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ASSESSMENT OF THE ROLE OF AFLATOXIN IN THE AETIOLOGY OF CARCINOMA OF THE MUCOSA OF THE ETHMOID

By
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THESIS

Submitted in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

Faculty of Veterinary and Animal Sciences
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Centre of Excellence in Pathology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR

1995

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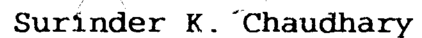

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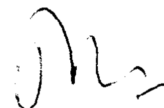

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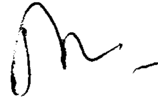

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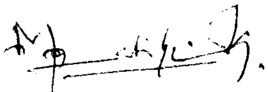

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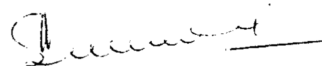

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Dedicated to my

Wife & Children

CONTENTS

Chapter	Title	Page No.
I	INTRODUCTION	1
II	REVIEW OF LITERATURE	4
III	MATERIALS AND METHODS	42
IV	RESULTS	56
V	DISCUSSION	88
VI	SUMMARY	105
	REFERENCES	112
	ABSTRACT	

LIST OF TABLES

Table No.	Title	Page No.
1.	Xenotransplantation experiment	52
2.	Average body weight (kg) of experimental pigs on various intervals of treatment	57
3.	Aflatoxin residues in blood and mucosa of experimental pigs	65
4.	Mean colony forming efficiency (CFE) of ethmoid mucosa cells in soft agar	77
5.	Colony forming efficiency (CFE) of ethmoid mucosa cells in 10 per cent and 1 per cent serum media	79
6.	Cytochemical assay of gamma-glutamyl transpeptidase (GGT) activity of *in vitro* aflatoxin B_1 treated ethmoid mucosa cells	80
7.	Aflatoxin B_1 (AFB_1) residues in the blood of tumour bearing animals	86

LIST OF FIGURES

Figure No.	Title
1.	Average body weight (kg) of experimental pigs at various intervals of treatment
2.	Ethmoid mucosa - Aflatoxin treated pig - 9th month - Degenerated glands with dense infiltration of lymphocytes and neutrophils
3	Ethmoid mucosa - Aflatoxin treated pig - 12th month - Focal aggregates of lymphocytes and oedema
4	Ethmoid mucosa - Aflatoxin treated pig - 12th month - Lymphnode like aggregates - Oedema
5	Ethmoid mucosa - Aflatoxin treated pig - 12th month - Focal glandular hyperplasia and mononuclear cell infiltration
6	Ethmoid mucosa - Aflatoxin treated pig - 15th month - Proliferating glandular epithelium forming acinar and tubular patterns
7	Ethmoid mucosa - Aflatoxin treated pig - 18th month - Glandular proliferation showing papillary projections
8	Ethmoid mucosa - Aflatoxin treated pig - 18th month - Focal squamous metaplasia - Lymphocytes and macrophage infiltration
9	Ethmoid mucosa - Ethmoid tumour extract instilled pig - 15th month - Pseudostratified columnar epithelium and mild oedema
10	Electron micrograph - Ethmoid mucosal cells from AFB_1 treated pig showing well developed endoplasmic reticulum containing secretory products - Degenerated mitochondria and lysosomal granules - Prominence of nuclear envelope with evagination of outer membrane

Figure No.	Title
11	Electron micrograph - Ethmoid mucosal cells from AFB_1 treated pig showing proliferation of rough and smooth endoplasmic reticulum - Cytoplasmic organelles degeneration
12	Electron micrograph - Epithelial cells from the ethmoid mucosa of AFB_1 treated pig showing prominent nucleus with a few indentations - Nucleoli show margination - Predominance of euchromatin in the nucleus - Lymphocyte and macrophage infiltration is seen
13	Electron micrograph. Epithelial cells from the ethmoid mucosa of AFB_1 treated pig showing nucleus with irregular nuclear membranes and deep indentations. Cytoplasmic organelles sparse
14	Electron micrograph. A macrophage located intravascularly and Lymphocyte perivascularly in the ethmoid mucosa of AFB_1 treated pig
15	Electron micrograph. The macrophage from ethmoid mucosa of AFB_1 treated pig showing well developed endoplasmic reticulum and Mitochondria with electron-dense matrix and partial loss of cristae
16	Electron micrograph. A part of cell from the ethmoid mucosa of AFB_1 and tumour extract administered pig showing well developed endoplasmic reticulum and prominent mitrochondria - Some of mitochondria show partial loss of cristae - Few dense granules seen - Nuclear chromatin is mostly euchromatin type
17	Electron micrograph - Ethmoid mucosa from the tumour extract instilled pig showing a layer of columnar cells
18	Ethmoid mucosa - Primary culture - A mixture of spindle shaped fibroblast - like cells and a few polygonal epithelial cells - 2nd passage - May-Grunwald & Giensa

Figure No.	Title
19	Ethmoid mucosa - Control Epithelial Culture - The epithelial cells displaying growth of closely adherent polygonal cells in mosaic-like sheet - 3rd passage - May-Grunwald & Geimsa
20	Ethmoid mucosa - Mixed culture - 10 days exposure to AFB_1 (0.05 ug/ml of medium) - Fibroblast-like cells showing degeneration and simultaneous proliferation of epithelial cells - May-Grunwald & Giemsa
21	Ethmoid mucosa - Mixed culture - 20 days exposure - Epithelial cell monolayer with a few strands of degenerating fibroblast-like cells - May-Grunwald & Giemsa
22	Ethmoid mucosa - Epithelial culture - 30 days exposure - The 6th passage - Nonadherent pleomorphic cells with cytoplasmic extensions - May-Grunwald & Geimsa
23	Ethmoid mucosa - Epithelial culture - 45 days exposure - 7th passage - Pleomorphic cells - Cytoplasmic vacuolation - A few binucleated cells - May-Grunwald & Geimsa
24	Ethmoid mucosa - Epithelial culture - 30 days after withdrawal of AFB_1 treatment - 13th passage - Small and compact epithelial cells with pleomorphism - May-Grunwald & Geimsa
25	Ethmoid mucosa - Epithelial culture - 60 days after withdrawal of AFB_1 treatment - 15th passage - the cells showing tendency to pile up - May-Grunwald & Geimsa
26	Ethmoid mucosa - Epithelial culture - 60 days after withdrawal of AFB_1 treatment - 15th passage - the cells showing multiple nucleoli and mitotic figures - May-Grunwald & Geimsa
27	Ethmoid mucosa - Epithelial culture - Dimethyl sulfoxide (DMSO) treated control - 5th passage - closely adherent polygonal cells in mosaic-like sheet - May-Grunwald & Geimsa

Figure No.	Title
28	Ethmoid mucosa - Fibroblast-like cells - 4th passage - spindle shaped cells growing in random orientation - May-Grunwald & Geimsa
29	Ethmoid mucosa - Fibroblast-like cells - 45 days AFB_1 exposure - 7th passage - cells showing enlarged nucleus with nucleolar fragmentation - slight cytoplasmic vacuolation - May-Grunwald & Geimsa
30	Ethmoid mucosa - Fibroblast-like cells - 45 days after AFB_1 treatment - 14th passage - A compact monolayer of fibroblast like cells - May-Grunwald & Geimsa
31	Electron micrograph. Epithelial culture cells showing cell junction
32	Electron micrograph. AFB_1 treated epithelial culture showing two cells with electron-dense and one cell with electron-lucent cytoplasmic contents - Mitochondria showing deformed and lytic cristae
33	Electron micrograph. A part of AFB_1 treated epithelial cell showing mitochondria with deformed and swollen cristae and partial degranulation of endoplasmic reticulum -Presence of fragmented filamentous structures seen
34	Electron micrograph. AFB_1 treated epithelial cell showing nucleus with irregular nuclear membrane and two prominent nucleoli - Ruffled plasma membrane is seen
35	Electron micrograph. A part of AFB_1 treated epithelial cell showing nucleus and cytoplasmic organelles with retrogate changes
36	Electron micrograph. AFB_1 treated epithelial cell showing invaginated nuclear membrane and nucleolar margination

Figure No.	Title
37	Electron micrograph. AFB_1 treated epithelial cells showing microvilli - The free ribosomes and glycogen in the cytoplasm - Nucleolar margination is also seen
38	Mean colony forming efficiency (CFE) of ethmoid mucosa cells in soft agar
39	Mean colony forming efficiency (CFE) of ethmoid mucosa cells in 10 and 1% serum media
40	Electron micrograph. Neoplastic cells showing dilated endoplasmic reticulum containing electron lucent material - Mitochondria with partial loss of cristae - Numerous glycogen particles in the cytoplasm
41	Electron micrograph. Neoplastic cell showing well developed endoplasmic reticulum - Interchromatin granules and nucleolar margination
42	Electron micrograph. Neoplastic cells showing cell junctions
43	Electron micrograph. A neoplastic cell showing association with lymphocyte
44	Electron micrograph. A dilated blood vessel with well differentiated endothelial cell in neoplastic tissue
45	Electron micrograph. A cell with prominent cytoplasmic organelles from retropharygeal lymphnode with metastasis
46	Electron micrograph. Intracellular retroviral-like particles in the cytoplasm of neoplastic cell
47	Electron micrograph. Extracellular retroviral-like particle close to the apical surface of neoplastic cell
48	Electron micrograph. Retroviral-like particles in cell free ethmoid tumour extract

Introduction

INTRODUCTION

In recent years the carcinoma of the mucosa of the ethmoid has emerged as one of the important neoplastic conditions affecting cattle. The prevalence of this tumour was reported to occur in an endemic form in the Scandinavian countries in the beginning of this century, although it seems to be non-existent in those countries now. Subsequently, the incidence of this neoplastic condition was reported from various other countries of the world.

In India, the occurrence of ethmoid carcinoma in cattle was reported as early as 1930 by Muthappa from the former Madras Province. The tumour of the mucosa of the ethmoid was first recorded in Kerala in 1960 (Rajan *et al*. 1972). The prevalence of this tumour has since been reported from Andhra Pradesh (Narayana, 1960; Sastri and Rao, 1964), Tamil Nadu (Damodaran *et al*. 1974),Karnataka (Balasubramaniam, 1975), Orissa (Nayak *et al*. 1979), Haryana (Pruthi *et al*. 1982) and Uttar Pradesh (Swarup *et al*. 1987). Although, in the earlier periods the incidence was confined to the southern part of India, now it is evident from the reports published that this kind of tumour is prevalent all over the country and the geographical barrier has been broken.

Since the last several years investigations have been carried out in a systematic manner on various aspects of the problem at the Centre of Excellence in Pathology, Kerala Agricultural University, Mannuthy. The symptoms, diagnostic criteria and immunological features of the tumour bearing animals have been well documented. Extensive pathological studies on a large number of tumour bearing animals have been made and the pathological features of this tumour have been very well described. Epidemiological studies have shown that this tumour has established itself in an endemic form in Kerala and has spread to other parts of the country, causing great concern to the farmers and persons associated with profitable livestock production programmes as this malady is causing serious economic loss. It is also pertinent to observe that the incidence is high in cross-bred animals in the midway of their life span, which is the period of maximum productive performance.

Although, various aspects of this tumour have been well elucidated, the aetiology of this cancer is still obscure. A viral aetiology has been suspected, but this has not been confirmed.

Since affected animals sometimes show clinico-pathological features of aflatoxicosis, a role of aflatoxin B_1 (AFB_1) in the development of ethmoid carcinoma has been

proposed (Lewis *et al*. 1967; Rajan *et al*. 1981; Zhang, 1981; Pospischil *et al*. 1982). However, this has not been proved. This proposal appears to be attractive and there is need to assess the role of aflatoxin in a seemingly multi-factorial genesis of the ethmoid carcinoma. The approach to assess the role of aflatoxin in the causation of ethmoid carcinoma should involve both *in vitro* and *in vivo* studies. The *in vitro* studies will certainly offer the possibility of elucidating the mechanisms of malignant conversion in a simplified, easily manipulative and readily observable way. Therefore, keeping in-view the economic importance of this cancer and the need to establish the aetiology of the carcinoma of the mucosa of the ethmoid in domestic animals, the present study was undertaken to elucidate the role of aflatoxin and virus/viruses in the aetiology of ethmoid carcinoma.

Review of Literature

REVIEW OF LITERATURE

2.1 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; Stenstrom, 1915 and Magnusson, 1916). Since then, there have been many reports on the incidence and clinico-pathological features of this neoplastic condition in various species of animals from different parts of the world (Jackson, 1936; Cohrs, 1952, 1953; Cotchin, 1956; Young *et al*. 1961; Amaral and Nesti, 1963; Nazario *et al*. 1966; Duncan *et al*. 1967; Rubaj and Woloszyn, 1967; Becker *et al*. 1972; Tokarnia *et al*. 1972; Madewell *et al*. 1976; Yonemichi *et al*. 1978; Njoku *et al*. 1978; Pospischil *et al*. 1979; Zhang, 1981; Njoku and Chineme, 1983; Steen *et al*. 1985; Rings and Rojko, 1985; Charry *et al*. 1985; Heras *et al*. 1991, Gazquez *et al*. 1992.

In India, Muthappa (1930) was the first to record a case of neoplasm in the ethmoid region in cattle from the former Madras Province. Subsequently, David and Venkataraman (1940), Nair and Sastry (1954), Narayana (1960), Sastry and Rao (1964), Rajan *et al*. (1972), Damodaran *et al*. (1974),

Balasubramaniam (1975), Jayaraman et al. (1979), Nayak et al. (1979), Viraraghavan et al. ((1980), Pruthi et al. (1982), Rameshmurthy (1984), Kornel et al. (1984), Singh and Singh (1984), Muralimanohar et al. 1986, Swarup et al. (1987), Chakraborty et al. (1988) and Muralimanohar (1988) also reported the occurrence of this tumour in different species of domestic animals from different parts of the country.

2.2 Pathology

2.2.1 Clinical symptoms

Intermittent nasal discharge, epistaxis, dyspnoea, unilateral or bilateral exophthalmos were the common clinical symptoms (Moussu, 1906; Stenstrom, 1915; Muthappa, 1930; David and Venkataraman, 1940; Narayana, 1960; Tokarnia et al. 1972 and Jose et al. 1985). Abdominal type of respiration characterised by snoring was a feature in the advanced cases (Rajan et al. 1972; Nair, 1973; Damodaran et al. 1974; Balasubramaniam, 1975; Njoku et al. 1978; Jayaraman et al. 1979). Most of the animals were in the first or second trimester of pregnancy when they manifested the symptoms of the disease (Rajan et al. 1972). Circling movements, perforation of frontal bone with swelling of the forehead and cachexia were reported by Nayak et al. 1979; Pospischil et al. 1979; Sreekumaran, 1980; Rajan et al. 1981 and Pruthi et al.

1982. Swelling of the submaxillary lymphnodes was reported by Kornel *et al*. (1984). Muralimanohar (1988) observed nasal discharge, epistaxis, exophthalmos, frontal swelling, dyspnoea, and nervous symptoms.

2.2.2 Gross pathology

Bergman (1914) and Stenstrom (1915) observed tumour mass originating unilaterally or bilaterally from the mucosa of the ethmoid as a pedunculated mass and filled the nasal cavity and extended into 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). Posteriorly, it occasionally extended into the cranial cavity perforating the horizontal plate of the ethmoid and invaded into the brain. Downward the tumour extended into the pharynx and blocked it. Anteriorly, the tumour invaded into the frontal bone, perforated it and bulged out as a tumour mass into the subcutaneous tissue (Pospischil *et al*., 1979; Rajan, 1987 and Gazquez *et al*., 1992). Jose *et al*. (1985) observed keratitis, corneal opacity, purulent discharge and glaucoma in those cases having exophthalmos.

The growths were generally greyish yellow and fleshy in consistency. Focal areas of necrosis, suppuration and cystic degeneration were often noticed (Rajan *et al.* 1972). Metastases were found in the regional lymphnodes (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), in lungs (Stenstrom, 1915; Rajan *et al.* 1972; Nayak *et al.* 1979 and Sreekumaran, 1980) and in liver (Balasubramaniam, 1975). Atrophy of the spleen was observed in the later stages of the tumour growth (Reddy and Rajan, 1982).

2.2.3 Histopathology

2.2.3.1 Cattle

Histologically the tumour was 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; Rajan *et al.* 1972; Nair, 1973; Damodaran *et al.* 1974; Balasubramaniam, 1975; Pospischil *et al.* 1979; Jayaraman *et al.* 1979; Sreekumaran, 1980; Rajan *et al.* 1981; Rajan and Sulochana, 1982; Chaudhary and Rao, 1982; Sreekumaran and Rajan, 1983; Muralimanohar, 1988). The primary tumour was considered as adenocarcinoma and it was clarified that it

progressed through a transitional stage to squamous cell carcinoma (Rajan, 1987).

2.2.3.2 Other species

There was no difference in the histological types of the tumours encountered in the different species of animals. Young <u>et al</u>. (1961) and Duncan <u>et al</u>. (1967) reported intranasal tumours of epithelial, origin in sheep. Yonemichi <u>et al</u>. (1978) grouped the intranasal tumours of sheep as papillary adenoma or adenocarcinoma. Njoku <u>et al</u>. (1978) also reported papillary growths of epithelial cells in the nasal cavity arising from the mucosa of the ethmoid bone in sheep. Rajan (1980) observed adenocarcinoma, papillary adenocarcinoma and squamous cell carcinoma in goats. Both adenocarcinoma and squamous cell carcinoma were encountered in pigs (Rajan <u>et al</u>. 1981). Mckinnon <u>et al</u>. (1982) classified enzootic nasal tumours of sheep histologically as adenomas, adenopapillomas or adenocarcinomas and regarded them as neoplasms of low grade malignancy because metastases had not been found. Wendt (1989) reported papillary adenoma, fibro-adenoma and adenocarcinoma from the nasal cavity of sheep. Low grade adenocarcinomas of nasal glands were reported in 38 goats by Heras <u>et al</u>. (1991). Gazquez <u>et al</u>. (1992) diagnosed nasal adenocarcinomas in a group of 25 verata goats. The

neoplasm contained two clearly defined zones, one cystic and the other compact.

2.3 Ultrastructural studies

Eventhough the light microscopic features of this neoplasm have been investigated in detail there are only a few reports on the ultrastructural features.

Yonemichi et al. (1978) studied the ultrastructure of the neoplasm of the ethmoid olfactory mucosa of sheep. The structure of the adenoma consisted of epithelial acini lined with cuboidal or columnar epithelium bounded by the basement membrane. The structure of the adenocarcinoma, with the exception of cytological changes in malignancy, was almost similar to that of adenoma. Infiltration of many plasma cells and lymphocytes and evidence of proliferation of fibroblasts were seen in the stroma. The nucleus of the tumour cell was spherical and had prominent nucleoli. Clumps of condensed chromatin were located adjacent to the nuclear envelope and were dispersed throughout the nucleoplasm. Adjacent cells frequently had peculiar interdigitation and desmosome-like structures. Only a small number of microvilli were observed at the cell surfaces.

The striking feature of the cytoplasm in the tumour cells of all sheep was the presence of secretory granules,

although their number varied widely. The golgi apparatus was well developed. Some of the cisternae were dilated. Some of the tumour cells had bundles of filaments in the cytoplasm. In the tumour cells a large number of coated vesicles and small vesicles of smooth surfaced endoplasmic reticulum were seen throughout the cytoplasm. The rough-surfaced endoplasmic reticulum developed in the basal portion of the cells.

Pospischil et al. (1979) described differentiating ultrastructural features of undifferentiated carcinoma, adenocarcinoma and squamous cell carcinoma of the ethmoid mucosa in cattle. Electron microscopically the undifferentiated carcinomas consisted of clusters of round to polyhedral cells with few interdigitations. Stromal tissue containing collagen fibrils could be found between the tumour cells without basement membrane formation. Two main cell types could be distinguished; light or electron lucent mainly with irregular shaped nucleus and a clumpy distribution of chromatin around the nuclear membrane. The content of the endoplasmic reticulum varied considerably. In the second type the endoplasmic reticulum was striking, occasionally forming whorls of concentrically arranged tubules. Some cells contained a few bundles of tonofibrils. Adjacent cell membranes were joined together by desmosomes which could not be seen in the cell types described first.

The ultrastructure of the well differentiated adenocarcinoma showed densely packed regular cylindrical cells with straight borders and few simple interdigitations. The cytoplasm had an electron lucent matrix containing abundant ribosomes and few straight elongated cisternae of mainly smooth surfaced endoplasmic reticulum. Occasionally the golgi apparatus was prominent. Mitochondria were scarce and contained distinct cristae without ramification. The close cellular contact was caused by numerous desmosomes and tight junctions between adjacent cell borders. Electron microscopically, squamous cell carcinoma consisted of elongated cells similar to those of the adenocarcinoma. The nucleus was irregularly shaped and the nucleoplasm was electron lucent. The cytoplasm contained abundant ribosomes, few mitochondria and sometimes a well developed golgi apparatus. Tonofibrils as well as myelin figures could be seen. The contents of the smooth and rough-surfaced endoplasmic reticulum varied. The adjacent cell membranes were joined by fully developed desmosomes.

Nair (1980) and Nair et al. (1987) described the ultrastructure of the neoplastic cells of the carcinoma of the mucosa of ethmoid in cattle and pigs. The cells showed a highly differentiated organellar components even with secretory granules. Mitochondria revealed numerous structural

aberrations. Nucleus was highly pleomorphic and predominantly euchromatinic. Occasionally nuclear bodies were encountered. The quantum of rough surfaced endoplasmic reticulum showed variation and depended on the anaplastic nature.

Heras et al. (1991) studied ultrastructural features of enzootic intranasal tumour in thirty-eight goats and confirmed the glandular character of the neoplasm. Tumour acini and tubules were composed of cuboidal or columnar cells. The cells had tight junctions, invagination with desmosomes between them, and a few microvilli on the apical surface. Proteinaceous structures like secretory granules, rough endoplasmic reticulum, and many mitochondria were characteristic components of the cytoplasm. The golgi apparatus was generally well developed and occasionally some bundles of filaments could be seen. Additionally, infrequent intracellular canaliculi and loose whorls, composed of a smooth endoplasmic reticulum, were observed. The nucleus of the neoplastic cell was round, slightly indented, and had prominent nucleoli. Clumps of condensed chromatin were situated both adjacent to the nuclear envelope and dispersed throughout the nucleoplasm. The stroma was infiltrated mainly by plasmacytes but also by lymphocytes and macrophages. Scanning electron microscopic observation of the surface of the tumour revealed many protrusions and depressions, but no

evidence of ciliated or goblet cells. The surface of the tumour was characterized by uniform, dome-shaped cells with some microvilli. Similar ultrastructural features were reported by Gazquez et al. (1992) in a group of 25 verata goats affected with adenocarcinoma of the ethmoid olfactory mucosa.

2.4 Etiopathology

2.4.1 General

Three aetiological factors namely, genetic predisposition, mycotoxin and infectious agents have been attributed to this condition. In a report of ethmoidal tumour in sheep in Germany, Cohrs (1952; 1953) postulated the possibility of a hereditary basis. Rubaj and Woloszyn (1967) attributed a genetic role in the enzootic adenopapilloma in the nasal cavity of sheep. Jayaraman et al. (1979) observed a definite genetic predisposition to sinus neoplasms in bovines in Tamil Nadu Livestock Farms. In contrast to this Young et al. (1961) and Duncan et al. (1967) could not establish a hereditary predisposition to this condition.

2.4.2 Role of aflatoxin in the etiopathogenesis of carcinoma of the mucosa of ethmoid

Among the seven sheep given aflatoxin contaminated groundnut feed two animals developed ethmoidal tumours, while

in one of the animals liver carcinoma was observed (Lewis *et al*. 1967).

Rajan *et al*. (1972) and Pospischil *et al*. (1979) reported occurrence of sinus neoplasms in animals associated with the presence of mycotoxins in the feed. They stated mycotoxins, particularly aflatoxin, as a carcinogenic factor has to be taken into account, though direct evidence for the involvement of these toxins in sinus neoplasm was lacking.

Adamson and Sieber (1979) observed one case of a nasal olfactory tumour in addition to nine liver carcinomas and single case of haemangioendotheliomas of the liver and pancreas among a population of 45 monkeys given aflatoxin B_1.

Goerttler *et al*. (1980) reported eight cases of nasal cavity tumours out of a total of 197 tumours in 483 test rats which had been exposed to aflatoxin B_1 transplacently or during early postnatal life.

Zhang (1981) observed epistaxis and circular movements among some of the breeding sows which consumed mouldy groundnut cake. The postmortem examination of the affected animals revealed tumourous growths in the ethmoidal sinuses and liver. The aflatoxin B_1 content of the groundnut cake assayed by thin layer chromatography was found to be in the range of 250-301 ppb.

In an epidemiological study in Dutch oil press workers, industrially exposed to aflatoxins, one case of nasal cancer was observed among 11 cancers in a group of 55 workers (Hayes *et al*. 1984).

Larsson *et al*. (1989) carried out *in vitro* study on the comparative capacity of bovine olfactory mucosa and liver to metabolise aflatoxin B_1 and reported that the nasal mucosa had much higher capacity than the liver to form lipid soluble, water soluble and tissue bound aflatoxin B_1 metabolites. High resolution microautoradiography showed a strong localization of tissue bound metabolites in the sustentacular cells in the apical portion of the olfactory surface epithelium and in Bowmen's glands in the olfactory lamina propria mucosa. The higher metabolism of aflatoxin B_1 in the nasal olfactory mucosa was attributed to its high cytochrome P5: cytochrome P450 ratio as compared to the liver.

Larsson *et al*. (1990) demonstrated a pronounced accumulation and retention of ^{3}H-labelled aflatoxin B_1 (^{3}H-AFB$_1$) in the nasal glands in C57 BI-mice using whole body autoradiography. At long survival intervals the labelling of the nasal glands was much higher than that of the liver.

Tjalve *et al*. (1992) reported that microsomal preparations of the bovine olfactory mucosa had a much higher

ability than liver microsomes to induce covalent binding of AFB_1 to calf thymus DNA and to microsomal proteins. The major DNA adduct formed was 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-aflatoxin B_1. Incubation of microsomal preparations of bovine nasal olfactory mucosa with glutathione (GSH) and cytosolic fractions of nasal mucosa resulted in decreased AFB_1-DNA binding A more pronounced decrease was observed when cytosolic fractions of mouse liver were added to the incubations. Supernatant preparations (900 g) of the bovine nasal olfactory mucosa incubated with AFB_1 were shown to have the capacity to induce a strong genotoxic response both as regards to the induction of gene mutations in Salmonella typhimurium TA 100 and the induction of sister chromatid exchanges in chinese hamster ovary cells, whereas the preparation of bovine liver (900 g) showed much lower ability to induce these effects.

2.4.3 Infectious agents

The etiology of this tumour has not been established but some evidence suggests an infectious cause. Tumours had been reproduced by the intranasal instillation of either a crude suspension of tumour tissue or bacteria-free or cell free filtrates (Cohrs, 1952; 1953). Miliary mucosal proliferations were observed in one ram 10 months after the aerosol inoculation of antibiotic treated tumour filtrate and, in another ram, a solitary ethmoid carcinoma was found

14 months after aerosol inoculation of untreated tumour infiltrate (Njoku *et al*. 1978).

Yonemichi *et al*. (1978) detected viral particles, which were morphologically similar to visna-maedi virus in all of the 12 intranasal tumours of the ethmoid olfactory mucosa of sheep and 3 of 4 cultures examined. The particles had an eccentrically located electrondense core and numerous spikes on their surfaces. Viral particles similar to Herpes virus were also detected in culture. Sulochana *et al*. (1982) isolated seven haemagglutinating agents from the tumour tissues by chicken embryo inoculation. Nair *et al*. (1981) observed budding viral particles and enveloped virus in tumours of the mucosa of the ethmoid in cattle. The particles were numerous and more or less uniform in size (95-140 nm) and had identical morphological features. They contained electrondense spots, possibly nucleocapsids and were covered with peplomer like structures. Mckinnon *et al*. (1982) observed retrovirus like particles within the cytoplasm of tumour cells of one sheep out of three subjected to electronmicroscopic examination.

Moreno-Lopez *et al*. (1989) isolated a herpes virus from the tumours of the ethmoidal mucosa in two of the three head of cattle in the state of Kerala. The virus designated M49 was cytopathic for a variety of cultured bovine and

porcine cells and it did not kill suckling mice or chicken embryos. Experimental infection of goats with the M40 virus did not result in development of tumours.

Numerous retroviral-like particles were found in the apical surface in six of the eight tumours in goats. They were located in extracellular spaces and between microvilli close to the apical cell membranes. They were round in shape, about 90-135 nm in diameter, and presented an electron-dense nucleoids, centrally or eccentrically located, some of which were bar or annular shaped. The core was surrounded by an electron lucent zone and outer spiky unit membrane (Heras et al. 1991).

Heras et al. (1992) reproduced intranasal tumours by the intranasal/intrasinusal injection with 20 fold concentrated nasal fluids in kids, collected from natural cases of enzootic intranasal tumours of goats.

Gazquez et al. (1992) observed viral particles, which were morphologically similar to Visna-Maedi virus, in ethmoid tumour tissue in verata goats. The particles were present in both extracellular and intracellular spaces and always in necrotic cells. The particles had an eccentrically located electrondense core. The diameter of the virus was about 90 nm and it showed an envelope with numerous spikes on the surface.

Heras *et al*. (1993) isolated retrovirus from the nasal fluids of two sheep with symptoms of enzootic intranasal tumour and from a sheep with pulmonary adenomatosis. They also examined this virus by SDS-polyacrylamide-gel-electrophoresis and Western blotting using a goat antiserum to Mason-pfizer Monkey virus P27. The antiserum gave clear reaction with a polypeptide of MV 25,000 in pellets from all samples. They further suggested that demonstration of a MV 25,000 protein is evidence for the association of a type-D-like retrovirus with this tumour.

Heras *et al*. (1995) described successful experimental transmission of enzootic intranasal tumour (EIT) from goat to goat. Ten kids, less than 48 hours old, from a flock free of the disease and seronegative for ruminant lentiviruses were inoculated intranasally or intrasinusally with either nasal fluid from goats with naturally occurring EIT or EIT retrovirus concentrated from such fluids. EIT was induced in three kids after 12 to 24 months. The EIT retrovirus was demonstrated in tumour materal from each of the three kids by western blotting and electron microscopy.

2.5 Aflatoxins: An overview

2.5.1 General

The aflatoxins were isolated from peanut meal in 1961

(Schoental, 1961) during the investigation of an epizootic of "Turkey X" disease in England. It was shown that these toxins were metabolites of some strains of Aspergillus flavus and that they were the etiological agents of the disease in turkeys (Blount, 1961).

The aflatoxins are a family of closely related chemical compounds. Toxigenic strains of Aspergillus flavus and Aspergillus parasiticus growing on corn, peanuts, cottonseed, and several other oilseeds and nut or food products may produce several related bisfuranocoumarin compounds known as aflatoxins. The four major aflatoxins are B_1, B_2, G_1 and G_2. Individual fractions are so designated because of their fluorescence and R_F values on thin layer chematography plates. Cattle and other farm animals are exposed to aflatoxins primarily via contaminated cereals, molasses and other concentrated feed-stuffs. The toxin production is favoured by warm and moist environments and aflatoxin contamination is therefore most common in tropical and subtropical latitudes (Patterson, 1973).

2.5.2 Toxicity and carcinogenecity

2.5.2.1 Cattle

The first report of poisoning in cattle by Brazilian peanut (groundnut) meal was that of Loosmore and Markson

(1961). Calves, 3-9 months of age, had eaten for at least six weeks a compounded food containing 15 per cent Brazilian peanut meal. The livers of animals exhibited areas of fibrosis and biliary proliferation.

Clegg and Bryson (1962) reported an outbreak occurring at about the same time in cattle of 1.5-2 years old. The affected animals showed symptoms and lesions similar to those described by Loosmore and Markson (1961).

Allcroft and Lewis (1963) investigated experimental poisoning of calves and older cattle by compounded food containing 2.0 ppm of aflatoxin. Progressive biliary proliferation, an increase in connective tissue, and some degeneration of centrilobular hepatic cells were observed after 4 months of exposure. The liver of animals killed after 11 weeks on the diet had complete disruption of lobular pattern and an increase of connective tissue which coursed throughout the liver lobule; many of the central veins were partially or completely obliterated by fibrous tissue. Throughout the lobule, parenchymal cells were isolated by strands of connective tissue. Structures resembling small bile ducts were scattered throughout the lobule, and there was a mild necrosis and pleomorphism of parenchymal cells located away from the periportal area, but mitotic figures were not

seen in either the parenchymal or biliary cells of the material examined.

Pier (1981) reported icterus and haemorrhages of the mucosal surfaces in a calf given 0.5 mg aflatoxin B_1 per kg daily. At necropsy, the liver was usually pale to yellow and the gall bladder was enlarged. Histopathologic examination revealed fatty changes in the hepatocytes, periportal fibrosis and extensive bile duct proliferation.

Maryamma et al. (1989) detected aflatoxin B_1, G_1 and M_1 in the skeletal muscle of the thigh, liver, kidney and bile collected from carcasses of nine cows analysed. They also detected aflatoxin B_1 in four samples out of 90 samples of cow's milk analysed.

Aflatoxin B_1, G_1 and M_1 were detected in the blood and urine samples of four out of nine cows suspected to suffer from spontaneous aflatoxicosis. Aflatoxin M_1 was present in the milk of three cows. Blood and urine of one bull calf given aflatoxin at the rate of 240 ug/kg bodyweight for a period of two weeks revealed the presence of aflatoxin B_1 and M_1 (Maryamma et al. 1991).

2.5.2.2 Pig

2.5.2.2.1 Acute toxicity

In acute cases normal handling of pigs led to massive subcutaneous haemorrhage (Hauser et al. 1971). Gross haemorrhage occurred in many parts of the body, especially in the ham areas. The increased pressure in the gluteal muscles led to ataxia, and animals presented a dog-like sitting posture with tachypnoea and panting (Edds, 1979).

2.5.2.2.1.1 Gross lesions

The principal lesions were liver damage and haemorrhage. The liver was swollen, congested and friable; occasional petechiae were visible on the liver surface, and animals surviving beyond 24 hours had ascites and hydrothorax. The gall bladder was oedematous and the mucosa was petechiated and echymotic. The gall bladder was atrophic in some cases (Burnside et al. 1957; Loosmore and Harding, 1961; Annau et al. 1964; Wilson et al. 1967; Gagne et al. 1968; Keyl and Booth, 1971; Edds, 1979; Lu and Ho, 1982; Osuna and Edds, 1982; Nair,1986 and Rajan et al. 1989).

Microscopically early liver changes were seen in about three hours. Disorganization of hepatocytes with fatty changes occurred. By six hours, the cells were swollen and centrilobular congestion and necrosis were evident. These

changes were accompanied by karyorrhexis and pyknosis. Infiltration by neutrophils and lymphocytes occurred by 12 hours in the necrotic areas. Congestion continued to increase and was accompanied by leucocyte infiltration. Bile duct hyperplasia and bile casts in canaliculi were also evident. These changes were in accordance with the observations made by Sippel et al. 1953; Burnside et al. 1957; Loosmore and Harding, 1961; Allcroft et al. 1961; Harding et al. 1963; Wilson et al. 1967; Patterson, 1973; Armbrecht, 1978; Miller et al. 1982, Nair, 1986 and Rajan et al. 1989.

2.5.2.2.2 Subacute toxicity

Lower dosages extended over a few weeks produced many of the features described. However, reticulum and collagen fibre proliferation and bile duct hyperplasia were observed along with intracellular glycogen depletion and lipid infiltration (Shalkop et al. 1967; Sisk et al. 1968).

2.5.2.2.3 Chronic toxicity

2.5.2.2.3.1 Gross lesions

In chronic toxicity, grossly the liver developed hard fibrous texture and the entire organ was dark yellow with scattered raised brown lumps and coarse nodularity. The gall bladder was atrophic and the wall was oedematous. The bile

was sometimes dark and had a thick consistency (Wilson *et al*. 1967; Iwasaki *et al*. 1974; Armbrecht, 1978; Nair, 1986 and Rajan *et al*. 1989).

2.5.2.2.3.2 Microscopic lesions

Microscopically the kind of liver lesion and its degree of extensiveness were dependent on the tissue and dosage rate relationship that preceeded the examination. There was pronounced centrilobular necrosis. The cytoplasm of the cells was granular and vacuolated or completely absent. There was pronounced karyomegaly of the surviving hepatic cells. Increased proliferation of reticular fibres, pseudolobulation with regenerating islands of hepatic cells, bile duct proliferation and progressive increase in collagen fibres were also evident. Most of the regenerating cells contained neutral fat globules. As the lesions progressed there were numerous foci of lymphocytes and few eosinophils and large nodules of regenerating liver cells with a collagen capsule extending throughout the parenchyma and above the liver surface (Sippel *et al*. 1953; Burnside *et al*. 1957; Loosmore and Harding, 1961; Harding *et al*. 1963; Shalkop *et al*. 1967; Sisk *et al*. 1968; Miller *et al*. 1981; Nair, 1986 and Rajan *et al*. 1989). Hepatocellular carcinoma was observed by Shalkop and Armbrecht (1974) and Chauhan *et al*. (1984).

Maryamma *et al*. (1992) investigated the influence of dietary protein levels on aflatoxin induced hepatocarcinogenesis in pigs. Hepatic carcinoma was recorded in 66 per cent of the animals that survived one year on high protein diet while incidence of hepatic carcinoma was only 16 per cent in the low protein group.

2.5.2.2.4 Aflatoxin residues

Maryamma *et al*. (1989) detected aflatoxin residues in the liver, kidney and skeletal muscles of three pigs given aflatoxin in feed at the level of 25 ug aflatoxin B_1 per kg bodyweight for a period of 36 weeks. The animals were sacrificed after a toxin-free period of five weeks.

Aflatoxin B_1, G_1 and M_1 were detected in the tissues of three pigs out of seven examined suffering from clinical aflatoxicosis. Blood and urine of these three pigs also revealed the presence of aflatoxin B_1 and M_1 (Maryamma *et al*. 1991).

2.5.2.3 Rat

Following the recognition of aflatoxin poisoning among farm animals, the rat has been used extensively to study the acute toxicity and carcinogenicity of the aflatoxins.

2.5.2.3.1 Toxicity

In most experiments, the rats usually died between three and seven days after exposure; mature females were considerably more resistant (Burnside et al. 1957). Lesions induced by an LD50 dose of aflatoxin B_1 included periportal zone of necrosis accompanied by marked biliary proliferation. These lesions developed three days post-treatment. Two weeks after treatment, prominent biliary proliferation persisted but the striking feature was the development of enlarged hyperchromatic nuclei. Biliary and oval cell proliferation of a magnitude that distorted the normal lobular pattern was seen in some of the animals after one month. Many of the parenchymal cells had large bizarre nuclei, some of which were located in an occasional small regenerative nodule (Butler and Barnes, 1963; Butler, 1964; Newberne et al. 1964).

2.5.2.3.2 Carcinogenesis

The first report of the carcinogenic effect of aflatoxin contaminated peanut meal was that of Lancaster et al. (1961). The peanut meal responsible for field outbreaks of aflatoxicosis in poultry and contained 0.4 ppm of aflatoxin (Barnes and Butler, 1964), was shown to induce hepatic carcinomas in rats. The incidence of the tumour was 100 per cent when peanut meal containing 4-5 ppm of aflatoxin

was fed. Aflatoxin levels as low as 0.7-0.8 ppm resulted in an incidence of 100 per cent also, but there was a longer latent period (upto 82 weeks). At these low levels, the early lesions were much less obvious and were seen only after many weeks on the diet. Lesions included mild oval cell proliferation and a few parenchymal cells with enlarged nuclei. At a later stage, when carcinomas were observed, there was no evidence of cirrhosis (Butler and Barnes, 1963; Newberne *et al*. 1964).

When purified aflatoxin became available, it was confirmed that the carcinogenic action of the peanut meal was a result of contamination with aflatoxin (Barnes and Butler, 1964). Subsequently, Carnaghan, 1967; Newberne and Wogan, 1968; Newberne and Butler, 1969; Novi, 1977; Morimura *et al*. 1990 and Gopal Naidu and Sehgal, 1992 reported aflatoxin B_1 induced hepatocellular carcinoma in rats.

2.5.2.3.3 Electron-microscopic study

Electron microscopic study of liver carcinogenesis in rats after aflatoxin B_1 administration revealed two patterns of modification in the hepatocytes depending on their location within the liver lobule. While periportal hepatocytes rapidly degenerated, more peripherally located parenchymal cells first showed a high proliferation of endoplasmic reticulum, of the

type observed in hepatocytes actively engaged in drug metabolism, and then peripheral hepatocytes underwent degenerative changes of a pattern similar to that observed in periportal hepatocytes. Endoplasmic reticulum appeared to be affected first, then depletion of glycogen and alteration of the golgi zones occurred, followed by damage to mitochondria and cell membrane (Novi, 1977).

2.5.2.4 Non-human primates

Monkeys have been shown to be susceptible to the acute toxicity of both purified aflatoxin and contaminated peanut meal. Doses of 500 ug for 18 days followed by doses of 1 mg/day to Rhesus monkeys resulted in deaths at 32 and 34 days. Histologically, the liver showed fatty infiltration, biliary proliferation, and portal fibrosis (Madhavan et al. 1965). Cuthbertson et al. (1967) studied the effects of contaminated peanut meal on cynomologus monkeys and described liver cell damage and biliary proliferation at dietary levels of 5 ppm of aflatoxin. At lower dietary levels (1.8 ppm of aflatoxin) animals survived three years. One animal had a coarse nodular cirrhosis, while the other monkey exhibited irregular size of parenchymal cell nuclei. Alpert and Serck-Hanssen (1970) and Deo et al. (1970) also recorded similar aflatoxin induced hepatic lesions in African and Rhesus monkeys respectively.

Adamson *et al*. (1970; 1973) evaluated carcinogenic effects of aflatoxin B_1 in long term studies using Rhesus monkeys and reported that 3 of 42 monkeys (7%) necropsied after receiving treatment for longer than 2 years developed malignant liver tumours. Sieber *et al*. (1979) reported an update of this study and recorded an overall tumour incidence of 28 per cent. Five of the neoplasms were primary liver tumours, and 2 cases of osteogenic sarcoma were found. Other tumours diagnosed were 6 carcinomas of the gall bladder or bile duct, 3 tumours of the pancreas or its ducts and one papillary Grade I carcinoma of the urinary bladder.

Mathur *et al*. (1991) investigated the effect of dietary restriction of protein on monkeys fed 1.0 ppm of aflatoxin B_1 in their daily diet. They observed that by 38-40 weeks, liver of monkeys in the low protein group exhibited large areas of hepatocyte necrosis, whereas those on high protein diet showed neoplastic nodules in the liver.

2.5.2.5 Man

There was strong circumstantial evidence linking aflatoxin intake in the human diet and the occurrence of hepatic carcinoma in man (Peers *et al*. 1976). For this reason there is considerable anxiety concerning entry of aflatoxin B_1 or M_1 into the human food supply. Apparently the majority of

human exposure is from direct ingestion of plant products rather than animal products (WHO, 1979). Some human exposure may occur from routes other than ingestion, e.g., inhalation and skin and mucous membrane contact in environment such as grain storage bins, where the air is laiden with aflatoxin contaminated grain dust, constitute a potential hazard (WHO, 1979).

The African and Asian data on the possible etiology of aflatoxin in human primary hepatic carcinoma cannot preclude the co-existence of hepatitis B virus in those populations. But research data predicted hepatoma rate due to aflatoxin alone to be far above that was actually observed due to all other causes in South East United States where hepatitis B infection does not exist (Bruce, 1990).

Cusumano (1991) screened sera from patients with lung cancer and from healthy donors for the presence of aflatoxins. He recorded significant difference in the levels of aflatoxins between the two groups. Only 1 of the neoplastic patients with aflatoxins in the serum was a smoker.

2.6 *In vitro* cytotoxicity and carcinogenicity

2.6.1 Aflatoxin B_1

Smith (1963) briefly referred to vacuolation of monkey-kidney cells growing in monolayers after aflatoxin B_1

was incorporated in the growth medium and to inhibition of growth and cell destruction with higher concentrations of the toxin. Juhasz and Greczi (1964) reported that extracts of groundnut meal samples contaminated with aflatoxin destroyed calf-kidney cells in culture. Legator and Withrow (1964) noted that aflatoxin suppressed mitosis in humans diploid and heteroploid embryo lung cells in tissue culture. Legator _et al_. (1965) using heteroploid embryo lung cells observed that both crude aflatoxin and aflatoxin B_1 suppressed the synthesis of DNA and inhibited mitosis. Giant cell formation occurred and it was suggested that this could be accounted for by the enlargement of non-dividing cells.

Zuckerman _et al_. (1966) investigated the effect of purified aflatoxin B_1 on the liver cells. Marked changes were observed after 16 hours exposure of cells to 10 ug/ml of aflatoxin B_1. The overall dimensions of the hepatic cells were reduced. There was complete loss of orange (RNA) fluorescence from the cytoplasm, and the cytoplasm became opaque and fluorescenced deep green. The nucleus also showed marked changes and the death of the cells followed.

Engelbrecht and Purchase (1969) reported that aflatoxin produced specific cytological alterations in African green monkey kidney epithelial cell cultures after 24 and 48 hours of exposure. There was decrease in mitosis. The

fragmentation of the nucleolus, as well as nonspecific changes such as cytoplasmic vacuolation and pyknosis or karyorrhexis were also observed.

Toyoshima et al. (1970) made an attempt to transform NLW cells, derived from the liver of a newborn Wistar rats, by means of aflatoxin B_1 in vitro. After 161 days cultivation through twelve subcultures in Eagle's minimum essential medium supplemented with 10 per cent calf serum, the cells were exposed to aflatoxin B_1 for 5 to 7 days at the concentration ranging from 10 to 0.01 ppm in the medium, and further cultivation was carried out in the maintenance medium. Delayed cytotoxic effect was observed for several weeks after the exposure, most remarkably at two weeks, then survived cells gradually presented morphological transformation in all the experimental groups. Growth of fibrosarcoma was recognised in wistar rats transplanted with the cells cultured for more than 87 days after the exposure.

Umeda (1971) showed that primary rat liver parenchymal cells were more susceptible in their reaction to aflatoxin B_1 than other cells. Cardeilhac et al. (1972) prepared tracheal organ cultures from day-old chick and determined lethal concentration 50 (LC 50) for 9 mycotoxins. LC 50 for aflatoxin B_1 was found to be 0.2 ug/ml.

Williams <u>et al</u>. (1973) exposed epithelial like cells from rat liver to aflatoxin B_1, dimethylnitrosamine, N-nitrosomethylurea, N-hydroxy-N-2-fluorenylacetamide or 7-12-dimethylbenz(a)anthracene. Microscopic observations revealed several morphological changes like enlarged and more prominent and numerous nucleoli, pleomorphism, overlapping in almost all of the treated sublines. The injection of 5 to 20 x 10^6 treated cells into new born or x-irradiated syngeneic rats yielded tumours, usually after latent period of 2 to 8 months on an average of 7-8 months. The tumours were diagnosed as carcinomas.

Aflatoxin B_1 treatment of long-term culture initiated from primary liver cultures resulted in morphological transformation accompanied by an increased growth in soft agar and an increased frequency of 8-azaguanine-resistant mutants (Williams, 1976).

Coulombe <u>et al</u>. (1986) used short-term tracheal explant cultures from rabbits to study the metabolism of the carcinogen aflatoxin B_1 (AFB_1) and to determine the cell types that are susceptible to damage by AFB_1 and their relative contents of mono-oxygenase enzymes. Ultrastructural evaluation of cultured trachea showed degenerative changes exclusively in non-ciliated secretary cells after 4 hours in culture. Extensive non-ciliated secretory cell necrosis was

evident by 12 hours. Ciliated cells did not show degenerative changes until 12 hours and appeared much more viable after 24 hours exposure to AFB_1 relative to non-ciliated cells. Tracheal sections stained to demonstrate rabbit lung cytochrome P-450, forms 2 and 5, and cytochrome P-450 reduced nicotin-amide adenine dinucleotide phosphate reductase by immunoperoxidase technique showed intense staining selectively within non-ciliated cells.

Wilson <u>et</u> <u>al</u>. (1990) compared the response to aflatoxin B_1 (AFB_1) in cultured tracheal epithelium from species with abundant (rabbit and hamster) and scarce (rat and monkey) distributions of smooth endoplasmic reticulum in non-ciliated tracheal epithelial cells. Cultures derived from rabbits were most active in metabolic conversion and formation of AFB_1-DNA adducts, followed by those from hamster, rats and monkeys. Rabbit tracheal epithelium formed a significantly greater proportion of glutathione conjugates, while that from hamster formed a greater amount of AFB_1-dihydrodiol, compared to rats and monkeys. The monkey formed significantly greater proportions of aflatoxin Q_1. The rabbit formed more aflatoxicol as compared to the other species. There was selective degeneration and accumulation of labelled material in non-ciliated cells in both rabbits and hamsters but not in rats or monkeys. Explants from rabbit trachea were much

more susceptible to cytotoxic injury and had higher autoradiographic grain densities than explants from hamsters.

Morimura et al. (1990) established cell lines from Aflatoxin B_1 induced rat hepatoma (Kagura-1 and Kagura-2). The chromosome counts were 34-45 and 40-130 respectively in the tumour cells of both the cell lines. The hepatoma cell lines expressed the two tumour markers glutathione-s-transferase-P and gamma glutamyl transpeptidase.

2.6.2 Other chemical carcinogens

Malignant transformation *in vitro* by chemical carcinogens was reported first by Barwald and Sachs (1965) who exposed culture cells derived from whole hamster embryo to carcinogenic hydrocarbons.

DiPaolo and Donovan (1967) produced tumorigenic cell lines from whole hamster embryo fibroblast cultures treated with polycyclic hydrocarbons. The tumorigenic cells were similar to untreated control cells in cellular and clonal morphology but were aneuploid and grew to saturation densities 10-20 times greater than controls.

Production of epithelial tumours *in vitro* was attempted by Heidelberger and Iype (1967) using culture of untreated C3H mouse prostates. Following treatment of cells

with methylcholanthrene for 1 to 6 days the cultures were then maintained for a further 2-3 weeks in carcinogenic free medium. Transformed colonies then appeared as heaped up, randomly orientated cells with intense basophilic staining against a confluent background of untransformed cells.

Attempts with 4-nitroquinoline-1-oxide were done by Kuroki and Sato (1968) and by Kakunaga and Kamahora (1968) independently, and both were able to transform hamaster embryonic cells in culture into malignant cells with this chemical carcinogen.

Electron microscopic studies on cultured rat-liver cells transformed by 4-nitroquonoline-1-oxide revealed vacuolization of golgi bodies, irregular nucleus, and swelling of round endoplasmic reticulum and mitochondria (Koshiba *et al*. 1970).

The primary cultures from the 7-12-dimethyl benzanthracene exposed tracheal explants of rats and the subsequently developed cell lines all exhibited morphological characteristics of keratinizing squamous epithelium. These characteristics included epitheloid cell morphology, multilayering and sloughing of orangeophilic squamous cells, and the presence of keratohyalin granules (Marchok *et al*. 1977).

Slaga *et al*. (1978) used cultures of epidermal cells obtained from new born BALB/C mice to study *in vitro* transformation of epithelial cells with N-methyl-N-nitro-N-nitrosoguanidine, 3-methylcholanthrene and 3-methyl cholanthrene-11, 12-epoxide. The treated cells were characterized by rapid growth, loss of visible keratinization and subculturability, having been passaged 12 times, in contrast to untreated cells, which were not subculturable. Electron microscopic studies did not reveal any true desmosomes, but junctional complexes were present in all of the cell strains examined. The injection of 10^6 cells from the various cell strains into athymic "nude" or sygeneic mice resulted in rapidly growing solid tumours, which were characterized as highly anaplastic "undifferentiated" tumours.

In vitro exposure of tracheal epithelium to the tumour promoting agent 12-0-tetradecanoyl-phorbol-13-acetate resulted in a marked increase in growth capacity. The growth changes were manifested in an increased rate of cell division and growth in primary cultures and in the establishment of permanent epithelial cell lines. Such changes did not occur in control cultures. Fourteen of the cell lines inoculated into immunosuppressed recipients, and all were nontumorigenic (Steele *et al*. 1978).

2.6.3 Transformation markers

2.6.3.1 Gamma glutamyl transpeptidase (GGT)

2.6.3.1.1 General

The enzyme GGT is present in many tissues (Glenner *et al*. 1962; Rutenberg *et al*. 1969). This enzyme is involved in the drug detoxification mechanism. The increased activity was reported in foetal and neonatal liver but relatively low in adult organs. GGT activity was demonstrated in precancerous lesions and carcinoma of liver (Fiala *et al*. 1972; Fiala and Fiala, 1973; Fiala *et al*. 1976), oral, pharyngeal and laryngeal mucosa in human beings (Calderson and Solt, 1985) and recently in ethmoid carcinoma of cattle (Gangadharan and Rajan, 1992).

2.6.3.1.2 Marker in chemical carcinogenesis and cells in culture

The expression of GGT was a common finding in liver lesions induced by aflatoxin B_1, a genotoxic carcinogen (Kalengayi *et al*. 1975). Because of this increase in GGT activity in carcinogen induced lesions interest has focussed on using GGT as a marker for neoplastic cells in culture.

San *et al*. (1979) detected GGT activity by cytochemical assay in tumorigenic liver cell lines as well as

in the lines derived from hepatocarcinomas, whereas nontumorigenic lines from normal rat liver were consistently negative. Similar observations were made by Huberman et al. (1979) and Morimura et al. (1990).

2.6.3.2 Colony forming efficiency (CFE) in soft agar

Normal cells do not form colonies when suspended as monodispersed suspensions in media converted to a soft gel by the inclusion of 0.3 to 0.5 per cent agar. But transformed cells may acquire the ability to grow progressively and form colonies in soft agar medium (Macpherson and Montagnier, 1964).

Growth in soft agar and correlation with tumorigenicity had been reported in both fibroblasts (Macpherson and Montagnier, 1964; DiPaolo and Donovan, 1967; Kakunaga and Kamahora, 1968; Macpherson, 1970; DiMayorca et al. 1973; Kakunaga, 1973; Reznikoff et al. 1973; Freedman and Shin, 1974; Pollack et al. 1974; Styles, 1977; Barrett et al. 1979 and San et al. 1979) and epithelial cells (Borek, 1972; Marshal et al. 1977; Montesano et al. 1977; Colburn et al. 1978; Knowles and Franks, 1978; Marchok et al. 1978; San et al. 1979 and Lin et al. 1990 and 1993).

San *et al*. (1979) reported that tumorigenic liver cell lines designated ARL6 and ARL17 exceptionally had low colony forming efficiency and were found to be tumorigenic.

2.6.3.3 Colony forming efficiency in 10 and 1 per cent serum media

Reduced serum requirement for growth had been associated with tumorigenicity of fibroblast like cells (Holley and Kiernan, 1968; Dulbecco, 1970; Smith *et al*. 1971; Oshiro and DiPaolo, 1973; Bertram, 1977; Barrett *et al*. 1979 and San *et al*. 1979). However, the dependency on serum for growth was reported to be less for epithelial cells in comparison to cells of mesenchymal origin (Castor, 1968; Dulbecco, 1970). In confirmation of this observation Dulbecco (1970), Jainchill and Todaro (1970) and San *et al*. (1979) reported that colony forming efficiency of tumorigenic epithelial like cells was not as inhibited in 1 per cent serum medium as compared to non-tumorigenic cells. They further reported that differences in the relative colony forming efficiencies in one and 10 per cent serum of non-tumorigenic and tumorigenic lines were not significant.

Materials and Methods

MATERIALS AND METHODS

3.1 Production of aflatoxin

Aspergillus flavus a known toxigenic strain obtained from the Central Food and Technological Research Institute, Mysore was used to produce aflatoxin B_1 (AFB_1) in the laboratory by the method of Shotwell *et al*. (1966) with minor modifications with respect to extraction solvent. A crude product containing aflatoxins was isolated by acetone extraction and precipitation with hexane from concentrated solution. AFB_1 was purified and analyzed by thin layer chromatography employing minimum fluorescence extinction method (AOAC, 1975).

3.2 Cell free ethmoid tumour extract

Among the tumour bearing animals, representative samples of tumour tissue were collected at random from animals bearing fresh healthy tumour under sterile conditions in Hanks Balanced Salt Solution (HBSS) supplemented with 200 IU of penicillin and 100 ug of streptomycin and 100 units of mycostatin (Sigma) per ml. Tissues were minced into very small pieces with a sterile pair of scissors and transferred to sterile grinding tube of mechanical homogenizer to prepare a 10-15 per cent suspension. After 3 cycles of freezing and

thawing the suspension was clarified by centrifugation at 2000 g for 15 minutes at 4°C and used for transmission studies.

3.3 Experimental design

Thirty-two, clinically healthy, Large White Yorkshire piglings of either sex of 2-3 months age were procured from the University Pig Breeding Farm, Mannuthy and divided into four groups of 8 each. The pigs were kept under observation for two weeks before commencement of the experiment during which period they were screened for common parasitic diseases and other ailments. The experimental animals were fed standard pig diet given at the farm. Also the farm schedule of feeding was followed. Every consignment of the feed was screened for the presence of aflatoxin before feeding it to the pigs.

The groupwise treatments were as shown below:

Group I: Intravenous administration (ear vein) of AFB_1 (0.070 mg/kg b.wt/inoculum) dissolved in 250 µl of dimethyl sulfoxide (DMSO), at weekly intervals for 6 months.

Group II: AFB_1 was administered as mentioned above. After 3 months of treatment with aflatoxin they were inoculated intranasally with 2 ml of cell free ethmoid tumour extract per

pigling (6 doses at 15 days interval), by intubation using fine bore polythene tube. Before intubation, nasal mucosa was desensitized by spraying 0.2 to 0.5 ml of 4 per cent Lignocaine hydrochloride (Gesicain 4%, Topical, S.G. Pharmaceuticals) into the nasal cavity to prevent sneezing.

Group III: The piglings in this group were inoculated intranasally with only cell free ethmoid tumour extract at the rate of 2 ml/pigling at 15 days interval for 3 months as described above.

Group IV: The animals in this group were administered DMSO (250 µl) through the intranasal route at 15 days interval for three months.

The animals in all the four groups were closely observed daily, for the appearance of clinical signs of illness, if any. The body weight of the animals was recorded before commencement of the experiment and subsequently at monthly intervals during the treatment period of six months followed by at three month interval after the termination of respective treatment.. After six months of the respective treatment in all the groups, two randomly selected pigs were sacrificed at three months interval and subjected to detailed necropsy examination. Appropriate tissue specimens were

collected, in duplicate, for light and electronmicroscopic investigation.

3.4 Aflatoxin residues in blood and ethmoid mucosa

The blood collected at 3, 7, 10, 15 and 30 days after the discontinuation of the respective treatments and thereafter at three months interval and the ethmoid mucosa collected from experimental pigs sacrificed at specified intervals were subjected to aflatoxin B_1 estimation by adopting the method recommended by Stubblefield and Shotwell (1981). The blood and ethmoid mucosa samples collected at various intervals were analysed for Aflatoxin M_1 by the method of International Dairy Federation (1991).

3.5 Pathological studies

Detailed postmortem examination was performed on all animals sacrificed during the course of the experiment. The gross lesions were recorded.

For light microscopy, representative samples of tissues collected from various organs of all the animals were fixed in 10 per cent neutral buffered formalin. These were processed by routine paraffin embedding technique and sections cut at 4-5 µ thickness were stained with Harri's haematoxylin and Eosin (Luna, 1968).

For electronmicroscopic examination, selected areas from the ethmoid mucosa of the experimental pigs were removed and cut in blocks of 1 cubic mm size in cold 2.5 per cent glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and transferred in same fixative at 4°C. Pieces were post-fixed with 1 per cent osmium tetroxide (in 0.1 M phosphate buffer, pH 7.4), dehydrated through a series of graded acetone and embedded in Polar-Bed (Bio-Rad). Ultrathin sections were cut with glass knives using Reichert microtome and routinely stained with uranyl acetate and lead citrate, and examined with Hitachi-H-600 A electronmicroscope at 50 KV.

3.6 *In vitro* carcinogenicity

3.6.1 Tissue culture growth medium

Various growth media were evaluated to cultivate and maintain the bovine ethmoid turbinate mucosal cells in cultures during the course of the study. The survival of bovine ethmo-turbinate mucosal cells was found to be most consistent in growth medium based on the following formula:

Dulbecco's Modified Eagle's Medium (Sigma)	45%
Ham's Nutrient Mixture, F-12 (Sigma)	45%
Foetal calf serum	10%
Penicillin	100 units/ml
Streptomycin sulphate	50 µg/ml
Nystatin (Sigma)	100 units/ml

3.6.2 Aflatoxin

Purified AFB_1 (Sigma) was dissolved in DMSO and diluted with growth medium to give two separate final concentrations of 0.05 µg and 0.1 µg per ml of the medium. Growth medium with DMSO alone was used as control.

3.6.3 Culture cells

The nasal olfactory mucosa was obtained from one-day-old male bovine calf born at the University Livestock Farm, Mannuthy. The ethmoid region was reached by sawing the head of the calf transversally in two planes one just in front of the eyes and one just behind the eyes. The ethmoturbinate region was punched out with the lining mucosa using a scissor and rat-toothed forceps. The punched out - ethmoturbinates were immediately placed in Hank's Balanced Salt Solution (HBSS) containing 4 times the normal concentration of antibiotics and Nystatin (100 units/ml).

To prepare the primary culture, the mucosa covering the ethmoturbinates was peeled off and washed three times using HBSS. After cutting the tissue into very small pieces, it was subjected to trypsinization as per the method described by Zuckerman _et al_. (1966). The dispersed cells thus obtained were suspended in growth medium in prescription bottles with loose rubber stoppers, and incubated at 37°C in an atmosphere

of O_2 : CO_2 (95:5) and a relative humidity of approximately 100 per cent. The medium was usually renewed twice a week and subculture of the cells was done at 15 days interval with 0.25 per cent Trypsin-Versene-Glucose (TVG) solution in phosphate buffered saline free from Ca and Mg salts.

3.6.4 Differential enzymatic digestion

When the bovine ethmo-turbinate mucosa was cultured, the monolayer consisted of epithelial and fibroblast-like cells. To have a comparatively pure culture of epithelial and fibroblast-like cells the mixed culture was subjected to selective enzyme treatment as described by Al-Yaman and Willenborg (1984).

Cultures were washed twice with trypsin diluent and then subjected to a series of 1-2 minutes exposures to trypsin-versene-glucose solution (final concentrations of trypsin-versene were 0.25 per cent and 0.1 per cent respectively) followed by washing in trypsin diluent. By repeating this procedure, most of the fibroblasts were removed and epithelial clusters were left to grow. The epithelial islands were rinsed three times with the complete medium, fed, and incubated at 37°C. This procedure sometimes needed to be repeated 2-3 times a week before a population of epithelial cells devoid of fibroblasts was established.

3.6.5 Exposure to AFB_1

The mixed culture, epithelial and fibroblast-like cells were cultivated for 60 days through four subcultures in the growth medium. These cells were then exposed to 0.05 µg and 0.1 µg of AFB_1 per ml of the medium for 90 days. Six subcultures were made during this exposure period. Further cultivation was carried out for another 60 days through four subcultures in growth medium.

3.6.6 Morphological observations

The carcinogen treatment was evaluated by light microscopic observation of morphology, growth pattern and cytotoxicity if any, by staining the coverslip culture prepared simultaneously at various intervals of the experimentation by May-Grunwald-Geimsa stain (Labzoffsky, 1974). For electron microscopy, the cells were harvested by scrapping the prescription bottle with a rubber policeman and packed them into a pellet by light centrifugation. The cell pellet was fixed with 2.5 per cent glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, containing 8.5 per cent sucrose and 0.002 per cent Calcium chloride. The pellet was then post-fixed in 1 per cent osmium tetroxide. Following dehydration to propylene oxide in a graded series of alcohols, the pellet was infiltrated and embedded in Polar-Bed (Bio-rad). Ultra-thin sections were cut on a Reichert microtome, stained with

uranyl acetate and lead citrate and examined with a Hitachi-H-600 A electronmicroscope operated at 50 KV.

3.6.7 Assay for transformation

To test the extent of transformation of these culture cells, colony forming efficiency (CFE) in soft agar, cytochemical assay of Gamma-glutamyl transpeptidase (GGT) activity, colony forming efficiency (CFE) in 10 and 1 per cent serum media as well as transplantation experimentation into weaned mice were performed at 14th passage as discussed below.

3.6.7.1 CFE in soft agar

All the lines were tested for the ability to grow in soft agar according to the method developed by Macpherson and Montagnier (1964) with modifications. The base layer of the agar medium was prepared by mixing an equal volume of stock agarose and double concentrated growth medium supplemented with 11 per cent foetal calf serum (Sigma) to give final concentrations of 0.5 per cent agarose, 0.2 per cent bacto-peptone, 0.05 per cent sodium chloride and 0.01 per cent Na_2HPO_4. 2.5 ml of the base agarose was poured in 35 mm petri-dish and allowed to solidify. After 5 minutes, 0.3 per cent agarose in the same medium containing a different number of cells were layered on top of each petri-dish. The seeding density included 10^2 and 10^3 cells per dish respectively, each

in triplicate. After hardening the top layer at room temperature, the cultures were incubated at 37° with 5 per cent CO_2 and humidity. On day 10, colonies in the soft agar were scored.

3.6.7.2 CFE in 10 and 1 per cent serum media

Cells were seeded at a density of 10^2 cells/35 mm petridishes. Following an attachment period of 24 h in growth medium containing 10 per cent foetal calf serum, the cells were refed with either 10 or 1 per cent foetal calf serum. On day 8, the culture were fixed in methanol and stained with May-Grunwald-Geimsa stain. Colonies with 32 or more cells were scored for computation of CFE as described by San *et al.* (1979).

3.6.7.3 Cytochemical assay of GGT activity

Cells were seeded at densities of 5×10^4 on to 18 mm x 18 mm sterile coverslips in 35 mm petri-dishes. The culture were fixed after 5 days by immersion in acetone at 4°C for 2 h. GGT activity was demonstrated cytochemically by the procedure of Rutenberg *et al.* (1969).

3.6.7.4 Xenotransplantation experiment

Thirty, weaned mice were procured from the small Animal Breeding Station, KAU Mannuthy and divided into

5 groups of six each. These mice were immunosuppressed by administrating cyclosporine (Sandimune-Sandoz) at the dose rate of 15 mg/kg body weight, orally, 1 day previous and 15 days after the inoculation of the cells.

0.1 ml aliquot containing 10^6 viable cells from various cell lines (Table 1) were inoculated subcutaneously on the dorsal surface just posterior of the neck of mice.

Group	Cell type	Treatment	Dose	Passage number
I	Epithelial	AFBI	0.05 µg/ml of medium	14th
I	Epithelial	AFBI	0.1 µg/ml of medium	14th
III	Epithelial	DMSO (negative control)	1 µl/ml of medium	7th
IV	Fibroblast-like	AFBI	0.05 µg/ml of medium	14th
V	Fibroblast-like	DMSO (negative control)	1 µl/ml of medium	8th

The inoculation sites were observed visually as well as by palpation daily from the 3rd day onwards after inoculation for the evidence of any growth at the site of injection.

3.7 Study in spontaneous cases of the carcinoma of the mucosa of the ethmoid

This study was carried out between 1991 and 1994 on a total of 50 tumour bearing cows, which were brought to the Centre of Excellence in Pathology, College of Veterinary and Animal Sciences from different parts of the Kerala state after obtaining information from the respective veterinary clinics.

The tumour bearing animals were euthanised by exsanguination after stunning with captive bolt pistol. The head was bisected into two halves with an electric saw. Healthy tumour tissue devoid of necrotic areas was dissected out from the deeper portions under aseptic precautions. Head lymphnodes like retropharyngeal, parotid and mandibular were examined for metastatic growth, if any. Detailed post-mortem examination was conducted to find out other lesions in the animal.

3.7.1 Ultrastructural study

For electron-microscopic examination, selected areas from 20 of the tumours were fixed in 2.5 per cent glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C. The specimens were post-fixed with 1 per cent osmium tetroxide. Tissues selected for transmission electron-microscopic study were dehydrated through a series of graded acetone and

embedded in polar-bed (Bio-Rad). Sections were cut with glass knives using Reichert ultramicrotome.

Ultrathin sections were stained with uranyl acetate and lead citrate, and observed with a Hitachi-H-600 A electron-microscope at 50 KV. Tissues selected for scanning electron-microscopic studies were dehydrated in graded alcohols and acetone for subsequent critical point drying in liquid carbon dioxide. They were glued to aluminium stubs and coated with gold in a vacuum evaporator. Observations were made with a Hitachi scanning electronmicroscope.

3.7.2 Electronmicroscopy of cell free ethmoid tumour extract

The cell free ethmoid tumour extract was prepared as discussed earlier. This was concentrated by ultracentrifugation. The cell free extract was spun at 10,000 g for 1 h, the supernatent further centrifuged at 45,000 g for 2 h, the pellet suspended in 0.5 ml phosphate buffer saline (PBS), pH 7.2 and centrifuged at 5000 x g for 20 minutes. The clear supernatent was collected in a small siliconised vial and preserved at 4°C till use.

Copper grids (300 mesh) were coated with 0.33 per cent formwar in chloroform as described by Horne (1967). After drying at 30°C, the concentrated material was charged on to the grid and after about 2 h, stained with 2 per cent

phosphotungstic acid (pH 7.0) as per the method detailed by Labzoffsky (1974). The grid was screened under 60,000 x to 1,20,000 x magnification in Hitachi-H-600 A transmission electromicroscope for the presence of virus, if any.

3.7.3 Aflatoxin residues in blood

Venous blood was collected by venipuncture from 21 ethmoid tumour bearing animals. AFBI was estimated by the method described by Stubblefield and Shotwell (1981).

Results

RESULTS

The observations made on the carcinogenic response of piglings dosed with aflatoxin B_1 (AFB_1) and/or ethmoid tumour extract are detailed below.

4.1 Quantification of AFB_1

The concentration of AFB_1 produced on rice culture and quantified by thin layer chromatography employing minimum fluorescence extinct method on an average ranged from 105-190 µg/g of rice.

4.2 Clinical signs

All animals appeared healthy and no clinical manifestations of the carcinoma of the mucosa of ethmoid were observed in any of the pigs in groups I, II, III and IV. The pigs which were administered AFB_1 showed some degree of depression as compared to healthy controls and ethmoid tumour extract instilled animals. During the experimental period of 18 months, one pig of group II died on the 95th day of experimentation.

4.3 Growth response

The data on average body weights at various intervals

Table 2. Average body weight (kg) of experimental pigs on various intervals of treatment

Group	Interval (Months)										
	0	1	2	3	4	5	6	9	12	15	18
Ia	10.87 ± 1.64	17.50 ± 4.53	24.68 ± 4.99	36.12 ± 5.24	43.06 ± 4.20	48.68 ± 2.90	53.56 ± 3.39	75.68 ± 3.26	92.33 ± 3.31	105.87 ± 4.53	114.00 ± 5.65
IIa	10.62 ± 2.44	15.12 ± 3.31	22.50 ± 3.42	31.25 ± 4.65	41.93 ± 9.47	49.07 ± 7.59	54.50 ± 6.29	71.71 ± 5.70	89.60 ± 3.30	105.66 ± 1.89	114.50 ± 0.00
IIIa	10.25 ± 1.28	19.12 ± 3.11	31.81 ± 5.16	42.18 ± 5.65	50.75 ± 6.62	57.50 ± 7.18	63.4 ± 7.32	85.37 ± 7.08	103.00 ± 4.38	112.62 ± 7.53	118.75 ± 12.37
IVb	11.56 ± 1.59	21.18 ± 1.48	33.81 ± 2.35	45.00 ± 4.35	50.62 ± 2.92	57.62 ± 2.92	65.68 ± 2.37	82.00 ± 1.85	101.33 ± 1.16	110.37 ± 6.40	113.00 ± 13.43

Groups having the same superscripts are not significantly ($P<0.05$) different

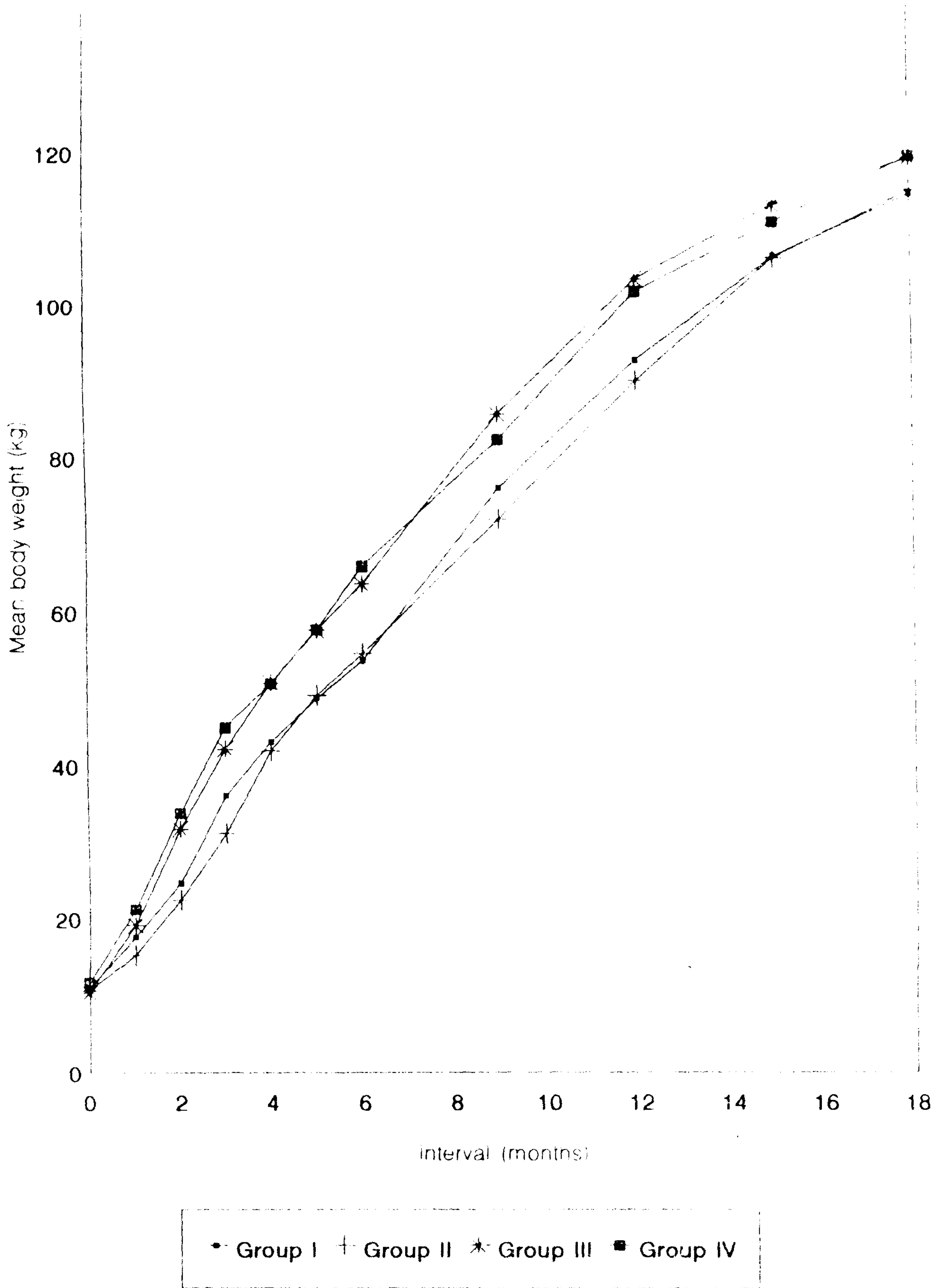


FIG. 1 AVERAGE BODY WEIGHT (kg) OF EXPERIMENTAL PIGS AT VARIOUS INTERVALS OF TREATMENT

of experiment starting from month 0 to 18 are summarized in Table 2 and presented graphically in Fig.1.

Animals in the control group showed a gradual increase in body weight from an initial 11.5625 ± 1.59 kg to 119.00 ± 13.43 kg by the 18th month.

The group I animals showed a gradual increase in body weight from 10.875 ± 1.642 kg to a maximum weight of 114.00 ± 5.65 kg by the 18th month. There was a significant ($P<0.05$) reduction in the weight from that of the control group from the month 1 to 12 of the experiment and thereafter values were more or less comparable with those of control animals.

The body weight of group II animals increased gradually from an initial value of 10.625 ± 2.44 kg to 114.5 ± 5.65 kg by the 18th month. The reduction was significant ($P<0.05$) from that of the control pigs from the month 1st to 12th of the observation period.

Similarly, the animals of group III showed a steady increase in their body weights from an initial value of 10.25 ± 1.28 kg to 118.75 ± 12.37 kg by the 18th month. Although, the average body weight of the animals of this group at various intervals of the experiment was invariably lower than that of the age matched controls, the difference was not significant at any stage of the experiment.

4.4 Pathological studies

4.4.1 Gross lesions

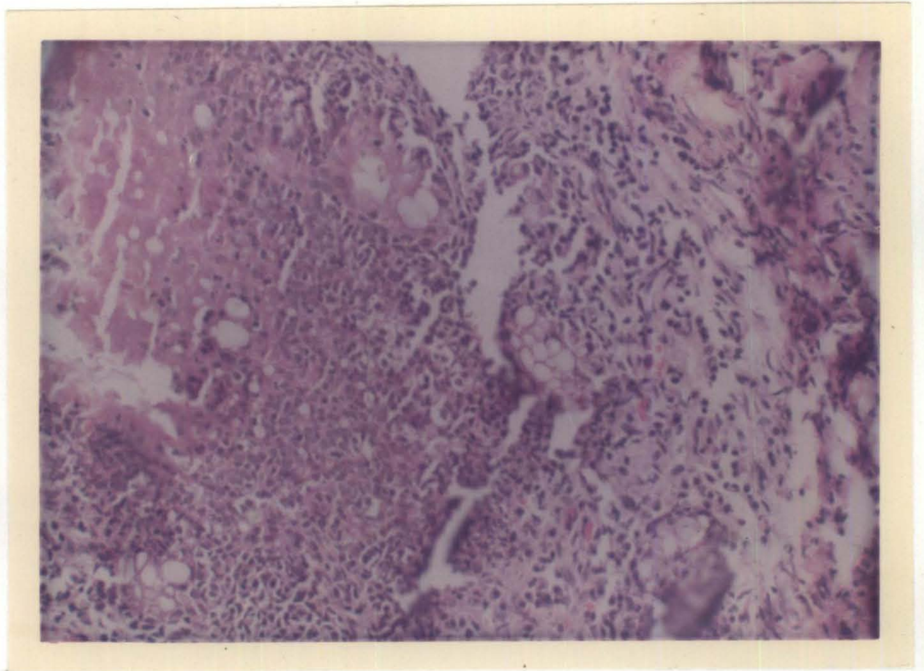
At the ninth month, eight pigs, two from each group, were sacrificed. The ethmoid turbinate and nasal mucosa of pigs from group I and group II revealed mild to moderate hyperaemia. In some portions of the ethmoid mucosa, a few small, somewhat pale elevations were observed. No gross lesion was observed in the pigs in groups III and IV.

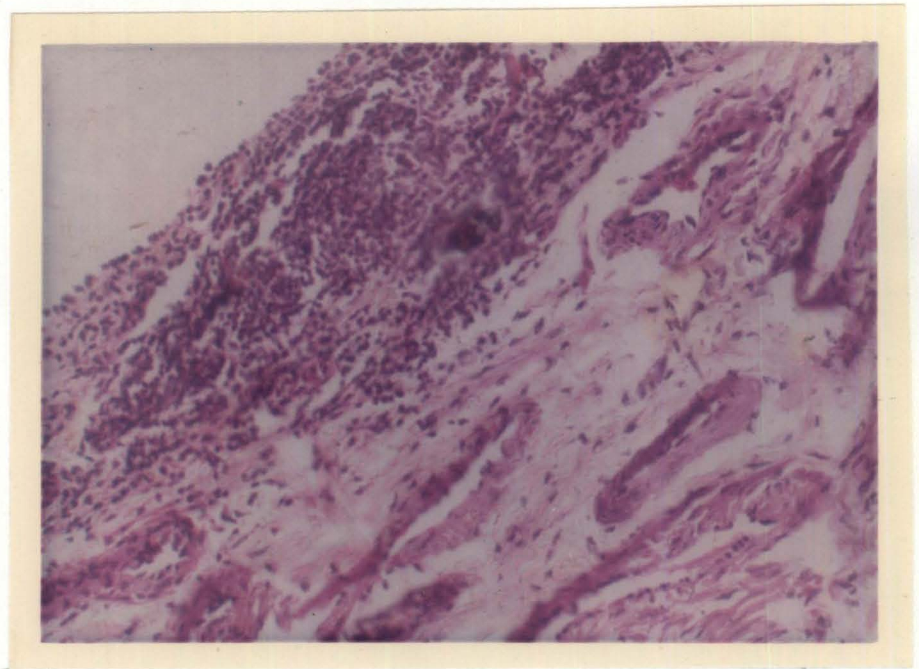
At 12th month, another eight pigs, two from each group, were sacrificed. The lesions in the ethmoid turbinates and nasal mucosa of the group I and II were similar to those observed at 9 months, but were more marked. No macroscopic lesions could be detected in the pigs in groups III and IV. The ethmoidal area appeared soft, grey-white and oedematous with the surface covered by mucus in the pigs sacrificed at 15th and 18th months of observation. No appreciable neoplastic growth was seen in any of the animals.

No gross lesions could be detected in the nasal or ethmoidal mucosa of the pigs of group III except mild thickening when the animals were sacrificed at 15th and 18th months of the experiment. Likewise, no macroscopic lesion was observed in the pigs of group IV at any stage of experimentation.

Fig.2 Ethmoid mucosa - Aflatoxin treated pig - 9th month - Degenerated glands with dense infiltration of lymphocytes and neutrophils - H&E x 400

Fig.3 Ethmoid mucosa - Aflatoxin treated pig - 12th month - Focal aggregates of lymphocytes and oedema - H&E x 400





4.4.2 Histopathology

4.4.2.1 Group I

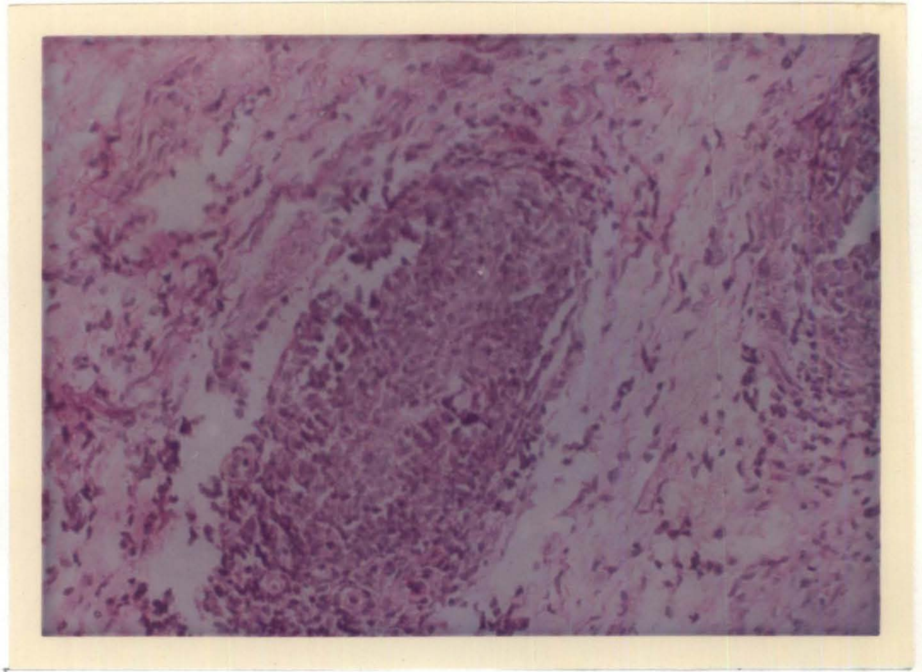
The histological examination confirmed the macroscopical findings. The blood vessels were dilated and engorged with erythrocytes. Slight to marked infiltration of lymphocytes in the mucosa and lamina propria was recorded. A varying degree of fatty degeneration of lining cells of submucosal glands was observed in the ethmoid mucosa of pigs sacrificed at the 9th month (Fig.2). In addition, there was degeneration and sloughing of the lining epithelium of the ethmoid mucosa.

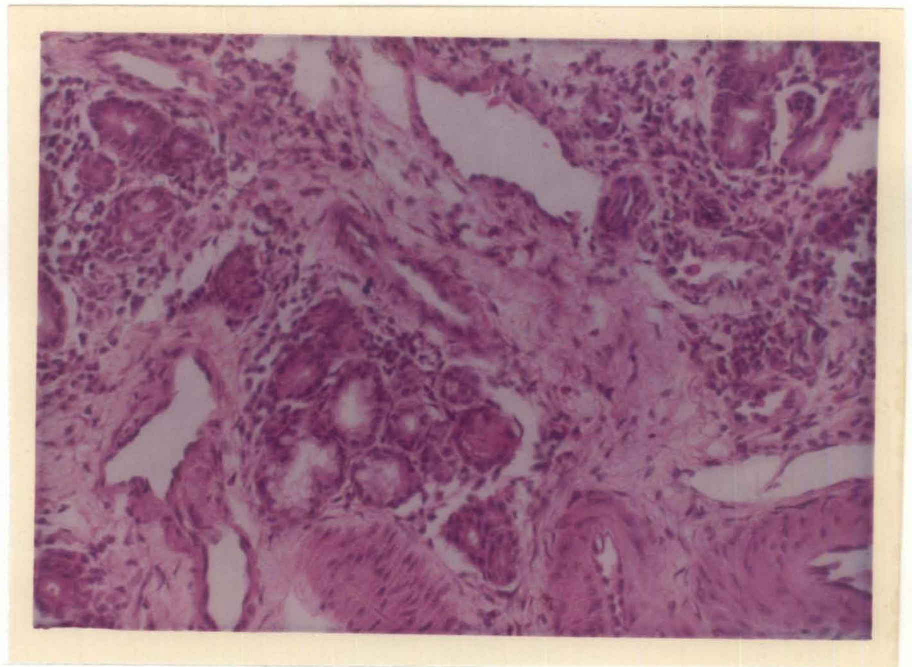
Similar microscopic lesions were observed in the pigs sacrificed at 12th month, but the lesions were more extensive. The macroscopically observed elevations were small almost lymphnode-like, aggregates of lymphocytes with occasional macrophages and plasma cells (Fig.3 & 4). Associated with these changes some degree of proliferation of submucosal glands (Fig.5) and oedematous lamina propria with hyperemic vessels were seen. Necrosis and sloughing of the ethmoid epithelium were more conspicuous at this period of observation.

The ethmoid mucosa of the pigs sacrificed at 15th and 18th month revealed proliferation of mucous glands, which were

Fig.4 Ethmoid mucosa - Aflatoxin treated pig - 12th month - Lymphnode like aggregates - Oedema - H&E x 400

Fig.5 Ethmoid mucosa - Aflatoxin treated pig - 12th month - Focal glandular hyperplasia and mononuclear cell infiltration - H&E x 400





arranged into acinar, tubular or papillary patterns. The stroma was scanty and infiltrated with lymphocytes, plasma cells and a few macrophages (Fig.6). There was tendency of the surface epithelium to form papillary projections at this stage of observation (Fig.7). In focal areas, squamous metaplasia of the ethmoid mucosa of three pigs was also noticed (Fig.8).

4.4.2.2 Group II

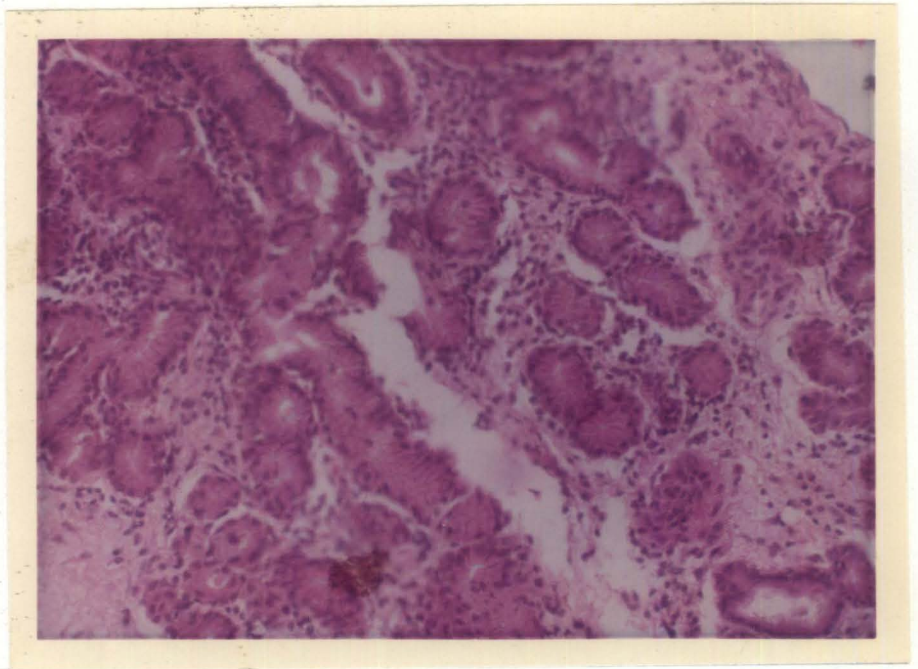
The histopathological findings in the ethmoid mucosa of the pigs of group II were more or less comparable to those of group I, but an increase of the connective tissue was more marked particularly at 15th and 18th month of investigation.

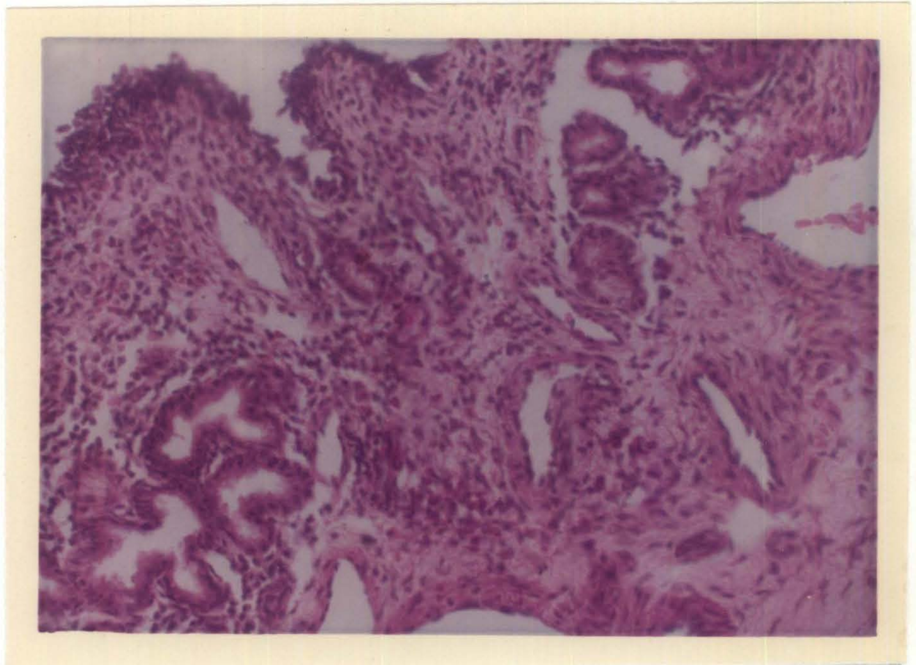
4.4.2.3 Group III

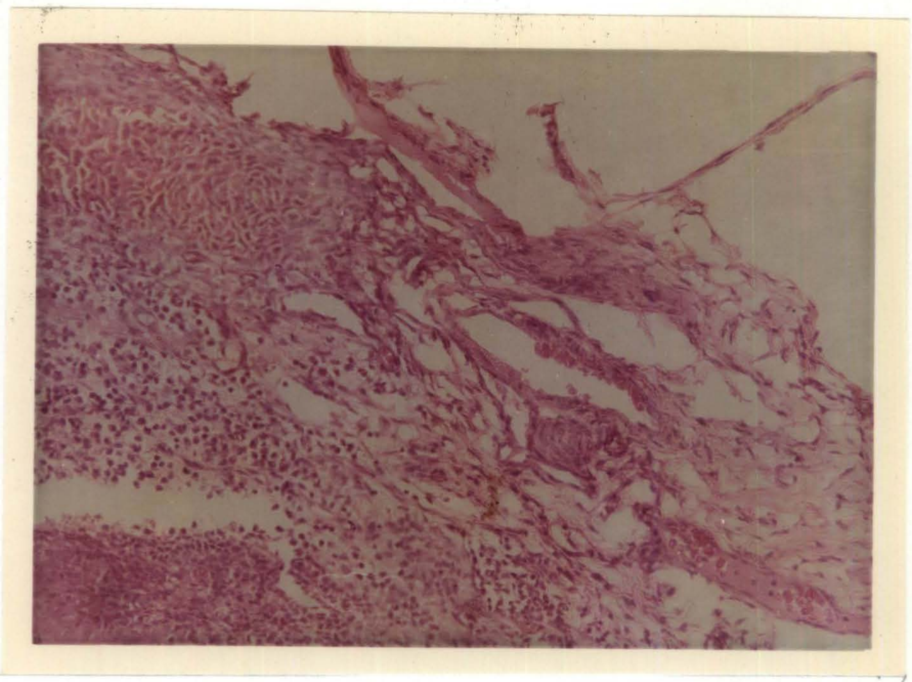
There was no significant morphological alteration in the pseudostratified columnar epithelium lining the ethmoid mucosa sacrificed at various intervals. The submucosal oedema was observed in the early stages. Besides the oedematous changes, increase in the connective tissue and mononuclear cell infiltration were noticed (Fig.9). The progressive increase in the connective tissue elements and mild to marked infiltration of mononuclear cells were the marked microscopic observations in the later stages of the experiment.

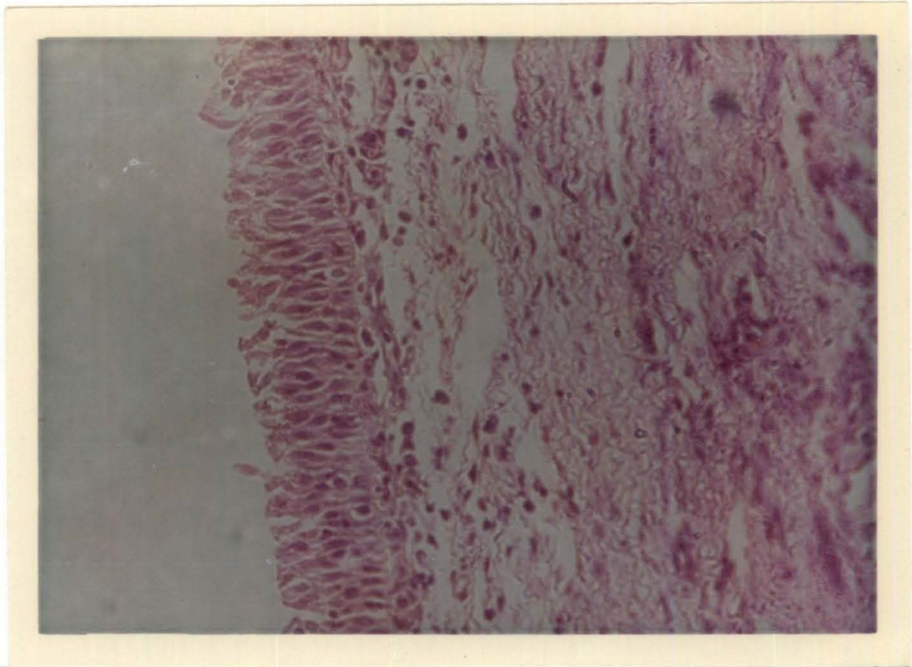
Fig.6 Ethmoid mucosa - Aflatoxin treated pig - 15th month - Proliferating glandular epithelium forming acinar and tubular patterns - H&E x 400

Fig.7 Ethmoid mucosa - Aflatoxin treated pig - 18th month - Glandular proliferation showing papillary projections - H&E x 400









4.4.2.4 Group IV

Examination of the ethmoid mucosa from the control pigs did not reveal pathological change at any interval/stage of the experiment.

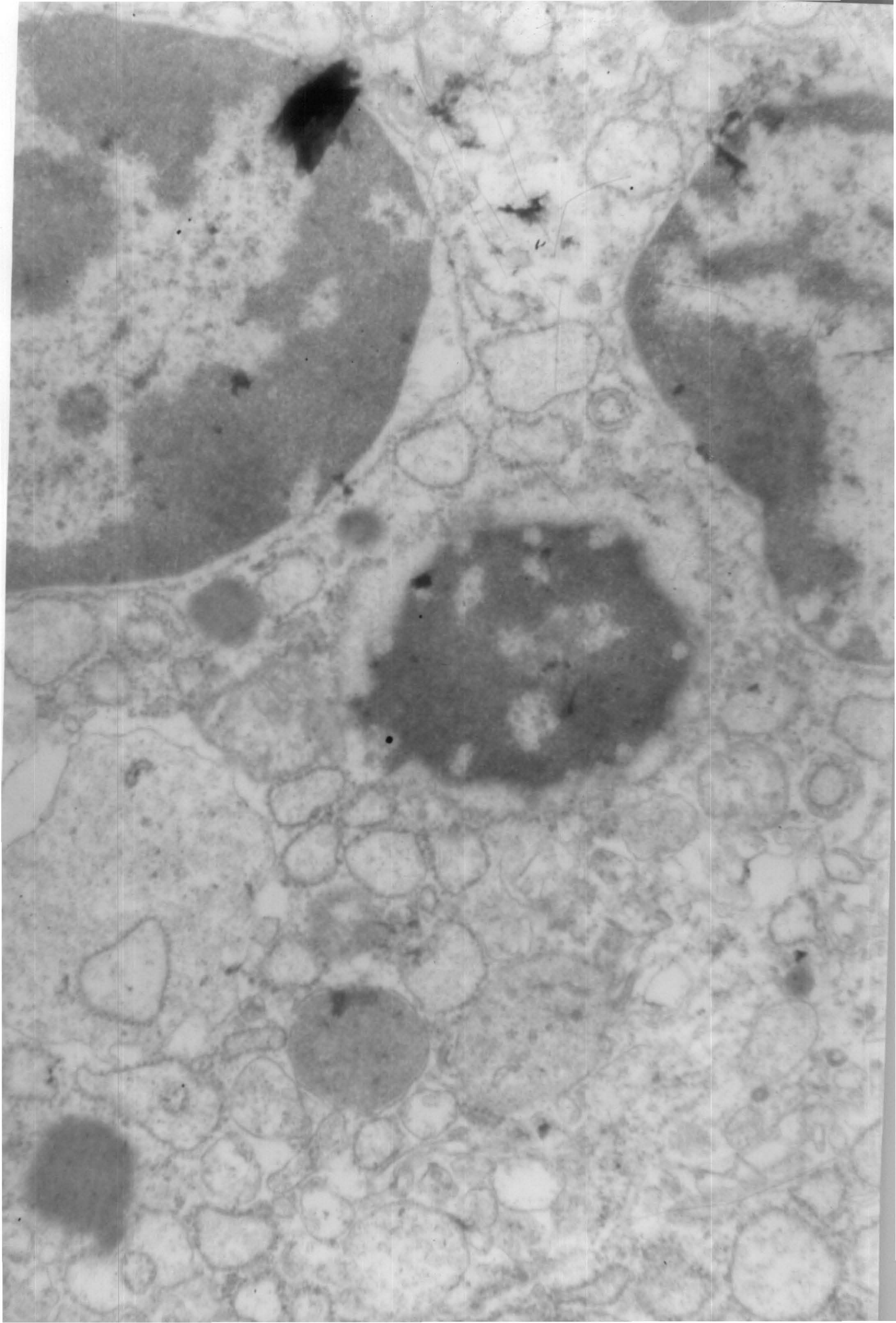
4.4.3 Ultrastructural Pathology

The principal ultrastructural alterations were observed in the lining epithelium and in the cells of the submucosal glands of the ethmoid mucosa of the experimental pigs of different groups sacrificed at various intervals of investigation.

4.4.3.1 Group I

At 9 months, primarily the changes were recorded in the secretory cells. There was marked proliferation of smooth endoplasmic reticulum. The rough endoplasmic reticulum were dilated. Mitochondria varied in number, size and shape. Transverse or ring-shaped cristae were noticed in some mitochondria, while in other there was complete disorganization and dissolution. Electron-dense structures, presumably lipids, lysosomes and a few secretory granules, were also observed. Nucleus was round to oval. At times, nucleus with irregular contour was also noticed. At focal areas, evagination of outer nuclear membrane forming a

Fig.10 Electron micrograph - Ethmoid mucosal cells from AFB_1 treated pig showing well developed endoplasmic reticulum containing secretory products - Degenerated mitochondria and lysosomal granules -Prominence of nuclear envelope with evagination of outer membrane - x 25,000



bleb-like structure was observed. In general, heterochromatin was predominant (Fig.10). Occasionally the necrosis of cells characterised by pyknosis and karyorhexis was apparent.

At the 12th month, the changes in the cells were not appreciably different from those observed in the cells of the ethmoid mucosa of pigs sacrificed at 9 month interval (Fig.11).

At the 15th month of the experiment, the cells were characterized by the atypical morphology of the nucleus which was irregular in shape and possessed deep cytoplasmic invagination. The cells had one or two nucleoli, in which granular or filamentous nucleolenema was more or less conspicuous. The margination of the nucleoli was also a characteristic feature (Fig.12). The cells were round to polyhedral with interdigitations. The cellular contact was caused by closely applied plasma membranes. The characteristic junctional complexes could not be appreciated. A few mitochondria with distinct cristae, varying amount of the rough endoplasmic reticulum at various stages of disorganization and free ribosomes were the prominent cytoplasmic organelles observed at the 15th and 18th month. Tonofibrils were also noticed predominantly in perinuclear region. The characteristic nuclear changes along with predominance of interchromatin granules were the other

Fig.11 Electron micrograph - Ethmoid mucosal cells from AFB_1 treated pig showing proliferation of rough and smooth endoplasmic reticulum - Cytoplasmic organelles degeneration - x 20,000

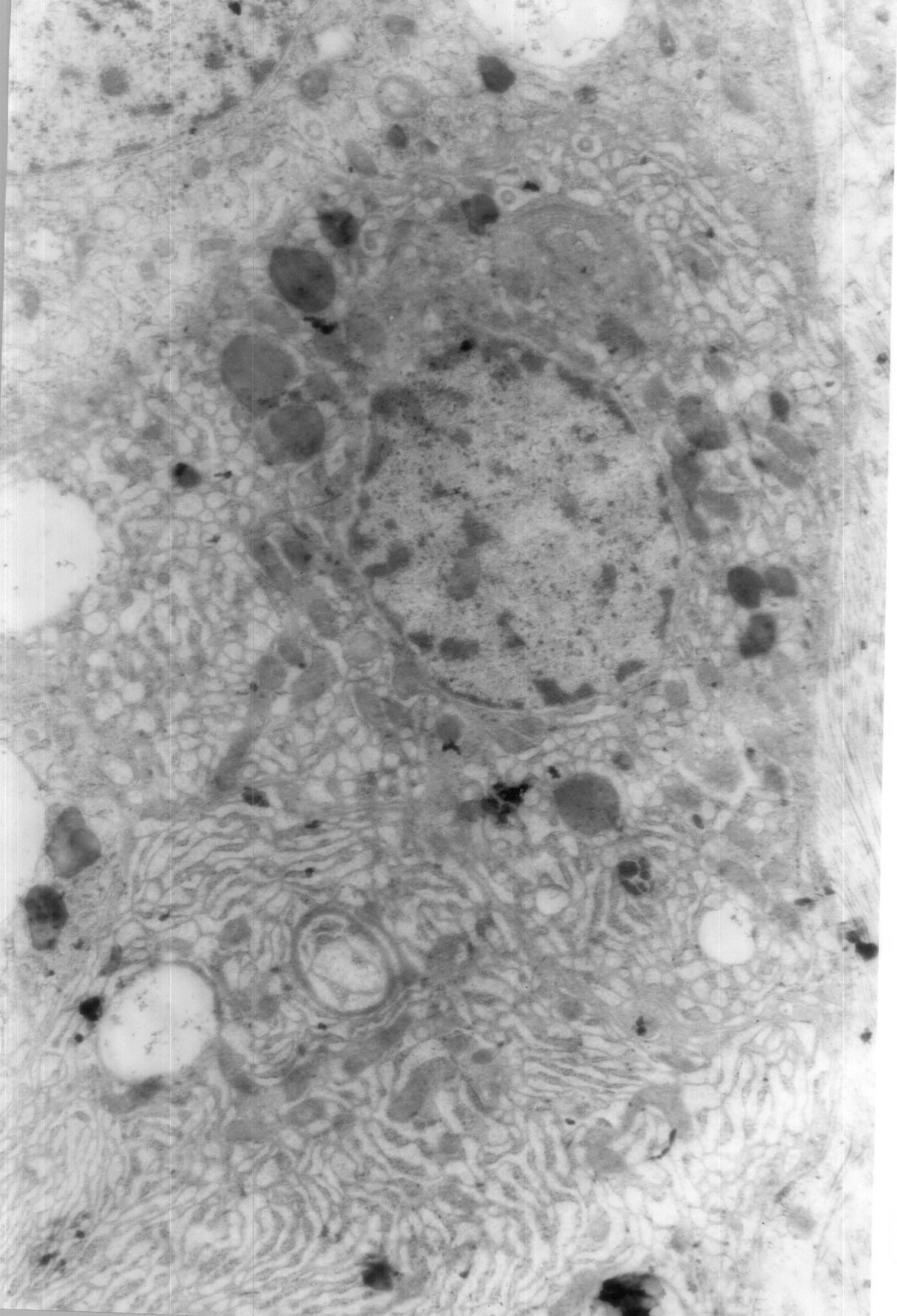
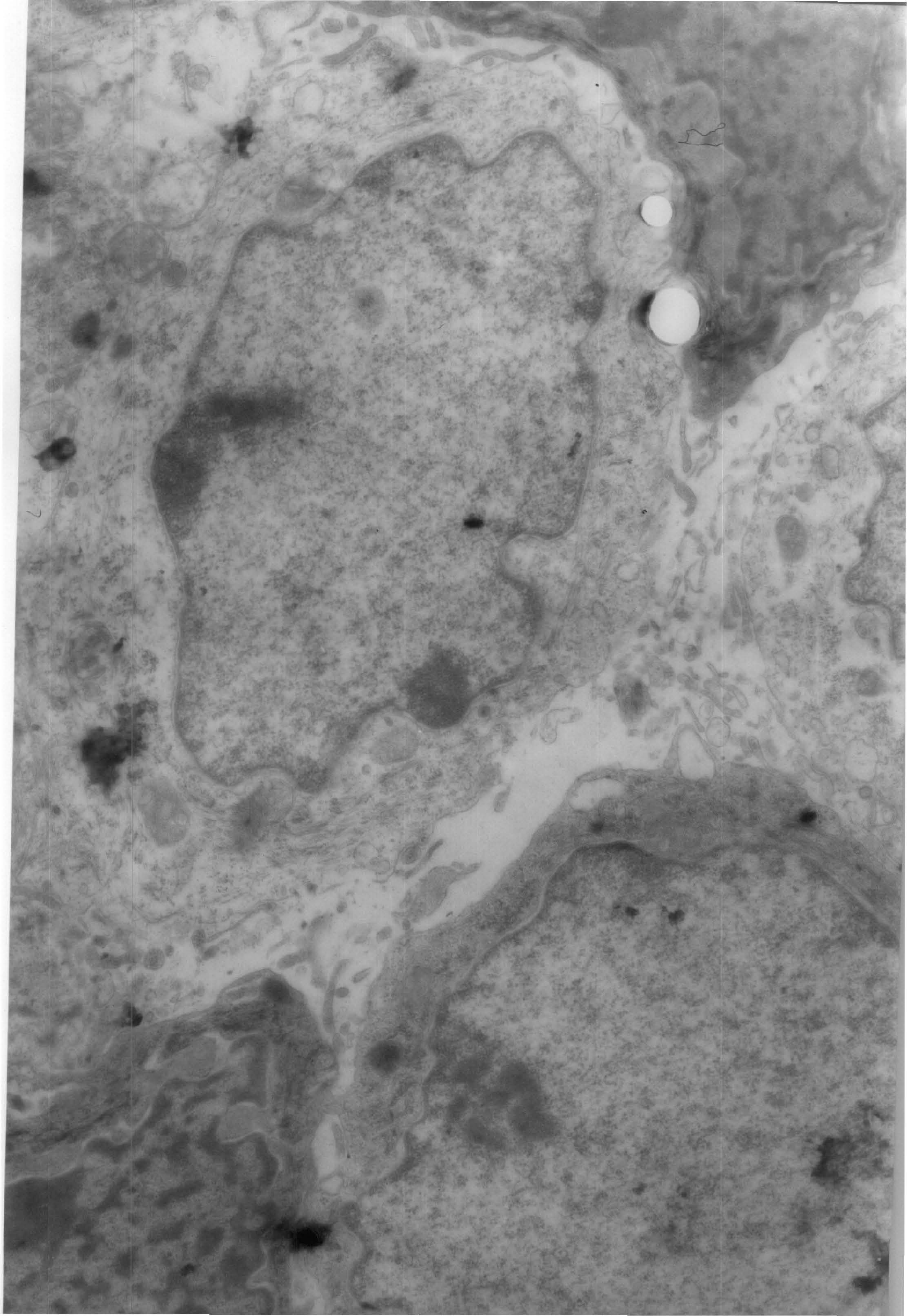


Fig.12 Electron micrograph - Epithelial cells from the ethmoid mucosa of AFB_1 treated pig showing prominent nucleus with a few indentations - Nucleoli show margination - Predominance of euchromatin in the nucleus - Lymphocyte and macrophage infiltration is seen - x 30,000



ultrastructural observations in the ethmoid mucosa of the pigs at the 18th month of experiment (Fig.13).

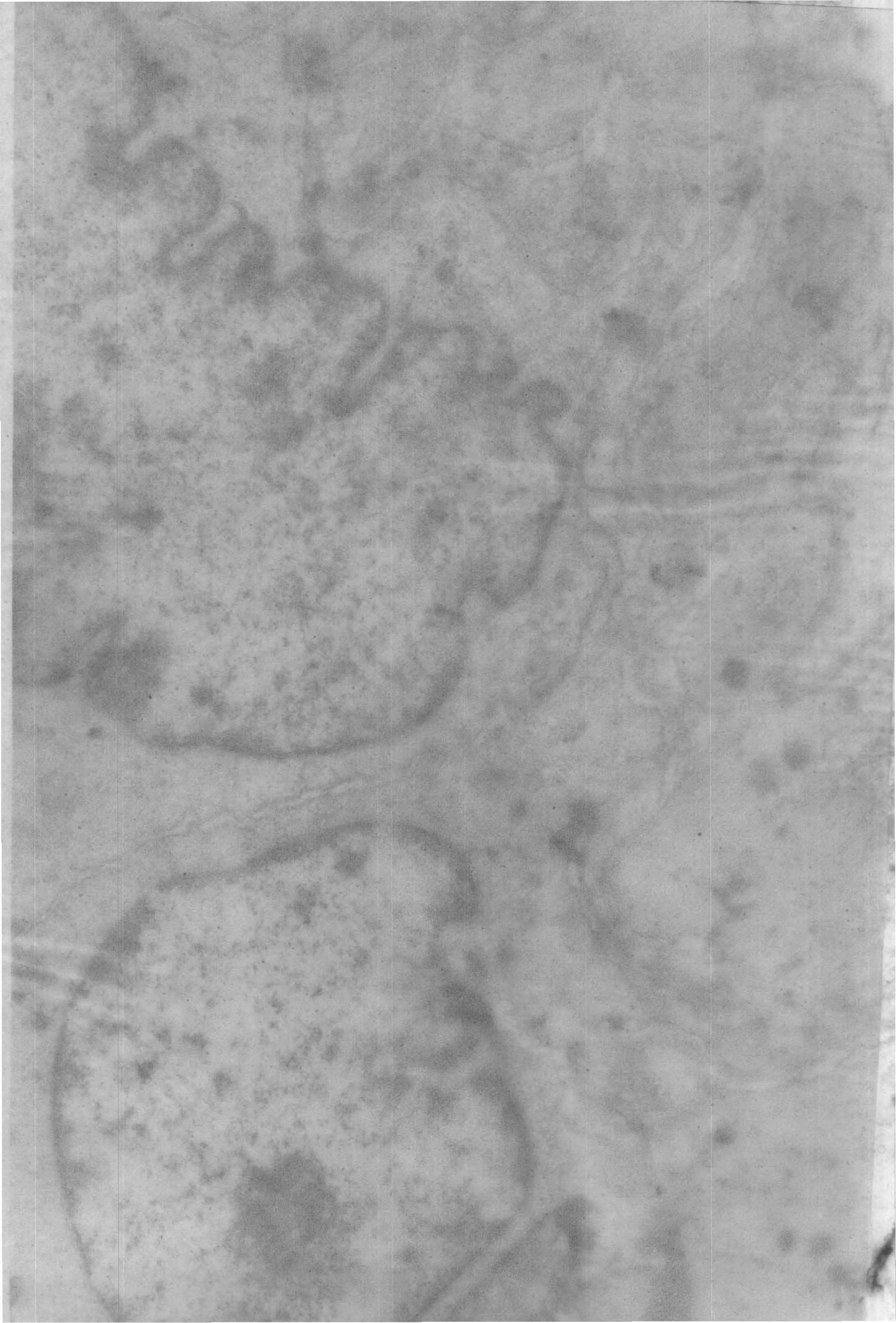
The presence of well differentiated secretory cells presumably mucus secretory cells and fibroblasts along with varying amount of collagen fibrils was noticed in the later stages. There was also infiltration of lymphocytes, macrophages and plasma cells (Fig.14 & 15).

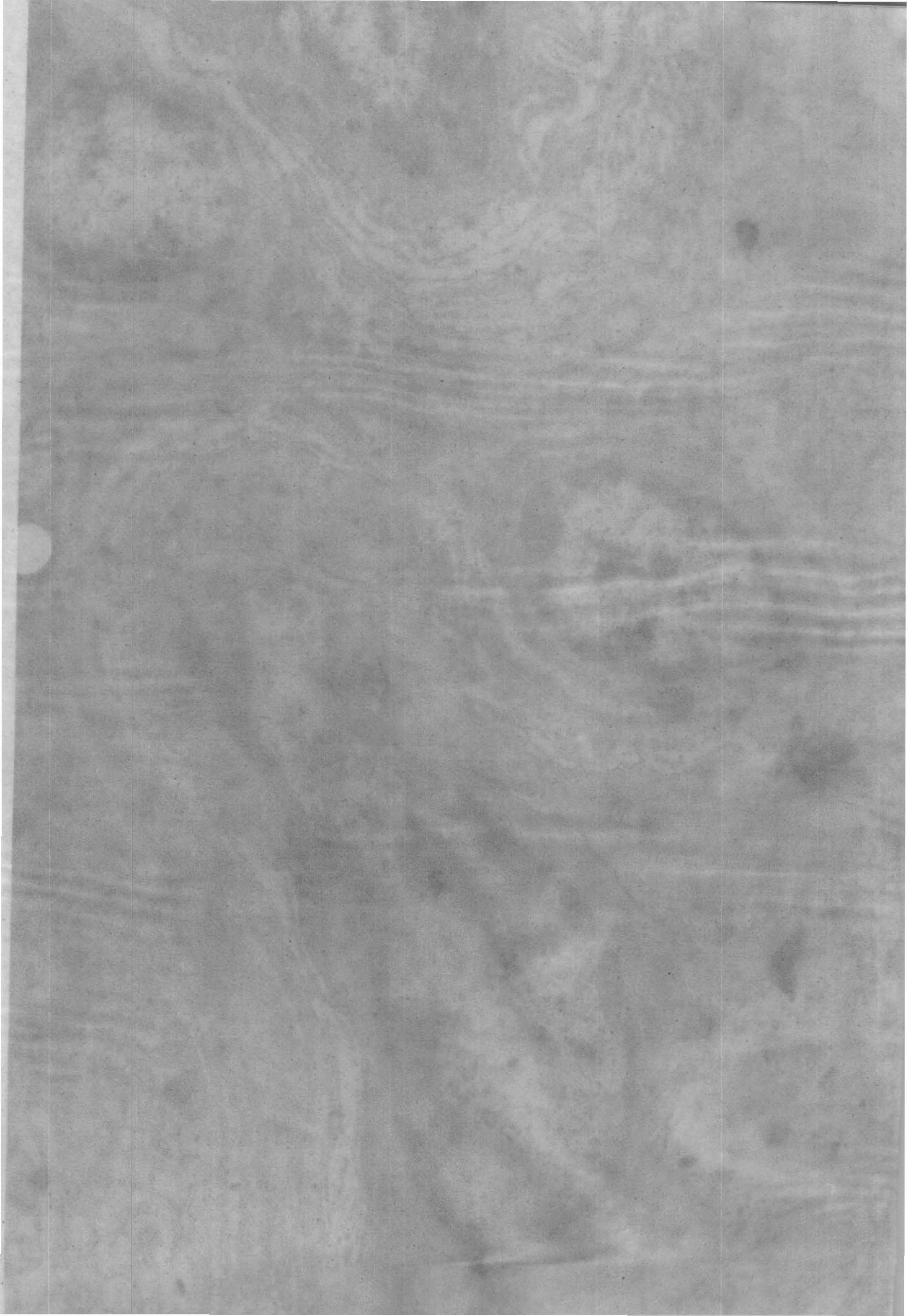
4.4.3.2 Group II

The fine structure of the cells of the ethmoid mucosa of the pigs of group II sacrificed at various stages of the experiment was comparable to those of pigs of group I (Fig.16).

4.4.3.3 Group III

The electron microscopic features of the cells of the ethmoid mucosa of pigs from group III given ethmoid tumour extract were more or less comparable to those of the control groups. The cells were predominantly columnar type. These cells had tight junctions and invaginations. The cytoplasmic organelles consisted of round to oval mitochondria, strands of rough endoplasmic reticulum and free ribosomes. The golgi apparatus was poorly developed and invariably inconspicuous. The nucleus was elongated and had prominent centrally placed





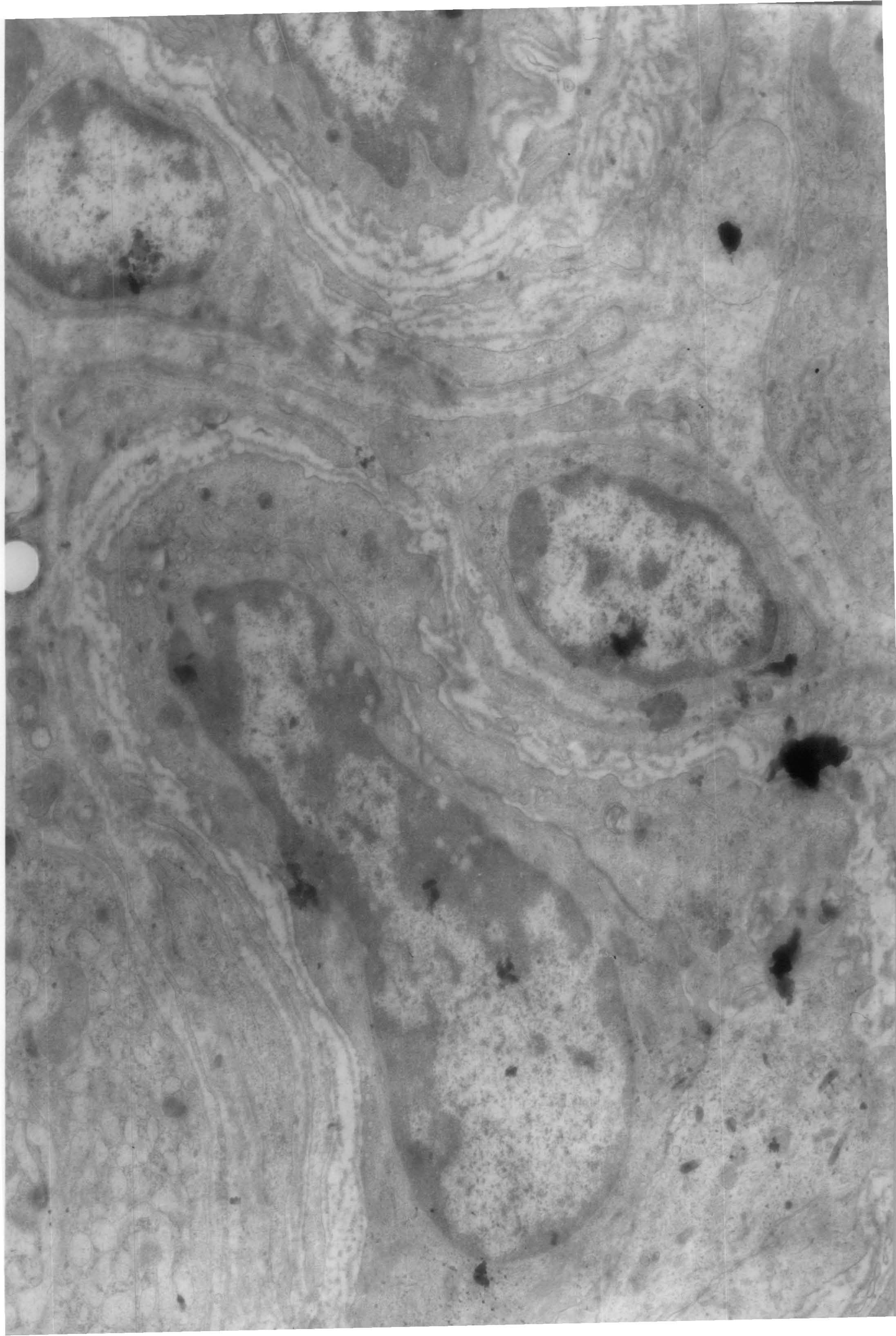


Table 3. Aflatoxin residues in blood and mucosa of experimental pigs

Group	AFL	No. of pigs positive for aflatoxins Interval (Days)																	
		183 (3)*		187 (7)		190 (10)		195 (15)		210 (30)		270 (90)		360 (180)		450 (270)		540 (360)	
		Blood	Mucosa	Blood	Mucosa	Blood	Mucosa	Blood	Mucosa	Blood	Mucosa	Blood	Mucosa	Blood	Mucosa	Blood	Mucosa	Blood	Mucosa
I	Bl	8/8	NT	8/8	NT	5/8	NT	0/8	NT	0/8	NT	0/8	0/2	0/6	0/2	0/4	0/2	0/2	0/2
	Range ppb	80-160	-	40-160	-	40-120	-	20-40	-	-	-	-	-	-	-	-	-	-	-
	Ml	1/8	NT	0/8	NT	0/8	NT	0/8	NT	0/8	NT	0/8	0/2	0/6	0/2	0/4	0/2	0/2	0/2
	Range ppb	42	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
II	Bl	7/7	NT	7/7	NT	5/7	NT	0/7	NT	0/7	NT	0/7	0/2	0/5	0/2	0/3	0/2	0/1	0/1
	Range ppb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ml	0/7	NT	0/7	NT	0/7	NT	0/7	NT	0/7	NT	0/7	0/2	0/5	0/2	0/3	0/2	0/1	0/1
	Range ppb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
III	Bl	0/8	NT	0/8	NT	0/8	NT	0/8	NT	0/8	NT	0/8	0/2	0/6	0/2	0/4	0/2	0/2	0/2
	Range ppb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ml	0/8	NT	0/8	NT	0/8	NT	0/8	NT	0/8	NT	0/8	0/2	0/6	0/2	0/4	0/2	0/2	0/2
	Range ppb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IV	Bl	0/8	NT	0/8	NT	0/8	NT	0/8	NT	0/8	NT	0/8	0/2	0/6	0/2	0/4	0/2	0/2	0/2
	Range ppb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ml	0/8	NT	0/8	NT	0/8	NT	0/8	NT	0/8	NT	0/8	0/2	0/6	0/2	0/4	0/2	0/2	0/2
	Range ppb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Seeding density per petridish

Mean values having the same superscripts are not significantly ($P<0.05$) different

Fig.15 Electron micrograph. The macrophage from ethmoid mucosa of AFB_1 treated pig showing well developed endoplasmic reticulum and Mitochondria with electron-dense matrix and partial loss of cristae - x 30,000

nucleoli. There was predominance of euchromatin with clumps of condensed chromatin situated both adjacent to nuclear envelope and dispersed throughout the nucleoplasm (Fig.17).

The stroma was infiltrated predominantly with fibroblasts and occasional lymphocytes. There was considerable amount of collagen. These changes were more prominent in the ethmoid mucosa of pigs sacrificed at 15th and 18th month.

4.4.3.4 Group IV

None of the pigs from the group IV showed any ultrastructural pathology throughout the experimental period of 18 months.

4.5 Aflatoxin residues in the blood and ethmoid mucosa of experimental pigs

Aflatoxin B_1 (AFB_1) in the range of 40-160 ppb was detected in the blood of all the pigs (8) of group I and group II (7 pigs), at 3 and 7 days post-treatment. Blood samples of five pigs each from both the groups revealed fluorescence characteristics of AFB_1 (40-120 ppb) at 10 days post-treatment. Thereafter, the blood and ethmoid mucosa samples were invariably negative for AFB_1 (Table 3).

Efforts were also made to detect aflatoxin M_1 (AFM_1) in the blood and ethmoid mucosa of experimental pigs at

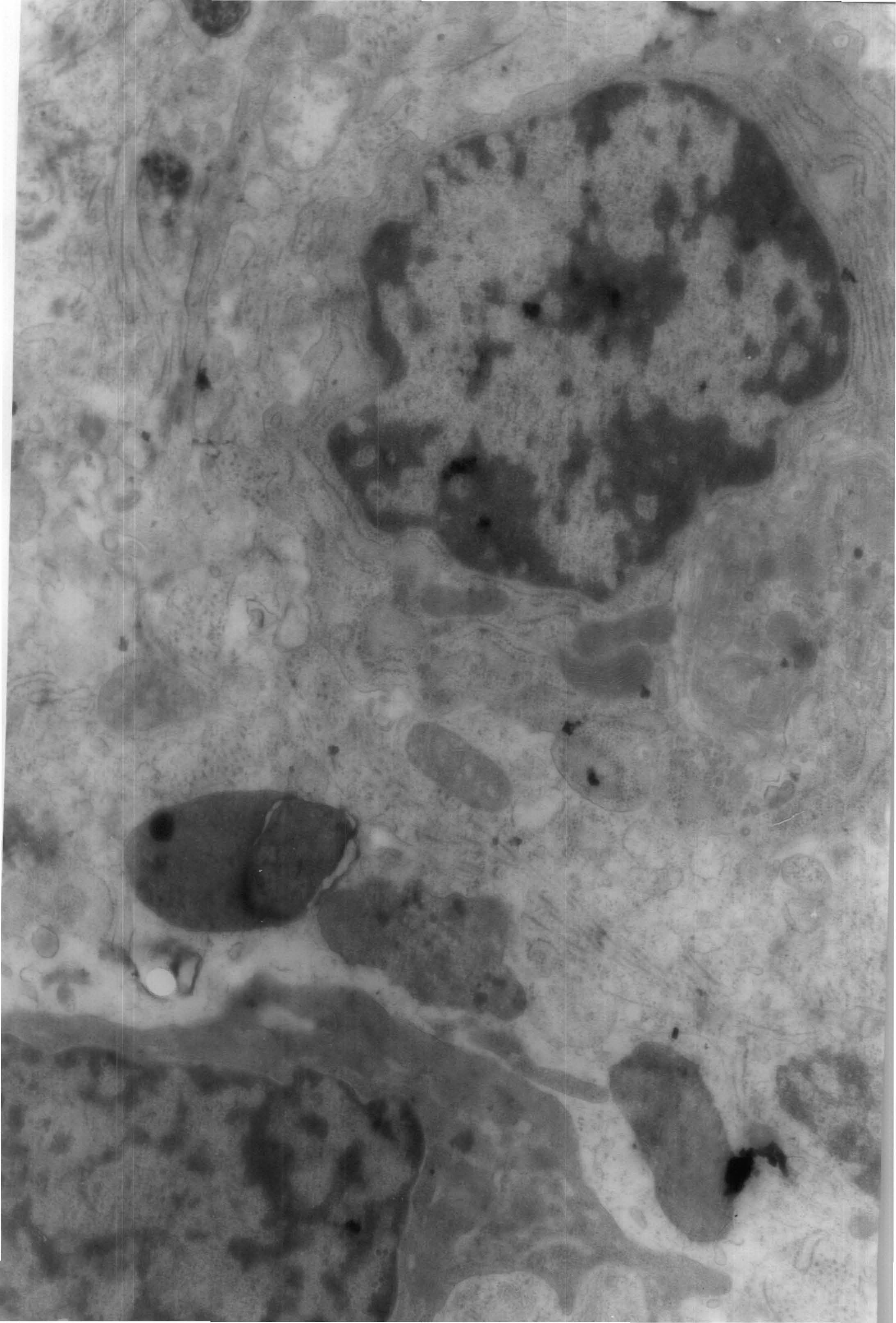


Fig.16 Electron micrograph. A part of cell from the ethmoid mucosa of AFB_1 and tumour extract administered pig showing well developed endoplasmic reticulum and prominent mitrochondria - Some of mitochondria show partial loss of cristae - Few dense granules seen - Nuclear chromatin is mostly euchromatin type - x 35,000

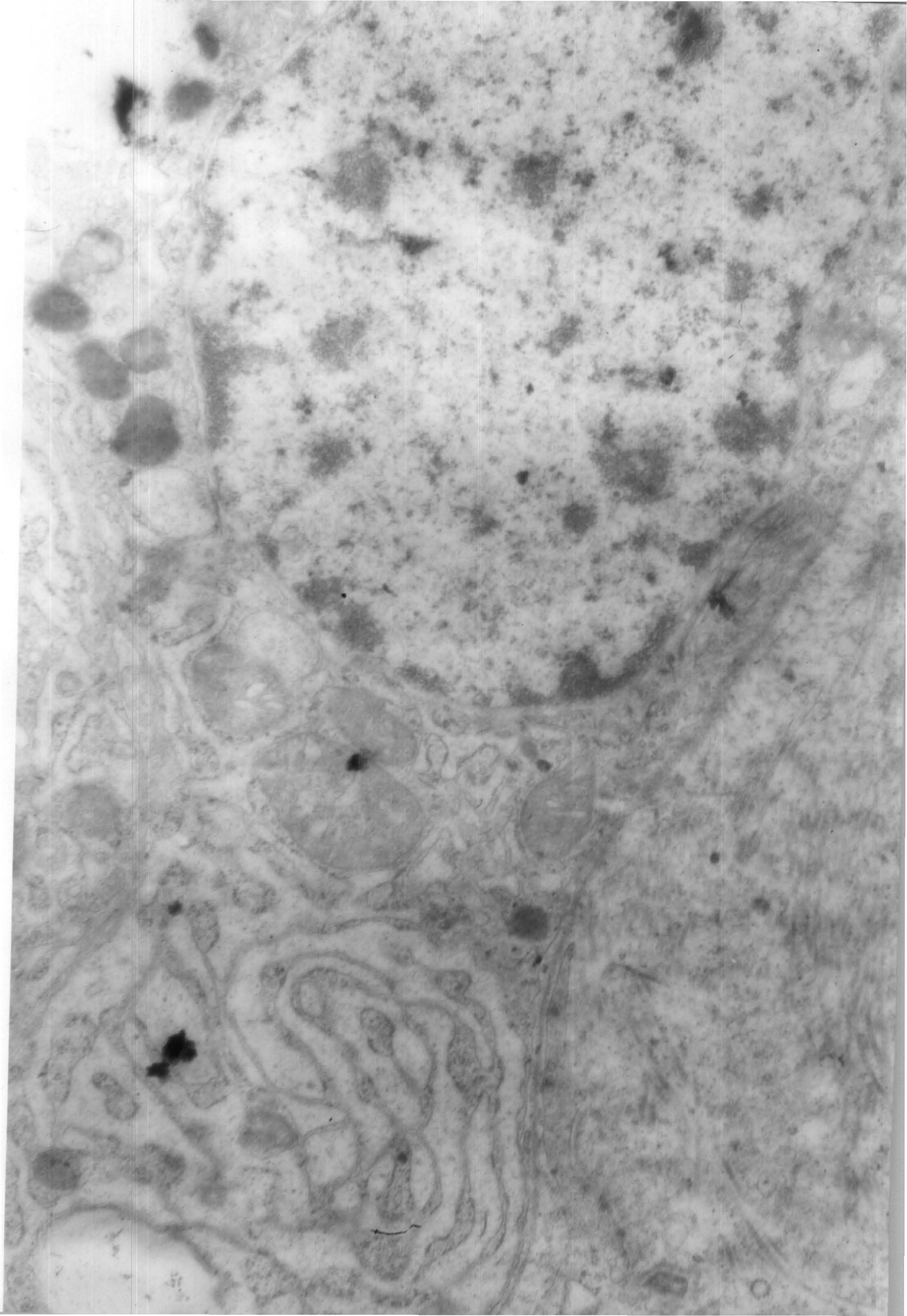
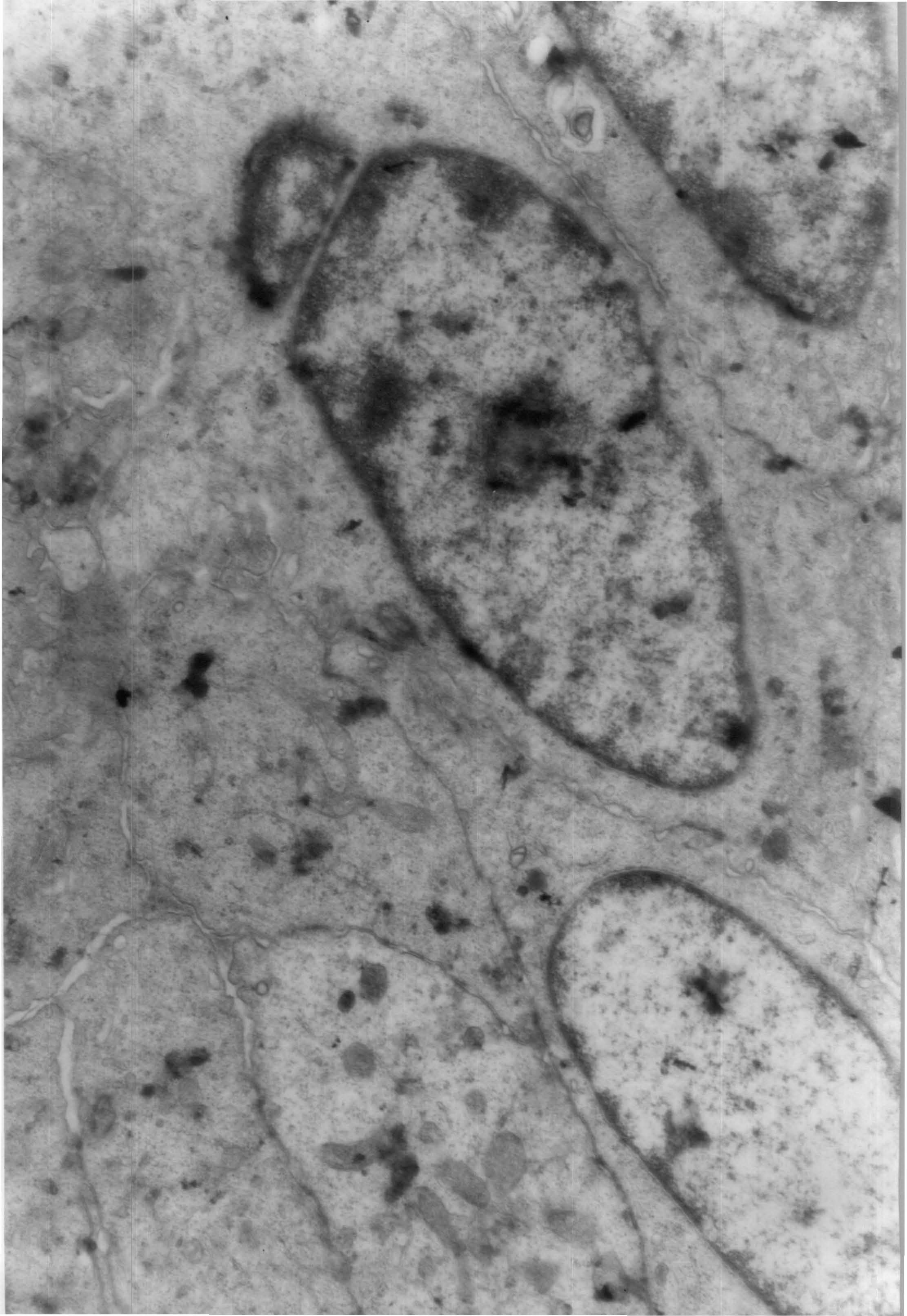


Fig.17 Electron micrograph - Ethmoid mucosa from the tumour extract instilled pig showing a layer of columnar cells - x 30,000



various intervals. The blood sample of one pig of group I was positive for AFM_1 (42 ppb).

Blood and ethmoid mucosa from the pigs of group III and IV collected at specific intervals were found to be consistently negative for AFB_1 and AFM_1 throughout the observation period of one and a half year.

4.6 In vitro carcinogenicity

4.6.1 Morphological observations

4.6.1.1 Culture cells

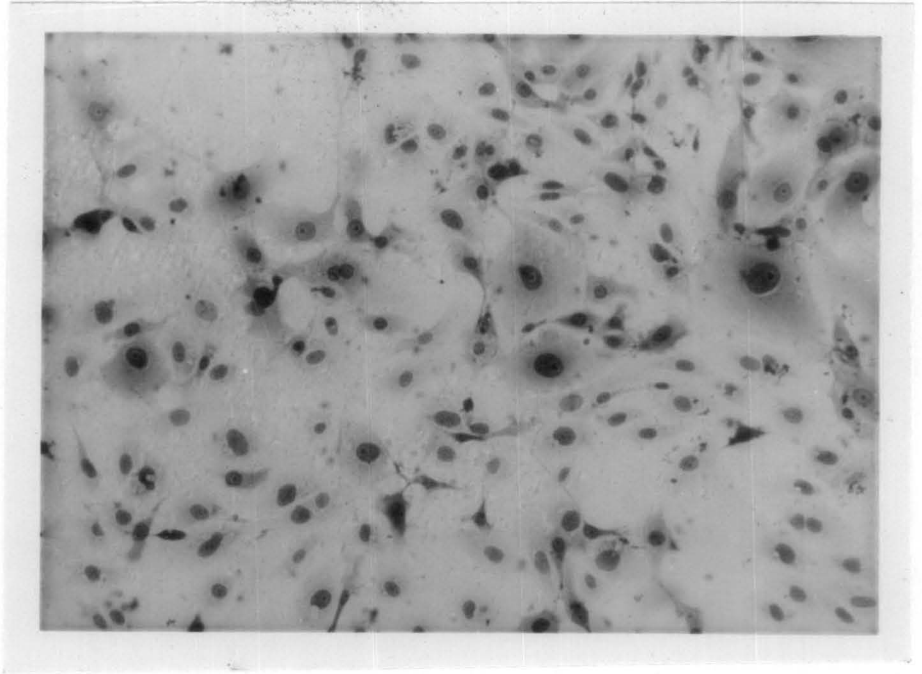
When the ethmoid mucosa was brought into culture, predominantly the cells were composed of fibroblast-like cells and a few epithelial cells (Fig.18). Through successive four subcultures in the growth medium, the quantitative balance between these two kinds of cells remained approximately similar to that in the early stage of the culture.

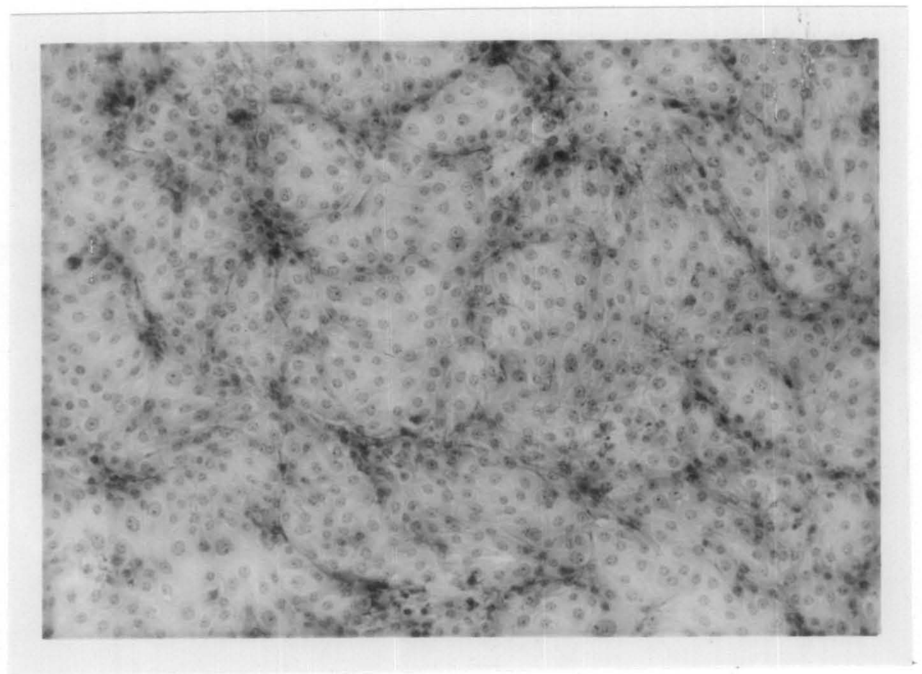
4.6.1.2 Differential enzymatic digestion

The differential enzymatic digestion, to have a comparatively pure culture of epithelial and fibroblast like cells, resulted in more rapid detachment of the fibroblastic cells than the epithelial cells. By repeating this procedure 2-3 times a week, most of the fibroblasts were removed and

Fig.18 Ethmoid mucosa - Primary culture - A mixture of spindle shaped fibroblast - like cells and a few polygonal epithelial cells - 2nd passage - May-Grunwald & Giensa x 250

Fig.19 Ethmoid mucosa - Control Epithelial Culture - The epithelial cells displaying growth of closely adherent polygonal cells in mosaic-like sheet - 3rd passage - May-Grunwald & Geimsa x 250





cultivated separately and epithelial clusters were left to grow. It took about 30-45 days to form more or less a complete monolayer. On successive subcultures, these cells behaved as epithelial cells displaying growth of closely adherent polygonal cells in mosaic-like sheets (Fig.19).

4.6.1.3 Aflatoxin B_1 (AFB_1) exposed culture

4.6.1.3.1 Mixed culture

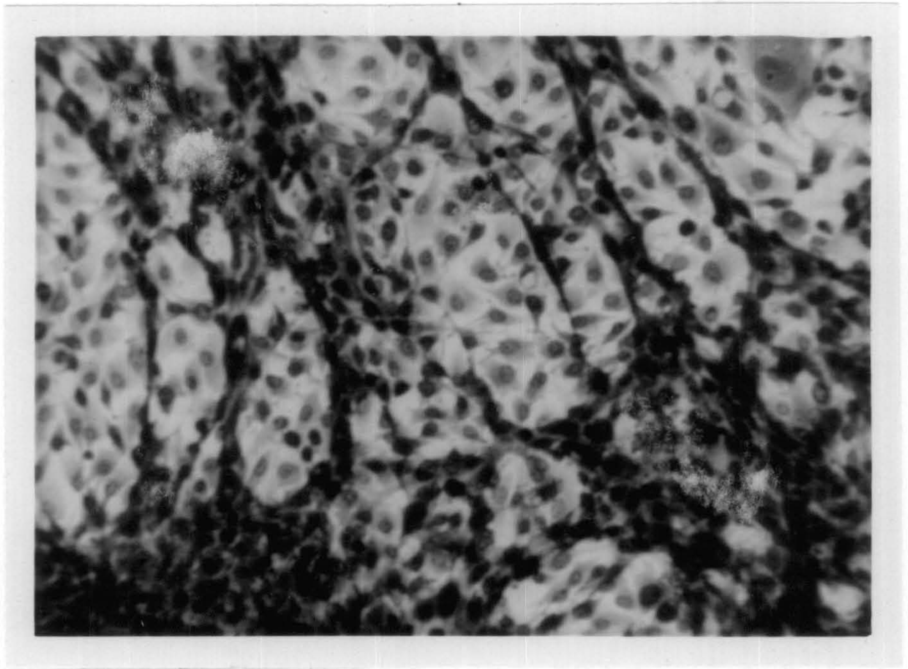
After the mixed culture was exposed to AFB_1, the cell damage was more marked in fibroblast-like cells than in the epithelial cells. The fibroblast like cells underwent degeneration and gradually decreased in number in accordance with the concentration of AFB_1 in the medium (Fig.20). This resulted in the difference of the numerical balance between epithelial and fibroblast-like cells. Selective cytotoxic effect was so severe that only few fibroblast-like cells could survive in both the experimental groups of cells treated with 0.05 µg and 0.1 µg of AFB_1/ml of media respectively, after about 20-25 days of exposure (Fig.21).

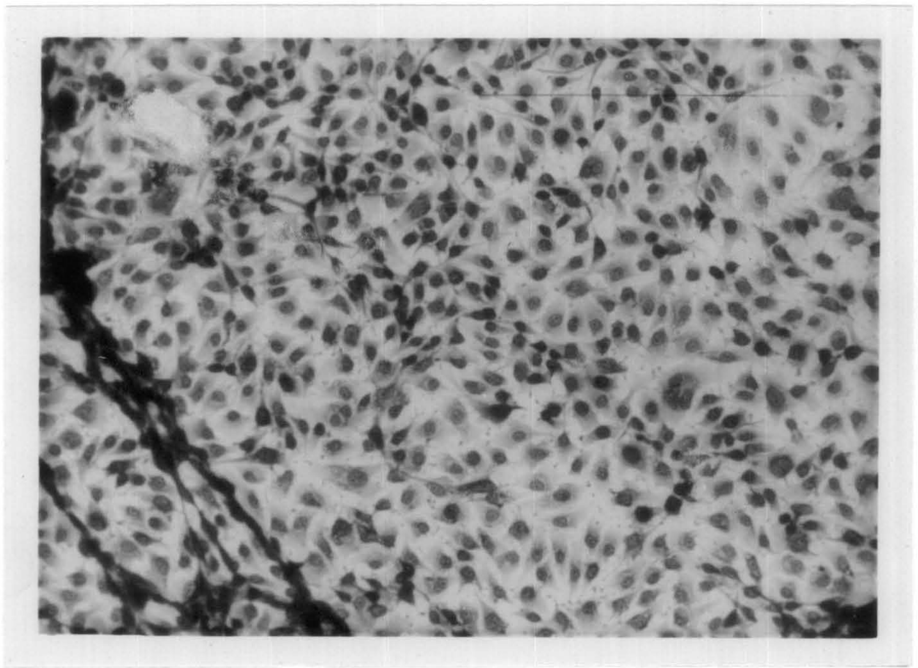
4.6.1.3.2 Epithelial culture

The cell damage in the epithelial culture, obtained after differential enzymatic digestion as well as that resulted after the mixed culture was exposed to AFB_1, was not

Fig.20 Ethmoid mucosa - Mixed culture - 10 days exposure to AFB_1 (0.05 µg/ml of medium) - Fibroblast-like cells showing degeneration and simultaneous proliferation of epithelial cells - May-Grunwald & Giemsa x 250.

Fig.21 Ethmoid mucosa - Mixed culture - 20 days exposure - Epithelial cell monolayer with a few strands of degenerating fibroblast-like cells - May-Grunwald & Giemsa x 250





so evident but when they were subcultured, it became remarkable. Subsequently, the progressive, cumulative cytotoxic effects of AFB_1 were noticed throughout the exposure period of 90 days through 6 subcultures.

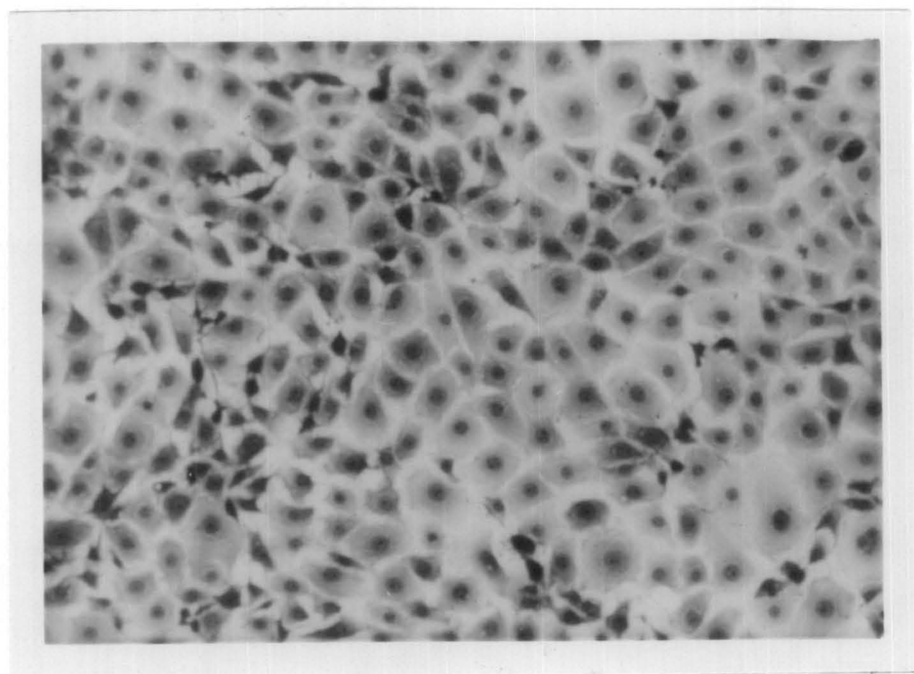
The most prominent alteration was the marked heterogeneity of cells ranging from small polygonal cells to larger cells with long cytoplasmic extensions in the epithelial sheets. In such cultures the cells also had conspicuous nucleus with 1 or two nucleoli (Fig.22).

The degenerative changes on successive subcultures were more prominent and characterized by karyorrhexis, pyknosis, increased cytoplasmic vacuolation and varying amount of cell debris. The nucleoli seemed to be separated or fractured into 2 or more parts. These "fractured" nucleoli were small and conspicuous (Fig.23). Although, some cells had two or more nucleus, mitosis was not appreciated during the treatment period of 90 days.

The morphology of the cells was fairly consistent, even after the AFB_1 treatment was discontinued. The pleomorphism was still apparent but cytoplasmic and nuclear degenerative changes were comparatively less extensive. The cells were smaller and more compact than they appeared when under AFB_1 exposure (Fig.24).

Fig.22 Ethmoid mucosa - Epithelial culture - 30 days exposure - The 6th passage - Nonadherent pleomorphic cells with cytoplasmic extensions - May-Grunwald & Geimsa x 250

Fig.23 Ethmoid mucosa - Epithelial culture - 45 days exposure - 7th passage - Pleomorphic cells - Cytoplasmic vacuolation - A few binucleated cells (arrows) - May-Grunwald & Geimsa x 250



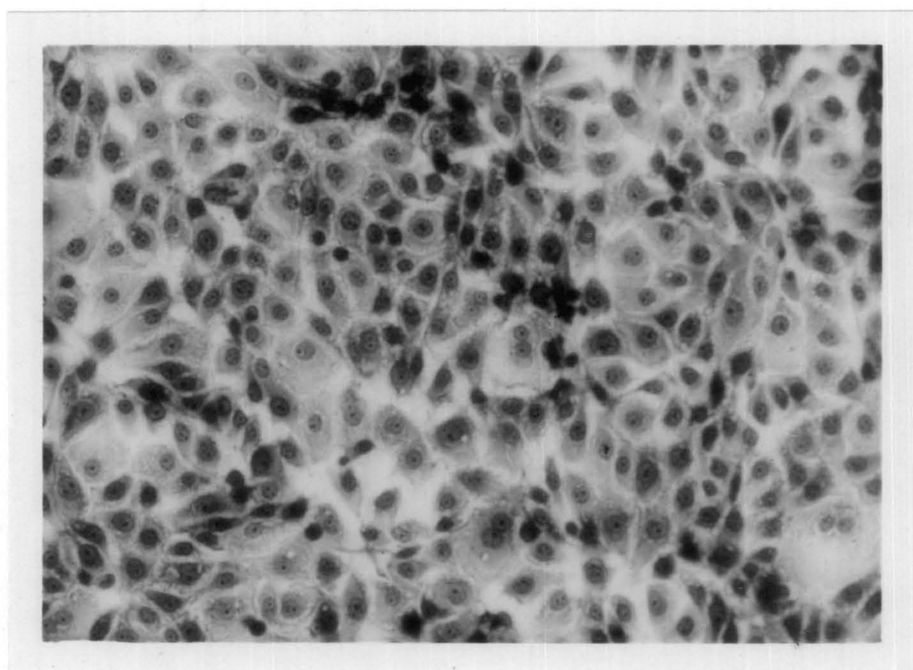
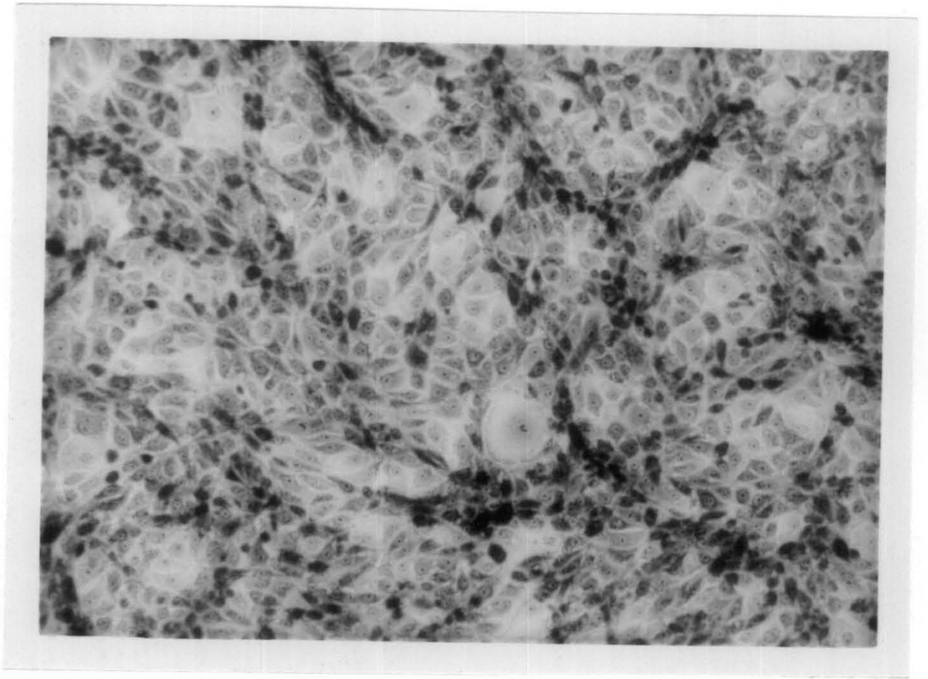
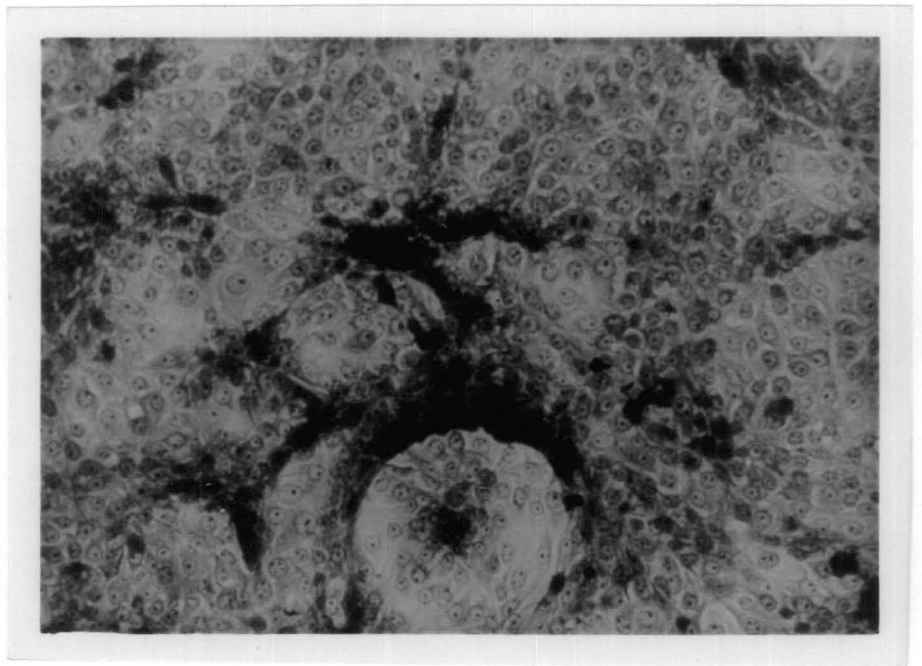


Fig.24 Ethmoid mucosa - Epithelial culture - 30 days after withdrawal of AFB_1 treatment - 13th passage - Small and compact epithelial cells with pleomorphism - May-Grunwald & Geimsa x 250

Fig.25 Ethmoid mucosa - Epithelial culture - 60 days after withdrawal of AFB_1 treatment - 15th passage - the cells showing tendency to pile up - May-Grunwald & Geimsa x 250





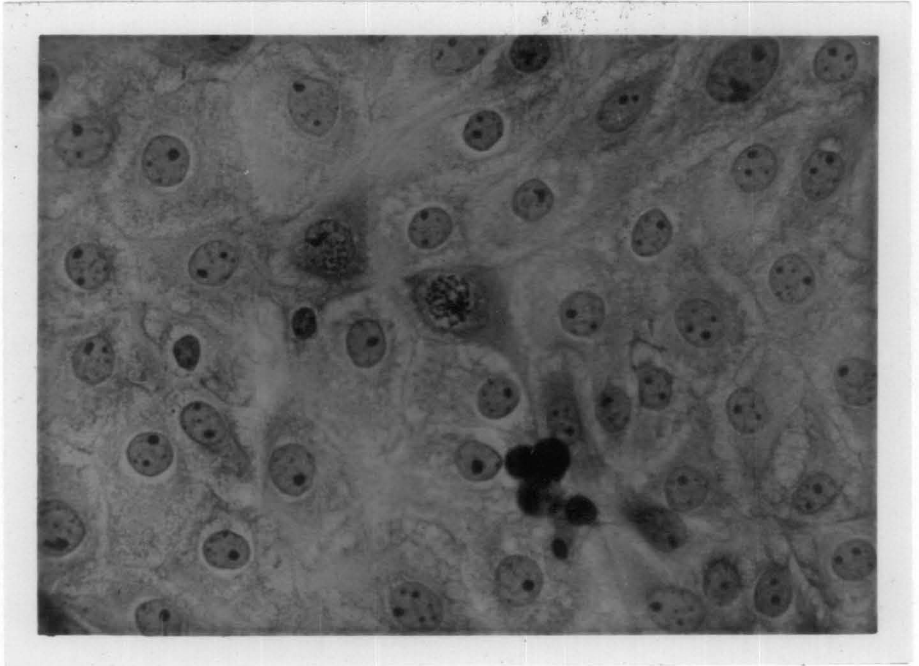
The AFB_1 initially produced a slight retardation in growth, which prolonged the interval between the subcultivation by several days. Later, the growth rates exceeded those of the control cultures, and subcultivation had to be performed every 7 to 10 days. This observation was also supported by the fact that the trypsinized cells were initially split 1:3, subsequently the cells were cultured 1:5 to 1:7, before reaching confluency every 7-10 days. The tendency of the cells to pile up (Fig.25) was conspicuous. The frequent mitotic cells (Fig.26) could be seen after the 12th subcultivation.

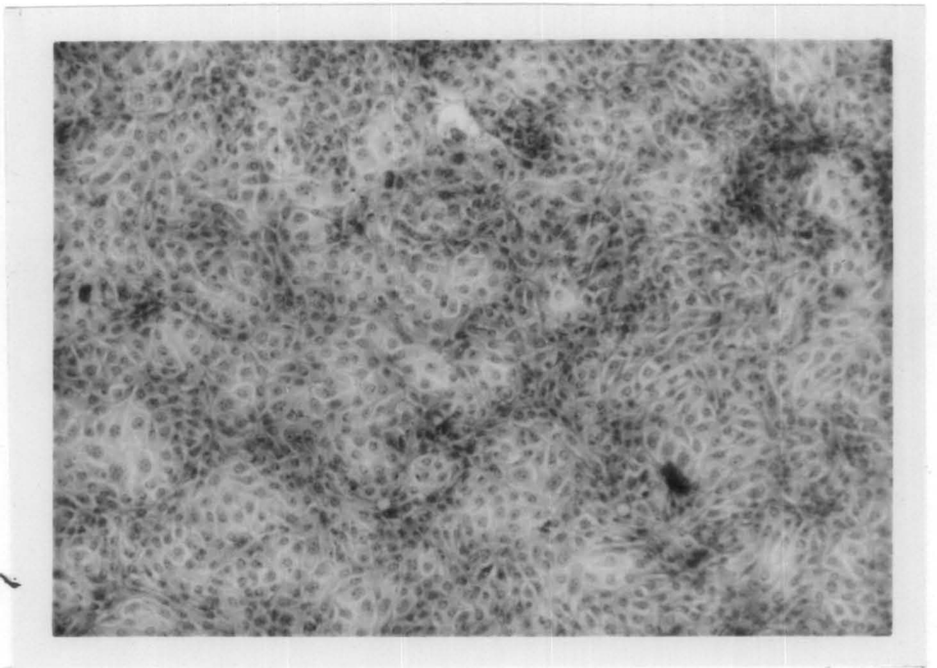
The sequential morphological alterations and/or developments in the epithelial cell line grown in the presence of AFB_1 at the concentration of 0.1 µg/ml of the media was comparable to that of the cell line treated at the dose level of 0.05 µg/ml, but the cytoxic changes were more extensive during the exposure period of AFB_1 for 90 days.

In the control groups, the cells grew as monolayer and no morphological alteration was recognised (Fig.27) and the cells could not be passaged after the 8th subcultivation under the similar experimental conditions.

Fig.26 Ethmoid mucosa - Epithelial culture - 60 days after withdrawal of AFB_1 treatment - 15th passage - the cells showing multiple nucleoli and mitotic figures - May-Grunwald & Geimsa x 400

Fig.27 Ethmoid mucosa - Epithelial culture - Dimethyl sulfoxide (DMSO) treated control - 5th passage - closely adherent polygonal cells in mosaic-like sheet - May-Grunwald & Geimsa x 160





4.6.1.3.3 Fibroblast-like culture

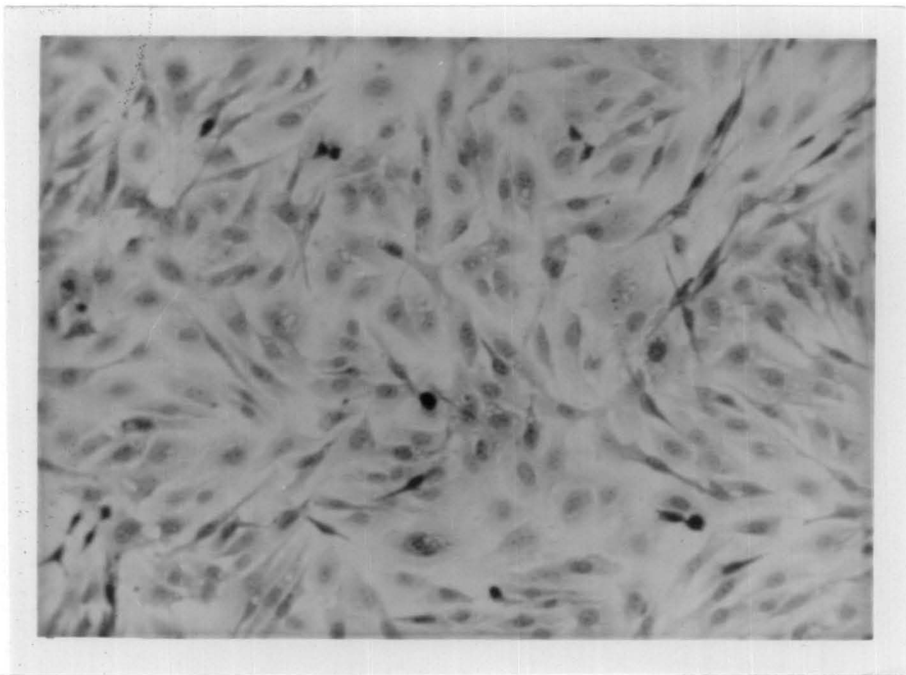
The spindle shaped fibroblast-like cells tended to grow in random orientation (Fig.28).

The fibroblast-like cells treated with 0.05 µg of AFB_1 per ml of the medium exhibited cell detachment within 48 hours of exposure period. However, small populations of the viable cells when subcultured began to proliferate into colonies. These cells on subsequent treatment with AFB_1 went through a similar crisis. But on every occasion, the viable cells which survived grew on subculture. Subsequent four cultivation of the viable cells, even in the presence of AFB_1, resulted in the recovery of growth rate and there was no marked cell detachment from the glass surface. Occasionally, small empty spaces appeared in the cell sheets suggesting localized cell degeneration. In addition, a variation in size and shape of the nucleus was noticed. There was marked karyorrhexis with conspicuous fragmentation of nucleoli. Some degree of cytoplasmic vacuolation was also recorded (Fig.29).

These morphological alterations persisted even after AFB_1 treatment was terminated. However, the cells were smaller and more compact than they appeared to be in the beginning of the study (Fig.30). The growth rate of the treated fibroblast-like cells was comparable to that of the

Fig.28 Ethmoid mucosa - Fibroblast-like cells - 4th passage - spindle shaped cells growing in random orientation - May-Grunwald & Geimsa x 250

Fig.29 Ethmoid mucosa - Fibroblast-like cells - 45 days AFB_1 exposure - 7th passage - cells showing enlarged nucleus with nucleolar fragmentation - slight cytoplasmic vacuolation - May-Grunwald & Geimsa x 250



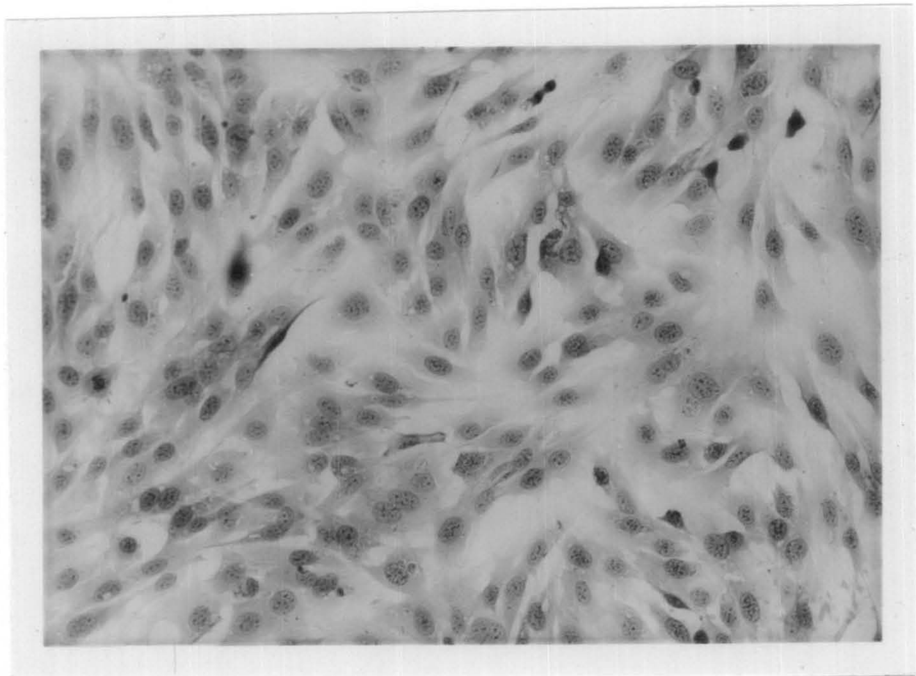
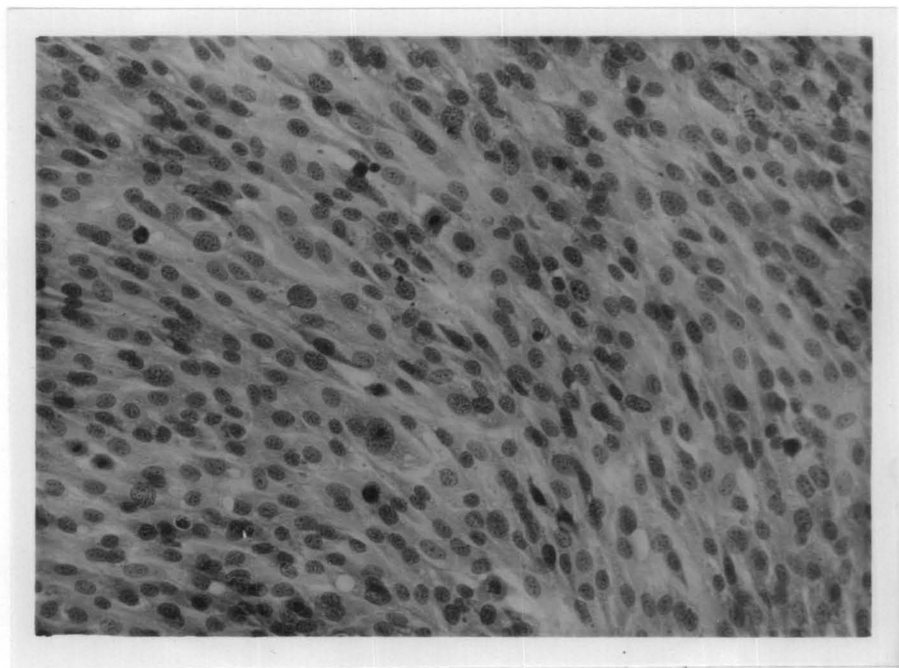


Fig.30 Ethmoid mucosa - Fibroblast like cells - 45 days after AFB_1 treatment - 14th passage - A compact monolayer of fibroblast like cells - May-Grunwald & Geimsa x 250



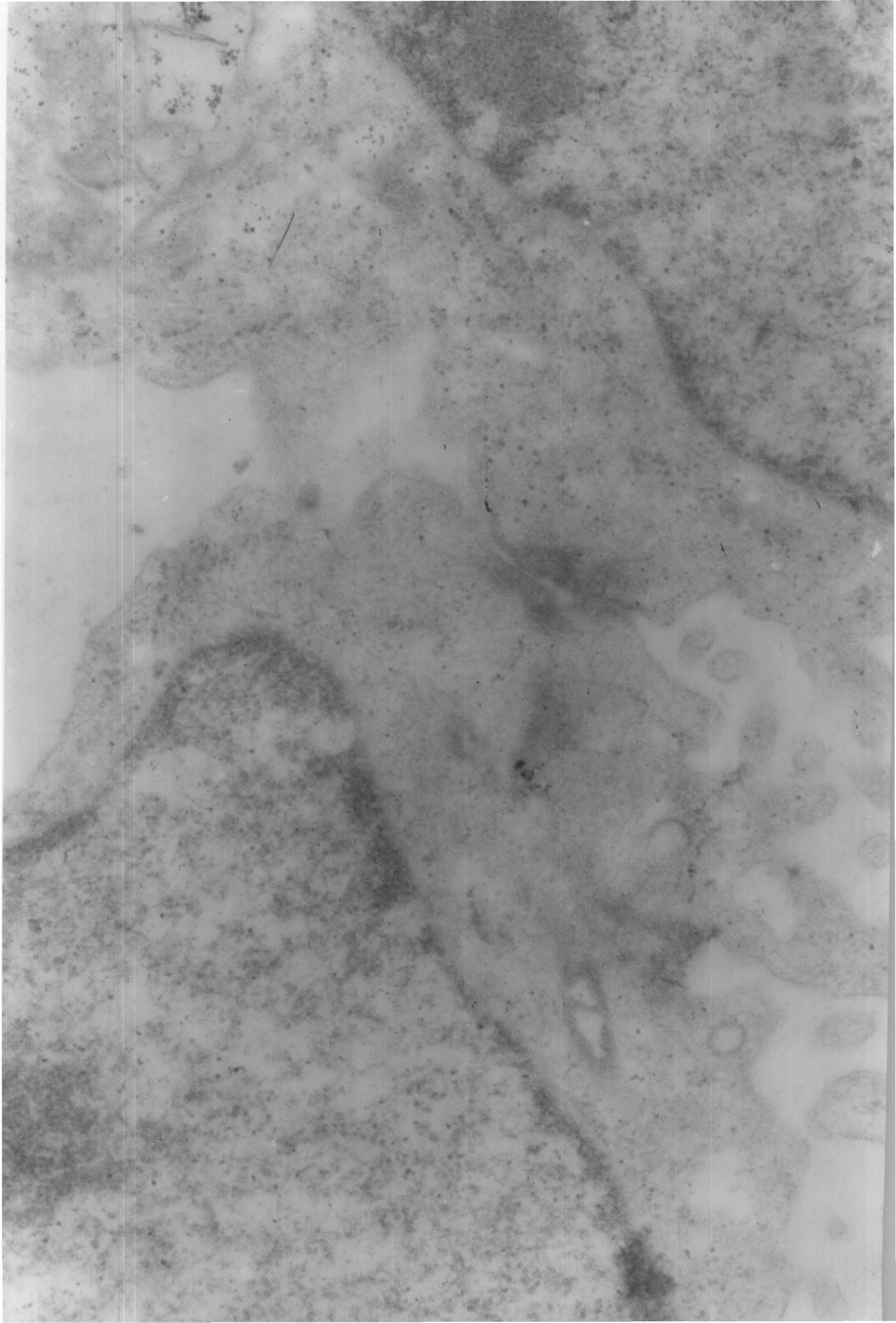
control. There was no significant increase in the split ratio of trypsinized cells at any stage of subcultivation which remained invariably constant at 1:3. The tendency to pile up as seen in treated epithelial cells after the 12th passage was not evident in the treated fibroblast-like cells at any stage of *in vitro* growth.

However, the vehicle treated fibroblast-like cells were characterised by the absence of morphological changes. However, after the 9th subcultivation, the cells enlarged, showed signs of senescence and they died after being about 4 months *in vitro*.

4.6.2 Ultrastructural morphology

The cells proliferating in clusters or aggregates had wavy irregular plasma membranes. The surface of these cells, as well as those growing singly often showed formation of microvilli-like extensions of the plasma membrane. Although no true desmosomes were observable, these cells had tight junctions, a characteristic of the epithelial cell (Fig.31). The cytoplasmic ground substance in between the cell organelles appeared as markedly dense and finely granular. The endoplasmic reticulum was moderately developed and randomly distributed throughout the cytoplasm. Vacuoles were seen near poorly developed golgi apparatus. Mitochondria were

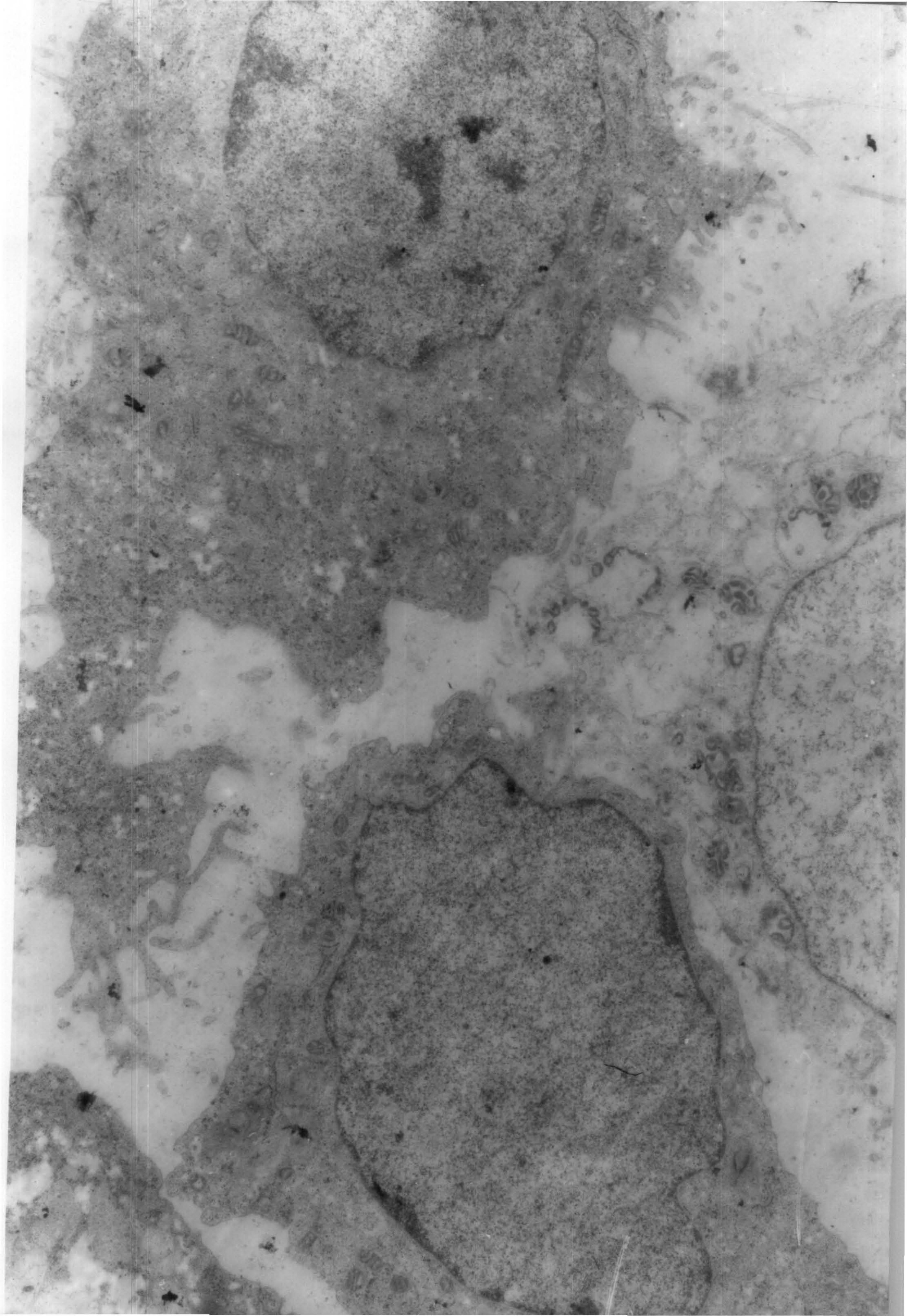
Fig.31 Electron micrograph. Epithelial culture cells showing cell junction - x 25,000



abundant and evenly distributed in the cytoplasm. They showed considerable variation in size and form. Most of the mitochondria were oval or elongated with short cristae. Nucleus was round or oval with prominent nucleoli. Other cytoplasmic structures like lipids were occasionally observed.

The ultrastructural morphology of the ethmoturbinate epithelial cells cultured in the presence of 0.05 µg of AFB_1 was characterized by marked degenerative changes. The presence of abundant dark cells, considered as degenerated cells, was the consistent feature of epithelial cells examined at various intervals of AFB_1 treatment (Fig.32). The dissolution of the plasma membrane was also observed particularly in later stages of AFB_1 exposure. The mitochondria showed intracristal swelling. In the early stages, this intracristal swelling was not associated with overall increase in the size of the mitochondria. But in later stages, a severe degree of intracristal swelling was observed, where matrix formed an electron-dense band at the periphery of the mitochondria. At times, one portion of the mitochondria showed intracristal swelling and dense matrix and the remainder showed swelling of the matrix chamber. Varying degree of dilatation and vesiculation of the rough endoplasmic reticulum was seen, which was more conspicuous in the terminal stages of acute toxicity of AFB_1. At focal areas, the

Fig.32 Electron micrograph. AFB_1 treated epithelial culture showing two cells with electron-dense and one cell with electron-lucent cytoplasmic contents - Mitochondria showing deformed and lytic cristae - x 20,000



degranulation of the rough endoplasmic reticulum was also very characteristic (Fig.33). The cytoplasmic vacuoles, lysome like bodies free ribosomes, cytoplasmic filaments and glycogen were also oberved in some of the cells, but other cytoplasmic organelles were not so conspicuous as those of control cells.

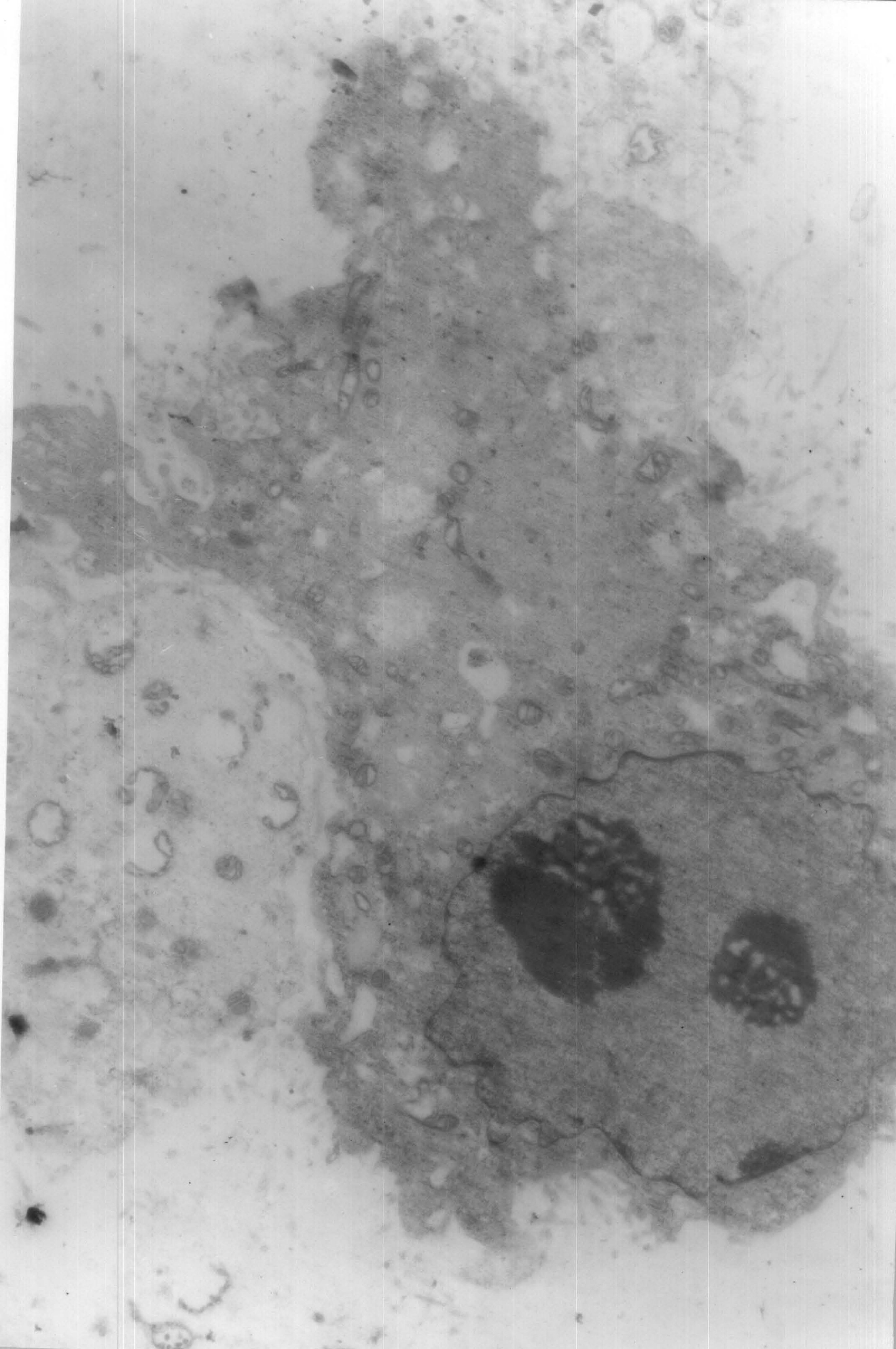
The nucleus was markedly enlarged, had one or two nucleoli (Fig.34). Nucleolar segregation was also conspicuous where dense filamentous and light granular zones were easily discernable. Other characteristic nuclear feature observed in these AFB_1 exposed cell was predominance of euchromatin with little heterochromatin.

The ultrastructural features of epithelial cells derived from bovine ethmoid mucosa and passaged in the presence of 0.1 µg of AFB_1 per ml of medium were comparable to those of 0.05 µg of AFB_1 treated cells, but the extent of the involvement of subcellular organelles and the intensity of the cytotoxic changes were more marked (Fig.35).

The cytotoxic features more or less persisted even after the withdrawal of AFB_1. Dilatation of the cisternae of rough endoplasmic reticulum and focal degranulation of ribosomes was noticed even at 60 days after the withdrawal of AFB_1 treatment. The nuclear changes with respect to the

Fig.33 Electron micrograph. A part of AFB_1 treated epithelial cell showing mitochondria with deformed and swollen cristae and partial degranulation of endoplasmic reticulum - Presence of fragmented filamentous structures seen - x 45,000

Fig.34 Electron micrograph. AFB_1 treated epithelial cell showing nucleus with irregular nuclear membrane and two prominent nucleoli - Ruffled plasma membrane is seen - x 20,000



irregularity of the nuclear contour, margination of the nucleoli, abundance of euchromatin and occasional nuclear body were the characteristic observations of epithelial cells especially after the 12th passage (Fig.36). Glycogen and free ribosomes were also observed in the cytoplasm at this stage of experiment (Fig.37).

The fine structure of the fibroblast-like cells of the ethmoid mucosa origin and growth in vitro showed pleomorphism with respect to shape and size. They were round to spindle shaped with irregular plasma membrane. The cells had sparse cytoplasm and irregular nucleus, which were suggestive of their mesenchymel origin. But the abundance of the rough endoplasmic reticulum, a characteristic of the fibroblast cells, was not seen in these cells.

The ultrastructural changes in these cells treated with AFB_1 at the concentration of 0.05 µg per ml of medium were mainly characterized by cytoplasmic vacuolization. These cytoplasmic vacuoles and vesicles were composed of smooth membranes and most of them presumably as were dilatated smooth endoplasmic reticulum. The enlargement of nucleus and necleolar segregation were also observed in these cells. These toxic changes persisted even after the termination of AFB_1 treatment. No change suggestive of in vitro transformation was seen in these fibroblast-like cells at any

Fig.35 Electron micrograph. A part of AFB_1 treated epithelial cell showing nucleus and cytoplasmic organelles with retrogate changes - x 25,000

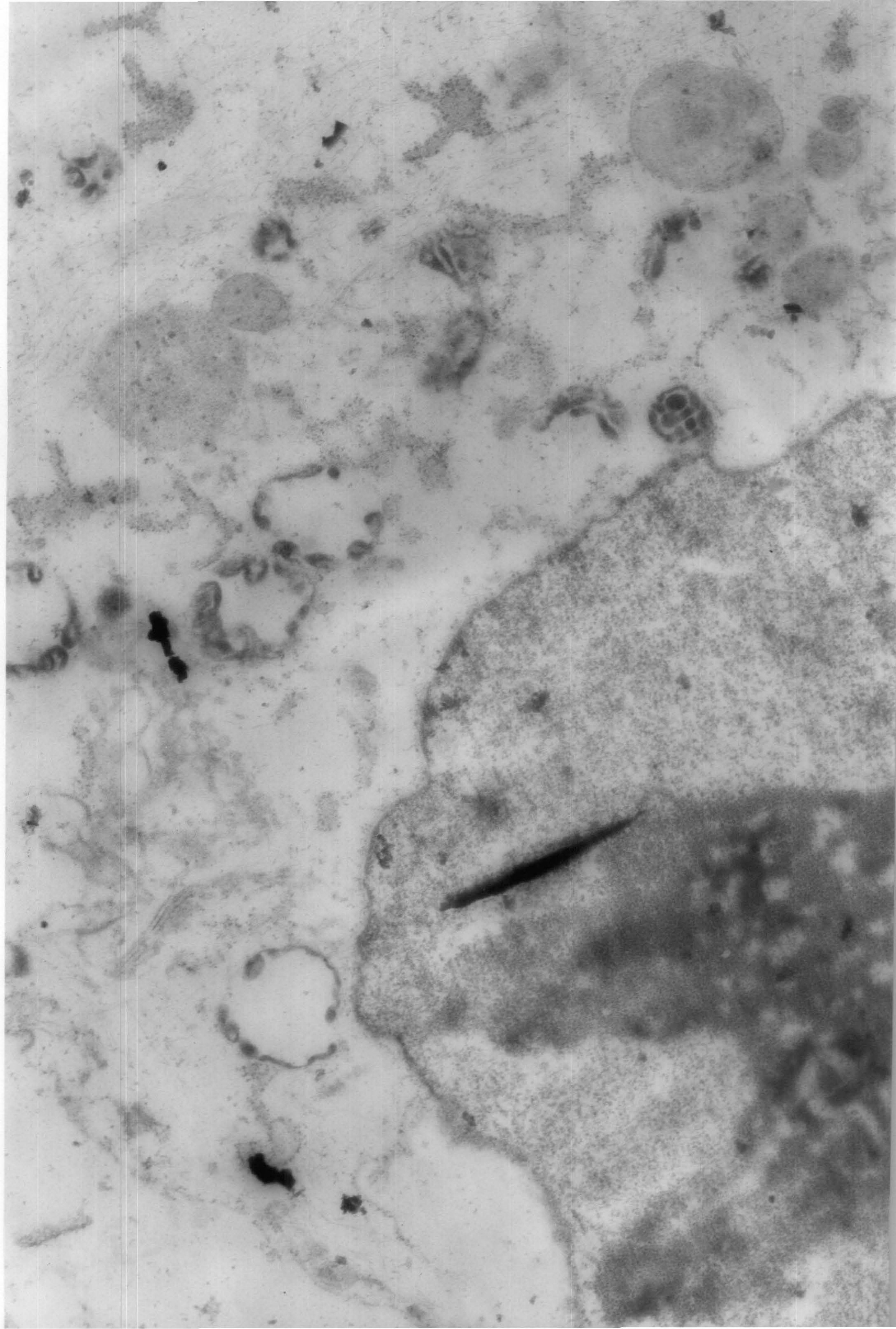


Fig.36 Electron micrograph. AFB_1 treated epithelial cell showing invaginated nuclear membrane and nucleolar margination - x 15,000

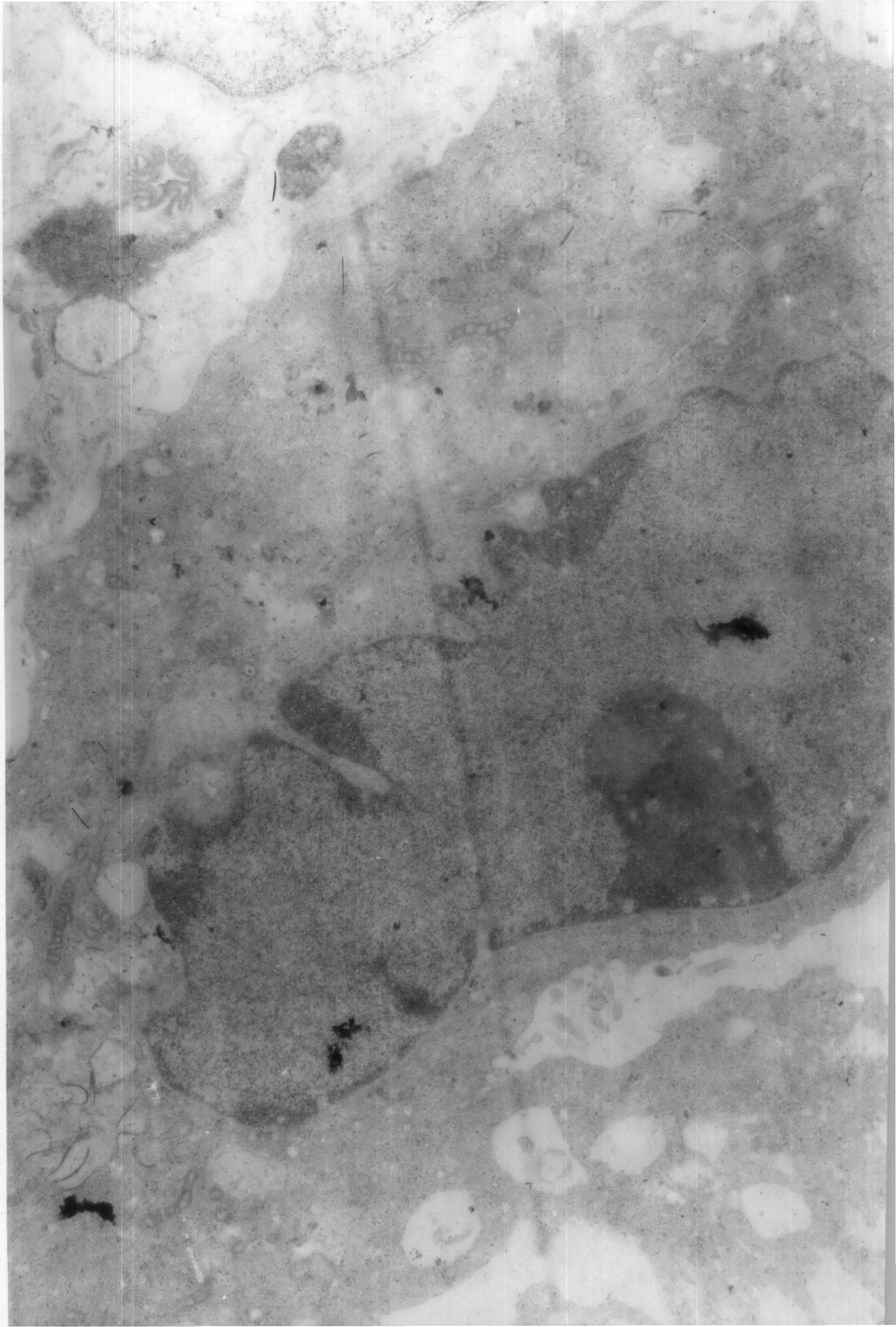
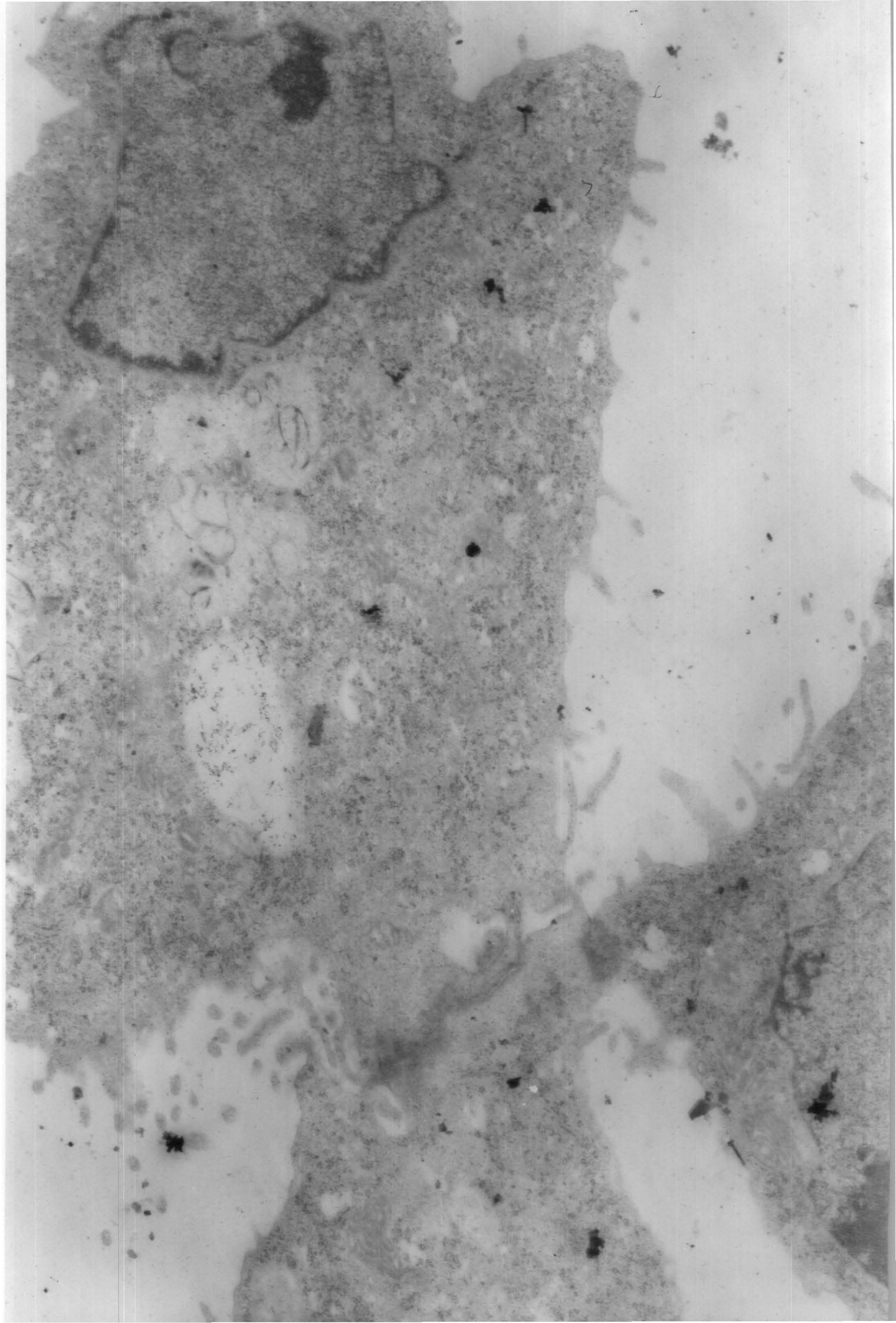


Fig.37 Electron micrograph. AFB_1 treated epithelial cells showing microvilli - The free ribosomes and glycogen in the cytoplasm - Nucleolar margination is also seen - x 25,000



stage of the cultivation cells for 180 days through 14 passages.

4.6.3 Assay for transformation

4.6.3.1 Colony forming efficiency (CFE) in soft agar

A correlation between transformation _in vitro_ and growth in soft agar was confirmed using assay of Macpherson and Montagnia (1964) with minor modifications (Table 4; Fig.38).

The percentage of the CFE in soft agar, of AFB_1 treated epithelial cells at a concentration of 0.05 µg and 0.1 µg/ml of media varied from 15.2 to 27 per cent (mean 21.06 $\pm$ 4.52%) and 13.9 to 29 per cent (mean 20.70 $\pm$ 6.75%) respectively. The corresponding values in the untreated controls ranged from 0.00 to 0.2 (mean 0.03 $\pm$ 0.08). The overall difference in the mean percentage of CFE's in treated and untreated control epithelial cells was statistically significant ($P<0.05$), thereby indicating the _in vitro_ transformation by AFB_1 treatment of epithelial cells of the bovine ethmoid mucosal origin.

A slight increase in the percentage of CFE'S in soft agar of fibroblast-like cells (2.0-4.1%) exposed to AFB_1 at the dose level of 0.05 µg/ml of medium was observed as

Table 4. Mean colony forming efficiency (CFE) of ethmoid mucosa cells in soft agar

Culture	Aflatoxin B1 (µg/ml of medium)	Duration of treatment (Days)	Duration in culture (Days)	Passage No.	CFE %						Mean
					102*			103*			
					P_1	P_2	P_3	P_1	P_2	P_3	
Epithelial	0.05	90	210	14th	25	18	27	15.2	18.7	22.5	21.06 ± 4.52^{a}
Epithelial	0.1	90	210	14th	17	22	29	13.9	14.1	28.2	20.70 ± 6.78^{a}
Epithelial	-	-	120	7th	0	0	0	0	0.2	0	0.03 ± 0.08^{b}
Fibroblast-like	0.05	90	210	14th	4	2	3	2.2	2.0	3.9	2.85 ± 0.92^{b}
Fibroblast-like	-	-	122	8th	0	0	0	0	0	0	0.01 ± 0.04^{b}

* Seedling density per petridish

Mean values having the same superscripts are not significantly ($P<0.05$) different

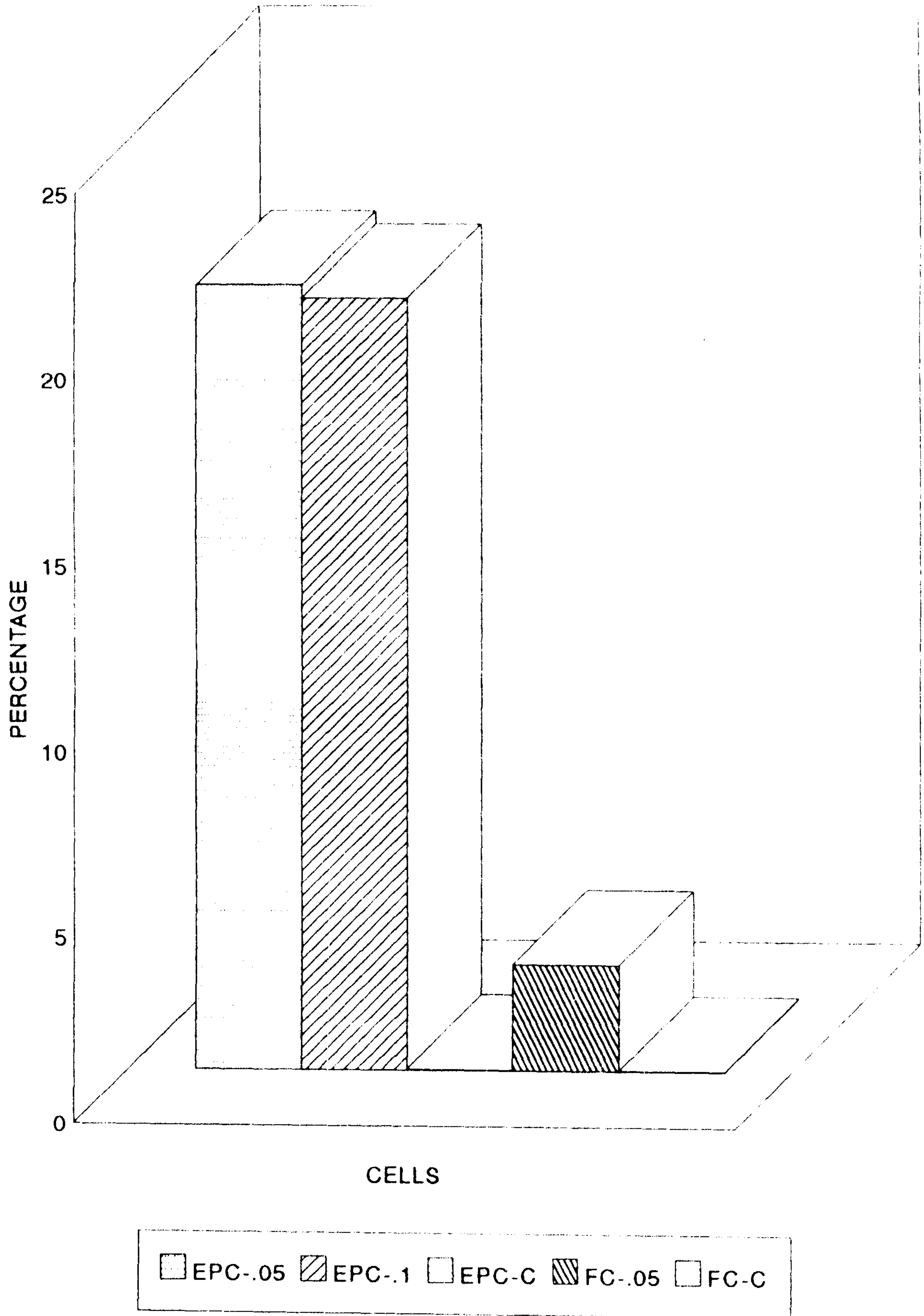


FIG. 38 MEAN COLONY FORMING EFFICIENCY (CFE) OF ETHMOID MUCOSA CELLS IN SOFT AGAR

compared to untreated control cells (0.00-0.10%). But the overall difference in the mean values of CFE in the treated (2.85 $\pm$ 0.92%) and control cells (0.016 $\pm$ 0.04%) was not significant.

4.6.3.2 Colony forming efficiency (CFE) in 10 and 1 per cent serum media

The untreated epithelial as well as fibroblast-like cells had lower CFE's in medium containing 10 or 1 per cent foetal calf serum (FCS) than the treated cells. But the depression in CFE's was more significant in fibroblast-like cells as compared to epithelial cells. The relative CFE's of the epithelial cells in 1 per cent FCS compared to 10 per cent FCS were high with no significant difference between treated and control cells, while this difference was significant in the case of fibroblast-like cells (Table 5; Fig.39).

4.6.3.3 Cytochemical assay of Gamma-glutamyl transpeptidase (GGT) activity

The non-treated epithelial and fibroblast-like cells as well as treated fibroblast-like cells, derived from normal bovine ethmoid mucosa were consistently negative for GGT, whereas epithelial cells treated with AFB_1 at the dose level of 0.05 µg and 0.1 µg per ml of media respectively had levels of activity varying from isolated foci of positivity to

Table 5. Colony forming efficiency (CFE) of ethmoid mucosa cells in 10 per cent and 1 per cent serum media

Culture	Aflatoxin B1 (ug/ml of medium)	Duration of treatment (Days)	Duration in culture (Days)	Passage No.	CFE % 10% FCS (10^2)* P_1	P_2	P_3	Mean ± SD	1% FCS (10^2)* P_1	P_2	P_3	Mean ± SD	Relative CFE (1%/10%)
Epithelial	0.05	90	210	14th	70	69	73	$70.66^{a} \pm 2.08$	43	52	59	$51.33^{aa} \pm 8.02$	72.64
Epithelial	0.1	90	210	14th	73	59	84	$72.00^{a} \pm 12.52$	56	47	60	$54.33^{aa} \pm 6.65$	74.45
Epithelial	-	-	120	7th	43	41	38	$40.66^{b} \pm 2.51$	20	22	27	$23.00^{bb} \pm 3.60$	56.56
Fibroblast-like	0.05	90	210	14th	52	63	47	$54.00^{c} \pm 8.18$	24	20	19	$21.00^{cc} \pm 2.64$	38.88
Fibroblast-like	-	-	122	8th	49	38	29	$38.66^{b} \pm 10.01$	20	18	27	$21.66^{bb} \pm 4.72$	56.02

Mean values having the same superscripts are not significantly ($P<0.05$) different

* Figures in the parentheses indicate seeding density per petri dish

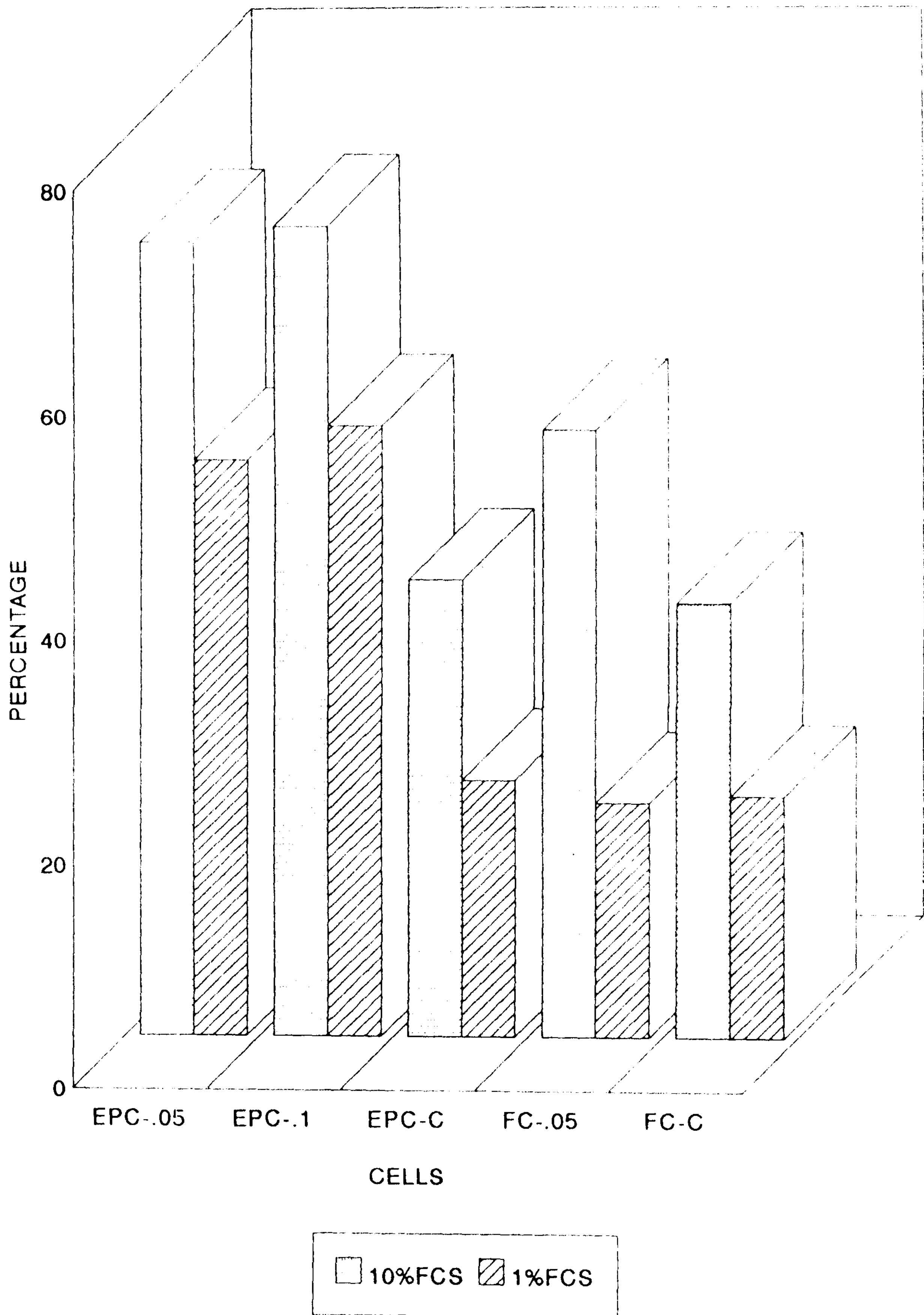


FIG. 39 MEAN COLONY FORMING EFFICIENCY (CFE) OF ETHMOID MUCOSA CELLS IN 10 AND 1% SERUM MEDIA

Table 6. Cytochemical assay of gamma-glutamyl transpeptidase (GGT) activity of *in vitro* aflatoxin B_1 treated ethmoid mucosa cells

Culture	Aflatoxin B_1 (µg/ml of medium)	Duration of treatment (Days)	Duration in culture (Days)	Passage No.	Cytochemical assay*	
					Semiconfluent	Confluent
Epithelial	0.05	90	210	14th	0 F	++
Epithelial	0.1	90	210	14th	0 F	++
Epithelial	-	-	120	7th	0	0
Fibroblast-like	0.05	90	210	14th	0	0
Fibroblast-like	-	-	122	8th	0	0

* GGT activity expressed as ++++ 100% cells show the activity
+++ 60-100%
++ 20-60%
\+ <20%
F activity only in foci
O No activity detected

moderate activity (Table 6). The level of activity was higher in cultures grown to confluency than in those tested at semiconfluency.

4.6.3.4 Xenotransplantation of *in vitro* treated cells

Out of six immunosuppressed mice, the two mice inoculated with epithelial cells treated *in vitro* with AFB_1 at the concentration of 0.1 µg per ml of medium and one mouse inoculated with epithelial cells exposed to AFB_1 at the dose level of 0.05 µg per ml of medium, developed palpable growth within 3 days after the inoculation. The growth persisted upto the 9th day after inoculation but subsequently it gradually disappeared. The histological examination of the tissue taken from the site at 60th day showed mild fibrosis, but no cell was seen.

There was no gross or microscopic evidence of "take" in the mice inoculated with treated fibroblast-like cells and non-treated control epithelial and fibroblastic cells at any stage of 60 days observation period.

4.7 Study in spontaneous cases of the carcinoma of the mucosa of ethmoid

4.7.1 Ultrastructural study

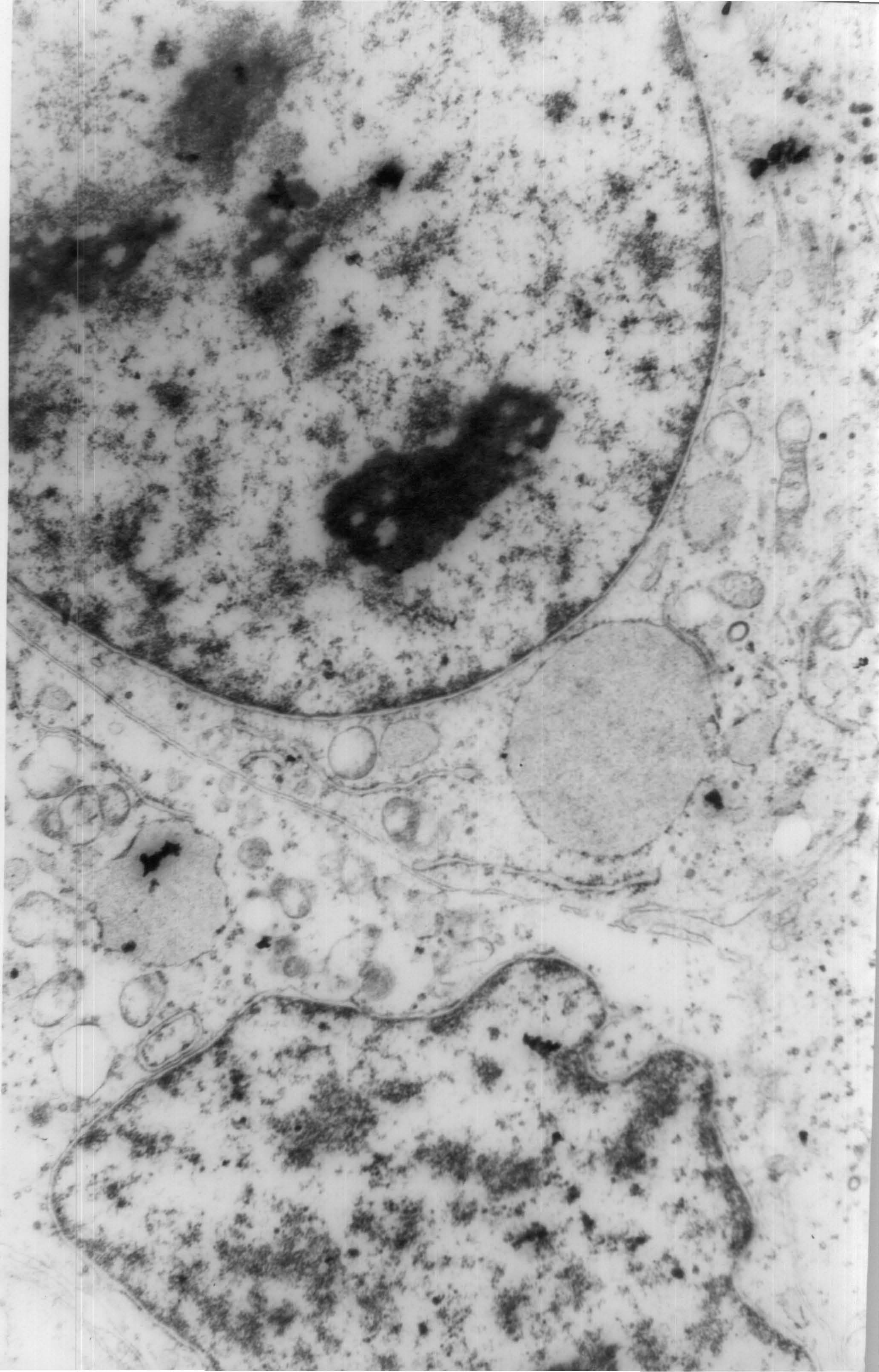
Ultrastructural examination of the ethmoid tumour

confirmed the epithelial nature of the neoplastic cells. Three main cells could be distinguished based on the size, shape and cytological variations.

Light or electron lucent round to polyhedral cells. These cells had more or less a regular plasma membrane with few interdigitation. The desmosomes could not be seen in the cell type described. In some areas, these cells were separated from the surrounding stromal tissue by a structure like the basement membrane. The few mitochondria observed had cristae with varying degree of disorganization and dissolution. The content of the endoplasmic reticulum were dilated and arranged either in the form of sinuous sacs or spherical structures. The golgi apparatus was encountered rarely. Abundant free ribosomes in the form of polyribosomes and glycogen were consistent features of these cells. The nucleus of the neoplastic cells was round, slightly indented and had one or two prominent nucleoli. The nucleoli were found close to the nuclear membrane in some cells. The euchromatin was predominant and heterochromatin could be seen as small aggregates along the inner nuclear membrane and dispersed throughout the cytoplasm (Fig.40).

The second type of neoplastic cells encountered during the electron-microscopic examination were secretory in nature. The cells were comparatively electron-dense and cuboidal or

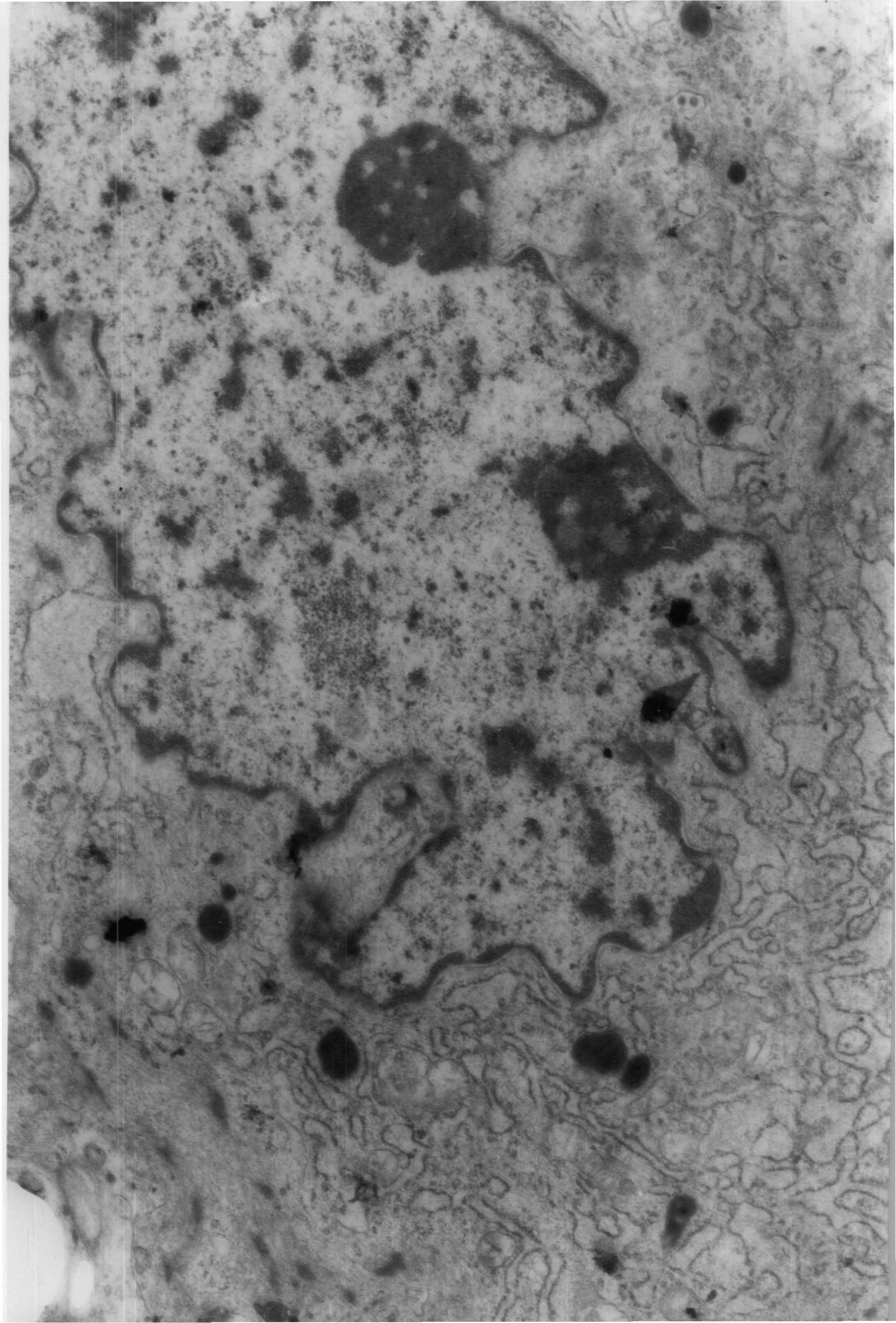
Fig.40 Electron micrograph. Neoplastic cells showing dilated endoplasmic reticulum containing electron lucent material - Mitochondria with partial loss of cristae - Numerous glycogen particles in the cytoplasm - x 25,000



columnar in shape. The cells had tight junctions and invagination between them. The cluster of cells formed lumina and had microvilli at the terminal surface. The mitochondria were scarce and randomly distributed throughout the cytoplasm, but invariably they were in various stages of degeneration. The close association between rough surfaced endoplasmic reticulum and mitochondria was also seen. The well developed prominent rough surfaced endoplasmic reticulum was a common finding. They were dilated and contained flocculent material. Occasional cell had electron-dense inspissated proteinaceous material trapped in rough endoplasmic reticulum. The secretory granules, which were round, and of size ranging from 0.1 to 0.8 µm, were uniformly electron-dense and bounded by a unit membrane, were characteristic components of the cytoplasm. Some of the cells had bundles of filaments which were 6-8 nm thick and randomly oriented throughout the cytoplasm. The cell nucleus was large, elongated and had irregular nuclear membrane. Two or more prominent nucleoli, predominance of enchromatin with little heterochromatin were also the characteristic features of these cells. The interchromatin and perichromatin granules could be clearly visualised (Fig.41).

The third type of cells found in the neoplasm of the mucosa of ethmoid were elongated and had irregular and ruffled

Fig.41 Electron micrograph. Neoplastic cell showing well developed endoplasmic reticulum - Interchromatin granules and nucleolar margination - x 20,000



plasma membrane. The adjacent plasma membranes were joined by fully developed junctional complexes and had few interdigitations (Fig.42). The extensively dilated rough surface edoplasmic reticulum and many round, swollen mitochondria with intact cristae were the characteristic features of these cells. Free ribosomes, glycogen, tonofibrils and myelin figures were the other cytoplasmic structures encountered. The nucleus was irregularly shaped and the nucleoplasm was electron lucent containing prominent nucleoli.

The stroma was infiltrated with lymphocytes, plasma cells and varying amount of connective tissues (Fig.43). Irrespective of the cell type, occasionally, nuclear bodies were seen in the nucleus. Dilatation of blood vessels with well differentiated endothelial cells was also observed (Fig.44).

Electronmicroscopic examination of retropharyngeal lymphnodes with metastasis revealed the presence of neoplastic cells with ultrastructural features similar to those observed in the cells of the primary ethmoid tumour (Fig.45).

The scanning electron microscopic investigations revealed that the surface of the tumour was characterized by uniform, domed shaped cells, but there was no evidence of cilia or microvilli on the cell surface. The oval to

Fig.42 Electron micrograph. Neoplastic cells showing cell junctions - x 45,000

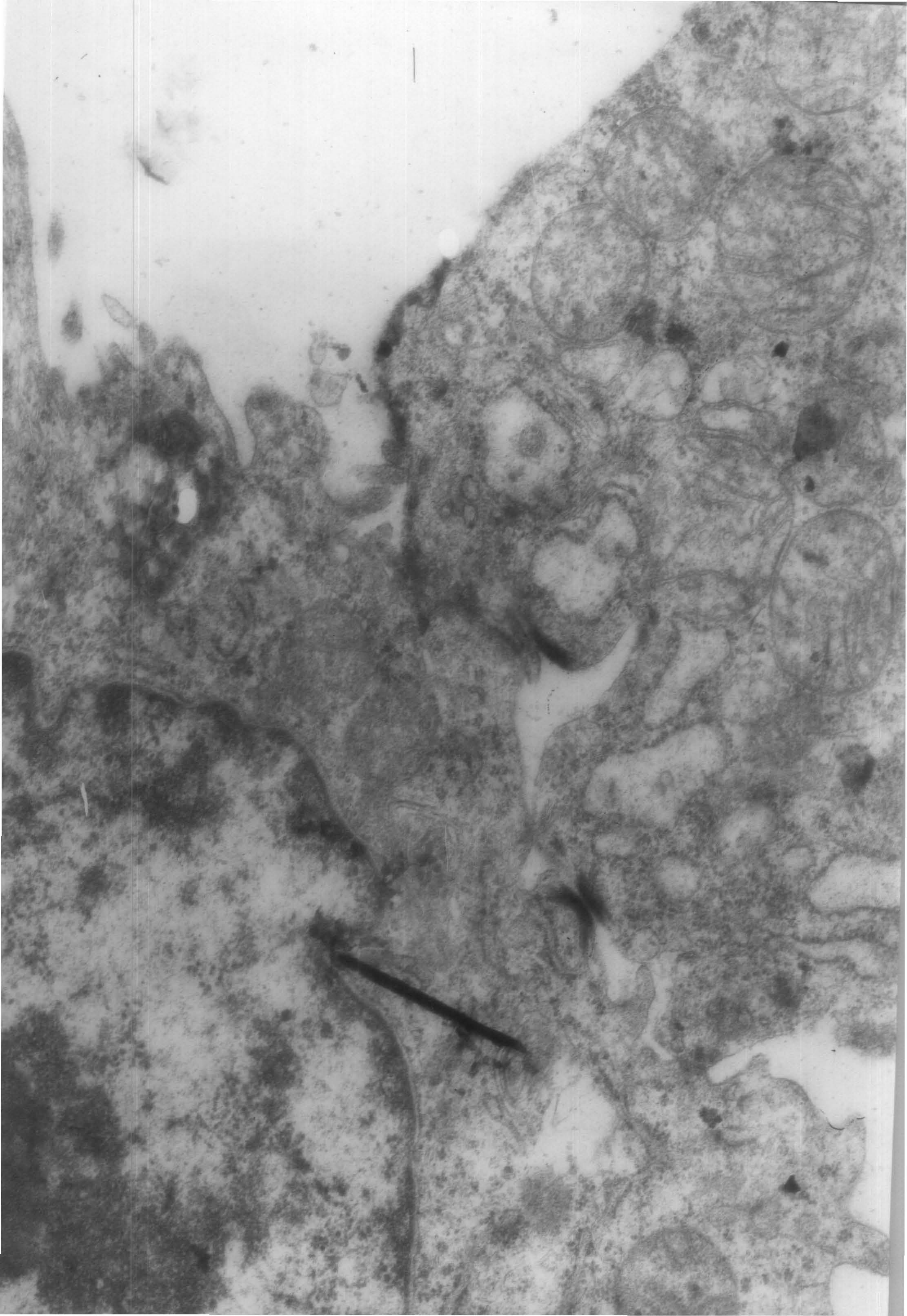


Fig.43 Electron micrograph. A neoplastic cell showing association with lymphocyte - x 25,000

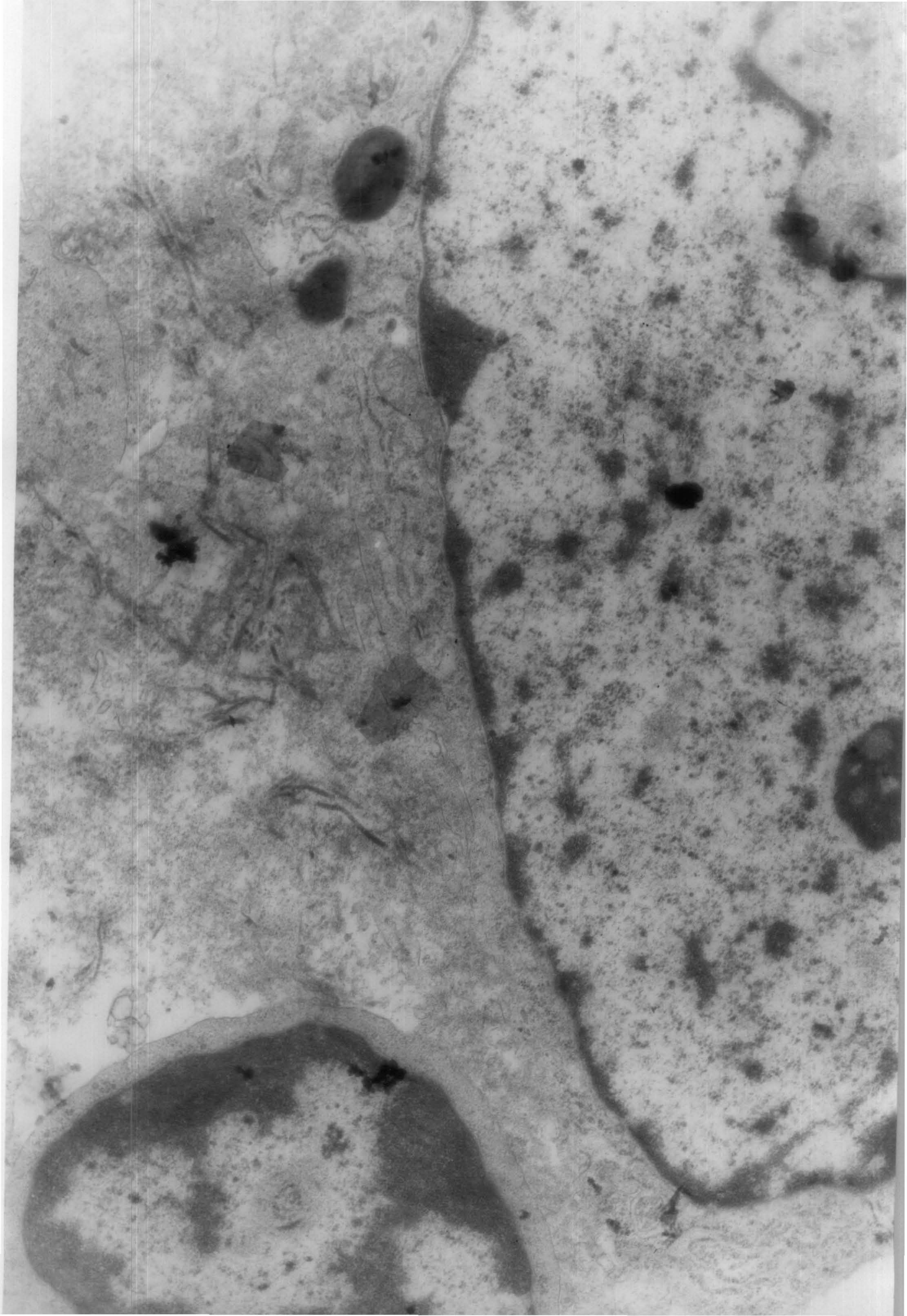
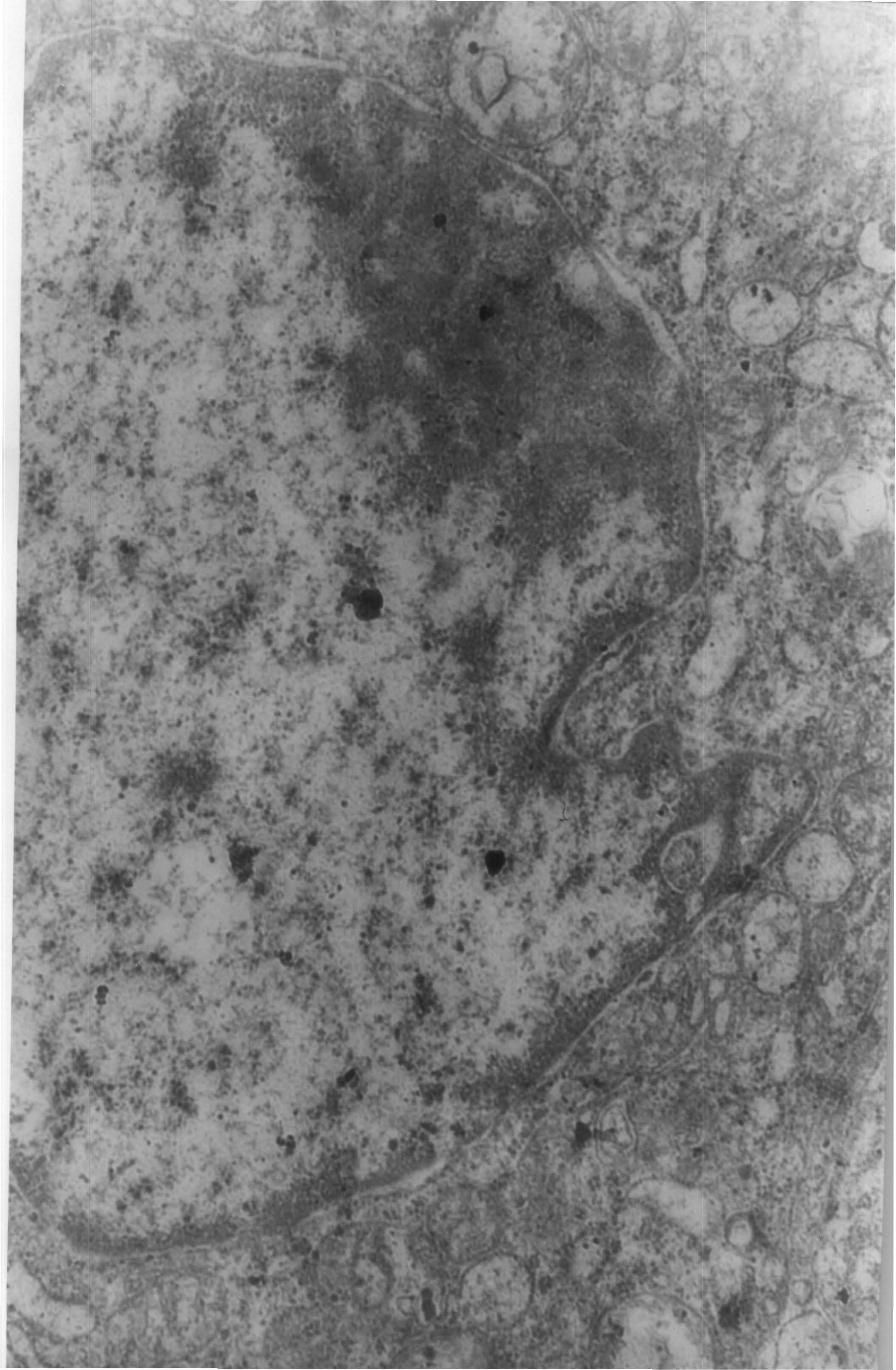


Fig.44 Electron micrograph. A dilated blood vessel with well differentiated endothelial cell in neoplastic tissue - x 25,000

Fig.45 Electron micrograph. A cell with prominent cytoplasmic organelles from retropharyngeal lymphnode with metastasis - x 30,000



elongated cells with stack suggestive of goblet or mucus secretory cells were also observed.

4.7.1.1 Viral particles *in vivo*

The retroviral-like particles predominantly in intracellular and occasionally in extracellular space were found in the neoplastic cells of 7 of 20 tumours examined. But this was not a consistent feature. They were round in shape, about 90-97 nm in diameter and these particles showed numerous spikes on its surface (Fig.46). They had an electron-dense nucleoids, centrally or eccentrically located, some of which were bar or annular shaped. Similar budding structures were also observed on the apical microvilli (Fig.47).

4.7.1.2 Viral particles in cell free tumour extract

The electron microscopy of cell free ethmoid tumour extract revealed viral particles similar to those observed *in vivo* in 3 of 21 tumour tissues examined (Fig.48). Herpes virus like particles with the size ranging from 160-230 nm were also seen in one of the tumour extracts. These particles had electron-dense core with envelope. The spikes on the surface were not observed.

Fig.46 Electron micrograph. Intracellular retroviral-like particles in the cytoplasm of neoplastic cell - x 45,000

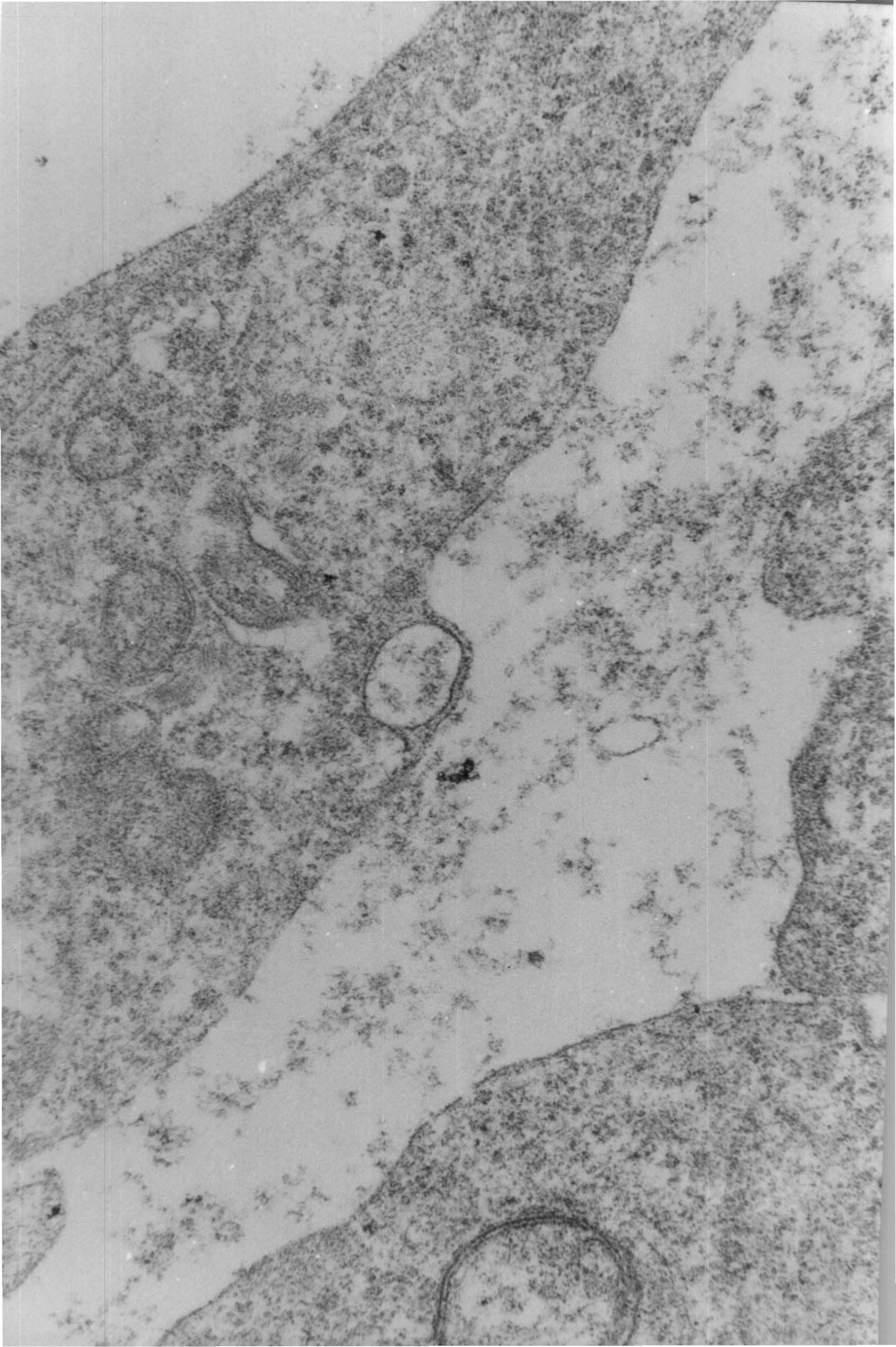


Fig.47 Electron micrograph. Extracellular retroviral-like particle close to the apical surface of neoplastic cell - x 40,000

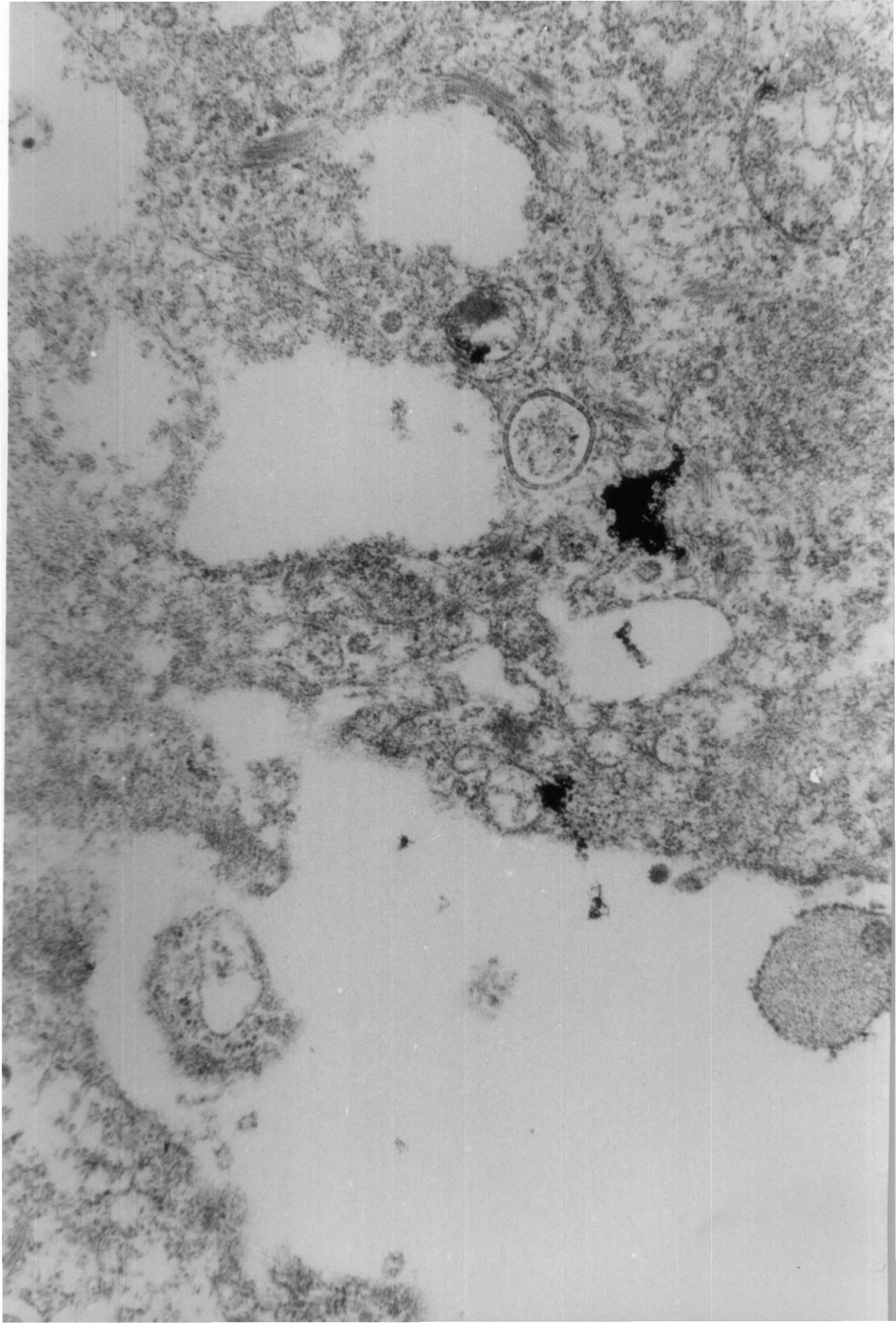


Fig.48 Electron micrograph. Retroviral-like particles in cell free ethmoid tumour extract - x 2,00,000

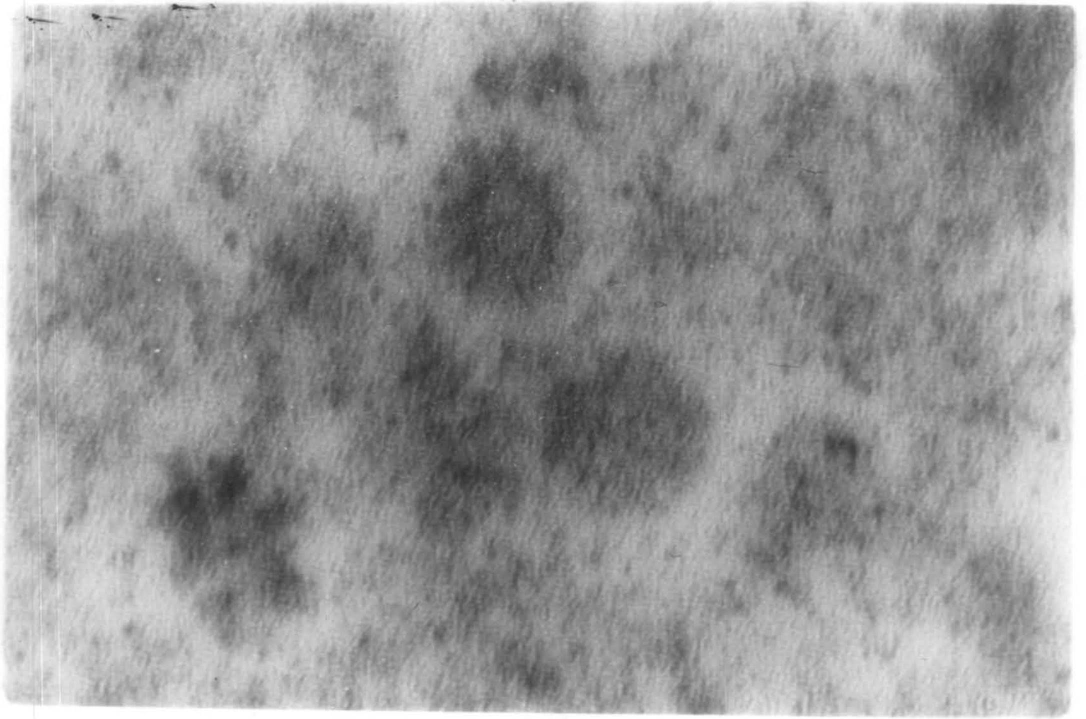


Table 7. Aflatoxin B_1 (AFB_1) residues in the blood of tumour bearing animals

Sl. No.	Cow No	Level (ppb)	Sl. No.	Cow No	Level (ppb)
1.	ET 4193	-	12.	ET 8993	92.4
2.	ET 15193	109.12	13.	ET 16693	-
3.	ET 16293	-	14.	ET 131093	-
4.	ET 1393	139.43	15.	ET 27194	121.92
5.	ET 8493	79.00	16.	ET 31194	-
6.	ET 12593	-	17.	ET 11295	-
7.	ET 13593	44.57	18.	ET 28394	43.12
8.	ET 18593	43.45	19.	ET 8495	45.60
9.	ET 29593	-	20.	ET 16494	-
10.	ET 18693	-	21.	ET 21494	77.50
11.	ET 24793	102.12			

Range	Positive for AFB_1 (%)
43.12-92.40 ppb	33.33
102.12-1339.43 ppb	19.04
43.12-139.43 ppb	52.37

4.8 Aflatoxin residues in blood

Out of 21 blood samples, from tumour bearing animals analysed, 33.33 per cent contained aflatoxin B_1 (AFB_1) ranging from 43.12-92.40 ppb and 19.04 per cent contained 102.12 to 139.43 ppb (Table 7).

Discussion

DISCUSSION

The carcinoma of the mucosa of the ethmoid occurs in an endemic form in the State of Kerala and less frequently in other parts of India. It has also been reported from other developing countries of the world. The aetiology of the ethmoid carcinoma is still obscure, although viral aetiology has been suspected. Since affected animals sometimes show signs of aflatoxicosis, aflatoxin was suggested as a possible factor in tumorigenesis (Lewis *et al*. 1967; Rajan *et al*. 1981; Zhang, 1981 and Pospischil *et al*. 1982). Animals may inhale high concentration of aflatoxin through respirable feed-dust particles leading to high exposure of the nasal epithelium, and this may increase the risk of carcinogenesis of this tissue (Burg *et al*. 1981; Sorenson *et al*. 1981). The hypothesis was also supported by reports indicating selective retention and increased bioactivation of aflatoxin B_1 in the olfactory mucosa as compared to liver of cattle (Larsson *et al*. 1989; Tjalve *et al*. 1992). The present study, was therefore, undertaken to assess the role of aflatoxin B_1 (AFB_1) in a seemingly multifactorial genesis of the endemic ethmoidal tumours in domestic animals.

The long-term experiment was designed in such a way as to minimize the early toxic effects of AFB_1 in pigs. The

dosage regimen that was previously shown to produce minimal toxic effects was adopted. The fact that prolonged and repeated administration of small doses of AFB_1 enhanced the carcinogenic response as compared to short term dosing with large doses (Wogan and Newberne, 1967) was also taken into account while deciding the dose and duration of the treatment in this experiment.

Although there was a gradual increase in the body weight of the experimental pigs of all the four groups, the average body weight of AFB_1 treated pigs continued to be significantly lower than that of healthy controls and tumour extract instilled pigs. The depression in the body weight gain may be attributed to the hepatotoxic effect of AFB_1. The reduced rate of protein synthesis due to aflatoxicosis may also explain to some extent the slowing of growth rate in pigs treated with AFB_1. The observation, therefore, confirms the report of earlier workers (Harley et al. 1969; Sarasin and Moule, 1973).

At the 9th month, grossly there was a mild to moderate hyperemia with areas of pale elevations in the mucosa of ethmo- turbinates and nasal mucosa of pigs given AFB_1. During the next three months, the intensity of these lesions increased. In the pigs sacrificed at 15th and 18th months, the grey-white, soft and oedematous appearance of the ethmoid

mucosa were very characteristic. These observations would suggest that there was progressive proliferative response in the mucosa of the ethmoid indicating a surmise that a preneoplastic change has been induced by aflatoxin.

The histological evidence of progressive degenerative, inflammatory and focal proliferative changes clarified the basic changes involved in the neoplastic process. The progressive increase in the number of regularly and irregularly shaped glandular structures lined with a single and occasionally double rows of low cuboidal epithelial cells in the submucosal area of the ethmoid mucosa associated with occasional squamous metaplasia just adjacent to the ethmoid lining epithelium and tendency of the surface epithelium to form papillary projection are evidences that would clarify again the conclusion that there has been aflatoxin induced preneoplatic changes. More or less similar microscopic lesions have been observed at the junction between the tumour tissue and normal ethmoid mucosa in enzootic nasal adenocarcinoma of sheep (Mckinnon et al. 1982), enzootic intranasal tumour in goats (Heras et al. 1991) and in solid adenomatous growth in the nasal cavity of sheep (Njoku et al. 1978), thereby suggesting that this might be a stage prior to the clinical development of tumour; a precancerous stage. In this context it is relevant to point out that the incidence of

spontaneous tumours involving the ethmoid mucosa of pigs was reported in the age group of 2-4 years (Rajan et al. 1981). Therefore, it would appear that the observation period of 18 months in the present study may be inadequate to develop clinically apparent neoplasm in the experimental pigs. The gross and histological observations clarified that aflatoxin has induced preneoplastic to neoplastic changes in the mucosa of ethmoid. Metaplasia observed in the present investigation may be a transitional stage, since in spontaneous cases, the primary ethmoid tumour is considered as adenocarcinoma and subsequently it progressed through a transitional stage to squamous cell carcinoma (Rajan, 1987).

It is evident from the ultrastructural changes observed in this study that productive and degenerative changes of subcellular structures are related to the ethmoid mucosa carcinogenesis. The productive changes consisted mainly of increase in the quantity of smooth and rough endoplasmic reticulum especially in the cells of secretory type. These intracellular modifications may be related to the higher metabolism of AFB_1 in the cells of the Bowman's glands of the olfactory mucosa and are to be considered as manifestations of its toxic and carcinogenic effects (Larsson et al. 1989). Furthermore, the AFB_1 is capable of inducing a

net synthesis of microsomal enzymes required for its metabolism (Schabort and Steyn, 1972).

From the electron microscopic observations, it appeared that degenerative changes could affect all the cellular structures, the involvement of which followed a well-defined sequential pattern. Endoplasmic reticulum appeared to be affected first and this was followed by damage to the mitochondria and the cell membrane. The disorganisation of the rough endoplasmic reticulum accompanied by degranulation seem to suggest that membranes, per se, are the major target of AFB_1 injury (Novi, 1977). The changes in the mitochondria may not necessarily reflect the primary effect of AFB_1 at the subcellular level. They could very probably result from altered intracellular metabolism following inhibition of protein synthesis as a result of disorganisation of the rough endoplasmic reticulum. For instance, lipid deposits have been attributed to decrease in available protein for triglyceride transport from the liver (Robinson and Seakins, 1962; Hamilton et al. 1967). In the present study, the morphological evidence supports a similar conclusion, since disruption of the rough endoplasmic reticulum has been found associated with lipid accumulation.

The electron microscopic investigations of the ethmoid mucosa of the AFB_1 treated pigs have further clarified that

appearance of productive and degenerative modifications in the secretory cells of the ethmoid mucosa seems to represent the "switching mechanism" towards the cell proliferation. Similar observations were made by earlier investigators (Pound and Lawson, 1974; Craddock, 1976) while investigating the liver carcinogenesis by sub-necrogenic dose of AFB_1. The presence of poor cytoplasmic contents, many polyribosomes along with irregular contour of the nucleus, invagination of the nuclear envelope and nucleolar margination in the ethmoid mucosa of AFB_1 administered pigs at 15th and 18th months observation are common features seen in neoplastic cells. It is now abundantly clear that immature or undifferentiated cells such as stem cells, blast cells, embryonic cells and the cells in culture have, as a rule, a poor compliment of rough endoplasmic reticulum as compared with their normal, mature functioning counterparts. Such immature cells, particularly fast growing population of cells, generally have more of free polyribosomes in the cytoplasm. This presumably reflects the active synthesis of endogenous proteins needed for cell growth and division (Ghadialy, 1982). These observations are further evidences to conclude that neoplastic transformation has taken place.

The absence of residual AFB_1 in the blood after 10 days of withdrawal of AFB_1 treatment and ethmoid mucosa

collected at various intervals, 3, 6, 9 and 12 months after the termination of AFB_1 treatment, at necropsy, could be because of bioactivation of AFB_1 primarily in the liver and olfactory mucosa, the organs considerably rich in drug metabolizing enzymes (Longo _et al_. 1991). Similar observations were made by Larsson _et al_. (1990), who could detect very little radioactivity in the nasal mucosa and liver one week after intravenous injection of radiolabelled ($3H-AFB_1$) AFB_1 in mice. Quick metabolism in the present experiment may also be more or less correlated with intravenous route of administration of AFB_1 in the experimental pigs. This observation is also supported by the detection of AFM_1 a metabolite of AFB_1, in the blood of one experimental pig. The insensitivity of the method used to detect micromolar concentration (<20 ppb) of AFB_1 and AFM_1 in this study may be the other possible explanation of our findings.

The successful establishment of stable ethmoid mucosa cells _in vitro_ in this investigation needs to be emphasised. It may be pointed out that by using Dulbecco's Modified eagle's medium and Ham's Nutrient Mixture (F 12) in the ratio of 1:1, an excellent growth of epithelial as well as fibroblast-like cells was established.

The *in vitro* exposure of cells of the ethmoid mucosa origin to micromolar concentration of AFB_1 can be considered as an environmentally relevant model, especially in the light of the fact that ethmoid mucosa may be directly exposed to the concentration of AFB_1 in the grain dusts in the parts per thousand range (Sorenson *et al*. 1981).

The present investigation describes for the first time the sequential transformation changes in the *in vitro* growth behaviour of ethmoid mucosa epithelium exposed to two different dose level of AFB_1. In an effort to quantify the degree of transformation in AFB_1 exposed ethmoid mucosa cells, they were tested for various transformation markers like colony forming efficiency (CFE) in soft agar, CFE in 10 and 1 per cent serum media and cytochemical assay of gamma-glutamyl transpeptidase (GGT) activity. The combined use of these markers provided reliable tools for identifying transformation in AFB_1 exposed cells of the ethmoid mucosa.

The selective cytotoxicity in mixed culture during AFB_1 treatment manifested by degeneration and sloughing of fibroblast-like cells, and allowing the clones of the epithelial cells to proliferate is of particular interest. This features more or less mimics the *in vivo* situation since carcinogens generally do not cause direct transformation of

normal cells to cancer cells but instead act by selection of clones of cells which are otherwise dormant.

The morphologically altered colonies composed of small, compact and pleomorphic cells along with the tendency to pile up and increased split ratio were the features observed in the *in vitro* treated epithelial cell of the ethmoid mucosa origin. This is in accordance with the results obtained by other investigators, who emphasized that the acquisition of an irregular outline of islands and piling up in subconfluent cultures are the definite morphological changes suggestive of *in vitro* transformation (Borek, 1972; Williams, 1973; Borenfreund *et al*. 1975; Weinstein *et al*. 1975).

The ultrastructural features of the *in vitro* AFB_1 treated ethmoidal epithelial cells were more or less as observed *in vivo* in AFB_1 given experimental pigs. But the degenerative changes were comparatively more. This severe cytotoxicity may be possibly because of greater formation of AFB_1-DNA adducts. The association between the binding of AFB_1 to nuclear DNA and cytotoxicity in susceptible species have important implications for the potential of AFB_1 as a respiratory carcinogen (Wilson, 1990). The nucleolar enlargement and segregation observed in the AFB_1 exposed epithelial culture in the present study also reflect DNA

binding. Similar observations were made by various workers (Simard and Bernhard, 1966; Reddy and Svoboda, 1968). They further postulated that nucleolar segregation produced a decrease in the activity of RNA polymerase, an enzyme known to catalyze the synthesis of RNA, because of loss of template activity of DNA. The persistence of subcellular changes induced by AFB_1 even after the withdrawal of the exogenous supply of the carcinogen may be attributed to the endogenous mechanism of storage and release of AFB_1 (Novi, 1977).

The poor content of endoplasmic reticulum, presence of ribosomes, tonofilaments associated with indentation of the nucleus and nucleolar margination in the AFB_1 treated epithelial cells in later passages, clarified the *in vitro* transformation of these cells. These ultrastructural findings are identical with the observations of Lin *et al*. 1990 in cell lines derived from human nasopharyngeal carcinoma. The significance of the nuclear bodies, which were occasionally seen in the *in vitro* treated epithelial cells is not clear. It has been reported that when cells are stimulated to activity by a variety of means, an increase in number, size and complexity of the nuclear bodies are observed (Ghadially, 1982). The nature of tonofilaments observed in these cells is not understood but it has variously been suggested as a

phenomenon of squamous metaplasia or an abnormal aggregation of proteins (Svoboda, 1964).

The significant increase in CFE's in soft agar of AFB_1 treated epithelial cells is an observation which would confirm the in vitro transformation of epithelial cells of ethmoid origin. Williams (1976) reported that in vitro transformed liver cells with AFB_1 consistently produced an increase in colony forming cells.

In the present study, although the treated epithelial cells had high CFE in 10 and 1 per cent serum media than the untreated cells, the difference was not marked. This possibly may be due to less dependency on serum for growth in case of epithelial cells in comparison to cells of mesenchymal origin (Castor, 1968; Dulbecco, 1970). In confirmation with the obsevations made in the present study Dulbecco (1970), Jainchil and Todaro (1970) and San et al. (1979) reported that CFE of tumorigenic epithelial cells was not as inhibited in 1 per cent serum medium as compared to non-tumourigenic cells.

The GGT activity was not detectable by cytochemical assay in untreated control epithelial cells but was present at least focally in the AFB_1 treated epithelial culture at the 14th passage. In the present experiment, the increased GGT activity observed in the transformed cells in culture is

identical with those described by San *et al*. (1979). They detected GGT activity by cytochemical assay in tumorigenic liver cell lines as well as in the lines derived from hepatocarcinomas, whereas non-tumorigenic lines from normal rat liver were consistently negative. Similar observations were made by Huberman *et al*. (1979) and Morimura *et al*. (1990). Incidentally, the increase in GGT activity in neoplastic ethmoid tissue similar to that which occurred in ethmoid mucosa derived AFB_1 treated epithelial cells in culture provides additional support to the existing evidence that a xenotoxic carcinogen may be involved in the carcinogenesis (Gangadharan and Rajan, 1992).

Although, observations with regard to morphological pattern, CFE's in soft agar as well as cytochemical assay of GGT activity have revealed the acquisition of transformed properties in AFB_1 exposed epithelial cells, xenotransplantation of these *in vitro* treated cells in immuno suppressed mice was unsuccessful. This does not mean that these cells may not, at a later time, become tumorigenic. There are reports that the ability of various cell lines to form colonies in soft agar was detected on several occasions before the lines were observed to be tumorigenic (San *et al*. 1979). Similar observations were made by Steele *et al*. (1979). They reported that the character of the cell line populations

during passage is changing towards increased malignancy. They further observed that five cell lines were negative for tumorigenicity at early passage but acquired the capacity to produce tumours by the 20th passage. Another possible explanation of this xenotransplantation failure may be graft rejection mechanism. Perhaps AFB_1 treatment of cells in vitro made them more antigenic. This possibility cannot be ruled out because palpable size growth persisted between three to nine days after the inoculation of treated cells in three mice out of twelve inoculated.

The study of the malignant transformation in vitro, demands attention to spontaneous transformation in the control cells. No morphological or malignant transformation was evident in the control cells. Moreover, the control cells showed degeneration and they could not be maintained under the similar experimental conditions beyond 8th passage.

The present studies have confirmed the induction of morphological changes by AFB_1 in cells of the ethmoid mucosa maintained in vitro and have established an association with other phenotypic changes. But it remains to be demonstrated that the quantity of morphologically altered cells correspond to the level of other quantified changes and the morphologically altered cells are, in fact, neoplastic. Nevertheless, the inducibility of quantifiable phenotypic

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changes offers some opportunities for the study of the mechanism of AFB_1 carcinogenesis in bovine ethmoid mucosa derived cells.

In the present investigation, the fibroblast like cells did not show any evidence of *in vitro* transformation at any stage of the experimentation. AFB_1 treated fibroblast-like cells exhibited severe degeneration and cell detachment within 48 hours. However, small population of cells survived when subcultured began to proliferate into colonies and on subsequent subcultivations there was recovery of growth rate. These findings are surprisingly different because most investigators have utilized fibroblast culture (Berward and Sachs, 1965; Borenfreund *et al*. 1966; Sato and Kuroki, 1966; Dipaolo and Donovan, 1967) because of their ease of cultivation. But Williams *et al*. (1973) noted that the fibroblast cultures have displayed sensitivity to limited classes of carcinogens. The failure to transform the fibroblast like cells *in vitro* with AFB_1 treatment may also be explained by the fact that these cells may be lacking in enzymes required for biotransformation of AFB_1. The oxidative and non-oxidative drug metabolizing enzymes are predominant in the epithelial cells of the olfactory and respiratory mucosa of cattle (Longo *et al*. 1990).

The ultrastructural studies on spontaneous cases of tumours of the mucosa of the ethmoid in cattle revealed the epithelial nature of the tumour. Between animals and within the individual neoplasms there was variation in structure. They were either well differentiated secretory structures or undifferentiated or differentiated squamous cells. These findings confirm the report of previous workers (Pospischil *et al*. 1979; Nair *et al*. 1987).

It is significant to point out that virus with morphological features of retrovirus was demonstrated on ultrastructural screening in seven of the twenty cases examined and in the cell free tumour extract of 3 of 21 extracts examined. Similar retrovirus like particles in association with ethmoidal tumours in sheep and goats have also been reported (Yonemechi *et al*. 1978; McKinnon *et al*. 1982; Heras *et al*. 1991). The inconsistency to demonstrate the presence of viral particles in the neoplasm arising from the mucosa of ethmoid in cattle more or less may be due to the advance stage of growth when the tumour tissue was examined and/or collected for various investigations. The other explanation for this inconsistency may be that the oncogenic viruses act only transiently and the genes involved are required only in early stages of tumour evolution and unknown

selective pressures against the virus eliminate it during tumour progression.

The tumour was experimentally transmitted in sheep by intranasal inoculation of homogenized tumour, free of bacteria (Cohrs, 1953). Recently, Heras et al. (1995) reported successful experimental transmission of enzootic intranasal tumour from goat to goat using the nasal discharge. If at all, retrovirus is involved, either alone/or in association with some unknown co-factors, in the carcinogenesis of the mucosa of ethmoid in cattle and pig, the failure to transmit the condition may be possibly due to the use of comparatively old pigs in the present investigation. It has been demonstrated that retrovirus associated carcinomas can be successfully induced using neonatal lambs (Rosadio et al. 1988) and kids (Heras et al. 1995). It has been further suggested that the virus may be transmitted in nature during the neonatal period. The age susceptibility of neonatal lambs and kids to develop retrovirus associated malignancies may be related to the immaturity of immune response.

The negative results of limited transmission studies using ethmoid tumour extract in pigs do not support the assumption that the virus is involved in the aetiology of endemic ethmoid tumours in Indian cattle and pigs. However, this conclusion has limitations. It has to be borne in mind

that the source of the material for this study was from cattle, the age of the pigs used was higher - the limiting factors in the present study. In this investigation, the material from pigs could not be used as ethmoid tumours are not encountered in the pigs now. The pig was used as model because of its aflatoxin sensitivity and the earlier records of ethmoid tumours in pigs. There is scope to make transmission studies using either neonatal calves or kids.

The histological evidence of early neoplastic transformation in the cells of the mucosa of the ethmoid in aflatoxin treated animals, the demonstration of neoplastic transformation in the cells of the mucosa of the ethmoid *in vitro* by AFB_1, and the detection of retroviral particles in a few spontaneous cases of ethmoid tumour and the establishment of an association between retrovirus and ethmoid carcinoma in goats (Heras *et al*. 1995) would also support the involvement of virus and aflatoxin in the carcinogenesis of the mucosa of ethmoid. Therefore, the proposed hypothesis appears to be true.

Summary

SUMMARY

An experimental study was designed taking pig as a model to assess the role of aflatoxin and/or virus in the aetiology of ethmoid carcinoma in the deomestic animals.

Thirty-two, Large White Yorkshire piglings of two-three months age were procured from the University Pig Breeding farm, Mannuthy and divided at random into four groups of eight each.

The pigs in group I and group II were administered aflatoxin B_1 (0.070 mg/kg b.wt/inoculum by intravenous route at weekly interval for six months) and/or ethmoid tumour extract (2 ml/pig/inoculum, intranasally, at fortnight interval for three months). The pigs in group III were administered ethmoid tumour extract alone, while the pigs in group IV were kept as negative controls.

The experimental pigs were examined for clinical manifestations and growth response. Blood and ethmoid mucosa samples were collected at different intervals of the experimentation to analyse the residual aflatoxin B_1 (AFB_1) and aflatoxin M_1 (AFM_1).

The sequential pathological changes were studied by sacrificing two randomly selected pigs from the treatment and control groups at 9th, 12th, 15th and 18th months interval.

There was no clinical manifestation of the development of ethmoid carcinoma in any of the experimental pigs. The AFB_1 administered pigs were depressed and the body weight recorded at various intervals was significantly low when compared to the control as well as those animals who were treated with ethmoid tumour extract.

Gross and microscopic lesions were seen in the ethmoid mucosa of AFB_1 treated pigs. The ethmoid mucosa of pigs which were instilled tumour extract did not show any lesion.

The pigs dosed with AFB_1 revealed congestion and scattered small pale elevation in the mucosa of the ethmoid at the 9 month of observation. In the later stages, the ethmoidal area appeared soft, grey white and oedematous.

Three months after the termination of the treatment, the ethmoid mucosa on histopathological examination showed infiltration of lymphocytes, fatty degeneration of the submucosal glands and degeneration and sloughing of the ethmoid mucosa. At subsequent intervals, the lesions were more marked and characterized by lymphnode-like aggregates of mononuclear cells, and extensive proliferation of mucus glands

showing acinar, tubular or papillary arrangements. The tendency of the surface epithelium to form papillary projections and focal areas of squamous metaplasia was also observed occasionally.

The electronmicroscopic studies revealed predominance of the smooth and rough endoplasmic reticulum especially in the secretory cells of the ethmoid mucosa of AFB_1 exposed pigs, at 9 months. The degenerative changes consisting of disorganization of the rough endoplasmic reticulum and mitochondria were also observed at 9 as well as 12 months stage. A few cisternae of the rough endoplasmic reticulum and free ribosomes in the cytoplasm along with the irregular contour of the nucleus and nucleolar margination were the consistent features of the cells of the ethmoid mucosa at 15th and 18th months of observation indicating a preneoplastic to neoplastic transformation. No ultrastructural features suggestive of neoplastic change were seen in the ethmoid mucosa of pigs given ethmoid tumour extract.

AFB_1 in the range of 40-160 ppb was consistently detected in the blood of all the pigs of group I and group II from 3-7 days after termination of respective treatments. Thereafter AFB_1 (40-120 ppb) could be detected in the blood of 5 pigs from both the groups at 10 days post-treatment. On

subsequent sampling, blood and ethmoid mucosa were consistently negative for AFB_1.

On analysing the blood samples from 21 tumour bearing cattle, AFB_1 in the range of 43.12-139 ppb was detected in 52.37 per cent of the animals.

On analysing the blood and ethmoid mucosa samples for AFM_1 from experimental pigs at various intervals, the blood sample from one pig of group I was found to have AFM_1 (42 ppb) only upto three days after the withdrawal of AFB_1 treatment.

By concerted efforts the cells of the mucosa of the ethmoid were established in culture for the first time. The mixed culture of epithelial and fibroblast-like cells from the bovine ethmoid mucosa was exposed in cell culture to AFB_1. There was more marked cell damage in the fibroblast like cells than epithelial cells. The selective cytotoxicity resulted in degeneration and sloughing of fibroblast-like cells and progressive proliferation of the epithelial cell clones.

The cytotoxic effects of AFB_1 consisted of pyknosis, karyorrhexis and cytoplasmic vacuolation developed gradually in the epithelial culture during the exposure period of 90 days and persisted even after the withdrawal of the treatment. The pleomorphism, tendency to pile up and increased split

ratio gave evidence for the neoplastic transformation of the epithelial cells.

Severe degeneration and detachment from the glass surface were observed within 48 hours of treatment of AFB_1 in fibroblast-like cells. However, gradual recovery of the growth rate on subsequent passages was observed, but none of these cells, showed any indication of *in vitro* transformation.

The *in vitro* transformation was proved by electron microscopic studies. There was marked dilatation and vesiculation of the endoplasmic reticulum, characteristic intracristal swelling of the mitochondria, dissolution of the plasma membrane associated with enlarged nucleus and nucleolar segregation. These changes continued even after the AFB_1 treatment was terminated suggesting that there was endogenous storage and release of AFB_1. The nuclear changes like enlarged nucleus with ruffled nuclear membrane, nucleolar margination, predominantly euchromatin and occasional nuclear body were the consistent ultrastructural changes observed in the treated epithelial culture in later passages. Occasionally these nuclear changes were also seen in the fibroblast like cells.

The colony forming efficiency studies in soft agar clarified and confirmed the neoplastic transformation of cells.

The untreated epithelial as well as fibroblast-like cells had lower colony forming efficiency (CFE) in 10 or 1 per cent foetal calf serum than the treated cells. But the depression in CFE's was more significant in fibroblast-like cells as compared to the epithelial cells.

The demonstration of gamma-glutamyl transpeptidase (GGT) activity in the epithelial cells and absence of this activity in fibroblast-like cells further gave proof to the genotoxic and carcinogenic effect of AFB_1 and gave indication to the aetiological role of AFB_1 in ethmoid carcinoma.

The xenotransplantation of the transformed cells of the ethmoid mucosa was not successful. Although palpable size growth was observed in 3 out of 12 mice inoculated between 3-9 days after the inoculation of treated epithelial cells, but there was no progressive growth during the subsequent period.

The ultrastructural analyses of the carcinoma of the ethmoid mucosa in cattle revealed the epithelial nature of the tumour. Between animals and within the individual neoplasms there was variation in structure. They were either well differentiated secretory structures or undifferentiated or differentiated squamous cells.

The electronmicroscopic studies revealed the presence of small particles which had the morphology of retrovirus in

the occasional neoplastic cells *in vivo* and in the ultracentrifuged cell free tumour extract from cattle indicating the possible involvement of the virus in the causation of ethmoid carcinoma.

The elegant demonstration of AFB_1 induced preneoplastic to neoplastic transformation of the cells of the ethmoid mucosa *in vitro* and *in vivo* and the detection of retroviral particles in seven of twenty spontaneous cases of ethmoid tumour in the present study were defnite evidences to conclude that a retrovirus and aflatoxin are involved in the causation of the carcinoma of the mucosa of the ethmoid in domestic animals.

References

REFERENCES

Adamson, R.H., Cooper, R.W. and O'Gara, R.W. (1970). Carcinogen induced tumours in primitive primates. J. Natl. Cancer Inst. 45: 555-560.

Adamson, R.H., Correa, P. and Dalgard, D.W. (1973). Occurrence of a primary liver carcinoma in a Rhesus monkey fed aflatoxin B1. J. Natl. Cancer Inst. 50: 549-553.

Adamson, R.H. and Sieber, S.M. (1979). The use of nonhuman primates for chemical carcinogenesis studies. In Coulston, F. (ed). Regulatory aspects of carcinogenesis and food additives. The Delaney Clause. Academic Press, New York, Vol.2, pp.275-302.

Allcroft, R., Carnaghan, R.B.A., Sargeant, K. and O'Kelley, J. (1961). A toxic factor in Brazilian groundnut meal. Vet. Rec. 73: 426-428.

Allcroft, R. and Lewis, G. (1963). Groundnut toxicity in cattle: Experimental poisoning of calves and a report on clinical effects in older cattle. Vet. Rec. 75: 487-493.

Alpert, E. and Serck-Hanssen, A. (1970). Aflatoxin-induced hepatic injury in the African monkey. Arch. Envir. Hlth. 20: 723-728.

Al-Yaman, F. and Willenborg, D.O. (1984). Successful isolation, cultivation and partial characterization of naturally occurring ovine squamous cell carcinomata. Vet. Immun. Immunopath. 5: 273-288.

Amaral, L.B.S. and Nesti, A. (1963). Incidencia de cancer em bovinos e suinos. Biologico. 29: 30-31.

Annau, E., Coiner, A.H., Magwood, S.E. and Jerieho, K. (1964). Electrophoretic and chemical studies on sera of swine following the feeding of toxic groundnut meal. Can. J. Comp. Med. Vet. Sci. 28: 264-270.

AOAC (1975). Natural Poisons. In: Official methods of analysis of Assoc. Off. Anal. Chem., Ed. Horwitz, W., Washington, D.C., Sects. 26.004-26.012. 12th Ed., pp.463-465.

Armbrecht, B.H. (1978). Mycotoxicosis in swine. In mycotoxic fungi, Mycotoxins and Mycotoxicosis. An encyclopedic hand book. Eds., Wyllie, T.D. and Morehouse, L.G., New York, Vol.2, pp.227-236.

Balasubramaniam, M. (1975). Studies on the pathology of the neoplasm of the paranasal sinuses of bovines with special reference to histological and histochemical features. M.V.Sc. Thesis. University of Agricultural Sciences, Bangalore.

Barnes, J.M. and Butler, W.H. (1964). Carcinogenic activity of aflatoxin to rats. Nature. 202: 1016.

Barrett, J.C., Crawford, B.D., Mixter, L.O., Schechtman, L.M., Ts'o, P.O.P. and Pollack, R. (1979). Correlation of in vitro growth properties and tumorigenicity of syrian hamster cell lines. Cancer Res. 39: 1504-1510.

Becker, M., Pohlenz, J. and Aumannmann, M. (1972). Nasal tumours in cattle. Schweizer Arch. Tierheilk. 114 (8): 404-412.

Bergman, A.M. (1914). Cited by Balasubramaniam, M. (1975). Studies on the pathology of the neoplasm of the paranasal sinuses of bovine with special reference to histological and histochemical features. M.V.Sc. thesis. University of Agricultural Sciences, Bangalore.

Bertram, J.S. (1977). Effects of serum concentration on the expression of carcinogen-induced transformation in the C3H/10T Y2 CL8 cell line. Cancer Res. 37: 514-523.

Berwald, Y. and Sachs, L. (1965). In vitro transformation of normal cells to tumor cells by carcinogenic hydrocarbons. J. Natl. Cancer Inst. 35: 641-661.

Blount, W.P. (1961). Turkey "X" Disease. Turkeys. 9: 52.

Borek, C. (1972). Neoplastic transformation in vitro of a clone of adult liver epithelial cells into differentiated hepatoma-like cells under conditions of nutritional stress. Proc. Natl. Acad. Sci. USA. 69: 956-959.

Borenfreund, E.K.M., Sanders, F.K., Sternberg, S.S., Bendich, A. 1966. Malignant conversion of cells in vitro by carcinogens and viruses. Proc. Natl. Acad. Sci. USA. 56: 672-679.

Borenfreund, E., Higgins, P.J., Steinglass, M. and Bendich, A. (1975). Properties and malignant transformation of established rat liver parenchymal cells in culture. J. Natl. Cancer Inst. 55: 375-384.

Bouillant, A.M.P. and Becker, S.A. (1984). In: Heras, M.D.L., Garcia de Jalon, J.A., Balaguer, L. and Badiola, J.J. Retrovirus-like particles in enzootic intranasal tumours in spanish goats. Vet. Rec. 123: 135.

Bruce, R.D. (1990). Risk assessment for aflatoxin. II implication of human epidemiology data. Risk Analysis 10 (4): 561-569.

Burg, W.R., Shotwell, O.L. and Saltzman, B.E. (1981). Measurements of airborne aflatoxin during the handling of contaminated corn. Am. Ind. Hyg. Assoc. J., 42: 1-11.

Burnside, J.E., Sippel, W.L., forgacs, J., Carl, W.T., Atwood, M.B. and Doll, E.R. (1957). A disease of swine and cattle caused by eating mouldy corn. II. Experimental production with pure culture of mould. Am. J. Vet. Res. 18: 817-824.

Butler, W.H. (1964). Acute toxicity of aflatoxin B_1 in rats. British J. Cancer 18: 756-762.

Butler, W.H. and Barnes, J.M. (1963). Toxic effects of groundnut meal containing aflatoxin to rats and guinea pigs. British J. Cancer. 17: 699-710.

Calderson, S.C. and Slot, D.B. (1985). Gamma-glutamyl transpeptidase in precancerous lesions and carcinoma of oral, pharyngeal and laryngeal mucosa. Cancer. 56: 138-143.

Cardeilhac, P.T., Nair, K.P.C. and Colwell, W.M. (1972). Tracheal organ cultures for the bioassay of nanogram quantities of mycotoxins. J. Assoc. Anal. Chem. 55: 1120-1121.

Carnaghan, R.B.A. (1967). Hepatic tumours and other chronic liver changes in rats following a single oral administration of aflatoxin. British J. Cancer. 21: 811-814.

Castor, L.N. (1968). Contact regulation of cell division in an epithelial-like cell line. J. Cell Physiol. 72: 161-172.

Chakraborty, A., Nayak, N.C. and Maity, B. (1988). Tumours of the ethmoid region in cattle in West Bengal. Indian Vet. J. 65 (12): 1132-1133.

Charry, J., Aman, N. and Tanoh, K.G. (1985). Outbreak of adenocarcinoma of the olfactory mucosa in West African dwarf ewes. Revue d' Elevage et de Medicine Veterinaire des Pays Tropicax. 38 (4): 406-410. Abst. Vet. Bull. 57: 947.

Chaudhary, C.H. and Rao, M.R.K.M. (1982). The incidence and clinicopathology of the tumours of the mucosa of nasal and paranasal sinuses in cattle and buffaloes in Andhra Pradesh. Cheiron. 11 (5): 245-248.

Chauhan, H.V.S., Jha, G.J., Singh, P.N., Singh, K.K. (1984). Hepatocellular carcinoma associated with aflatoxicosis in pigs. Indian Vet. J. 61: 1009-1014.

Clegg, F.G. and Bryson, H. (1962). An outbreak of poisoning in store cattle attributed to Brazilian groundnut meal. Vet. Rec. 74: 992-994.

Cohrs, P. (1952). Ubertragbare Adenome der Riechschleimhaut beim Schaf. Z krebsforsch. 58: 682-692.

Cohrs, P. (1953). Infektiose Adenopapillome der Riechrchleimhaut Schat. Berl Munch Tierarztl Wochenschr. 66: 225-228.

Colburn, N.H., Vorder Bruegge, W.F., Bates, J.R., Gray, R.H., Rossen, J.D., Kelsey, W.H. and Shimada, T. (1978). Correlation of anchorage-independent growth with tumorigenicity of chemically transformed mouse epidermal cells Cancer Res. 38: 624-634.

Cotchin, E. (1956). Neoplasms of the domestic mammals - a review. Review series No.4 of the Commonwealth Bureau of Animal Health. 17-19.

Coulombe, R.A., Wilson, D.W., Hsieh, D.P.H., Plopper, C.G. and Serabjit-Singh, C.J. (1986). Metabolism of aflatoxin B_1 in the upper airways of the rabbit: role of the nonciliated tracheal epithelial cell. Cancer Res. 46: 4091-4096.

Craddock, V.M. (1976). Cell proliferation and experimental liver cancer. In: Cameron, H.M., Linsell, D.A. and Warwick. eds. Liver cell cancer, Elsevier, Amsterdam. 152-201.

Cusumano, V. (1991). Aflatoxins in sera from patients with lung cancer. Oncology. **48**: 194-195.

Cuthbertson, W.F.J., Laursen, A.C. and Pratt, D.A.H. (1967). Effect of groundnut meal containing aflatoxin on Cynologus Monkeys. British J. Nutr. **21**: 893-908.

Damodaran, S., Ramakrishnan, R. and Parthasarathy, K.R. (1974). Neoplasms of the ethmoidal mucosa in bovines. Cheiron. 3 (1): 1-7.

David, J.D. and Venkataraman, K. (1940). Malignant growth in the frontal sinus. Indian Vet. J. **17** (3): 153-154.

Deo, M.G., Dayal, Y. and Ramalingswami, V. (1970). Aflatoxin and liver injury in Rhesus monkeys. J. Pathol. **101**: 47-56.

Dimayorca, G., Greenblatt, M., Trauthen, T., Soller, A. and Giordano, R. (1973). Malignant transformation of BHK 21 Clone 13 cells in vitro by nitrosamines - a conditional state. Proc. Natl. Acad. Sci. U.S.A. **70**: 46-49.

Dipaolo, J.A. and Donovan, P.J. (1967). Properties of syrian hamaster cells transformed in the presence of carcinogenic hydrocarbons. Expt. Cell Res. **48**: 361-377.

Dulbecco, R. (1970). Topoinhibition and serum requirement of transformed and untransformed cells. Nature (Lond.). 227: 802-806.

Duncan, J.R., Tyler, D.E., Van Der Maaten, M.J. and Andersen, J.R. (1967). Enzootic nasal adenocarcinoma in sheep. J. Am. Vet. Med. Assoc. 151: 732-734.

Edds, G.T. (1979). Biological effects of aflatoxins in swine. Proc. ASAS and ADSA, National Academy of Sciences, Washington, D.C. pp.67-76.

Engelbrecht, J.C. and Purchase, I.F.H. (1969). Changes in morphology of cell cultures after treatment with aflatoxin and ochratoxin. South African Med. J. 43: 524-528.

Fiala, S., Fiala, A.E. and Dixon, B. (1972). Gamma-glutamyl transpeptidase in transplantable chemical induced rat hepatomas and in "spontaneous" mouse hepatomas. J. Natl. Cancer Inst. 48: 1393-1401.

Fiala, S. and Fiala, E.S. (1973). Activation by chemical carcinogens of gamma-glutamyl transpeptidase in rat and mouse liver. J. Natl. Cancer Inst. 51: 151-158.

Fiala, S., Mohindru, A., Kettering, W.G., Fiala, A.E. and Morris, H.P. (1976). Glutathione and gamma-glutamyl transpeptidase in rat liver during chemical carcinogenesis. J. Natl. Cancer Inst. 57: 591-598.

Freedman, V.H. and Shin, S.I. (1974). Cellular tumorigencity in nude mice and correlation with cell growth in semi-solid medium. Cell. 3: 355-359.

Gagne, W.E., Dungworth, D.L. and Moulton, J.E. (1968). Pathological effects of aflatoxin in pigs. Pathol. Vet. 5: 370-384.

Gangadharan, B. and Rajan, A. (1992). Assessment of efficacy of certain tumour markers in ethmoid carcinoma. Indian J. Vet. Pathol. 16 (2): 73-75.

Gazquez, A., Roncero, V., Redondo, E., Duran, E., Masot, J. and Gomez, L. (1992). Adenocarcinoma of ethmoid olfactory mucosa: a histopathological and ultrastructural study with evidence of virus-like particles. J. Vet. Med. A. 39: 609-615.

Ghadially, F.N. (1982). Ultrastructural Pathology of the cell and metrix. 2nd ed. Butterworths. London.

Glenner, G.G., Folk, J.E. and Mcillan, P.J. (1962). Histochemical demonstration of a gamma-glutamyl transpeptidase activity. J. Histochem. Cytochem. 10: 481-489.

Goerttler, K., Lohrke, H., Schweizer, H.J. and Hesse, B. (1980). Effects of aflatoxin B_1 on pregnant inbred sprague-Dawley rats and their F_1 generation. A contribution to transplacental carcinogenesis. J. Natl. Cancer Inst. 64: 1349-1354.

Gopal Naidu, N.R. and Sehgal, S. (1992). Hepatic tumours following prenatal and perinatal exposure to aflatoxin B_1 metabolites - An experimental study in rats. In Proceedings: Advances in animal cancer research, Tamil Nadu Veterinary & Animal Sciences University, Madras.

Hamilton, R.L., Regen, D.M., Gray, M.E., Lequire, U.S. (1967) Lipid transport in liver. I. Electron microscopic identification of very low density lipoproteins in perfused rat liver. Lab. Invest. **16**: 305-319.

Harding, J.D.J., Cona, J.T., Lewis, G. and Allcroft, R. (1963). Experimental groundnut poisoning in pigs. Res. Vet. Sci. **4**: 217-229.

Harley, E.H., Rees, K.R., Cohen, A. (1969). A comparative study of the effect of Aflatoxin B_1 and actinomycin D on Hela cells. Biochem. J. **114**: 289-298.

Hauser, R.H., Harland, E.C. and Rubin, H.L. (1971). Haemorrhagic syndrome in swine consuming moldy corn. University of Florida, Mineoserier, ARCLO, 71-73. Cited by Osuma, O. and Edds, G.T. (1982).

Hayes, R.B., Nieuwenhuize, J.P.V., Raatgever, J.W. and Kate, F.J.W.T. (1984). Aflatoxin exposures in the industrial setting: an epidemiological study on mortality. Food Chem. Toxic **22**: 39-43.

Heidelberger, C. and Iype, P.T. (1967). Malignant transformation in vitro by carcinogenic hydrocarbons. Science **155**: 214-217.

Heras, M.D.L., Garcia de Jalon, J.A. and Sharp, J.M. (1991). Pathology of enzootic intranasal tumour in thirty-eight goats. Vet. Pathol. **28**: 474-481.

Heras, M.D.L., Garcia de Jalon, J.A. and Sharp, J.M. (1992). Experimental transmission of enzootic intranasal tumour in goats. In. Booklet of abstracts, Association of Veterinary Teachers and Research Workers. Abst. No. A39. p.10.

Heras, M.D.L., Sharp, J.M., Ferrier, L.M., Garcia de Jalon, J.A. and Cebrian, L.M. (1993). Evidence for a type D-like retrovirus in enzootic nasal tumour of sheep. Vet. Rec. 132 (17): 441.

Heras, M.D.L., Garcia de Jalon, J.A., Minguijon, E., Gray, E.W., Dewar, P. Sharp, J.M. (1995). Experimental transmission of enzootic intranasal tumours of goats. 32: 19-23.

Holley, R.W. and Kiernan, J.A. (1968). "Contact inhibition" of cell division in 3T3 cells. Proc. Natl. Acad. Sci. USA. 60: 300-304.

Horne, R.W. (1967). Electron microscopy of isolated virus particles and their components. In. Methods in Virology, Vol. II, Eds. K. Maramorosch and H. Koprowsky, Academic Press, New York and London. pp. 522-575.

Huberman, E., Montesano, R., Drevon, C., Kuroki, T., St. Vincent L., Pugh, T.D. and Goldfarb, S. (1979). Gamma-glutamyl transpeptidase and malignant transformation of cultured liver cells. Cancer Res. 39: 262-271.

International Dairy Federation (1991). Milk and dried milk determination of aflatoxin M_1 content, International IDF standard III A: 1990 IDF, General Secretariat, Brussels, Belgium.

Iwasaki, H., Okubo, K. and Iwase, K. (1974). Studies on an outbreak of cirrhosis of the liver in pigs. III. Long term feeding experiment with aflatoxin in pigs. J. Japanese Vet. Med. Assn. 27: 225-229.

Jackson, C. (1936). The incidence and pathology of tumours of the domestic animals in South Africa. Onderstepoort J. Vet. Res. 6: 131-134.

Jainchil, J.L. and Todaro, G.J. (1970). Stimulation of cell growth in vitro by serum with and without growth factor. Relation to contact inhibition and viral transformation. Exp. Cell Res. 59: 137-146.

Jayaraman, M.S., Padmanabhan, V.D., Masillamony, P.R. and Nachimuthu, K. (1979). Epidemiological and virological studies on sinus neoplasms of the upper respiratory tract of bovines in Tamil Nadu. Cheiron. 8 (1): 34-49.

Jose, C.J., Ramachandran, K.M., Sreekumaran, T. and Rajan, A. (1985). Clinical features of ophthalmic lesions in ethmoid carcinoma. Kerala J. Vet. Sci. 16 (1): 141-148.

Juhasz, S. and Greczi, E. (1964). Nature (Lond.). 203: 861. Cited by Zuckerman, A.J., Tsiquaye, K.N. and Fulton, F. (1966).

Kakunaga, T. (1973). A quantitative system for assay of malignant transformation by chemical carcinogens using a clone derived from BALB/3TC. Int. J. Cancer 12: 463-473.

Kakunaga, T. and Kamahora, J. (1968). Properties of hamster embryonic cells transferred by 4-nitroquinolone 1-oxide in vitro and their correlations with the malignant properties of the cells. Biken J. 11: 313-332.

Kalengayi, N.M.R., Ronchi, G. and Desmet, O.J. (1975). Histochemistry of GGT in rat liver during aflatoxin induced carcinogenesis. J. Natl. Cancer Inst. 55: 579-588.

Keyl, A.C. and Booth, A.N. (1971). Aflatoxin effects in livestock. J. Am. Oil Chem. Soc. 48: 599-604.

Knowles, M.A. and Franks, L.M. (1978). Ultrastructure and biological markers of neoplastic change in adult mouse epithelial cells transformed in vitro. Br. J. Cancer 37: 603-611.

Kornel, D., Rajulu, K.V. and Chhabra, A.D. (1984). Incidence of ethmoturbinate neoplasms in Jersey herd. Indian Vet. J. 61 (4): 276-279.

Koshiba, K., Namba, M. and Oda, T. (1970). Electron microscopic studies on cultured rat liver cells transformed by 4-nitroquonoline 1-oxide. GANN 61: 233-238.

Kuroki, T. and Sato, H. (1968). Transformation and neoplastic development *in vitro* of hamster embryonic cells by 4-nitro-quinoline 1-oxide and its derivatives. *J. Natl. Cancer Inst.* **41**: 53-71.

Labzoffsky, N.A. (1974). Microscopy. In. *Virology Manual*. Ed. N.A. Labzoffsky, Ministry of Health, Ontario. 227-246.

Labzoffsky, N.A. (1974). Electron microscopy. In. *Virology Manual*. Ed. N.A. Labzoffsky, Ministry of Health, Ontario. 265-325.

Lancaster, M.C., Jenkins, F.P. and Philp, J. Mcl. (1961). Toxicity associated with certain samples of groundnut. *Nature* **192**: 1095-1097.

Larsson, P., Hoedaya, W.L. and Tjalve, H. (1990). Deposition of ^{3}H-aflatoxin B_1 in mice: Formation and retention of Tissue Bound Metabolites in Nasal Glands. *Pharm. Toxic*. 67: 162-171.

Larsson, P., Petterson, H. and Tjalve, H. (1989). Metabolism of aflatoxin B_1 in the bovine olfactory mucosa. *Carcinogenesis* **10** (6): 1113-1118.

Legator, M.S. and Withrow, A. (1964). *J. Assoc. Offic. Agr. Chemists*. **47**: 1007. Cited by Zuckerman, A.J., Tsiquaye, K.N. and Fulton, F. (1966).

Legator, M.S., Zuffante, S.M. and Harp, A.R. (1965). Aflatoxin: effect on cultured heteroploid human embryonic lung cells. *Nature* **208**: 345-347.

Lewis, G., Markson, L.M. and allcroft, R. (1967). The effect of feeding toxic groundnut meal to sheep over a period of five yers. Vet. Rec. 80: 312-314.

Lin, C.T., Chan, W.Y.,Chen, W., Huang, H.M., Wu, H.C., Hsu, M.M., Chuang, S.M. and Wang, C.C. (1993). Characterization of seven newly established nasopharyngeal carcinoma cell lines. Lab. Invet. 68 (6): 716-724.

Lin, C.T., Wong, C.I., Chan, W.Y., Tzung, K.W., Ho, J.K.C., Hsu, M.M. and Chuang, S.M. (1990). Establishment and characterization of two nasopharyngeal carcinoma cell lines. Lab. Invest. 62 (6): 713-724.

Longo, V., Mazzaccaro, A., Naldi, F. and Gervasi, P.G. (1991). Drug metabolizing enzymes in liver, olfactory and respiratory epithelium of cattle. J. Biochem. Toxicol. 6: 123-128.

Loosemore, R.M. and Harding, J.D.J. (1961). A toxic factor in Brazilian groundnut causing liver damage in pigs. Vet. Rec. 73: 1362-1364.

Loosemore, R.M. and Markson, L.M. (1961). Poisoning of cattle by Brazilian groundnut meal. Vet. Rec. 73: 1362-1364.

Lu, M.R.S. and Ho, C.C. (1982). Experimental aflatoxicosis in meat type hybrid pigs. II. Effect of higher level of aflatoxin on young pigs: pathological study. J. Chinese Soc. Vet. Sci. 8: 113-120.

Luna, L.G. (1968). Manual of histological staining methods of the Armed Forces Institute of Pathology. 3rd ed. McGraw Hill Book Company, New York.

MacPherson, I. (1970). The characteristics of animal cells transformed in vitro. Adv. Cancer Res. 13: 169-215.

MacPherson, I. and Montagnier, L. (1964). Agar suspension culture for the selective assay of cells transformed by polyoma virus. Virology 23: 291-294.

Madewell, B.R., Priester, W.A., Gillette, E.L. and Snyder, S.P. (1976). Neoplasms of the nasal passages and paranasal sinuses in domestic animals as reported by 13 Veterinary Colleges. Am. J. Vet. Res. 37: 851-856.

Madhavan, T.V., Tulpule, P.G. and Gopalan, C. (1965). Aflatoxin induced hepatic fibrosis in Rhesus Monkeys. Arch. Pathol. 79: 466-469.

Magnusson, H. (1916). Endemische Geschwulste im Siebbein. Z Infekt Krankh Haustiere 17: 329-344, 355-392.

Marchok, A.C., Rhoton, J.C., Griesemer, R.A. and Nettesheim, P. (1977). Increased in vitro growth capacity of tracheal epithelium exposed in vivo to 7,12-dimethylbenz(a)anthracene. Cancer Res. 37: 1811-1821.

Marchok, A.C., Rhoton, J.C. and Nettesheim, P. (1978). In vitro development of oncogenicity in cell lines established from tracheal epithelium pre-exposed in vivo to 7,12-dimethylbenz(a)anthracene. Cancer Res. 38: 2030-2037.

Marshall, C.J., Franks, L.M. and Carbonell, A.W. (1977). Markers of neoplastic transformation in epithelial cell lines derived from human carcinomas. J. Natl. Cancer Inst. 58: 1743-1751.

Maryamma, K.I., Rajan, A. and Nair, M.G. (1991). Clinical diagnosis of aflatoxicosis by detection of aflatoxins in blood, urine and milk. Indian Vet. J. 68: 824-828.

Maryamma, K.I., Rajan, A., Nair, M.G., Ismail, P.K., Manomohan, C.B. and Gangadharan, B. (1989). Aflatoxin residues in animal tissues and animal products. Kerala J. Vet. Sci. 20 (1): 116-124.

Maryamma, K.I., Rajan, A., Nair, M.G. and Manomohan, C.B. (1992). Effect of dietary protein levels on aflatoxin-induced hepatocarcinogenesis in pigs. In Proceedings International symposium on advances in Animal cancer research, Tamil Nadu Veterinary and Animal Sciences University, Madras.

Mathur, M.,Rzvi, T.A. and Nayak, N.C. (1991). Effect of low protein diet on chronic aflatoxin B_1-induced liver injury in Rhesus monkeys. Mycopathologia 113: 175-179.

McKinnon, A.Q., Thorsen, J., Hayes, M.A. and Misener, L.R. (1982). Enzootic nasal adenocarcinoma of sheep in Canada. Can. Vet. J. 23: 88-94.

Miller, D.M., Crowell, W.A., Stuart, B.P. (1982). Acute aflatoxicosis in swine: clinical pathology, histopathology and electromicroscopy. Am. J. Vet Res. 43: 273-277.

Miller, D.M., Stuart, B.P. and Crowell, W.A. (1981). Experimental aflatoxicosis in swine: morphological and clinicopathological results. Can. J. Comp. Med. 45: 343-351.

Montesano, R., Drevon, C., Kuroki, T., St. Vincent, L., Handleman, S., Sanford, K.K., Defeo, D. and Weinstein, I.B. (1977). Tests for malignant transformation of liver cells in culture: cytology, growth in soft agar, and production of plasminogen activator. J. Natl. Cancer Inst. 59: 1651-1658.

Moreno-Lopez, J., Goltz, M., Rehbinder, C., Valsala, K.V. and Ludwig, H. (1989). A bovine herpes virus (BHV-4) as passenger virus in ethmoidal tumours in Indian cattle. J. Vet. Med. B. 36: 481-486.

Morimura, S., Tashiro, F. and Ueno, Y. (1990). Establishment and characterization of cell lines (Kagura-I and Kagura-2) from aflatoxin B_1 induced rat hapatoma. Clin. Pharm. Bull. 38 (2): 460-463.

Moussu, G. (1906). Des tumurs des cavites nasales chez les animaxu de lepsece bovine. Rec. Med. Vet. 83: 610-623.

Muralimanohar, B. (1988). Studies on ethmoidal neoplasms in animals. Ph.D. thesis, Tamil Nadu Veterinary and Animal Sciences University, Madras.

Muralimanohar, B., Sunderraj, A., Thanikachalam, M. and Muhalingam, P. (1986). Ethmoid carcinoma in a goat. Cheiron 15 (3): 100-102.

Muthappa, A.M. (1930). A case of fibroma in the frontal sinus of a cow. Indian Vet. J. 7: 175-176.

Nair, K.P.C. and Sastry, G.A. (1954). A survey of animal neoplasms in Madras State in bovines. Indian Vet. J. 30: 325-338.

Nair, K.V.N. (1973). A study of the common neoplasms of domestic animals in Kerala. M.Sc. thesis, Kerala Agricultural University, Mannuthy.

Nair, M.G. (1986). Immunopathological response of pigs in aflatoxicosis. M.V.Sc. thesis. Kerala Agricultural University, Mannuthy.

Nair, M.K. (1980). Electron microscopic studies of the neoplasms of the ethmoid mucosa of cattle. Pro. Symp. Tumours of Head, Kerala Agricultural University.

Nair, M.K., Rajan, A. and Ramachandran, K.M. (1987). Electron microscopic observations on the carcinoma of ethmoid mucosa in domestic animals. Kerala J. Vet. Sci. 18 (1): 89-104.

Nair, M.K., Sulochana, S., Rajan, A. Sreekumaran, T., Rehbinder, C. and Karlsson L. (1981). Virus-like particles in tumours of the mucosa of the ethmoid in Indian cattle. Acta Vet. Scand. 12: 143-145.

Narayana, J.V. (1960). Carcino-sarcoma in a bull. Indian Vet. J. 37 (4): 174-178.

Nayak, B.C., Rao, A.T., Das, B.C., Chakravorty, A. and Parthi, N.K. (1979). Tumours of bovine nasal cavity in Orissa. Indian J. Vet. Path. 3: 29-31.

Nazario, W., Valente, F.A.T., Portugal, M.A.S.C., Amaral, L.B.S. and Nesti, A. (1966). Carcinoma in the ethmoidal sinus of bovines and swines. Proceedings of the 5th Panamerican Congress on Veterinary Medicine and Zootechnics, Caracas, Vol.2. 232-833.

Newberne, P.M. and Butler, W.H. (1969). Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals: A Review. Cancer Res. 29: 236-250.

Newberna, P.M., Carlton, W.W. and Wogan, G.N. (1964). Hepatomas in rats and hepatorenal injury in ducklings fed peanut meal or Aspergillus flavus extract. Pathol. Vet. (Basel). 1: 105-132.

Newberne, P.M. and Wogan, G.N. (1968). Sequential morphological changes in aflatoxin B_1 carcinogenesis in the rat. Cancer Res. 28: 770-781.

Njoku, C.O. and Chineme, C.N. (1983). Neoplasms of the nasal cavity of cattle and sheep. In: Nasal tumours in animals and man Vol.II. Tumour Pathology. Eds. Reznik, G.V. and Stinson, S.F. CRC Press Inc. Boca Raton Florida. 181-198.

Njoku, C.O., Shannon, D., Chineme, C.N. and Bida, S.A. (1978). Ovine nasal adenopapilloma. Incidence and clinicopathologic studies. Am. J. Vet. Res. 39: 1850-1852.

Novi, A.M. (1977). Liver carcinogenesis in rats after aflatoxin B_1 administration. Current Topics in Pathology. Eds. Grundmann, E. and Kirsten, W.H. 65: 115-164.

Oshiro, Y. and DiPaolo, J.A. (1973). Loss of density - dependent regulation of growth of BALB/3T3 cells chemically transformed in vitro. J. Cell Physiol. 81: 133-138.

Osuna, O. and Edds, G.T. (1982). Toxicology of aflatoxin B_1, warfarin and cadmium in young pigs. Metal residues and pathology. Am. J. Vet. Res. 43: 1395-1400.

Patterson, D.S.P. (1973). Metabolism as a factor in determining the toxic action of the aflatoxins in different animal species. Food Cosmet. Toxicol. 11: 287-294.

Peers, F.G., Gilman, G.A. and Linsell, C.A. (1976). Dietary aflatoxins and human liver cancer. A study in Swaziland. Internat. J. Cancer. 17: 167-176.

Pier, A.C. (1981). Mycotoxins and Animal Health. Adv. Vet. Sci. Comp. Med. 25: 185-243.

Pollack, R., Risser, R., Conlon, J. and Rifkin, D. (1974). Plasminogen activator production accompanies loss of anchorage regulation in transformation of primary rat embryo cells by simian virus 40. Proc. Natl. Acad. Sci. USA. 71: 4792-4796.

Pospischil, A., Haenichen, T. and Schaeffer, H. (1979). Histological and electronmicroscopic studies of endemic ethmoidal carcinomas of cattle. Vet. Pathol. 16: 180-190.

Pospischil, A., Weiland, F., Sandersleben, J., Haenichen, T., Schaeffler, H. (1982). Endemic ethnoidal tumours in cattle. Sarcomas and carcinomas. A light and electronmicroscopic study. Zentbl. Vet. Med. 29 (8): 628-636.

Pound, A.W. and Lawson, T.A. (1974). Effect of partial heptactomy on carcinogenecity, metabolism and binding to DNA of ethyl carbamata. J. Natl. Inst. 53: 423-429.

Pruthi, A.K., Mishra, S.K., Sadana, J.R. and Paul Gupta, R.K. (1982). Fibrosarcoma of nasal region in a bullock. Haryana Vet. 21 (2): 139-141.

Rajan, A. (1980). Incidence and pathology of tumours of the mucosa of the ethmoid in domestic animals. Proc. Symp. Tumour Head, KAU. 1-6.

Rajan, A. (1987). Carcinoma of the mucosa of the ethmoid in domestic animals. Ann. Rech. Vet. 18: 13-17.

Rajan, A., Maryamma, K.I. and Nair, M.G. (1989). Aflatoxin induced hepatopathy in pigs and ducks. J. Toxicol. Toxin Reviews. 8 (1921): 255-263.

Rajan, A., Sivadas, C.G., Nair, M.K. and Maryamma, K.I. (1972). Incidence and pathology of tumours of the paranasal sinuses in domestic animals. Kerala J. Vet. Sci. 3: 83-101.

Rajan, A. and Sulochana, S. (1982). Tumours of the mucosa of the ethmoid in domestic animals - Incidence and pathological features. Cheiron. 11 (1): 1-4.

Rajan, A., Sulochana, S., Reddy, M.V., Valsala, K.V., Ramachandran, K.M. and Maryamma, K.I. (1981). Tumours of the mucosa of the ethmoid in pigs. Indian J. Cancer 18: 202-205.

Rameshmurthy, S. (1984). Incidence of ethmoturbinate neoplasms in Jersey herd of Red Dane Project (Jersey) substation in Karnataka. Indian Vet. J. 61 (6): 528.

Reddy, J. and Svoboda, D.J. (1968). The relationship of nuclear segregation to ribonucleic acid synthesis following the administration of selected hepatocarcinogens. Lab. Invest. 19: 132-137.

Reddy, M.V. and Rajan, A. (1982). Pathology of the spleen in cattle bearing carcinoma of the mucosa of the ethmoid region. Kerala J. Vet. Sci. 13 (2): 219-222.

Reznikoff, C.A., Bertram, J.S., Brankon, D.W. and Heidelberger, C. (1973). Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitine to postconfluence inhibition of cell division. Cancer Res. 33: 3239-3249.

Rings, D.M. and Rojko, J. (1985). Naturally occurring nasal obstructions in 11 sheep. Cornell Vet. 75 (2): 269-276.

Robinson, D.S. and Seakins, A. (1962). The development in the rat of fatty livers associated with reduced plasma lipoprotein synthesis. Biochem. biophys. Acta. 62. 163-165.

Rosadio, R.H., Lairmore, M.D., Russell, H.I. and DeMartini, J.C. (1988). Retrovirus associated ovine pulmonary carcinoma (Sheep Pulmonary Adenomatosis) and lymphoid interstital pneumonia I. Lesion development and age susceptibility. Vet. Pathol. 25: 475-483.

Rubaj, B. and Woloszyn, S. (1967). Adenopapilloma enzooticum jamy nosowej u owiec. Madycyna Wet. 23: 226-229.

Rutenberg, A.M., Kim, H., Fischbein, J.W., Hanker, J.S., Wasserkrug, H.L. and Seligman, A.M. (1969). Histochemical and ultrastructural demonstration of gamma-glutamyl transpeptidase activity. J. Histochem. Cytochem. 17: 517-526.

San, R.H.C., Shimada, T., Maslansky, C.J., Kreiser, D.M., Laspia, M.F., Rice, J.M. and Williams, G.M. (1979). Growth characteristics and enzyme activities in a survey of transformation markers in adult rat liver epithelial-like cell culture. Cancer Res. 39: 4441-4448.

Sarasin, A., Moule', Y. (1975). Translational step inhibited in vivo by aflatoxin B_1 in rat liver polysomes. Europ. J. Biochem. 54: 329-340.

Sastry, G.A. and Rao, S.P. (1964). Carcinosarcoma in a bullock. Indian Vet. J. **41** (10): 16.

Sato, H. and Kuroki, T. (1966). Malignization in vitro of hamster embryonic cells by chemical carcinogens. Proc. Jap. Acad. **42**: 1211-1216.

Schabort, J.C. and Steyn, M. (1972). Aflatoxin B_1 and phenobarbital inducible Aflatoxin - 2 - hydration by ret liver microsomes. Biochem. Pharmacol. **21**: 2931-2933.

Schoental, R. (1961). Liver changes and primary liver tumours in rats given toxic guinea pig diet (M.R.C. diet 18). British J. Cancer. **15**: 812-815.

Shalkop, W.T. and Armbrecht, B.H. (1974). Carcinogenic response of brood sows fed aflatoxin for 28-30 months. Am. J. Vet. Res. 35: 623-627.

Shalkop, W.T., Geleta, J.N. , Armbrecht, B.H. and Wiseman, H.G. (1967). Experimental aflatoxicosis in feeder pigs. Cited by Armbrecht (1978).

Shotwell, C.L., Hesseltine, C.W., Stubblefield, R.D. and Sorenson, W.G. (1966). Production of aflatoxin on rice. Appl. Microbiol. **14**: 425-428.

Sieber, S.M., Correa, P., Dalgard, D.W. and Adamson, H. (1979). Induction of osteogenic sarcomas and tumours of the hepatobiliary system in nonhuman primats with aflatoxin B_1. Cancer. Res. **39**: 4545-4554.

Simard, R. and Bernhard, W. (1966). LePhenomene de La segregation nucleolaire specificite 'd' action de certains antimetabolites. Int. J. Cancer. 1: 463-466.

Singh, B. and Singh, N. (1984). Neoplasms in Indian buffaloes. Indian Vet. J. 61 (8): 639-643.

Sippel, W.L., Burnside, J.E. and Atwood, M.B. (1953). A disease of swine and cattle caused by eating moldy corn. Proc. Book Am. Vet. Med. Assoc. 174-181.

Sisk, D.B., Carlton, W.W. and Curtin, T.M. (1968). Experimental aflatoxicosis in young swine. Am. J. Vet. Res. 29: 1591-1602.

Slaga, T.J., Viaje, A., Bracken, W.M., Buty, S.G., Miller, D.R., Fischer, S.M., Richter, C.K. and Dumont, J.N. (1978). In vitro transformation of epidermal cells from Newborn mice. Cancer Res. 38: 2246-2252.

Smith, H.S., Scher, C.D. and Todaro, G.J. (1971). Induction of cell division in medium lacking serum growth factor by SV40. Virology. 44: 359-370.

Smith, R.H. (1963). The influence of toxins of Aspergillus flavus on the incorporation of (C 14) leucine into proteins. Biochem. J. 88 (2): 50.

Sorenson, W.G., Simpson, J.P., Peach, M.J., Thedell, T.D. and Olenchock, S.A. (1981). Aflatoxin in respirable corn dust particles. J. Toxicol. Environ. Health., 5: 669-672.

Sreekumaran, T. (1980). Pathobiology of the neoplasms involving the paranasal sinuses in bovines. Ph.D. thesis. Kerala Agricultural University, Mannuthy.

Sreekumaran, T. and Rajan, A. (1983). Histology and histochemistry of endemic ethmoid carcinoma in bovines. Indian J. Cancer 20 (1): 10-14.

Steele, V.E., Marchok, A.C. and Nettesheim, P. (1978). Establishment of epithelial cell lines following exposure of cultured tracheal epithelium to 12-0-tetradecanoyl-phorbol-13-acetate. Cancer Res. 38: 3663-3365.

Steele, V.E., Marchock, A.C. and Nettesheim, P. (1979). Oncogenic tranformation in epithelial cell lines derived from tracheal explants exposed in vitro to N-methyl-N[1]-nitro-N-nitrosoguanidine. Can. Res. 39: 3805-3811.

Steen, M., Rehbinder, C. and Morner, T. (1985). Nasal tumour in a fallow deer (Dama dama L.). A case report. Acta Vet. Scand. 26: 461-465.

Stenstrom, O. (1915). Enzootisches Auflreten Von Geschwulsten bei Rind Und Pferd. Veroffentlichung der med Staatsanstalt in Stockholm, 1915.

Stubblefield, R.D. and Shotwell, O.L. (1981). Determination of aflatoxins in Animal tissues. J. Assoc. Off. Anal. Chem. 64 (4): 964-968.

Styles, J.A. (1977). A method for detecting carcinogenic organic chemicals using mammalian cells in culture. British J. Cancer. 36: 558-563.

Sulochana, S., Rajan, A., Sreekumaran, T. and Reddy, M.V. (1980). Isolation of haemagglutinating agents from tumours of the mucosa of the ethmoid in cattle. Kerala J. Vet. Sci. 11: 229-237.

Svoboda, D.J. (1964). J. Natl. Cancer Inst. 33: 315. Cited by Koshiba, K., Namba, M., Oda, T. (1970).

Swarup, D., Singh, G.R., Sharma, M.C. and Dwivedi, S.K. (1987). Ethmoid tumour in two dairy cow-clinical and radiological features. Indian J. Vet. Med. 7 (2): 122-124.

Tjalve, H., Larsson, P., Andersson, C. and Busk, L. (1992). Bioactivation of aflatoxin B_1 in the bovine olfactory mucosa. DNA binding, metagenicity and induction of sister chromatin exchanges. Carcinogenesis 13 (8): 1345-1350.

Tokarnia, C.H., Dobereiner, J., Canella, C.F.C. (1972). Tumor ethmoidal enzootico em bovinos no Estado do Rio de Janeiro. Perq Agropec Bras Ser vet. 7: 41-46.

Toyoshima, K., Hiasa, Y., Ito, N. and Tsubura, Y. (1970). In vitro malignant transformation of cells derived from rat liver by means of aflatoxin B_1. GANN. 61: 557-561.

Umeda, M. (1971). cytomorphological changes of cultured cells from rat liver, kidney and lung induced by several mycotoxins. Japanese J. Exptl. Med. **41**: 195-207.

Viraraghavan, K., Masillamony, P., Kesavalu, L. and Ramakrishnan, R. (1980). Survey of incidence of neoplasms in the upper respiratory tract of bovines in Tamil Nadu. Proc. Symp. Tumour Head, KAU. 20-26.

Weinstein, I.B., Orenstein, J.M., Gebert, R., Kaighan, M.E., Stadler, U.C. (1975). Growth and structural properties of epithelial cell cultures established from normal rat liver and chemically induced hepatomas. Cancer Res. 35: 253-263.

Wendt, M. (1989). Clinical aspects and diagnosis of ethmoidal tumours (adenopapillomatosis) in sheep. Tierarztliche Umscho. **44** (9): 540-547.

Williams, G.M. (1976). The use of liver epithelial cultures for the study of chemical carcinogenesis. Am. J. Pathol. 85 (3): 739-752.

Williams, G.M., Elliott, J.M. and Weisburger, J.H. (1973). carcinoma after malignant conversion in vitro of epithelial-like cells from rat liver following exposure to chemical carcinogens. Cancer Res. 33: 606-612.

Wilson, B.J., Teer, P.A., Barney, G.H. and Blood, F.R. (1967). Relationship of aflatoxin to epizootics of toxic hepatitis among animals in Southern United States. Am. J. Vet. Res. **28**: 1217-1230.

Wilson, D.W., Ball, R.W. and Coulombe, R.A. (1990). Comparative action of aflatoxin b_1 in mammalian airway epithelium. Cancer Res. **50**: 2493-2498.

Wogan, G.N. and Newberne, P.M. (1967). Dose-response characteristics of aflatoxin B_1 carcinogenesis in the rat. Cancer Res. **27**: 2370-2376.

World Health Organization (1979). Environ. Health Criter. 11: 1-127. Cited by Pier, A.C. (1981).

Yonemichi, H., Ohghi, T., Fijimoto, Y., Okada, K., Onuma, M. and Mikami, T. (1978). Intranasal tumor of the ethmoid Olfactory mucosa in sheep. Am. J. Vet. Res. 39: 1599-1606.

Young, S., Lovelace, S.A., Hawkins, W.W. and Catlin, J.E. (1961). Neoplasms of the olfactory mucous membrane of sheep. cornell Vet. **51**: 96-112.

Zhang, Yu-Hui (1981). Preliminary report on adenocarcinoma of the ethmoidal sinus and liver cancer of swine on a breeding farm in Dehha Counts Fujien, Province. Chinese J. Vet. Med. **7** (1): 2-8.

Zuckerman, A.J., Tsiquaye, K.N. and Fulton, F. (1966). Tissue culture of human embryo liver cells and the cytotoxicity of aflatoxin B_1. Br. J. Exptl. Pathol. **48**: 20-27.

ASSESSMENT OF THE ROLE OF AFLATOXIN IN THE AETIOLOGY OF CARCINOMA OF THE MUCOSA OF THE ETHMOID

By

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ABSTRACT OF A THESIS

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ABSTRACT

The present investigations were planned to assess the role of aflatoxin B_1 (AFB_1) and/or virus in the aetiology of ethmoid carcinoma using pig as a model *in vivo* and bovine ethmoid mucosa culture *in vitro*.

Thirty-two, Large White Yorkshire piglings of two-three months age were procured from the University Pig Breeding farm, Mannuthy and divided at random into four groups of eight each.

The pigs in group I and group II were administered aflatoxin B_1 (0.070 mg/kg b.wt/inoculum by intravenous route at weekly interval for six months) and/or ethmoid tumour extract (2 ml/pig/inoculum, intranasally, at fortnight interval for three months). The pigs in group III were administered ethmoid tumour extract alone, while the pigs in group IV were kept as negative controls.

During the period of observation of 18 months all the pigs of different groups given AFB_1 and/or ethmoid tumour extract appeared healthy and no clinical manifestation of the carcinoma of the mucosa of ethmoid was observed. However, there was appreciable reduction in the weight and mild degree of depression.

In the AFB_1 treated pigs, sacrificed at 9, 12, 15 and 18 months of investigation, the ethmoid mucosa had greyish white, soft and oedematous appearance along with scattered small pale elevations at necropsy. Histologically, the ethmoid mucosa exhibited hyperaemia, varying degree of mononuclear cell infiltration and fatty degeneration in the initial stages. In the later stages, there was proliferation of mucous glands showing acinar, tubular or papillary arrangements. Occasionally papillary projection of the surface epithelium and focal squamous metaplasia were also observed. Ultrastructural features of the cells of the ethmoid mucosa consisted of both productive and degenerative changes. The cells had sparse cytoplasmic organelles. The poor cytoplasmic contents and irregular nucleus with nucleolar margination were the other electron microscopic features observed in the ethmoid mucosa of AFB_1 treated pigs.

AFB_1 in the range of 43.12-139.43 ppb could be detected in the blood of 52.37 per cent of the ethmoid tumour bearing cattle analysed in the present study.

The blood samples from the AFB_1 treated pigs were positive for AFB_1 (40-160 ppb) upto 10 days after the withdrawal of treatment whereas AFM_1 could be detected in blood sample of one pig only upto 3 days after the treatment. The ethmoid mucosa analysed after 3 months and at subsequent

specified intervals was consistently negative for AFB_1 and AFM_1.

By concerted efforts cells of the mucosa of the ethmoid were established <u>in vitro</u>. AFB_1 treatment of long term epithelial cultures initiated from the primary culture of bovine ethmoid mucosa origin resulted in morphological transformation accompanied by increased growth in soft agar and cytochemical positivity of gamma-glutamyl transpeptidase. This confirmed the tumourigenicity of AFB_1. The xenotransplantation of these <u>in vitro</u> transformed epithelial cells in mice was not successful.

Electron microscopic studies of the cells of the carcinoma of the ethmoid mucosa in spontaneous cases of cattle revealed varying ultrastructural features. The neoplasticcells were either well differentiated secretory structures or undifferentiated ones. Desmosomes and tight junctions were seen between the epithelial cells. Endoplasmic reticulum and mitochondria varied in their contents and degree of disorganization. Nucleus was highly pleomorphic and predominantly euchromatinic.

The retroviral like particles were demonstrated intracellularly and occasionally in extracellular spaces in the neoplastic cells of 7 tumour bearing cattle. Similar particles were also seen in the cell free ethmoid tumour extract in three of 21 tissues examined.