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# Cytopathology and short-term culture of malignant tumours in Ibadan

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**Summary**

This report is a retrospective review of early studies (1965-70) on the cytology and short-term tissue culture of fresh specimens from 1643 patients under investigation for cancer at the University College Hospital, Ibadan. A total of 580 specimens were positive for malignant cells. The technical procedures are described in some detail and were found to be particularly useful as aid to laboratory diagnosis of 310 childhood tumours. The relative frequency of Burkitt's lymphoma, retinoblastoma, neuroblastoma and Wilm's tumour in the series was 20:1:1:0.5 respectively. Adoption of the technique as part of routine diagnostic service of teaching hospitals of developing countries is recommended.

**Résumé**

Ceci est une compte rendu rétrospectif des études effectuées de 1965 à 1970 à l'University College Hospital, Ibadan. Ces études portent sur la cytologie et la culture à court terme des tissus frais qui ont été prélevés de 1643 patients étudiés pour déterminer le cancer. 580 cas contiennent des protoplasmes malignes. Les procédés techniques ont été décrits en quelques détails et ils ont été particulièrement utiles pour diagnostiquer 310 tumeurs de l'enfance. La fréquence relative de lymphome, de neuroblastome de Burkitt et celle de la tumeur de Wilm dans les séries étaient de 20:1:1:0.5 respectivement. L'adoption de ces techniques comme faisant une partie intégrale du service diagnostique dans les hôpitaux des pays en voie de développement est recommandée.

## Introduction

As pointed out by exponents of the examination of living pathological tissues, the parameters which microscopic examination of living cells offers for the characterization of cell-types are manifold (Pulvertaft, 1959; Pulvertaft & Humble, 1962). After preliminary studies with emphasis on Burkitt's lymphoma (Pulvertaft, 1964, 1965; Osunkoya, 1966) the techniques of phase contrast cytology and tissue culture of suitable specimens were adopted by the department of Pathology, University College Hospital, Ibadan as routine diagnostic investigation of patients admitted to the hospital with a provisional diagnosis of cancer. This report is a retrospective review of such laboratory practice during the period 1965-1970. The report does not include exfoliative cytology procedures.

## Materials and methods

All the materials studied were specimens obtained from in-patients of the University College Hospital, Ibadan, during the period September 1965 to December 1970 inclusive. Preference was given to specimens taken from patients suspected clinically of suffering from malignant neoplastic diseases, especially childhood tumours, soft tissue tumours and lymphomas. Table 1 lists the types of materials studied. These comprise biopsies of solid tumours and lymph nodes, peritoneal, pleural, bone marrow and splenic aspirates, as well as cerebro-spinal fluid obtained usually by lumbar puncture and occasionally by cisterual puncture. In all, 1643 samples were studied by the author in the Pathology department of the hospital (Table 1).

All specimens were obtained by aseptic techniques and collected into sterile containers. Solid tumour biopsies and cerebrospinal fluids



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**Table 1.** Incidence of malignant cells in specimens processed for cytology and tissue culture University College Hospital, Ibadan (1965–1970)

| Type of specimen | No. of specimens studied | No. and % positive for malignant cells | No. equivocal for malignant cells | No. negative for malignant cells | No. not reported |
|---|---|---|---|---|---|
| Tumour biopsy | 381 | 185(48.6%) | 13 | 49 | 134 |
| Tumour aspirate | 220 | 98(44.5%) | 6 | 75 | 41 |
| Lymph node biopsy | 124 | 41(33.0%) | 2 | 31 | 50 |
| Bone marrow aspirate | 26 | 2 (7.7%) | 1 | 15 | 8 |
| Ascitic fluid | 541 | 175(32.3%) | 13 | 283 | 70 |
| CSF | 209 | 56(26.8%) | 2 | 135 | 16 |
| Pleural fluid | 130 | 22(16.9%) | 4 | 85 | 19 |
| Pericardial fluid | 12 | 1 (8.3%) | 0 | 10 | 1 |
| Total | 1643 | 580(35.3%) | 41 | 683 | 339 |

were collected into dry universal bottles. Aspirates (5–20 ml) from serous cavities were collected into heparinized bottles, while fine needle biopsies from spleen and bone marrow aspirates were collected in universal containers containing 5 ml heparinized tissue culture medium (TC 199). The final concentration of heparin in samples ranged from 5–20 units/ml.

Processing of specimens for microscopic examination and tissue culture was as a rule undertaken within 30 min of collection. In a few instances it was not possible to do this, and specimens, particularly fluid samples were found to give good results even after standing at 4°C for 1–2 h. Aseptic precautions were taken throughout the period of preparation of materials and processing of specimens.

*Culture Medium*

The basal medium used throughout these studies was TC 199. It was found necessary to augment the antibiotic content of the medium with neomycin (30 units/ml) and mycostatin (50 units/ml). TC 199 was used as the suspension medium for dispersal, washing and phase microscopy of cells.

The definitive culture medium (in which all cultures were set up and maintained) was a 25–30% concentration of human serum in TC 199 supplemented with 0.4% chick embryo extract (Difco).

Human serum was obtained from blood bank donors at UCH, Ibadan. About 20 ml blood was collected from each donor, and serum separated by centrifugation within 36 h of collection. The serum samples were pooled in pairs usually as 20 ml aliquots, and were deep frozen until use, after addition of neomycin and mycostatin, and inoculation into nutrient broth. Each sample was judged sterile and safe for use if there was no growth in the nutrient broth culture after 48 h.

*Processing of solid tissues*

Biopsies of solid tissues were divided into four portions for

(i) routine diagnostic histology,
(ii) impression (touch) smear cytology,
(iii) phase contrast cytology and tissue culture and
(iv) other studies such as stained-smear cytology, histochemistry, electron microscopy, animal innoculation, etc.

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*Phase contrast cytology and culture*

The portion for phase cytology and culture was placed in a 2–5 ml pool pf TC 199 in a petri dish, and the cells dispersed into the medium by gentle squeezing and teasing of the biopsy tissue between a pair of forceps and scalpel. With most cellular tissues, cells were released into the medium in numbers adequate for microscopic examination and culture. Occasionally, it was necessary to expose small fragments of the biopsy to 0.25% trypsin in TC 199, at 37°C for 1–2 h to effect a reasonable cell-dispersal.

A drop of the cell suspension was placed on a glass slide, covered with a coverslip and examined under a phase contrast microscopic, to obtain a quick general assessment of the biopsy material. For more detailed cytological studies, the cell suspension was transferred into a centrifuge tube, and allowed to stand for about 2 min, during which time large tissue fragments would sediment to the bottom of the tube. The supernatant was then transferred into another centrifuge tube, and the contained cells spun down at 250 × g for 3 min. A debris-free cell suspension was prepared by washing the cells twice in TC 199 by centrifugation. This permits optimal optical conditions for phase contrast cytology of the preparation. Features such as cell-size, cell-shape, degree of coherence between cells, pattern of cell aggregation, identification of different cell types, nuclear and cytoplasmic characteristics were readily observed.

In most cases a diagnosis was readily made at this stage. In difficult cases, or when desired, a prolonged observation was further made on the cells. More information such as longevity in TC 199, capacity for and type of motility, type of pseudopodal activity, ability to spread and adhere to glass may then become evident. For this purpose, the cover slip preparation was sealed with paraffin and microscopy continued.

The remaining cells in suspension were centrifuged, and portions of the cell deposit seeded into tissue culture chambers.

*Processing of fluid specimens*

Ascitic, pleural, pericardial and cerebrospinal fluids were simply centrifuged, the cell deposit resuspended in TC 199, and a drop of the cell suspension examined microscopically as sealed coverslip preparation. If the specimen is adequate in cells, cultures were set up as for solid tissue biopsies.

Bone marrow samples were handled in a different manner. The suspension of marrow aspirate in TC 199 was decanted into a Petri-dish, and marrow fragments (which appear as pale yellowish-white specks) were picked up with a Pasteur pipette and inoculated on agar slides, or examinated as sealed coverslip preparation.

*Stained-smear cytology preparations*

Two types of smear preparations were made from specimens for microscopy, and filing as permanent records. One type is the imprint (or touch) smear preparations made from tumour biopsies according to the method of Wright (1963). The second is the thin smear of washed cell deposits of centrifuged serous effusion specimens or dispersed cells from tumour biopsies. Multiple smears were prepared from each specimen. A slide was fixed in methanol, stained with May-Grunwald-Giemsa and mounted for microscopy; the rest were kept for other studies such as immunofluorescence, enzyme histochemistry etc. All slides were labelled appropriately and stained preparations filed as permanent records for reference and teaching.

*Stained preparations of cultured cells*

Multiple cultures of cell preparations from each specimen were set up in plastic rings mounted on glass slides as described by Osunkoya (1966, 1970), and monitored by phase contrast microscopy. Such monolayer cultures were terminated after periods ranging from 2 to 106 days (Table 3).

The culture slides were generally fixed in methanol and stained by May-Grunwald-Giemsa. When multiple cultures are available as in some cases, other fixatives such as Bouin's fluid or formalin, and stains such as iron haematoxylin and eosin were used for the additional culture slides. All stained slides were examined by microscopy and kept for photomicrography or permanent records.

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## Results

During the period reviewed and as shown in Table 1, a total of 1643 specimens were subjected to phase contrast cytology, microscopy of strained preparations of cell smears, as well as short-term cell cultures. The specimens were of necessity procured and processed by asceptic procedures as outlined in the preceding section. Such specimens were of two categories, (a) biopsies or aspirates of tumour mass, and (b) fluid specimens from serous cavities or cerebrospinal space.

Definite diagnosis of malignancy was made in specimens from 580 (35.3%) cases. Such diagnosis was reported on the day of sampling in most cases, and confirmed later by routine histopathology in biopsy cases. On the whole, phase contrast cytology was found to be most useful as a diagnostic aid for the differential diagnosis of childhood tumours. The four commonest malignant tumours in Nigerian children (Burkitt's lymphoma, retinoblastoma, neuroblastoma and Wilm's nephroblastoma) featured prominently in the study and accounted for 310 cases (Table 2). Burkitt's lymphoma was by far the commonest (250 cases), and the relative frequency ratio of the four childhood tumour was about 20:1:1:0.5 respectively.

The features of freshly dispersed cells from each of the four childhood tumours by phase contrast mircoscopy have been well described (Osunkoya, 1967, 1966, 1970). Briefly, the malignant cells from Burkitt's lymphoma patients appeared as non-coherent, round, monomorphic lymphoid blast cells with little variation in size (10 microns) and few but prominent cytoplasmic lipid granules. A small proportion of the tumour cell population include small lymphocytes and histocytic cells loaded with cellular debris. Retinoblastoma cells were seen as chains of monomorphic cuboidal cells with large round nucleus. Neuroblastoma cells appeared as small groups of tightly coherent large faceted cells with a hemispherical nucleus, abundant cytoplasm and numerous mitochondria. In contrast to these three childhood tumours, Wilm's nephroblastoma cells are pleomorphic cells aggregated as an admixture of round, oval and spindle embryonic cells with varying size and scanty cytoplasm.

Cultures in plastic ring chambers on glass slides were effective in providing cell monolayers for microscopy and other studies. The duration of such cultures are analysed in Table 3 which shows clearly that the technique generally permits maintenance of cultures for a few days up to a few weeks. The culture system selects glass adherent cells from the explant of tumour cells. Such cells were abundant in retinoblastomas, neuroblastomas, malignant melanomas and mucinous adencarcinomas, etc. Burkitt's lymphoma and Wilm's nephrosblastoma also yielded monolayer cultures, obviously from the minority of cell sub-population in the initial tumour biopsy or serous fluid specimen.

Most cultures were harvested or terminated within 2 weeks of set up, by which time diagnostic features were manifested by some malignant tumours. Such features include neurobirils in neuroblastomas, and melanin pigment in malignant melanomas. Although there were a few exceptional cultures that survived longer, the maximum duration of monolayer cultures was generally about 6 weeks.

## Discussion

The value of cytological examination of specimens as aid to the diagnosis of malignant tumours has been well highlighted (Papanicolaou, 1954; Pulvertaft & Humble 1962; Soderstrom, 1966) and is now generally recognized. The advantage of the diagnostic investigation over routine histopathology is the quick and definite information which can be provided on relatively small samples of solid tissue or fluid specimens. During the present study, major exploratory surgery such as laparatomy, thoracotomy or craniotomy became unnecessary after definite diagnosis of malignancy on appropriate specimens from abdominal, thoracic and cerebrospinal cavities respectively.

The disadvantage of loss of topography and need for special apparatus, experience and skill which the technique involves are far outweighed by the wealth of information which may be gathered. Such information includes most criteria used in orthodox histology and cytology such as nuclear and cellular configuration and size, nuclear-cytoplasmic ratio, presence and prominence of nucleoli, presence or

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**Table 2.** Nature of specimens positive for malignant cells of 310 childhood tumours University College Hospital, Ibadan, (1965–1970)

| Type of specimen | Total No. positive for malignant cells* | No. Positive[1] | | | |
|---|---|---|---|---|---|
| | | Burkitt's lymphoma | Retino-blastoma | Neuro-blastoma | Nephro-blastoma (Wilm's tumour) |
| Tumour biopsy | 185 | 85 | 14 | 4 | 4 |
| Tumour aspirate | 98 | 55 | 3 | 5 | 3 |
| Lymph node biopsy | 41 | 3 | 0 | 1 | 1 |
| Bone marrow aspirate | 2 | 2 | 0 | 0 | 0 |
| Ascitic fluid | 141 | 41 | 1 | 1 | 1 |
| Peritoneal aspirate | 34 | 21 | 0 | 2 | 4 |
| Cerebrospinal fluid | 56 | 38 | 8 | 8 | 0 |
| Pleaural fluid | 12 | 5 | 0 | 0 | 0 |
| Pleaural aspirate | 10 | 0 | 0 | 0 | 0 |
| Pericardial fluid | 1 | 0 | 0 | 0 | 0 |
| Total | 580 | 250 | 26 | 21 | 13 |

*All age groups.
[1]Childhood tumours (1–15 years).

**Table 3.** Number of short-term cell cultures categorized by duration of culture University College Hospital, Ibadan (1965–1970)

| Duration of cultures (days) | No. of cultures |
|---|---|
| 1–5 | 103 |
| 6–10 | 122 |
| 11–20 | 154 |
| 21–30 | 77 |
| 31–40 | 25 |
| 41–50 | 7 |
| 51–60 | 3 |
| 61–70 | 1 |
| 71–80 | 3 |
| 81–90 | 0 |
| 91–100 | 0 |
| 101–110 | 1 |
| Total | 496 |

absence of intracytoplasmic foreign debris and granules, etc. In addition, readily observed properties of viable cells such as presence and type of motility, capacity for phagocytosis, and degree of cell adhesion to glass or other surfaces can be observed readily. Sub-cellular structures such as mitochondria and cytoplasmic granules can also be observed.

Furthermore, the degree of cohesion cells, and the pattern of aggregation formed by coherent cells are readily observed. Lastly, when the cells under study are allowed to remain in a standard *in vitro* environment, their longevity in such environment, their pattern and rate of growth, the degree of stability of their morphology, and the type of cell death manifested (karyolysis, cytolysis, cytoplasmic sequestration, nuclear vesciculation etc.) can be observed.

During the studies reported here, phase contrast cytology was particularly useful not only in the definitive diagnosis of childhood

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tumours, but also in excluding other tumours with similar clinical features on presentation. The microscopic appearance of such tumour cells and the pattern of cohesion between freshly dispersed cells are so characteristic that they make differential diagnosis easy. The only additional special equipment needed in a department of hispathology for this laboratory test to be included in routine service is a light microscope with phase contrast accessories. The hospital service which is thereby provided is analogous to the prompt diagnostic investigation by cryostat (frozen) section of fresh biopsies.

Laboratory diagnosis of malignant neoplastic diseases is of course mandatory before therapy is commenced, for ethical reasons and record purposes. In this regard, photomicrography provides good records of phase contrast cytology observations but is of limited routine application in practice. However, cell smears prepared from biopsies or fluid specimens can be filed as permanent records for future reference and teaching, when such smears are stained and mounted. The two types of cytology techniques (phase contrast cytology and stained smear cytology) are indeed complimentary and should be available for diagnostic service and professional training purposes especially in teaching hospitals of this and similar developing countries.

As mentioned in the introductory section, exfoliative cytology is excluded from this report. This is because in certain patients the technique and anatomical site of procurement of specimens for microscopy in such cases do not permit asepsis. In particular, specimens of urine, gastric aspirates and bronchial washings are unsuitable for tissue culture and are therefore not included in this report, although some were positive for malignant cells.

Specimens obtained by aseptic techniques provided the opportunity to supplement cytology examination with cell culture procedures, provided the respective specimen is adequate in quantity. Initially, the short term cultures provided good material for morphological studies on the cell types from the tumour explant that survive in the *in vitro* environment provided by the culture medium. However, tumour cells from a small proportion of Burkitt's lymphoma patients (Osunkoya & Mottram, 1967) and retinoblastoma patients were established as continuous cultures and propagated for several months or years. Such continuous cell lines can be maintained indefinitely or cryopreserved (Osunkoya, 1965). They are also of value in providing readily available human cells for diverse studies in cell biology, cytogenetics, membrane biochemistry, *in vitro* propagation of some protozoal parasite and viruses, molecular biology etc.

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