Incisional Biopsy and Seeding in Hamster Buccal Pouch Carcinoma

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INCISIONAL BIOPSY AND SEEDING IN
HAMSTER BUCCAL POUCH CARCINOMA

BY
Ibrahim M. Safour

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
JULY
1983
DEDICATION

To my parents, Mohamed Safour and Mabruka Yossif, in appreciation for their encouragement and support throughout my educational years.
ACKNOWLEDGMENTS

I would like to express sincere gratitude to Dr. Norman K. Wood, D.D.S., M.S., Ph.D. who offered his advice and direction in planning this study.

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I also thank all who contributed to accomplishing this work.
VITA

The author, Ibrahim M. Safour, is the son of Mohamed Safour and Mabruka Yossif. He was born July 15, 1953 in Umalaranib, Libya.

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In September, 1981, he was granted a scholarship to pursue his graduate studies at Loyola University of Chicago.
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Cancer of the lip, tongue, floor of the mouth, buccal mucosa, palate, gingiva and oral pharynx account for approximately 5% of all malignancies. Biopsy is a helpful procedure in establishing an accurate diagnosis not only for tumors but for other lesions as well. When doing an excisional biopsy, the surgeon usually successfully removes the entire lesion in one piece along with a suitably wide margin of normal tissue. The incisions are made in non-lesional (normal) tissue in order to avoid cutting across a potentially malignant tumor. It has been generally assumed that if the incision passes through malignant tissue, the scalpel will become contaminated and possibly spread the tumor beyond the margins of the original tumor. Theoretically, this concern should be greater with incisional biopsies because traditionally the procedure is completed so that the biopsy section contains some tumor tissue and some normal tissue at the periphery. Hence, the incision is made from tumor to normal tissue and visa versa. This is especially of concern in plaque-like lesions which may be intra-epithelial malignancies or minimally invasive carcinomas in their very early stages. Incisional biopsy of these early treatable lesions could therefore carry malignant cells into normal tissue and thus spread the lesion.

Workers such as Stuteville (1966) and Warpeha (1979) indicated that
incisional biopsy could have harmful effects. Other studies of a large number of cases of head and neck tumors have failed to substantiate this concern: Pomeranz and Stahl (1953), Santis and Shklar (1964), King and Coleman (1965), Medak, et al. (1967) and Guinta, et al. (1969). However, most of the tumors in the latter studies had reached advanced stages prior to initial treatment. Thus, one would expect a high rate of recurrence anyway, regardless of whether incisional biopsy was done or not.

Also, post-surgical irradiation is routinely used in advanced cases and so cells which may have been disseminated by incisional biopsy would very probably be destroyed by the irradiation anyway. So the present concern is rather with the use of incisional biopsy in early lesions which often show malignant changes that are restricted to the epithelium. Theoretically such lesions could be completely removed by wide excisional biopsy and a 100% cure rate anticipated. However, if incisional biopsy is used first and there is a delay in complete excision of the lesion harmful dissemination of tumor cells could possibly result.
A. CHEMICAL CARCINOGENESIS IN THE HAMSTER

1. Polycyclic Hydrocarbons as Carcinogens:

One of the most intriguing problems in cancer research concerns the mechanism by which polycyclic hydrocarbons initiate tumors. Hill (1761) reported on the development of nasal cancer as a consequence of excessive use of tobacco snuff. Pott (1775) reported on the unusually high incidence of cancer of the skin of the scrotum among young men who had worked as chimney sweeps and related the cancers to the soot to which they were daily exposed.

The modern era of experimental carcinogenesis research was initiated by Yamagiwa and Ichikawa (1915) who developed the first experimental model of chemically induced cancer. They induced skin carcinomas in the ears of rabbits by repeated topical applications of coal tar.

Tsutsui (1918) and Passey (1922) induced skin cancer in mice using tars and ether extracts of tars respectively.

Kennaway and Hieger (1930) demonstrated the carcinogenicity of dibenzanthracene by application of 1:2, 5:6-dibenzanthracene to the skin of mice. Since then it has become generally accepted that certain polycyclic hydrocarbons are capable of inducing cancer.

The biological effects produced by polycyclic hydrocarbons such
as mutagenesis and malignant transformation are now believed to be due to the metabolic activation of these relatively inert compounds. However, a general acceptance of the molecular mechanism by which these compounds cause malignant transformation has not yet been achieved. Evidence is accumulating to show that the active metabolites which cause these effects are epoxides. The metabolism of polycyclic hydrocarbons takes place principally on the microsomes of the endoplasmic reticulum and is catalyzed by the mixed-function oxidases. These enzymes are NADPH-dependent and are present in most tissues of mammalian species and serve to detoxify foreign compounds. Evidence for this was provided by the work of Holtzman, et al. (1967).

Several attempts to explain the role of polycyclic hydrocarbons in the process of malignant transformation have been undertaken by many investigators: Boyland and Green (1962) and Liquori, et al. (1961) thought that the physico-chemical binding of hydrocarbons to nucleic acids might be sufficient to account for their biological effects. Heidelberger and Davenport (1961) and Brooks and Lawley (1964) indicated that metabolic activation of polycyclic hydrocarbons occurred in the cell and that these active metabolites became covalently bound to the macromolecules of the cell. Miller (1978) reported that metabolic derivatives of hydrocarbons could cause permanent alterations in cell phenotype by reacting with genetic material.

Toto (1983) stated that polycyclic hydrocarbons when applied to mammalian tissues in vivo or in vitro are metabolized by the tissues
producing intermediate products such as epoxides, diols and phenols. Such products bind to the nuclear DNA and induce mutagenesis and initiate cancer.

2. The Hamster as an Experimental Animal:

Waterhouse (1839) first described the golden Syrian hamster and wrote that they are native to Syria, smaller than the common European species and covered by smooth short fur which is golden-brown dorsally and light-gray ventrally. The female is consistently larger than the male, but rarely exceeds seven inches in length and two-hundred grams in weight.

Alder (1948) presented this animal to the scientific community as a valuable experimental model in 1930. Arnold (1942) suggested the potential value of the Syrian hamster in dental research.

Keyes and Dale (1942) described the buccal pouch of the Syrian hamster as a balloon-like structure located beneath the dermis along the lateral side of the head and neck and consisting largely of dense fibroelastic tissue. Each pouch is suspended by a thin muscle slip which is inserted into the lumbar fascia in the mid-dorsal line. The pouch opens into the mouth through the diastema between the incisors and the molars.

Salley (1957) stated that the cheek pouch is derived embryologically from the primitive buccal cavity so the histology of the pouch apart from the absence of adnexal structures is identical to the rest of the oral cavity. Walker (1970) disagreed with Salley and stated that the pouch is considerably thinner than the rest of the oral mucosa and has a
unique submucosal connective tissue.

Shklar, Eisenberg and Flynn (1979) reported that the major advantages of the hamster pouch carcinoma model include: (1) the similarity between the pouch mucosa of hamster and the keratinizing human oral mucosa in terms of histology, histochemistry and ultrastructure; (2) the absence of spontaneously occurring carcinoma in the pouch of this animal which would confuse the data on carcinogenesis; (3) 100% consistent production of carcinoma with a potent carcinogen; (4) the development of precancerous dysplastic lesions comparable to human leukoplakia preceding the development of true epidermoid carcinoma; (5) the consistent time pattern of tumor induction with potent carcinogens; and, (6) the induced tumor is influenced by systemic and environmental factors such as immune response, hormones, vitamins and various drugs.

MacDonald (1981) undertook a study to compare the epithelial dysplasia of the hamster cheek pouch with that which occurs in human mucosa. He concluded that there is close similarity in the histological features. He added that, this conclusion should promote the hamster pouch carcinoma model to be used in more detailed analyses of the features of epithelial dysplasia.

In contrast, other investigators object to the use of the cheek pouch model in studies on intraoral carcinogenesis. Kolas (1955) stated that the cheek pouch could not be considered representative of the oral cavity proper as it is not subjected to the same environmental influences as the rest of the mouth. Stormby and Wallenius (1964) also objected to
the use of the cheek pouch on the basis of major anatomical and histological differences between the pouch and other oral mucosa. Billingham, et al. (1960) and Williams, et al. (1971) gave objections that were based on the fact that the pouch is a site of immunologic privilege, but Mohammad and Mincer (1976) and Marshack, et al. (1977) showed that contact hypersensitivity reactions can be induced in the cheek pouch after skin contact with dinitrochlorobenzene: a simple allergen which is a non-specific stimulator of T-lymphocytes.

3. Hamster Cheek Pouch Carcinoma:

Wantland in a personal communication to Salley (1954) reported the first documented attempt to produce tumors in the cheek pouch of the hamster by spraying and painting 20-methylcholanthrene, 1:2, 5:6 dibenzanthracene and 2-acetylaminofluorine for six weeks. Results showed only hyperplasia of the pouch. Salley (1954) successfully produced malignant epithelial tumors in the cheek pouch of the golden Syrian hamster. Using a #4 camel's hair brush, the epithelial surface of the pouch was painted with 9, 10-dimethyl-1,2-benzanthracene (DMBA) in either benzene or acetone solution, 20-methylcholanthrene (20-MC) or 3,4-benzpyrene (3,4-BP). Each pouch was painted three times per week for sixteen weeks and subsequently observed for nine weeks. The tumors which developed ranged from benign papillomas to invasive squamous cell carcinomas with lymph node metastases. Also it was shown that DMBA was better when dissolved in acetone than in benzene; however, in a subsequent study Salley (1955) noted that, using mineral oil as a solvent instead of acetone decreased
the tumor induction-time of six to seven weeks to four and one-half weeks.

Salley (1957) also studied the histologic changes in the hamster cheek pouch during early painting of polycyclic hydrocarbons as carcinogenic agents. Results showed that one application of 0.5% DMBA in mineral oil produced inflammation and three applications caused degeneration and necrosis with some sloughing. Epithelial regeneration followed after four to five applications, papillomas developed after fifteen applications and squamous cell carcinomas were found after seven and one-half weeks of DMBA applications.

Santis, et al. (1964) and MacDonald (1978) stated that the malignant tumors produced in the buccal pouch of Syrian hamsters were preceded by a hyperkeratosis and dysplastic lesions comparable to human leukoplakia.

Studies on how DMBA gets into the epithelial cells and the underlying connective tissue were done by many investigators. Goldhaber (1958) suggested the possibility that a pathological portal of entry may be necessary and he proposed ulceration of the mucosa as a mechanism by which the carcinogen could enter the tissue. It was found that topically applied carcinogen became localized in high concentrations in the ulcerated areas. Morris (1957), however, reported no evidence of ulceration of the hamster pouch mucosa treated with DMBA during the premalignant phase.

Salley and Kreshover (1959) compared the carcinogenicities of DMBA and 3,4-benzpyrene when they were applied to the ear, pouch and palate of the hamster. Both chemicals produced malignant tumors of the ear and of the pouch mucosa in 100% of the cases and palatal tumors in 54%.
The authors questioned the necessity of physiological or pathological portals of entry in oral carcinogenesis compared to skin carcinogenesis in which accessory epidermal structures tended to enhance penetration of the carcinogen.

Salley (1961) used fluorescence microscopy to investigate the distribution of DMBA and benzpyrene when they were topically applied to four areas of the hamster: skin, tongue, pouch and palate. In the skin, the carcinogen was observed early in the sebaceous glands and there was a heavy concentration of carcinogen in the epidermis after two to three applications. In the pouch, the carcinogen became incorporated in the keratin layer after a single application where it fluoresced with a brilliant yellow color. The color changed to yellow-blue and large fluorescent bodies were detected in the lamina propria and along the basement membrane after four applications of carcinogen.

Listgarten, et al. (1963) studied the ultrastructural changes which took place when the pouch mucosa was painted with DMBA. They noted widening of the intercellular spaces as early as the second application. They believed this widening facilitated the penetration of the carcinogen into the underlying connective tissue.

Kendrick (1964), and Meskin and Woolfrey (1964) used fluorescence microscopy and DMBA labeled with $^{14}$C to investigate the portal of entry of DMBA through the pouch mucosa. They concluded that DMBA penetrates an intact epithelium within hours of its application.

Morris and Reiskin (1965) attempted to determine at what point or
stage in the carcinogenic process the tissue became irreversibly altered. They exposed hamster pouch mucosa to three applications per week of 0.5% DMBA. Results showed that three weeks of painting were necessary to produce tumors in all animals; however, the latent period for tumor production was longer than that of animals painted for four weeks. They concluded that the critical changes of malignant transformation took place in all animals following three to four weeks of DMBA application in spite of the fact that no gross or microscopic evidence of tumor development could be seen at that time. However, Levij, et al. (1968) demonstrated that when 0.5% DMBA was applied locally three times a week to hamster pouches for six weeks and then discontinued, no cancer developed. However, continued painting of another group of hamsters did result in cancer development. Eisenberg (1977) concluded that irreversible alteration of epithelial cells of pouch mucosa by DMBA application occurs by the 10th to 12th week.

Odukoya and Shklar (1982) described a two-phase mechanism of chemical carcinogenesis in hamster buccal pouch which agreed with the original concept of Berenblum and Shubik (1947 and 1949). The latter postulated that early treatment with DMBA initiated and the later applications sustained the carcinogenic process.

4. Factors Modifying Hamster Pouch Carcinoma:

Rusch (1944) wrote that one of the most fundamental discoveries in the entire field of cancer research was the observation that certain chemical or physical agents are capable of inducing tumors when brought
in contact with living tissue. He added that the carcinogenicity of chemical agents like the hydrocarbons is influenced by the medium in which they are dissolved. Hydrocarbons produced tumors in fewer animals and required a longer period of time for induction when applied as a powder, crystals or as pellets than when given in oily solution. Salley (1954 and 1955) used different DMBA solvents: acetone, benzene and mineral oil. Results showed that acetone is better than benzene but that mineral oil was the best in that it decreased the tumor induction-time from six or seven weeks to four and one-half weeks.

Morris (1961) studied the effect of age, concentration of carcinogen and frequency of application. Lesions in old animals required a greater length of time to develop than in younger animals and five weeks of age appeared to be ideal for experimental oral carcinogenesis. A 0.5% concentration of DMBA was the optimal concentration for the rapid production of malignant tumors. Exposure to the carcinogen three times a week required less time for tumor development than exposure only twice weekly. He also noted that the responses did not vary according to the sex of the animals.

Renstrup, et al. (1962) demonstrated that when a level of chronic mechanical irritation which was capable of causing ulceration was combined with DMBA application, it took only four weeks instead of ten weeks for tumors to develop.

Reiskin and Berry (1968) found that different hamster strains showed variable latent periods for tumor induction: 7.3 weeks for in-bred,
dark-eared, partial albino (DEA) hamsters; 10 weeks for random-bred golden hamsters; and 10.75 weeks for random-bred cream hamsters. The average growth rate of the tumors was higher in the DEA hamsters than in the other strains.

In addition to the above mentioned studies, the influence of various natural and synthetic materials upon the carcinogenic process in the hamster pouch has been studied by many investigators. Table I lists factors which augment tumor induction while Table II lists those factors which depress tumor induction.

5. Metastasis in the Hamster:

Coman (1944 and 1947) planned an experiment to measure quantitatively the force necessary to separate neoplastic squamous cells compared to that of normal squamous cells. Results showed that normal cells had relatively greater adhesiveness. The ability of the neoplastic cells to be invasive was not solely dependent on decreased adhesiveness, but also on the ameboid movement of the liberated cells and production of spreading factors, such as, hyaluronidase.

Salley (1954) demonstrated metastasis to the cervical lymph nodes subsequent to induction of squamous cell carcinoma in the hamster pouch treated with DMBA.

Lemon and Smakula (1955) investigated the most favorable circumstances for the transplantation of tumors and metastases in hamsters with special reference to the age of the host and the effect of repeated inoculation. They concluded that metastases to lymph nodes developed
following primary implantation, whether or not the primary graft was later excised, but that there were no metastases to lungs, liver, spleen, peritoneum or kidneys. All cervical lymph node metastases exhibited rapid growth. There was, however, a significant influence of age on the frequency of metastasis and upon the time interval between primary implantation of the tumor and the clinical manifestations of metastasis. In young animals palpable metastases had developed within three to five weeks of tumor implantation in thirteen out of twenty-five hamsters, in contrast to only one out of thirty-three adult hamsters.

Listgarten, et al. (1963) stated that topical application of DMBA resulted in desmosomal disruption and peripheral clumping of the tonofibrils. Desmosomal disruption has been used to confirm decreased adhesiveness of tumor cells.

Rwomushana, et al. (1970) observed one case of metastasis out of ten animals treated with vinblastin (cytostatic drug) which was applied locally in combination with DMBA.

Fujita, et al. (1973) studied the induction of tongue tumors in the hamster. Various sites of the hamster's tongue were first scratched using a pulp-canal cleaner (a barbed broach) and then 0.5% DMBA in acetone was applied to the traumatized areas using a #4 sable brush, three times a week while under ether anesthesia. The period required for tumors to develop was relatively short for the lateral border of the middle third of the tongue, longer for the ventral surface, still longer for the tip of the tongue and longest for the midportion of the dorsum
of the tongue. Tumor infiltration into the muscle of the tongue was demonstrated in 96.5% and 12.9% showed metastases to the regional lymph nodes.

Craig stated through personal communication with Eveson (1981) that cervical lymph node metastases could be produced by extending the duration of the tumor-bearing period. In contrast Shklar (1966, 1967, 1968, and 1970) reported that although the carcinoma induced in the buccal pouch showed morphologic and histologic similarities to human oral carcinoma, it did not metastasize or develop as a histologically anaplastic lesion. He related the absence of metastasis in the hamster carcinoma model to the fact that buccal pouch carcinomas were of the well-differentiated type.

Mesrobian and Shklar (1969) who shortened the time for inducing gingival tumor by using cyanoacrylate tissue adhesive to hold pure DMBA powder in place, reported neither gross nor microscopic evidence of distant tumor metastases.

Woods (1969) studied the effect of antilymphocyte serum on hamster buccal pouch carcinoma and showed that it speeded up the initiation of the tumor and increased the total number of tumors, but he was unable to find metastasis in any of the hamsters.

Levij, et al. (1967), Giunta and Shklar (1971), Sheehan, et al. (1971) and Freedman and Shklar (1978) all indicated that they never found metastases in their studies.

Marefat and Shklar (1977), and Eveson and MacDonald (1981) reported
that epidermoid carcinoma could be consistently produced in the hamster's tongue, but no metastases were detected in any of the lymph nodes in spite of a meticulous search.

B. INCISIONAL BIOPSY

1. Biopsy in Humans:

It is mandatory and of practical value to supplement clinical diagnosis of oral lesions particularly cancerous lesions with histological examination. Incisional biopsy is the most common method used for making a diagnosis of cancer and has been accepted as a reliable and accurate means in clinical diagnosis. The assumption that incisional biopsy may contribute to the spread of tumor cells is in dispute among scientists and health professionals.

Hellwing (1932) wrote that "the most serious objection to biopsy is the special danger pertaining to the incision into a tumor with stimulation of growth and/or dissemination of tumor cells through the blood or lymph vessels."

Miller (1946) believed that the possible hastening of metastasis by local irritation from a biopsy procedure should be considered to be only theoretical.

Dingman (1948) reported that biopsy if done with care need not be a matter of great concern as far as dissemination of tumor cells was concerned. He went on to say that it may be dangerous to manipulate and palpate the tumor unnecessarily during examination and that treatment should be instituted as early as possible following excision of tissue for biopsy.
Thoma (1949) advised the use of chemical or heat cauterization following the biopsy procedure to prevent the spread of cells via the lymph or blood streams.

Bernier (1950) stated that biopsy may disturb a quiescent tumor and that the dissemination of malignant cells is a theoretical possibility. He stressed, however, that this seldom occurs in practice. In his opinion this possibility is minimized by surgery or irradiation treatment immediately following the biopsy.

Bourgoyne (1954) emphasized that the occurrence and the severity of complications are slight when compared with the value of the knowledge obtained by the biopsy.

Boyle (1955) reported that palpation, massage and incision have been shown to favor early and widespread metastasis of highly malignant tumors in experimental animals. He also stated that cancer appears to be localized temporarily at the primary site by the surrounding normal tissue which acts as a natural barrier (granulation tissue of repair) and that surgical incision may destroy this barrier and thereby promote spread of the tumor. He advised that the operator should be aware of the potential dangers to the patient, that prompt treatment is of great importance and that biopsy should be performed only by qualified specialists.

Shira (1963) wrote about the dangers of biopsy, such as, implantation of tumor cells, formation of tumor embolism and disruption of the natural barrier provided by the surrounding host tissue. He stressed that the possibility of these complications should not interfere with an
indicated biopsy because the benefits of biopsy far outweigh any of the potential dangers.

It is generally believed (Tiecke, 1965, Kerr, et al. 1974 and Tyldesley, 1978) that the spreading of tumor cells along lymphatic and vascular channels following a biopsy procedure is a possibility but if this procedure is done with care, the spread of tumor cells will be minimized.

Rosai and Ackerman (1979) reported that if incisional biopsy is to be done, a definitive surgery must include the previous site of incisional biopsy.

2. Biopsy in Experimental Animals:

There have been relatively few previous experimental approaches to determine the effect of incision upon dissemination of tumor cells.

Lubarsch who was cited by Hellwing (1932) studied extensively what effect mechanical forces could exert on the rate of tumor growth. He inoculated tumor cells into mice and rats and subsequently traumatized the tumors with forceps. He did not observe any increase in the rate of growth of these tumors.

Tyzzer (1913) attempted to determine whether surgical operations increase or decrease the incidence of metastasis. He implanted tumors in Japanese Waltzing mice and showed that the incision of these implanted tumors did not increase the incidence of metastasis. However, he believed that metastasis may be artificially enhanced by vigorous manipulation and factors, such as, the biological characteristics of the tumor, the
duration of its growth, the size of the primary mass and any peculiar conditions furnished by the host tissues.

Nather who was cited by Hellwing (1932) implanted mouse carcinoma intramuscularly into 30 mice and later excised biopsies in one-half of the animals. Four days afterwards, he weighed the biopsied animals and found a 5% increase in their weights. He attributed the increase in body weight to tumor growth stimulated by the diagnostic incision.

Wood (1925) studied the incidence of metastasis after biopsy by inoculating 400 rats with Flexner rat carcinoma which normally metastasizes to the lungs. In one-half of the animals, biopsies were excised from the tumors and the other half served as controls. At the end of ten days, the tumors were excised from both groups to prevent further metastasis. The animals were then maintained for several months before autopsy. The percentage of metastasis to the lungs was practically the same in both groups. He concluded that when the biopsy procedure was carefully executed there was no increase in the incidence of metastasis of a Flexner carcinoma. Knox (1929) similarly studied transplanted Flexner-Jobling carcinomas in mice. He gently massaged the transplanted tumor in some of the animals for one to two minutes on two to three successive days and then surgically removed the tumors from all animals. His results, contrary to those of Wood, showed that the percentage of metastasis to the lungs was increased in those animals in which the tumors had been massaged.

Shklar (1968) used the hamster cheek pouch to study the effect of
incision and manipulation of a DMBA-induced carcinoma upon its growth. He concluded that a biopsy incision of three mm in length and one mm in depth together with massage and compression of the tumors with tissue forceps does not promote spread or increased rate of tumor growth. This study is the only one reported in the literature which investigated the effect of incisional biopsy on tumor growth and metastasis in the hamster pouch carcinoma model.
### TABLE I

Factors augmenting carcinogenesis in the hamster pouch

<table>
<thead>
<tr>
<th>Modifying factor</th>
<th>Investigator</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Systemic:</strong></td>
<td></td>
</tr>
<tr>
<td>Vitamin A deficiency</td>
<td>Rowe and Gorlin (1959)</td>
</tr>
<tr>
<td>Cortisone (S.C.)</td>
<td>Shklar (1966 and 1967)</td>
</tr>
<tr>
<td>Ethanol 30% (oral)</td>
<td>Henefer (1966)</td>
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<tr>
<td>Ethanol 10% (oral)</td>
<td>Freedman and Shklar (1978)</td>
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<tr>
<td>Sodium methotrexate (S.C.)</td>
<td>Shklar (1966)</td>
</tr>
<tr>
<td>Thiamine deficiency</td>
<td>Salley, et al. (1962)</td>
</tr>
<tr>
<td>&quot; &quot; (I.P.)</td>
<td>Giunta and Shklar (1971)</td>
</tr>
<tr>
<td>&quot; &quot; (I.P.)</td>
<td>Giunta, et al. (1974)</td>
</tr>
<tr>
<td><strong>Local:</strong></td>
<td></td>
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<tr>
<td>Acetone as DMBA solvent</td>
<td>Salley (1954)</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>Polliack and Levij (1967 and 1969)</td>
</tr>
<tr>
<td>Croton oil</td>
<td>Silberman and Shklar (1963)</td>
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<tr>
<td>Cortisone</td>
<td>Sabes, et al. (1963)</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>Elzay (1967)</td>
</tr>
<tr>
<td>Mineral oil as DMBA solvent</td>
<td>Salley (1955)</td>
</tr>
<tr>
<td>Sodium orthophosphate</td>
<td>Rubin and Levij (1975)</td>
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</table>

S.C. : subcutaneous  
I.M. : intramuscular  
I.P. : intraperitoneal
## TABLE II

Factors depressing carcinogenesis in the hamster pouch

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<td>Azathioprine (oral)</td>
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<td>Aspirin and Indomethacin (oral)</td>
<td>Perkins and Shklar (1982)</td>
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<td>Excess dietary zinc (oral)</td>
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<td>Levamisol (oral)</td>
<td>Shklar (1982)</td>
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<tr>
<td>Testosterone propionate (I.M.)</td>
<td>Poswillo and Cohen (1971)</td>
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<td>Edward (1976)</td>
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<td></td>
<td>Levij, et al. (1970)</td>
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<td></td>
<td>Eisenburg and Shklar (1977)</td>
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<td>Cottone, et al. (1979)</td>
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<td>Giunta and Shklar (1974)</td>
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<td>Polliack, et al. (1970)</td>
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<td>Chloroform as DMBA solvent</td>
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<td>Chlorpromazine</td>
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<td>Cortisone acetate</td>
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<td>Vitamin D$_2$, D$_3$</td>
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<td>Dimethyl sulfoxide</td>
<td>Sandres, et al. (1966)</td>
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<tr>
<td>&quot;</td>
<td>Siegel and Shklar (1969)</td>
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<tr>
<td>&quot;</td>
<td>Shklar, et al. (1969)</td>
</tr>
<tr>
<td>Dinitrochlorobenzene (skin contact)</td>
<td>Mohammad and Mincer (1976)</td>
</tr>
<tr>
<td>&quot;</td>
<td>Mohammad (1979)</td>
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<tr>
<td>Phenyl phosphate</td>
<td>Rubin and Levij (1975)</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>Levij, et al. (1970)</td>
</tr>
<tr>
<td>Irradiation</td>
<td>Shklar, et al. (1970)</td>
</tr>
<tr>
<td>Castration</td>
<td>Weatherred and SaIley (1964)</td>
</tr>
<tr>
<td></td>
<td>Levij, et al. (1969)</td>
</tr>
</tbody>
</table>

S.C. : subcutaneous
I.M. : intramuscular
CHAPTER III

STATEMENT OF THE OBJECTIVE

The objective of this study was to determine the possibility of seeding tumor cells to other sites, if such sites are incised with a scalpel contaminated with tumor cells from a chemically induced hamster buccal pouch carcinoma. Incisions were made at four different sites in the same animal. Later these sites were examined grossly and histologically to determine if seeding of tumor cells and/or tumor growth had occurred. The cervical lymph nodes were also examined for metastasis.
CHAPTER IV

MATERIALS AND METHODS

A. ANIMALS

A total of forty-nine male Syrian golden hamsters (Cricetus auratus) approximately three months of age and averaging 100 grams in weight, were used as experimental animals. The animals were housed four in a plastic cage with a stainless steel lid and were maintained in a temperature of 65-70°F. The animals were fed Purina Lab Chow and tap water ad libitum. After one week of acclimatization, carcinogenic painting was commenced.

B. TUMOR INDUCTION

The right pouches of forty animals were painted three times per week (Monday, Wednesday, and Friday) for fourteen weeks with a 0.5% solution of 9,10-dimethyl-1,2-benzanthracene (Sigma Chemical Co., St. Louis, Mo.) in heavy mineral oil (U.S.P.), using a #4 camel's hair brush (Fig. 1). The brush was dipped in the dimethyl-benzanthracene solution (DMBA), excess solution was allowed to drip off and then the brush was gently introduced into the pouch and rotated after the angle of the animal's mouth had been retracted by the thumb of the left hand which also held the animal steady (Fig. 2 and 3). The left pouches served as controls for the DMBA-treatment.

Clinical examination was done weekly on randomly selected animals to observe the alteration of the pouch mucosa associated with DMBA.
application and to compare these changes to untreated mucosa of the left pouch (Fig. 4). Both right and left pouches of the DMBA-treated animals were everted for examination using two blunt forceps. One forceps was used to retract the cheek and the other forceps was introduced into the pouch, the pouch base was grasped and then everted.

C. BIOPSY AND SEEDING PROCEDURES

After fourteen weeks of DMBA application, all treated animals (40) were examined clinically and the six animals with the largest tumors were operated on two days after the final day of DMBA application, and those animals which had smaller tumors at this time were operated on at later dates.

Tumors in the right pouches of the DMBA-treated animals were subjected to incisional biopsy and various sites were seeded in each animal with the same scalpels that were used to incise their tumors. Forty DMBA-treated animals and nine untreated (normal) animals were subjected to the seeding procedure. Immediately following each cut in the tumor, the scalpel was used to seed either a site in the same treated animal or in an untreated one. Between each cut the scalpel was rinsed in saline. By repeating these procedures incisions with a tumor-contaminated scalpel were made in the following four sites: (1) ventral surface of the tongue; (2) untreated pouch (left); (3) mucosa of lower lip; and, (4) muscle of the animal's back. The sequence of seeding was varied from animal to animal. Seeding in these sites was made in the following ways: (1) the tongue was gently lifted and stretched using a blunt forceps; a vertical
incision with the contaminated scalpel was made into the mucosa of the ventral surface and deep into the muscle; (2) the untreated pouch (left) was everted and stretched with two blunt forceps; an incision with the contaminated scalpel was made through the pouch mucosa; (3) the lower lip was stretched using two blunt forceps; a horizontal incision with the contaminated scalpel was made between the two labial frena and deep into the mucosa; and, (4) an incision was made in the back muscle by passing the contaminated scalpel 2-3 times through the prepared site (previously the animal had been shaved in this location and an incision was made through the skin). The latter incision was closed using 4-0 plain gut.

For each tumor chosen for biopsy a smear from the contaminated scalpel was made on a glass slide, stained and studied microscopically. After the biopsy and seeding procedures were completed for the six animals which had the largest tumors and for two untreated (normal) animals, three of these treated animals were randomly selected and were sacrificed by ether overdose at one, two and three hours subsequent to the surgery. The other animals were examined periodically for any gross tumors and finally were sacrificed after seven weeks and the seeding sites were excised and prepared for histologic study.

The rest of the animals (thirty-four DMBA-treated and seven untreated) were subjected to the above procedures during the subsequent three weeks. Thus a total of twenty DMBA-treated animals were surgurized and sacrificed within hours of the surgery, and twenty DMBA-treated and nine untreated (normal) animals were kept alive for long-term observation
for gross tumor growth at the seeding sites, and for later histologic
study.

After each animal was sacrificed the four incision sites were ex­
cised. In addition a longitudinal incision was made in the skin of the
neck and the skin was reflected. The submandibular and parotid salivary
glands along with the associated lymph nodes were excised in one mass and
prepared for histologic study.

D. PREPARATION OF THE HISTOLOGIC SECTIONS

The biopsy specimens, the excised surgical sites (pouch, tongue,
lower lip and the back muscle) and the lymph nodes were fixed in 10% form­
alin, embedded in paraffin, sectioned serially at 6 microns, stained with
Hematoxylin and Eosin (H&E) and examined using light microscopy. The
smears from the contaminated scalpels were made on glass slides, fixed
immediately in ether-alcohol and stained with Papanicolaou stain.

The prepared slides were examined under light-microscopy to study:
(1) the histology of the biopsies of the cheek pouch growths in order to
establish malignancy and degree of differentiation; (2) the presence or
absence of tumor cells in the seeded sites shortly after the incisions
were made (1, 2, and 3 hours); (3) the presence or absence of malignant
tumors in the various seeded sites in those animals which were not sacri­
ficed immediately following the biopsy and seeding procedures; (4)
determine if the tumors which were found in the seeded sites were contin­
uous with the overlying mucosa or were completely separate from the surface
mucosa; and, (5) the presence of metastasis in the regional lymph nodes.
FIGURE 1
Carcinogenic solution and #4 camel's hair brush.
FIGURE 2
Introduction of carcinogenic solution into pouch.

FIGURE 3
Rotation within pouch of brush containing carcinogenic solution.
FIGURE 4

Normal (untreated) pouch after eversion and stretching.
CHAPTER V

RESULTS

A. CARCINOGENESIS

1. Macroscopic Examination

Erythematous changes were observed as early as three days following the initial painting. The first three weeks of painting caused inflammatory processes which were recognized clinically as ulceration, edema and bleeding. These inflammatory responses peaked during the second and third weeks of painting and were observed mostly at the base of the treated pouches.

By the end of the third week of painting, the inflammatory response usually had subsided and the animals were not as aggressive as they were previously. The animal's calmness may have been due to the animals getting used to the painting procedure or the pain disappearing subsequent to the subsiding of the inflammatory response.

After four and one-half weeks of the DMBA application, the pouches of most animals were almost normal in appearance, but those of some animals still showed inflammation and in those animals it was very difficult to evert the pouches for examination. Possibly the walls of the pouch adhered to each other as a result of ulceration and fibrosis.

By the end of the fifth week, most of the animals showed whitening and thickening of the pouch mucosa. The earliest papillomatous lesions
were detected in three animals by the end of the sixth week as small, red and/or white overgrowths of the pouch epithelium.

By the end of 8 weeks most of the animals had papillomatous lesions ranging in size from 1-3 mm in diameter. These papillomatous lesions were scattered over the pouch mucosa--- some were at the pouch orifice, others at the pouch base and still others on the lateral and medial walls of the pouch mucosa. These lesions had a verrucous appearance, occurred as sessile and/or pedunculated tumors and bled easily when handled.

Examination of the painted pouches on the tenth week showed that all forty animals had tumors except one which showed only a white, thickened pouch without any outgrowth of the pouch mucosa. In three animals, the tumors were fixed to the pouch base which made the procedure of evert­ing the pouch difficult. Most of the small papillomatous growths detected earlier became large, necrotic and had an unpleasant smell.

From the end of the tenth week to the end of the fourteenth week, the tumors became larger (Figure 5 and 6), the animals became weaker and most of the animals developed skin tumors over the painted pouches.

The animals which were not sacrificed immediately after the biopsy and seeding procedures seemed to recover well during the first days, but later on they showed gradual deterioration of their health. The neo­plasms in their pouches rapidly increased in size during the first week after the biopsy and seeding procedures and they continued to increase in size until some animals had difficulty keeping their heads in an upright position. These tumors made the animal look like its right pouch was
filled with food. Palpation of the swollen pouches revealed the hard consistency of neoplasms. The procedure of everting the cancerous pouches was not easy because the mucosa of the pouches could not be held by the forceps. If the pouch could be everted it was seen to be necrotic and ulcerated, bled easily and pieces of the pouch mucosa tended to slough off.

2. Microscopic Examination

Figure 7 represents a cross-section of normal pouch wall which consists of several layers. The epithelial lining is a stratified squamous type similar to that lining the oral cavity proper which is 2-4 cells in thickness. It has some keratinization on the surface and is devoid of rete ridges and epithelial accessory structures. There is a thin layer of rather dense connective tissue lacking glands and other accessory structures under the surface epithelium. A layer of longitudinal striated muscle fibers and an outer layer of loose fibrous connective tissue, are noted next.

Figure 8 is a section of the excised biopsy from the pouch tumor which displays reactive and neoplastic changes. Reactive changes were in the form of hyperkeratosis, acanthosis where the prickle cell layer was more than ten cells thick and hyperplasia of the basal layer. Neoplastic changes such as downward growth of the basal cell layer which was either in the form of long and thin rete ridges or bosslated ones. The basal and suprabasal cells stained deeply basophilic with numerous mitotic figures. Malignant changes included a loss of polarity of the basal cell
layer, complete disappearance of the basement membrane in some areas, and cells displayed one or more of the following: pleomorphism, alteration of the nuclear-cytoplasmic ratio, hyperchromatism, prominent nucleoli, irregular nuclei with sharp edges, uneven distribution of chromatin material within the nuclei, disappearance of cytoplasm in some cells and bizarre mitotic figures. These cellular atypicalities were not confined to the surface but were also detected in the underlying connective tissue where neoplastic epithelial cells were arranged in nests, cords and sheets with ortho- and para-keratin production. A mononuclear cell infiltrate could be seen around the neoplastic epithelium. T-lymphocytes were assumed to be one of the constituents of this mononuclear infiltrate which would indicate that the animals had responded immunologically to the neoplastic changes. There were also numerous sinusoidal spaces and edema of the connective tissue.

B. TRANSFER OF TUMOR CELLS

1. Cytological Smears

Examination of the freshly collected cytological smears which were made from the tumor-contaminated scalpels that were used to excise the biopsy specimens from the induced tumors in the cheek pouches revealed that all the smears contained histological evidence of malignant epithelial cells (Figure 9). The diagnosis of squamous cell carcinoma was established on the basis of cellular abnormalities commonly associated with this type of tumor. The morphological features of the cellular abnormalities ranged from minor atypia to cells which were so atypical
that they could not be identified as epithelial in origin.

The changes within these cells were mainly in the nuclei and included alterations in the nuclear-cytoplasmic ratio which was probably due to an increase in the size of the nucleus, condensations of nuclear chromatin into aggregates which were unevenly distributed either in the center of the nucleus or at the nuclear border, irregularities of the nuclear border with sharp angles and indentations, enlarged and numerous nucleoli, anisonucleosis, hyperchromatism, eccentrically located nucleus, and densely packed nuclei with little or no cytoplasm.

Cornification was common; the cytoplasm of the cornified cells stained eosinophilic and the nuclei had different shapes and stained deeply basophilic. The cytoplasm and the nuclei of other cells stained basophilic. The differences in staining was related to the origin of the cells either from the surface or from the basal layer.

Besides neoplastic cells, normal cells were also recognized in the smears as clusters of squamous cells that demonstrated cohesiveness and normal nuclear-cytoplasmic ratios or single squamous cells with their nuclei either fragmented or whole with homogenous chromatin distribution, smooth nuclear borders and a centrally located nucleus.

2. Clinical and Histological Examinations of the Seeding Sites:

Clinical Examination:

Examination of the seeding sites (tongue, lower lip, left normal pouch and muscles of the animal's back) was done weekly and revealed that in most of the animals these sites were swollen with pus accumulation
Whereas the others showed only redness around these incision sites. The infection of the seeding sites gradually subsided.

Single, small, red or white papillomas were observed in the seeded normal pouches in only 7 out of 17 animals (41%). The seeding sites had completely healed and could not be detected clinically, but these papillomatous growths in the normal pouches presumably arose at the seeding sites. These small papillomas were seen only in the DMBA-treated animals.

In the untreated seeded animals, all the seeded sites showed some inflammation but the incisions healed rapidly and there were no gross lesions. These animals remained in good health until they were sacrificed at the end of the experiment.

**Histological Examination**

Neoplastic cells could be identified histologically at the seeding sites in many of the animals which were sacrificed within hours after the biopsy and seeding procedure (Figures 10-A to 13-B). In other animals an unidentified basophilic material (possibly necrotic tumor tissue) was seen. In still other animals neither tumor cells or basophilic material was observed. Table III summarizes the results of cellular transfer to the seeded sites.

In the animals which either succumbed or were sacrificed during the seven weeks subsequent to the biopsy and seeding procedures, serial sections of the seeded sites were examined histologically and revealed neoplastic growths in the tongue, lip and left pouch. In the tongue and lower lip, these lesions were not continuous with the overlying surface.
mucosa (Figures 14-A, 14-B and 15), but were seen as nests of neoplastic epithelium within the muscular structure of the tongue and the lamina propria of the lip and were growing inward rather than outward. In the left pouch, the diagnosed exophytic lesions were continuous with the surface mucosa. In the skeletal muscle of the animal's back, there was no indication of neoplastic growth and the examined sections showed only histiocytic reaction and muscle regeneration. Table IV displays the results of tumor growth at the various seeded sites.

In the untreated seeded animals, the histologic study of the seeded sites revealed no indication of tumor growth. Table V displays these negative results.

C. HISTOLOGIC EXAMINATION OF THE CERVICAL LYMPH NODES

In this study, nine out of seventeen animals (53%) which were kept alive after the biopsy and seeding procedures showed metastasis to the lymph nodes associated with parotid and submandibular salivary glands. In two of these nine animals with metastasis, gross metastatic lesions were seen clinically after reflection of the neck skin on the right side. The histology of the diagnosed metastatic lesions showed squamous cell carcinoma which was well-differentiated in some animals but highly anaplastic in others (Figures 16-A to 17-B). Table VI summarizes the results of the histologic examination of the lymph node.
TABLE III

Transfer of tumor cells from the pouch tumor to the seeded sites in the animals which were sacrificed within hours of the seeding procedure.

<table>
<thead>
<tr>
<th>Animal #</th>
<th>Tongue</th>
<th>Lower lip</th>
<th>Left pouch</th>
<th>Back muscle</th>
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<tr>
<td>1</td>
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<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
<td>?</td>
<td>?</td>
<td>-</td>
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<tr>
<td>%</td>
<td>47%</td>
<td>21%</td>
<td>32%</td>
<td>21%</td>
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</table>

+ : Transferred tumor cells were observed in the seeding sites.
- : No transferred tumor cells at the seeding sites.
? : Unidentified basophilic material at the seeding sites.
* : Animal died during the anesthesia.
** : Technical problem (fixation and/or sectioning).
**TABLE IV**

Tumor growth in the seeded sites of the DMBA-treated animals

<table>
<thead>
<tr>
<th>Animal #</th>
<th>Tongue</th>
<th>Lower lip</th>
<th>Left pouch</th>
<th>Back muscle</th>
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<tbody>
<tr>
<td>21</td>
<td>+</td>
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+: Tumor growth at the seeding sites.
-: No tumor growth observed at the seeding sites.
*: Animal died and no tissues were taken for histologic study.
# TABLE V

Tumor growth in the seeded sites of the untreated animals

<table>
<thead>
<tr>
<th>Animal #</th>
<th>Tongue</th>
<th>Lower lip</th>
<th>Left pouch</th>
<th>Back muscle</th>
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- : No tumor growth observed at the seeding sites.
### TABLE VI

Metastasis to the cervical lymph nodes in the DMBA-treated and untreated, seeded animals

<table>
<thead>
<tr>
<th>DMBA-treated animals</th>
<th>DMBA-treated animals</th>
<th>Untreated animals</th>
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</thead>
<tbody>
<tr>
<td>Animals sacrificed within hours after surgery.</td>
<td>Animals living for 3-7 weeks after surgery.</td>
<td>Normal seeded animals</td>
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<tr>
<td>Animal #</td>
<td>Metastasis</td>
<td>Animal #</td>
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+ : Metastasis to the regional lymph nodes.
- : No metastases were observed to the regional lymph nodes.
* : Animal died and no tissues were taken for histologic study.
FIGURE 5

Tumor mass within pouch treated with DMBA for 13 weeks.

FIGURE 6

Multiple tumor growths in the everted pouch treated with DMBA for 13 weeks.
FIGURE 7
Photomicrograph of normal hamster cheek pouch showing stratum corneum, stratum spinosum, stratum germinativum, lamina propria, and muscular layer (Hematoxylin and eosin stain---magnification X144).

FIGURE 8
Photomicrograph of excised biopsy from the pouch tumor demonstrating hyperkeratosis, malignant transformation of the pouch epithelium and invasion of the underlying lamina propria (Hematoxylin and eosin---magnification X58).
FIGURE 9

Cytological specimen from tumor-contaminated scalpel. Note the cells from the epidermoid carcinoma of the pouch. Nuclei are enlarged, have abnormal chromatin distribution and show alteration of nuclear/cytoplasmic ratio and hyperchromatism (Papanicoiaou stain—magnification X360).
FIGURE 10-A

Photomicrograph of the incision site in the ventral surface of the tongue within hours following the biopsy and seeding procedures. Note the mass of tumor cells at the seeding site (arrow) which were transferred by the tumor-contaminated scalpel (Hematoxylin and eosin stain---magnification X144).

FIGURE 10-B

Higher magnification of the transferred tumor cells seen in Figure 10-A. The nuclei are hyperchromatic and of variable shapes and sizes (Hematoxylin and eosin stain---magnification X1134).
FIGURE 11-A
Photomicrograph of the incision site in the mucosal surface of the lower lip within hours following the biopsy and seeding procedures. Note the mass of seeded epithelial cells (arrow) from the pouch tumor, single epithelial cells and unidentified, darkly stained material (Hematoxylin and eosin stain---magnification X144).

FIGURE 11-B
Higher magnification of the epithelial mass seen in Figure 11-A (Hematoxylin and eosin stain---magnification X360).
FIGURE 12-A

Photomicrograph of the incision site in the untreated (normal) pouch within hours following the biopsy and seeding procedures. Note a keratin mass at the seeding site (arrow), which had been transferred by the tumor-contaminated scalpel (Hematoxylin and eosin stain—magnification X144).

FIGURE 12-B

Higher magnification of the keratin mass seen in Figure 12-A. Darkly stained nuclei can be seen in the center of this mass (Hematoxylin and eosin stain—magnification X360).
FIGURE 13-A

Photomicrograph of the incision site in the skin and the underlying muscle of the animal's back within hours following the biopsy and seeding procedures. Note a mass of epithelial cells at the incision site (arrow) which had been transferred by the tumor-contaminated scalpel (Hematoxylin and eosin stain---magnification X58).

FIGURE 13-B

Higher magnification of the epithelial mass seen in Figure 13-A (Hematoxylin and eosin stain---magnification X360).
FIGURE 14-A
Photomicrograph of the ventral surface of the tongue five weeks subsequent to the biopsy and seeding procedures. Visible in this section is a tumor mass which is not continuous with the overlying mucosa as substantiated by a study of serial sections (Hematoxylin and eosin stain---magnification X58).

FIGURE 14-B
Higher magnification of the tumor mass seen in Figure 14-A. Note the dysplasia of the epithelial cells (Hematoxylin and eosin stain---magnification X144).
FIGURE 15

Photomicrograph of the lower lip four weeks subsequent to the biopsy and seeding procedures. Visible in this section is a tumor mass within the lamina propria. Study of serial sections revealed no connection of the tumor with surface epithelium (Hematoxylin and eosin stain---magnification X144).
FIGURE 16-A

Metastatic lesion of well-differentiated squamous cell carcinoma in a cervical lymph node five weeks subsequent to the biopsy and seeding procedures (Hematoxylin and eosin stain---magnification X144).

FIGURE 16-B

Higher magnification of the metastatic lesion seen in Figure 16-A (Hematoxylin and eosin stain---magnification X360).
FIGURE 17-A
Metastatic lesion of highly anaplastic type of squamous cell carcinoma in cervical lymph node six weeks subsequent to the biopsy and seeding procedures. Much of the nodal architecture was replaced by the metastatic tumor (Hematoxylin and eosin stain---magnification X144).

FIGURE 17-B
Higher magnification of the metastatic lesion seen in Figure 17-A, showing lack of differentiation (Hematoxylin and eosin stain---magnification X360).
CHAPTER VI

DISCUSSION

The potential value of the hamster as an experimental model in dental research was suggested by Arnold in 1942 and this animal continues to serve well for a variety of experiments. Of particular significance has been its use to study the biology of oral cancers following the successful attempts by Salley (1954) to induce buccal pouch carcinoma. However, researchers such as Kolas (1955), Stormby and Wallenius (1964), Walker, et al. (1970) and Williams, et al. (1971) criticized the use of cheek pouch carcinoma model in studies of intra-oral carcinogenesis because the cheek pouch could not be considered representative of the oral cavity proper as it is not subjected to the same environmental influences as the rest of the mouth. In addition the cheek pouch is anatomically and histologically different from other areas of the oral cavity---the pouch mucosa has a unique submucosal connective tissue and the pouch mucosa is a site of immunologic privilege and will accept heterografts of both normal and neoplastic tissues.

In spite of the criticism of this model, hamsters continue to be used to study many questions about human oral cancer. In this experiment, the question of possible dissemination of tumor cells subsequent to incisional biopsy of hamster buccal pouch carcinoma was studied. Shklar (1968) investigated the same question and reported that biopsy
incision and physical manipulation of the tumor did not appear to contribute to the spread of tumors either in the local region or to remote organs. Shklar's negative results could be related to the following: all the incisions were made after only 12 weeks of DMBA treatment yet 50% of the pouches had no clinically apparent tumors by that time. He did not take biopsies and so it is very probable that the incisions in many of the animals were not made through malignant tissue at all. This is likely because it is believed that many exophytic lesions induced by 12 weeks of DMBA treatment are only benign papillomas. In addition, Shklar's 1 mm depth of incision through the exophytic lesion (even if these exophytic lesions were malignant) would be insufficient depth to transfer cells into sublesional normal tissue.

In this study, the animals were painted with DMBA for a longer period, 14 weeks; biopsies were taken from selected lesions (red lesions) in each case and studied histologically with the indication that all the excised biopsies were malignant; and, the time of biopsy and seeding was dictated by the size of the tumor rather than a set period after the commencement of DMBA application. Before the biopsy and the seeding procedures the animals were divided into groups according to the size of their tumors. Assuming that larger tumors are more aggressive than smaller ones, the animals with larger tumors were operated upon first.

In this experiment, the scalpel which was used to excise biopsy tissue from the hamster pouch carcinoma did carry neoplastic cells to the selected seeding sites (Table III) and to the glass slides (Figure 9).
All forty smears made from the contaminated scalpels displayed viable appearing neoplastic cells. The most prominent histologic features of these cells were the change in the nuclear-cytoplasmic ratio and hyperchromatism. Incisional biopsy presents a danger not only of an increased tumor spread at the primary site, but also the increased likelihood of tumor spread to the other tissues as indicated by the presence of metastatic lesions in the cervical lymph nodes.

Transplantation of hamster cheek-pouch carcinoma cells to other organs of the same species was studied by Merk, et al. (1979) and Meng, et al. (1982). Their results showed that buccal pouch carcinoma could be serially transplanted through five generations. They were also able to maintain a stable anaplastic carcinoma which could be transplanted into non-immunosuppressed animals. The transplanted carcinoma could also metastasize. In the present study neoplastic cells from the pouch carcinoma were transferred to other sites of the same animal with the contaminated scalpel that was used to excise the biopsy specimens. This conclusion is based on the following observations:

(1) Neoplastic cells could be identified histologically at the seeding sites in many of the animals which were sacrificed immediately after the biopsy and seeding procedures. In some animals, however, there was no apparent transfer of cells. The negative results could have been due to the fact that the neoplastic cells remaining on the tissue surface instead of being seeded deep into the tissues and subsequently being washed away.
(2) In those animals which were not sacrificed immediately after the biopsy and seeding procedure, gross lesions were noticed in the left normal pouch as small papillomas of red and/or white color. However, a solid conclusion cannot be drawn that these papillomatous lesions were a result of seeding neoplastic cells. Floating neoplastic cells from the carcinomatous pouch could have been carried with food or saliva to the left normal pouch or some of the carcinogenic chemical applied to the right pouch could have reached the normal pouch and caused these lesions.

(3) Also in those animals which were not immediately sacrificed after the biopsy and seeding procedures, microscopic examinations of the contaminated sites revealed neoplastic lesions in the tongue, lower lip and the untreated pouch. The back muscles, however, showed neither macroscopic nor microscopic growth of transferred neoplastic cells. The neoplastic changes in the ventral surface of the tongue and the mucosal surface of the lower lip were associated with other reactive changes of the surface mucosa of these two organs. These neoplastic changes are believed to be the direct result of the seeding procedure. All the neoplastic changes in the tongue were seen only on the ventral surface of the tongue and were usually prominent at one area which was assumed to be the site of the seeding, although some reactive changes could be seen in other areas of the ventral mucosa of tongue as well. Histologically these neoplastic changes were seen either as epithelial nests which manifested carcinomatous changes within the tongue structure and were not connected to the surface mucosa, or as hyperkeratosis, acanthosis and mild
atypicality of the epithelial cells at a definite area of the surface mucosa.

Also in support of the seeding possibility, it should be noted that the ventral surface of the tongue is highly resistant to the action of chemical carcinogens. Eveson and MacDonald (1977) were unable to induce carcinoma of the ventral surface of the hamster tongue by directly applying a water-soluble carcinogen, 4-nitro quinoline N-oxide (4NQO) even though Wallenius and Lekholm (1973) by painting the palatal mucosa of rats three times a week with the same solution had been able to produce carcinoma of the palate in 100% of the animals and carcinoma of dorsal surface of the tongue in 75% of the animals. It took Fujita, et al. (1973) seven weeks to produce small nodular or papillary growths of the tongue mucosa and thirteen to twenty-five weeks to produce grossly carcinomatous lesions; they used DMBA dissolved in acetone and applied the carcinogen and traumatized the tongue three times each week. They also reported that only four out of fifteen animals developed squamous cell carcinoma by the twenty-seventh week if trauma was not combined with the application of the carcinogen. Marefat and Shklar (1977) reported that painting the hamster tongue with DMBA in mineral oil subsequent to trauma with a barbed broach produced hyperkeratosis after twelve to thirteen weeks and hyperkeratosis with dysplasia after fifteen to sixteen weeks. In one animal the changes were sufficiently severe, so that the diagnosis of carcinoma in situ could be made. Unfortunately nothing was mentioned about the effects, if any, if the DMBA was applied without trauma. Eveson and
MacDonald (1981) first noticed a gross tumor of the ventral lingual mucosa during the tenth week of treatment with 0.5% DMBA in acetone solution. The animals were anesthetized and lightly scratched with a barbed broach before DMBA application. However, in our study although the ventral surface of the tongue was not painted with the carcinogen and the tongue was not traumatized, six out of seventeen animals showed malignant changes in the ventral side of the tongue subsequent to contamination with scalpel that was used to excise the biopsy specimen from the pouch carcinoma. In this study also, the duration of the experiment (23 weeks) was comparable to that of Fujita, et al. (1973), and to that of Eveson and MacDonald (1981).

It is also important to mention as support of the seeding possibility that the animals at the time of the biopsy and seeding procedure which followed fourteen weeks of DMBA application, had large tumors, were debilitated and likely suffered a significant loss of immunological competence. The transferred neoplastic cells would then have a greater chance to proliferate in a less hostile environment. The suggestion that the DMBA-treated animals were probably immunologically compromised due to the induced tumors and the surgical procedures is supported by the observation that the non-treated seeded animals showed no growth or any other indication of neoplastic cells in the seeding sites. It is assumed that the latter animals were immunologically competent and thus were able to destroy the transferred foreign cells before they were able to proliferate. Shklar (1966 and 1967) stated that systemically administered cortisone
appeared to augment the action of DMBA, so that more rapid neoplastic transformation ensued with the carcinomas tending to be much larger and more invasive. Woods (1969) reported a short latent period of tumor induction and an increased total number of tumors when hamsters were treated with antilymphocyte serum. Scully (1982) reported that patients who were given immuno-suppressing drugs in order to inhibit rejection of organ grafts have an increased risk of malignancy of eighty times that of matched controls.

In this study the fact that no tumor growth occurred in some of the treated animals could have been due to drying of the transferred cells during transfer to the seeding sites. Also some animals may have been immunologically more competent than others and thus able to destroy the transferred cells. Local infection may also have played a role.

In this experiment, besides what is believed to be seeding of transferred neoplastic cells in the tongue, there were reactive changes of the ventral surface of the tongue seen as hyperkeratosis, acanthosis and slight cellular atypicality. These reactive changes may have been due to the technique used in this experiment to apply the carcinogen to the treated pouches which allowed some of the DMBA to escape into the oral cavity proper. This suggestion is in agreement with Salley (1954) who noted carcinomatous changes on the palate, tongue as well as esophagus and forestomach of hamsters which he had treated with DMBA in acetone to induce pouch carcinoma. Fujita, et al. (1973) also reported that when the carcinogen was applied to the ventral surface and to the tip of the
tongue, carcinomas also developed elsewhere in the oral cavity. In this study, carcinomatous changes were seen only in the tongue, lower lip, left untreated pouch (seeding sites) and the skin over the painted pouch. No gross tumors were detected in the floor of the mouth. However, Eveson and MacDonald (1981) reported that twelve out of eighteen animals developed tumors in the floor of the mouth (some were gross) when they studied the induction of tongue carcinoma subsequent to three times weekly application of 0.5% DMBA in acetone with a #3 camel's hair brush. They suggest that the carcinogen may pool in the floor of the mouth before being swallowed and/or that the mucosa of the floor of the mouth is especially sensitive to chemical carcinogens.

In this study, the back muscle was also contaminated, but there were neither gross nor histologic indications of tumor growth. This negative result could have been due to the fact that the back muscle was a hostile environment for the transferred cells or the back muscle had not come in contact with the chemical carcinogen as the other contaminated sites might have.

node metastasis subsequent to the induction of buccal pouch carcinoma—two are already reported in the literature and the third is the result of the present study. Salley (1954) stated that eighteen out of nineteen animals developed lymph node metastasis. He used acetone and benzene as vehicles for DMBA; however, when he used liquid paraffin as solvent, no mention was made of lymph node metastasis (1955). Rwomushana, et al. (1970) reported that one case of lymph node metastasis in a hamster with DMBA-induced cheek pouch carcinoma was observed during a study of the effect of a locally applied cytostatic drug, vinblastine in liquid paraffin, on the hamster pouch carcinoma. This case was the only one observed out of 562 carcinogen-treated hamsters. In the present experiment, nine out of seventeen animals showed metastasis to the lymph nodes. In two of these nine animals with metastasis, a gross metastatic lesion in the neck could be seen after skin reflection. Histologically the lymph nodes of these nine animals showed squamous cell carcinoma, highly differentiated in some animals and anaplastic in others.

The difference between the results of this study and what is reported in the literature concerning the lymph node metastasis could be related to the following: the metastases in this study could have been the result of incisional biopsy upon the cheek pouch carcinoma or a longer tumor-bearing period. The latter suggestion is in agreement with Craig who stated through personal communication with Eveson (1981) that cervical lymph node metastasis could be produced by extending the duration of the tumor-bearing period. Creasey (1981) on the other hand
stated that many soft tissue tumors became immobilized by biopsy-induced fibrosis if there is a delay of five days or more between the biopsy and the surgical removal of the tumor. In this study, one animal had metastasis four weeks following cessation of DMBA application; in the others it took five to eight weeks. In the literature, animals lived for similar periods, but no metastases were reported (Renstrup, et al. 1962, and Shklar, 1966 and 1968). The trauma of the surgical procedures (biopsy and seeding) and the induced tumors may have lowered the animal's resistance to metastasis of the tumor to the regional lymph nodes.

Further studies will be needed to: (1) elucidate with certainty that the neoplastic changes which were seen in the seeded sites were the direct result of growth of transferred neoplastic cells; (2) determine to what extent carcinogen escapes from the pouch during the painting procedure and causes neoplastic changes elsewhere within the oral cavity proper; and (3) determine if incisional biopsy increases the spread of the tumor to either the regional lymph nodes or internal organs.
SUMMARY AND CONCLUSIONS

Forty-nine male Syrian hamsters, three months of age were used to study the effect of incisional biopsy upon the process of chemical carcinogenesis of the buccal pouch. Forty animals had the right buccal pouch painted three times weekly with a 0.5% solution of 9, 10-dimethyl-1,2-benzanthracene in heavy mineral oil. Twenty animals were randomly selected and were sacrificed immediately following the biopsy and seeding procedures. The rest of the animals either succumbed or were sacrificed during the following seven weeks.

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

(1) The scalpel which was used to excise tissue from the pouch carcinoma did carry neoplastic cells to the glass slides from each tumor chosen for biopsy.

(2) Neoplastic cells were identified histologically at the seeding sites (tongue 47%, lower lip 21%, left pouch 32% and the skeletal muscle of the animal's back 21%) in the animals which were sacrificed immediately after the biopsy and seeding procedures.

(3) Microscopic examinations of the seeded sites in the DMBA-treated animals which either succumbed or were sacrificed
during the seven weeks following the biopsy and seeding procedures revealed neoplastic lesions in the tongue (36%), lower lip (41%), and left pouch (41%), but not skeletal muscle (0%).

(4) The seeded, untreated animals showed no indication of neoplastic growths at any of the seeded sites.

(5) Microscopic examination of the cervical lymph nodes in animals which lived 3-7 weeks subsequent to the biopsy and seeding procedures revealed metastatic lesions in nine out of seventeen animals (53%).

Biopsy is a mandatory procedure in establishing the diagnosis of oral lesions and in clinical practice, generally, early carcinomatous lesions, erythroplakia and carcinoma in situ, are not followed by post-operative radiation. In such cases malignant cells which might have been transferred to normal tissue during the incisional biopsy procedure could act as seeds for new tumor growth.

It is advisable, where possible to do a complete excisional biopsy of these early lesions and thus avoid seeding the surrounding normal tissue with the contaminated blade. In cases where an incisional biopsy must be done (large size of lesion) a total surgical excision of the oral tumor should be done as soon as possible after the biopsy and the surgery must include the site of the biopsy incision.
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APPROVAL SHEET

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

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Oral Biology.

July 22, 1983

Directors Signature