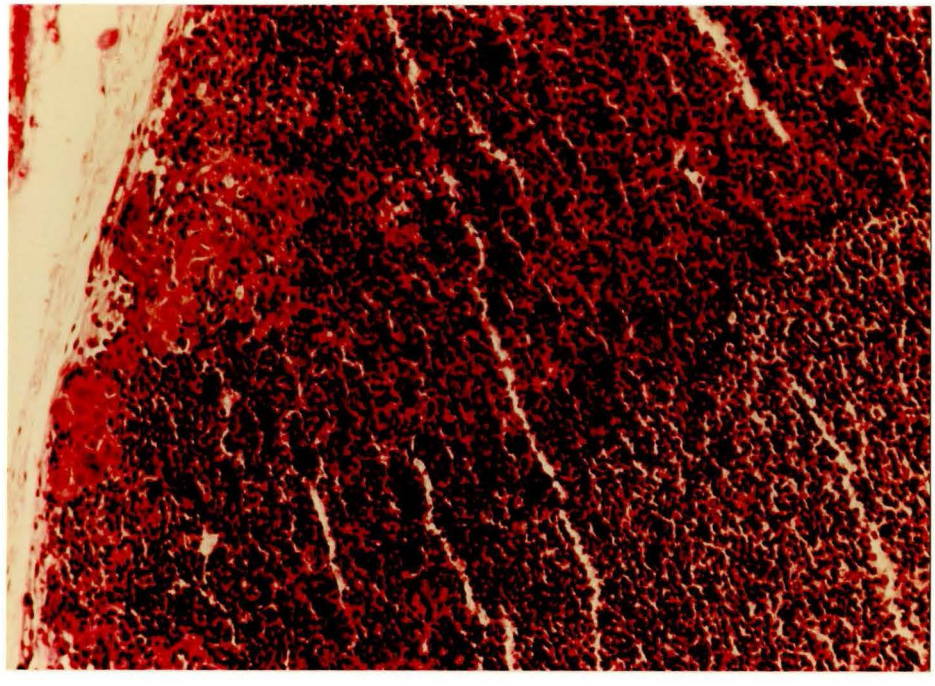


FIGURE 16

Photomicrograph of an early metastatic carcinoma in a cervical lymph node showing tumor cells present in the peripheral lymphatic sinusoids just beneath the fibrous capsule (H & E stain--- magnification X144).

FIGURE 17

Early metastatic lesion extending from the periphery of the lymph node (H & E stain--- magnification X360).



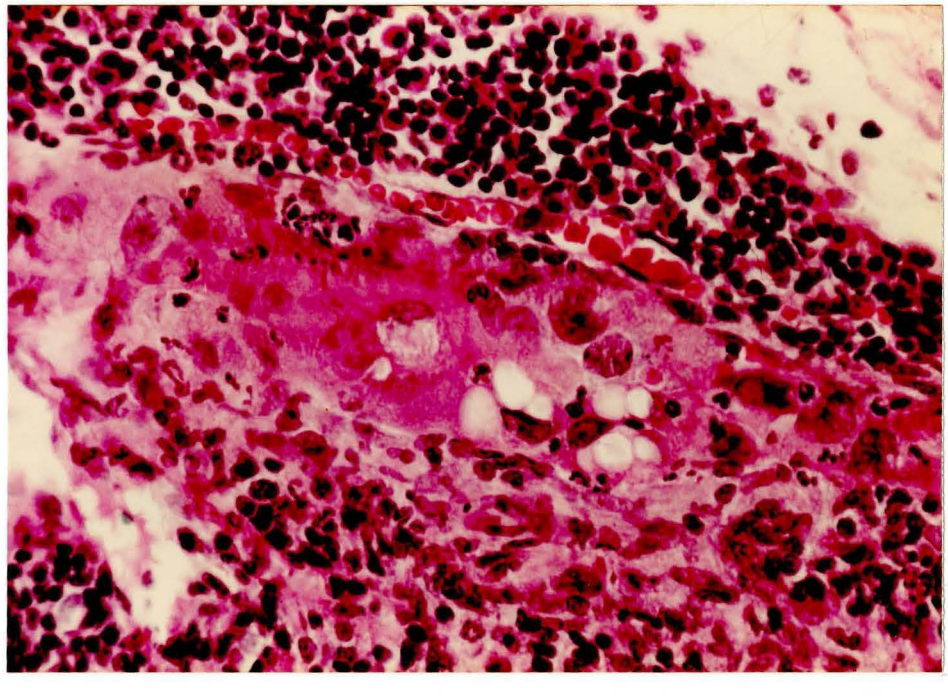
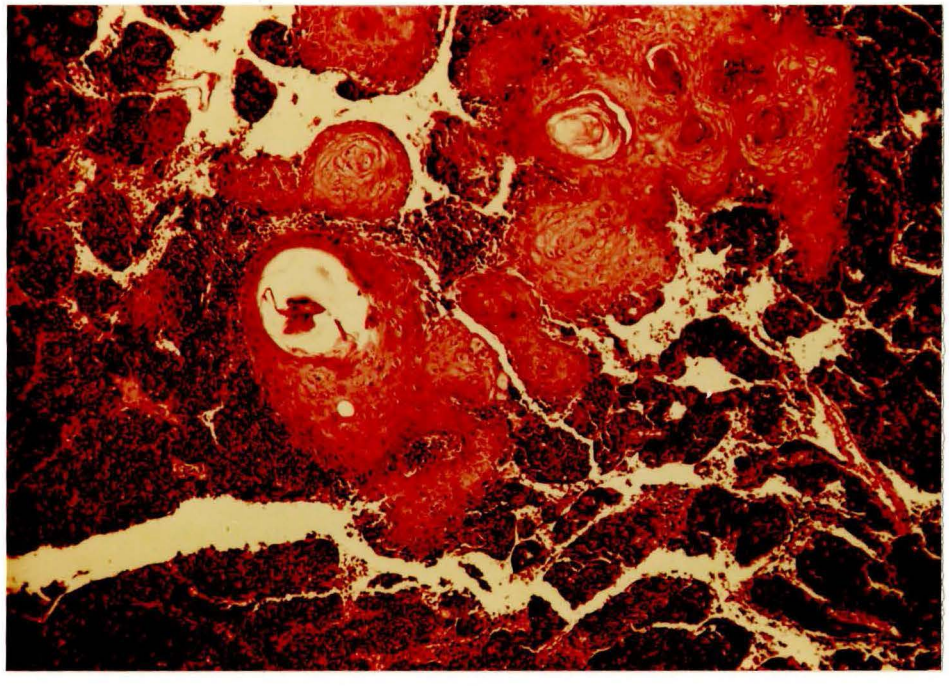


FIGURE 18

Metastatic tumor mass replacing a large area of the nodal architecture (H & E stain--- magnification X58).

FIGURE 19

Metastatic tumor mass occupying almost the entire lymph node (H & E stain--- magnification X58).



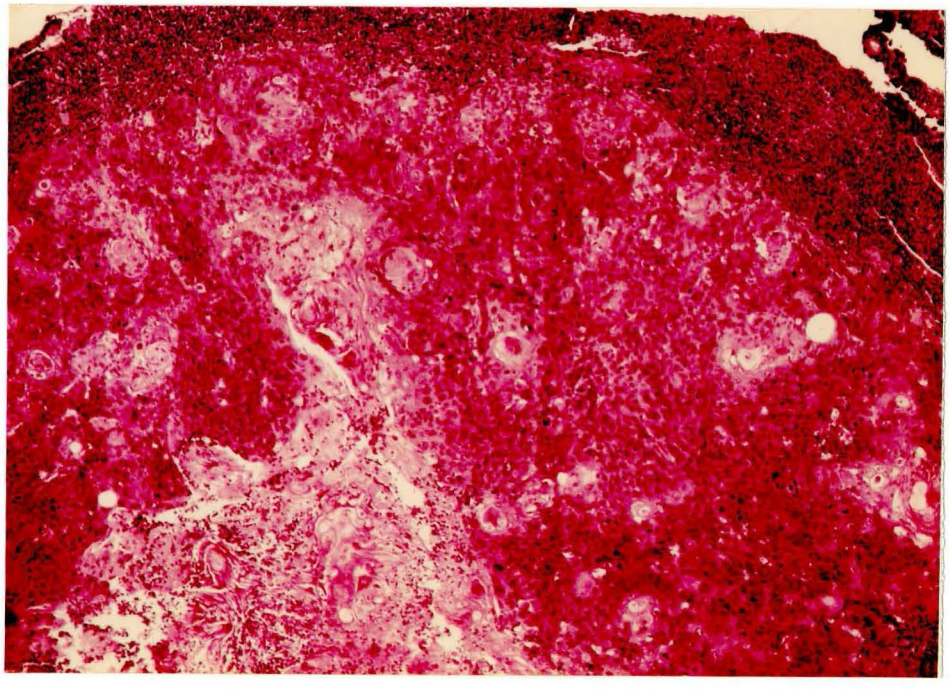
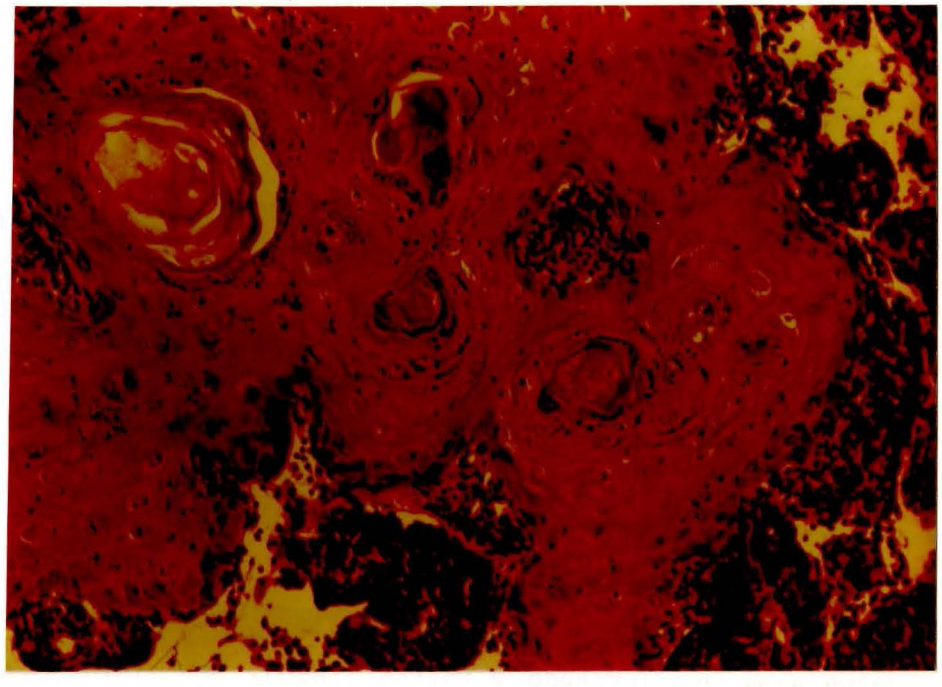


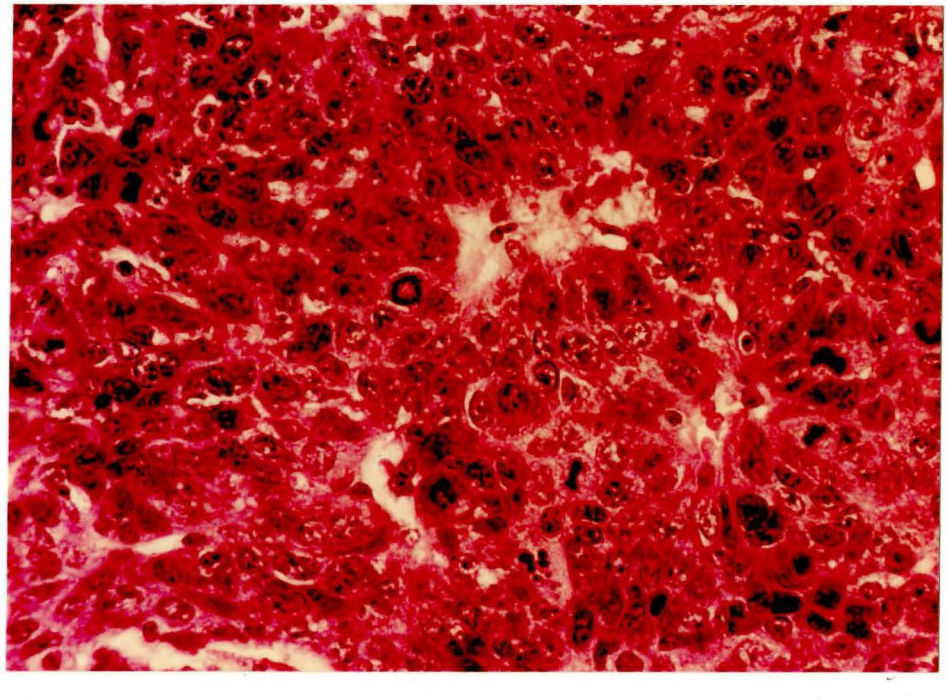
FIGURE 20

Metastatic lesion of a well differentiated type of squamous cell carcinoma in a cervical lymph node (H& E stain--- magnification X144).

FIGURE 21

Metastatic lesion of highly anaplastic type of squamous cell carcinoma in a cervical lymph node showing great degree of atypia, hyperchromatism and abnormal mitotic figures (H& E stain---
-magnification X360).





CHAPTER V

DISCUSSION

After the discovery of the carcinogenic chemicals and their experimental use by Yamagiwa and Ichikawa (1918), much research has been carried out on the study of experimental neoplasia in specific sites and tissues. The experimental pathology of oral mucosal carcinoma began with the investigations of Salley (1954,1957) who demonstrated that squamous cell carcinomas could be produced in the buccal pouches of hamsters by multiple paintings with chemical carcinogenic agents, such as, dimethyl-benzanthracene (DMBA). Salley's observations were extended by Morris (1961) who made an attempt to standarize the experiment by providing data on the optimal age of the animals and concentration of the carcinogen. Since then hamster buccal pouch carcinoma has served as an excellent experimental model in studying different problems of human oral neoplasia.

However this system has received some criticism because of the unique anatomical structure of the pouch and the relative absence of saliva deep within the pouch and it was thought by some investigators (Kolas 1955, Stormby and Wallenius 1964, Walker, et al. 1970, Williams, et al. 1971) that it could not be considered as representative of the human oral cavity. In spite of this criticism and because experimental carcinomas in areas other than the pouch are more difficult or impossi-

ble to induce, hamster buccal pouch carcinoma has continued to serve as the most accurate model in studying different parameters of oral carcinogenesis.

Carcinomas of the hamster pouch are similar histologically with the well differentiated type of human oral carcinomas. These pouch carcinomas have the tendency to proliferate on the surface mucosa rather than invading deeply into the underlying tissues. Despite the fact that many attempts have been made to develop an experimental model of oral carcinoma which would be more anaplastic and consistently metastasize to lymph nodes or distant organs, most of them were unsuccesfull (Shklar 1972, Merk, et al. 1979).

In this study we were able to show: (1) that incision of the exophytic tumors increases the local growth of the neoplasms, (2) systemic administration of cortisone results in more invasive neoplasms and (3) metastases to the regional lymph nodes in 15 to 38 percent of the cases.

LEFT NORMAL POUCH

In most studies of the hamster pouch carcinogenesis the unpainted pouch of the animal usually serves as the control for the carcinogenesis. This means that this pouch remains normal until the end of the experiment and no reactive or dysplastic changes are found. In the present study the left untreated pouch did not show any macroscopic or microscopic change. Only in 2 out of the 54 animals which ended the experiment, a small papillomatous exophytic growth was detected in the otherwise normal pouch. Histologically these tumors were well differentiated squamous cell carcinomas. The histologic examination of the

other unpainted pouches showed a normal mucosa. In a very few cases reactive or hyperplastic but no dysplastic changes were found. We believe that the development of the two exophytic tumors at the untreated pouch was due to the escape of small amounts of the carcinogen solution from the opposite pouch into the oral cavity. This suggestion is in agreement with the initial observations of Salley (1954) who noted dysplastic changes on the palate, tongue and upper portion of the gastrointestinal tract of hamsters in which DMBA was applied to the pouch. Similar observations were reported by Fujita, et al. (1973) who found carcinomas in several areas in the oral cavity when the carcinogen was applied to the ventral surface of the tongue in order to induce lingual carcinomas.

CARCINOMAS OF THE RIGHT POUCH

CLINICAL CHANGES

The early changes observed on the painted pouches of the animals were identical for all groups. The inflammatory response which appeared clinically as erythema and edema lasted approximately until the end of the second week and then the pouch appeared normal for the next one or two weeks. These observations are in agreement with Salley's study (1957) who found that the initial inflammatory response and degeneration of the treated pouch were observed until the fifth application of the carcinogen and was followed by epithelial regeneration and a return to normal appearance of the pouch.

Santis, et al. (1968) and McDonald (1978) were able to demonstrate the existence of a hyperkeratotic and dysplastic lesion on the pouch

comparable to human oral leukoplakia. This leukoplakic lesion usually preceded the development of the carcinomas and was an obvious precancerous condition. In this study leukoplakic areas on the right pouches appeared in all animals during the sixth week of the DMBA applications just before the development of the exophytic tumors.

The first exophytic papillomatous growths were observed at the sixth week in three animals of the cortisone-injected groups (C and D). At the same time no tumors were observed in the non-cortisone groups (A and B). During the following two weeks although papillomatous tumors were observed in all groups by this time, the tumors of the animals that were receiving cortisone appeared larger in size and more necrotic. These findings suggest that systemic administration of cortisone decreases the induction time of the chemically induced hamster buccal pouch carcinomas. There is only one study reported in the literature dealing with the effect of the systemic administration of cortisone on the pouch carcinomas (Shklar 1966, 1967). In this study the investigator reported that the animals which received cortisone developed earlier, larger and more invading neoplasms. In the present study earlier and larger neoplasms were found in the cortisone treated animals only at the beginning of the experiment. During the course of the study and until the end of the DMBA applications these tumors were not different from the non-cortisone groups.

POUCH WEIGHT

After the sacrifice of the animals, the cancerous pouches were removed and weighed. The pouch weight although it has not been reported previously in the literature, was thought to be an accurate method of comparing the difference in the size and growth of the exophytic neoplasms. Of course weight of the pouch can not be used as a method of studying the degree of invasion or the degree of anaplasia which can be determined only by microscopic examination. It was also thought that the more invasive the neoplasm is, the less exophytic or necrotic it will be and the less pouch weight it will demonstrate. On the other hand the superficially invading neoplasms proliferate on the surface, form large areas of keratin or necrosis and for this reason will exhibit a heavier pouch.

Group D animals which received systemic administration of cortisone showed the lowest values of pouch weight. The highest values were found in Group B in which incisional biopsy was performed but cortisone was not administered. The difference of the mean values of these groups was statisically significant ($P<0.05$). This finding suggests that the exophytic growth of the neoplasms is enhanced by the incision of the tumors and inhibited by the systemic administration of cortisone. The tumors in cortisone-treated animals showed a great degree of invasion into the underlying tissues instead of proliferation on the surface. Shklar (1966, 1967) reported that at the end of the experiment, animals that had received cortisone exhibited larger and poorly demarcated margins. On the other hand Baserga and Shubik (1954) reported that systemic

administration of cortisone inhibited the primary growth of transplated adenocarcinoma and of the induced skin carcinoma of mice. Inhibition of the primary tumors has also been reported by topical application of cortisone on the hamster buccal pouch (Polliack, et al. 1970).

DEGREE OF INVASION

Histologically 42 to 46 percent of the tumors of the cortisone groups were characterized as deeply invading. Deeply invading tumors in the control group were found only in 18 percent of the cases. This difference which was statistically significant suggests that systemic administration of cortisone results in the development of more endophytic neoplasms and this observation agrees with the reports of other investigators (Shklar 1966 and 1967, Zimel, et al. 1977, Cameron, et al. 1982). Fisher, et al. (1976) suggested that the systemic administration of cortisone induces immunosupression either by inhibiting some functions of the macrophages or by inducing a secondary lymphocytopenia affecting mostly the T lymphocytes. This depressed immunologic reactivity is probably an explanation for the greater degree of invasion reported in the literature and also observed in this study. So in cortisone-treated animals the growth of the pouch carcinoma meets little or no resistance to deep penetration of the tissues and invades in a greater extent rather than proliferating on the surface of the pouch.

The assumption that surgical trauma or incisional biopsy may contribute to the local spread of tumors is in dispute among the investigators. Shklar (1968) reported that neither biopsy incision or massage and compression of the hamster buccal pouch carcinomas promoted spread

or increased growth rate of the tumors. Shklar's negative results could be related to the following: at the time of the incision 50 percent of the animals did not have clinically detectable tumors; biopsies were not taken and it is therefore possible that the incisions were not made through malignant tissue; also a single incision was performed and in only 1mm in depth. In the present study incisions were made in clinically detectable tumors and an effort was made to select a large neoplasm (5 mm or more in diameter) which would not be necrotic or keratotic. Biopsies were obtained and the histologic examination proved that the incision was made through malignant tissue in every case. Also multiple incisions were performed and in greater depth in order to reach the muscularis layer of the pouch.

A rapid increase in the size of the tumors was observed during the two weeks that followed the incision in Group B and C animals. The possible spread of tumor cells into the adjacent tissues along with the inflammatory reaction which followed the incisions probably is the reason for the enhancement of the local tumor growth. The autopsy results confirmed the clinical observations. Most of the tumors of Group B animals had grown to such extent that they occupied the whole pouch and this group showed the highest values of the pouch weight. Also Group C animals which received cortisone and also had biopsies performed showed 25 percent higher values than Group D which received cortisone but did not have biopsies performed. Microscopically the tumors of the right pouch of the biopsied animals were not different from the tumors of the controls. Most of the tumors were of the well differentiated type and

although they exhibited more invasion than the control group, this finding was not statistically significant.

LYMPH NODE METASTASES

The most important finding in this study was the detection of metastases to the regional lymph nodes in 15 to 38 percent of the animals. Most of the previous studies in the literature have reported lack of metastases to the lymph nodes or distant organs from buccal pouch carcinomas (Shklar 1966, 1967, 1968, 1970, 1980, Levij, et al. 1970, Giunta and Shklar 1971, Freedman and Shklar 1978, Merk, et al. 1979, Eveson 1981). Sheehan, et al. (1971) reported that not a single metastasis was observed in their laboratory in more than one thousand cases. Shklar (1972) reported that buccal pouch carcinomas have the tendency to proliferate on the surface and metastasis does not occur.

There are only few exceptions in the literature which indicated lymph node metastases subsequent to the induction of pouch carcinomas. Salley (1954) in his first report stated that eighteen out of nineteen animals developed lymph node metastases. This finding however was not confirmed by Salley's later work (1955,1957,1961) or by other investigators. It was not until 1970 when the next report about lymph node metastasis appeared in the literature (Rwomushana, et al. 1970). This report concerned a single case found in one hamster treated with DMBA and vinblastine (cytostatic drug). Craig (1980) extending the duration of the tumor-bearing period found lymph node metastases in 48 percent of his animals. Safour (1983) found regional lymph node metastases in 53 percent of the animals in which biopsy of the tumors and seeding proce-

dures at several sites were performed.

From the reported metastases only in Salley's study did the animals receive the carcinogen without the addition of any other factor. In the present study, metastases were found in 21 percent of the control group. This finding is important as it indicates that DMBA-induced carcinomas have the ability to metastasize to the lymph nodes without the cocarcinogenic effects of any additional factors. Salley obtained metastases in 95 percent of the animals where in the study reported here the incidence was only 21 percent. A possible explanation for the difference in the metastatic yield is probably related to the vehicle of the carcinogen. Salley originally used acetone as a solvent for the DMBA but subsequently (Salley 1957) employed heavy mineral oil and was unable to detect metastases at the end of a somewhat shorter experimental period. More recently Marefat and Shklar (1977) have shown that carcinoma of hamster tongue can be much more readily and rapidly induced with DMBA in acetone as opposed to DMBA in mineral oil.

A common feature of all the reported cases of metastases is the relatively longer lasting experiment which results in extending the tumor-bearing period. Salley painted his animals for 16 weeks and sacrificed them 9 weeks later, Craig let the animals to survive more than 35 weeks and the animals which showed metastases in Safour's study were sacrificed between the twentieth and twentyfourth week of the experiment. Most of the studies in the literature which reported lack of metastases were terminated between the fourteenth and eighteenth week. The findings of the present study indicate that twenty weeks are sufficient

in detecting metastases from hamster buccal pouch carcinomas. Clinically detected metastases have never been reported in the literature. In this study grossly detected metastatic tumors were found in five cases.

INCISION AND METASTASES

Although surgical incision of the pouch carcinomas significantly increased the local superficial growth of the neoplasms, it did not enhance the rate of metastasis. Histologically the primary tumors of these animals showed large necrotic areas but they did not exhibit greater atypia or invasion than the controls and this may be the reason why metastasis was not enhanced. One has to consider also that in this study a single surgical procedure was performed during the course of the experiment. In the two previous studies (Craig 1980, Safour 1983) where the metastasis incidence was higher than this study, multiple surgical procedures were performed. Craig removed every tumor greater than 0.5 cm in diameter and Safour surgically exposed several areas of the animal. Several other studies have shown that extensive surgical trauma stimulates the formation of metastases after the injection of tumor cells into experimental animals (Agostino and Clifton 1965, Fisher, et al. 1967, Ivarsson 1976). On the oter hand in studies where a single incision was performed the metastatic rate was not increased (Tyzzer 1913, Shklar 1968).

CORTISONE AND METASTASES

Despite the fact that cortisone induced more invading malignant neoplasms it did not promote metastases. This finding is in dissagreement with the findings of most studies reported in the literature where systemic administration of cortisone favored metastatic spread of the experimental neoplasms (Pomeroy 1954, Baserga and Shubik 1954, Bloom, et al. 1963, Stoker 1969a,1969b, Zimmel, et al. 1973, Cameron, et al. 1982). A possible explanation of the difference between this study and that previously reported could be related to the following: (1) most of the other studies were dealt with the metastatic spread of intravenously injected cells and not of primary induced tumors as in the hamster buccal pouch carcinomas; (2) it may be that the dose of cortisone used in this study was not high enough to produce significant immunosupression of the animals; (3) in most of the other studies high doses of cortisone were administered just before the injection of the tumor cells and an increased metastatic rate was observed a few days later as an effect of the cortisone. In this study cortisone was administered from the beginning of the experiment, many weeks before the possible initiation of the metastatic spread which we assume began after the eighteenth week. Also the general condition of the animals did not permit the administration of higher doses during the two or three last weeks of the experiment as was scheduled at the beginning.

It was interesting that the animals receiving cortisone and also having incision of the tumors showed the highest incidence of metastases (38%). Similar findings have been reported by Mosley, et al. (1978)

where large doses of cortisone associated with surgical trauma significantly increased pulmonary metastases of the transplanted Lewis tumor on mice. The explanation of this observation may be that the surgical trauma further increases the resistance of the already immunosuppressed animal. Also the stress of the surgical procedure has been implied as a strong factor of inducing metastatic spread (Zimal, et al. 1977).

Further studies are needed to determine if the metastatic incidence of the hamster buccal pouch carcinomas will be increased: (1) by extending further the tumor bearing period; (2) by administrating higher doses of cortisone; and (3) by performing multiple surgical incisions in cortisone-treated animals.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The purpose of this study was to examine if the hamster buccal pouch carcinoma has the ability to metastasize to the regional lymph nodes or to distant organs and also to determine the effect of cortisone and incision on the primary tumors and on the metastatic rate.

Sixty four male Syrian hamsters, 3 months of age and weighing approximately 100 grams were divided into four equal groups. The right pouch of all animals of all groups was painted three times weekly with a 0.5 percent solution of DMBA in heavy mineral oil (USP) for 13 weeks. Group A animals, received no other treatment and served as the controls; in Group B animals, incisional biopsy of the tumors was performed after the end of the DMBA applications; in Group C animals, incisional biopsy was performed as in Group B and also cortisone was administered subcutaneously from the beginning until the end of the experiment; Group D animals, in addition to the DMBA, received cortisone as in Group C. Clinical examination of the right pouch of all animals was performed weekly. At the end of the experiment (20 weeks) the animals were killed by inhalation of an ethyl ether overdose and both pouches, the right and left salivary glands with the associated lymph nodes, the lungs and the livers were removed for gross and microscopic examination.

Clinically an increased tumor growth rate was observed following

the incisional biopsy. At the end of the experiment these animals showed large, exophytic and more necrotic tumors than the animals in the other groups. Although tumors appeared earlier in the cortisone-treated animals, during the course of the study and by the end of the experiment no significant differences in the size of the tumors between cortisone and control groups were observed. Histologically most of the tumors in all groups were of the well differentiated type but the tumors of the cortisone groups showed a significant greater degree of invasion than the tumors of the non-cortisone groups.

Metastases to the cervical lymph nodes were found in 21 percent of the animals in Group A, in 21 percent in Group B, in 38 percent in Group C and in 15 percent in Group D. The differences between groups were not statistically significant. In five animals a large metastatic tumor was present in the submandibular area. No metastatic lesion was found in the lungs or the livers of the animals.

Under the conditions of the experiment described here it is concluded that:

(1) Incision of the malignant neoplasms of the hamster buccal pouch resulted in larger but superficially growing neoplasms.

(2) Systemic administration of cortisone resulted in more deeply invading neoplasms.

(3) Metastasis to the cervical lymph nodes was found in 15 to 38 percent of the animals. In 5 out of 13 total metastatic cases a metastatic tumor observed grossly in the right

submandibular area of the animals.

(4) Metastasis to the lungs or livers of the animals were not observed in any case.

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APPENDIX A

Histopatholigical Evaluation Sheet

GROUP ANIMAL POUCH WEIGHT

Right Pouch	Left Pouch
Gross	Gross
Microscopic description	Microscopic
differentiation	
invasion	

Right Side of the Neck	Left Side of the Neck
Gross	Gross
Microscopic Salivary glands	Microscopic Salivary glands
Lymph nodes	Lymph nodes
Metastases	Metastases
Capsular invasion	Capsular invasion
Other findings	Other findings

Lungs	Livers
Gross	Gross
Microscopic	Microscopic

APPROVAL SHEET

The thesis submitted by Kostas Tsiklakis has been read and approved by the following committee:

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