BIOLOGY OF THE NEOPLASTIC CELLS OF ETHMOID CARCINOMA

By

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THESIS

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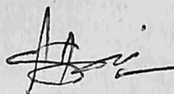
DECLARATION

I hereby declare that this thesis entitled "BIOLOGY OF THE NEOPLASTIC CELLS OF ETHMOID CARCINOMA" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.



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CERTIFICATE

Certified that this thesis, entitled "BIOLOGY OF THE NEOPLASTIC CELLS OF ETHMOID CARCINOMA" is a record of research work done independently by Sri. Madan Singh Karki under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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DEDICATED

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LATE PARENTS

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Introduction

INTRODUCTION

Carcinoma of the mucosa of the ethmoid is an important neoplastic condition affecting different species of domestic animals. The prevalence of this tumour was first reported by Moussu (1906) from Sweden and subsequently by Stenstrom (1915). Since then the incidence of the tumour has been reported from different parts of the world, although, the tumour is no longer encountered in Scandinavian countries.

From India carcinoma of the mucosa of the ethmoid was first reported in cattle by Muthappa in 1930 from Madras State. Subsequently the prevalence of this tumour has been reported from Kerala, Tamil Nadu, Andhra Pradesh, Karnataka and Orissa. In recent years, there has been considerable increase in the incidence of neoplasms arising from the mucosa of the ethmo-turbinate region. The high incidence of this tumour and its occurrence in different species of domestic animals and occasionally in captive wild animals have caused considerable interest among scientists engaged in research in the field of oncology. Their observations ruled out the possibility of any breed predisposition in the occurrence of the tumour and also threw light on the fact that there is no species barrier for this tumour. The published reports have shown that females at the peak of

their production are more affected and particularly they show the symptoms when they are in the first or second trimester of pregnancy. These are points which would highlight the economic importance of the tumour.

In Kerala, since the first record of the tumour in 1960, there has been steady increase in the incidence of the tumour. Now the tumour has established itself in an endemic form in the State of Kerala and is encountered in cattle, goats, pigs and occasionally in wild animals like the deer.

During the last seven years investigations were undertaken in a systematic manner in the department of Pathology to understand various aspects of this tumour. The symptomatology was chalked out, early diagnostic features were formulated and the immunological background of the tumour bearing animals was monitored. Efforts were made to identify the aetiology and to formulate effective therapeutic measures. The work is being continued.

An understanding of the nature and behaviour of the neoplastic cell is very essential to understand the mechanism of cancer development and to chalk out an effective strategy to control the unmanageable proliferative character of the cancer cell. Cell biology study is now being widely used as a tool to understand the nature of the neoplastic process in oncology. _In vitro_ cultivation of the cancer

cell and reproduction of neoplastic state in experimental and natural hosts are the two major widely accepted methods involved in cell biology studies.

The advancements made in tissue culture techniques have greatly helped to study the biology of the individual neoplastic cell. The cell culture studies and tumour transplantation studies have been considered as effective tools to discover the biological process which induce and maintain the cancer state and to help in identifying means of controlling the neoplastic state once it has begun.

In the field of oncology it is the endeavour of the scientist to grow the tumour cell in artificial media and to establish a cell line. This would not only help to assess the biological behaviour of the tumour cell but also gives an opportunity to clarify the effect of various chemotherapeutic agents on the cancer cells. For formulating an effective therapeutic schedule the information gained by these studies will be of utmost value.

The growth of the tumour cell in the artificial media alone will not speak of the behaviour of the tumour cell in the host system. All over the world, oncologists have been trying to transplant the tumour in the homologous and heterologous hosts. Conditioned and unconditioned host systems have been extensively employed to achieve this objective and

the results have been variable with different types of tumours.

No systematic efforts have been made to culture the ethmoid carcinoma cells in artificial media and to transplant them in homologous and heterologous host systems. So far results obtained by limited studies carried out by scientists at different places have not been very satisfactory. An attempt, was therefore, undertaken to culture the ethmoid carcinoma cells in artificial media and to transplant the tumour cells in autologous, homologous and heterologous hosts.

Review of Literature

REVIEW OF LITERATURE

1. Incidence and Epidemiology

The tumour of the ethmoturbinate mucosa was known to exist in cattle during the beginning of this century. Moussu (1906) reported the incidence in two cows from Sweden. Subsequently detailed reports appeared on the clinical and pathological features (Bergman, 1914 and Stenstrom, 1915). Muthappa (1930) reported the occurrence and symptomatology of the tumours of the nasal cavity in bovines in India. David and Venkatraman (1940) reported the occurrence of malignant growths in the frontal sinus of cattle. In a survey study, Nair and Sastry (1954) recorded 18 cases of ethmoid tumour out of total 2003 cases of neoplasm in cattle and buffaloes encountered by them in Madras State. Cotchin (1956), in his review of the neoplasms of the domestic animals cited 160 reports of ethmoid cancer in cattle and 24 in horses in Sweden. Narayana (1960) reported a case of tumour of the nasal passage and frontal sinus in an eight year old Ongole breeding bull. Subsequently the incidence, epidemiology, clinical and pathological features of ethmoid neoplasia were described in cattle, buffaloes, pigs, goats, dogs and captive deer by different workers (Rajan et al., 1972; Becker et al., 1972; Tokarnia et al., 1972; Nair, 1973; Damodaran et al., 1974; Balasubramaniyam, 1975; Legendre et al., 1975; Brownstein et al., 1975; Stunzi

and Hauser, 1976; Madowell *et al*., 1976; Brown *et al*., 1977; Njoku *et al*., 1978; Prasad and Kohli, 1978; Jayaraman *et al*., 1979; Rajan *et al*., 1981; Rajan and Sulochana, 1982; Howard *et al*., 1982; Chaudhary and Rao, 1982; Pruthi *et al*., 1982; Rameshmurthy, 1984; Kornel *et al*., 1984; and Rings and Rojko, 1985).

Sreekumaran and Rajan (1983a) observed that the tumour has established itself in an endemic form in Kerala. They recorded high incidence in cross-bred Jersey cattle particularly in the age group of 7-10 year. The cases were recorded from all districts in the State and there was no significant difference in the distribution of incidence between districts. Similarly Rameshmurthy (1984) found this tumour in Jersey cattle in the age group of 4-11 years and Kornel *et al*. (1984) reported the incidence in a pure-bred herd of Jersey cattle. The affected animals were mostly in the age group of 5-11 years.

2. Symptomatology

Initial clinical features manifested by the tumour bearing animals were profuse nasal discharge, sometimes blood tinged, dyspnoea and unilateral or bilateral exophthalmos (Moussu, 1906; Stenstrom, 1915; Muthappa, 1930; David and Venkatraman, 1940; Narayana, 1960; Tokarnia *et al*., 1972 and Jose *et al*., 1985). Snoring and abdominal type of respiration were found in advanced cases (Rajan *et al*., 1972;

Nair, 1973; Damodaran *et al.*, 1974; Balasubramaniyam, 1975; Njoku *et al.*, 1978; Jayaraman *et al.*, 1979). Other manifestations noted were circling movement, cachexia, perforation of the frontal bone and bulging of fore-head (Nayak *et al.*, 1979; Pospischil *et al.*, 1979; Sreekumaran, 1980; Rajan *et al.*, 1981; Pruthi *et al.*, 1982) and swelling of the sub maxillary lymph nodes (Kornel *et al.*, 1984).

3. Clinical Pathology

3.1. Haematology

Nair (1973) carried out haematological studies on 25 animals bearing neoplasms of the ethmoid mucosa. He found anaemia, moderate leucocytosis and marked eosinophilia. Sreekumaran (1980) described anaemia and slight to moderate leucocytosis with occasional neutrophilia and moderate lymphocytosis in all the cases.

3.2. Serum proteins

Sreekumaran and Rajan (1982b) found decrease in albumin percentage and reduction in albumin globulin ratio associated with increase in gamma globulin and alpha-1 globulin in tumour bearing animals.

3.3. Calcium and phosphorus ratio (Ca:P)

A relative increase in serum phosphorus with low calcium level was detected by Sreekumaran (1980) in the animals having rarefaction and perforation of the frontal bone.

3.4. Cerebrospinal fluid

Vijayan and Rajan (1982a) evaluated the cerebrospinal fluid of animals bearing ethmoid carcinoma and recorded slight lymphocytic pleocytosis.

3.5. Exfoliative cytology

Nair (1973) observed neoplastic cells in the nasal discharge. The nucleus of the cells were hyperchromatic with clumping of chromatin, anisokaryocytosis and anisocytosis. Cytoplasm, occasionally was vacuolated and the cells in mitotic division were also seen. Masilliamony et al. (1980) advocated staining of cells obtained from nasal washings by Acridine Orange, indirect fluorescent antibody technique and Papanicolaou's method of staining for detecting and picking up cases of sinus neoplasms in bovines. Vijayan and Rajan (1982b) described that the early diagnosis and classification of the tumours can be made by studying the cytomorphological features of the exfoliated cells. They perfected the technique and gave a detailed account of the neoplastic cells and formulated the criteria for early diagnosis of the neoplasm.

3.6. Ehrlich test

As a diagnostic test Rajan and Vijayan (1981) employed ehrlich test. When the Ehrlich's reagent was added to the

plasma of the animals to be tested, immediately a dense white precipitate was formed. During incubation in water bath, the colour of the suspension became purple blue and more darker colour was observed in cancer positive animals. The highest mean optical density was observed in advanced stages of the tumour growth. They suggested that this test could be used as one of the battery of tests to be employed for diagnosing the tumour.

3.7. Mucus block technique

Vijayan and Rajan (1982c) employed mucus block technique for the diagnosis of ethmoid tumour. Mucus was collected by a nasal scoop and by aspiration to prepare paraffin blocks. Four to six micrometer thick Haematoxylin and eosin stained sections were examined microscopically. Aspiration method gave satisfactory results. An organoid pattern of the tumour tissue was evident and a precise diagnosis of the tumour was possible.

4. Pathological features

4.1. Gross pathology

Bergman (1914) and Stenstrom (1915) observed that the growth originated from the ethmoid mucosa, it extended and filled the frontal, sphenopalatine and maxillary sinuses. They pointed out that it occasionally extended into the orbital cavity and sometimes destroyed the lamina cribrosa and entered the

cranial cavity. Similar features were observed by Muthappa (1930), David and Venkatraman (1940), Narayana (1960), Becker *et al.* (1972), Tokarnia *et al.* (1972) and Pruthi *et al.* (1982). In addition to the above features, rarefaction of the frontal bone and bulging of the forehead were described (Rajan *et al.* 1972; Sreekumaran, 1980; Rajan and Sulochana, 1982 and Choudary and Rao 1982). Jose *et al.* (1985) observed keratitis, corneal opacity, purulent discharge and glaucoma in cases having exophthalmos. The tumour mass was fleshy, cauliflower-like, firm and had focal areas of necrosis, suppuration and cystic degeneration and metastases were found occasionally in the liver (Balasubramaniam, 1975). Metastases were found more commonly in the regional lymph nodes (Rajan *et al.* 1972; Tokarnia *et al.* 1972; Damodaran *et al.* 1974; Pospischil *et al.* 1979; Sreekumaran, 1980; Rajan *et al.* 1981 and Pospischil *et al.* 1982) and in the lungs (Stenstrom, 1915; Rajan *et al.* 1972; Nayak *et al.* 1979 and Sreekumaran, 1980). Atrophy of the spleen was observed in the later stages of tumour growth (Reddi and Rajan, 1982a).

4.2. Histopathology

4.2.1. Cattle

Histologically the tumours were found to be epithelial in origin. The most common histological types encountered

in bovines were "adenocarcinoma", "squamous cell carcinoma" and "undifferentiated carcinoma" (Stenstrom, 1915; Nair and Sastry, 1954; Narayana, 1960; Sastry and Rao, 1964; Rajan et al. 1972; Nair, 1973; Damodaran et al. 1974; Balasubramaniyam, 1975; Pospischil et al. 1979; Sreekumaran, 1980; Rajan et al. 1981; Rajan and Sulochana, 1982; Choudary and Rao, 1982 and Sreekumaran and Rajan, 1983b). In some cases transitional cell carcinoma (Nair, 1973 and Balasubramaniyam, 1975), osteoma and myxosarcoma (Moussu, 1906) were also reported. In addition to these, rarely, fibroma (Muthappa, 1930), mixed cell sarcoma (David and Venkatraman, 1940), carcinosarcoma (Narayana, 1960 and Sastry and Rao, 1964), fibromyxo-chondro osteoma, myxochondro osteosarcoma and fibro-osteo chondroma (Becker et al. 1972), histiocytic tumour, malignant lymphoma and reticulum cell sarcoma (Balasubramaniyam, 1975), fibrosarcoma (Madewell et al. 1976, and Choudary and Rao, 1982), osteoma (Prasad and Kohli, 1978), atypical osteoma (Rumbaugh et al. 1978), myxosarcoma (Nayak et al. 1979) and mesenchymal blastomas i.e. osteorhabdomyo and osteoblastic carcinoma were also documented (Pospischil et al. 1982).

Ultrastructural studies

Nair (1980) described the ultrastructure of the neoplastic cells of the carcinoma of the mucosa of the ethmoid and confirmed the epithelial nature of the neoplastic cell.

The cells were either well differentiated secretory structures or undifferentiated or differentiated squamous epithelium. There was great variation in the size and shape of the nucleus. The nucleus had irregular nuclear membrane and more than one nucleoli. The relative proportion of chromatin varied from cell to cell. The mitochondria were swollen with matrix having granular appearance and complete disorganisation and dissolution of the cristae. The rough endoplasmic reticulum was dilated and contained flocculent electron dense material. Structures very much similar to the viral particles were seen in the cytoplasm of the neoplastic cells.

4.2.2. Sheep

Intranasal tumours of epithelial origin have been reported (Young et al. 1961 and Duncan et al. 1967). Yonemichi et al. (1978) grouped the intranasal tumours of sheep as papillary adenomas or adenocarcinomas. The fine structure of tumour cells was characterised by the presence of numerous secretory granules. Njoku et al. (1978) also reported papillary growths of epithelial cells in the nasal cavity arising from the mucosa of the ethmoid bone in sheep.

4.2.3. Other species

In other species of animals, the histological types encountered were adenocarcinoma in dog (Cho et al. 1974),

adenocarcinoma in captive Eld's deer (Brownstein *et al.* 1975). Stunzi and Hauser (1976) classified the nasal tumours of domestic animals histologically as surface epithelial, glandular epithelial, undifferentiated carcinoma, tumours of bone and cartilage, lymphoid tissue and unclassified tumours. Brown *et al.* (1977) described a papillary adenocarcinoma in a Taiwan macaque monkey. Confer and De Paoli (1978) observed squamous cell carcinoma, undifferentiated carcinoma, chondrosarcoma and undifferentiated sarcoma in the nasal cavity of dogs and adenocarcinoma in pigs was observed by Rajan *et al.* (1981).

Rajan and Reddy (1981) evaluated the adrenal glands of tumour bearing animals and found haemorrhages, necrosis and degenerative changes in the cortex and medulla.

5. Immunology

5.1. T-Cell response

Sulochana *et al.* (1982) evaluated the CMI response of the cattle affected with ethmoid tumour by employing leucocyte migration inhibition (LMI) and skin hypersensitivity tests. From their studies on large number of tumour bearing animals they concluded that there was reduced CMI response in these animals. Reddy and Rajan (1983a) found lower counts of ANAE positive (T-cells) cells in the tumour bearing animals in advanced stages of tumour growth when compared to those of healthy animals.

5.2. Lymph node reaction

Sreekumaran and Rajan (1982c) observed hyperplasia of the paracortical region and sinus histiocytosis indicating enhanced general CMI response on intranodal administration of BCG. Reddy and Rajan (1983b) observed marked lymphoid follicle hyperplasia, increased size of paracortical region and pronounced sinus histiocytosis in the lymph nodes of vaccinated animals bearing ethmoid cancer. Sreekumaran and Rajan (1984) found that the animals having lymph nodes with histiocytic response and germinal centre activity had no secondary foci whereas the animals with unstimulated lymph nodes had high incidence of lymph node metastases. In vaccinated tumour bearing animals, sinus histiocytosis and macrophage activity was more pronounced and quicker in the lymph nodes stimulated by tumour antigen than with BCG (Reddy and Rajan, 1984) whereas in unvaccinated animals, BCG was found to be better immunostimulant than the tumour antigen. Reddy and Rajan (1985) noticed stimulation of T-Cell dependent areas in the lymph nodes of tumour bearing animals in response to tumour antigen and BCG.

5.3. Reaction of the spleen

Reddy and Rajan (1982a) observed severe lymphoid cell depletion in the spleen of the tumour bearing animals in late stages of tumour growth. They observed that there was immunological deficiency at this stage.

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5.4. <u>Response of peripheral blood lymphocytes to tumour antigen and BCG</u>

Tumour antigens and BCG stimulate the lymphocytes and enhance the CMI response. Several reports have appeared describing the immune reactivity of lymphocytes in tumour bearing animals.

Lindsay *et al.* (1978) observed inhibition of cell migration of peripheral blood lymphocytes isolated from cows with ocular squamous cell carcinoma, when autochthonous tumour homogenates were used to stimulate the lymphocytes. Jun *et al.* (1979) did not find blastogenic response of peripheral blood lymphocytes (BRPL) to tumour extract in sheep with ovine squamous cell carcinoma. Prolonged increase in blastogenic response of peripheral lymphocytes to tumour antigen after removal of the tumour was observed by Jun and Johnson (1979) in ovine squamous cell carcinoma. This was significantly greater in sheep with mature than with early tumours. Challenge of sheep, from which tumour mass had been removed surgically, with tumour antigen 15 weeks after operation was associated with a significant anamnestic type short term surge in BRPL which was significantly greater in sheep having advanced tumour growth. Sreekumaran (1980) found that tumour bearing animals were immunologically competent when stimulated with tumour antigen and BCG. Reddy (1981) found a higher rate of lymphocyte blast transformation

with tumour antigen and better immunostimulation with BCG in the vaccinated animals bearing tumours of ethmoid mucosa. Reddy and Rajan (1983c) found higher lymphocyte blast transformation rates to tumour antigens in early stages of tumour growth.

5.5. Lymphocyte response to phytomitogens

Phytomitogens stimulate lymphocyte blast transformation *in vitro* and intra dermal injection bring about skin response. Lymphoproliferative response and inhibition of cell migration of lymphocytes of animals with ocular squamous cell carcinoma was noticed by Lindsay *et al.* (1978). Jun *et al.* (1979) described significant differences in BRPL to different stages of tumour growth in ovine squamous cell carcinoma. There was fall in blastogenesis in advancing maturity of tumours. Jun and Johnson (1979) observed prolonged increase in BRPL to Phytohaemagglutinin-P (PHA-P) in advanced tumour growth. Sreekumaran and Rajan (1982a) found a significant reduction in blastogenic response of peripheral lymphocytes to phytohaemagglutinin-M (PHA-M). Jones and Amoss (1982) observed reduced proliferative capacity of lymphocytes to all mitogens (PHA, Con-A and Pockweed mitogen) in swine bearing cutaneous melanoma.

5.6. Macrophage response at remote inflammatory site

Inflammation induced by Dextran sulphate in animals

bearing tumours of ethmoid region revealed that the accumulation of macrophages and number of ingested particles in the cytoplasm were comparatively less in tumour bearing animals than those of control animals (Reddy and Rajan, 1983d). The tumour bearing animals were suggested to be immunodeficient.

5.7. Response of tumour bearing animals to specific immunotherapy

To evaluate the general immune status of the animals bearing carcinoma of the mucosa of the ethmoid, Reddy and Rajan (1982c) employed ultra violet irradiated tumour tissue suspension in Freund's adjuvant as vaccine. They found marked infiltration of large number of lymphocytes, macrophages and epithelioid cells at the site of vaccination. The epithelioid and giant cell reaction was less in animals with advanced tumour growth. In early stages, there was lymphocytic leucocytosis within a fortnight after vaccination which remained for 45-60 days whereas no significant rise in total leucocyte count was observed in the animals in advanced stages of tumour growth (Reddy and Rajan, 1982b). Extensive necrosis in deeper parts of tumour tissue with massive infiltration of lymphocytes and macrophages was observed in vaccinated animals (Reddy and Rajan, 1982d). The infiltration was much less in advanced stages of tumour growth.

6. Propagation and Transmission studies

6.1. Cell culture studies

One of the main reasons for development of animal cell and tissue culture techniques has been the conviction that, it could provide a means to overcome the problems of cancer. In cancer research, tumour cell culture is attempted to provide a source of virus, the study of tumour cell metabolism and material to study sensitivity to anticancer agents.

Roux in 1885 first performed the explantation of living tissue by isolating the medullary plate of the chicken embryo. Jolly (1903) described the division of amphibian leucocytes cultivated for one month in a hanging drop preparation. These were followed by Harrison's (1906-1907) remarkable experiments on the living developing nerve fibres of the frog, thus establishing the basis for the science of tissue culture. Genuine attempt on animal tissue culture started in 1906 when Beebe and Ewing described the cultivation of an infectious canine lymphosarcoma in blood from resistant and susceptible animals (dogs). Since then, cell culture has been a tool more frequently in the study of human and animal oncology. More extensive use of cell culture appeared in animal oncology during late fifties of this century. Murray (1959) reported *in vitro* culture of normal

embryonic and adult cells as well as cancer cells of human and animal origin. Sykes *et al*. (1959) studied the behaviour and properties of cells derived from bovine ocular squamous cell carcinoma. Unlike those from benign precursors plaque and papilloma lesions, cytoplasmic inclusions were seldom observed in these cells. Multiple nuclei and margination of chromatin were found only in rare instances. Changes normally were associated with cells from cultures of plaque and papilloma. Sykes *et al*. (1961) cultivated cells from bovine ocular squamous cell carcinoma. Cells from some of the lesions were maintained for more than 8 months and 173 serial passages. Twentysix per cent of the carcinoma specimens showed characteristic changes after minimum of five or more passages. The changes consisted of cytoplasmic vacuolation, sometimes accompanied by formation of cytoplasmic inclusions indicating the possible involvement of a viral agent. None of these changes occurred in cultures derived from tissues of the limbal region of the eyes of healthy cattle.

Duncan *et al*. (1967) attempted *in vitro* propagation of neoplasms of the nasal mucosa of sheep and did not succeed. Cleaver *et al*. (1972) cultivated epidermal cells and fibroblasts to determine if ocular squamous cell carcinoma was due to an enzymatic defect in the repair synthesis of DNA damaged by ultra violet light in Hereford and

Aberdeen Angus cows. Differences were not found in sensitivity of their cells to UV light or in the level of DNA repair synthesis. In ovine squamous cell carcinoma, Jun et al. (1978) obtained pure strains of epithelial cells by fragment explantation following a selective trypsinization procedure. Yonemichi et al. (1978) observed the greater RNA dependent DNA polymerase activity in the tumour or the cultured tumour cells from the intranasal tumours of the ethmoid olfactory mucosa in sheep. Virus particles similar to herpes virus were also detected in one culture.

Attempts for in vitro cultivation of the tumour cells from endemic ethmoid neoplasms of bovines were not successful (Jayaraman et al. 1979; Sulochana, 1980 and Pospischil et al. 1982). However, Sulochana (1980) was able to get growth in some cases for the first week but subsequently the cells died out. Changing the media or subculturing did not rescue the cells.

Kleinschuster et al. (1979) obtained cultures rich in epithelial cells and almost devoid of fibroblasts which were suppressed by using modified TICL-15[4] medium in case of bovine ocular carcinoma. Meeney et al. (1983) also cultivated in vitro monolayers from bovine ocular squamous cell carcinoma cells obtained from clinical cases. Al-Yaman and Willenborg (1983) successfully established cultures from ovine squamous cell carcinoma when tumour tissue was directly

explanted rather than treated enzymatically. The success in establishing cultures appeared to be related to the site on the body from which the tumour biopsy was taken, with tumours derived from the nose being most readily cultivated.

6.2. Transplantation studies

There has been an increasing interest in the growth of human tumours in immunologically defective animals or in the immunologically unreactive sites. However, due to difficulties involved in using these sites for transplantation studies, attempts were made to grow human tumours in normal animals which had been immunosuppressed by various treatments.

A few cases of successful transfer of canine tumours have been reported (Allam *et al.* 1955). Spontaneous canine thyroid carcinoma could successfully be transplanted in mixed-bred puppies after conditioning with cortisone in addition to X-irradiation (Allam *et al.* 1954, 1955, 1957). Canine osteosarcoma and melanoma cell cultures and fresh canine cells from a mammary carcinoma and from transmissible venereal tumour were successfully transplanted subcutaneously into nude mice (Oughton and Owen 1974). The histological appearance of the osteosarcoma in mice resembled that seen when these cell cultures were transplanted in immunosuppressed dogs but some differences were apparent in the melanoma and

the transmissible venereal tumour resembled that of the donor dog.

Several attempts were made for transplantation of the ethmoturbinate neoplasms of the domestic animals into experimental animals i.e., calves, rabbits, pigs, guinea pigs and mice with or without immunosuppression without success (Duncan et al. 1967; Rajan et al. 1972; Nair, 1973; Jayaraman et al. 1979; Sulochana, 1980 and Pospischil et al. 1982).

Several reports have appeared describing transplantation of bovine ocular squamous cell carcinoma into nude mice and cows (Hoffmann et al. 1977; Irvin et al. 1977 and Dennis et al. 1984). Hoffmann et al. (1977) used bovine squamous cell carcinoma as an experimental model system in studies of neoplasia. The minced tumour tissue suspension in medium 199 was injected subcutaneously on the back of one group of mice at the rate of 0.2 ml per mice. Single cell suspension was also injected to another group at the rate of 0.1 ml each. Growth of neoplastic cells was observed in the mice inoculated with minced cell suspension whereas inoculation of single cell suspension failed to initiate the growth. Irvin et al. (1977) inoculated established cell culture subcutaneously into irradiated and nonirradiated athymic (nude) mice. Tumour developed in all the irradiated mice but failed in non-irradiated ones.

invation of surrounding tissue or metastasis was not observed. Further passage in mice revealed a similar growth pattern. Dennis *et al.* (1984) used cows for autotransplantation. No transplant was successful. However, in two of the five cows given autograft, a pure viable tumour cell suspension, there was marked regression of the primary tumour after the transplantation.

Al-Yaman and Willenborg (1983) reported successful cultivation of ovine squamous cell carcinoma cells. The cell lines were successfully transplanted to nude mice and the growth pattern observed was similar to that seen in the original host. Al-Yaman and Willenborg (1984b) implanted ovine squamous cell carcinoma from various sites of sheep into nude mice. Of the 25 samples, 10 were successfully transplanted and serially passaged. An association was found between the site of the tumour growth in the sheep and its acceptance as a xenograft, most successful growth was obtained in the case of tumours arising from the skin of the nose. Expansive and invasive growth pattern and histological appearance of the xenografts were similar to that seen spontaneously in sheep, though faster growth rates were observed after the tumour was serially passaged in the nude mice. Successful heterotransplantation of experimentally induced sheep lung adenomatosis in nude mice was described by Zimber *et al.* (1984).

Materials and Methods

MATERIALS AND METHODS

In the present study efforts were made to cultivate the ethmoid carcinoma cells *in vitro* so as to establish a cell line. Attempts were also made to transplant the tumour cells in laboratory animals and tumour bearing animals to study the biological behaviour of the neoplastic cells and to establish a model system for further investigation.

1. Source of tumour bearing animals

The tumour bearing animals, for study, were brought to the College of Veterinary and Animal Sciences from different parts of the Kerala State after obtaining informations from the respective veterinary clinics. Three female goats positive for ethmoid carcinoma were obtained from the AICRP on Goats, Mannuthy.

2. Cell culture studies

2.1. Glasswares

Tissue culture bottles, milk diluting bottles and test tubes containing cover slips were used for seeding the culture.

2.2. Media

The following media were used in this study:-

2.2.1. Hank's balanced salt solution (HBSS)

This was supplemented with 0.5% Lactalbumin hydrolysate, 0.1% yeast extract, 10% calf serum and antibiotics.

2.2.2. TC 199 (Difco) media

It was supplemented with 10% calf serum and antibiotics.

2.2.3. Dulbecco's modified Eagle's media in Hank's base(Difco) supplemented with 10% calf serum and antibiotics.

2.3. Trypsin

0.25% trypsin (1:250 Difco) in phosphate buffered saline (Ca and Mg free) was used for primary dissociation of the cells.

2.4. Calf serum

2.4.1. New born calf serum

It was collected from unsuckled new born calf obtained from the University Livestock Farm, Mannuthy. It was bled and serum was collected aseptically. It was sterilised by filtration through Seitz filter, inactivated by heating at 56°C for 30 minutes, dispensed in small aliquot and stored at -20°C.

2.4.2. TC-Foetal calf serum (desiccated Difco)

This was reconstituted in 30 ml of sterile distilled water before use.

2.5. Antibiotics

The following antibiotics in the following concentrations of per ml of growth media were used:

Penicillin	..	200 I.U./ml
Streptomycin	..	100 ug/ml
Mycostatin	..	100 I.U./ml
Gentamycin	..	80 I.U./ml

2.6. Growth promoting factor (Insulin)

In two cases, 0.1 I.U. per ml of growth media, insulin (Boots) was used to facilitate adhering of the cells on glass surface.

3. Techniques

All the procedures were carried out in sterile conditions. To control the infection, all the tumour bearing animals were given "Dicrysticin"-Large (Sarabhai) one vial intramuscularly for three days before collection of the tumour tissue.

3.1. Collection of the tumour tissue

The tumour tissues for the studies were collected by two methods:

3.1.1. Six cows and two goats, bearing tumour of the mucosa of the ethmoid were subjected to euthanasia by exsanguination after stunning with the help of captive-bolt pistol. Immediately the head was cut into two halves. A few cubes of tumour mass was dissected out with the help of a sterile scalpel. Care was taken to avoid necrotic area and to collect only fresh healthy tumour tissue. The harvest of

the tumour tissue was collected into a sterile beaker containing Hank's balanced salt solution (BSS) with antibiotics. Retropharyngeal lymph nodes having metastatic lesions were also collected separately from the two of the euthanised cows in the same solution. The tissue was washed several times with BSS antibiotic solution. Subsequently a suitable piece from the original tissue was transferred to a Petri-dish containing BSS antibiotic solution. The superficial facia was removed from the tumour mass. Fat and capsule from the lymph node were dissociated and separated. The tissue was then cut into small cubes with the help of sterile scissors and washed several times with BSS antibiotic solution.

3.1.2. Three tumour bearing cows were operated for collecting tissues. The frontal and nasal part of the face was shaved, washed thoroughly with soap and water and disinfected with tincture of iodine. A linear incision was made on the forehead extending down towards the nasal bone. The bone was cut and paranasal sinus at the side having tumour growth was exposed. After dissecting out the tumour mass, the surgical wounds were sutured. The tumour mass was dissected out aseptically and collected in BSS antibiotic solution. It was processed as described above.

3.2. <u>Tissue dissociation</u>

0.25 per cent trypsin (Difco) in Hank's BSS with antibiotics was used for the primary dissociation of tissue.

A few small cubes of tissue after washing with BSS antibiotic solution were transferred to a flask containing 100 ml of 0.25% trypsin solution in BSS. Teflon coated sterile magnetic stirring paddle was put inside the flask. The flask was placed over the magnetic stirrer. After ten minutes of stirring, the supernatant was decanted and replaced with fresh 100 ml of 0.25% trypsin in BSS. The flask was placed again over the magnetic stirrer and stirred for 45 minutes. This fractional stirring was done to avoid the possibility of presence of cytotoxic factors in the tissue.

3.3. Preparation of cell suspension

A little quantity (5%) of the serum was added in the trypsinization flask to inactivate the trypsin and to prevent the adverse effect on cell population. The suspension was then sieved through double layer of muslin cloth and collected in a sterile flask. This was transferred to the centrifuge tubes and spun at 1000 rpm for 5 minutes. The supernatant was poured off and fresh BSS with antibiotics was added. The cell pellet was mixed well and spun at 1000 rpm for 5 minutes. This procedure was repeated for once more. The final suspension in small amounts of BSS with antibiotics solution was prepared and pooled in one flask. It was finally suspended in TC 199, HBSS and Eagle's media for seeding. The media was supplemented with 10% calf serum, 200 IU/ml of penicillin, 100 ug/ml of streptomycin, 50 IU/ml

of gentamycin, 100 IU/ml of nystatin. The pH was adjusted to 7.2-7.4. In addition, in two experiments in Hank's BSS and Eagle's media, insulin at the rate of 0.1 IU/ml was added to facilitate the glucose transport by the cells as a growth promoter.

3.4. Seeding of the cells

The suspension of the cells in growth media, i.e., TC 199, Hank's BSS and Eagle's media was made in such a way that each ml contained 10^7 cells. The suspension was dispensed into 100 ml tissue culture bottles at the rate of 10 ml per bottle, into milk diluting bottles at the rate of 12 to 15 ml per bottle and into the test tubes 1 to 1.5 ml per tube for a monolayer culture. The tubes were placed in slanting position at about 30° and incubated at 37°C.

3.5. Control

"VERO" and "WISH" cell lines were obtained from the Amala Cancer Hospital and Research Centre, Trichur. The cells were harvested by using trypsin versine glucose (TVG) solution and suspended in Eagle's media supplemented with 5% calf serum and antibiotics. The cells were suspended at the concentration of approximately 10^5 cells per ml of the growth media. The final suspension was seeded at the rate of 10-12 ml per tissue culture bottle and 1-1.5 ml per test tube as per monolayer protocol.

3.6. Observations

All the tubes and bottles in the experimental and control groups were observed under the microscope daily from 48 hours of initial incubation. The coverslips from the tubes were taken at 48, 72, 96 and 120 hour intervals and washed with BSS solution and fixed in acetic acid alcohol solution (1:3). The cover slips were rehydrated in descending grades of alcohol, stained with haematoxylin stain. Dehydration was done in ascending grades of alcohol and cleared with xylol two changes of 5 minutes each, mounted in DPX for microscopic examination.

4. Transplantation studies

Attempts were made for autologous, homologous and heterologous transplantation of the tumour tissue in various experimental animals. The following animals were used for this study:

4.1. Cows: The tumour bearing cows obtained from the different parts of the State.

4.2. Rabbits: Four young rabbits were obtained from the small Animal Breeding Station, Mannuthy.

4.3. Mice: Twenty young male mice were obtained from the Small Animal Breeding Station, Mannuthy.

4.4. Hamsters: Two male and two female young golden hamsters were procured from University of Agricultural Sciences, Bangalore.

4.5. Goats: Five healthy and three tumour bearing goats were obtained from the AICRP on Goats, Mannuthy.

5. Techniques

All the experiments were carried out in sterile conditions.

5.1. Immunosuppression

Cyclophosphamide (Endoxan-ASTA-Khandelwal) and hydrocortisone (Roussel) were used as immunosuppressive agents in the case of rabbits and mice at the following dosage and routes.

5.1.1. Endoxan:

Rabbits: 20 mg per rabbit intramuscularly 3 days prior to tumour cell inoculation.

Mice: 2 mg per mice intramuscularly three days prior to tumour cell inoculation to 10 mice.

5.1.2. Hydrocortisone: 0.1 ml subcutaneously to 10 mice the day previous to the tumour cell inoculation and 0.5 ml per rabbit intramuscularly to four rabbits the third day following endoxan administration. No immunomodulators were used for Hamsters, goats and cows.

5.2. Collection of tumour tissue

5.2.1. Six cows and a goat were euthanised by exsanguination after stunning, the head was opened and the tumour tissue was collected in BSS with antibiotics.

5.2.2. Three cows were operated and the tumour tissue was collected in BSS antibiotics solution.

5.3. Preparation of the tissue

The tumour tissue was washed thoroughly a couple of times with BSS antibiotics solution and four preparations were made.

5.3.1. Graft

The tumour tissue was cut into small cubes and kept in BSS antibiotic solution for subcutaneous implantation.

5.3.2. Homogenate

The small cubes of the tissue were collected in a clean sterile tube containing BSS antibiotic solution. The tissue was homogenised under sterile conditions with the help of tissue homogenizer. An equal volume of BSS antibiotic solution was added and kept for inoculation.

5.3.3. Filtrate

A portion from the homogenate was sieved through muslin cloth and used for inoculation.

5.3.4. Cell suspension

The tumour cell suspension was prepared by trypsinisation using the same technique as described for cell culture.

5.4. Autotransplantation

Attempts were made to transplant the tumour in the same

tumour bearing animals at a site far away from the primary location.

5.4.1. Inoculation of the tumour cells

The tumour tissue homogenate and filtrate were inoculated subcutaneously on the right side of the dewlap at the dosage of 5 ml each, to two cows.

5.4.2. Observation of growth

The inoculated sites were observed visually and by palpation daily from the third day onwards after injection for the evidence of any growth at the site of injection.

5.5. Homologous transplantation

Tumour cells obtained from one animal were transplanted to other tumour bearing animals of the same species.

5.5.1. Inoculation of the tumour tissue

Different preparations were inoculated at different sites.

a) Tumour tissue fragments

The tumour tissue fragments were implanted subcutaneously on the right side of the gluteal region in three cows. The area was cleaned, shaved and disinfected with tincture of iodine. A linear incision about two centimeters in length was made. The skin was lifted and the tumour tissue was inserted inside. The incision was closed by applying suture.

b) Cell suspension

The dissociated (Trypsinised) tumour cells were suspended

in TC-199 medium with antibiotics. Five ml each of this suspension was injected subcutaneously on the left gluteal region to three cows.

c) Homogenate

The homogenate was injected subcutaneously to three tumour bearing cows at the dosage of 5 ml each. Similarly 2 ml of the tumour tissue homogenate from a goat was inoculated subcutaneously to another tumour bearing goat.

d) Filtrate

Three tumour bearing cows were injected subcutaneously on the right side of the dewlap at the dose rate of five ml each.

3.5.2. Observation of growth

The implantation and inoculation sites were observed daily from the third day onwards, for the evidence of growth.

3.5.3. Histopathology

The skin, at the site of inoculation and implantation was excised along with the neighbouring tissue at weekly intervals from first week onwards. After gross examination, the tissue was fixed in 10 percent formaldehyde solution for 3 days. It was processed by routine procedures for paraffin embedding. Five micrometer (um) thick sections were cut and stained by hematoxylin and eosin stains for histopathological examination.

5.6. Heterotransplantation

The tumour tissue obtained from a tumour bearing cow was inoculated into five goats, four rabbits, 20 mice, and two hamsters.

5.6.1. Tumour cell inoculation

a) Rabbits

The tumour cell suspension at the rate of 1.0 ml each, was injected subcutaneously on the right flank of the four rabbits.

b) Mice

Twenty mice were injected with 0.2 ml each of the cell suspension subcutaneously on the back.

c) Hamsters

Tumour tissue filtrate was injected on the cheek pouch of two hamsters at the rate of 0.25 ml each.

d) Goats

Five healthy goats were inoculated with tumour tissue homogenate into the frontal sinus and on the right side of the neck subcutaneously at the dose rate of 1.0 ml and 2.0 ml respectively.

5.6.2. Observation of growth

All the inoculated animals were observed daily from third day onwards after inoculation for any change at the

site of inoculation. The goats were observed for any nasal discharge.

5.6.3. Histopathology

a) Rabbits

Tissue, at the site of injection was taken 45 days post-inoculation. It was fixed in 10 per cent formaldehyde and processed by routine procedures for paraffin embedding. Sections at 5 um thickness were cut and stained with haemotoxylin and eosin for histopathological examination.

b) Mice

One mice each from the groups immunosuppressed by hydrocortisone and cyclophosphamide respectively were sacrificed every third day after inoculation. The tissue from the site of injection was collected, fixed in 10 per cent formaldehyde solution and processed by routine procedures for paraffin embedding. Sections cut at 5 um thickness were stained with haematoxylin and eosin for histopathological examination.

Results

RESULTS

To carry out *in vitro* cell culture and transplantation studies, tumour tissue was obtained from nine cows and three goats . *In vitro* culture of tumour cells was tried using Hank's balanced salt solution (BSS), TC-199 medium and Eagle's medium. All the media were supplemented with 10 to 20 per cent calf serum. The transplantation was attempted on different natural hosts and experimental laboratory animals.

1. Cell culture study

The tumour cells from cows did not show active monolayer formation or proliferation. The details of the culture made are shown in table 1. However, for the first 48 hours, the cells were seen adhering on the glass surface but subsequently they got detached. Though the cells were adhering, monolayer was not formed and mitotic activity of the cells was not seen within the period of 48 hours. Viable cell count made employing dye exclusion technique, using trypan blue showed increased number of dead cells in the suspension. Addition of insulin in two cases did not show any improvement in the cell activity. There was no difference in the survival and growth of the cells in any of the three media used. Replacement with fresh medium after 48 hours of seeding also did not favour the cell rescue.

Bacterial and fungal contamination were seen although in very few instances in spite of using antibiotics at the maximum permissible level. All other cultures remained free from bacterial or fungal contamination.

In two cultures employing tumour tissue from goats, cell growth in monolayer was obtained upto a maximum period of nine days. The cells were seen growing in a thin sheet. Most of the cells on stained coverslip cultures at 72 hours were seen as large flattened polygonal cells (Fig. 1 and 2). They had well defined borders and the cytoplasm was distinct. The nucleus was ovoid or round. Many of the cells were showing marked mitotic activity. The daughter cells were irregular in size and shape with regard to the nucleus and cytoplasm. A moderate number of fibroblasts were also seen. From 72 hours onwards, some of the cells showed rounding and grouping showing no mitotic activity. A few cytoplasmic vacuoles appeared in some of the cells. There was increase in the cytoplasmic granulation. At this stage the cells became shrunken and detached from the glass surface and came down in the suspension. Cell sheet was broken at several points from the glass surface and cell groups detached from each other. Change of the media and addition of fresh media in the beginning when rounding started, did not improve the condition. On sub-cultivation on the fourth day, the cells did not adhere to the glass surface. On the ninth day

Fig. 1. Tumour cells - Goat - 72 hours after culture in
Hank's Balanced salt solution - proliferating cells
are seen in groups - Harri's haematoxylin x 400

Fig. 2. Tumour cells - Goat - 72 hours after culture in
Hank's Balanced salt solution - Neoplastic cells
in isolated groups, dividing cells also seen -
Harri's haematoxylin x 400

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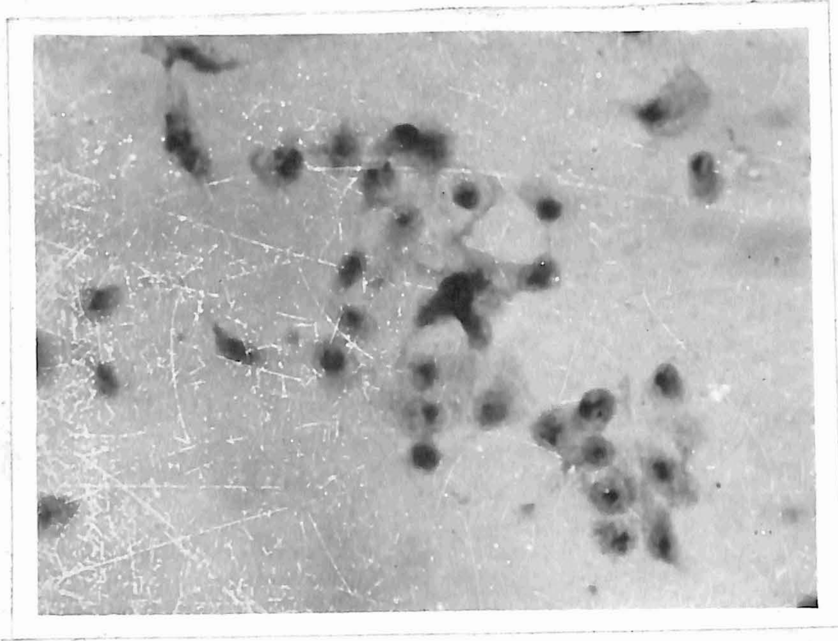


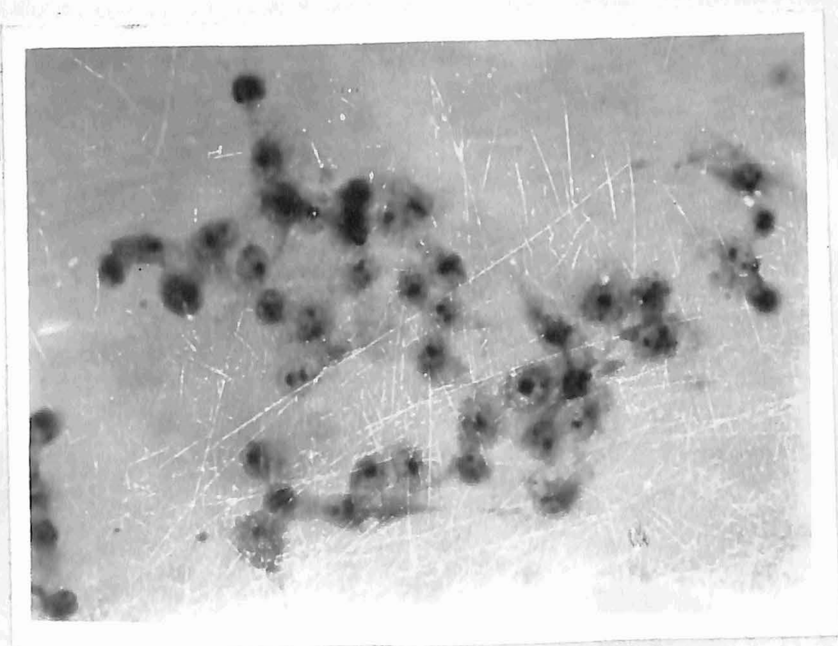


Table 1

Cell culture study

Experiment No.	Source of the tissue	Media used	Calf serum %	Insulin per ml	Number of seeded: Bottles	Number of seeded: Tubes	Growth observed	Contamination: Bacterial	Contamination: Fungal
1	Cow	Hank's BSS	15	-	1	20	-	8 t,1b	3t,1b
2	Cow	TC 199 (Difco)	20	-	1	16	-	16t, 1b	-
3	Goat	TC 199 (Difco)	15	-	3	15	16	-	-
4	Cow	TC 199 (Difco)	10	-	3	18	-	-	1b
5	Cow	TC 199 (Difco)	10	-	5	10	-	-	1b
6	Cow	Eagle's media (Difco)	10	-	5	-	-	-	-
		Hank's BSS	10	-	-	10	-	-	-
7	Cow	Eagle's medium (Difco)	10	-	-	16	-	-	-
		Hank's BSS	10	0.1 IU	5	-	-	-	-
8	Cow	Eagle's medium (Difco)	10	0.1 IU	5	12	-	-	-
9	Cow	Eagle's medium (Difco)	10	-	5	5	-	-	-
10	Cow	Eagle's medium (Difco)	10	0.1 IU	3	16	-	-	-
11	Goat	Hank's BSS	10	-	4	5	4b, 5 t	4b	-
		TC 199 (Difco)	10	-	3	-	3b	3b	-
12	Goat	TC 199 (Difco)	10	-	6	5	-	-	-

Legend: b = bottles; t = tubes

bacterial contamination appeared in eight seeded culture bottles. There was no significant difference in the growth of cells on any of the media used i.e., Hank's BSS or TC-199.

As a control, parallel cultivation of established cell lines "Vero" and "WISH" was done in the medium 199 supplemented with 10% calf serum. The "Vero" cells derived from normal adult "African Green Monkey" kidney, grew well on the media showing usual fibroblast-like morphology in monolayer with irregular spindle shaped fibres. The "WISH" cell line derived from human amnion tissue produced monolayer on the glass surface showing epithelial-like morphology (Fig.3). The cells were seen to form continuous mosaic-like sheets of closely adherent polygonal cells with very little intercellular substance which is normal for this type of cell line. There was no evidence of growth inhibition or contamination even after four successive passages at an interval of three days for each passage.

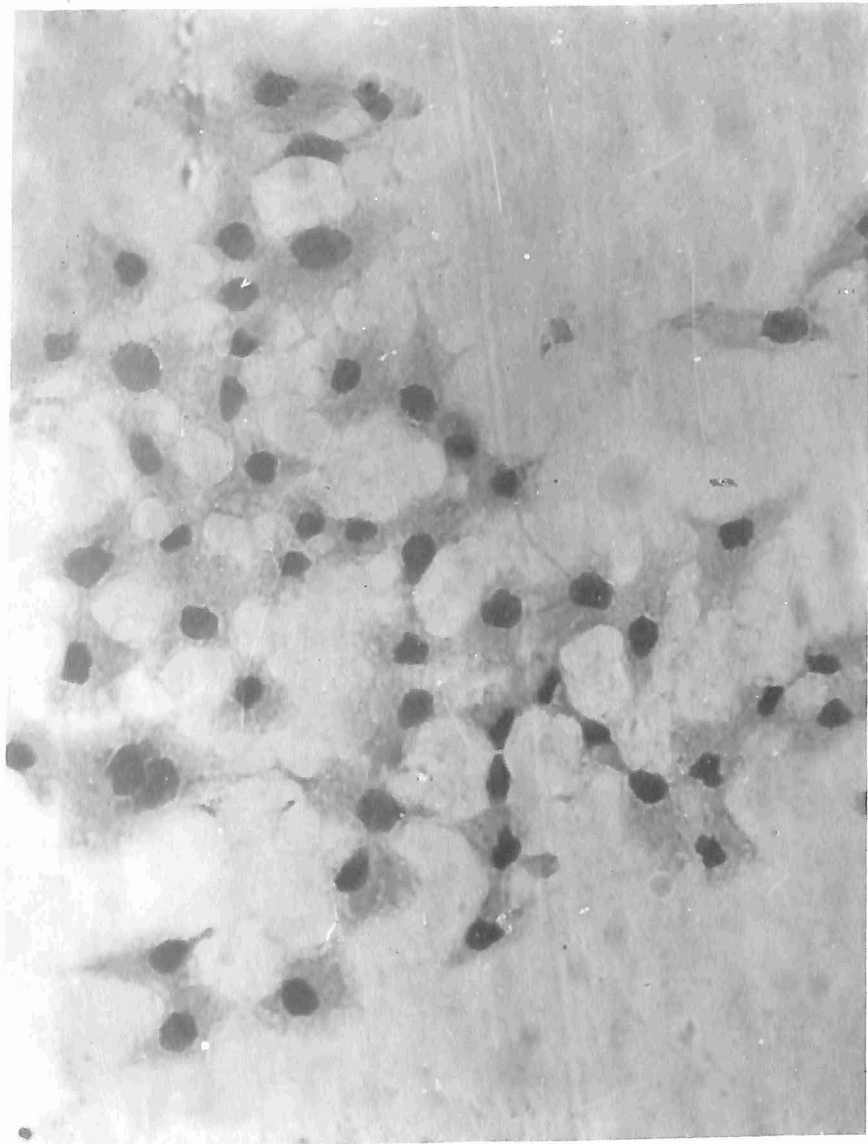
2. Transplantation studies

The results of transplantation experiments carried out in natural and experimental animals were variable.

2.1. Autotransplantation

The study was carried out in two cows by injecting their own tumour tissue on the dewlap by subcutaneous route. A painful, hot swelling was noticed at 24 hour post-inoculation.

Fig. 3. WISH cells - cover slip culture in Eagle's medium - mosaic-like sheets of polygonal cells with little intercellular substance - Harri's haematoxylin x 400.



This subsequently regressed and completely subsided within a week (Table 2).

2.2. Homologous transplantation

Different preparations of the tumour tissue were injected in six tumour bearing cows at different sites. The results are presented below and on table 3.

2.2.1. Implantation of tumour tissue fragments

Small pieces of solid fresh tumour tissue were implanted into three tumour bearing cows subcutaneously. The cows developed painful swelling after 24 hours. It increased progressively in size. For the first week, the swelling was soft and fluctuating which remained for three weeks. In one cow, the swelling ruptured and discharged pus. Gradually it started healing. The swelling subsided completely in all the three cows and left only a scar. Histologically no evidence of neoplastic cells was observed.

2.2.2. Homogenate and filtrate

All the three cows which were administered the filtrate and homogenate subcutaneously, showed a hard painful swelling at the site of injection by 48 hours post-inoculation. After 96 hours, the pain subsided and the swelling increased in size (Fig.4). The pathological features observed at different intervals, were:-

Table 2

Autotransplantation

Experiment No.	Animals inoculated		Preparation of inoculum	Administration			Period of observation (days)	Observations
	Species	Number		Dose (ml)	Route	Site		
1	Cow	1	Homogenate	5	S/C	Dewlap	7	No growth
			Filtrate	5	S/C	Dewlap	7	No growth
2	Cow	1	Homogenate	5	S/C	Dewlap	7	No growth

Table 3

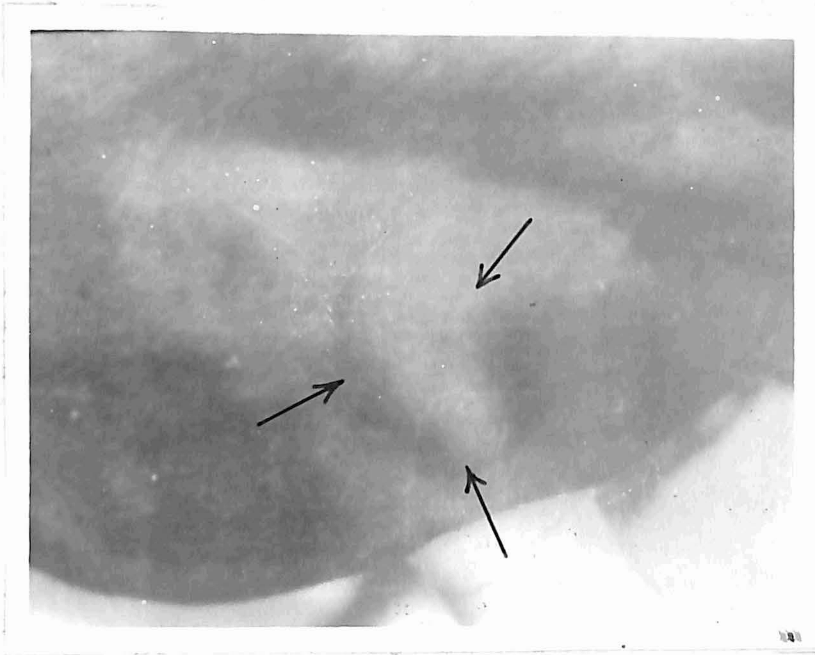
Homologous transplantation

Experiment No.	Animals inoculated		Preparation of inoculum	Administration			Period of observation (days)	Observation of growth
	Species	No.		Dose	Route	Site		
1	Cows	3	Solid tumour fragments	-	S/C	Gluteal region	90	No growth, suppurative lesion developed within a week
			Cell suspension	5 ml	S/C	Gluteal region	90	No growth
2	Cows	2	Homogenate	5 ml	S/C	Dewlap	*	Soft painful swelling appeared on the third day
			Filtrate	5 ml	S/C	Dewlap	*	Firm painless swelling persisted for three weeks
3	Cows	1	Filtrate	5 ml	S/C	Dewlap	*	Swelling appeared at 48 hours, gradually became hard and mobile by a week
4	Goat	1	Homogenate	1.5 ml	S/C	Neck	60	Oedema appeared after 24 hours and gradually subsided

*The observations were taken at third, seventh, fifteenth and twentyfirst days.

Fig. 4. Cow - skin 16 days after administration of tumour tissue homogenate - tumour development at the site of inoculation

Fig. 5. Cow - Administered homogenate - seven days post inoculation - proliferating neoplastic epithelial cells seen amidst an oedematous zone - H & E x 250.



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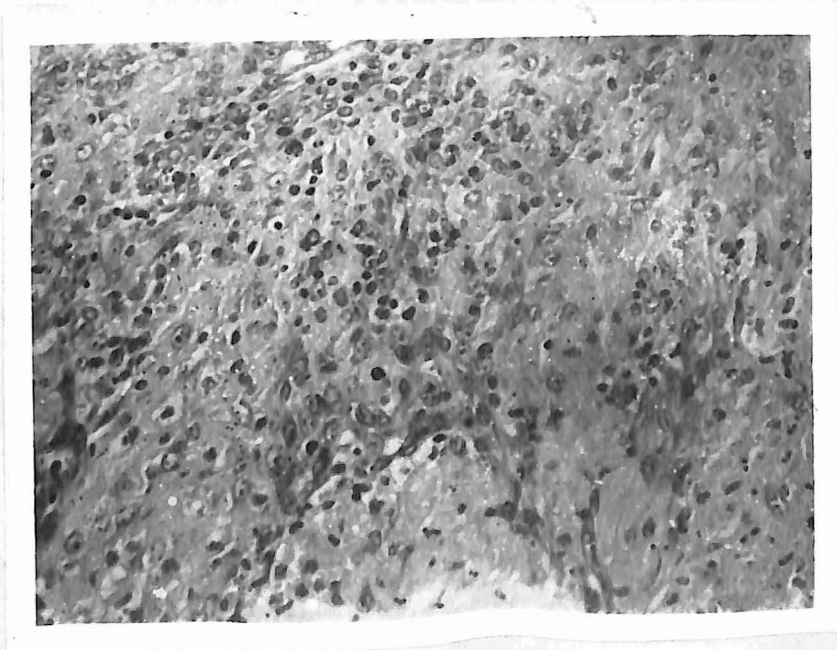


a) At three days post inoculation

In the subcutaneous tissue, grossly there was a thick brownish grey mass with a narrow vascular zone at the periphery. Histologically there was oedema and a fibrinous exudate consisting of a few neutrophils and lymphocytes. There was no evidence of neoplastic cell proliferation, although a few implanted cells were seen scattered.

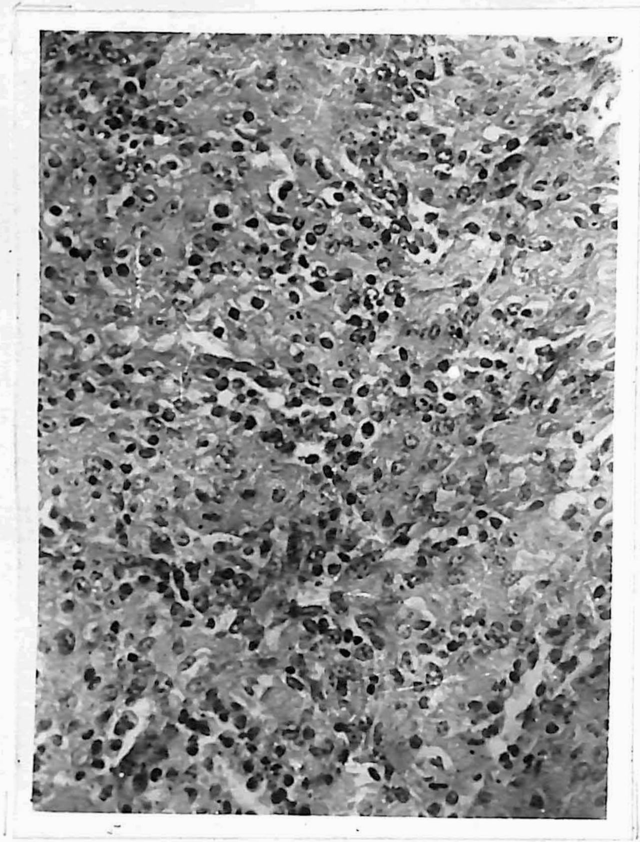
b) At seven days post inoculation

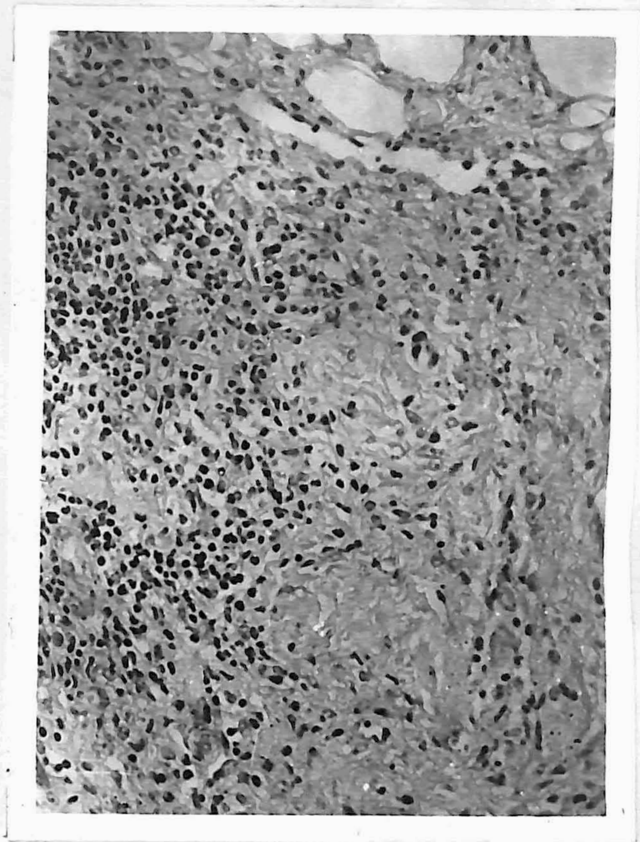
On gross examination a moderately firm brownish grey mass about 3 cm to 4 cm in size was seen embedded in the subcutis. In certain foci in the mass there was relatively dark brown vascular zones.

Histologically there was severe oedema in the dermis. There were focal areas of proliferating sheets of epithelial cells (Fig. 5 and 6). These cells were spherical to oval in shape and hyperchromatic. The cytoplasm was scanty but the cells had compact hyperchromatic large nucleous which almost filled the cytoplasm. Some of the cells were seen in prophase and metaphase of mitosis (Fig.7). Focal areas of dense infiltration of lymphocytes and macrophages were also present. There were focal areas of necrosis surrounded by neutrophilic reaction. Amidst the proliferating cells, in certain areas, there were lymphoid foci. Fibroblastic proliferation was evident in the dermal tissue around the proliferating zone.

Fig. 6. Cow - Administered homogenate - seven days post inoculation - proliferating neoplastic cells forming closely packed sheets of cells - H & E x 250.

Fig.7. Cow - Administered homogenate - seven days post inoculation - dense fibroblasts, infiltrating inflammatory cells and a few neoplastic cells - H & E x 250.





c) At fifteen days post inoculation

The swelling was small but was very firm on palpation. On incision of the encapsulated mass small amount of pus was seen within the capsule. Histologically there was moderate infiltration with lymphocytes, macrophages and plasma cells amidst a proliferating zone of fibrous tissue. In focal areas, a few groups of hyperchromatic epithelial type cells were seen (Fig.8 and 9).

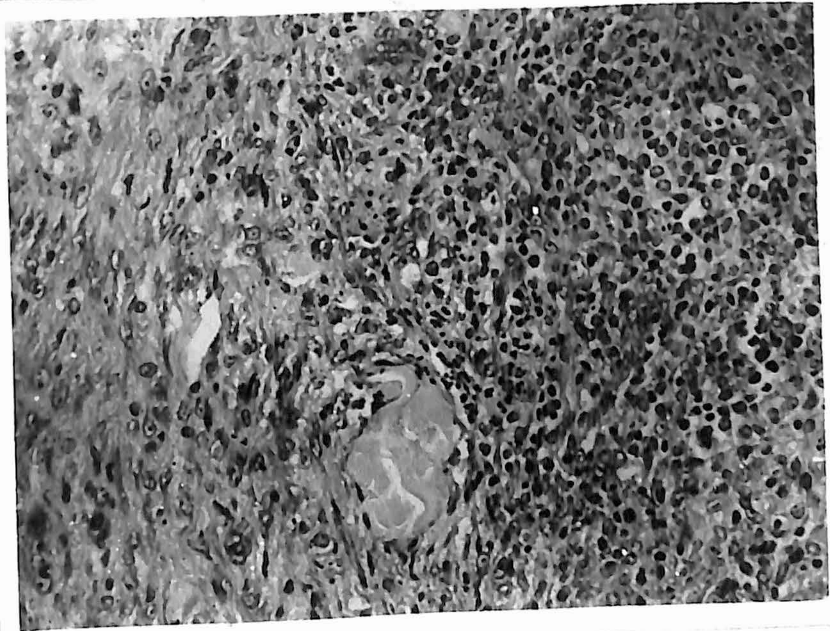
d) At 21 days post inoculation

On gross examination the brownish-grey growth was firm and measured about 3 cm x 5 cm.and was embedded in the sub-cutaneous tissue. On incision, the mass looked brownish-grey and encapsulated. Around this lesion sparse amount of pus was seen accumulated. The capsule was thick and highly vascular.

Histologically there were scattered small islands of proliferating epithelial cells, spheroid to oval in shape (Fig.10). Some of these cells were very much hyperchromatic and were in varying stages of mitosis (Fig.11). There were focal areas of necrosis and granulation tissue formation. Capillaries showed perivascular accumulation of lymphocytes and a few macrophages.

Fig. 8. Cow - Administered homogenate - 15 days post inoculation - Groups of neoplastic cells, fibroblasts and infiltrating inflammatory cells - H & E x 400.

Fig. 9. Cow - Administered homogenate - 15 days post inoculation - actively proliferating epithelial cells - H & E x 400



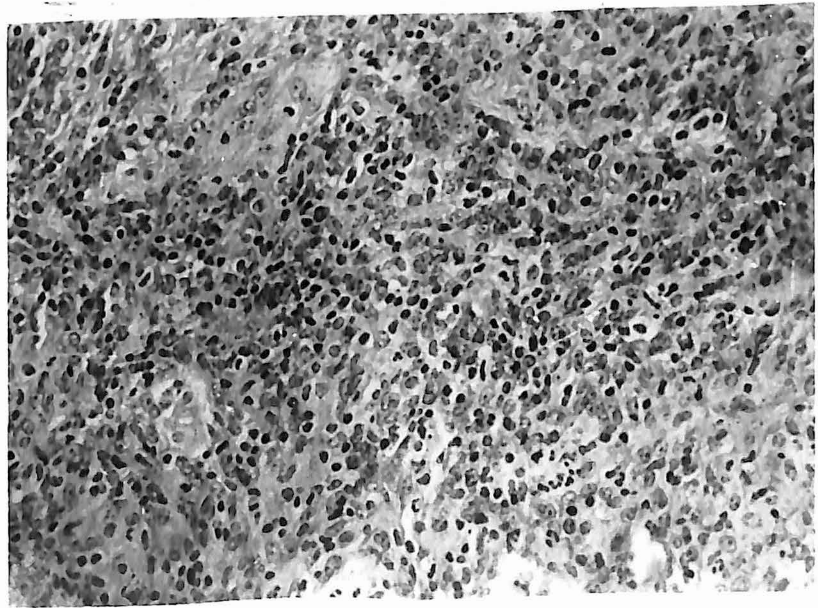
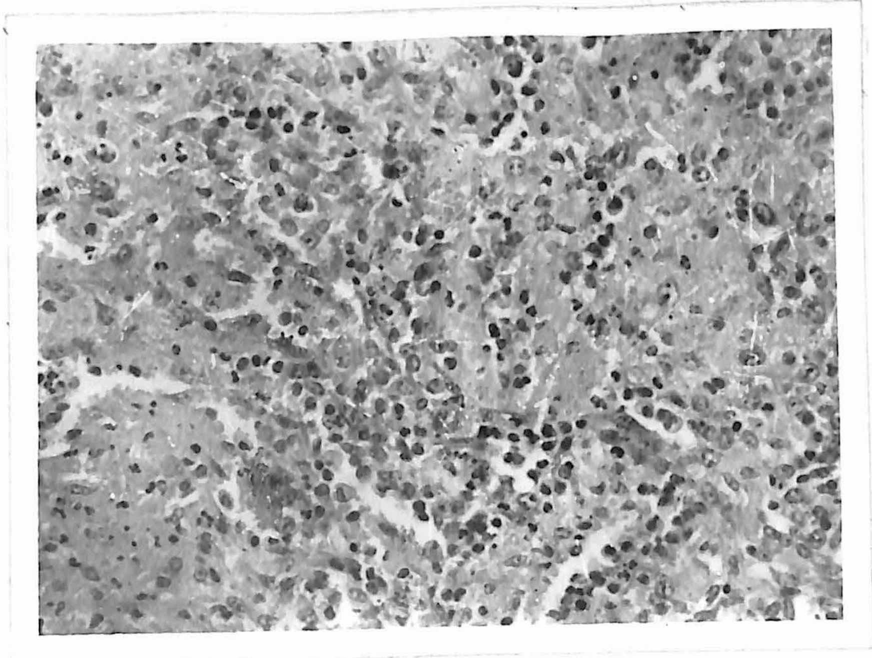


Fig. 10. Cow - Administered filtrate - 21 days post inoculation - sheets of proliferating epithelial cells with some in stages of mitosis - H & E x 400.

Fig. 11. Cow - Administered filtrate - 21 days post inoculation - proliferating sheets of epithelial cells - H & E x 400.



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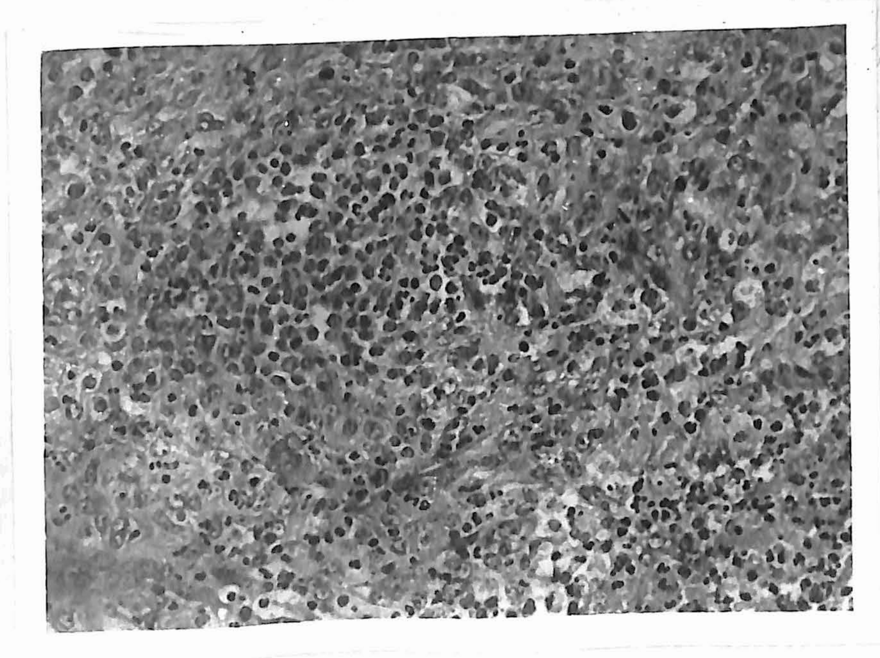

2.2.3. Tumour cell suspension

Three tumour bearing cows which received tumour cell suspension subcutaneously showed a painful swelling at the site of injection by 24 hours post-inoculation. The swelling gradually regressed and subsided completely within one week leaving no traces behind. Histological examination of the tissue from the site of inoculation did not reveal any reaction or neoplastic cells.

2.3. Heterotransplantation

The response shown by the animals inoculated with homogenate, filtrate and tumour cell suspension is described below. The findings are shown in table 4.

2.3.1. Goats

Intra sinus and subcutaneous inoculation of tumour tissue homogenate failed to show any evidence of tumour growth in all the five goats within an observation period of 60 days. Painful swelling developed at the site of subcutaneous injection by 24 hours in all the goats. This swelling gradually transformed into suppurative lesion which became caseated within three weeks. The swelling gradually regressed. No goat showed signs of tumour development in the frontal sinus or any infection even during an observation period of 60 days. Histologically only a suppurative reaction was seen. There was no evidence of the proliferation of neoplastic cells.

2.3.2. Rabbits

The four immunosuppressed rabbits which were administered tumour cell suspension subcutaneously did not show any evidence of growth at the site of injection even after 90 days. Histological examination did not reveal any inflammatory response and there was no indication of the presence of any neoplastic cell.

2.3.3. Mice

No gross lesions were observed at the site of injection in all the twenty mice which were administered tumour cell suspension subcutaneously. Histological examination of the tissue taken from the site showed slight fibrosis. No neoplastic cells were seen.

2.3.4. Hamsters

There was no gross or microscopical evidence of neoplasia in the hamster cheek pouch even after 60 days observation.

Discussion

DISCUSSION

The neoplasm arising from the mucosa of the ethmoid in domestic animals is an important emerging problem. The aetiology is not yet fully understood and the knowledge on the biological behaviour of the neoplastic cell and its relationship with the host is still incomplete. The present study was an attempt to propagate the cancer cells *in vitro* and also to establish the growth in susceptible natural and experimental animals.

The tumour tissue was obtained from two species of animals for *in vitro* cell culture and transplantation. The tumour cells obtained from nine cows and three goats were seeded for *in vitro* culture. It was not possible to grow the cancer cells from cows successfully in the artificial media. The cancer cells in the case of bovine tumour although adhered, did not show any evidence of proliferation on glass surface and they showed disintegration by 24 to 48 hours. Failure of the bovine cancer cells to grow *in vitro* was observed in all the nine experiments. Earlier workers had attempted to grow the bovine ethmoid carcinoma cells in culture media (Jayaraman *et al*., 1979; Sulochana, 1980 and Pospischil *et al*. 1982) and they were not able to establish the growth successfully. No reports have so far appeared on the successful cultivation of the cancer cells from bovine

ethmoid carcinoma. As the tumour is located in the para-nasal sinuses and it has access to the air that is inhaled and exhaled, the tumour tissue often gets contaminated with microbes. Besides this, the forced respiratory movements made by the ailing animals as a result of blockage of the nasal passage, favours more contamination of the tumour tissue. Therefore, to prevent the contamination of bacterial growth heavy dose of antibiotics had to be used. This, perhaps would have interfered with the growth of the cancer cells.

Sulochana (1980) attributed the failure of the growth of the tumour cells in culture media due to the absence of certain essential unknown factors required by the tumour cells for growth. It seems obvious that these factors are only present in the primary hosts but not in the media, which may possibly cause failure of the growth. However, the tendency of the cells to adhere to the glass surface for the first 48 hours is an indication of the presence of viable cells and ability of the cells to grow and multiply. The condition of the tumour tissue taken for the culture would have also contributed to the death of cells. The disease is diagnosed only at a late stage and by this time most of the tumour tissue would have undergone necrosis and cells from the necrotic area may perhaps liberate certain substances toxic to the viable cells thereby causing their death.

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All the common media employed for culturing the tumour cells successfully were used in the study. But they did not support the growth of the cancer cells. To test whether the media were deficient to support the growth of the cancer cells, parallel culture of established cell lines was done in the same media under the same situations as those employed for the culture of ethmoid cancer cells. The cells grew well. This observation perforce leads to the conclusion that ethmoid cancer cells might require certain essential nutrient factors for growth. Insulin was shown to have a growth stimulatory effect on cells when grown in culture media (Gey and Thalhimer, 1924). Leslie et al. (1957) showed that an increase in nucleic acid synthesis accompanied insulin treatment. On three occasions insulin was incorporated in the media but the cultures did not show any improvement in the growth potentials.

Several other carcinomas of bovine origin have been reported to have successfully cultivated in vitro. Sykes et al. (1959, 1961) and Cleaver et al. (1972) cultivated cells from bovine ocular squamous cell carcinoma successfully using Eagle's minimum essential medium (MEM). Since eye cancer is a superficially placed tumour, its diagnosis is early and tissue can be taken for culture at a very early stage when the cells are very much active.

On two occasions tumour cells obtained from goats were cultivated and maintained for nine days. Duncan _et al._ (1967) reported failure to propagate the cells _in vitro_ from the neoplasms of the nasal mucosa of sheep whereas Jun _et al._ (1978) were successful in cultivating epithelial cells from the specimens of ovine squamous cell carcinoma located at different sites in sheep. Yonemichi _et al._ (1978) used MEM for culturing the cells from the tumours of the ethmoid olfactory mucosa of sheep. They described the presence of virus particles in these cultured cells. Al-Yaman and Willenborg (1983) reported successful cultivation of cells from naturally occurring squamous cell carcinoma of sheep using Dulbecco's modified Eagle's medium. In the present study also culture of the cancer cells was attempted in Dulbecco's modified Eagle's medium but the cells did not show any significant difference in either adhering property to the glass surface or mitotic activity.

Al-Yaman and Willenborg (1983) found that the establishment of culture was most successful when the tumour tissue was explanted rather than treated enzymatically. Sulochana (1980) also attempted cell culture from ethmoid carcinoma of bovine by explantation as well as by enzymatic digestion. The media used were HBSS and TC 199. She did not succeed in establishing the growth of the tumour cells by either of the techniques employed or media used.

Death of the cells after the ninth day accompanied by bacterial contamination was observed in one case. This suggests the presence of pre-existing bacterial flora in the tumour tissue. However, in most of the cases bacterial or fungal contamination of the culture was not a major problem. The clumping and rounding of some of the cells in the monolayer suggested cytopathic effect due to some infection. This observation would suggest the possibility of the tumour cells harbouring some endogenous infective agents. But this could not be ruled out. If the diagnosis is made early and efforts are made to culture the tumour cells at this stage, it may prove fruitful in establishing the growth of the cells in culture media. In spite of taking every effort to locate the cases at a very early stage, it was not possible to get the tumour bearing animals at an early stage.

Transplantation of the tumour cells in suitable experimental animals was attempted in an effort to establish *in vivo* growth of the cancer cells. Autologous, homologous and heterologous species of animals either with or without immunosuppression were used. The cancer cells were also inoculated into the immunologically deficient cheek pouch of the hamster.

Autotransplantation was not successful in both the cows inoculated with their own tumour tissue homogenate

subcutaneously on the dewlap. Complete disappearance of the swelling was observed at the site of injection by the third day post-inoculation. Dennis et al. (1984) tried autotransplantation of bovine ocular squamous cell carcinoma in cows. No autograft was successful. Al-yaman and Willenborg (1984a) described detection of both humoral and cellular cytotoxic activities to autochthonous tumour cells in sheep bearing squamous cell carcinoma. These activities did not cause the death of the inoculated tumour cells while primary tumour was in situ but a secondary immune response was observed following the removal of the primary tumour. They concluded that the presence of primary tumour interfered with pre-existing host immunity. They achieved growth of the transplant successfully when they implanted the tumour tissue fragments subcutaneously on different parts of the body while primary tumour was in situ. The swelling that appeared at the site of injection, during the present study, was only an inflammatory reaction initiated by tumour cells favouring the accumulation of leucocytes and thereby destruction of the tumour cells was brought about. In this study about 90% of the primary tumour was removed and therefore, there was no interference of the host immune response. Besides this, there might have been a secondary anamnestic immune response caused by inoculated autochthonous tumour homogenate suspension which probably might have resulted in complete destruction of inoculated tumour cells.

Homologous transplantation was tried in the tumour bearing cows by implanting solid tumour fragments, injecting homogenate, filtrate and cell suspension subcutaneously. It is well understood that the presence of primary tumour in the tumour bearing hosts interferes with the existing host immunity (Whitney et al. 1974; Al-Yaman and Willenborg, 1984a) and the animals which are in advanced stages of tumour-growth, are immunodeficient (Sulochana et al. 1982; Sreekumaran and Rajan, 1982a; Reddi and Rajan, 1983a, 1983c) and the recipient animals were presumed to accept the transplant. In three cows inoculated with homogenate and filtrate simultaneously, successful transplantation of the neoplastic cells was possible. This was characterised by a firm swelling. Histologically the proliferation of neoplastic cells was associated with an immune reaction. The tissue reaction observed was indicative of an immunological response manifested by the animal against the transplanted neoplastic cells. This is an observation which will support the conclusion that the animals were still not completely immunodeficient.

In three cows, which received solid tumour implants subcutaneously, manifested a painful swelling after 24 hours which later became a suppurative lesion. The reason for the failure of the solid tumour to establish growth might be due to the presence of some of the degenerating cells in the

implanted mass which would have initiated neutrophilic reaction resulting in pus formation.

Single cell suspension also failed to produce neoplastic growth in cows. The possible explanation for this failure may be that the tissue was dissociated enzymatically into single cell suspension. These individual cells are easily destroyed by the macrophages by eliciting a CMI response. Another reason may be that the site of implantation is unable to provide a suitable graft bed for the neoplastic cells to grow. It has been shown that the ability of a tumour graft to grow is partly dependent on the site of transplantation (Auerbach *et al.* 1978).

Inoculation of a homogenised suspension of tumour tissue and filtrate of this suspension containing antibiotics resulted in growth. It might be because encapsulation of the inoculated tumour preparation during growth might have resulted in failure to sensitize the immune system adequately due to inadequate antigen release from the site. It is also possible that the animals were in various stages of tumour growth and they were partially immunodeficient and this favoured the transplant to grow. It is likely that the tumour tissue may contain cells capable of producing antibodies. This will block the exposure of antigen to the cells of the host's immune system. Al-Yaman and Willenborg (1984a)

explained the possibility for the successful growth of the tumour tissue fragments as autografts due to the cells producing antibodies in the tumour tissue which blocked or masked the tumour antigen; so the recipient host's immuno system failed to respond to the graft. In the present study, the homogenate also might have acted in the same way.

Allam et al. (1954, 1955, 1957) successfully transplanted spontaneous canine thyroid carcinoma in mixed-bred puppies after conditioning them with cortisone in addition to X-irradiation. Several attempts to transplant the ethmoid carcinoma in calves were unsuccessful (Duncan et al., 1967; Rajan et al. 1972; Nair, 1973; Jayaraman et al. 1979 and Sulochana, 1980). The animals used for the study in this investigation were not immunosuppressed but only considered to be immunodeficient. The proliferating tumour cells were invaded by macrophages and they rejected the transplant. It is worth while to attempt to create an environment of immunological paralysis in experimental animals by giving massive doses of the immunosuppressive agents before and during transplantation because the dose regime employed in this study did not seem to cause adequate immunosuppression to establish the growth.

Heterotransplants made by different routes failed to grow in all the attempts. All the five goats used in this study remained fairly normal. No immunosuppressive treatment

was given to the goats. They were healthy and immunocompetent. Therefore, the transplant was readily rejected. Nair (1973) tried transplantation of bovine sinus tumour to pigs and white mice by intra peritoneal routes but did not succeed. Similarly in the present study all the four rabbits, 20 mice and two hamsters did not show any sign of growth even after 60 days. These findings agree with the results of transplantation studies of Sulochana (1980). Though, rabbits and mice were subjected to immunosuppression by endoxan and hydrocortisone, inoculation of cell suspension did not initiate growth. This supports the opinion that certain host factors may be operating to cause transplant rejection. Injection of filtrate into the hamster cheek pouch also did not result in establishment of tumour growth. It may be due to lack of certain unknown factors required by the tumour cells to grow which this recipient host could not provide.

However, a variety of tumours of animal origin have been reported successfully transplanted into nude mice. Oughton and Owen (1974) described successful transplantation of naturally occurring canine tumours into nude mice. In a previously recorded successful transplantation of cultured bovine lymphosarcoma cells into nude mice (Irvin et al. 1977) whole body irradiation was required prior to inoculation of single cell suspension. Hoffmann et al. (1977) transplanted

Summary

SUMMARY

In vitro propagation and transplantation studies in different species of animals were carried out to understand the biological behaviour of the neoplastic cell and its relationship with the host so as to lay out a model system for further investigation.

1. The ethmoid cancer cells obtained from nine cows were cultured in different culture media. The cells were grown in HBSS, medium 199 (TC) and Dulbecco's modified Eagle's medium supplemented with 10 to 20 per cent calf serum. The cells were seen adhering on the glass surface for the first 48 hours but there was no growth and prolifo-ration subsequently.

2. Tumour cells from three goats were cultured in HBSS and medium 199 supplemented with 10 and 15 per cent calf serum. The cells grew and were maintained upto nine days. Stained coverslip cultures showed neoplastic cells growing in sheets.

3. As a control, continuous cell lines "Vero" and "WISH" were cultured in the medium 199 along with ethmoid tumour cells. The cell lines yielded satisfactory monolayer even after four successive passages.

4. HBSS, TC-199 and Dulbecco's modified Eagle's media were used for culture of tumour cells. There was no

difference in the growth potential. Addition of insulin to the media also did not improve the growth of the tumour cells.

5. Transplantation studies were carried out in autologous, homologous and heterologous species of animals using tumour tissue fragments, tissue homogenate, filtrate and cell suspension.

6. Autotransplantation attempted in two cows failed to initiate the growth. Oedema developed by 24 hours post-inoculation at the site of injection which later on subsided leaving no traces behind.

7. Homologous transplantation was tried in six tumour bearing cows by implanting tumour tissue fragments, injecting homogenate and filtrate subcutaneously. Administration of homogenate and filtrate resulted in the growth of neoplastic cells in the tissue at the site of inoculation. A well defined circumscribed growth was evident at the site. Histologically proliferating sheets of epithelial cells were seen at the site of inoculation. This was associated with a CMI response characterised by infiltration of lymphocytes and macrophages.

8. Attempts at heterotransplantation of the ethmoid tumour with and without immunosuppression was made using the tumour tissue fragments, homogenate, filtrate and the

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BIOLOGY OF THE NEOPLASTIC CELLS OF ETHMOID CARCINOMA

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ABSTRACT

Attempts were made to propagate the ethmoid carcinoma cells *in vitro* to study the biological behaviour of the neoplastic cells and their relationship with the host. Efforts were also made to transplant the neoplastic cells in laboratory animals and tumour bearing natural hosts so as to lay out a model system for further investigation.

In vitro propagation was tried by obtaining the tumour tissues from nine cows and three goats. Cells were grown in Hank's balanced salt solution (HBSS), TC-199 and Dulbecco's modified Eagle's medium with 10 to 20 per cent calf serum. The cell suspension was seeded into tissue culture bottles, milk diluting bottles and test tubes and incubated at 37°C. No growth was observed in the cultures made from the tumour tissue of cows. The tumour cells from two goats grew in monolayer and were maintained for nine days. There was no difference in growth of cells in different media employed. Insulin supplementation in the media did not cause any difference in the growth of the cells. Subsequently the cells died even after subculturing and changing of the media. Absence of certain unknown factors required by the neoplastic cells for growth was considered responsible for failure of the growth.

Transplantation studies were carried out in autologous, homologous and heterologous species of animals using the tumour tissue fragments, homogenate, filtrate and cell suspension. No autograft or heterotransplant were successful. Homologous transplantation resulted in a firm painless growth at the site of injection. The growth initiated a cell-mediated immune response at the site of implantation. Failure of neoplastic cells to grow in the other hosts was considered to be due to rejection of the transplants by the host's immune system.