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TUMOUR INDUCTION BY VIRUSES ISOLATED FROM *MANSONIA AFRICANA**

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Summary

A group of viruses originally isolated from different pools of *Mansonia africana* induced 'tumours' in swiss albino mice when injected intraperitoneally, intracerebrally and intravenously.

Tumours which developed in close proximity to points of initial inoculation were round, single or bilobular and usually appeared to adhere to underlying structures.

Extracts of the tumours yielded similar tumours in freshly inoculated animals. Clarified extracts caused cytopathogenecity in cell cultures and agglutinated a variety of red blood cells.

Tissue culture fluids from infected cell cultures also agglutinated red blood cells.

Tumours were not transplantable by embedding the materials in a new set of animals, but appear to be transmissible via cannibalism.

Résumé

Un groupe de virus isolés à l'origine de différents groupes d'insectes (*Mansonia africana*) provogna des tumeurs chez les souris suisses albinos après injection dans le péritoine, dans le crâne ou dans les veines. Les tumeurs qui se développèrent tout près des points d'inoculation initiale étaient rondes, uniques, et bilobaires, et paraissaient généralement adhérer aux structures sous-jacentes.

Des extraits de tumeurs produisirent des tumeurs semblables chez des animaux récemment inoculés. Des extraits clarifiés causèrent une cytopathogenicité des cultures cellulaires et agglutinèrent une variété de globules rouges du sang. Les fluides de la culture de tissus obtenus de cultures de cellules contaminées agglutinèrent aussi les globules rouges de sang.

Il n'a pas été possible de transplanter la tumeur en greffant des morceaux de tissu de la tumeur sur d'antres groupes d'animaux. Il semble cependant que le cannibalisme permette sa transmission.

Introduction

Mansonia, like Phlebotomus and Dacus is a unique mosquito in that it has two spermatheca instead of the one that anopheles has and in comparison with the three that Culex and *Aedes egypti* have. It is a common blood-sucking mosquito, seen primarily in the late evenings, generally between the hours of 4 and 7 p.m. (Boorman, 1960). The viruses used in these studies were isolated from different pools of the mosquitoes (*Mansonia africana*) by Draper and his colleagues (personal communication) between 1961 and 1963. The viruses were labelled Yaba virus with the designation of Yaba 1, 2 etc. Y5 and Y9 were used in these studies. Y5 has been confirmed as a strain of Bunyamwera (Simpson & Williams, 1964; J.S. Porterfield & Shope; personal communications). Y9 on the other hand has been confirmed as a virus related to the Chickungunya and Onyong-nyong groups (Simpson and Williams, 1964). The findings

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reported here initially were detected purely by accident. Attempts were made in the early 1970s to grow out some of the viral materials that had been stored frozen for nearly a decade and some of the cages used in this exercise were overlooked, and were not harvested when they should have been. It was discovered after about 45 days post inoculation that some of the mice particularly the females in these cages had developed some unusual growth in areas close to the point at which they were initially inoculated. The growths which occured in 16–33% of the animals were excised and investigated.

New sets of mice inoculated as in the previous cases using fresh viral preparations were set up. Tumour development in these cases varied from 1.5 to 15 months and tumours were similar to those observed earlier on. These and other observations made during the ensuing investigations are the subject of this paper.

Materials and methods

Virus source

The viruses used were taken from a group of viruses available in the laboratory of the Medical Research Council of Nigeria (now National Institute for Medical Research). They were rehydrated and inoculated into several animals.

Animals

Monkeys, rabbits, guinea pigs, hamsters, rats, chicken and swiss albino mice were used in the investigation. Generally a group of 2–3 of the large animals were used. Six hamsters and mice were employed per group.

Inoculum and inoculation procedure

Intracerebral, intraperitoneal and intravenous routes were employed in injecting measured quantities of each virus preparation into chosen animals. The initial inocula were rehydrated preparations of Draper's group (West African Council for Medical Research, 1962–1964; Draper personal communication). Subsequent inocula included freshly harvested and processed brains, tumour extracts and tissue culture fluids following development of a cytopathic effect by the extracts in cell cultures. Tissue culture fluid and cell extracts obtained from cells cultured from tumours were also used. Two-tenths of the preparation were inoculated into groups of six mice at concentrations varying from undiluted to 10^{-8}. In the case of the tumour extracts, 0.2 cc of a 20% suspension of the tumour from Y9 or Y5 was inoculated into mice at concentrations of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. All were set up with appropriate controls i.e. groups of mice treated in every respect as the inoculated ones, except that they received saline solution in place of the virus inoculum. All other animals were similarly inoculated.

Confirmatory experiments

Every rehydrated virus, or those produced from other sources were always checked out to confirm that the same virus was being used. This was done by checking for the cytopathic effect in tissue culture, and via haemmaglutination titres and pattern as well as via the fluorocarbon test. (Theiller, 1957; Sunaga, Taylor & Henderson, 1960).

The nature of the tumour was also used as a criterion.

Brain and tumour extract

Mouse brain harvests were prepared according to the method discussed by Clarke & Casals (1958). For tumours, 20% extracts were prepared by grinding pieces of the tissue which were then treated according to the methods used for the brains.

Determination of tumour size

Harvested tumours were placed in sterile petri dishes and the size measured with a ruler.

Preparation and processing of tumour cells for culture

Harvested tumours were minced and either ground and filtered as previously stated or directly placed in tissue culture media and allowed to grow in suspension or as monolayer cultures.

Sites of tumours

Tumours generally developed at regions very

close to the point at which viral materials were inoculated.

The nature of the tumour and its cell components

After cutting open the usually single or bilobular tumours, no unusual structures were macroscopically visible nor was fluid seen. Tumour suspensions cultured in microbiological media did not show growth of any microorganisms. Results of histological studies will form a part of further reports in our study. Cells prepared for cell cultures however gave a general idea of the cell components of the tumour. Those that grew as monolayer cultures were principally large cells with large cytoplasm, while those that did not attach to a glass surface were pleomorphic. It has not been possible to obtain a continous cell line from the tumours as they died off after a few generations. The longest surviving preparations died off after the fifth transfer — a period of about 12 weeks.

Transmission of tumour

Apart from the conventional methods of tumour transferences, the possibility of these tumours being transmitted via sexual engagements was exploited. It did not appear that this was the case.

Transplantation of tumour

Deliberate attempts were made to transplant the tumour by embedding pieces of the tumour in the mice. During the experimental period sexual activities of the mice continued. Some of the infected male mice mated with their sisters (also infected). Each female that became pregnant as a result was separated along with a male, and allowed to continue normal life.

Following delivery, both parents and the offsprings were allowed to continue to live together in their respective cages.

In some other cases, some mothers ate their inoculated suckling babies. Such cases were also separated for observation.

Infectivity and hemagglutination titrations

A selected number of medium free tubes and bottles of different cell strains (primary and secondary) were inoculated with 0.2 cc of viral preparation. Unabsorbed virus was removed by removing the fluid on the cell, after a cell-virus interaction at 37°C for 2–4 h. Fresh maintenance medium (EE2FC), was added and incubation continued at 37°C. Observation for CPE which included balooning, granulation, rounding off, detachment of cells from glass surface and giant cell formation with multinucleation were recorded at intervals generally for a period 1–2 weeks. The cells used included the following secondary and primary lines; HEp–2, BHK–21, KB, HeLa; and mouse embryo Fibroblast as well as sheep embryo kidney.

Hemagglutination

These were carried out as described by Porterfield (1957). Red cells from goose, crownbird, pelican, house rats and day-old chicks were used.

Buffers, media and other solutions

Phosphate buffered saline was prepared according to the method of Dulbecco & Vogt (1954). Borate Buffer was prepared in the conventional way.

Tissue culture media

Growth media contained 10–20% foetal calf serum while maintenance media contained 0–2% foetal calf serum. Eagles' basal medium was used throughout the studies.

Results

Tumour induction by Y5 and Y9

Figure 1 shows the development of tumours in inoculated mice. It futher shows that tumours develop in close proximity to the point of initial viral injection.

Tumour size and shape

A typical bilobular tumour is shown in Fig. 2. Generally the tumour varied in size and shape. They were roundish, either single of bilobular and measured 1–2 cm.

Cytopathic Effect of Y5 and Y9

The different forms of changes in cells infected by these viruses, are shown in Fig. 3a–d.

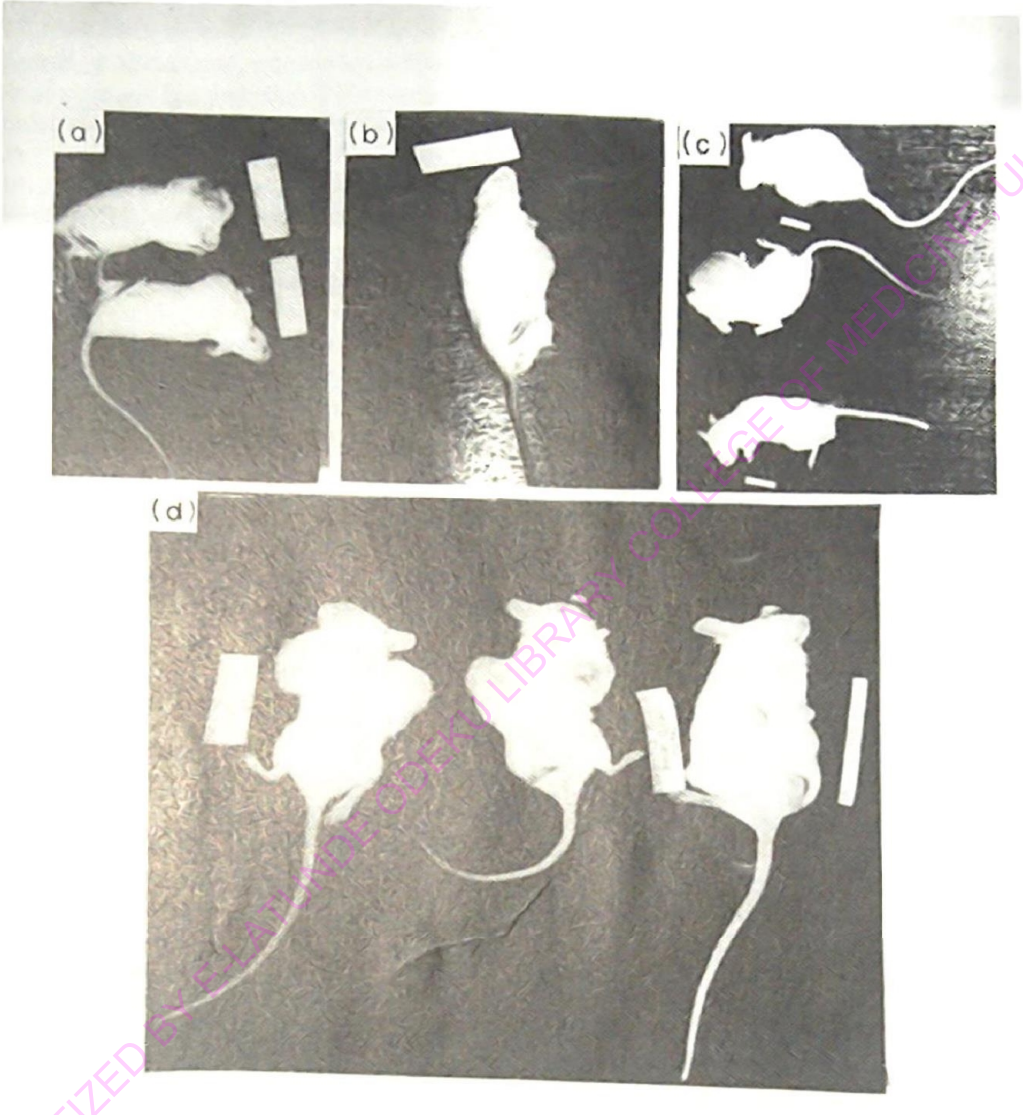


FIG. 1. (a) Mice inoculated intracranially with Y9 virus.
(b) Mouse inoculated intraperitoneally with Y5 virus.
(c) Top mouse inoculated intraperitoneally; middle mouse inoculated intracranially; bottom mouse inoculated intravenously.
(d) Left: Dorsal view of mouse inoculated intraperitoneally. Middle: Ventral view of same mouse. Right: Control, uninfected mouse.
Note development in regions close to point of initial virus inoculation.

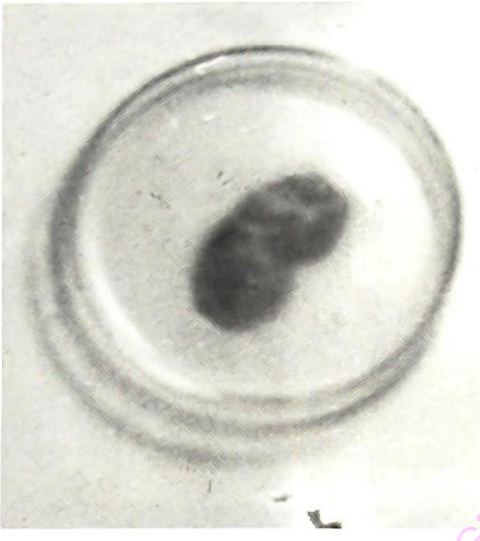


FIG. 2. Bilobular tumour excised from an infected mouse.

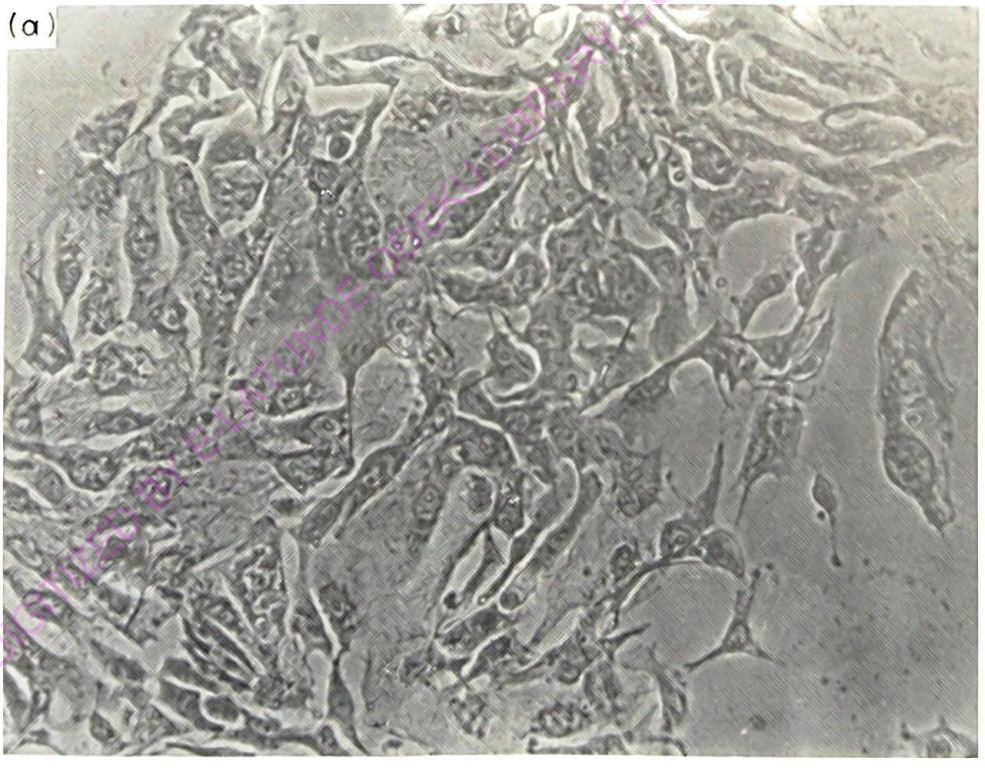


FIG. 3. Effect of tumour extracts on cell cultures of mouse embryo fibroblasts (MEF).

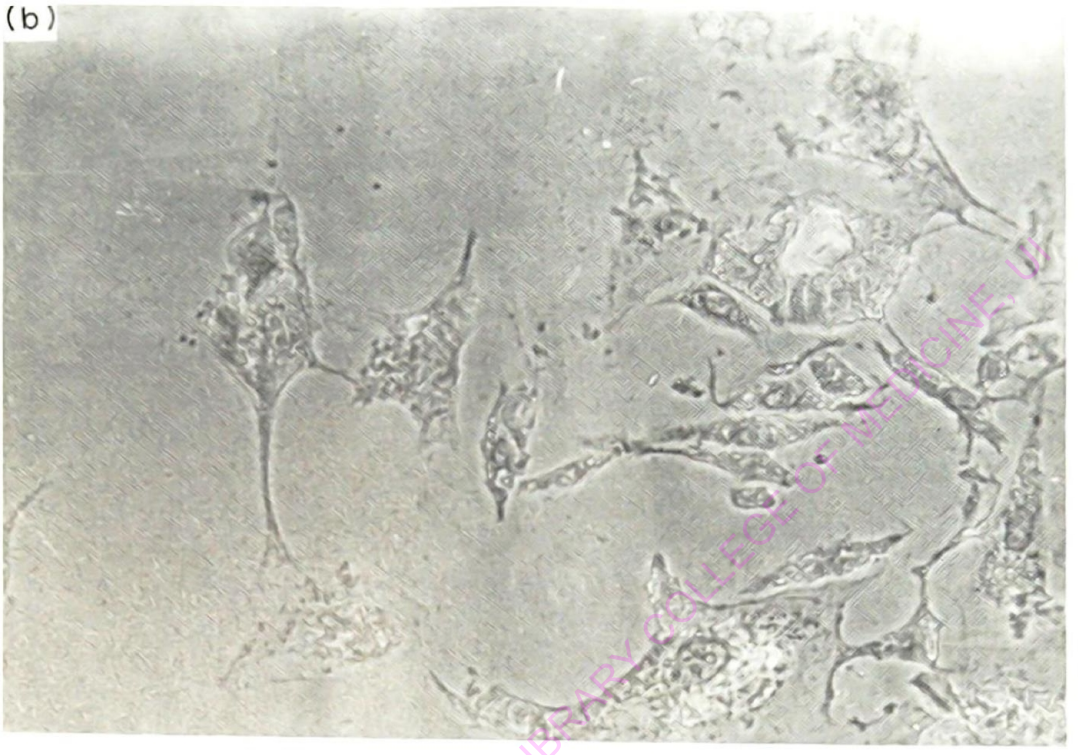
(a) Control uninfected cells.

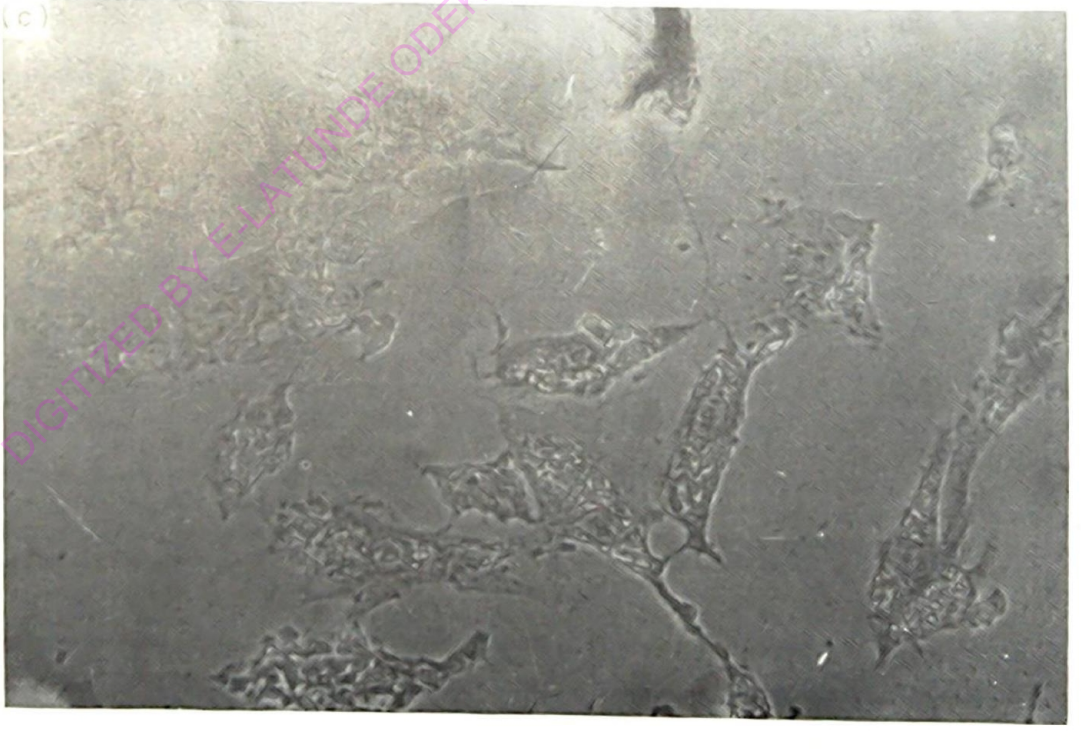
(b) Infected cells showing vacoulation, ballooning and pseudopodia-like structure.

(c) Infected cells with large cytoplasm in giant cells; note also deterioration in the cell structure.

(d) Infected cells; note giant cells appearing to undergo degeneration

(a), (b), and (c) were taken under phase contrast microscopy, magnification × 100; (d) was taken by ordinary microscopy, magnification × 100.





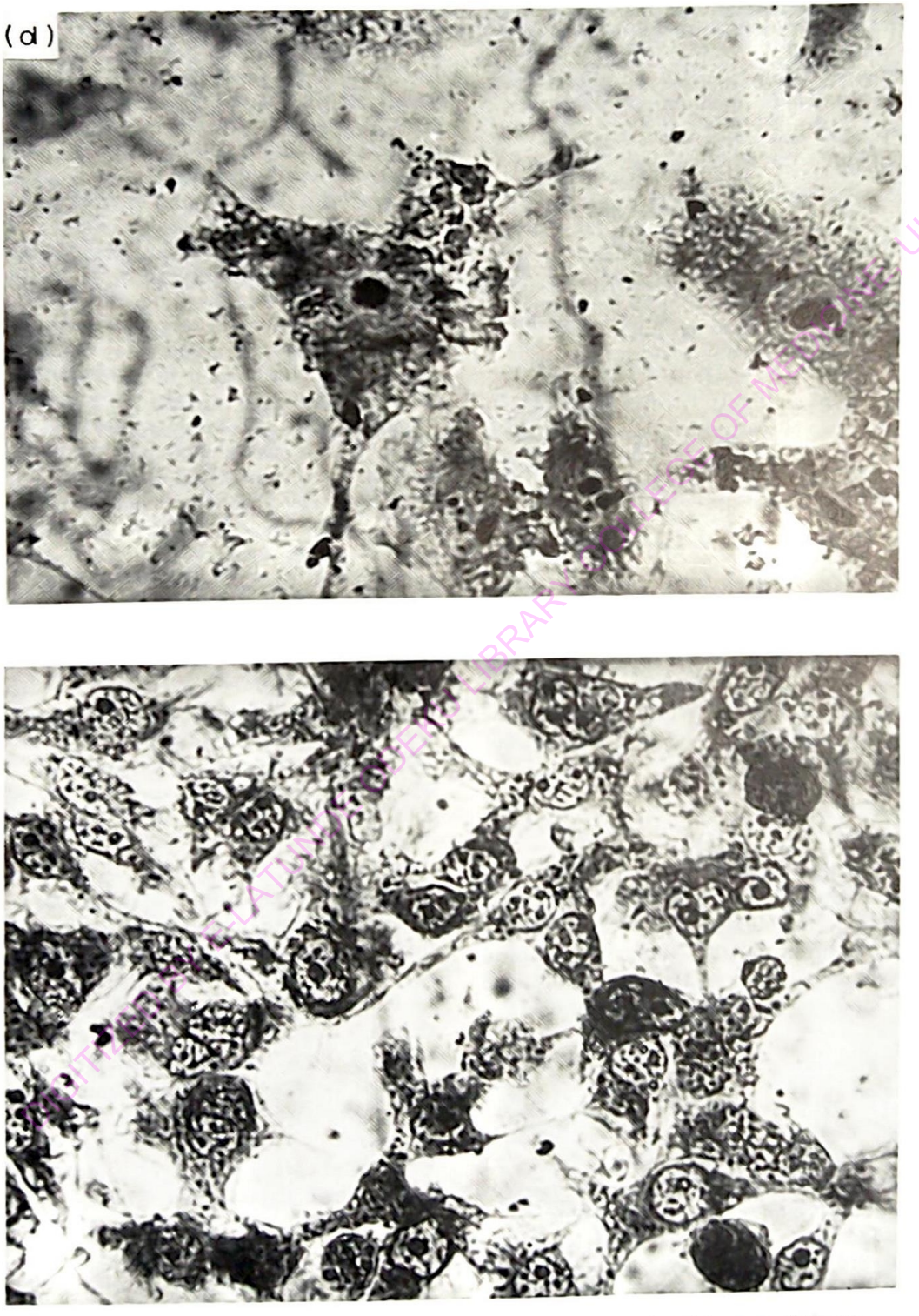


FIG. 4. Trypsinized tumour cells growing on monolayer cultures (3rd generation). Note some cells' larger size of cytoplasm in relation to rest of the cell. Ordinary microscopy, magnification × 100.

Tumour cells in culture

Figure 4 shows typical tumour cells growing as monolayers.

Tumour cells growing in suspension cultures, actually floated in the medium.

Hemagglutination (HA) by tumour extracts

The titres obtained when extracts from Y5 and Y9 tumours were checked for HA activity varied from 1 : 64 to 1 : 512, which compares favourably with those of dehydrated or fresh brain-harvest preparations. The pattern of agglutination for tissue culture fluid from cultures where the tumour extracts produced a distinct cytopathic effect are less spectacular, generally being around 1 : 128. Cells agglutinated included those of goose, crown-bird and a few others while those of giant rats were not agglutinated.

Occurences of tumour

Tumours were more common in female mice than in males, the ratios being, on the average, four females to one male.

Tumour development following cannibalism followed an interesting pattern, but tumours were not very common. When they occurred, they were usually found randomly located, but always viscerally situated.

Cytopathic effect

A cytopathic effect was induced by the tumour extracts. The manifestation included balooning, formation of pseudopodia-like structure, lifting off of the glass surface etc. Typical results on mouse embryo fibroblasts are shown in Fig. 3 a-d.

Discussion

The mechanisms underlying tumour initiation in animals remain largely unknown.

The role of carcinogens in the development of tumours continues to generate debate. Both laboratory induced and spontaneously generated tumours are being investigated by others to elucidate the mechanism of tumour formation. The Arboviruses are a large group of viruses which are known to cause many human diseases and discomforts but tumour initiation has not been described as one of them (Burkitt, 1969; Kafuko & Burkitt, 1970). It is to be understood that the word 'tumour' in this paper is being used in a wide sense, without a direct indication that the growths observed and described are true neoplasms (cf. Dalldorf, 1962; Dalldorf *et al.*, 1964). A large part of the present study was conducted after the initial accidental observation of unusual growth in animals that were inoculated to produce more seed virus. The curiosity of the author was aroused when the tumour production was reproduced by directly inoculating laboratory animals. A number of interesting observations were made: (1) The tumours developed in close proximity to the region of inoculation of the viruses, indicating that they may be true neoplasms – carcinoma, fibroma and fibrosarcomas. (2) They are more common in females, especially when virus was inoculated intraperitoneally. (3) They are transferrable by cannibalism; mother-mice that ate their inoculated suckling babies also developed tumours. (4) It has been shown that some of the cells making up the tumours grew both in monolayer and suspension cultures, although in either case, the cells were short-lived. (5) The extracts made from the tumours did not only induce fresh tumors, they caused cytopathic effects on a number of primary and secondary cell lines. The extracts also agglutinated some red blood cells. (6) Serum free-cell culture fluids from which the tumour cells grew were shown also to agglutinate red blood cells.

Surprisingly, no tumours were observed in animals receiving either a transplant of the tumours or those inoculated with tissue culture fluids of cultured tumours. It did not appear that the tumours are also sexually transmissible.

Tumours arose primarily in the subcutaneous tissues. Once initiated — first as small nodules — they grew rather rapidly and fungated through the skin. They were found to adhere to underlying structures. No encapsulation was observed.

Tumours were usually of roundish shapes, either single or bilobular. On sectioning, no visible structures were recognizable. Aseptically produced suspensions were cultured in bacteriological and mycological media and no fungus or bacterium were found to grow.

Tumour materials were sent to pathologists for histological examinations. It was however

clear from materials prepared for cell cultures, that the cells making up the tumour were heterogeneous, the predominant cells being large with relatively extensive cytoplasm — these were the ones that grew in cell cultures. With bi-weekly passages and frequent medium changes, up to five generations were obtained. The second abundant type of cells were rather pleomorphic. This set failed to adhere or attach to a glass surface in culture; they floated. Later they were grown in suspension cultures. They became non-viable generally, after the third transfer, each transfer being 5–7 days apart.

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