STUDIES ON THE BURKITT AND LYMPHORETICULAR TUMOURS IN NIGERIA

by

B. O. OSUNKOYA. M.B., B.S. (Lond).

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

of

The University of Ibadan

December, 1966.

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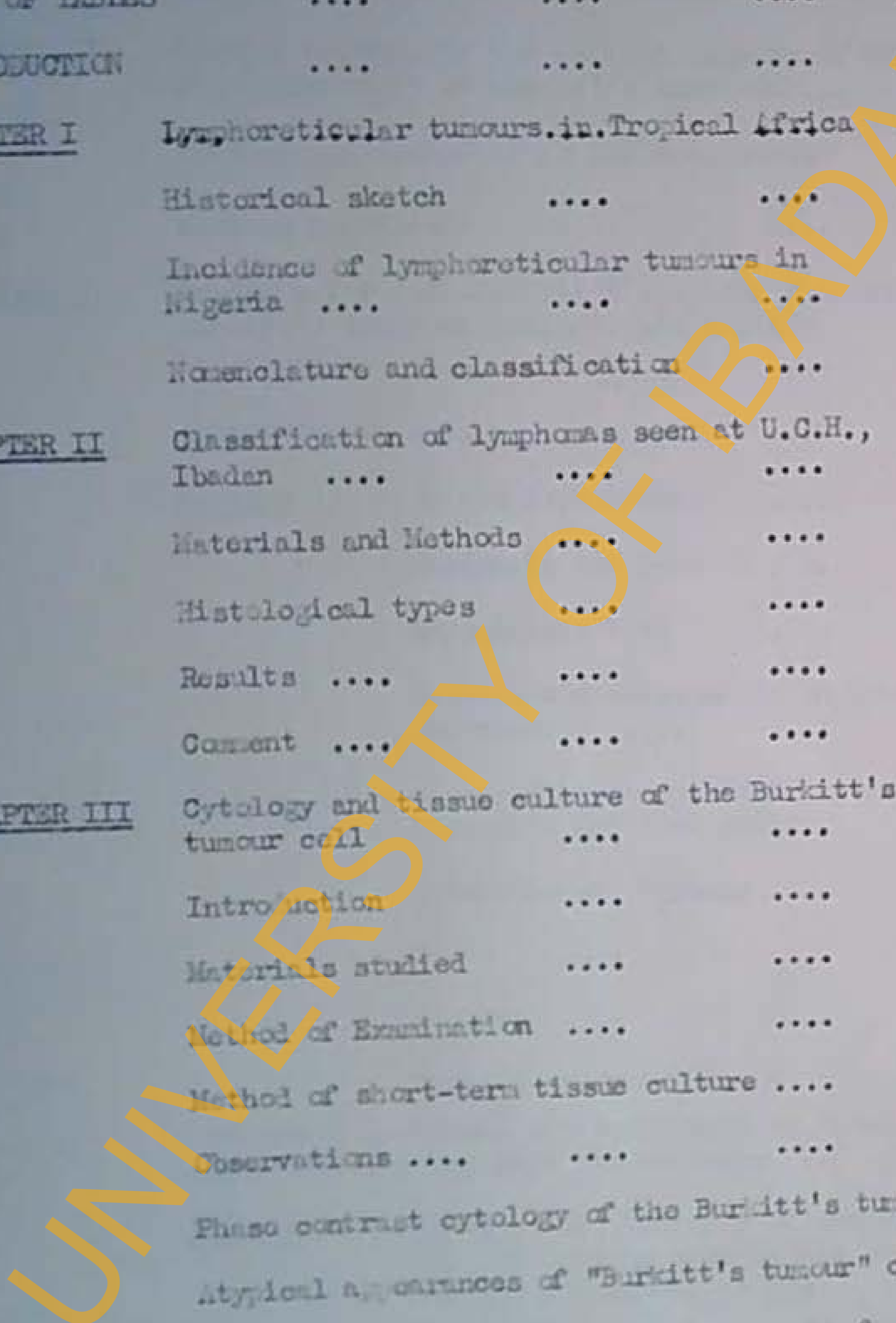

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SUMMARY.

Burkitt's tumour exemplifies the popular belief that lymphoreticular tumours are relatively more common in Africa than elsewhere. At the time the present studies were contemplated, it's exact histogenesis was controversial, it's aetiology and pathogenesis were unknown. It was however clear as a result of the pioneer work of Dr. Denis Burkitt, that the tumour is predominantly a childhood cancer with remarkable predilection for the jaws, and preponderate in wet tropical Africa.

Morphological studies were therefore undertaken using histological and phase contrast criteria, to characterise the tumour and classify it within the framework of modern nomenclature for lymphoreticular tumours and against the background of other solid lymphoreticular and childhood tumours seen at U.C.H.Ibadan.

From a histological review of biopsies of lymphoreticular tumours seen in the Department of Pathology, U.C.H., Ibadan, it became evident that a histological diagnosis of Burkitt's tumour was possible in most, but certainly not all cases. A uniform histological cell-type is the rule, but the characteristic "starry-sky" pattern is not by itself unique to the tumour. A "starry-sky" appearance produced by closely packed, hyperchromatic, monomorphic blast cells, interspersed by large, pale, well-differentiated histiocytes is considered pathognomonic; the appearance is shared only by a hyperplastic lymphoid germinal centre.

The blast cells of Burkitt's tumour were characterised as lymphoblasts and distinguished from the cells of other round cell sarcomas of childhood by phase contrast cytology. The close similarity

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of the cells to phytohaemagglutinin transformed lymphocytes already observed by Pulvertaft (1964) and Wright (1966a) was confirmed. It was however, also noted that there is close resemblance between the cells and certain blast cells seen in preparations from reactive lymph nodes, and presumed to be blast cells from hyperplastic lymphoid germinal centres.

It is submitted that the lymphoblasts of Burkitt's tumour can be distinguished by phase contrast cytology from those of conventional childhood lymphosarcoma. This finding establishes the occurrence of both types of solid lymphoblastic neoplasias of childhood in Nigeria, and is in support of the contention of Burkitt and Wright . . . [illegible] (Burkitt and Wright 1966, Wright 1966b) that the two are distinct entities. The title "Burkitt's lymphoma" is in consequence adopted, to emphasise the lymphomatous origin of the tumour and to distinguish it from conventional childhood lymphosarcoma.

Eight lymphoblast cell lines (OB1, OB2, OB3, OB4, OB5, OB6, OB7, and MOB8) established from Burkitt's lymphoma provided readily available tumour material for use in experimental studies of the immunopathology of the tumour.

Immunofluorescent and in vitro cytotoxic tests as well as growth inhibition of Burkitt's lymphoma cells were the parameters used to provide evidence for the conclusion that Burkitt's lymphoma patients (untreated and in remission), as well as individuals with no history of the disease, do possess antibodies to antigens in Burkitt's lymphoma. Significant differences in the incidence of positive individuals in the various groups tested led to the conclusion that the antibody was against a ubiquitous infective antigen to which Nigerians (and presumably individuals in other areas where the tumour is 'endemic') are

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readily exposed, more so than Americans.

The conclusions support the virus-induced hypothesis of Davies, (1962), Burkitt (1962b) and Stanley (1966) for Burkitt's lymphoma. Speculative submissions are made on the pathogenesis of Burkitt's lymphoma. Finally, capacity of Burkitt's lymphoma cells for immunoglobulin synthesis and secretion, demonstrated in preliminary experiments in collaboration with the Burkitt's lymphoma Study Group at U.C.H. Ibadan, confirms the suspicion of existence of host-defence mechanisms, raises hope of immunotherapy, and encourages future search for specific infective antigens related to the aetiology of the tumour.

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I was priviledged to benefit by the advice, stimulating discussions, and cooperation of the members of the Burkitt's lymphoma study group, U. C. H., Ibadan, led by Prof. V. A. Ngu (Professor of Surgery, University of Ibadan).

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INTRODUCTION

Lymphoreticular tissues comprise two distinct classes of cells, the prototype of which are the histiocyte and the lymphocyte. The two cell series are aggregated together to form organs such as the spleen, thymus and lymph nodes, or specialised tissues in the bone marrow and submucosa of respiratory and gastro-intestinal tracts. Both cell series are present as free non-coherent cells in the blood. Cells of the lymphocyte series are sparesely scattered throughout the connective tissues of the body, while histiocytic cells are present unassociated with lymphocytes in the sinusoids of the liver, alveolar walls of the lungs, and serous lining of body cavities.

The histiocyte typifies cells classed into a functional system - the reticulo-endothelial system of Aschoff - characterised by capacity for phagocytosis of carbon, metallic and colloid particles as well as living organic matter. Macrophage, monocyte, reticulum ceell, littoral cell, and Kuppfer cells are but a few of the names given to cells of this system, all of which are descendants of the primitive mesenchymal "stem cell".

The lymphocyte is the chief cell-type of the lymphoid series of cells. This series include lymphocytes of various sizes, plasma cells, and poorly differentiated blast cells (lymphoblasts). The lymphoid blast cell is closely related to, and sometimes indistinguishable morphologically from the haemocytoblast of haemopoietic tissues or the mesenchymal stem cell of the reticuloendothelial system.

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These two series of cells constitute together a biological system the main function of which is the protection of the entire animal from the effects of alien, inanimate or living matter which may gain access into the body. This is achieved through the manifestation of an immune response, which essentially is a chain of reactions culminating in the production of humoral or cell-bound substances capable or reacting specifically with the provoking alien material.

The response by the lymphoreticular system to the presence of alien molecules or matter involves, at some stage, a proliferative activity of one or both of the cell classes mentioned above. This normal immunoproliferative activity of lymphoreticular tissues may, like all other biological processes become disordered and thereby manifest as disease, quite apart from, and in spite of the disease which may be provoked by the alien substance. Neoplastic forms of immuno-proliferative disorders constitute quite a large proportion of the derangements of normal lymphoreticular function which manifest as disease. The immunoneoplastic diseases include solid tumours of lymphoreticular tissues and some forms of leukaemias. It is with the former that this thesis is primarily concerned.

It is generally agreed that lymphoreticular tumours are common in Africa, and it was the conviction that "investigators have been struck by the fact that the neoplasia belonging to this group seem to be observed more frequently in Africa than elsewhere" (Roulet, 1964), which prompted a symposium on lymphoreticular tumours in Africa, held in Paris in 1963, under the auspices of the International Union Against Cancer. It is also known that there is widespread

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infections and infestations in Africa. The relatively high incidence in tropical Africa of solid malignant tumours of lymphoreticular origin may therefore be due to the probable "infective" nature of aetiological factors which produce or predispose to the production of such tumours. The normal immuno-hyperplastic reaction of lymphoreticular tissues to infection might predispose such tissues to neoplastic proliferation or increase the likelihood of manifestations of aberrant response to the effects of common infective agents.

In the first two chapters of this thesis are presented data which suggest an unduly high incidence of lymphoreticular tumours in Nigeria, a finding which confirms previous impressions. Lymphosarcomas, particularly a peculiar type seen almost exclusively in children and known as Burkitt's tumour, contributes largely to this overall impression. Burkitt's tumour was therefore regarded as the prototype for experimental search for evidences which may shed light on the role which environmental factors might play in the pathogenesis of this class of tumours. Intensive research on Burkitt's tumour is presently being carried out in many institutions all over the world because of the unique opportunities which this tumour offers for elucidation of the aetiogenesis of a human cancer believed to be precipitated by a natural environmental agent.

Despite its undoubted high incidence in tropical Africa, Burkitt's tumour was only recently recognised as a clinicopathological entity, and although knowledge about the tumour is advancing at a remarkable pace, many aspects such as histogenesis of the tumour are still controversial.

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A prerequisite to the full understanding of the aetiology, pathogenesis, epidemiology and cure of a disease entity is its characterisation in terms of clinical features, morbid anatomical peculiarities, as well as the development of diagnostic laboratory tests. Hence before embarking on purely experimental work on Burkitt's tumour, much time was spent in the study of the morphology of the tumour with the aim of characterising the tumour cell and thereby elucidating its histogenesis. Characterisation of the tumour cell would also facilitate both the recognition and diagnosis of the tumour as well as its distinction from other tumours (such as other round cell sarcomas of childhood) which may mimick it.

The morphological studies are embodied in chapters 2 and 3, at the end of which it was thought justifiable to adopt a more precise terminology for the tumour in place of the currently used non-committal eponymous title of Burkitt's tumour. During the morphological studies it became evident that Burkitt's tumour is not just a tumour, but a lymphoma and that the lymphomatous tumefaction is composed largely of primitive cells of the lymphocytic series which closely resemble the pyroninophilic blast cells of hyperplastic lymphoid germinal centres, or in vitro phytohaemagglutinin transformed lymphocytes.

The successful propagation of Burkitt's tumour cells in continuous long-term cell cultures offered unique opportunities for large scale experimental studies on the tumour. With such readily

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available "in vitro tumour material" it was possible to verify experimentally a hypothesis concerning the aetiogenesis and age-distribution of Burkitt's tumour. In these studies, immunological methods were combined with observations on tissue cultural behavior of the Burkitt's tumour blast cell.

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Chapter 1.

LYMPHORETICULAR TUMOURS IN TROPICAL AFRICA

1. Historical Sketch.

All the contemporary reports on the incidence and pattern of cancer in tropical Africa have emphasised the relatively high frequency of malignant tumours of lymphoreticular tissue (Davies, 1949, 1963; Davies and Wilson, 1954; Edington, 1956; Thys, 1957; Edington and Maclean, 1963, 1965). During the first quarter of this century, it was widely believed that neoplastic disease was rare or unknown in Africa, and that indigenous as well as immigrant inhabitants of Africa were somehow protected against cancer. These beliefs were of course unfounded because even during this period, a few physicians were able to recognise cancer through the thick and distracting screen of overwhelmingly prevalent infective, parasitic and nutritional diseases.

Though compiled at the end of the nineteenth century and during the first few decades of this century, the meticulously documented clinical notes of Dr. (now Sir)Albert Cook at Mengo Hospital in Uganda were not deficient in clinically diagnosed malignant tumours (Davies et al, 1964).

In 1922, Macfie gave a brief and unpretentious account of prevalent diseases in the Gold Coast. His impressions are largely and remarkably true of the disease pattern in most tropical African countries even today. He considered that tumours were "probably as ..

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common as elsewhere", and was impressed by the high incidence of "sarcomas".

It was however not until Smith and Elmes' publication in 1934 of their analysis and histological classification of 500 malignant tumour biopsies seen at the Medical Research Institute in Lagos, that the not infrequent occurence of cancer in Africa was firmly established. Vint (1935), reporting in the following year from Nairobi, confirmed this fact, and found a remarkably close similarity in the pattern of tumours seen in Kenya and Nigeria. He pointed out the strikingly high sarcoma/carcinoma ratio (2 : 3), a situation due to the high incidence of Kaposi's sarcoma, melanosarcomas, and tumours of probable lymphoreticular origin. The later group was classified as round-celled mixed-celled or myeloid sarcomas.

Attention was in subsequent years focussed on the then obvious fact that malignant neoplastic diseases have a different type-incidence in Africa. Analysing the records of Mengo Hospital in Uganda, Davies and colleagues (1964) showed that the pattern of cancer, as regards site and type, remained constant over a period of 60 years which extended as far back as the end of the last century.

Certain types of tumours were apparently seen more frequently than in Europe and America. Cutaneous squamous carcinoma, hepatoma and cervical carcinomas top the list in reports concerning malignant tumours of epithelial origin. Malignant melanoma and Kaposi's sarcoma were strikingly common. As a group, lymphoreticular tumours were however by far the commonest type of non-epithelial tumours seen (Camain and Lambert, 1963) and in Nigeria, certainly by far the commonest

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of all tumours (Edington and Maclean, 1965). The figures compiled by various authors from West, Central and East Africa, for the relative incidence of lymphoreticular tumours range from 8 to 23% of all malignant diseases seen in these areas (Table 1).

The problem of unavailable or at best unreliable and misleading vital statistics in tropical African countries has been raised by various authors in justifying the lack of cancer incidence rates for comparison with figures from more developed countries (Higginson, 1953; Davies and Wilson, 1954; Edington, 1956). Davies and Wilson (1954) aptly remarked that we need not wait for the full establishment of facilities for accurate recording of vital statistics before studying the cancer incidence or the effect of environment on carcinogenesis, because "we can be quite certain that this will only have come about by major changes in social system and structure that cannot have failed to influence the cancer situation". Almost all cancer surveys in Africa to date have been retrospective in approach. Nevertheless, these reports provide useful information, and after due consideration is given to the pitfalls inherent in compiling such incidence rates, it is still possible to claim that lymphoreticular tumours have quite a high incidence rate in Africa.

Cancer incidence figures emerging from the recently established cancer registries in Kampala and Ibadan, though crude statistically, confirm previous impressions, the relative frequency of lymphoreticular tumours shown to be actually higher than previously thought. The later situation arose from the recognition of the round-cell sarcoma of the jaws in children living in tropical Africa as a clinicopathological entity (Burkitt 1958; Burkitt and O'Conor, 1961) and its classification

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as a lymphoma (O'Conor and Davies, 1960).

Apart from the apparently relatively high incidence of malignant neoplastic diseases of lympho-haemopoietic tissues in Africa, the pattern of the types of tumours within this class differs from those found elsewhere. Thus leukaemia, particularly the acute lymphoblastic type is rare in children (Trowell and Jellife, 1958; O'Conor and Davies, 1960; Davies, 1965). Follicular lymphoma is uncommon (Oettle, 1963) while lymphomas of the Hodgkin's type occur more frequently in lower age-groups (Canain and Lambert, 1963; Edington and Maclean, 1963). The multicentric round cell sarcoma of childhood is quite common, accounting for more than half the total number of malignant tumours seen in children (O'Conor and Davies, 1960; Edington and Maclean, 1964).

2. Incidence of Lymphoreticular tumours in Nigeria.

Recently, a cancer rate survey covering the 3-year period 1960-1963 was carried out by Edington and Maclean (1965) at the Cancer Registry, U.C.H., Ibadan, An analysis of the 1920 malignant tumours registered within this period revealed that solid lymphoreticular tumours, excluding myelomas, accounted for one fifth (19.9%) of all malignant tumours registered.

Burkitt's tumour alone accounted for as much as 44% of all solid lymphoreticular tumours, and 8.8% of all malignant neoplasms registered. As an entity, it was second only to carcinoma of the cervix (9.4%) in relative ratio frequency, and was by far the commonest cancer

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Of the 105 malignant tumours seen in children below the age of 15 years, 51 (48.5%) were diagnosed as Burkitt's tumour. This is in marked contrast to the leukaemias, which together accounted for 8.6% of all childhood cancers seen.

As part of the preliminary studies to compilation of experimental data for this thesis, a study was made on the relative incidence of malignant tumours diagnosed histologically as lymphoreticular in origin in the Department of Pathology, University College Hospital, Ibadan, during the 6-year period July 1960 to June 1966. An analysis of figures obtained from the files of the Cancer Registry, on common malignant tumours registered during this period is shown in Table 2. There is close similarity to the results obtained by Edington and Maclean (1965).

In all, 4,570 malignant tumours were registered out of which 950 were solid tumours of lymphoreticular origin, including 30 myelomas. There were 183 cases of leukaemia.

As a group, the lymphoreticular tumours accounted for about one fifth (20.7%) of all malignant tumours accepted by the Cancer Registry. This group of neoplasms was seen as frequently as all the three commonest types of carcinomas - Cervix, Liver, and Breast - combined.

Of the 950 lymphoreticular neoplasms, 600 were diagnosed histologically as lymphosarcomas, (lymphoreticular tumours attributed to proliferation of cells of the lymphocytic series) accounting for as much as 13.1% of all malignant tumours.

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3. Nomenclature and Classification

The recognition of neoplastic proliferation of lympho-haemopoietic tissues, and their distinction from malignant tumours of other tissues is attributed to Rudolf Virchow who, a hundred years ago, grouped this class of malignant diseases under the generic name of "lymphosarcoma". Ever since, there has been increasing controversy over criteria for segregation of these diseases into recognisable clinico-pathological entities. The situation was further complicated by the confusion arising from the adoption of widely different terminology by individual investigators and schools in describing the proliferating cells involved.

Lymphosarcoma was coined by Virchow in 1864 to include malignant proliferations of lymph nodes and spleen, as well as leukaemia which he had described two decades earlier (Virchow, 1847). Already, Hodgkin (1832) and later Wilks (1856) had described a number of cases illustrating a fatal clinical syndrome the main features of which were progressive lymph node enlargement, and splenomegaly accompanied by anaemia. Such cases Wilks called Hodgkin's disease, and these were also considered by Virchow as belonging to the lymphosarcoma group of tumours.

Kundrat (1893) was credited with establishing the segregation of the malignant tumours of lymph nodes from the leukaemias. The former was regarded as focally invasive solid tumours lacking the hallmark of leukaemia, which is a diffuse proliferative bone marrow changes.

The first three decades of this century witnessed the controversy on the granulomatous versus neoplastic histogenesis of Hodgkin's disease. Consequent on increasing histopathological studies, and particularly as a result of the works of Coley (1908), Mallory (1914) and Callender (1934), it became clear that there were several distinco-

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pathological syndromes, including Hodgkin's disease, which fulfil the criteria for inclusion in Virchow's "lymphosarcoma". During this period, a few distinct groups of lymphoid tumours were described. Brill, Baehr and Rosenthal (1925) described a relatively benign form of lymphoid neoplasia, noting particularly the follicular pattern of the lesions. Roulet in 1930 and 1932 introduced the term "retothelsarcome" in describing yet another group of lymphoreticular neoplasms in which the cells were primitive reticular cells (see Klemperer, 1934)

It became necessary to find an appropriate terminology to embrace these apparently different types of primary tumours of lymphoid tissue. Lymphosarcoma suggested by Virchow was no longer adequate and its connotation has since suffered many changes. Presently it is being used more specifically to cover solid tumours produced by cells of the lymphocytic series. "Reticulosis" was first suggested by Letterer (1924) and echoed by Pullinger (1932). Robb-Smith (1938) retained this title for what he regarded as benign lymphoid tumours, and preferred 'Reticulosarcoma' for the malignant ones. The term 'malignant lymphoma' was originally proposed by Billroth (1871), but it was not until Gall and Mallory's publication on their clinicopathological studies of over 600 cases of lymphoreticular tumours that its present wide usage by American and British pathologists became established. The French schools led by Mathe, Benhard and Camain prefer the title of "haematosarcomas". Contemporary international opinion is however more liberal, and allowed room for flexibility by grouping these tumours together as "tumours of of haemopoietic and lymphoid tissues" (UICC illustrated tumour nomenclature, 1965).

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The phylogeny, ontogeny and biological functions of lymphoid tissue are different from those of the other main types of tissues, such as epithelial, connective and neural tissue. So is the biological behavior of their neoplastic lesions. It would appear therefore that all that is required is a generic label which is distinctive and at the same time compares in simplicity, specificity, and connotation with those already established and generally accepted for tumours of the other main types of tissues. The term which seems to fulfil all these criteria is "lymphoma". The future adoption of "lymphoma" as the generic terminology covering all solid malignant tumours of lymphoid tissues, and its usage in the same sense as carcinoma and sarcoma in tumour pathology, seems almost inevitable. Already, the trend in English speaking countries is towards this concept.

Several attempts have been made by various workers to envolve a satisfactory and generally acceptable scheme of classification of the lymphomas into clinico-pathological types. Gross anatomical, cytological and clinical criteria were used in various combinations and with different degrees of emphasis by some investigators (Callender, 1934; Robb-Smith, 1938). Others, (Lumb, 1954)based their classification mainly on Maximow's concept of the multipotent primitive mesenchyme cell, a concept which was amplified by Pullinger (1932) in discussing the histogenesis of lymphoid tumours.

In 1942, Gall and Mallory presented for the first time a scheme of classification based exclusively on cytological grounds. They

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argued that the cytologic classification finds validity in the fact that classification in tumour pathology is fundamentally cytologic, and by and large, there is correlation between the cytological appearance of a tumour and its biological and clinical behavior. In addition, they concluded from comparative studies on histological appearances of sequential biopsies that "cytologic types are remarkably constant", a point of view not shared by Custer and Bernhard (1948).

Cytological classification of the lymphomas is now generally accepted despite the limitations of histology in elucidating the true nature of poorly differentiated cell-types. This difficulty is recently being solved by the use of ancillary methods such as histochemistry, ultrastructural studies, and simple cytological techniques, in combination with orthrodox histopathology. The perennial problem of the adoption in different laboratories of widely different names for the same cell-type however remains.

The contemporary classification favoured and adopted (with some modifications) by the writer is that suggested by Rappaport (1963). This scheme of classification is based on cytological grounds, and embraces, as first suggested by Rappaport et al (1956), the two architectural patterns seen histologically in this class of tumours - the diffuse and nodular (follicular) pattern. (Table 3).

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Table 1. Review of published Cancer surveys in Tropical Africa showing relative frequency of lymphoreticular tumours.

Author and Year		Country	Period of survey	Type of material analysed	Total cancers analysed	Lymphoreticular tumours*	
						Total	% of all cancers
...ith and Elmes,	1934	Nigeria	1926-34	Biopsy	500	N.S	? 23.0
...t,	1935	Kenya	N.S	?Biopsy	546	N.S.	? 23.0
...es and Baldwin,	1947	Nigeria	1935-44	Biopsy	1,000	130	13.0
...ies,	1949	Uganda	1931-49	Biopsy and Autopsy	1,309	217	16.6
...ies and Wilson,	1954	Uganda	1952-53	Biopsy and Autopsy	796	65	8.2
...ngton,	1956	Gold Coast	1923-55	Biopsy and Autopsy	1,193	117	9.8
...s,	1957	Congo	1939-55	Biopsy and Autopsy	2,529	339	13.4
...ngton & Maclean,	1964	Nigeria	1960-61	Clinical and Biopsy	1,038	176	17.0
...ain and Lambert,	1964	French West Africa	1950-62	Biopsy and Autopsy	7,968	952	12.0
...ngton & Maclean,	1965	Nigeria	1960-63	Biopsy and Autopsy	1,920	382	19.9
...sent Study,		Nigeria	1960-66	Clinical, Biopsy and Autopsy	4,570	950	20.7

* Excluding leukaemias N. S - not stated.

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Table 2.

Common malignant neoplastic diseases registered at the Cancer Registry, Ibadan, during the period July, 1960 to June, 1966.

Type of neoplasm	Number of cases	Relative frequency
Carcinoma of Cervix	385	8.8%
Carcinoma of Liver	356	7.8%
Carcinoma of Breast	245	5.4%
Carcinoma of Stomach	117	2.6%
Leukaemias (all types)	183	4.0%
Lymphosarcoma (including Burkitt tumours)	600	13.1%
Reticulum cell sarcoma	143	3.1%
Hodgkin's disease	162	3.6%
Myeloma	30	0.7%
Unclassified ("Reticulosis")	15	-
Total Solid Lymphoreticular tumours	950	20.7%
Total malignant tumours registered.	4,570	

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Table 3.

Rappaport's classification of tumours of the lympho-haemopoietic tissues (Rappaport, 1963).

Predominating cell-type	TUMOUR (i) with diffuse or follicular pattern. (ii) with or without systemic involvement.
Primitive reticular cell	Malignant lymphoma, reticulum cell type, undifferentiated.
Histiocyte	Malignant lymphoma, reticulum cell type, histiocyte.
	Malignant lymphoma, Hodgkin's type.
Histiocyte and lymphocyte	Malignant lymphoma, mixed cell type (Histiocytic-lymphocytic)
Lymphoblast	-
Poorly differentiated lymphocyte	Malignant lymphoma, lymphocytic type, poorly differentiated.
Differentiated lymphocyte	Malignant lymphoma, lymphocytic type, well differentiated.
Plasma cell	Plasmacytoma
Tissue mast cell	Mastocytoma
Granulocyte	Chloroma.

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CHAPTER 2.

CLASSIFICATION OF LYMPHOMAS SEEN AT U.C.H. IBADAN.

In view of the relatively high incidence of the lymphoreticular tumours in Nigeria, an attempt was made to carry out a histopathological review of the lymphomas seen in Ibadan, with the aim of

(1) classifying them in the light of contemporary nomenclature and concepts, and

(2) determining from the pattern of relative frequencies of the various types, those which contribute largely to the apparently high incidence of these tumours.

MATERIALS AND METHODS

Prior to this study, slides of all lymph node biopsies reported in 1965 as "normal" or showing "non-specific reactive hyperplasia" were studied microscopically, to provide a useful background against which neoplastic lymphoid lesions could be assessed. In all 156 slides were studied. (see Appendix 1 and 2).

Histology slides of biopsy sections reported under the headings: Lymphosarcoma, reticulum cell sarcoma, Hodgkin's disease, reticulosis and myeloma during the 6-year period July 1960 to June 1966 were selected from the files of the Department of Pathology, University College Hospital, Ibadan, for microscopic examination.

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Haematoxylin and eosin stained sections from 528 biopsies were accessible for study. In a few cases additional slides stained for demonstration of reticulin and collagen fibres were also available.

Each slide was examined histologically, and if the appearances were compatible with the diagnosis of lymphoma, an attempt was made to classify it according to the predominating or proliferating cell-type(s). In most cases slides were of such quality as to permit classification without difficulty. In some cases however, sections have been so poorly processed or preserved that assessment of criteria for classification was not possible. Such slides were scored as "not classified". Sections were classified without prior knowledge of clinical data or the source of the respective biopsy material.

Observations on each slide were scored on proforma (Appendix 3), taking into consideration features such as degree of destruction of nodal architecture, pattern of distribution of proliferating cells, predominant cell type, other cell-types present, frequency of mitotic figures, absence or presence of capsular or pericnodal invasion, granulocyte infiltration, karyophagocytosis, fibrosis etc. Classifcation was into one of the 12 histologically distinguishable types listed in Table 5, which is a modification of the scheme recommended by Rappaport (1963a, 1963b)(Table 3).

After the histological review, the clinical data on each biopsy material were studied and duplication of slides from multiple sections or serial biopsies from the same patient noted. The final figures represent adjusted data to eliminate such duplications.

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By far the majority of patients were of Western Nigeria origin, living in Ibadan or referred from outside hospitals. The material studied also included patients or biopsies referred to U.C.H. Ibadan from other regions of the country.

HISTOLOGICAL TYPES

All solid malignant lymphoreticular neoplasms are produced by the proliferation of one or more of the four major classes of lymphoid cells, viz:-

(1) Cells of the lymphocytic series:

Lymphocytes (small medium and large)

lymphoblasts.

(2) Cells of the histiocytic series:

Reticulum cells

Histiocytes

Sinus-lining (Littoral) cells

(3) Undifferentiated reticular cells

(so-called stem cells)

(4) Plasma cells.

Histologic classification of the lymphomas into types is based on the predominating or proliferating cells in the tumour. Almost invariably, other cell types are also present; their presence may be incidental, reactionary or obligatory. It is incidental if the cells represent those normally present at the site of the tumour and have not yet been completely obliterated by the proliferating neoplastic cells.

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Plate 1. Lymphomas of the Histiocytic series

Fig. 1. Undifferentiated reticular cell lymphoma. (Stem cell lymphoma). Note pale staining cells with indistinct outlines due to syncytial pattern. Occasional lymphocytes present. H and E, x 490.

Fig. 2. Undifferentiated reticular cell lymphoma. (Stem cell lymphoma) Pale staining syncytium-forming cells interspersed by a good number of small lymphocytes. H and E. x 490.

Fig. 3. Reticulum cell lymphoma. Sheets of cells characterised by vesicular nucleus and prominent usually single nucleolus. Cell and nuclear outlines distinct. Mitosis common. H and E. x 490

Fig. 4. Fibrolymphocytic lymphoma Haphazard arrangement of ill-defined cells with fusiform or round hyperchromatic nucleus indistinguishable from nuclei of cells of lymphocytic series. Note fibrillar interstitial matrix. Photomicrograph of portion of the same biopsy material as that shown in Fig. 2. H and E. x 490.

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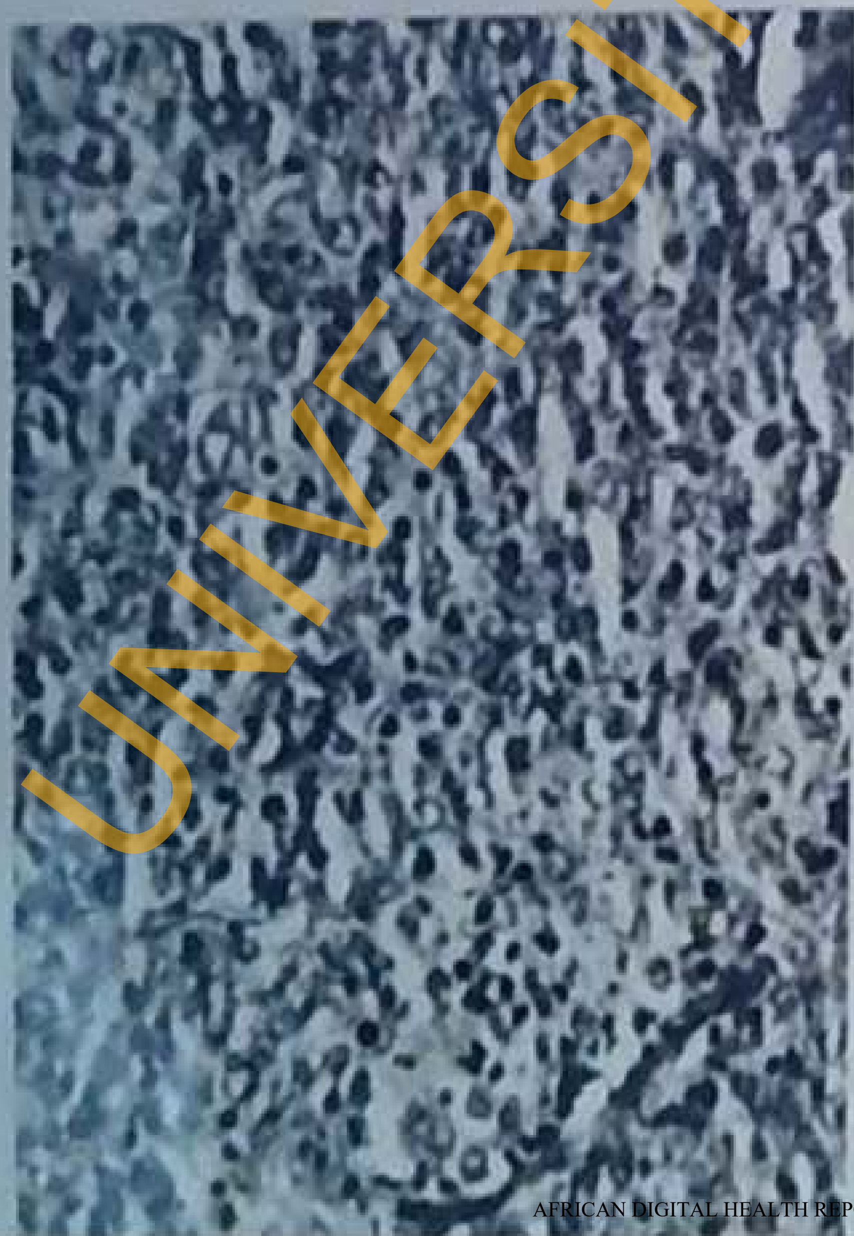

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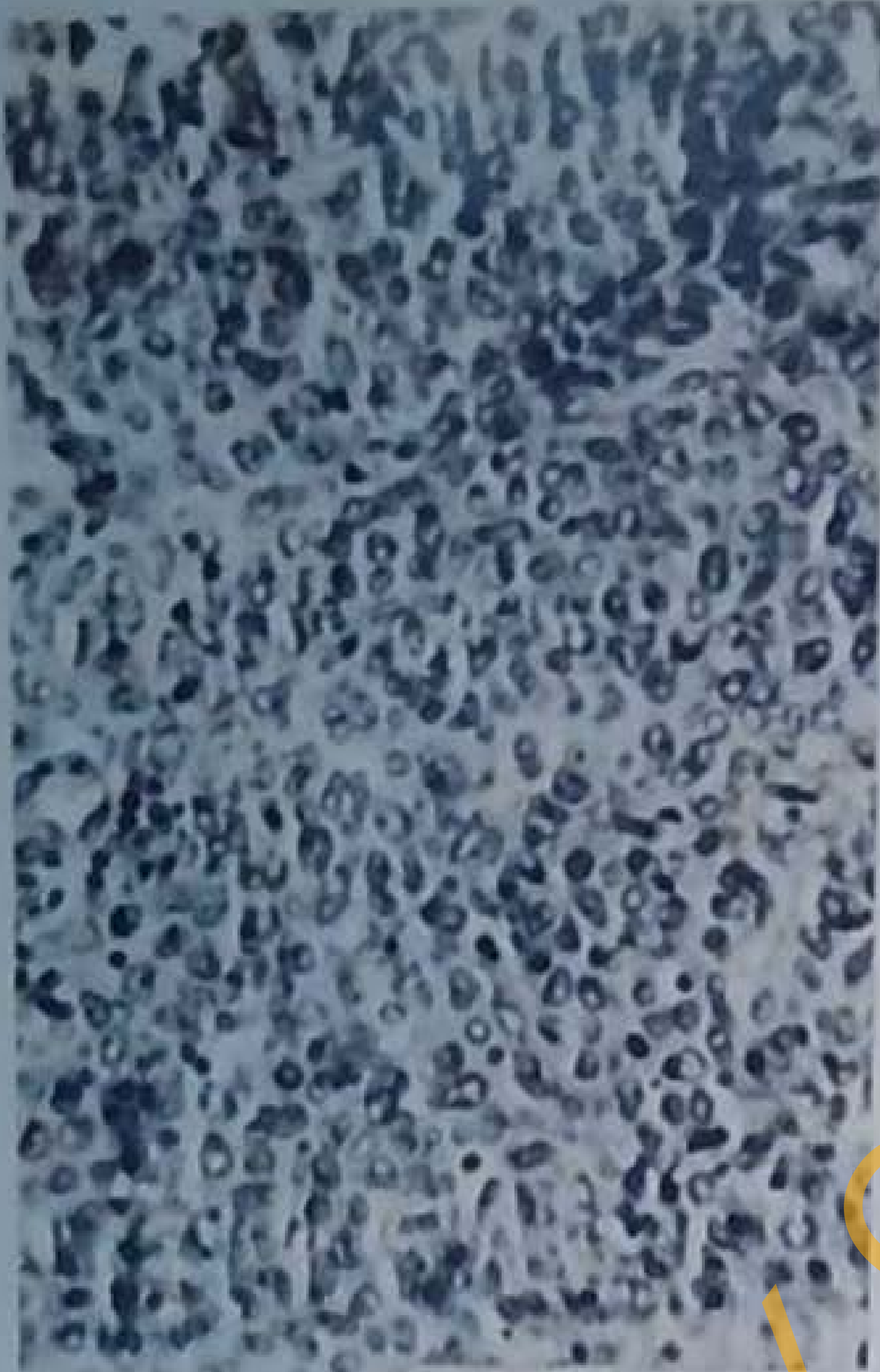

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It is reactionary if such cells are part of a host response to the neoplastic cells, or to the neoplastic stimulus, or both. It is obligatory if the "normal" cells, in some way, provide the oncogenic stimulus, or the necessary milieu for neoplastic proliferation. There is still however the possiblity of differentiation of the proliferating cells into the apparently normal and fewer cell types.

The material reviewed fell into twelve histologically distinguishable types, grouped into five subdivisions.

1. LYMPHOMA OF THE HISTIOCYTIC SERIES

1. Undifferentiated reticular cell lymphoma:

Synonym: Malignant lymphoma, reticulum cell type, undifferentiated. - (Rappaport, 1963b)

Stem cell lymphoma (Gall and Mallory, 1942)

Undifferentiated reticulosarcoma (Robb-Smith, 1938)

The predominating and proliferating cells are characterised by their chromophobic nature and syncytial pattern of growth, thereby resembling primitive or undifferentiated mesenchyme cells in appearance, (Fig. 1). The nucleus is moderately large, being about two to three times the size of a small lymphocyte, and show a remarkably uniform size and shape. It is generally round, though it may assume a slightly ovoid form. The nuclear membrane is thin, and the scanty chromatin is delicately scattered as small particles and short threads with no tendency to margination. Nucleoli are not evident in many nuclei, and when present, they are usually single, small and basophilic.

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The cytoplasm is very pale, abundant, faintly eosinophilic and featureless. Cell outlines are non-existent as a result of confluence of the cytoplasm of contiguous cells. Mitosis is infrequent. Small lymphocytes are always sparsely scattered between the proliferating cells.

Frequently there is gradual transition from areas showing the appearances described above to portions of the same biopsy material in which the nuclei become frankly fusiform in shape while the syncitial cytoplasm assumes a fibrillar pattern. The impression given is that of haphazardly arranged spindle cells. In such areas fairly abundant pale-staining interstitial matrix weakly positive for collagen but rich in reticulin fibres are readily demonstrable.

2. Fibrolymphocytic lymphoma

In some cases, the entire section shows the appearances just described for the atypical areas of some undifferentiated reticular cell lymphomas. The haphazardly arranged fusiform cells of which this type of tumour is composed are readily distinguished from fibroblasts of normal fibrous tissue or those of a fibrosarcoma. The nuclear-size and staining characteristic closely resemble those of small lymphocytes. Although most nuclei appear compressed into a fusiform or oval shape, many are perfectly round, these later being indistinguishable from the nucleus of small lymphocytes. There is hyperchromatism, with closely packed coarse chromatin granules obscuring other nuclear organelles. The overall impression is that of a tumour composed of fusiform cells bearing the nucleus of small lymphocytes. Mitosis in infrequent, there is no evidence of phagocytic activity, collagen fibres are not

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demonstrable, but reticulin fibres are abundantly present. Tumour giant cells are absent.

This histologic type may not be a distinct entity, but rather represents a later stage in the normal evolution of the undifferentiated reticular cell lymphoma. Rappaport (1963b) mentions that stem cell lymphomas may show "early histiocytic differentiation", and cited Ehrlich and Gerber's suggestion (Ehrlich and Gerber, 1935) that the lymphocytes and lymphoblasts frequently seen in stem cell lymphomas are manifestations of differentiation of the stem cells into cells of the lymphocytic series. Fibroblastic or similar differentiation of stem cell lymphomas have not found prominence in the literature. Several photomicrographs showing identical appearances as those described for fibrolymphocytic lymphoma have been published, but they have all been designated with the non-specific term - reticulum cell sarcoma.

It is considered from evidences on histological appearances alone that until more is known about the exact histiogenesis of this type of lymphoma, it is most convinient to regard it not as a distinct entity but rather as "stem cell lymphoma with fibrolymphocytic differentiation".

3. Reticulum cell lymphoma

Synonyms: Malignant lymphoma, reticulum cell type, histiocytic - (Rappaport, 1963b)
Reticulum cell sarcoma (Roulet, 1930; Lumb, 1954)
Clasmatocytic lymphoma (Gall and Mallory, 1942)

Perhaps the lymphoid cell-type with the most controversial definition is the reticulum cell. All are agreed that it belongs to the

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histiocyte-macrophage class of cells. Many believe it is derived from undifferentiated reticular (stem) cells, while others have claimed experimental evidences showing that large lymphocytes can modulate into reticulum cells (Rebuck, 1964). Generally reticulum cells are regarded as relatively primitive histiocytes, usually associated with agyrophil reticulin fibres, and with some capacity for phagocytosis. It is not established whether or not the reticulin fibres (believed to be precursors of collagen fibres) are the product of reticulum cells.

The cytological appearances of reticulum cells in histological preparations are charateristic. In the writer's experience, the main features are best exemplified by the proliferating histiocytic cells in Hodgkin's paragranuloma (Fig. 17). Reticulum cells are charatorised by large round frequently indented vescicular nucleus, with a distinct thick nuclear membrane accentuated by margination of the scanty chromatin content of the nucleus. There is usually a single central large prominent round intensely eosinophilic nucleolus which is frequently seen to be joined to peripheral chromatin by fine chromatin threads. The cytoplasm is fairly abundant, faintly eosinophilic and may exhibit phagocytosed particles. The cell outline is irregular due to multiple cytoplasmic processes some of which are seen to be continuous with adjacent cells of the same type.

Histologically reticulum cell lymphoma shows masses of haphazardly arranged cells with the appearances just described. Mitotic figures are common, and a few small lymphcytes may be scattered among the proliferating cells (Fig. 3). Phagocytosis may be evident.

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Plate 2. Lymphomas of the lymphocytic series.

Fig. 5. Lymphocytic lymphoma. (Well differentiated lymphosarcoma)
Masses of closely packed small lymphocytes.
H and E. x 490.

Fig. 6. Lymphoblastic lymphoma (poorly differentiated lymphosarcoma)
Sheets of closely packed hyperchromatic cells of various sizes, with large blast cells predominating. Small and large lymphocytes present. Mitosis numerous though inconspicuous. H and E. x 490

Fig. 7 Burkitt's lymphoma
Atypical appearance. Sheets of closely packed hyperchromatic round blast cells of uniform size and appearance with scanty inconspicuous histiocytic cells present. Mitosis frequent. H and E. x 490.

Fig. 8 Burkitt's lymphoma
Typical appearance. Closely packed monomorphic and hyperchromatic blast cells interspersed by large pale conspicuous histiocytes many of which show intracytoplasmic pyknotic nuclei and/or intact cells. Occasional small lymphocytes scattered among the blast cells. Mitosis is frequent.
The scattered pale staining histiocytes against a background of the closely packed blast cells, gives the "starry-sky" or "[illegible]-pot" effect.
H and E. x 490

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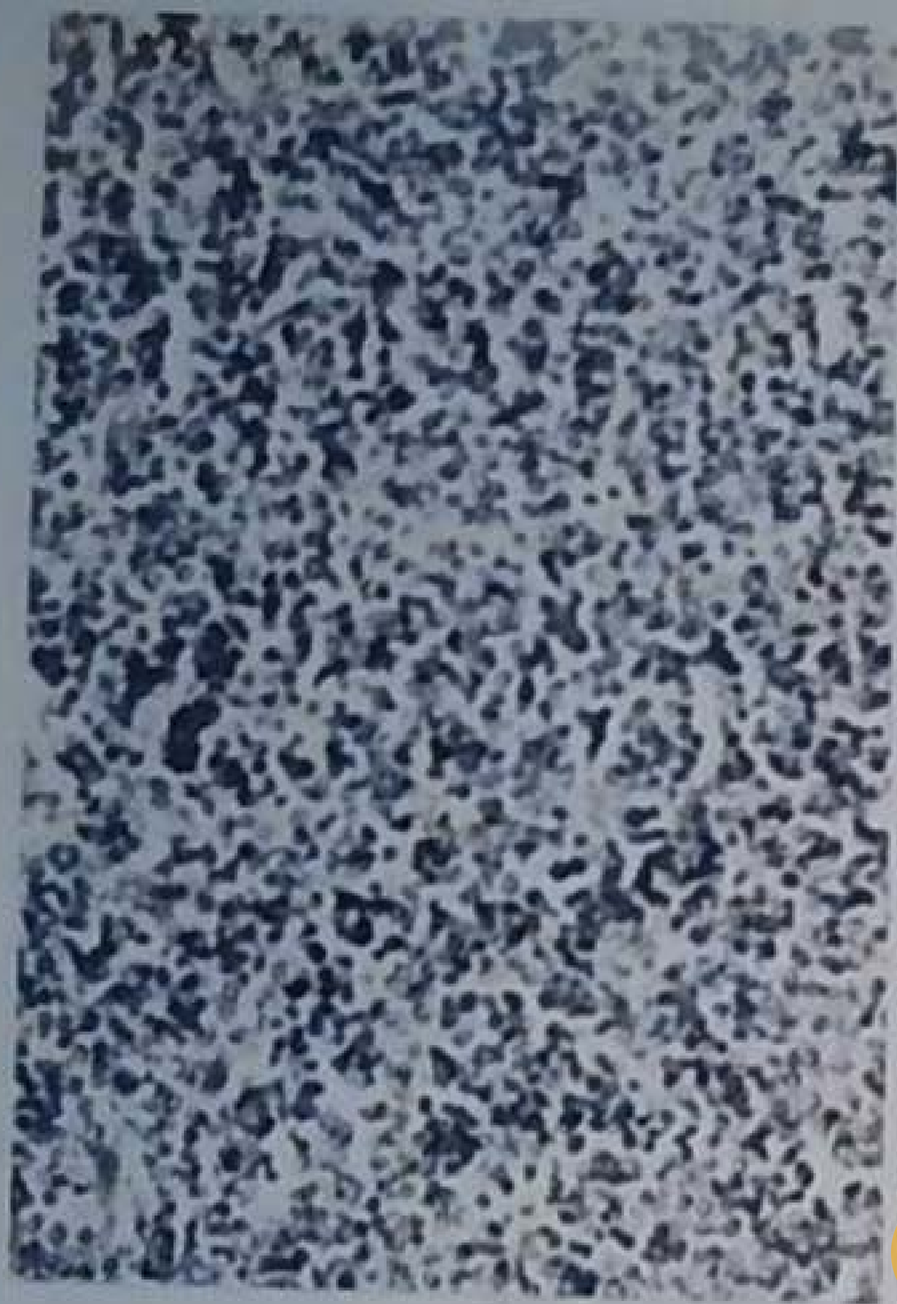

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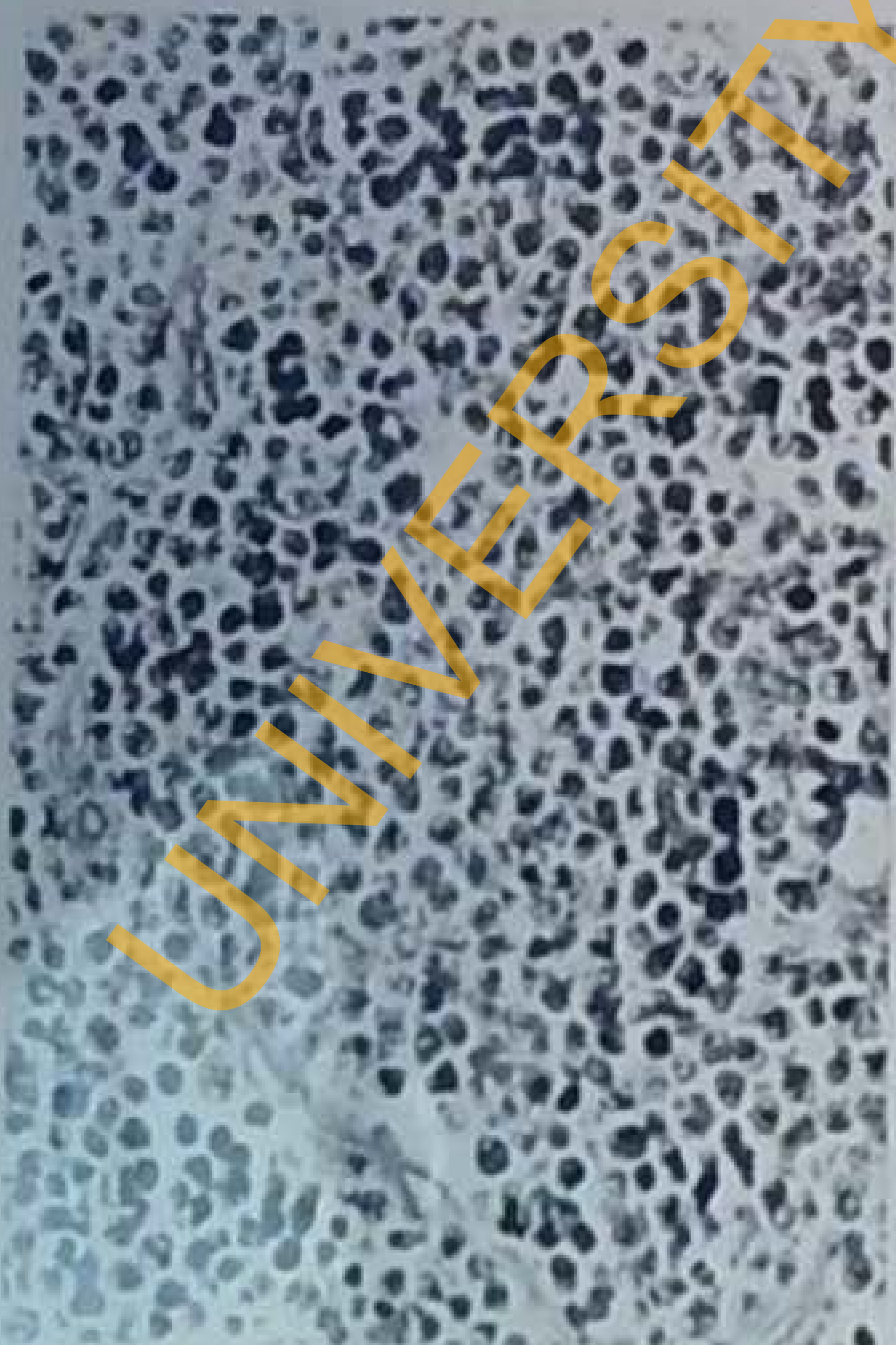

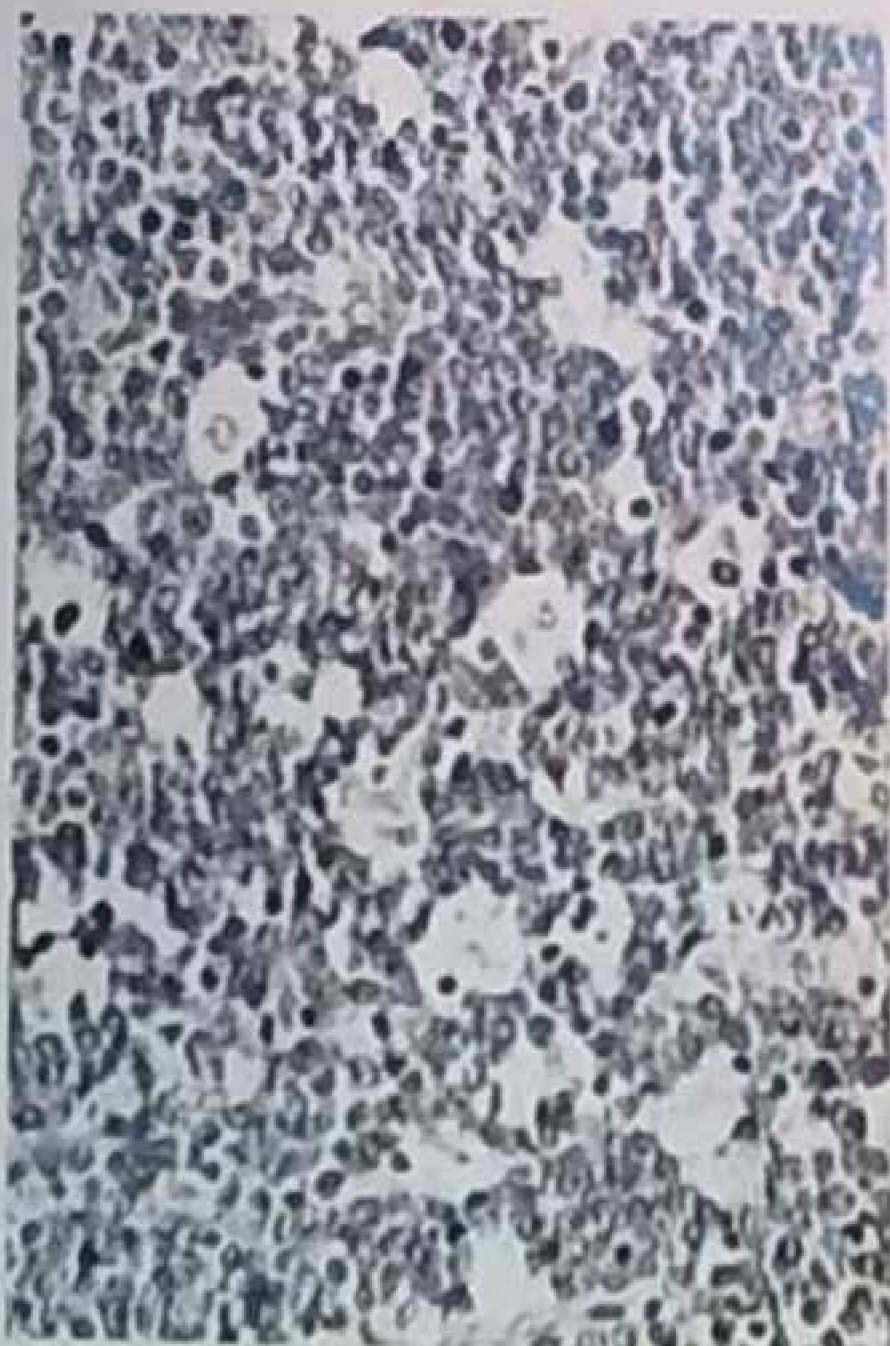

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Metalophilic 'reticulin' fibres are as a rule readily demonstrated in close association with the proliferating cells.

11. LYMPHOMAS OF THE LYMPHOCYTIC SERIES

In this subdivision, the predominating and/or proliferating cell type is one of the various morphological types of lymphocytes or the primitive blast cell of the series. Histologically recognisable types are the small lymphocyte, the large lymphocyte, and the lymphoblast.

The small lymphocytes is readily identified because of its small size (5-8 microns) made up almost entirely of a hyperchromatic nucleus with closely packed large chromatin clumps. Nucleolus and cytoplasm are hardly if ever visible.

The large lymphocyte is slightly larger (8-10 microns) shows less clumping of chromatin particles, and possess an eccentric rim of amphophilic cytoplasm. The nucleolus is frequently visible, small and basophilic. The cells are capable of division by mitosis.

The lymphoblast is a moderately large cell (12-20 microns) with round, sometimes indented nucleus and a high nuclear-cytoplasmic ratio. The chromatin is deeply staining, abundant, coarse and evenly dispersed in the nucleus. Nucleoli are intensely basophilic, large and usually multiple. The scanty cytoplasm is amphophilic, cell outline is distinct, and mitosis frequent.

The lymphomas of this sub-division are classified according to the proportion of lymphocytes and blast cells present. Two

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classical types are recognised - well differentiated lymphosarcoma, and poorly differentiated lymphosarcoma. To this two histologic types may be added the recently described variant of poorly differentiated lymphosarcoma in which apparently normal histiocytes are present in conspicuous numbers.

1. <u>Lymphocytic lymphoma</u>

Synonym: Malignant lymphoma, lymphocytic type, well differentiated - (Rappaport, (1963b)
Lymphocytic lymphosarcoma
Lymphocytoma.

This type of lymphoma is characterised histologically by sheets of closely packed small lymphoytes. Occasionally some large lymphocytes may be present, and it is in such cases that occasional mitotic figures may be seen. The commonest presentation is a diffuse pattern, but less commonly, a follicular pattern may be evident. The conspicuous presence of histiocytes is most unusual; a starry sky appearance seen in a lymphocytic lymphocytic lymphoma, as a result of presence of large well differentiated non-malignant histiocytes dispersed among small lymphocytes, was encountered in this series in a biopsy from a 60 years old man.

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2. Lymphoblastic lymphoma

Synonyms: Malignant lymphoma, lymphocytic type, poorly differentiated - Rappaport, (1963b)

Lymphoblastic lymphosarcoma

Lymphoblastoma

Poorly differentiated lymphosarcoma.

The predominant and proliferating cells are lymphoblasts, but invariably present and intimately mixed with the blast cells are many lymphocytes, both small and large (Fig. 6). The numbers of lymphocytes present vary from case to case, but is always such as to give an overall polymorphic appearance, and the impression of lymphocytes in various stages of differentiation with preponderance of blast forms. Cells of the histiocytic series are not a feature.

3. The Burkitt's Lymphoma

Synonyms: Burkitt's tumour

Childhood lymphoma

African lymphoma etc.

The authors of the first published histological study of the multicentric sarcoma prevalent in children living in tropical Africa regarded the neoplasm as a malignant lymphoma, of the poorly differentiated lymphocytic type (O'Conor and Davies, 1960).

Although now regarded as a type of lymphosarcoma, it presents a histological picture quite different from those described above for lymphocytic and lymphoblastic lymphomas. The predominating cell is

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Plate 3. Starry sky apperance in Burkitt's lymphoma

Fig. 9 Burkitt's lymphoma; starry-sky appearance.
Large pale well-differentiated histiocytes against a background of dark rapidly proliferating monomorphic blast cells. H and E x 250.

Fig. 10 Burkitt's lymphoma; starry-sky appearance.
Note uneven distribution of pale histiocytes, with almost none evident at top right corner.
Distinguish smaller polygonal histiocytes from large perfectly round fat cells.
H and E x 110.

Fig. 11 Burkitt's lymphoma; ovary.
Atypical starry-sky effect. Note few small pale histiocytes and very closely packed blast cells.
H and E x. 490.

Fig. 12. Burkitt's lymphoma; "negative starry-sky".
Typical starry-sky at lower third, but opposite effect in upper third as a result of scattered histiocytes appearing [illegible] more darkly stained than back-ground of blast cells. Higher magnification revealed large histiocytes loaded with pyknotic nuclei, intact blast cells and lymphocytes.
H and E x 490.

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a somewhat atypical lymphoblasts. Compared to the typical lymphoblast, its nucleus is more hyperchromatic, the chromatin much less coarse, but more closely packed with consequent masking of nucleoli. The nucleus shows a higher degree and number of indentations. The cytoplasm is less abundant and more basophilic. Careful scrutiny of sections may reveal cytoplasmic vacuolation. These atypical lymphoblasts are always closely packed, monomorphic and show remarkably little variation in size. Small lymphocytes usually present in the tumour are not as a rule conspicuous histologically. Mitosis is very common, but may be rendered inconspicuous by the intense hyperchromaticity of the blast cells.

An important histological characteristic of the Burkitt's lymphoma is the presence of large, well differentiated, "non-malignant" histiocytes scattered among the blast cells. This dispersal of conspicuous pale staining histiocytes against a background of darkly stained monomorphic blast cells is pathognomonic for Burkitt's lymphoma (Fig. 8, 9, and 10). It should be stressed that the "starry-sky" effect thus produced is not peculiar to Burkitt's lymphoma (Fig. 13). The histiocytes frequently show cytoplasmic vacuolation, and intra-cytoplasmic nuclear and other debris.

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Plate 4 Hodgkin's type lymphomas

Fig. 13. Hodgkin's paragranuloma. (Hodgkin's lymphoma, lymphocytic predominance type).

Sheets of small lymphocytes interspersed by large, pale malignant reticulum cells, including one Sternberg-Reed cell. Note mitotic figure in reticulum cell a little above and to the right of the field. Note "starry-sky" effect. H and E x 490

Fig. 14. Hodgkin's granuloma. (Hodgkin's lymphoma, mixed-cell type)

A pleomorphic picture. Haphazardly arranged admixture of many reticulum cells, tumour giant cells, lymphocytes, fibroblasts and collagenous intercellular matrix. A Sternberg-Reed cell lies in the centre of the field. H and E x 490

Fig. 15. Hodgkin's sarcoma. (Hodgkin's lymphoma, lymphocytic depletion type).

Overwhelming predominance of proliferating reticulum cells many of which are giant cells with marked nuclear atypia; many are typical Sternberg-Reed cells. Lymphocytes scanty. Note scanty fibrillar stroma. H and E x 490.

Fig. 16. Sternberg-Reed cell.

Giant binucleated malignant reticulum cell. Note mirro-image nuclei and giant nucleoli, giving an "owl's-eye" appearance. H and E x 900.

Fig. 17. Malignant reticulum cells of Hodgkin's paragranuloma.

Note pale cytoplasm with indistinct outline, vesicular nucleus with thick nuclear membrane, margination of chromatin, and large nucleolus. H and E x 900.

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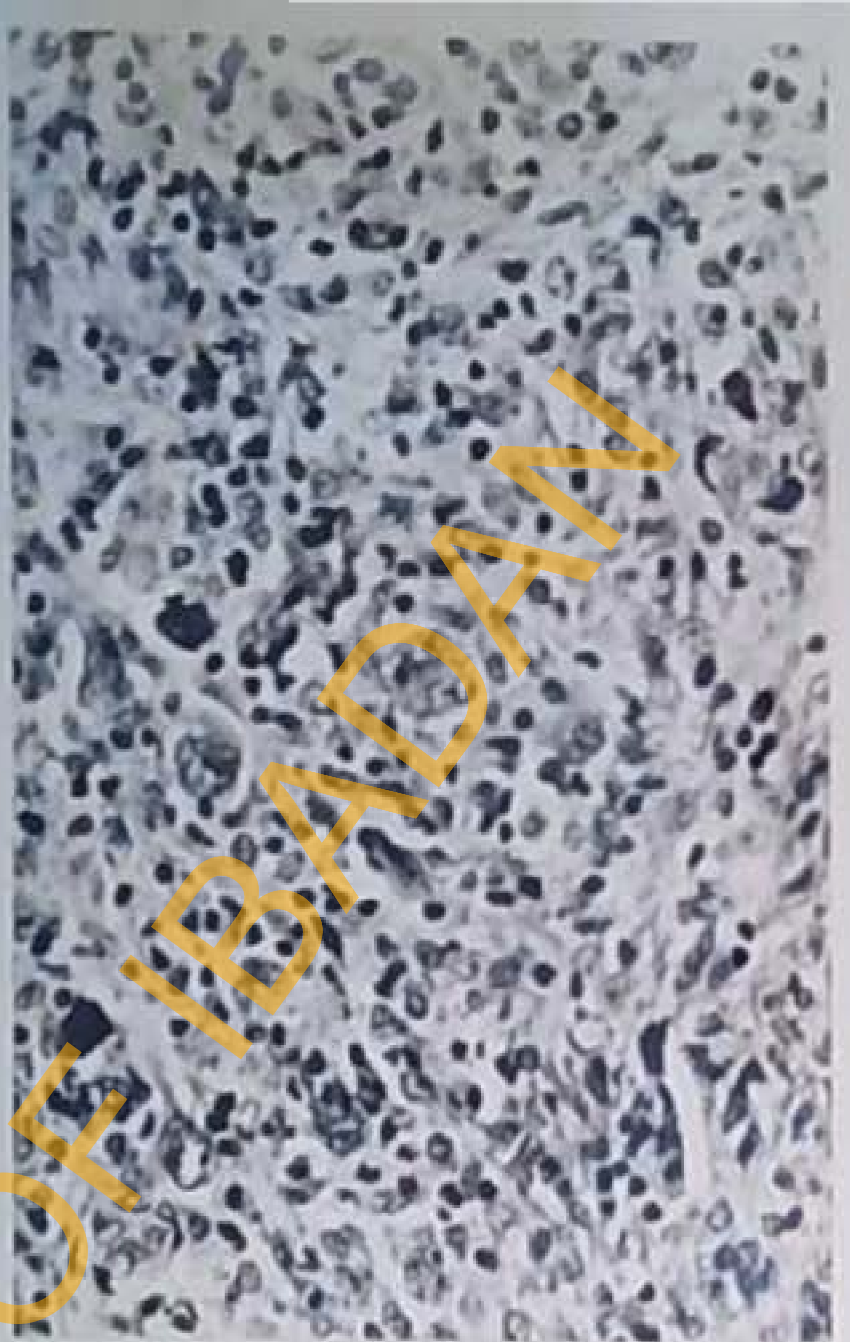

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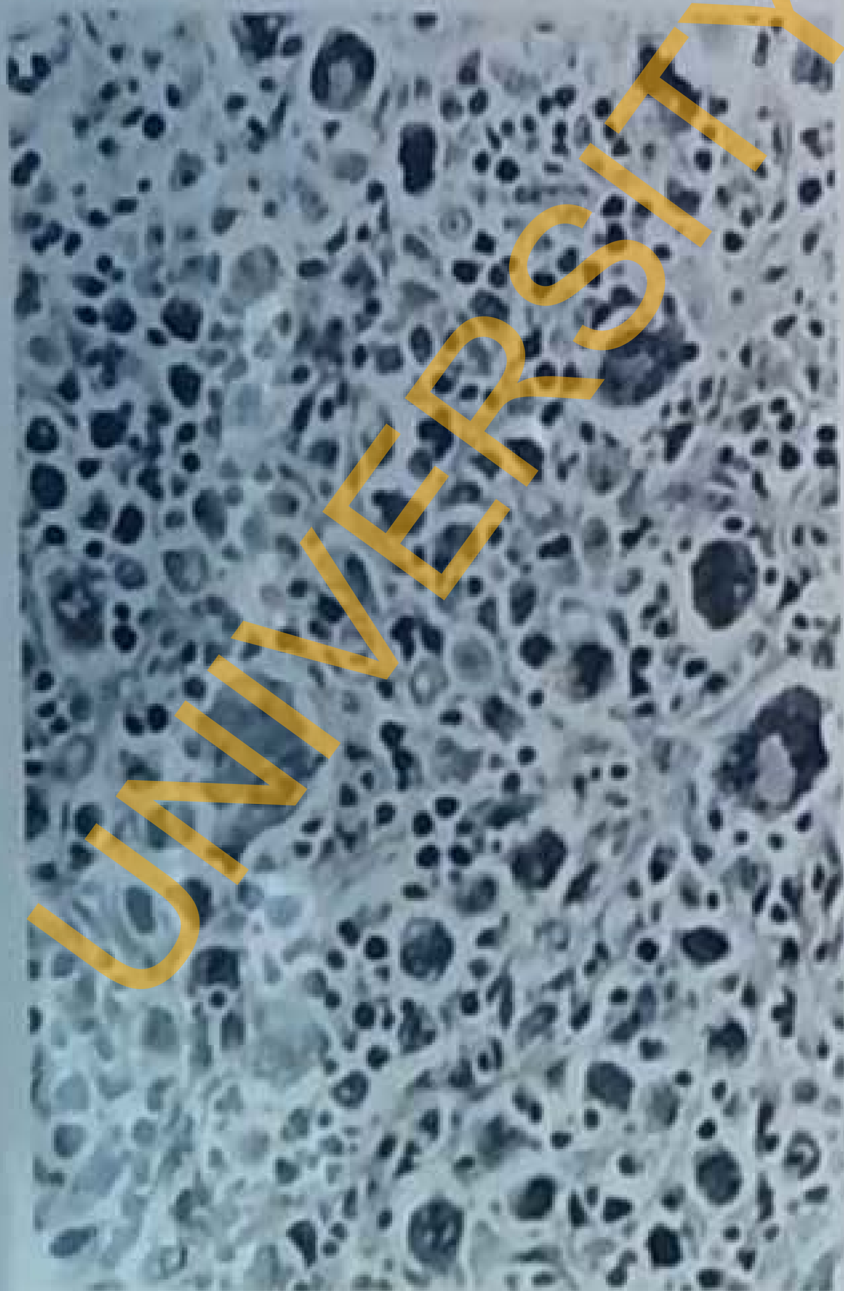

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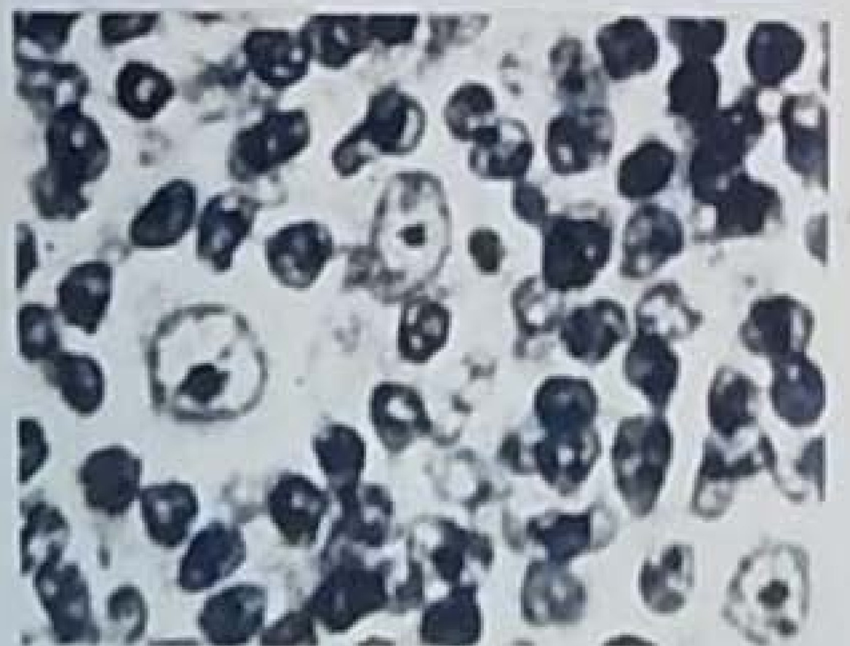

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111. HODGKIN'S DISEASE

Although an infective aetiology is increasingly suspected, the neoplastic nature of the lesions produced in this syndrome is now undoubted. Presently, Hodgkin's disease is considered as a clinico-pathological entity the lesions of which manifest a range of histological appearances which vary at different sites in the same patient, at different stages in the natural history of the disease, and from patient to patient.

All lesions share two features in common, - polymorphism and the presence of peculiar malignant reticulum cells, the binucleated giant forms of which have a distinct appearance and were first noted in the last quarter of the 19th century by several workers, including Sternberg (1898) and described in detail by Reed (1902). The cells are now known by various eponyms of which "Sternberg-Reed cells" is one. Their presence is pathognomonic for Hodgkin's disease (Fig. 16).

There is essentially a proliferation of malignant histiocytic cells associated in varying degrees with lymphocytes, plasma cells, granulocytes, fibroblasts and collagen fibres (Fig. 13-17). The relative proportion of Sternberg-Reed cells to lymphocytes is used as the basis for most histological sub-classification. Three main sub-classes are recognised, and the terminology adopted by various authors in describing these subclasses are shown in (Fig.13-15).

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Plate 5 Mixed lympho-histiocytic lymphoma

Fig. 18. Diffuse mixed lympho-histiocytic lymphoma.

Proliferating histiocytes (? sinus lining cells) with an intimate admixture of small lymphocytes. The lymphocytes are closely applied to the margins of the histiocytic cells; some are within the cytoplasm of the proliferating cells.
H and E. x 490

Fig. 19. Diffuse histiocytic lymphoma, with pseudofollicular pattern in places.

Note sheets of proliferating pale-staining sinus histiocytes obliterating dark lymphocytic cells. Three pseudofollicles present in the right half of the field.
H and E x 110.

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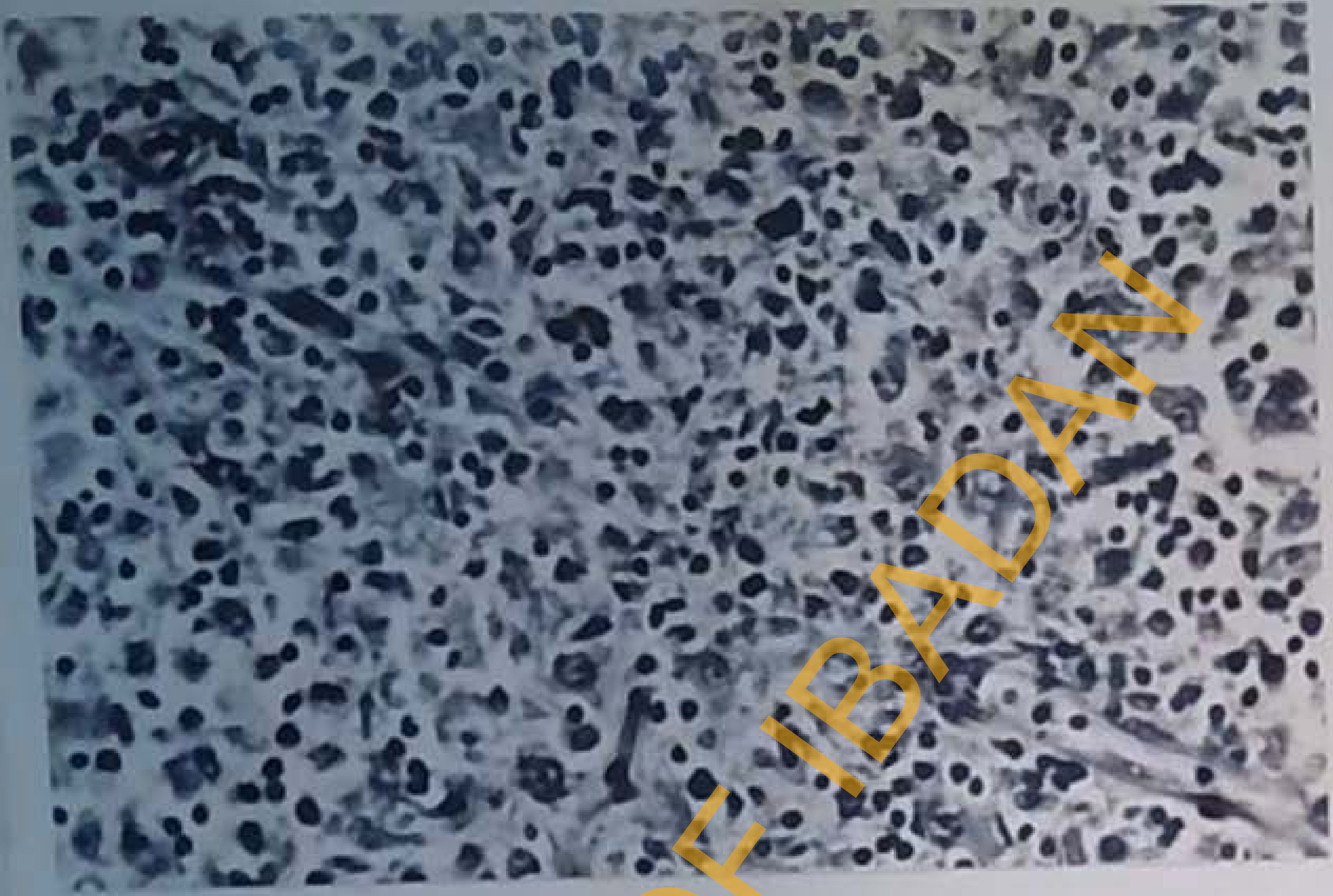

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IV. MISCELLANEOUS LYMPHOMAS

Some types of lymphoma occur, in which there is proliferation of well differentiated histiocytes, in association with small lymphocytes, the numbers of which vary from case to case. The histiocytes in this class are different in appearance from undifferentiated reticular cells and reticulum cells. They resemble the histiocytic cells which normally line and also form a network in the sinuses of lymphnodes and the sinusoids of the spleen and liver. They are characterised by very abundant eosinophilic cytoplasm and a relatively small nucleus with scanty finely dusted chromatin and a single tiny basophilic nucleolus; the shape is round or oval, and the nuclear membrane distinct but thin.

A striking feature is the presence in the histiocytes of intracellular pyknotic nuclear material as well as viable-looking small lymphocytes. Also prominent in most cases is the close approximation of small lymphocytes to the histiocyte cell margins. This appearances are reminiscent of in vitro phenomena of "emperipolesis" and "peripolesis" respectively. The impression therefore given is that of proliferating sinus histiocytes engaged in active biological interaction with normal lymphocytes.

1. Mixed lympho histiocytic lymphoma

The histologic appearance is that of proliferating well differentiated histiocytes associated with many lymphocytes, the later contributing passively to the neoplastic process (Fig. 18 and 19). Whether the lymphocytes represent the normal cells present in the

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lymphoid tissue before the neoplastic histiocytic proliferation, or an active accumulation of such cells following neoplastic transformation is not known.

2. Sinus cell histiocytic lymphoma

When sheets of proliferating sinus histiocytes are associated with only occasional lymphocytes, the impression is given of a monocellular lymphoma. It would however appear that such cases are variants of the so-called mixed lymphoma, in which emperipolesis and peripolesis is not marked.

V. PLASMA CELL TUMOURS

Normal plasma cells have a well known readily recognisable histologic appearance. Characteristically the cells are round or oval, of moderate size, with distinct outline, basophilic cytoplasm, a round eccentric nucleus, coarsely clumped chromatin, and inconspicuous nucleolus. Perinuclear cytoplasmic pallor and marginal arrangement of chromatin condensations, giving the "clock-face", "cart-wheel", or "spoke-wheel" appearance further characterises these cells.

A tumour composed largely or exclusively of such cells provides an easy histological diagnosis. Usually however, there is in such tumour, fairly remarkable variation in cell size and presence of atypical cells approximating more or less to large lymphocytes. Binucleated or trinucleated plasma cells and small quantities of intercellular homogeneous faintly eosinophil material may also be

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A tumour composed largely or exclusively of such cells provides an easy histological diagnosis. Usually however, there is in such tumour, fairly remarkable variation in cell size and presence of atypical cells approximating more or less to large lymphocytes. Binucleated or trinucleated plasma cells and small quantities of intercellular homogenous faintly eosinophil material may also be evident. Mitotic figures are usually rare, while normal small lymphocyte

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may be present in appreciable numbers. This picture typifies the solitary extraosseous tumours.

The osseous multiple myeloma although probably related to the extraosseous plasmacytoma, shows a more bizzare histologic picutre. The nucleus is large with prominent nucleolus, and mitotic figures are common.

RESULTS.

Of the 528 biopsies reviewed, 27 were in the opinion of the writer misdiagnosed as malignant lymphoma on histological grounds. Figure 6 shows the revised diagnosis on such cases. By far the commonest sources of error were labelling of non-malignant lesions such as reactive lymphoid hyperplasia and granulomatous, probably tuberculous, lesions as lymphomas. Next was the failure to distinguish anaplastic epithelial tumours from lymphoma. Two of such cases were secondary metastastic epidermoid carcinoma in lymph node, while two were nephroblastomas (Wilm's tumour) showing only a few foci of tubule formation.

Sections from a further 30 cases could not be classified because of poor quality of the slides. The malignant nature of these lesionswas evident from destruction of normal architecture, diffuse proliferation, or invasion of peri-nodal tissues. Most of the cases were in all likelihood lymphoblastic or stem cell lymphomas.

In all,471 biopsies were classified. These fall into one or other of the twelve histologic types already described (Table 5).

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By far the commonest type seen was the Burkitt-type lymphoma, constituting at least one-fifth of all lymphomas, and 44% of lymphomas of the lymphocytic cell series. In 9 cases, the age of the patient was over 20 years. 69% of cases in which the age was known were children aged 15 years and below (Table 7).

The next common lymphoma was the lymphoblastic type, accounting for 16.4% (Table 5) of all lymporeticular tumours and 31.8% (Table 7) of the "lymphosarcomas". Twenty four of such cases were seen in individuals below the age of 15 years, out of which 10 were from lymph node biopsies. Burkitt's lymphoma was exluded in the classification of the remaining 14, because histiocytes were inconspicuous (probably absent) and there was some degree of polymorphism as a result of the presence of many large and small lymphocytes.

Lymphocytic lymphomas accounted for 12.6% of all lymphomas. This type of lymphoma was encountered in 6 children aged 10 to 15 years.

Lymphoma of the stem cell type totalled 34, of which 10 belonged to the "stem cell with fibrolymphocytic differentiation" group. None showed evidence of differentiation into reticulum cells or lymphoblasts. The age of patients with stem cell lymphoma with or without fibrolymphocytic differentiation ranged between 30 and 71 years, with a median of 50 years. Those with partial or toal fibrolymphocytic differentiation also had a median age of 50 years, with a range of 30 to 60 years.

But

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Reticulum cell lymphoma accounted for 13.1%.Of all lymphomas, while Hodgkin's type lymphomas had a type frequency of 16.5% of the 78 cases of Hodgkin's type lymphoma, 9 were of the lymphocytic predominance (paragranuloma) type (Fig. 13), 53 were of the mixed cell (granuloma) type (Fig. 14), while the rest 16 cases were classified as the lymphocyte depletion (sarcoma) type in which there was a preponderance of Sternberg-Reed cells and bizarre reticulum cells with a wide variety of nuclear atypia and sparse distribution of lymphocytes (Fig. 15)

Twenty-seven cases were encountered in which there was admixture of large numbers of lymphocytes and lymphoblasts with a predominatly histiocytic cell population. Such cases were classified as mixed (lympho-histiocytic) lymphoma.

Diffuse proliferation of well differentiated histiocytes showing marked phagocytic activity, a good number of mitotic figures and complete obliteration of nodal architecture was seen in 10 cases. An additional case showed an apparently multifocal proliferation of such cells, with a pseudo-follicular appearance (Fig. 19). Careful scrutiny however revealed the pattern of distribution of such cells to coincide with the lymphnode sinuses, both peripheral and medullary. In all cases, there was persistence of normal lymphocytes many of which were evidently within the proliferating cells, together with pyknotic nuclei and other particulate matter (Fig. 18). The malignant nature of the lesion was undoubted, and although there was no clinical data to suggest the involvement of extra-nodal sites, some of these cases may well be instances of the so-called systemic malignant histiocytosis, otherwise

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known as malignant reticulohistiocytosis (Rappaport, 1963b). They were classified as sinus cell (histiocytic lymphoma).

Seventeen solitary plasma cell tumours were encountered. Seven were plasmacytomas of the upper respiratory tract, while the rest were solitary tumours associated with bones; clavicle (2), sternum (2) spine (2) maxilla (1), humerus (2) and femur (1).

Of the 471 lymphomas reviewed, only 4 showed a follicular (nodular) pattern. Three of these were lymphocytic in type, while the fourth was not a genuine follicular lymphoma, but rather a sinus cell (histiocytic) lymphoma in which there was as yet no confluence of the neoplastic sinuses, a transverse section of the neoplastic sinuses giving a pseudofollicular appearance (Fig. 19). No instance of classical giant follicular lymphoma of Brill-Symmers was seen.

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COMMENT

1. Apparent rarity of follicular lymphoma

The paucity of follicular lymphoma in this series reflects the rarity of this type of lymphoma in Nigerians. Similar findings in Uganda Africans was reported by Davies in 1963. Dorfman (1961), and Oettle (1963) have also commented on the apparent low incidence of lymphomas with a follicular architectural pattern in the Bantu people of South Africa as compared with individuals of caucasian origin living in the same area.

Follicular lymphoma (nodular lymphoma, Brill-Symmers disease, giant follicular lymphoblastoma) was for a long time accepted as a distinct clinico-pathological entity. Since Rappaport et al's evaluation of the position in a study of 253 cases of lymphomas with a follicular pattern (Rappaport et al, 1956), it is being increasinlgy accepted that such a segregation of follicular lymphoma is unjustified. The subject is still controversial. All are agreed however that lymphomas with a follicular or nodular pattern are relatively less malignant, slower-growing neoplastic lesions, many of which ultimately progress into a cytologically similar, more fulminating but diffuse form.

The apparent rarity of such tumours in Africans may be due to the usually late presentation at hospitals of African patients. Diagnostic biopsies are therefore likely to be undertaken after lymphomas with a nodular pattern have lost this pattern with time. On the other hand, it may be a genuine rarity due to either racial

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or environmental factors or both. Host resistance to lymphomas may be relatively low in Africans, in consequence of which the lymphomas may be more fulminating in appearance and behaviour, a surmise which as yet has no clinical or experimental proof. Lastly, and more plausible is the possiblity that the growth and natural history of lymphomas may be influenced by the dosage of the oncogenic stimuli precipitating or perpetuating these class of tumours. More frequent exposure and/or a high and persistent induction dose, may evoke a more fulminating tumour with consequent telescoping of certain stages in the natural history of the neoplasm.

2. Natural history of undifferentiated reticular cell lymphoma

The occurrence in this series of a peculiar histologic appearance which is sometimes associated with lymphomas of the 'stem cell' type, but which in several cases constituted the entire biopsy material, is remarkable only because it has not higherto found prominence in the literature. The accepted lines of differentiation of stem cell lymphomas are towards reticulum cells or lymphoblasts or both, the only evidence for this belief being association of the later cells with the 'stem cells' in histological preparations. There is as far as the writer is aware no claim for differentiation into haphazardly arranged spindle and filiform cells with a lymphocytic type of nucleus, and for which fibrolymphocytic sub-class of lymphomas is proposed.

The absence of typical reticulum cells as well as Sternberg-Reed or other malignant giant cells excludes by definition lymphomas of the reticulum cell and Hodgkin's type respectively.

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Spindle cell tumours (fibrosarcoma, leiomyosarcoma etc.) and normal endothelial cells are readily distinguished by their bigger, paler and more vesicular nucleus.

Primary lymphoreticular tumours with a fibro-lymphocytic histologic appearance are in all probabilities variants of undifferentiated reticular cell lymphoma. Apart from presency of both appearances in the same biopsy material, the age incidences are closely similar. It would appear that the fibrolymphocytic appearance represents yet another histological type into which a stem cell lymphoma may progress. Detailed study of sequential biopsies on this class of lymphoma should prove more elucidating.

3. Difficulties in histological diagnosis and classification of the lymphomas.

In the vast majority of cases a histological diagnosis of malignant lymphoma is readily made on an adequate and representative biopsy or autopsy material after good fixation and staining with haematoxylin and eosin. However, this is certainly not true in all cases, even in the hands of competent pathologists, and is exemplified by the results of the present histopathological review. There were 27 false positives out of the 528 biopsies reviewed, an observer error of the order of 5%. From the analysis of the 27 biopsies wrongly diagnosed as lymphomas, it may be concluded that not infrequently, there is difficulty in distinguishing between primary neoplasms of lymphoreticular tissues and non-specific hyperplastic inflammatory

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reactions in such tissues. Many authors have called attention to this difficulty. Perhaps Rappaport's recent note on the distinction between lymphoma and reactive lymphoid hyperplasia is the most elucidating, practical and comprehensive. (Rappaport, 1963b).

Metastatic anaplastic carcinoma in lymph node may be very difficult to distinguish from reticulum cell lymphoma. This is even more likely when replacement of lymphoid tissue by the metastatic deposit is extensive or complete, and when a primary site for the carcinoma is unknown.

When the tumour presents at extra nodal sites, the differential diagnosis of lymphoma on histological grounds takes the formidable attempt at identifying and distinguishing malignant cells of lymphoid origin from those of all forms of mesenchymal as well as other epidermal and neuroectodermal tumours. The controversy on the histogenesis of Ewing's sarcoma, and the histological limitations preventing the unequivocal distinction between Burkitt's lymphoma and other round cell blastomas presenting as jaw, orbital and retroperitoneal tumour, exemplifies this point.

Cytological methods that are now being introduced as ancillaries to orthodox histopathology should go a long way to help minimise the difficulties in the differential diagnosis and classification of lymphomas.

4. Histological features of Burkitt's tumour

This study confirms previous reports that in most cases, the diagnosis of Burkitt's lymphoma is readily made on histological grounds. The characteristic scattering of large, pale, well-differentiated histiocytes among masses of closely packed, hyperchromatic, monomorphic,

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rapidly proliferating primitive cells is a unique characterisitc of this type of malignant tumour. This histologic appearance is shared only by a hyperplastic germinal centre. The visual effect produced by the scattered pale histiocytes among sheets of dark blast cells has been described as the "starry sky" appearance (O'Conor and Davies, 1960).

The starry-sky effect is however not peculiar to Burkitt's lymphoma. It was encountered in some lymphocytic lymphomas in adults and is readily seen in Hodgkin's paragranuloma. In all these lymphomas, the cells forming the "sky" are invariably small lymphocytes, while the "stars", the scattered histiocytic cells, may be entirely malignant histiocytes, as in Hodgkin's paragranuloma. What distinguishes Burkitt's lymphoma is that the dark cells froming the "sky" are proliferating blast cells of uniform appearances, size and maturity.

It should however be stressed that the starry-sky appearance is not always evident in all biopsies of Burkitt's lymphoma. Neither is it always evenly distributed in all parts of the same biopsy material. Distinction of such atypical appearances from a non-Burkitt lymphoblastic lymphoma may often be impossible. The only useful distinctive criterion is the presence in lymphoblastic lymphoma (of the non-Burkitt type) of lymphocytic cells manifesting all gradation of differentiation, that is, a mixture of lymphoblasts with large and small lymphocytes.

It may be justified to regard lymphoblastic lymphoma with a monomorphic cell composition as Burkitt's lymphoma, whether or not histiocytes are prominent. This justification would be tenable only if it can be shown that the tumour cells in Burkitt's lymphoma are in fact monomorphic. However, the histological appearance of the blast cells in

Burkitt's

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lymphoma do show some differences from case to case, in respect of cell size nuclear configuration, apparent chromatin content and quantity of cytoplasm. These variations cannot be accounted for only by variation in technical procedures such as fixation and staining.

Notwithstanding gross underestimation due to the review of only histologically processed biopsy materials, the relative frequency of Burkitt lymphoma was still remarkably high, accounting for at least 21% of all lymphomas seen in Ibadan. Burkitt lymphoma is obviously a prototype for any investigation relating to the search for factors responsible for the unduly high incidence of lymphoreticular tumours in Nigeria and other tropical African countries.

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Table 4.

Histologic classification of Hodgkin's disease

Histologic features	Histologic type (after Lukes et al 1966)	Synonyms of Histologic type	Author and Year
1. Lymphocytes overwhelmingly predominant; scattered reticulum cells and Sternberg-Reed cells. (Fig. 13 and 17).	Hodgkin's lymphoma - lymphocytic predominance type.	Early Hodgkin's disease Benign Hodgkin's disease Indolent Hodgkin's disease Reticular lymphoma. Hodgkin's paragranuloma	Jackson 1937 Harrison 1952 Symmers 1958 Lamb 1954 Jackson and Parker 1944
2. Pleomorphic. Mixture of Sternberg-Reed cells, lymphocytes granulocytes, plasma cells and fibrosis. (Fig. 14).	Hodgkin's lymphoma - mixed cell type.	Hodgkin's granuloma Fibromyeloid medullary sclerosis	Jackson and Parker 1944 Robb-Smith 1938
3. Predominantly typical and atypical Sternberg-Reed cells; some reticulum cells, few lymphocytes (Fig. 15).	Hodgkin's lymphoma - lymphocytic depletion type.	Hodgkin's sarcoma Anaplastic sarcoma of lymphoid tissue.	Jackson and Parker 1944 Lamb 1954

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Table 5.

Histopathological review and classification of biopsies reported as malignant tumours of lymphoreticular origin. (U.C.H., Ibadan, 1960-1966).

Classic sub-division	Type of lymphoma	No. of cases	% of lymphoma	Sub-total
Reticulum cell sarcoma	1. Undifferentiated reticular cell lymphoma	26	5.5	96(20.3%)
	2. Fibrolymphocytic lymphoma	8	1.7	
	3. Reticulum cell lymphoma	62	13.1	
Lymphosarcoma	4. Lymphocytic lymphoma	59	12.6	
	5. Lymphoblastic lymphoma	77	16.4	242(51.5%)
	6. Burkitt's lymphoma	106	22.5	
Hodgkin's disease	7. Hodgkin's paragranuloma	9	1.9	
	8. Hodgkin's granuloma	53	11.2	78(16.5%)
	9. Hodgkin's sarcoma	16	3.4	
Reticulosis	10. Mixed lymphohistiocytic lymphoma	27	5.7	38(8.1%)
	11. Sinus cell (histiocytic lymphoma	11	2.4	
Plasma cell tumour	12. Plasmacytoma	17	3.6	17(3.6%)
		Total lymphomas	471	
		Not classified	30	
		Not lymphomas	27	
		TOTAL reviewed	528	

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Table 6.

Analysis of biopsies found misdiagnosed in a histopathological review of 528 cases of lymphomas.

Revised diagnosis	No. of cases
Non-specific reactive hyperplasia	8
Granuloma, with occasional Langhan's giant cell	5
Primary carcinoma	4
Metastatic carcinoma in lymph node	2
Soft tissue sarcoma	3
Granulation tissue	2
Others	3
TOTAL	27

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Table 7.

Analysis of 240 cases of "lymphosarcomas" according to cell-type, and age of patient.

Age (years).	Burkitt lymphoma	Lymphoblastic lymphoma	Lymphocytic lymphoma	TOTAL
0-15	90	24	6	120(49.6%)
16-20	4	4	0	8(3.3%)
21+	9	43	48	100(41.2%)
Age not known	3	6	5	14
	106(44.0%)	77(31.8%)	59(24.2%)	242

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CHAPTER 111.

CYTOLOGY AND TISSUE CULTURE OF THE BURKITT'S TUMOUR CELL.

INTRODUCTION

"Two discoveries, of phase contrast and antibiotics, have made the examination of fresh pathological tissue very simple" (Pulvertaft,1959).

Although now generally accepted as being of lymphoreticular origin (O'Conor and Davies, 1960; Edington et al, 1963; Wright 1963; 1966(a); Pulvertaft, 1964; Epstein et al 1965; Hutt, 1966), many of the clinicopathological features manifested by Burkitt's tumour contrast shaprply those which characterise the malignant lymphomas. Its focal geographical distribution; bizarre clinical presentation with apparent predilection for certain anatomical sites, particularly those normally devoid of lymphoid tissue; frequent involvement of the central nervous system; conspicuous rarity in superficial lymph nodes and spleen; and the peculiar age incidence have been well documented (Burkitt and Wright, 1963; Edington et al, 1963; Wright 1964; Janota, 1966). The commonly used non-committal eponymous title of "Burkitt's tumour", and the long list of synonyms that have been proposed (see Dalldorf et al, 1964; Janota, 1966 for review) are however evidence of uncertainty about the exact histogenesis of this tumour. Is the Burkitt's tumour cell a distinct morphological entity, as for example the mature eosinophil granulocyte?

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Or is it a cell-type with a narrow spectrum of cytomorphological diversity such as the myeloma cell?

In the previous chapter, it was evident that Burkitt's tumour was encountered relatively frequently in biopsies of lymphoreticular tumours available for study at the University College Hospital, Ibadan. The microscopic appearance of "starry-sky" effect in a predominantly lymphoblastic neoplastic proliferation was believed to be pathognomonic. Since the absence of "starry-sky" appearance does not exclude a diagnosis of Burkitt's tumour, the histological distinction between Burkitt's tumour and lymphoblastic lymphoma was found to be difficult and very frequently impossible. It would therefore be useful to search for other methods of laboratory investigations which would readily distinguish between the blast cells of Burkitt's tumour and those of the lymphomas particularly the lymphoblastic type, as well as cells of other childhood tumours.

As has already been pointed out by one of the leading contemporary exponents of the examination of living pathological tissues (Pulvertaft, 1959; 1965), the parameters which microscopic examination of living cells afford for the characterisation of cell-types are manifold. The disadvantage of loss of topography and requirements of special apparatus and skill which the technique involves together with experience which interpretation of observations requires are however far outweighed by the wealth of information which may be gathered for the characterisation of the cells or tissues under study. These include most criteria utilised in orthodox histology and stained-smear cytology, such as

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nuclear and cellular configuration and size; nuclear-cytoplasmic ratio; presence and prominence of nucleoli; presence or absence of intra-cytoplasmic foreign debris and granules etc. To these may be added readily observed properties of viable cells such as absence, presence and type of motility; capacity for phagocytosis and cellular adhesion to glass and other surfaces. Sub-cellular structures like mitochondria may be observed. In addition, the degree of cohesion between cells, and the pattern of aggregation formed by the coherent cells are readily observed. Finally, when the cells under study are allowed to remain in a standard in vitro environment, their longevity in such environment; the type of cell death manifested, (karyolysis, cytolysis, cytoplasmic sequestration, nuclear vesciculation etc.); the pattern and rate of growth as well as the degree of stability of cytomorphology with time may be observed.

The unsolved problem of the relative importance of hereditary and environmental factors in the aetiology of human cancer is highlighted by the apparently high incidence of lymphoreticular tumours in tropical Africa. In particular, Burkitt's tumour is yet another example of Steiner's dictum:

> "The more the racial cancers are studied the greater is the tendency for them as such to vanish. What appears at first to be racial in the genetical sense tends upon further examination to be racial only in the cultural or special environmental sense". (Steiner, 1954).

Malignant tumours with histological appearances indistinguishable from Burkitt's lymphoma are not found in African children alone. Tumours with clinical and histological features of Burkitt's lymphoma have been described in children of other races living in Africa (Burkitt, 1963),

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New Guinea (Burkitt, 1963; Ten Seldam et al, 1966), Brazil (Luisi et al, 1965), Colombia (Beltran, 1966), United States of America (O'Conor, 1963; O'Conor et al, 1965; Dorfman, 1965), and England (Wright, 1966). Tumours histologically resembling Burkitt's lymphoma occur in the cat (Squire, 1966) and the dog (Bras et al, 1965). Racial (hereditary) factors do not seem therefore to be of prime importance in the aetiogenesis of the tumour; environmental influences are more plausible. Such environmental factors are probably more prevalent, or more readily accessible to humans in Tropical Africa, the area in which Burkitt's lymphoma occurs in relatively "endemic" proportions.

It would therefore appear that Burkitt's tumour is a prototype in the search for the role of environmental factors in the apparent frequency of lymphoretic lar tumours in Africa. As a first step towards this search, and in the hope of shedding more light on the histogenesis of the tumour, it was thought necessary to study in more detail, the morphology and biological characteristics of the Burkitt's tumour cell.

Phase contrast cytology, short-term tissue culture and long-term tissue propagation were used in various combinations to achieve some of these aims. In this chapter is presented the use of phase contrast cytology and tissue culture in

(1) the characterisation of the Burkitt's tumour cell,

(2) the differentiation of Burkitt's tumour from other round cell sarcomas of childhood and

(3) the study of the behavior of the Burkitt's tumour cell in continous culture.

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MATERIALS AND METHODS

Preliminary studies

As a preliminary to the use of tissue culutre methods in experimental study of the morphology and biological properties of the Burkitt's tumour cell, the suitability of the working environment available to the writer, and the general behavior of a variety of normal and pathological tissues of human and murine origin were studied, using largely the tissue culture techniques of Prof. R. J. V. Pulvertaft. The cells studied included:-

(i) human thyroid, amnion and bone marrow;

(ii) phytohaemagglutinin transformed peripheral leucocytes;

(iii) few human malignant cells mainly from children suffering from retinoblastoma and Burkitt's tumour;

(iv) normal mouse spleen cells and peritoneal macrophages, as well as;

(v) cells from ascitic fluid samples from cases with clinical diagnosis of abdominal tuberculosis.

From these preliminary studies were evolved the techniques described below in the handling and observation of materials presented in this chapter.

Materials studied: sources, selection, types, and collection.

All the materials studied were specimens obtained from in-patients of the University College Hospital, Ibadan, during the 13-month period 1st September, 1965 to 30th September 1966, inclusive. Preference was given to specimens taken from patients suspected clinically of suffering from malignant neoplastic diseases, particularly childhood tumours, soft

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Table 8.

Analysis of materials studied by phase contrast cytology and short-term tissue culture.

…ase cytological diagnosis	Solid tumour biopsies	Peritoneal aspirates	Cerebrospinal fluid	Bone marrow aspirate	Pleural aspirate	Splenic aspirate	Total
…ymphoblastic lymphoma (mainly Burkitt's type)	25	5	11	2	2	1	46
…inoblastoma	3	-	2	-	-	-	5
…euroblastoma	3	1	-	-	-	-	4
…ms's nephroblastoma	4	2	-	-	-	-	6
…her lymphomas	21	-	-	-	-	-	21 [uncertain]
…her neoplasms	62	2	-	-	-	-	64
…n-malignant lesions	56	50	29	10	5	-	150
…tal	174	60	42	12	7	1	296

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tissue tumours, lymphomas and obscure ailments for which no clinical diagnosis was evident. Table 8 lists the types of materials studied. These comprise biopsies of solid tumours and lymph nodes, peritoneal, pleural, bone marrow and splenic aspirates, and cerebro-spinal fluid obtained usually by lumbar puncture, occasionally by cisternal puncture. In all, 296 samples were studied.

Most of the biopsies of solid tumours and lymph nodes were performed in the operating theatre, but some lymph node biopsies as well as needle biopsies of solid tumours were carried out in the Wards. Aspirates from serous and marrow cavities and lumbar puncture specimens were obtained in the Wards.

All specimens were obtained by aseptic techniques and collected (usually in person) into sterile containers. Solid tumour biopsies and cerebrospinal fluids were collected into dry universal bottles. Aspirates (5-20ml volumes) from serous cavities were collected into heparinised bottles, while needle biopsies and aspirates from spleen and bone marrow were collected in universal containers containing about 5 ml. heparinised balanced salt solution (TC 199). The final concentration of heparin in samples ranged from 5-20ml/units.

Processing of specimens for phase contrast cytology and tissue culture.

Processing of specimens for microscopic examination and tissue culture was as a rule undertaken within 30 mins. of collection. In a few instances it was not possible to do this, and specimens, particularly fluid samples have been found to give good results even after standing at 4°C for 1 to 2 hours. Asceptic precautions were taken throughout the

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the period of processing of materials.

1. Culture Medium:

The basal medium used throughout these studies was TC 199 (Glaxo) (Parker, 1964). It was found necessary to augment the antibiotic content of this medium with neomycin (30 unts/ml) and mycostatin (50 unts/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 U.C.H., Ibadan. About 25ml clotted blood was collected from each donor, and serum separated by centrifugation within 36 hours of collection. The serum samples were pooled in pairs usually as 25ml aliquots, and were deep frozen until use, after addition of neomycin and mycostatin, and innoculation into nutrient broth. Each sample was judged sterile and safe for use if there was no growth in the nutrient broth culture after 48 hours.

2. Processing of solid tissues:

Biopsies of solid tissues were divided into four portions for

(i) phase contrast cytology and tissue culture;

(ii) routine histology;

(iii) impression (touch) smear cytology;

(iv) other (usually collaborative) studies, such as histochemistry, electron microscopy, animal innoculation, immunopathology etc.

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It is with the first portion, that this chapter is concerned. The portion for phase cytology and culture was placed in a 2-5ml pool of TC 199 in a petri dish, and the cells dispersed into the medium by gentle squeezing and teasing of the biopsy 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. Very occasionally however, it was necessary to expose small fragments of the biopsy to 0.25% trypsin in TC 199, at 37°C for 1 to 2 hours to effect a reasonable cell-dispersal.

At this a stage drop of the cell suspension was placed on a glass slide covered with a coverslip and examined under the phase contrast microscope, 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 mins., during which time large tissue fragments would have sedimented to the bottom of the tube. The supernatant was then transferred into another centrifuge tube, and the contained cells spun down at 250g for 3 mins. A debris-free cell suspension in TC 199 was then prepared by washing the cells twice in TC 199 by centrifugation. This permits optimal optical conditions for phase contrast cytological studies on the cells. 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 more difficult cases, or when desired, a prolonged observation was made on the cells. Further information such as longevity in TC 199, capacity for and type of motility, type of pseudopodial activity, ability to spread and

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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 spun down, and portions of the cell deposit seeded into the various types of tissue culture chambers to be described below.

3. Processing of fluid specimens:

Fluid specimens such as ascitic, pleural, and cerebrospinal fluids were simply centrifugated, the cell deposit resuspended in TC 199, and drop of the cell suspension examined microscopically as sealed cover slip preparation. If the material 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 aspriate 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 innoculated on agar roller slides, or examinated in a coverslip preparation.

METHOD OF EXAMINATION

All slide preparations (coverslip preparations, ring cultures, agar-roller slide) were examined on the warm stage of a Tiyoda phase contrast trinocular research microscope complete with photomicrographic attachment and Leica (M1) camera. The stage is warmed by a removable perspex box built round the microscope and containing two thermostatically controlled heating bulbs set at 36°C. The whole apparatus sits on an asbestos sheet. (Appendix 4).

Slides or chambers may be subjected to continous or intermittent examination for periods varying from a few minutes to two days at a time

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Microscopic fields exhibiting valuable information were recorded by photomicrography.

SHORT-TERM TISSUE CULTURE

In some cases, cytological study of the biopsy material in cover-slip preparations does not provide enough distinctive parameters for a final diagnosis. By allowing the cells to remain in a more congenial in vitro environment, a few more characteristics may become evident, while those amenable to observation by the cover-slip preparation method still persist. Longevity in the definitive (more supportive) culture medium; capacity for growth (increase in cell size and cell population); rate and mode of growth, and change in cell morphology may be observed.

The techniques used for these diagnostic biological activities of living cells, were such as to permit a continuous and detailed observation on morphological and other features of the cells without undue interference with the culture. For this purpose three types of cell cultures were usually set up:

(1) Agar cultures on perspex roller slides advocated by Pulvertaft (1965) (Appendix 5)

(2) Cover-slip cultures in Klingerite ring chambers (Pulvertaft 1965) (Appendix 5)

(3) Suspension cultures in 15ml (McCartney's) or 60ml (Medical Flat) bottles.

All cultures were incubated at 37°C.

1. Agar roller slide culture:

Although cells were successfully maintained in vitro on agar-media in the early years of this century, (Lewis and Lewis, 1911) it was only recently that Pulvertaft and co-workers revived this technique by

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advocating the use of the method in short-term cultures of bone-marrow and lymphoid cells (Pulvertaft and Jayne, 1953; Humble et al, 1956; Pulvertaft 1965). It is particularly useful for cytological study of all types of cells capable of in vitro survival for hours or days, but do not adhere to or spread on glass.

The roller-slides used in this study are polished perspex plates, 7.5 x 5 x 0.5cm, in the middle of which is a 0.5cm. wide circular trough surrounding a central platform 2.5 cm in diameter, and opening on to one side of the plate through two narrow parralled vents. The level of the platform is 0.3 cm. below the surface of the plate, to allow room for the disc of agar on which cells are cultured.

The agar used is Bacto-Agar (Difco) kept as 3% stock concentration in distilled water. For use the melted stock agar, is allowed to cool to about 50°C and then further diluted 1 : 3 with definitive culture medium. The feeding vents of the perspex roller slide are sealed with parafilm and enough melted agar filled into the trough until a dome forms on the central platform. A glass slide is then laid gently on the agar, care being taken to avoid trapping of any gas bubbles. The agar is allowed to set at room temperature, and the agar-slide stored at 4°C until use.

In setting up a culture, the glass slide cover is removed, and the agar removed from the trough, care being taken to leave the disc of agar on the central platform undistributed. High vacuum silicone grease is smeared evenly on the top surface of the slide around the trough, and the feeding vents and trough completely cleared of agar. Minute quantities of cell deposits, tissue or marrow fragments are then explanted on to the surface of the agar, and a cover-slip (4 cm x 4 cm) pressed down on to the silicone.

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This compresses the explanted cells between coverslip and agar. The edge of the coverslip is sealed with parafilm and the trough charged with culture medium through the feeding vents.

The explanted cells are compressed and become flattened between coverslip and agar, revealing intracellular detail when examined by phase microscopy. They are bathed in a meniscus of culture medium, the circulation of which is facilitated by placing the perspex slide in a rack which is slowly rotated mechanically by a small motor erected in the incubator (Appendix 6)

The culture medium is changed on alternate days until observation is discontinued.

The agar roller slide was found particularly useful for the study of cytological details, cellular interactions (such as peripolesis and emperiopolesis), and biological processes (such as mitosis motility and phagocytosis) in marrow and lymph node cultures.

2. <u>Cover-slip cultures:</u>

These were set up in Klingarite (plastic) ring chambers. The ring chamber is assembled by fixing the plastic ring (thickness: $\frac{1}{8}$ ins., external diameter: large size $1\frac{1}{4}$ ins, small size 13/16 ins) (Appendix 5) on to a cover slip with high vacuum silicone grease. The chamber is then filled with suspension of cells under study, and the chamber sealed with a glass slide fixed with silicone grease on to the other surface of the ring.

The chamber is then incubated at 37°C with the coverslip downwards, and left undisturbed for at least 18 hours by which time the cells have settled on to the cover slip, and glass-adherent cells would have stuck

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to the surface of the coverslip. Feeding is by removal of the glass slide cover, decanting off the medium, and refilling the chamber with fresh medium. Non glass adherent cells such as red cells and lymphocytes are eliminated by this process, and a selection made for only cells that adhere to glass, and those that in turn adhere to these cells. Frequency of feeding depends on the rapidity of pH fall in the medium, and may vary from daily to weekly.

When the growing cells are well spread out and forming monolayers, the chamber is inverted with the cover-slip upwards, thus permiting the cells to be examined with the high power objectives.

This technique is obviously only of advantage in cultures of glass-adherent cells. Examinations are undertaken as frequently as convenient on a warm stage, noting any observed change in cell morphology.

Cover-slip cultures were routinely sacrificed when good monolayers have formed, and the cells fixed in Zenker's fluid for staining with haematoxylin and eosin. In some cases pahase effect was preserved using osmic acid-ethyl gallate method.

3. Bottle cultures:

The type of bottle used depends on the quantity of available cells for culture. The initial concentration of cells aimed at in setting up primary bottle cultures is 2 x 10^5/ml. Depending therefore on the total volume of the cell suspension, a bijou, Macarthney's or 60ml Medical flat may be used.

Such cultures were primarily set up as stationary suspension cultures and are particularly useful for serial estimations of cell population as an index of culture growth. Frequently a monolayer of cells grows on the bottom of the bottle, and these may then be maintained separately from

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from the non-glass adherent cells.

Bottle cultures were fed by allowing the suspended cells to sediment by gravitation to the bottom of the bottle, removing about half of the supernatant and then making up to the original volume with fresh medium. The removed supernatant was centrifugated, and the deposit of contained cells added back to the culture.

Apart from serial cell counts, serial cell smears may be made for staining. Also serial roller slide cultures may be set up from cell deposits of aliquots of the bottle culture removed at intervals.

Studies on most biopsies in this series fell under the category of phase contrast cytology of dispersed cells, and not tissue culture. Some difficult cases were subjected to "ephemeral in vitro cytopathological study (Pulvertaft, 1965b), by allowing them to survive in vitro just long enough to manifest some pathognomonic biological activity. Except when attempts were made to establish continuous cell lines from biopsy material, the time required for a diagnosis to be made on specimens were usually a matter of minutes. Not infrequently however, diagnosis would only be obvious after several days of observation, when a definite pattern of growth is manifested, such as growth of neurites from an explant of neuroblastoma, or the characteristic pavement pattern of epithelial cells. In this study therefore, cells were generally maintained in vitro only until when characteristic features for definite diagnosis was made, after which the cultures were usually discarded. Some tumours in which the writer was interested were maintained in long-term cultures. The only tumour in this category pertinent to this thesis is the Burkitt's tumour.

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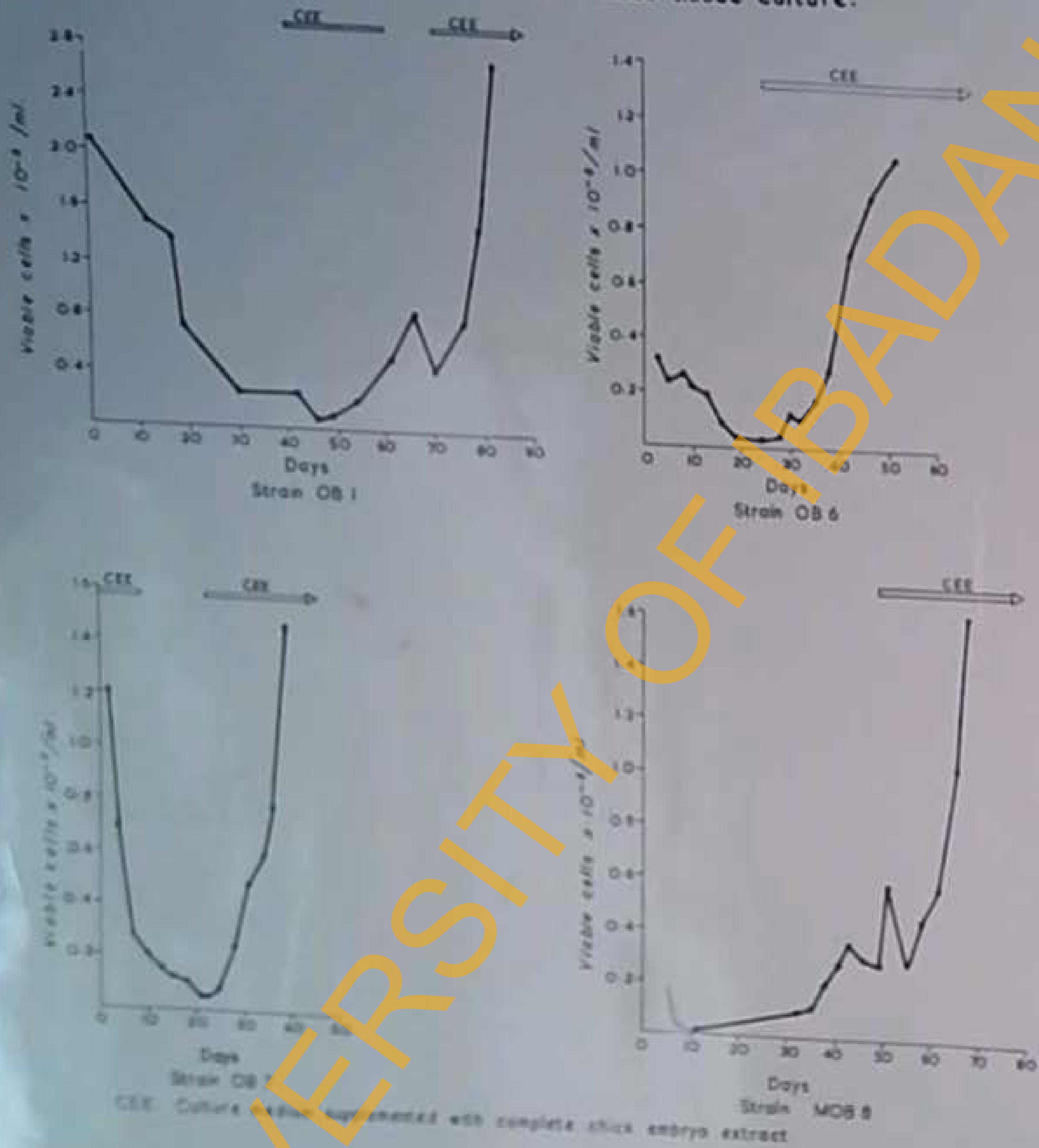

Fig. 20 Growth curves of primary cultures of 4 established Burkitt's lymphoma cell lines. (Plate 6)

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4. Establishment and propagation of Burkitt's tumour cells in long-term tissue culture.

On 14 occasions deliberate attempts were made to establish continuous cultures from Burkitt's tumour material. For this purpose, cultures were set up in 60ml medical flats using a heavy innoculum of cells (2 to 4 x 10^6 per ml)., and the cultures incubated stationary at 37°C. The volume of culture was maintained constant as long as the viable cell population remained above 2 x 10^5 cells/ml; when viable cell concentration fell below this figure, the volume of the culture medium was halved. The cultures were fed (as described for routine bottle cultures) every 2 to 4 days depending on the rapidity of pH fall.

Viable cell counts were carried out after each feeding. Counting was by simple haemocytometer method on a mixture of cell suspensions of culture (0.05 ml) and an equal volume of 0.025% vital toluidine blue (Gurr Ltd.) in TC 199. Viable Burkitt's tumour cells appear intense purple under a phase contrast microscope within 3 minutes of exposure to a dilute solution of this dye. Dead tumour cells do not take up stain, but dying cells may appear faintly stained.

Cultures were regarded established when a progressive increase of viable blast cell population, is maintained. This was usually three or more weeks after explantation, by which time viable blast cell population has greatly diminished (Plate 6). By this time also, a monolayer of histiocytes and fibroblasts would be growing on the bottom of the culture bottle. When the blast cell population rises to about 1 x 10^6 cells per ml., the culture volume is doubled and the suspension culture split and transferred into two bottles. The histiocyte monolayer

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is thence maintained separately for as long as desired. In one instance, blast cells were released into suspension, and harvested regularly from the histiocyte cultures for more than six months.

The established blast cell cultures were maintained as stationary suspension cultures in 120ml medical flats (Appendix 6). Feeding was by replacement of the upper half of culture with fresh medium When the culture becomes very turbid, indicating a high cell content, feeding was by doubling the culture with fresh medium, and dividing it into two bottles.

Failure of some cultures to establish was attributed to inadequate cell innoculum in primary cultures, infrequent or overenthusiastic feeding, "toxic" serum, and/or other factors of unknown nature.

OBSERVATIONS

Of the 296 specimens studied in this series, 150 showed no evidence of neoplasia. There were 66 malignant neoplastic lesions encountered in specimens obtained from individuals aged 20 years or less; of these 46 fulfilled most of the criteria diagnostic of Burkitt's tumour, 6 showed the typical features of Wilm's nephroblastoma, 5 were diagnosed unequivocally as retinoblastoma, while 4 showed characteristic features of neuroblastoma. Other childhood tumours encountered included rhabdomyosarcoma of bladder, ovarian dysgerminoma, granulosa cell tumour, angioblastoma of middle ear, and an embryonal sarcoma of the jaw (Table 8).

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Phase contrast cytology of the Burkitt's tumour.

Biopsies of Burkitt's tumour constantly yielded swarms of single free-floating, non-coherent, spherical, opaque, dark, small, non-motile, amoeboid(?) blast cells with very little variation in size (8 to 12 microns in diameter). A most characteristic feature was the presence in most cells of few but prominent superficially located lipoid granules, usually aggregated at one pole of the cell.

Gentle compression of the cells between cover-slip and glass, or mounting on an agar roller-slide revealed a very high nuclear-cytoplasmic ratio, very pale nucleus with multiple indentations, and large bizzare nucleoli. The scanty cytoplasm was dark and opaque rendering the few mitochondria present barely visible. The refractile cytoplasmic lipoid granules became even more conspicuous and were seen to be freely mobile, and to lie singly or in small aggregates of two or more spherules. Mitosis may be evident in some cells.

In addition to the tumour cells which were always predominant, clumps of dead cells, varying numbers of motile small lymphocytes, histiocytes many of which were loaded with dead as well as viable cells, sequestrated cytoplasmic fragments, scattered extracellular lipoid granules and, of course, red blood cells were invariably present (Fig. 22 and 26).

Atypical appearances of Burkitt's tumour cells.

The appearances described above were so characteristic of materials examined from biopsies of Burkitt's tumour as to make diagnosis in most cases unequivocal. In 6 cases however, atypical features were strikingly obvious in the tumour cells. Three of these were biopsies of solid

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tumours (jaw tumour, inguinal lymph node, cervical lymph node), one was a pleural fluid specimen and one a splenic aspirate. The atypical findings are described case by case and in detail below.

Case 1. TC 6. U.C.H. 137,011.

Boy aged 12 years.

Breathlessness; pleural effusion; abdominal swelling.

Pleural aspirate, haemorrhagic; yielded atypical Burkitt's tumour cells with "undue transparency, and scanty lipoid granules".

Case 2. TC 27. U.C.H. 147,326.

Boy aged 20 years.

Abdominal swelling and massive splenomegaly.

Splenic aspirate: yielded monomorphic lymphoblastic cells with pale transparent cytoplasm high nuclear-cytoplasmic ratio, richly indented nucleus and many lipoid granules. (Fig. 23).

Case 3. T.C. 47. U.C.H. 106,218.

Girl aged 14 years.

Third recurrence after previous surgery and chemotherapy for Burkitt's tumour. Biopsy of subcutaneous nodule on lateral chest wall: yielded lymphoblastic cells with wide variation in cell-size, abundant dark cytoplasm, round nucleus, and little or no cytoplasmic lipoid granules.

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Case 4. TC 87. U.C.H. 152,389.

Girl aged 12 years.

Abdominal tumour and superficial lymph node enlargement.

Biopsy of inguinal lymph node: "Yielded primitive lymphoid cells almost exclusively. The cells are slightly atypical for Burkitt's tumour cells; slightly larger in size with very transparent rather abundant cytoplasm. Cytoplasmic lipoid granules are present and nuclear configuration is similar to that of typical Burkitt's tumour cells"

Case 5. TC 94. U.C.H. 152,790

Boy aged 13 years.

Biopsy of maxillary tumour: Yielded lymphoblasts with some viariation in cell size, and conspicuously rare lipoid granules. The cytoplasm is dark and opaque, the nucleus round with no indentations. (Fig. 24).

Case 6. TC 327. U.C.H. 165,490

Boy aged 10 years.

Superficial lymphadenopathy and ascites.

Biopsy of cervical lymph node; "Yielded large numbers of lymphoblasts, a little larger than typical Burkitt's tumour cells, with abundant pale cytoplasm, and no lipoid granules".

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Atypical appearances peculiar to source of Burkitt tumour material

Although not without exceptions, certain peculiar features were associated with the site of sampling of Burkitt's tumour material.

(i) Cerebrospinal fluid.

Burkitt's tumour cells in most cerebrospinal fluid specimens examined showed a conspicuous absence of lipoid granules (Fig. 25). This peculiarity of Burkitt's tumour cells in cerebrospinal fluid was however not seen in two specimens with very high cell counts (over 800 tumour cells per cm.)

Normal lymphocytes were always present as well, and there may be cells showing features intermediate between the blast cells and lymphocytes. Macrophages were sometimes seen, but not the usual giant-size histiocyte commonly present in solid tumour biopsies. Almost all cells present in freshly taken specimens appear to be viable.

(ii) Ascitic fluid.

Normal small lymphocytes were usually present in large numbers, although the tumour cells were always predominant.

(iii) Bone marrow.

The tumour cells may assume a slightly bigger size than normal. Although most cells appeared viable when first examined, the rapid autolysis of Burkitt's tumour cells in the two marrow biopsies which contained them in this series was very striking. (Plate 11).

The differential diagnosis of Burkitt's tumour.

All the other childhood malignant tumours studied in this series were readily distinguished from Burkitt's tumour by phase microscopy of dispersed cells in suspension. These included retinoblastoma (5 cases),

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neuroblastoma (4 cases), nephroblastoma (6 cases); ovarian dysgerminoma, ovarian granulosa cell tumour, embryonal rhabdomyosarcoma angioblastoma of middle ear and embryonal sarcoma of jaw (1 case each). In addition, each of these types of tumours exhibited distinctive cytological features and growth characteristics of diagnostic value. Only the types in which more than one case has been studied, will be described.

1. Retinoblastoma: - In all cases, the dispersed tumour cells, or tumour cells present in cerebrospinal fluid were seen as small aggregates of monomorphic coherent round small cells arranged in a three-dimentional network of chains. (Fig. 27). The individual cells were about the size of Burkitt's tumour cells, with pale round nucleus and pale scanty cytoplasm with no lipoid granules present. They adhered poorly to glass but survived readily in short-term tissue culture.

All five cases studied showed identical cytomorphology, similar to those described by Pulvertaft and others (Pulvertaft, 1965; Ifekwunigwe et al, 1966). In one case, it was possible to maintain cells from a solid tumour in continuous culture. These cells were however glass-adherent and grew profusely as fusiform cells with bipolar long fine processes; they are at the moment of writing three months old in vitro. It is not evident whether these cells were stromal or tumoural in origin.

2. Neuroblastoma: - Indentical cytomorphological appearances and tissue culture behavior were observed in all 4 cases of neuroblastoma studied in this series. The cells were large coherent and monomorphic. They varied in size and formed large aggregates with no definite pattern of arrangement. The nucleus was enormous, very pale and hemispherical, with multiple shallow indentations. Nucleoli are multiple, prominent

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and bizzare (Fig. 28). Mitosis is infrequent. The cytoplasm is fairly abundant, and moderately dark owing to the presence of numerous particulate dark mitochondria. Lipoid granules were as a rule absent, although the occasional cell may be seen with few such granules. Mutual compression between the tightly packed cells imparts various shapes to the cells; a faceted appearance is typical in freshly dispersed preparation, while there is tendency of assuming a fusiform shape in culture.

Loose bundles of very long and extremely fine dark hair-like fibrils may be seen occasionally between the tumour cells in fresh preparations. They are probably non-myelinated nerve fibrils, since they are not digested by trypsin or collagenase. None has however, been seen arising directly from a cell-body.

Neuroblastoma cells survive readily in ring and agar-slide cultures. A most characteristic feature is the sprouting of many fine fibrils from the margins of cell aggregates, within one week in culture. The earliest period that such neurites were demonstrable was 72 hours after explantation. The cells take some days to spread out, but when they do, they adhere firmly to glass. Similar features were reported by Grant and Pulvertaft (1966).

3. Wilm's nephroblastoma: - Unlike the other round cell sarcomas of childhood, nephroblastoma presents a bimorphic phase cytology picture. Two distinct cell types are recognisable.

(a) closely packed large round dark malignant epithelial cells forming follicular aggregates. The nucleus is round, and the cytoplasm abundant and finely granular.

(b) loosely packed, very small, round or fusiform, dark supporting mesenchymal cells, almost devoid of cytoplasm (Fig. 29).

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Table 3. Data on established Burkitt's lymphoma cell lines maintained as continuous cell cultures.

PATIENT						CELL LINES		
Cell line	Name and Hosp. No.	Age (yrs)	Sex	Therapy at time of biopsy	Mode of diagnosis	Source of primary explant	Duration of propagation (Sept. 1966)	Mode of growth in suspension culture.
[illegible]	[illegible]	[illegible]	[illegible]	Untreated	Histology	Jaw tumour	24 months	Single cells
[illegible]	[illegible]	13	M	Untreated	Cytology Autopsy	Ascitic fluid	15 months	Large clumps
[illegible]	[illegible]	9	[illegible]	Untreated	Cytology	Ascitic fluid	13 months	Small clumps and many single
[illegible]	[illegible]	7	M	Untreated	Cytology Histology Autopsy	Thigh tumour	12 months	Large clumps
[illegible]	[illegible]	8	M	Second recurrence after treatment with [illegible]-Endoxan.	Cytology Histology Autopsy	C.S.F.	10 months	Small clumps
[illegible]	[illegible]	9	F	Untreated	Cytology Histology	Jaw tumour	7 months	Large clumps
[illegible]	[illegible]	6	M	Untreated	Cytology Histology	Jaw tumour	4 months	Small clumps
[illegible]	[illegible]	10 10	M	Untreated	Cytology Histology	Jaw tumour	5 months	Large clumps

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Table 9. Data on established Burkitt's lymphoma cell lines maintained as continuous cell cultures.

PATIENT						CELL LINE		
Cell line	Name and Hosp.No.	Age (yrs)	Sex	Therapy at time of biopsy	Mode of diagnosis	Source of primary explant	Duration of propagation (Sept.1966)	Mode of growth in suspension culture.
OB1	A.D.. 123,251	9	F	Untreated	Histology	Jaw tumour	24 months	Single cells
OB2	I.E 138,617	13	M	Untreated	Cytology Autopsy	Ascitic fluid	15 months	Large clumps
OB3	A.G 142,766	9	M	Untreated	Cytology	Ascitic fluid	13 months	Small clumps and many single
OB4	E.E 144,338	7	M	Untreated	Cytology Histology Autopsy	Thigh tumour	12 months	Large clumps
OB5	A.A 120,853	8	M	Second recurrence after treatment with [illegible] Endoxan.	Cytology Histology Autopsy	C.S.F.	10 months	Small clumps
OB6	L.A 151,997	9	F	Untreated	Cytology Histology	Jaw tumour	7 months	Large clumps
OB7	A.T 157,587	8	M	Untreated	Cytology Histology	Jaw tumour	4 months	Small clumps
MOB8	R.M 155,868	10 10	M	Untreated	Cytology Histology	Jaw tumour	5 months	Large clumps

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The later do not survive in vitro, but the epithelial cells grow readily on glass into enormous cells with the appearance of giant fibroblasts in short term tissue culture.

4. <u>Lymphoblastic lymphoma:</u> - There is a subtle distinction between the blast cells in Burkitt's tumour and classical lymphoblastic lymphoma. Those of lymphoblastic lymphoma are slightly larger, but distinctly paler than Burkitt's tumour cells. The cytoplasm is slightly more abundant, and unduely transparent. The nucleus is usually round and there is tendency to motility. Cytoplasmic lipid granules are also present.

It is of interest to note that the blast cells in some of the "atypical cases" of Burkitt tumour (see pp. 62 and 63) approximate in appearance to the above description. It is also note-worthy that the source of materials manifesting the atypia for Burkitt's tumour cells were superficial lymph nodes and pleural fluid, the later from a case with clinical signs of mediastinal compression, - features unusual for Burkitt's tumour, but characteristic of (non-Burkitt) childhood lymphosarcoma seen elsewhere (Rosenberg et al 1958; Wright, D. H. 1966).

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<u>The Burkitt's tumour cell in long-term tissue culture</u>

Eight cell lines of Burkitt's tumour blast cells (OB1, OB2, OB3, OB4, OB5, OB6, OB7 and MOB8) were established and have been maintained in continuous culture for periods ranging from four months to two years. Three histiocyte cell lines are also being maintained in parallel with their respective blast cell lines (OB6, OB7 and MOB8). Table 9, summarises relevant data of the source from which specimens were obtained for culture.

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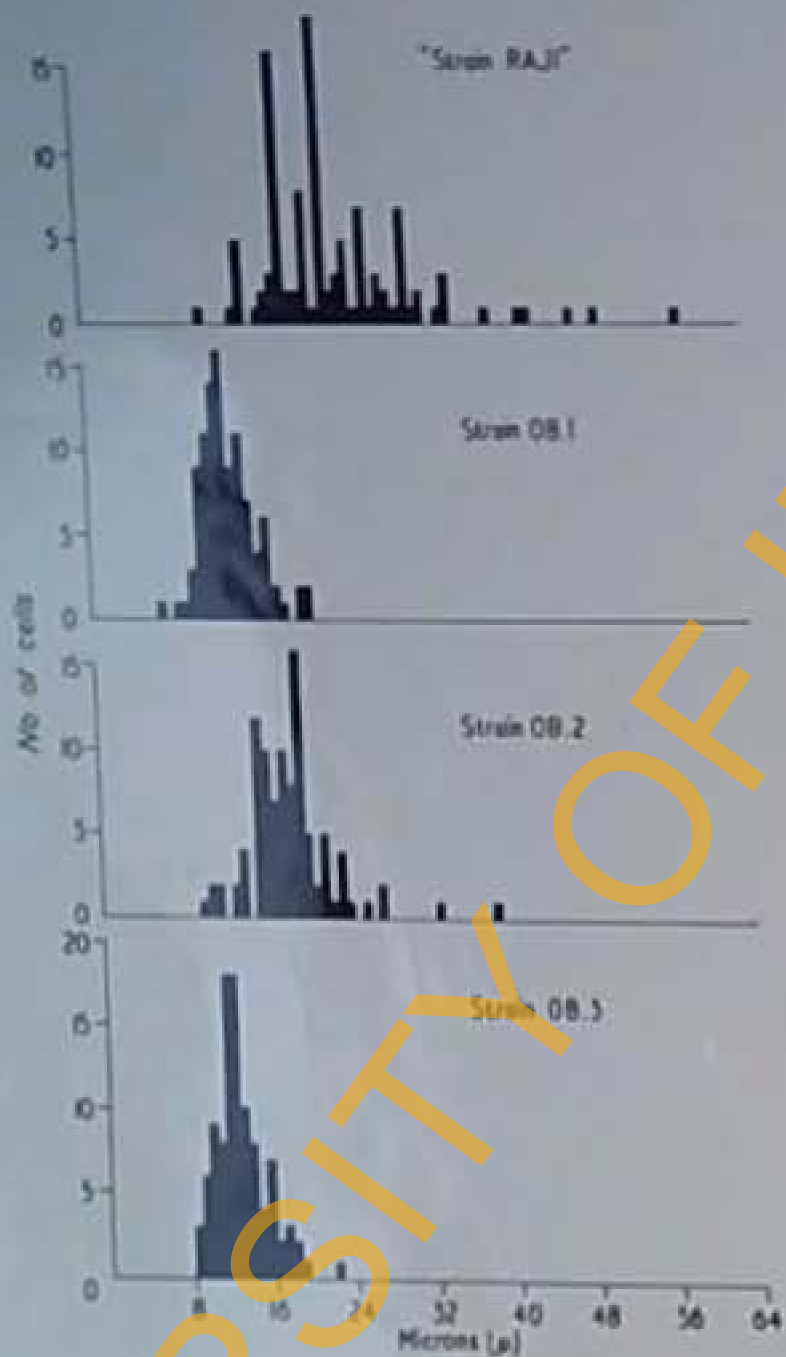
TABLE 10.

TABLE : COMPARATIVE MORPHOLOGICAL STUDY OF 4 ESTABLISHED STRAINS OF BURKITT TUMOR CELLS.

STRAIN	CELL DIAMETER (Microns)		NUMBER OF NUCLEI PER CELL						NUCLEAR INDENTATIONS	CYTOPLASMIC LIPOID GRANULES	MITOCHONDRIA
	Range	Mean Average	% distribution of cells						% cell with one or more nuclear indentations	% cell with lipoid granules	Predominant type
			Nuclei 1	2	3	4	5	6			
"Raji"1*	12.9 - 33.4	20.8	77	14	2	5	1	1	26	77	'Lead-shot' type
OB 1	6.3 - 21.1	14.1	97	3	0	0	0	0	24	92	'Lead-shot' type
OB 2	13.4 - 24.4	18.3	98	2	0	0	0	0	4	8	Filamentous type
OB 3	8.8 - 22.4	13.8	97	3	0	0	0	0	78	98	'Lead-shot' type

* Strain "Raji" isolated by Prof. R.J.V. Pulvertaft.



Distribution of cell size (diameter) of 4 established Burkitt's tumour cells, as seen on agar-roller-slide preparations, by phase contrast microscopy.

Fig. 21 Distribution of cell-size in 4 established Burkitt's lymphoma cells lines.

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All the blast cell lines share certain properties in common. They grow as suspension cultures with no attempt whatever at adhering to glass or forming monolayers. Growth rate depends largely on the batch as well as the concentration of (human) serum present in the culture medium. The cells are serum dependant, optimal growth being attained in 30-35% human serum in TC 199. Continuous cultures can just be maintained in 5-10% human serum medium. Growth is promoted by chick embryo extract, the presence of which is imperative in early passages. The cells transfer easily from human serum cultures, to media containing calf, horse (Burroughs Welcome) or foetal calf (Difco) sera, and survive and grow readily on agar, in plasma clot, and on monolayers (probably feeder layers) of histiocytes, fibroblasts thyroid cells and amnion cells. The cells are readily preserved under the protection of 15% dimethyl-sulphoxide at moderately low temperatures (-50°C) using simple apparatus and little effort (Osunkoya 1965). Cells have been revived after 11 months at -50°C.

Some differences in morphological and growth characteristics exist between cells of the different cell lines. These differences include varying degrees of anisocytosis (Fig. 21), motility, and cytoplasmic lipoid granule content (Table 10 and Plate 19). More obvious and perhaps of some significance is the difference in mode of growth. The cells grow either as single free-floating cells (Strain OB1) as large cell aggregates (Strains OB2, OB4, OB6) or as small cell aggregates with many single cells present (Strains OB3, OB5, OB7 and MOB8) (Plate 16). The strain growing as single cells (OB1) consists of monomorphic cells with a relatively narrow variation in appearance

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and size (Fig. 60, & 74). Those growing in clumps show gross anisocytosis and a marked degree of pleomorphism (Fig. 73 & 75).

The factors responsible for auto-agglutination of Burkitt's tumour cells with formation of small or large clumps in continuous cultures are not clear. It is of interest however to note that topography of the cells forming a "clump" appear to follow a pattern. Observation of an undisturbed large clump, ideally on an agar roller slide, shows a central zone of a few very large cells, surrounded by smaller cells with high nuclear-cytoplasmic ratio, active pseudopodal activity but little or no tendency to motility. Intimately mixed with the latter cell type, and predominating at the surface of the clump, is the distinctly motile cell type (Plate 18, 20 & 21). It should be mentioned that all cell types are capable of mitosis, but by far the commonest cell type in all strains is the rapidly mitosing non-motile blast cell of which the cell strain growing as single cells (strain OB1) is almost exclusively composed. Polyploidy and multipolar mitosis is largely limited to the large cells. (Plate 22).

There is some evidence that gigantism may not be due entirely to polyploidy in blast cells. In stained smears of Burkitt's tumour cells obtained from established continuous cultures, the large cells are seen to be of two types; one with cytoplasmic and nuclear staining appearance of the smaller blast cells, and another giant cell type with chromatin pattern and cytoplasmic pallor characteristic of cells of the histiocytic series. (Fig. 75). Adherence of smaller blast cells to such cells is reminiscent of the phenomenon of peripolesis (Plates 18 & 20).

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ON THE TISSUE CULTURE APPEARANCES OF BONE MARROW FROM BURKITT'S TUMOUR PATIENTS.

A few peculiar features seen in agar-roller slide cultures of bone marrow aspirates obtained from Burkitt's tumour patients deserve some attention. Only a brief summary of such features can be given in this presentation.

The freshly explanted marrow fragment regularly show non-specific but striking features such as marked lymphocytic and plasma cell infilteration (Plate 12), large numbers of eosinophil granulocytes and precursors, abundant intracellular yellow-orange coarse cystalline (probably iron) pigment, and black amorphous presumably malarial pigment within histiocytic cells.

Burkitt's tumour cells were present in large numbers in two out of the ten samples studied in there series. They were not present within the marrow fragments (haemopoietic centres) but lie free as single cells or agglutinated with erythrocytes (Plate 11). Many are seen in mitosis, but all die before cell division is accomplished, usually in metaphase. All the tumour cells die extremely rapidly(within 6 hours of explantation) by nuclear vacuolation in cells in interphase, and "pyknosis" in cells undergoing mitosis (Fig. 40).

Normoblasts, neutrophil granulocytes and precursors, and megakaryocytes are the next cell types to decrease in numbers, the later two by cell death, the former by maturation (of at least a few) to erythrocytes.

Fixed tissue cells (histiocytes, lipocytes, fibroblasts and capillary endothelial cells), motile phagocytic mononuclear cells (macrophages), plasma cells, lymphocytes and eosinophils all persist

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for at least two weeks, and may be present in small numbers after one month in vitro. By this time, there is eminent swamping of all other cell types by proliferating fibroblasts.

All previously described types of cellular association of the small lymphocytes were a common feature. Auto-agglutination, peripolesis, emperipolesis especially in histiocytes (Fig. 56), adhesion to megakaryocytes and "cutting of the cord" of dividing cells in late telophase were commonly seen (Humble et al, 1956; Pulvertaft and Humble, 1956; Synkovics, 1962) . In addition however, lymphocytes were found in intimate association with neutrophil myelocytes.

During the first few days, and as early as 4 hours after explantation, small lymphocytes, were occasionally seen apparently emperipoletic within neutrophil myelocytes. Only one lymphocyte was seen per myelocyte. The lymphocytes remain viable, occupying the centrosomal area of the host-myelocyte, and move continuously in a circle, the centre of which is formed by the centrosome (Figs. 55a & 55b). Such a lymphocyte - myelocyte interaction was watched intermittently for 48 hours during which time neither cells showed signs of dying.

The second rather unusual observation is the fact that the lymphocyte is not the only leucocyte which have affinity for histiocytes in vitro. Marrow cultures from Burkitt's tumour patients have on careful scrutiny consistently revealed eosinophils attracted to histiocytes with which they remain in close association for indefinite periods, sometimes in conjunction with small lymphocytes. (Fig. 57).

Lastly, the appearance in marrow cultures from Burkitt's tumour patients within 4 to 5 days, of rapidly mitosing primitive "Dark cells" was usually impressive. The nature of these dark cells remains obscure,

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although distinctive morphological features were present. These include eccentric reniform pale nucleus, abundant very dark cytoplasm, and a conspicuous thin small ring evident around the centrosome, from which radiate filamentous mitochondria (Plate 13). These cells persist in vitro for about one week after their first encounter. Some form of differentiation takes place in culture, the cells becoming darker, smaller and with some capacity for motility. Appearance of lipid vacuoles in the cytoplasm also becomes evident at this stage (Plate 14).

The origin of these "dark cells" is not known; they are distinguishable from primitive cells of the myeloid series, and appear to belong to the histiocytic class of cells. Transformation from lymphocytes or plasma cells appear remotely possible although there is no evidence. Likewise, derivation from multinucleated giant cells distinct from megakaryocytes and osteoclasts is also a possibility. The constancy and preponderance with which these cells appear after a few days culture of marrow from Burkitt's tumour patients, whether or not tumour cells are present in the aspirate, and their absence from cultures of the few "control" marrows studied in the preliminary part of this work raises the question of whether or not these cells are peculiar to Burkitt's tumour patients, and if so, what their relation to the aetiogenesis, pathogenesis, and natural history of the disease is. Is is undoubted that tissue culture studies of bone marrow from Burkitt's tumour patients is necessary and should be rewarding.

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Table 11. Classification of childhood lymphoblastic lymphomas seen in Ibadan.

Types	Numbers of cases	Age of patients (years)	Phase cytological characteristics			
			Variation in cell size	Nuclear configurations	Cytoplasmic lipoid granules	Cytoplasm
Type 1 Burkitt's lymphoma	40	3-14	Little	Multiple indentations	Conspicuous in most cells	Scanty. Dark and opaque.
Type 2. Atypical Burkitt's lymphoma	2	10+	Wide	Round	Inconspicuous in most cells	Abundant. Dark, and opaque.
Type 3. Classical childhood lymphosarcoma	4	10+	Moderate	Multiple indentations	Variable. conspicuous in many cells.	Scanty. Very pale and transparent

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COMMENT AND CONCLUSIONS

1. Childhood lymphoblastic lymphomas:

These studies support the classification of Burkitt's tumour as a lymphoma. All evidences point to the Burkitt lymphoma cell as belonging to the lymphocytic series of cells. The general phase contrast appearance of the cell, its non-adhesion to glass, the capacity of its more differentiated variant for "Hand-mirror" type of motility, and cell-death by nuclear vacuolation are a few of such evidences. The cell is by and large a very primitive lymphoblast.

O'Conor (1961) in a histopathological study of Burkitt's lymphoma seen in the East Africa, suggested that there were four histologic variants of the tumour. Wright (1963) using histochemical methods on materials from the same part of Africa concluded that Burkitt's lymphoma cells showed "a uniformity of cytological and histochemical pattern that do not justify their division into different histological types". He however considered that there were small viarations in cell maturity both between different tumours, and cells of the same tumour.

In the present study three cytologically distinct types of childhood lymphoblastic lymphomas were recognised by phase contrast microscopy. Table 11, summarises the distinguishing features of the three types. For ease of description and reference they may be designated Types 1,2 and 3.

Type 1. is by far the commonest in this series, with the blast cells exhibiting features which are regarded as typical of Burkitt's lymphoma. The cells have a remarkably uniform appearance.

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and size, and are characterised by the presence of prominent lipoid granules in most cells, as well as scanty cytoplasm with a relatively high refractive index, and thus appearing dark by phase. (Fig. 22).

Blast cells showing similar features were encountered during the present studies among fresh cells from lymph node biopsies with histological appearances of non-specific follicular hyperplasia. Attention has already been drawn to the cytological similarity betwen Burkitt's tumour cells and phytohaemagglutinin transformed lymphocytes. (Pulvertaft, 1964; Wright, 1966a), (see also Plate 24).

Type 2. blast cells show marked variation in size, and differ from Type 1 cells in the absence of lipoid granules in almost all cells, and by the possession of fairly abundant cytoplasm and more or less round nucleus, (Fig. 24). It should be distinguished from the usual appearance of Burkitt's lymphoma cells in the cerebro-spinal fluid, where the cells are no more than type 1 cells with inconspicuous lipoid granules. (Fig. 25).

Type 3. shows minimal variation in blast cell-size, but the cell is distinctly different from the first two because of the low refractive index of the cytoplasm which in consequence appears very pale and translucent under the phase microscope. It however, resembles type 1

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cells in nuclear configuration and presence of lipoid granules (Fig. 23).

The numbers of cases of type 2 and 3 studied in this series are too small for general conclusions to be made. It is perhaps of significance however to note that types 2 and 3 appear to be more frequent in the older child; all the six cases in the two groups were aged over 10 years.

It is now becoming evident that there are some differences in the natural history of Burkitt's lymphoma in the young child and adolescent; jaw tumours are more frequent in the younger age group, while abdominal tumours, particularly ovarian tumours in girls are commoner in the adolescent (Burkitt, 1966b). Whatever may be the cause or causes of such differences, it is possible that another expression of such causes may be the presence of a cytological variant of the typical Burkitt's lymphoma blast cell.

Osunkoya (1966a) concluded from a comparative cytomorphological study of four established Burkitt's lymphoma cell lines that "the malignant cells of Burkitt's tumour comprise a narrow spectrum of cells with variation in degree of differentiation. At one end of the spectrum is the cell variant with little or no tendency to nuclear indentation, scanty lipoid granules and definite tendency to motility. At the other end is the inert cell with a higher nuclear cytoplasmic ratio, large indented nucleus and a high lipoid granule content". He further contended that the cell variants at the first end of the spectrum were more differentiated. If this is the case, then lymphoblastic lymphoma type 2 is no more than a more differentiated type of Burkitt's lymphoma. In support of this concept is the fact that one of the two cases showing

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the type 2 appearance is a girl (case 3, pp. 63), who has had two previous remissions following chemotherapy; it is conceivable that the cells appearing in the recurrent tumour were drug-selected and probably more differentiated blast cells.

The relationship of type 3 lymphoblastic lymphoma to types 1 and/or 2 is not clear. Wright (1966) has drawn attention to the difference between the clinical presentation, gross anatomical tumour distribution, and geographical location of cases with Burkitt's lymphoma as distinct from classical childhood lymphosarcoma. The clinical presentation of the four cases in this series with a phase cytological appearance of type 3 lymphoblastic lymphoma, corresponds to those characteristic of the non-Burkitt-type of childhood lymphoblastic lymphoma (classical childhood lymphosarcoma).

This study therefore supports the distinction of Burkittt's lymphoma from childhood lymphosarcoma described in England and America. It establishes the occurrence of the later type of lymphoma in Nigeria, and demonstrates the ease of distinction between the two by phase contrast cytology.

2. Burkitt's lymphoma cells in continuous culture

Except for a few murine leukaemias, lymphoreticular tumours have been notoriously difficult to maintain in tissue culture (Fischer, 1958). With the successful establishment in continuous culture of the first Burkitt's lymphoma cell line from a Nigerian boy by Pulvertaft in 1964, and the simultaneous report by Epstein and Barr (1964) of similar success, yet another unique feature of the tumour became established.

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Table 12: Cell lines of lymphoblasts from Burkitt's lymphoma established in continous cultures.

Cell line	Investigators	Laboratory	Patient	
			Country	No.
Raji	Pulvertaft (1964)	U.C.H., Ibadan	Nigeria	1
Jiyoye.	Pulvertaft (unpublished)	U.C.H., Ibadan	Nigeria	2
Awo	Pulvertaft (Unpublished)	U.C.H., Ibadan	Nigeria	3.
Ogun	Pulvertaft (Unpublished)	U.C.H., Ibadan	Nigeria	4
EB1	Epstein et al (1964)	Middlesex Hosp. London	Ugandan	5
EB2	Epstein et al (1965(a)	Middlesex Hosp. London	Ugandan	6
EB3	Epstein et al (1965(b)	Middlesex Hosp. London	Ugandan	7
SL1	Stewart et al (1965)	N.I.H., Washington	Nigerian	8
AL1	Rabson et al (1966)	N.I.H., Washington	Nigerian	8
OR	O'Conor and Rabson (1965)	N.I.H., Washington	American	9
OB1	Osunkoya (see Table 9)	U.C.H. Ibadan	Nigerian	10
OB2	Osunkoya	U.C.H. Ibadan	Nigerian	2
OB3	Osunkoya	U.C.H. Ibadan	Nigerian	3
OB4	Osunkoya	U.C.H. Ibadan	Nigerian	4
OB5	Osunkoya	U.C.H. Ibadan	Nigerian	11
OB6	Osunkoya	U.C.H. Ibadan	Nigerian	12
OB7	Osunkoya	U.C.H. Ibadan	Nigerian	13
MOB8	Osunkoya	U.C.H. Ibadan	Nigerian	14

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SL1	Stewart et al (1965)	N.I.H., Washington	Nigerian	8
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OR	O'Conor and Rabson (1965)	N.I.H., Washington	American	9
OB1	Osunkoya (see Table 9)	U.C.H. Ibadan	Nigerian	10
OB2	Osunkoya	U.C.H. Ibadan	Nigerian	2
OB3	Osunkoya	U.C.H. Ibadan	Nigerian	3
OB4	Osunkoya	U.C.H. Ibadan	Nigerian	4
OB5	Osunkoya	U.C.H. Ibadan	Nigerian	11
OB6	Osunkoya	U.C.H. Ibadan	Nigerian	12
OB7	Osunkoya	U.C.H. Ibadan	Nigerian	13
MOB8	Osunkoya	U.C.H. Ibadan	Nigerian	14

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Several cell-lines have since been established from various lympho-haemopoietic cells (Iwakata and Grace, 1964; Foley et al, 1965), but no less than eighteen Burkitt's lymphoma cell lines have been established in long-term cultures (Table 12).

There is no doubt that the Burkitt's lymphoma cell can be maintained in tissue culture indefinitely. Good survival of the cells when preserved by freezing is an additional safeguard for perpetuation. There is thus ample opportunity, unprecedented in human lymphoreticular oncology for extensive immunological, morphological, virological, chemotherapeutic, biochemical and other studies, using these readily available Burkitt's lymphoma cells. Already, cell-lines maintained in the writer's laboratory are being used extensively for experimental studies, some of which will be presented in succeeding chapters.

SUMMARY

1. Phase contrast cytology and tissue culture provided a wide range of parameters for the characterisation of cell types and made possible an easy, quick, and definite differential diagnosis of the common round cell sarcomas of childhood seen at the University College Hospital, Ibadan. In particular, childhood lymphoblastic lymphomas were readily distinguished from retinoblastoma, neuroblastoma and Wilm's nephroblastoma.

2. The Burkitt's lymphoma cell was characterised cytologically as being a primitive cell of the lymphocytic series and constitutes an entity with a narrow spectrum of cytomorphological diversity, the more differentiated variant of which are more likely to be encountered

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in the older age group for the tumour, as well as in recurrent tumours.

The typical blast cells of Burkitt's lymphoma resemble pHA - transformed lymphocytes, as well as some blast cells encountered in reactive lymph nodes.

3. The blast cells of some cases presenting with clinical features typical for childhood lymphosarcoma of temperate countries possess features which distinguishes them by phase cytology from cells of typical Burkitt's lymphoma. It is submitted that these two types of lymphoblastic lymphoma in children are separate entities, and that both do occur in Nigeria.

4. Eight Burkitt's lymphoma blast-cell lines were established in continuous culture. Such cultures should provide useful material for a wide variety of experimental studies, some of which will be presented in succeeding chapters.

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Plate 7. Childhood lymphoblastic lymphoma cells.

Fig. 22. - Burkitt's lymphoma. Typical.

Fresh cells from maxillary tumour. Round non-coherent cells with scanty dark opaque cytoplasm and large pale nucleus with multiple indentations. Few refractile cytoplasmic lipid granules present in some cells.
Note normal motile small lymphocyte with "hand mirror" appearance.
Pressed Cover-slip preparation. Phase contrast. x900

Fig.23 Classical childhood lymphosarcoma.

Fresh cells from pleural effusion. Note profound pallor. Nucleus indented, lipoid granules present.

Pressed cover-slip preparation. Phase contrast. x900

Fig. 24. Burkitt's lymphoma. Atypical.
Fresh cells from maxillary tumour. Note round nucleus, fairly abundant dark cytoplasm with no lipoid granules.

Pressed cover-slip preparation. Phase contrast. x900

Fig. 25. Burkitt's lymphoma. Atypical.

Fresh cells from cerebrospinal fluid. Cells similar to those in Fig. 22, except for absence of lipoid granules.
Cover-slip preparation (not pressed). Phase contrast. x900

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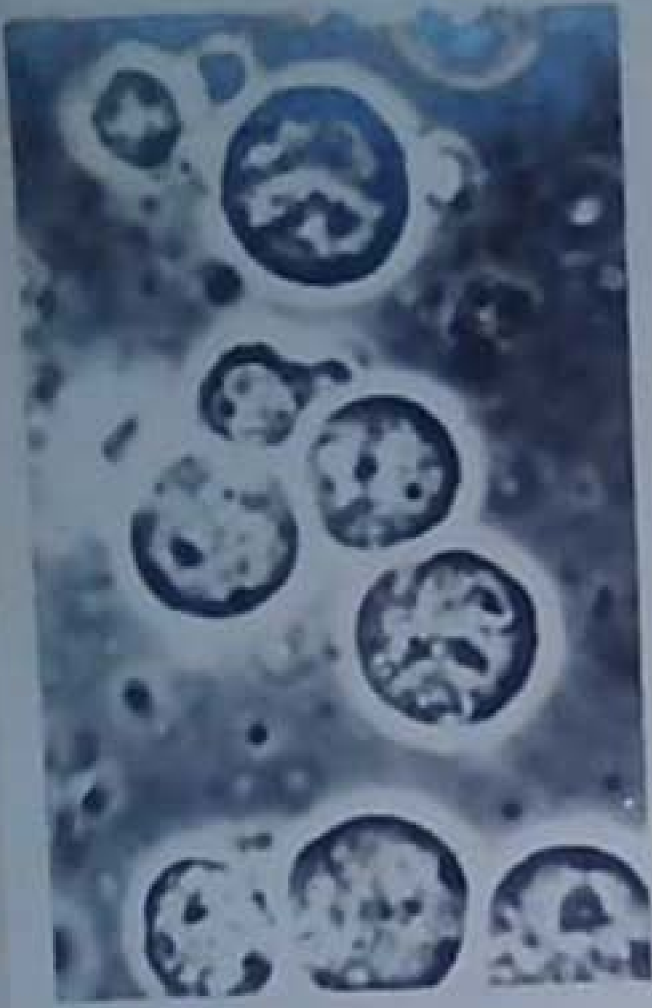

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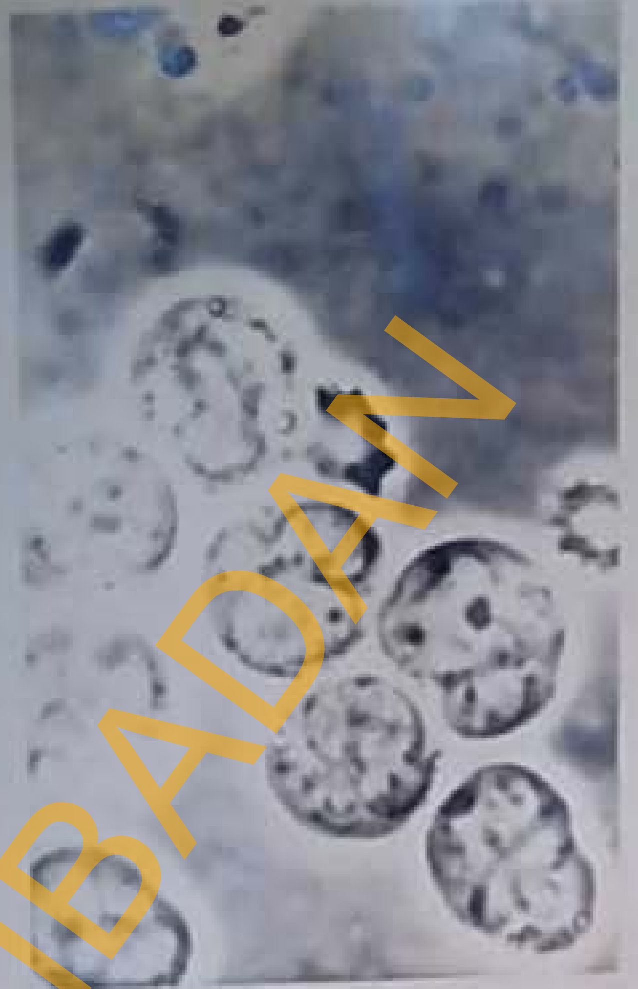

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Plate 8. Round cell sarcomas of childhood.

Fig. 26. Burkitt's lymphoma.

Fresh cells from maxillary tumour. Non-coherent round blast cells with very scanty cytoplasm and few refractile lipoid granules. Note also small lymphocytes, binucleated histiocyte with large phagocytic vacuole, and scattered sequestrated cytoplasm.
Coverslip preparation. Phase contrast. x 450.

Fig. 27. Retinoblastoma.
Fresh cells from C.S.F. Tightly coherent faceted monomorphic cells forming chains. Note large pale nucleus and scanty featureless cytoplasm.
Pressed coverslip preparation. Phase contrast. x 1000

Fig. 28. Neuroblastoma.
Fresh cells from ascitic fluid. Tightly coherent faceted monomorphic large pale cells with large richly indented nucleus, abundant cytoplasm with many mitochondria. Pressed coverslip preparation.
Phase contrast x 1000.

Fig. 29. Wilm's nephroblastoma.
Fresh cells from abdominal tumour.
Loosely aggregated cells of two types; dark round cells in clumps with supportive fusiform cells having little or no cytoplasm.
Pressed coverslip preparation. Phase contrast x 1000

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Plate 9. Malignant lymphomas; cell types.

Fig. 30. Lymphoblastic lymphoma
Three lymphoblasts and two small lymphocytes. All are capable of motility.
Pressed coverslip preparation. Phase contrast x 1000

Fig. 31. Mixed lymphohistiocytic lymphoma
Many small and large lymphocytes and a well differentiated histiocyte with intracytoplasmic pyknotic nuclear debris.
Coverslip preparation. Phase contrast x 1000

Fig. 32. Hodgkin's paragranuloma
Two malignant reticulum cells of Hodgkin's paragranuloma with two peripoletic lymphocytes. Note enormous folded nucleus, giant nucleolus and abundant refractile clumps of cytoplasmic lipoid granules.
Pressed coverslip preparation. Phase contrast x 1000.

Fig. 33 Hodgkin's granuloma
Large histiocytic cells with peripoletic large lymphocytes.
Pressed coverslip preparation. Phase contrast x 1000

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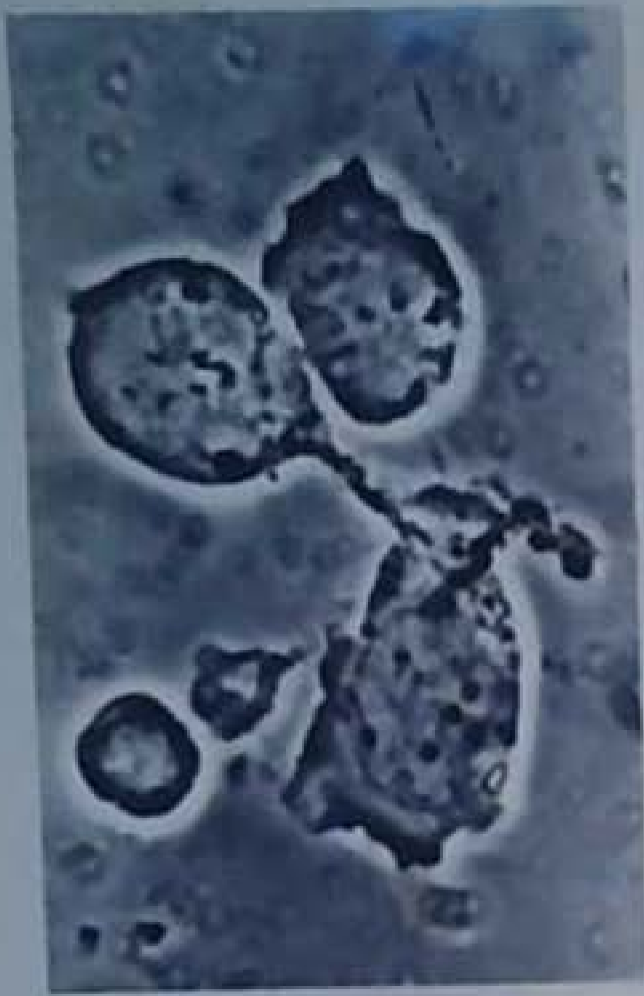

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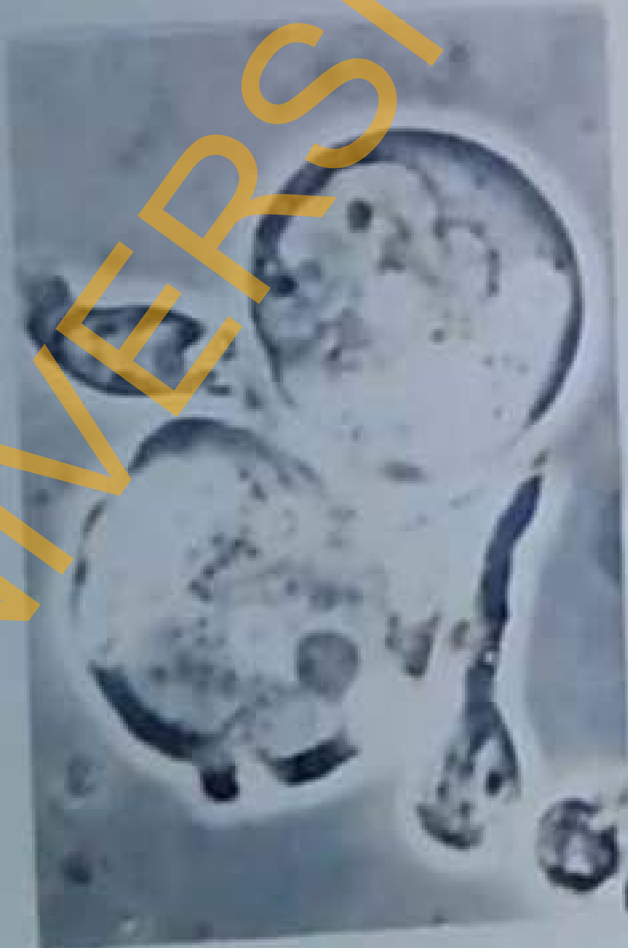

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Plate 10. Malignant lymphoma; cell types.

Fig. 34. Hodgkin's granuloma
Sternberg-Reed cell with 3 emperipolotic small lymphocytes.
Coverslip preparation. Phase contrast x 450.

Fig. 35-37 Reticulum cell lymphoma
"Reticulum cells" and many small lymphocytes.
Note close similarity of the reticulum cells to lymphoblasts
Coverslip preparations. Phase contrast x 450.

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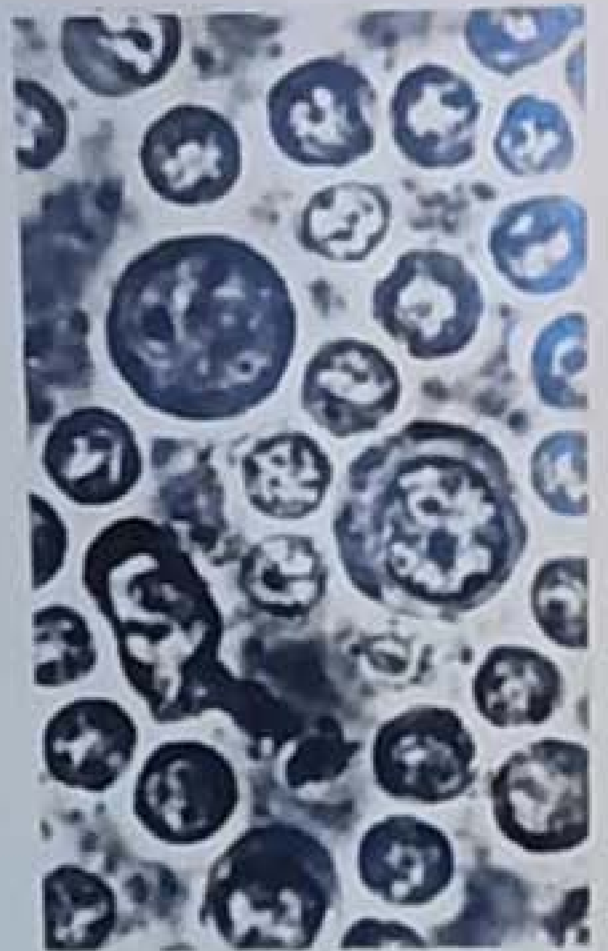

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Plate 11. Burkitt's lymphoma cells in bone marrow

Figs 38(a) and 38(b). Fresh Burkitt's lymphoma cells and red cells in marrow aspirate. Note large pale nucleus with trefoil appearance, dark scanty cytoplasm and few refractile lipoid granules.
Pressed coverslip preparations. Phase contrast x 1000

Fig. 39. Clump of fresh Burkitt's lymphoma cells surrounded by red cells. Agar roller slide. Phase contrast x 450.

Fig. 40. Same field 2 hours later, showing death of all lymphoma cells. Cell death is by nuclear vacuolation in resting cells, but cells in mitosis give a false "pyknotic nucleus" appearance, due to clumping and condensations of the chromosomes.

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Plate 12. The bone marrow in Burkitt's lymphoma. Lymphocytic and Plasma cell infiltration.

Fig. 42. Marked lymphocytic infiltration. An isolated aggregate of normal small lymphocytes.

Roller slide culture (4 days) Phase. x 1000

Fig. 43. Marked plasma cell infiltration. An isolated aggregate of plasma cells in the centre of a marrow fragment.

Roller slide culture (6 days) Phase. x 1000.

Fig. 44. Two plasma cells, one medium lymphocyte and portions of fibroblasts.
Roller slide culture (9 days) Phase. x 1000

Fig. 45. Centre of marrow fragment showing 4 plasma cells, 2 primitive cells and [illegible] fibroblasts.

Roller slide culture ([illegible] weeks) Phase. x 1000

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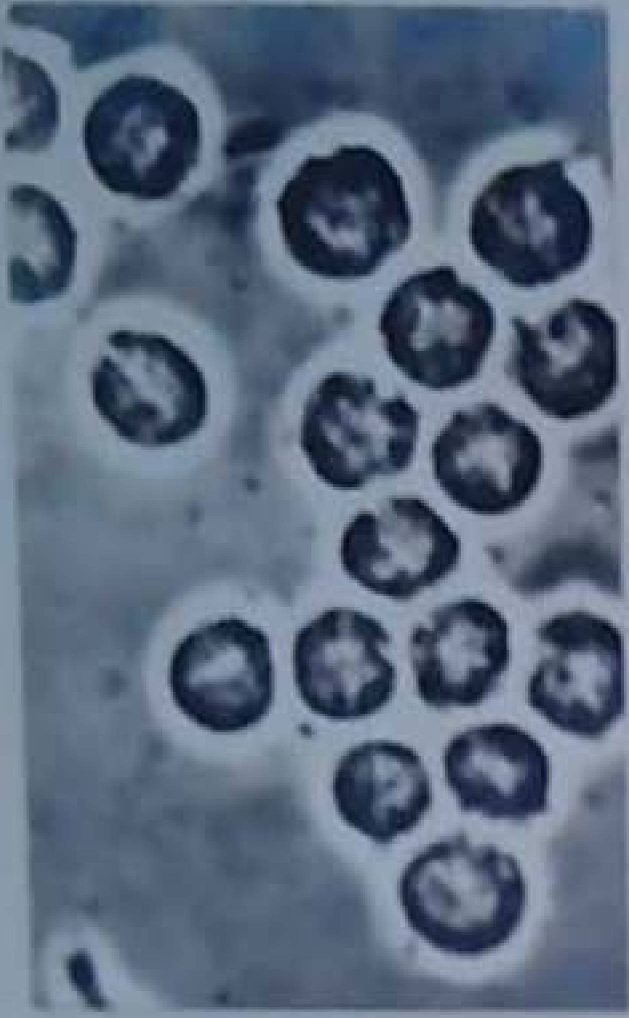

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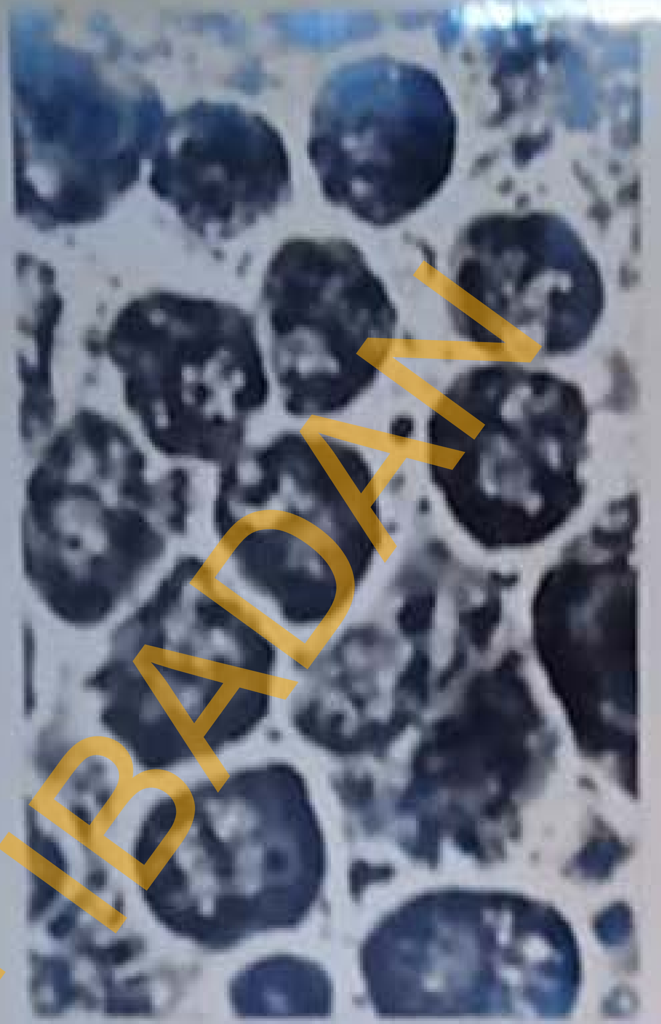

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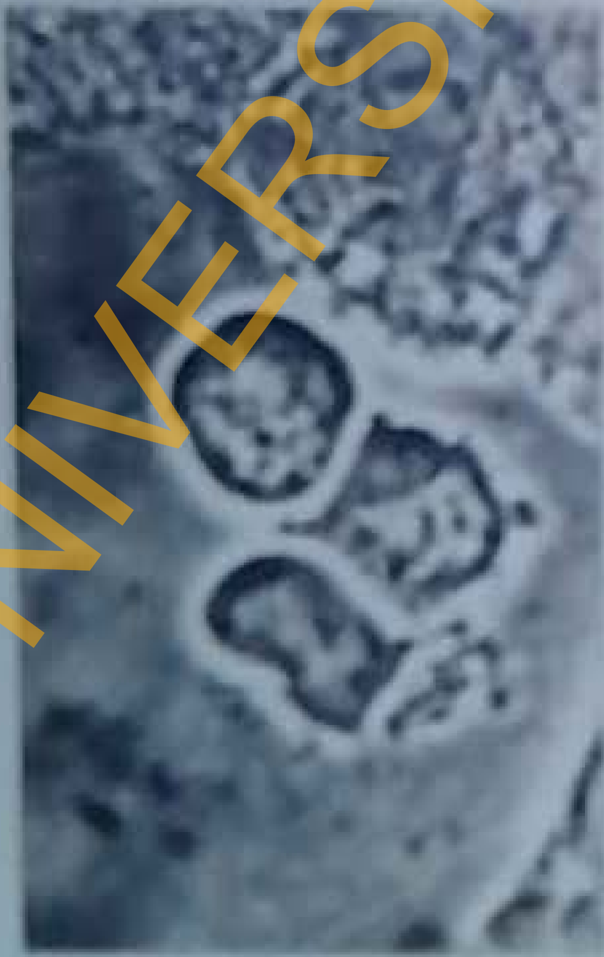

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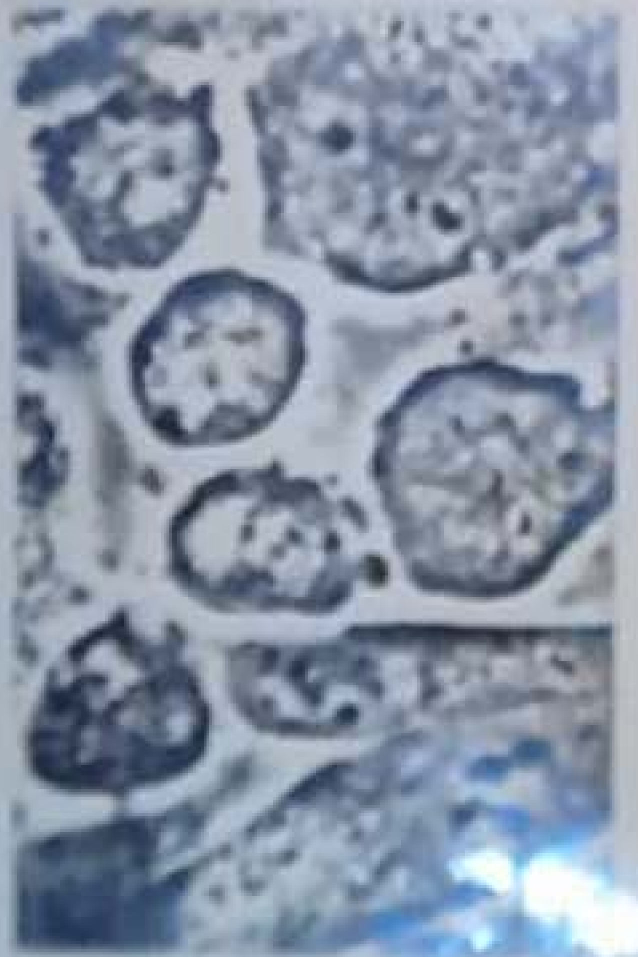

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Plate 13. The bone marrow in Burkitt's lymphoma. Proliferating dark primitive cells.

Fig. 46. Dark cell in early telophase
Roller slide culture (2 weeks) Phase. x 900

Fig. 47. Four dark cells and 2 plasma cells in centre of marrow fragment.

Fig. 48 & 49. Two Dark cells and portions of histiocytes. Note eccentric large pale reniform or sausage shaped with prominent nucleoli. Cytoplasm is abundant, dark loaded with mitochondria, and contains a curious centrosomal ring, from which radiate filamentous mitochondria.

Roller slide culture. (9 days) Phase contrast . x900

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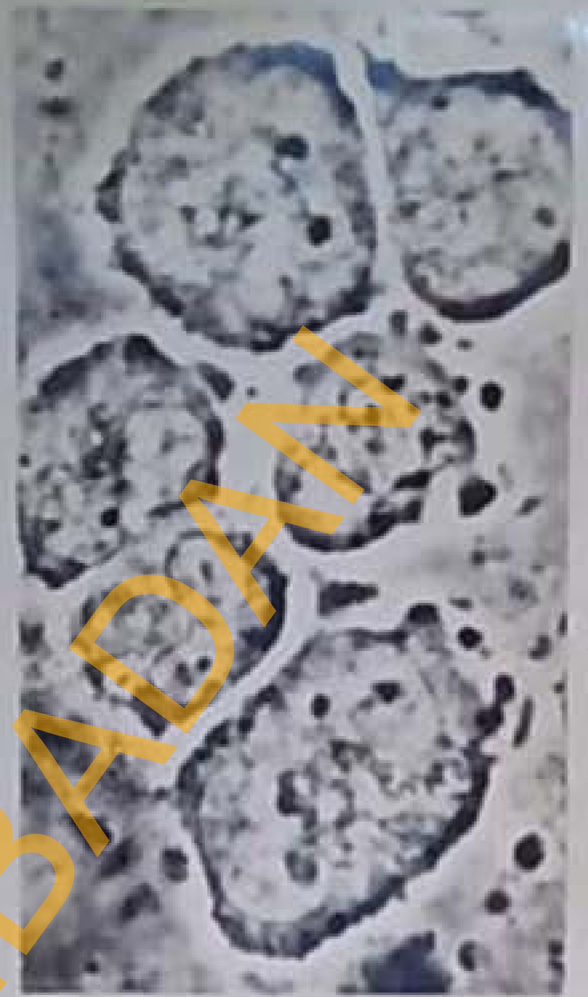

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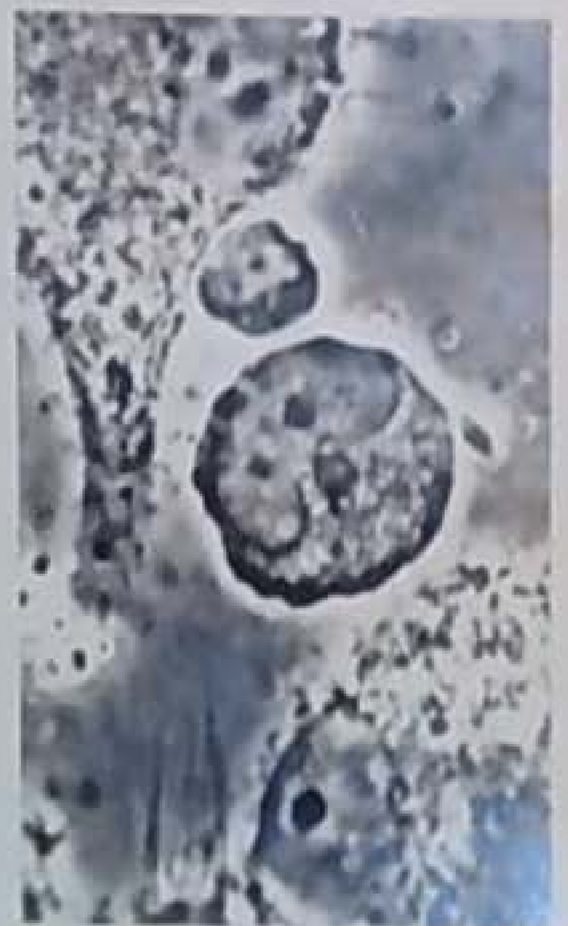

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Plate 14. The Bone marrow in Burkitt's lymphoma. Degerate 'Dark cells, and ? precursor cells.

Fig. 51 & 52.

The dark cells show signs of degeneration with time, but retain mitotic activity and acquire capacity for motility. Note vacuolated cytoplasm.

Roller slide culture (12 days) Phase contrast. x 900

Fig. 53 and 54.

Probable precursors of Dark cells. Non-motile, multinucleated giant cells which usually show synchronous mitosis in all nuclei, or divide into several large dark cells, with "centrosomal ring".

Roller slide culture (3 days) Phase. x 1000

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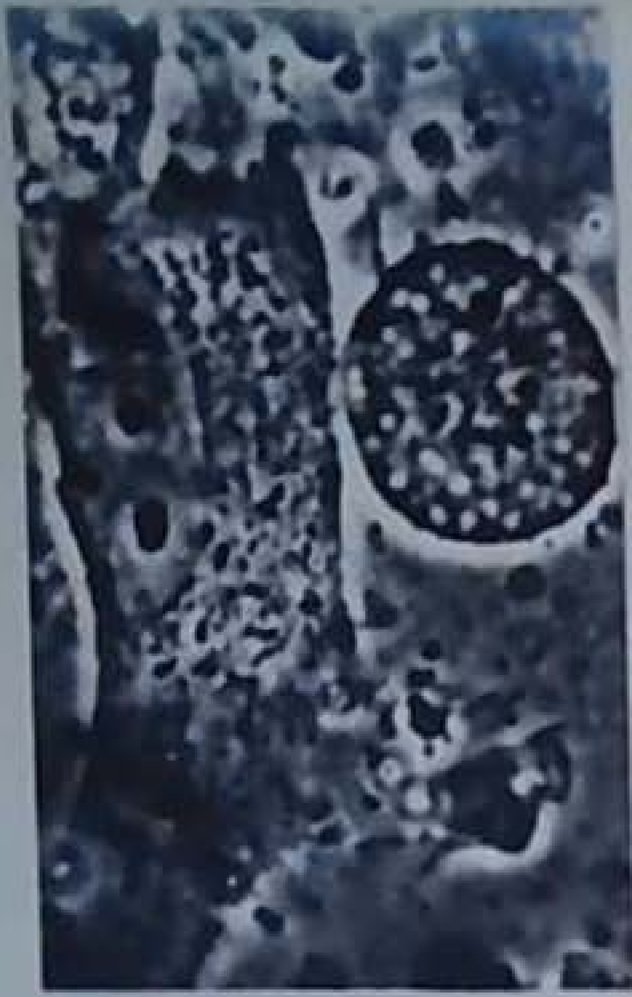

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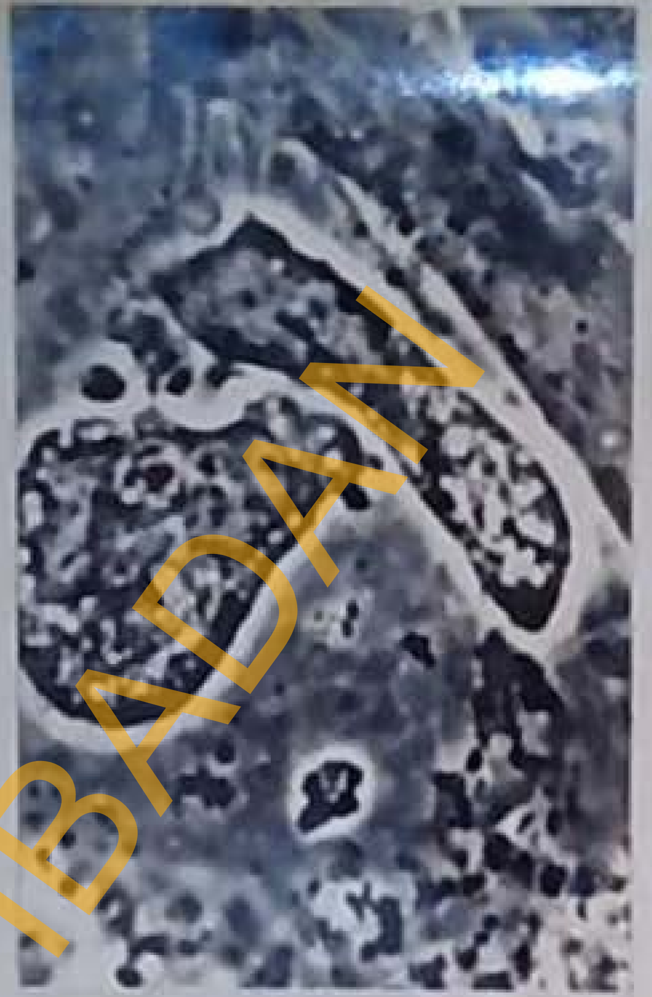

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Plate 15. The bone marrow in Burkitt's lymphoma. Cellular interactions.

Fig. 55(a)

Motile, viable small lymphocyte emperipoletic in neutrophil myelocyte.

Fig. 55(b)

Same field 10 minutes later demonstrating motility of lymphocyte.

Roller slide culture (2 days) Phase x 900.

Fig. 56. Emperipolesis.
Three viable motile lymphocytes in a histocyte.

Roller slide culture (2 days) Phase x 1000

Fig. 57. Emperipoletic eosinophil.
Motile Eosinophil granulocyte (arrow) on or in a large histiocyte.

Roller slide culture (5 days) Phase x 1000.

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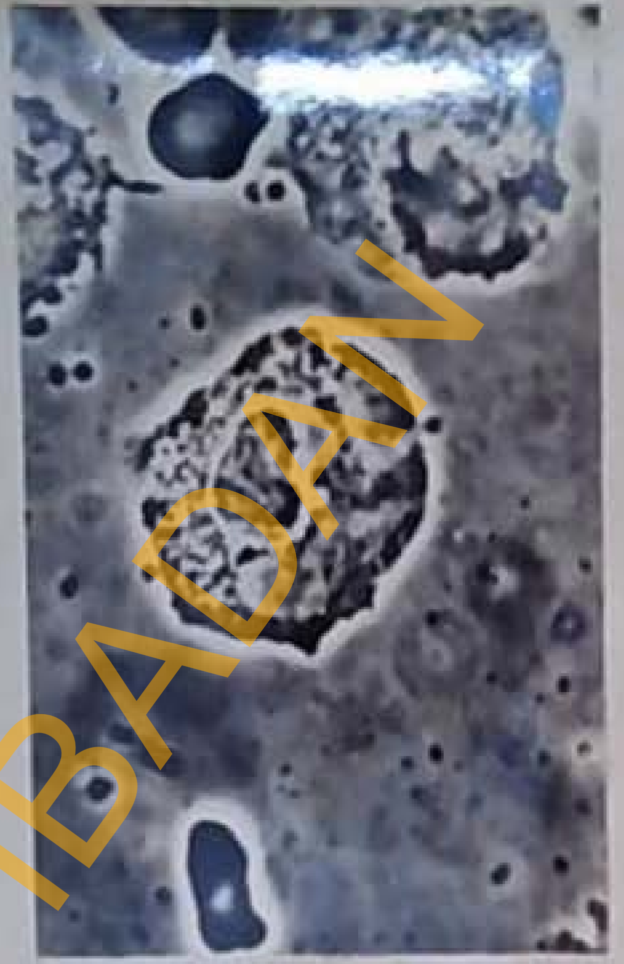

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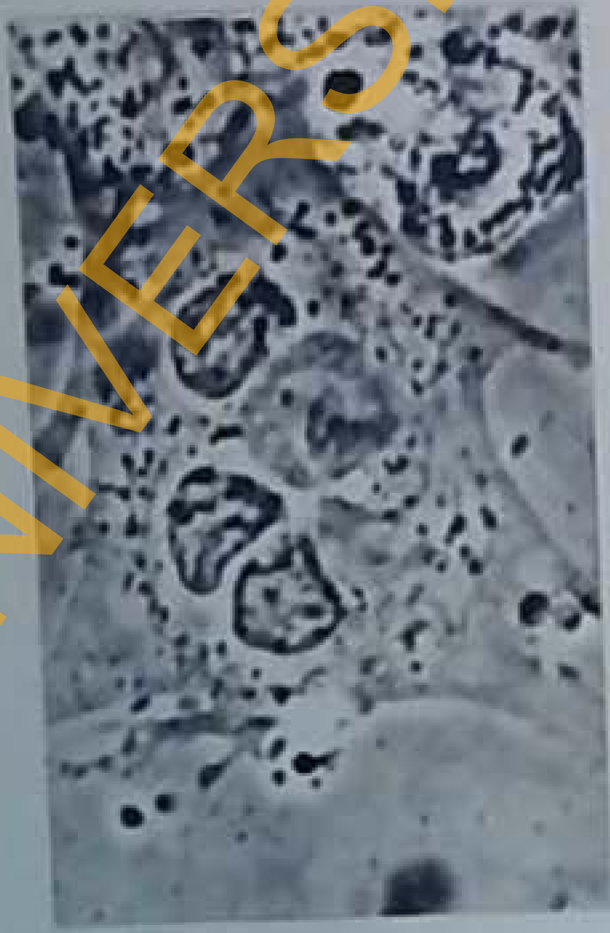

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Plate 16. Mode of growth of Burkitt's lymphoma cells in long-term cultures.

Fig. 58. Strain CB2. Large clumps of cells.

Fig. 59. Strain CB3. Small clumps and single cells.

Ring cultures. x 1000

Fig. 60. Strain CB1. Single cells.
Coverslip preparation. Phase contrast. x 200

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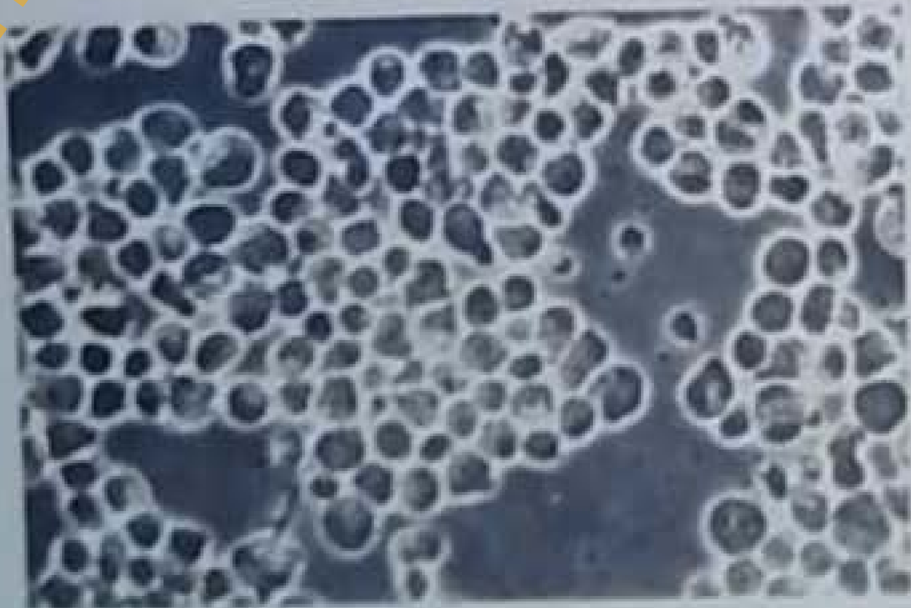

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Plate 17. Cytoplasmic lipoid granules of Burkitt's lymphoma cells.

Fig. 61. Imprint (touch) smear of tumour biopsy.
Note cytoplasmic vacuoles.
Methyl alcohol. May-Grunwald Giemsa x 1000

Fig. 62. Blast cells from established strain (CB1)
Refractile cytoplasmic lipoid spherules,
joined together by short, thin, rigid rods.
Pressed coverslip preparations. Phase contrast. x1000

Fig. 63. Blast cells from established strain (CB3)
Note numerous vacuoles.
Smear. Methyl alcohol. May-Grunwald Giemsa. x450.

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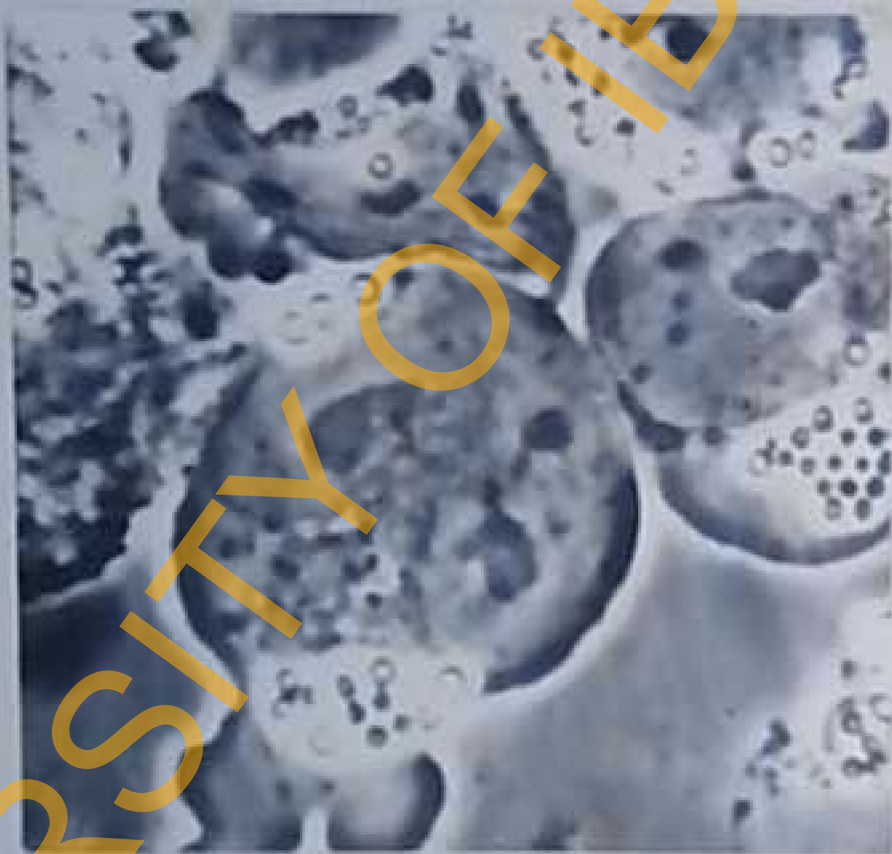

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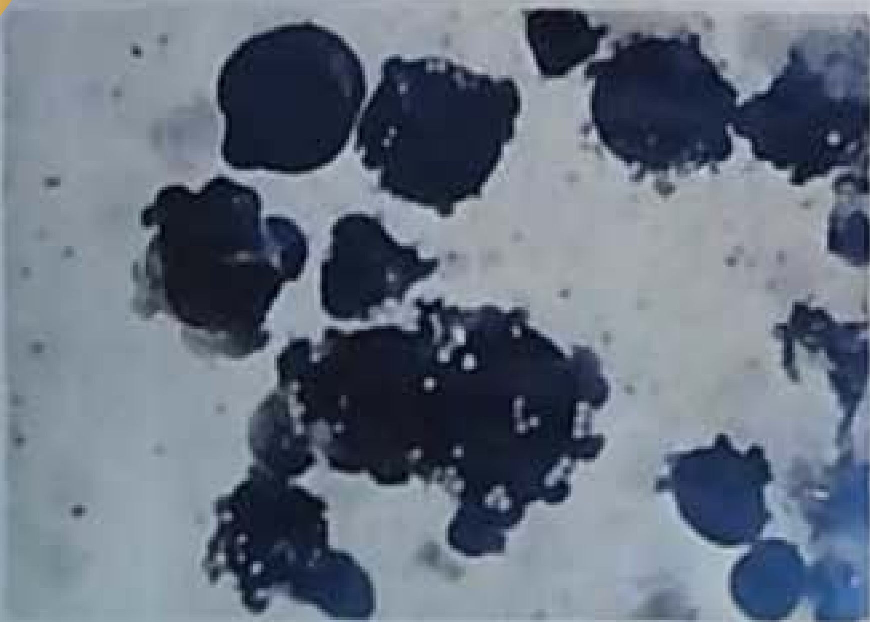

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Plate 18. Pattern of auto-agglutination in Burkitt's lymphoma cell cultures.

Fig. 64. Strain CB6. Large clump of cells.
Note giant cells in centre of clump with blast cells forming periphery of clump.

Roller slide culture. Out of phase. x 200

Fig. 65. Strain CB6. Small clump of cells.
Central large cell with "peripolastic" blast cells.
Note free motile cell at upper part of clump.

Roller slide culture. Out of phase x 200

Fig. 66. Strain CB6. Small aggregate of cells.
Mixture of cell types; very small lymphocytes mostly at surface of clump, many blast cells some in mitosis, and a giant cell showing multipolar mitosis, occupying a central position.

Pressed coverslip preparations. Phase contrast. x 450

Fig. 67. Strain CB2.
Two distinct cell types present; cells with dense hyperchromatic nucleus (?lymphoblasts and lymphocytes) surrounding three large pale staining cells with reticulated nuclei (? primitive histiocytic cells).

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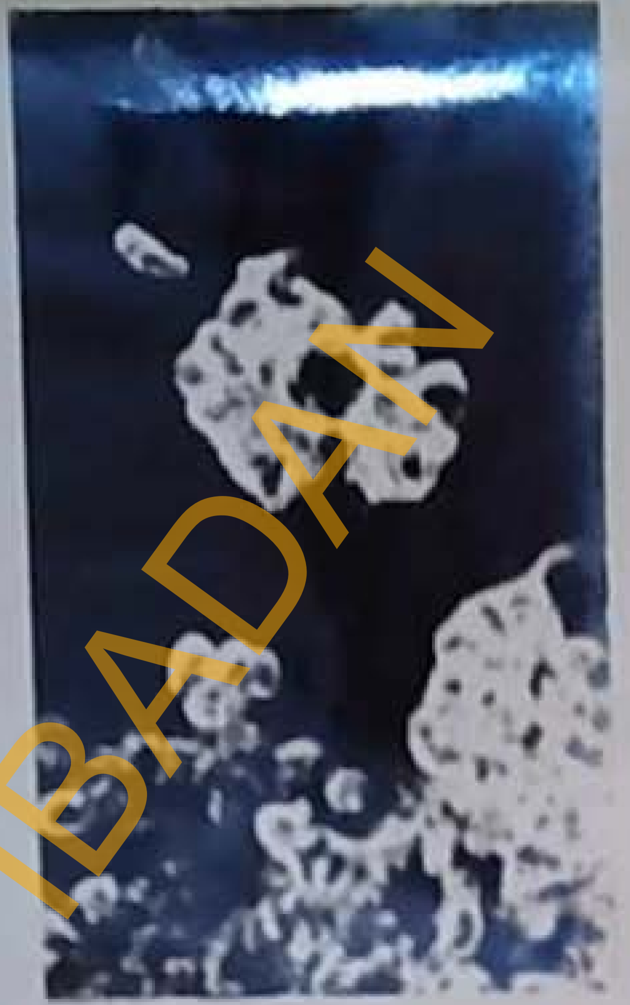
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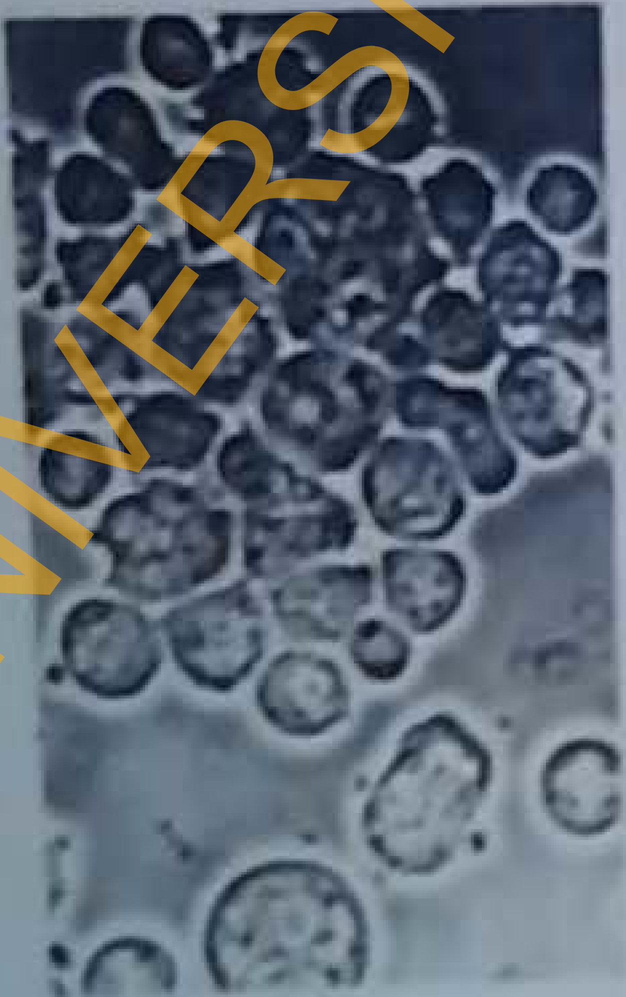
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Plate 15. Diversity of cell types in Burkitt's lymphoma cell cultures.

Fig. 68. Strain OB2. Two cell types distinguishable.
(a) Blast cells - Large, with high nuclear cytoplasmic ratio, large nucleus with indentations, many cytoplasmic lipoid granules.

(b) Lymphocytic cells - Smaller, with round nucleus, abundant cytoplasm and no cytoplasmic lipoid granules.

Roller slide culture. Phase contrast x 900

Fig. 69. Strain OB2.
Distinctly motile cell with hand mirror appearance characteristic of lymphocytes, but membranous pseudopodia reminiscent of histiocytic cells. Note round nucleus, and abundant cytoplasm free of lipoid granules.

Roller slide culture. Phase contrast x 900

Fig. 70 Strain OB1.
Non-motile blast cells with high nuclear-cytoplasmic ratio and many cytoplasmic lipoid granules.

Roller slide culture. Phase contrast x 900

Fig. 71. Strain OB1.
Two non-motile blast cells, and two showing slight tendency to motility.

Roller slide culture. Phase contrast. x 900

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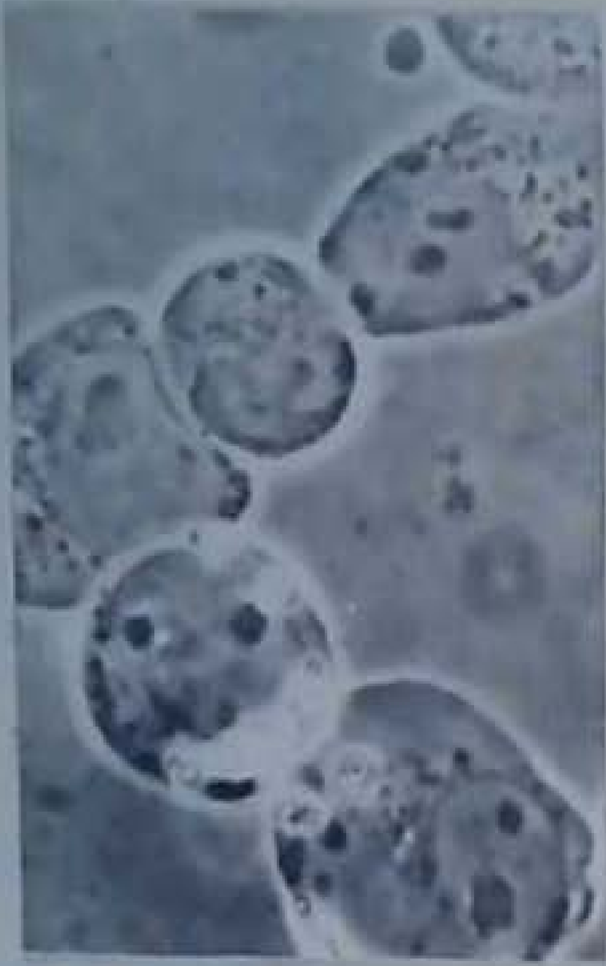

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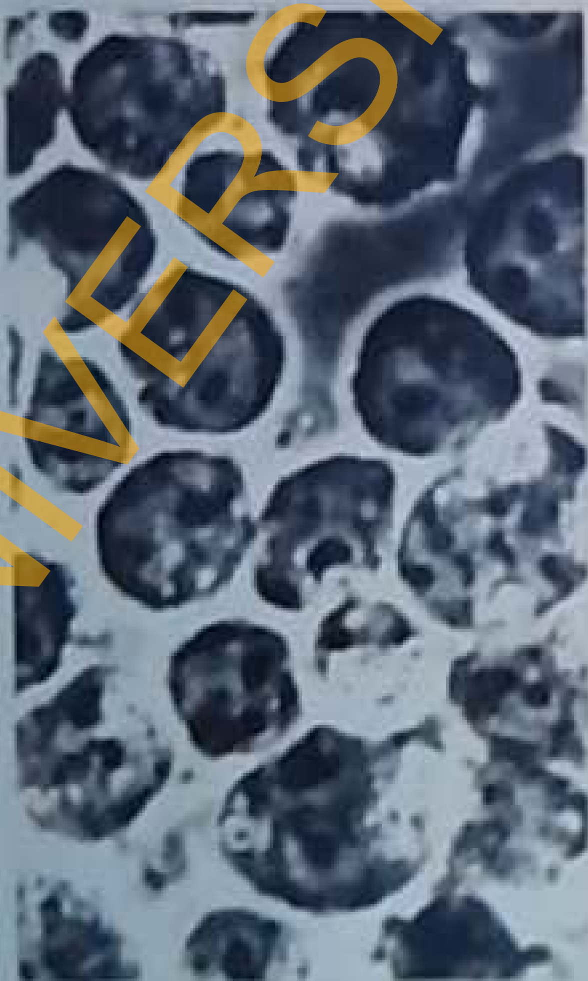

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Plate 20. Auto-agglutination of cultured Burkitt's lymphoma cells. - ? a peripolotic phenomenon.

Fig. 72. Strain OB3.
Small aggregate of cells giving the impression of a central large cells with adherent smaller cells.

Smear. May-Grunwald Giemsa. x 490.

Fig. 73. Strain OB6.
Large clump of cells with cytomorphological diversity. Cluster of small hyperchromatic cells in centre of clump obscuring a giant cell to which most of the other cells forming the clump are adhered.

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Plate 21 Burkitt's Lymphoma blast cells, maintained in vitro.

Fig. 74 Strain CB1.

Round monomorphic blast cells with high nuclear-cytoplasmic ratio, very scanty dark cytoplasm with lipid vacuoles. Note non-coherence, little variation in size and appearance. Smear. Methyla alcohol. May-Grunwald Giemsa x 1000.

Fig. 75 Strain CB4.

Pleomorphic, anisocytotic, coherent cells with giant forms present. Four cell variants distinguishable:

(i) small cells with dense round nucleus little or no apparent cytoplasm with no vacuoles (?lymphocytes)

(ii) medium sized cells with large notched nucleus, fairly abundant dark, richly vacuolated cytoplasm. Predominant type. (lymphoblasts);

(iii) field shows one very large binucleated cell, with nuclei of unequal size (one deeply notched), abundant dark richly vacuolated cytoplasm. (? giant heterophoid form of the blast cells, produced in vitro);

(iv) another very large cell, quite different in appearance from the binucleated one just described; note elongated reticulated nucleus and abundant very pale cytoplasm with very few large vacuoles. (? primitive cell of histiocytic series).

Smear. Methyl alcohol. May-Grunwald Giemsa x 1000.

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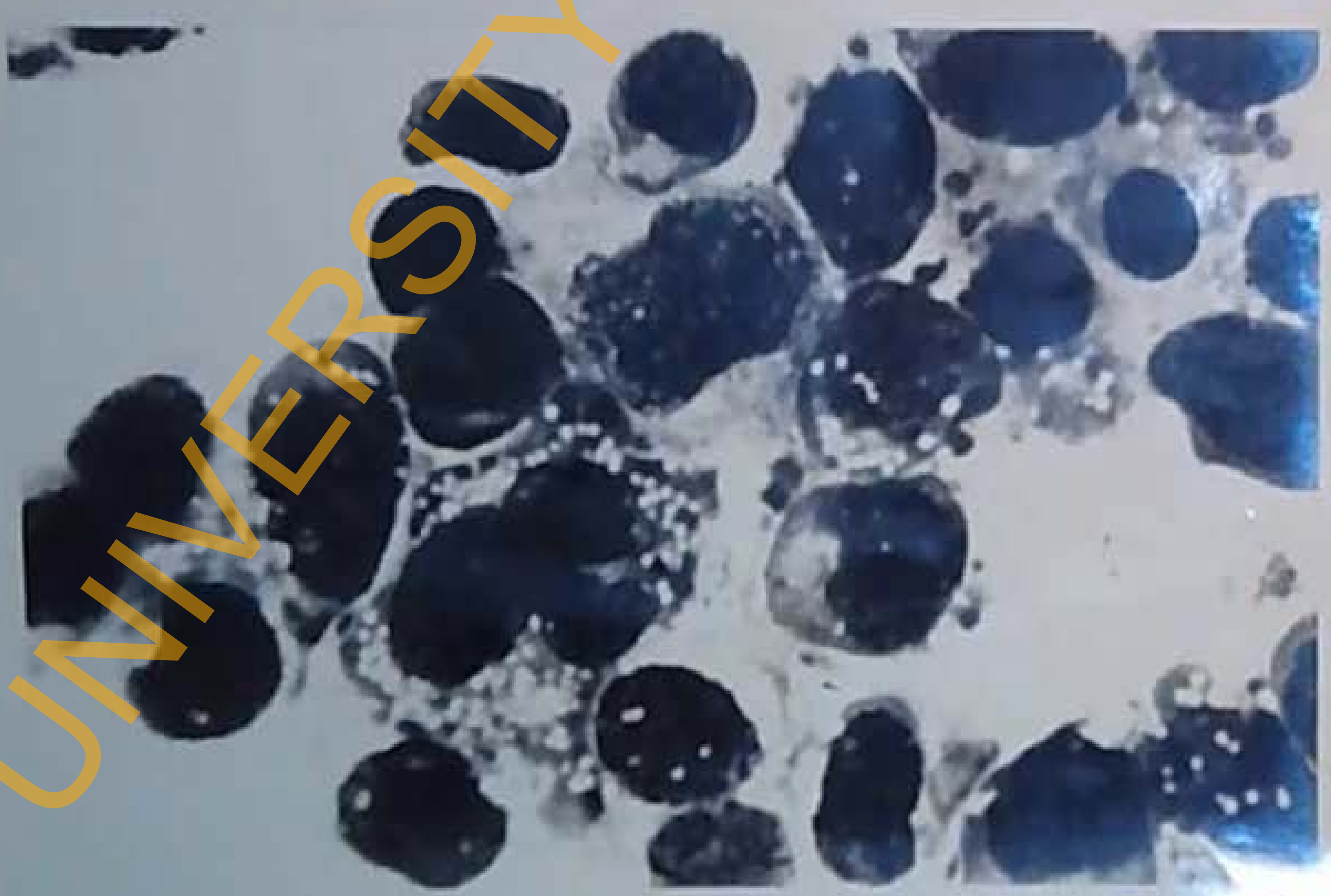

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Plate 22. Normal and multipolar mitoses in cultured Burkitt's lymphoma cells.

Fig. 83-86. Various sequential stages during mitosis in two Burkitt's lymphoma cells (OB2). Multipolar mitosis in giant cell and normal mitosis in smaller cell. Microphotograph taken at 30 minutes interval (37°C). Four daughter cells produced after 2 hours, one binucleated.

Roller slide culture (4 days) Phase contrast. x 900

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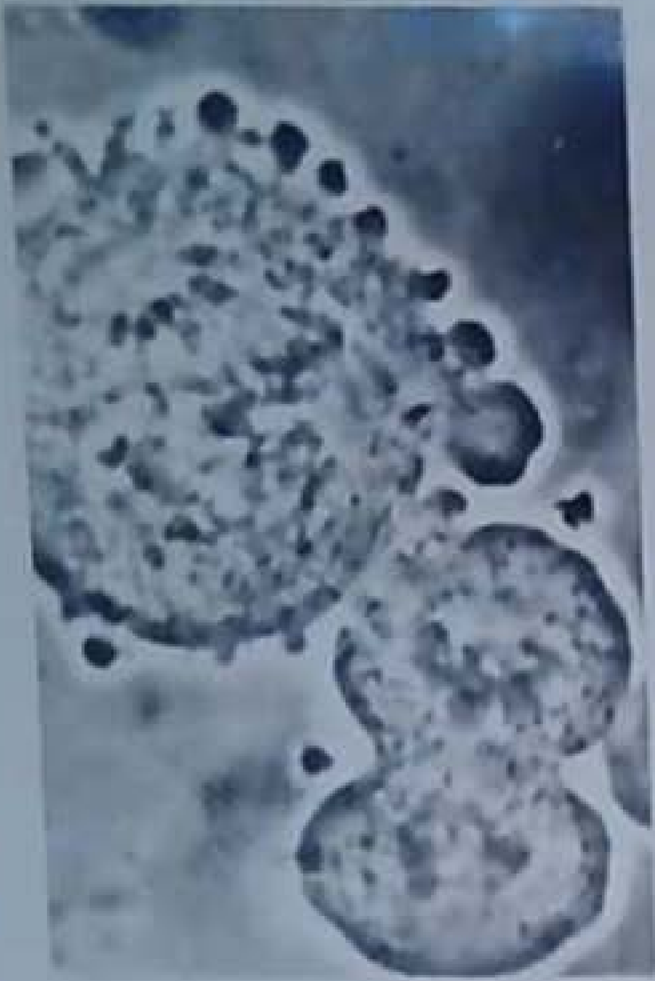

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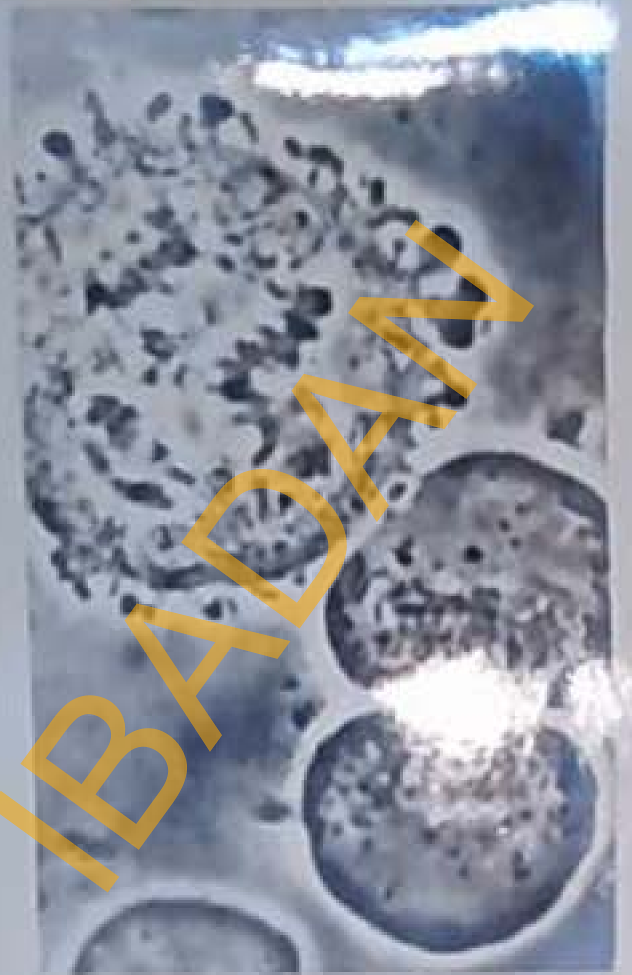

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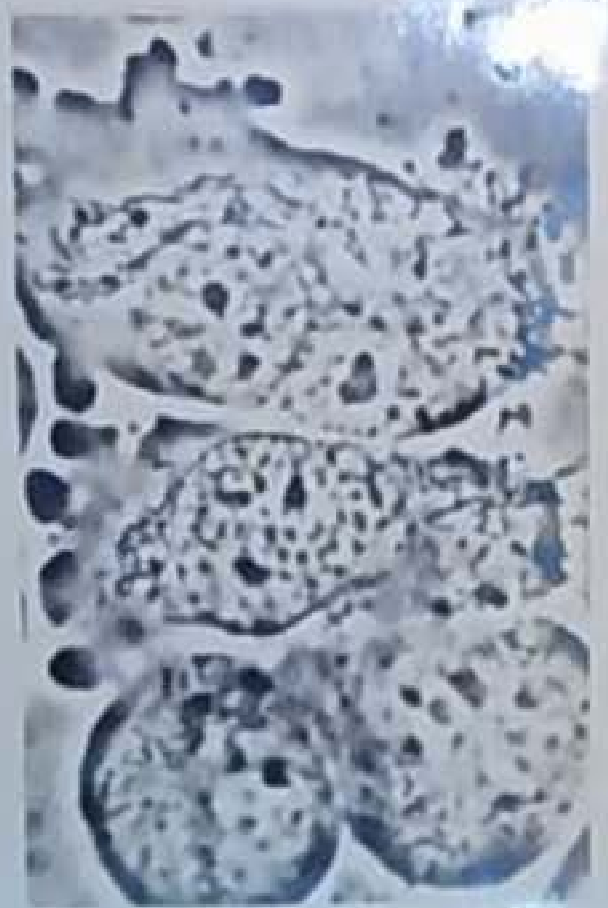

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Plate 23. Interaction of blast cells and histiocytes in Burkitt's lymphoma

Fig. 88. Fresh cells from maxillary tumour.
Large histiocyte loaded with cellular debris. Note many blast cells adherent to the histiocyte.

Cover slip preparations. Phase contrast x 200

Fig. 89. Over thirty viable Burkitt's lymphoma blast cells (OB1) emperipoletic in giant histiocyte from bone marrow of a Burkitt's case.

Ring culture (3 days) Phase contrast x 200

Fig. 90. Histiocyte with several intracellular pyknotic nuclei, and surrounded by blast cells.

Imprint (touch) Smear. May-Grunwald Giemsa x 900

Fig. 91. 'Starry-sky' appearance. Note large histiocyte containing many intact viable cells as well as dead (pyknotic) nuclei.

Section H and E. x 450.

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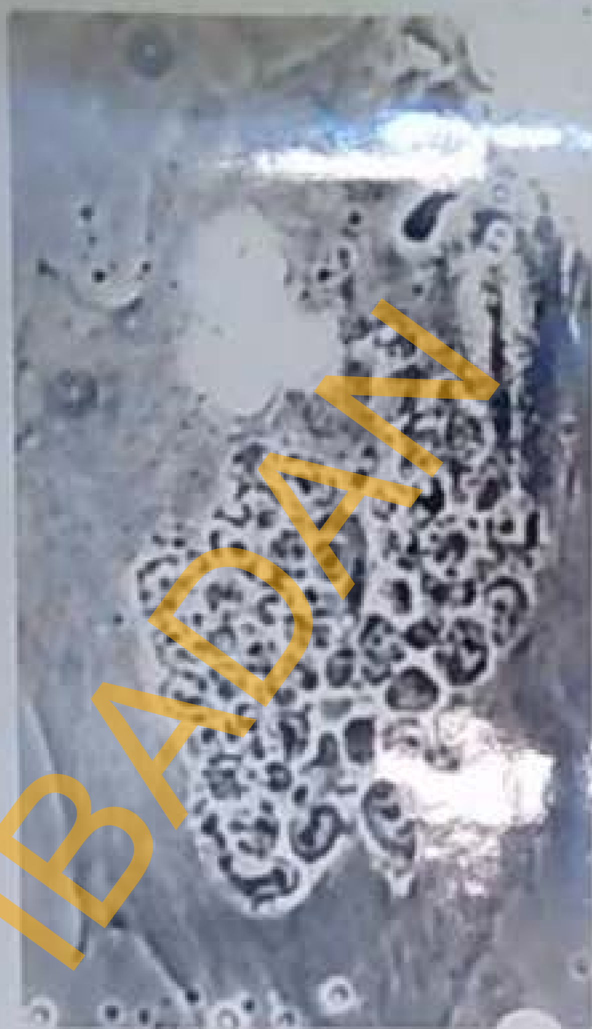
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Plate 24. Burkitt's lymphoma cells and transformed lymphocytes.

Fig. 92. Burkitt's lymphoma blast cells. (Strain OB3). Roller slide culture. (4 days) Phase contrast x 900

Fig. 93. Phytohaemagglutinin transformed lymphocytes. Peripheral blood culture. Note close similarity to Burkitt's lymphoma blast cells in Fig. 92.

Roller slide culture. (4 days). Phase contrast x 900

Fig. 94. Blast cell in a culture of peripheral blood lymphocytes from a case of Burkitt's lymphoma, to which a homogenate of fresh autochthonous tumour material was added.

Roller slide culture. (4 days). Phase contrast. x 900

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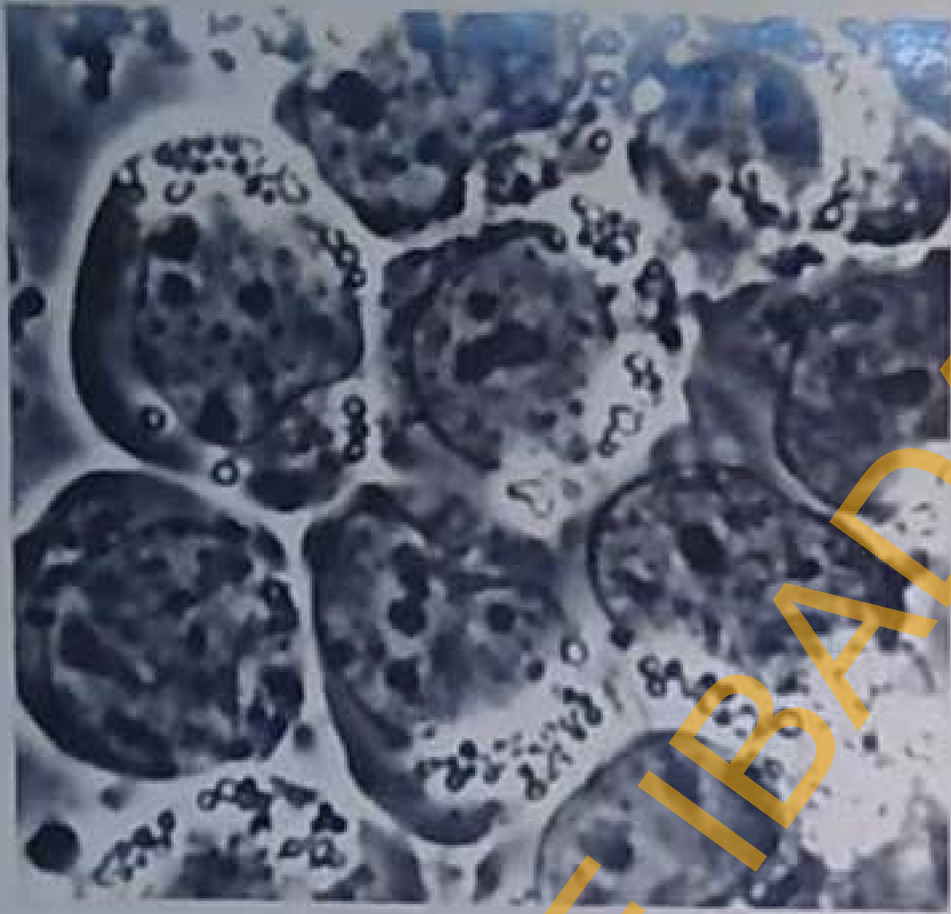

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CHAPTER IV

WORKING HYPOTHESIS FOR CERTAIN ASPECTS OF THE IMMUNOPATHOLOGY OF BURKITT'S LYMPHOMA.

During the preliminary studies on the phase cytology of Burkitt's lymphoma outlined in Chapter III, the freshly dispersed blast cells from the lymphoma were observed to undergo rapid lysis under certain conditions. Such rapid "autolysis" had been noted by Pulvertaft (Pulvertaft, 1964; Pulvertaft and Platt, 1963), who however thought it was due to the effect of trypsinisation.

Most cells were dead within 48 hours of non-enzymatic dispersal of cells from solid tumour biopsies, washing in TC 199, and then resuspending in "regular" culture medium, which contained 30% human serum obtained from donors at the U.C. H. Ibadan Blood bank. The blast cells from ascitic fluid or cerebrospinal fluid also manifested rapid death in regular culture medium, but appeared to remain viable for longer periods if left standing in the autologous serous fluid samples in which they were collected. It was also noticed that marrow aspirates containing Burkitt's lymphoma cells thrived better when incubated in suspension in the heparinised TC 199 into which the sample was collected, than when the cells were explanted on agar-roller-slides charged with regular culture medium.

Blast cells from Burkitt's lymphoma biopsy material collected, processed and cultured in TC 199 containing 30% autologous serum showed much better survival than when the same tumour biopsy was collected into

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and cultured in regular culture medium.

From these observations, it was strongly suspected that what was innocuous to the freshly dispersed blast cells was the human serum present in the processing or culture medium, since it was the only material common to all situations in which rapid cell death was observed. It was also thought that presence of serum from Burkitt's lymphoma patient was at least innocuous, and probably encouraged blast cell sruvival, actively.

On the basis of these impressions, it was thought necessary to carry out more careful and systematic studies to determine the effect, if any, of human sera on the survival and growth of Burkitt's lymphoma cells in vitro.

Preliminary Experiment 1.

Biopsy of a Burkitt's lymphoma of the jaw was collected into a dry universal bottle, and the cells dispersed into and washed twice in TC 199 by centrifugation at 250g for 5 minutes. The washed cells were resuspended in TC 199 the volume of which was adjusted to give a cell population of 6×10^6 cells per ml. of suspension. 0.1ml of the cell suspension was then added to each of three tubes containing 2ml of 30% adult human serum from a blood donor, serum from the patient from whom the jaw tumour biopsy was taken, and serum from a treated case of Burkitt's lymphoma in remission, respectively. The serum samples were fresh.

Serial viable cell counts were carried out on each culture at 6 hours, 54 hours and at 72 hours, the criteria of viability being phase contrast appearance and vital toluidine blue uptake.

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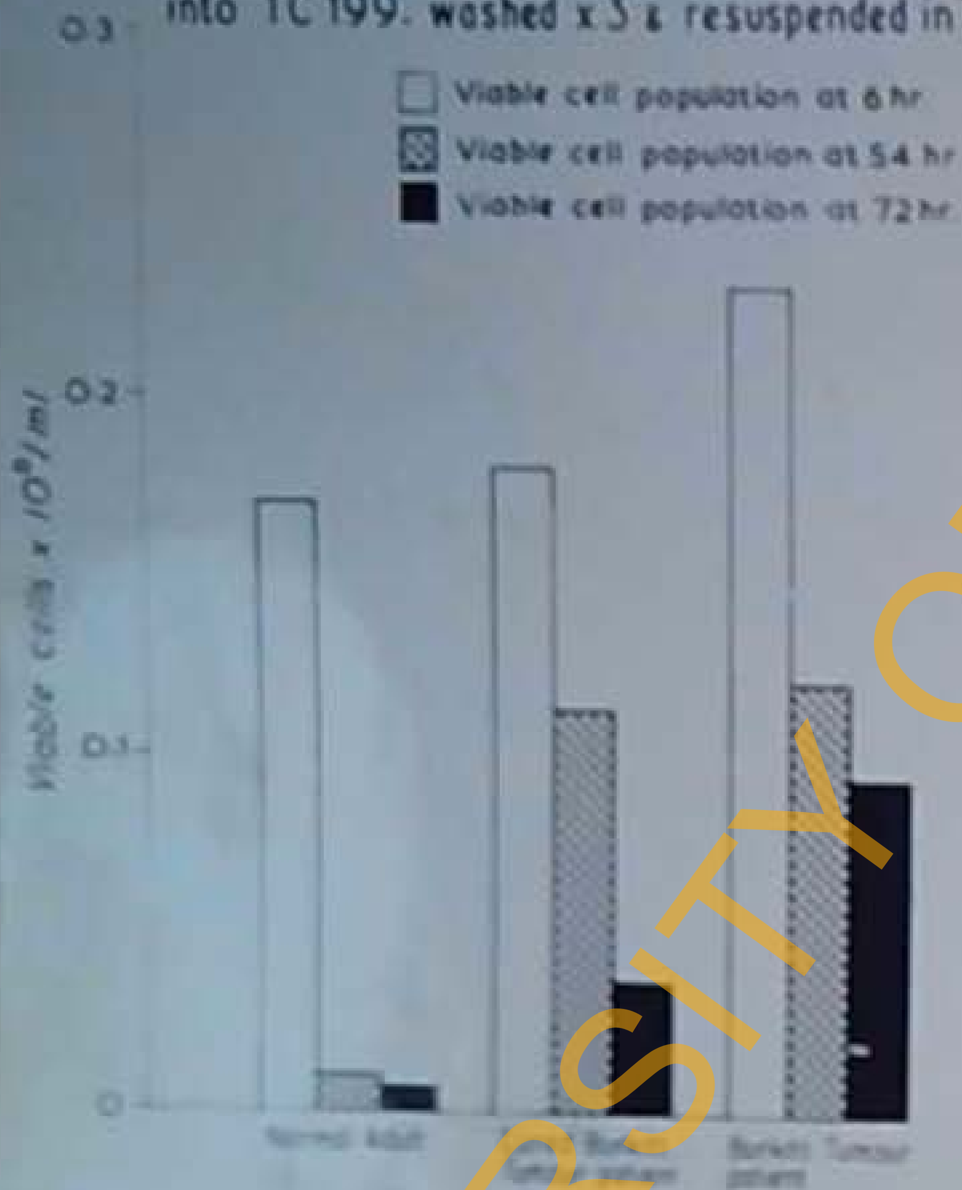
Biopsy Jaw Tumour: collected into dry sterile bottle & processed immediately. Cells squeezed into TC 199, washed x 3 & resuspended in TC 199



Survival of freshly isolated Burkitt tumour cells in Sera from normal indigenous adults and Burkitt tumour patients

Fig. 35 Survival of fresh Burkitt's lymphoma cells in sera from healthy indigenous adults and Burkitt's lymphoma patients. (Preliminary Expt. 4)

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Fig. 95 depicts the relative degree of blast cell survival in culture media containing the serum samples tested. The cells survived best in autologous serum, while the worst survival was in the indigenous adult human serum; almost all the cells were dead at 54 hours in the latter serum culture.

The results of this experiment confirms the previous suspicion of "toxic" effect of serum of indigenous adult Nigerians on freshly isolated Burkitt's lymphoma cells in vitro. The inhibition of cell-survival was much less in the serum samples from both the treated and untreated Burkitt's lymphoma patients.

The single samples tested in this experiment precluded generalisations, and as such, more serum samples were tested on the next available Burkitt's lymphoma biopsy.

Preliminary Experiment 2.

The serum sample tested were from 18 adult Nigerian blood donors, 9 untreated Burkitt's lymphoma patients including the patient (B20) from whom the target test cells were obtained, and 4 Burkitt's lymphoma patients in remission. All the sera except the autologous sample had been stored at 4°C for periods ranging from 2 weeks to 2 months. The autologous sample was fresh.

Processing of material was as described in the former experiment. Viable cell counts were carried out on each test culture at 0 hour and at 54 hours. The effect of the individual serum samples on the cells was estimated as the proportion of cells which survived at 54 hours expressed as a percentage of the numbers present at 0 hour, viz:-

$$\text{Percentage survival} = \frac{\text{viable cell population at 54 hours}}{\text{viable cell population at 0 hour}} \times \frac{100}{1}$$

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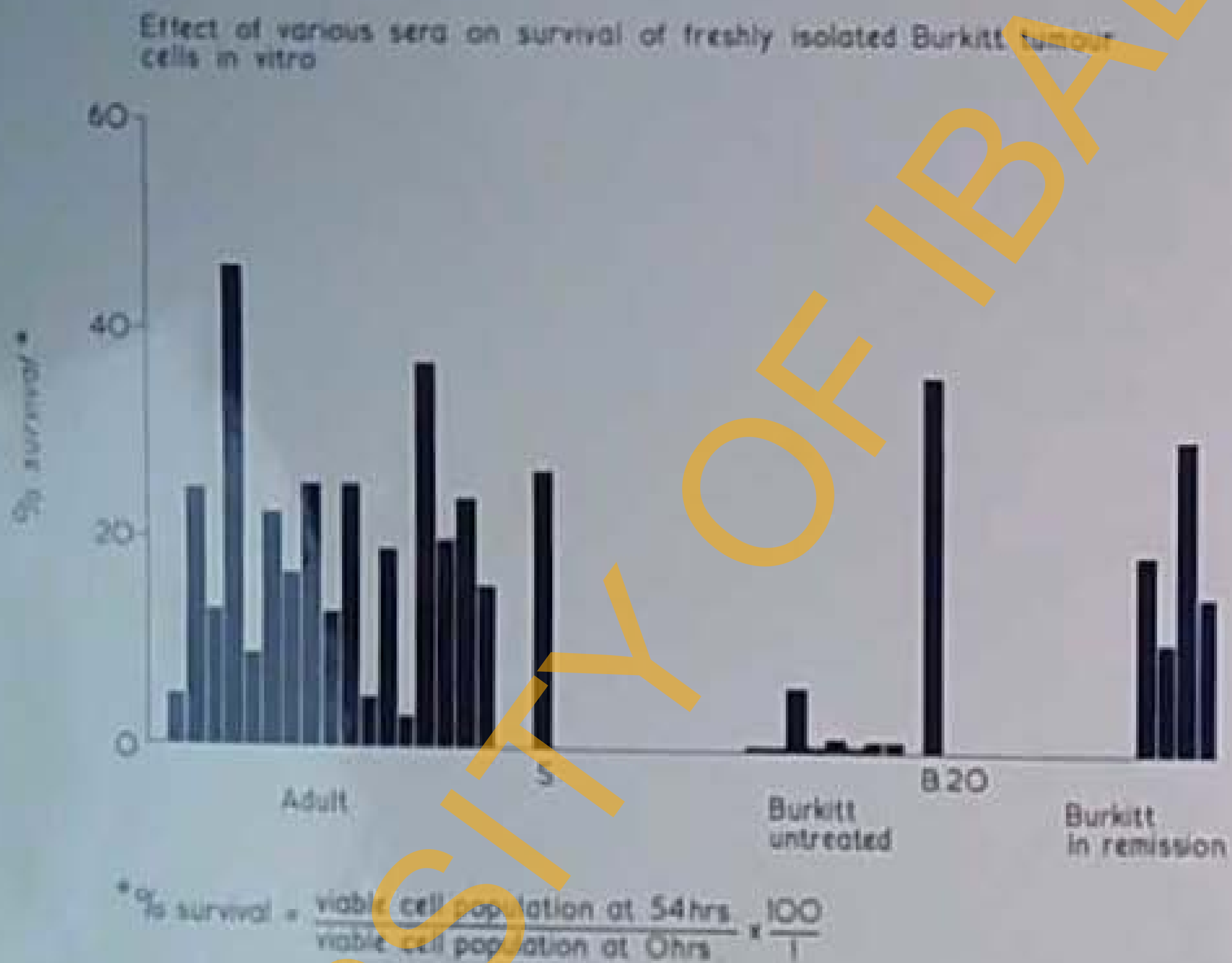

Fig. 96 Effect of various sera on survival of freshly isolated Burkitt's lymphoma cells in vitro. (Preliminary Expt. 2)

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The histogram (Fig. 96) shows the relative effects of the serum samples tested on the lymphoma cells. Unexpectedly, sera from untreated tumour-bearing Burkitt's lymphoma patients showed the greatest deleterious effects on the cells, although the autologous serum again showed relatively high supportive effects. There was however a fairly wide range of effects manifested by sera from healthy indigenous adults, some having profound deleterious effects on the cells. Serum from the Burkitt's lymphoma patients in remission also showed some degree of inhibition on the freshly isolated cells, but to a degree not as severe as sera from untreated tumour-bearing patients.

COMMENT: The influence of storage on the effect of serum samples is not known and may well be the reason for the differences between the effects of the long-stored Burkitt's sera and the fresh autologous serum. It is however also possible that the supportive effect shown by Burkitt's sera on fresh Burkitt's lymphoma cells is a property of autologous sera only, and that homologous Burkitt's sera are decidedly "toxic" to such cells.

It is therefore of interest to find out the effect of fresh homologous Burkitt's sera on the cells. This was attempted in the next experiment. In the same experiment, an attempt was also made to find out if sera from patients suffering from leukaemias and lymphomas behave in any way similar to Burkitt's sera.

Preliminary Experiment 3.

Fresh Burkitt's lymphoma cells were obtained from a 120 ml ascitic fluid specimen in a Yoruba girl aged 6 years (Hosp. No. 169,067) with abdominal Burkitt's lymphoma. The cells were predominantly tumour cells

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<u>Table 13.</u> Survival of fresh Burkitt's lymphoma cells in human serum samples. (Preliminary Experiment 3).

Code No.	Diagnosis	Survival index	Code No.	Diagnosis	Survival index.
R. 557	Ac. L. Leukaemia	16.2	B. 29	Burkitt's	49.6
804	Ac. L. Leukaemia	16.2	30	Burkitt's	48.4
542	Ac. L. Leukaemia	19.6	34	Burkitt's	51.4
524	Ac. L. Leukaemia	19.6	18*	Burkitt's	30.0
694	Ac. L. Leukaemia	12.0	21*	Burkitt's	17.2
117	Ac. L. Leukaemia	14.0	23*	Burkitt's	14.8
546	Ac. G. Leukaemia	18.8	27*	Burkitt's	14.4
789	Ac. G. Leukaemia	9.2	A. 4922	Blood donor	50.4
539	Ac. G. Leukaemia	22.8	4923	Blood donor	50.4
505	Ac. G. Leukaemia	22.0	4924	Blood donor	31.6
534	Ac. G. Leukaemia	18.2	4926	Blood donor	26.8
636	Erythroleukaemia	12.0	4927	Blood donor	13.6
466	Erythroleukaemia	5.6	4928	Blood donor	6.8
550	Chr. G. Leukaemia	30.0	4929	Blood donor	40.0
320	Chrn. G. Leukaemia	16.4	4930	Blood donor	16.0
[illegible]	Chr. L. Leukaemia	16.0	4931	Blood donor	45.2
639	Hodgkin's	14.4			
555	Myeloma	28.0			
862	Lymphosarcoma	16.0			
[illegible]	Lymphosarcoma	19.6			
633	Reticulum cell sarcoma	44.0			
230	Reticulum cell sarcoma	20.4			

*Serum stored at 4°C for over 3 months before use.

Survival Index = viable cell population at 48 hours [illegible] x 100

preserved Burkitt's sera.

Serum from indigenous adults showed a wide range of effects. Some have effects comparable to that of well preserved Burkitt's sera while others showed about the highest degree of inhibition of survival.

COMMENT: The above findings would suggest that:-

(1) Freshly isolated Burkitt's lymphoma cells survive best in serum from untreated Burkitt's lymphoma patients provided the serum is fresh or have been stored at -20°C. The supportive factor is labile and is destroyed at 4°C.

(2) Sera from patients with leukaemia or lymphoma other than Burkitt's type do not as a rule encourage the survival of the cells.

(3) Human sera of indigenous adult origin have all grades of supportive capacity for fresh Burkitt's cells. Many inhibit the cells.

It would appear therefore that the apparent rapid "autolysis" of freshly dispersed Burkitt's lymphoma cells in vitro was due at least in part, to the presence of human serum of the deletarious type in the medium in which the cells were collected, processed and/or cultured.

The extent to which possible blastic transformation of the 'normal' small lymphocytes (which are present in fresh tumour material) contributes to the population of surviving cells at 48 hours in the above experiments is not known. The possibility that the "survival index" of each serum sample is a reflection of the sample's capacity to induce blastic transformation of small lymphocytes from Burkitt's lymphoma patients cannot be ignored. Further investigations along this lines might be rewarding, especially in view of the appearance of a few blast cells 2 to 4 days after culture of peripheral blood lymphocytes from untreated Burkitt's lymphoma patients in autologous serum, (unpublished observation ; see Fig. 94).

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WORKING HYPOTHESIS

Many known biological effects of sera on cell survival can be postulated for the observed deleterious effect of some serum samples on Burkitt's lymphoma cells. Of prime importance in the consideration of factors responsible for such effects is the nutritional requirements of the cells. It is very likely that Burkitt's lymphoma cells require one or several essential nutrients for survival, the concentration of which vary widely in the serum of different individuals.

It is also possible that the effects manifested by the serum samples tested are those usually attributed to "toxic sera" in tissue culture work. However, apart from thermolability, little is known about the properties and true nature of the serum factors responsible for non-specific toxicity of sera to cells in tissue culture (Parker, 1964).

Lastly, immunological processes may well be the case of the observed effects of the sera. Immune cytolysis might result from presence in culture serum of isoantibodies to surface antigens on the cultured cells. In this respect blood group (ABO, Rh, MNS, etc.) or related antigens take pride of place, and the relatively good survival of cells in autologous sera lends support to this possibility.

The importance of serum complement for immune cytolysis is well known. Its different concentration in the serum of different individuals may be partly responsible for the varying degrees of survival of susceptible lymphoma cells. The possibility of a homologous immune reaction cannot however be excluded. The presence or absence of specific antibodies to antigens present in the blast cells may be playing an important part in the outcome of the observed longevity of cells in

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various serum samples.

Of relevance to this last possibility is the peculiar age incidence of Burkitt's lymphoma. The apparent rarity in infancy and post-adolescence of Burkitt's lymphoma (Burkitt and Wright, 1965) offers the attractive concept of "high incidence in the non-immune" as a possibility in the pathogenesis of the disease. This presupposes an antigenic, probably infective aetiology or aetiological co-factor to which infants are passively protected, and adults actively immune. Since Burkitt's lymphoma, if untreated, is almost invariably fatal, the alternative hypothesis of genetic susceptibility towards tumour formation as an abberrant response to a common agent to which individuals are readily exposed, permits the elimination of most tumour-susceptible individuals before adulthood. These two hypotheses are not mutually exclusive, and in either or both events, indigenous adults in areas where the lymphoma is "endemic" would posses humoral and/or cellular immunity against the hypothetical aetiological agent(s)

On the other hand, irrespective of the mode of pathogenesis of Burkitt's lymphoma, the possibility of tumour auto-immunity is being raised by the encouraging reports of increasing numbers of Burkitt's lymphoma patients asymptomatic many months or years after complete withdrawal of cytotoxic chemotherapy (Burchenal, 1966).

Does the Burkitt's lymphoma patient respond in any protective manner to his cancer? Burkitt et al (1965) considered that there was probable participation of immunological processes in the manifestation of certain clinical observations made on patients suffering from Burkitt's lymphoma.

In particular, the dramatic response of tumours to unusually low does of certain cytotoxic drugs, failure of subsequent tumours to appear at sites

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of completely regressed tumour, and the many cases of authentic long-term remissions were believed to be due, at least in part, to host defence mechanisms directed against the tumour. The concept was further supported by the independent reports by Ngu (1966) and Burkitt (1966c) of almost complete, though transient, regression of jaw tumours in two Burkitt's patients infused with plasma from cases in long-term remissions.

At present, the role of normal lymphocytes and phagocytic histiocytes which are invariably present in freshly dispersed tumour material or in serous fluids containing Burkitt's lymphoma cells is not known. Prognosis in Hodgkin's lymphoma has been correlated with the degree of lymphocytic infilteration of the tumefaction, "lymphocytic predominance" lesions having a better prognosis than the more malignant Hodgkin's sarcoma in which there is "lymphocytic depletion" (Lukes, 1964; Lukes et al, 1966). The normal lymphocytes present in Burkitt's lymphoma may therefore be evidence of an attempted host resistance to the tumour cells or, more precisely, to foreign antigens present in the tumour. It is however, equally possible that the lymphocytes ultimately undergo blastic transformation into the characteristic Burkitt's lymphoma cells, and as it were belong to the neoplastic tumour process rather than the host's resistance mechanisms.

Present concepts regard the apparently normal and well-differentiated histiocytes in Burkitt's lymphoma tissue as reactionary and defensive in nature. Similarly the presence of pyknotic and cell debris within histiocytes in a hyperplastic reactive lymphoid germinal centre is generally believed to be evidence of phagocytic activity of such cells. In this connection, and as mentioned earlier (pp. 40), it should be noted that there is striking histological similarity between Burkitt's

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lymphoma and Flemming's germinal centre of hyperplastic lymph nodes. Indeed, the lymphoma may well be a malignant lymphoid germinal centre. Although the intracytoplasmic prominence of nuclear and cell debris in the histiocytes in these two types of lesions gives the impression of a non-specific scavenger activity ridding the tissues of unwanted, deleterious or effete cells, the phenomenon may be an essential biological step in the cellular dynamics involved in secondary immune responses. The phenomena of emperipolesis and peripolesis suggest mutual, probably essential interaction between histiocytes and lymphocytes. It may permit reutilization of lymphocyte DNA (Hamilton, 1956; Medawar, 1957; Trowell, 1958; Loutit, 1960) and/or the transfer of processed antigens from macrophages to the interacting lymphocytes during the early phases of antibody production (Sharp and Burwell, 1960; Fishman et al, 1963; Schoenberg et al, 1963; Bartfeld and Juliar, 1964). While bearing the above considerations in mind it is still consistent with our present state of knowledge to regard the presence of lymphocytes and phagocytic histiocytes in Burkitt's lymphoma as depicting an anti-tumour host defence reaction.

The foregoing observations, citations and speculations led the author to search for experimental evidences in support of the premise that the Burkitt's lymphoma patient may well possess humoral and/or cellular immunological factors directed against antigens in the tumour, and that individuals with no evidence or history of the disease but who live in areas where the lymphoma is seen frequently might also possess similar immunological factors.

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The rationale, methods and results of the experimental studies are presented in the next chapter. Three techniques were proposed for the detection of humoral anti-Burkitt's lymphoma factors, while an experimental model for in vitro detection of delayed-type hypersensitivity was to be adapted for the Burkitt's lymphoma problem.

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Methods of experimental study in cancer immunology are based on the classical immunochemical techniques, modified usually to increase the sensitivity of the system under study. Methods which have been used for the detection of anti-tumour antibodies and tumour antigens ranged from complement fixation, haemagglutination, agar-diffusion precipitation, cytolytic and fluorescent antibody tests to transplantation resistance reactions and virus neutralization tests, the later in virus-induced tumours. Two techniques which have been used extensively, particularly in the study of animal leukaemia, leukosis and lymphoma are the cytotoxic tests of Gorer and O'Gorman (1956), and more recently the fluorescent antibody reaction of which Moller (1961) has developed a technique applicable to living cells. The membrance immunofluorescence test of Moller has all the advantages of immunological specificity of fluorescent antibody tests, with little or no interference by non-specific or auto-fluorescence. It is however applicable only to the detection and characterisation of cell surface antigens.

In this chapter is presented the use of two standard immunological techniques - in vitro cytotoxic test and fluorescent antibody reaction - in the search for anti-Burkitt's lymphoma antibodies. A third parameter which, as far as the author is aware, has no precedence in the literature is the use of data obtained from growth measurements in cell cultures as an index of presence of antitumour factors.

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SECTION 1.

GROWTH EXPERIMENTS.

As a prelude to comparing the growth effects of serum samples on Burkitt's lymphoma cells in vitro, it was considered necessary to study the growth kinetics and requirements of the cells in culture.

The sensitivity of growth experiments in measuring the difference between the growth effect of a variable should be maximal under optimal growth conditions. The optimal concentration of serum in TC 199 for growth of Burkitt's lymphoma cell lines has not been established by titration experiments. Neither is data available for minimal or optimal viable cell population for maximal growth rate of the cells. Other properties of Burkitt's lymphoma cell lines for which no data was available at the time these experiments were undertaken include the length of exponential growth of Burkitt's cell cultures and the influence of complement on cell growth. Finally it is desirable to determine the sensivity inherent in the sytem which may be adopted for the testing of growth effects of serum samples on Burkitt's lymphoma cells.

MATERIALS AND METHODS.

Burkitt's Lymphoma cells.

The experiments described in this section were carried out on Burkitt's lymphoma cells maintained as continous suspension cultures. The cell line used was OB1, the only cell line established by the writer at the time the preliminary experiments were undertaken. OB1 (see Table 9) had been in continuous propagation for nine months at the time this

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series of experiments were carried out. Growth behavior and cytomorphology of OB1 cells have not been observed to change since the cell line was established; this statement is indeed true up to the moment of writing.

OB1 cell line is particularly suited to the method of growth estimation adopted in this presentation, which is cell population estimation by counting. The cells grow in suspension as single cells with no tendency whatsoever to autoagglutination. Serial cell counts were therefore readily carried out on cultures without undue disturbance of the culture; - at least not to the same degree as would be expected in experiments with cell lines growing as clumps, in which vigorous agitation would be necessary to ensure even cell suspension before sampling.

Cells were harvested from stock cultures as required, and washed twice by centrifugation in TC 199 before innoculation into test cultures.

<u>Basal culture medium.</u>

The basal medium in all experiments was freshly prepared TC 199 (a chemically defined, buffered, isotonic medium containing glucose, amino-acids, vitamins, salts, phenol red, penicillin and streptomycin; Parker, 1964a) to which was added 50 unts/ml of mycostatin (Squibb, Liverpool) and 35 units/ml of neomycin sulphate (Boots, Nottingham).

An arbitrary total culture volume of 2 ml was chosen for all test cultures.

<u>Serum.</u> Unless otherwise stated, the source of human serum used were blood donors presenting at the Blood bank, U.C.H., Ibadan. About 20ml clotted blood was collected from each donor, and the serum separated within 36 hours. Serum samples were kept frozen at -10°C until use. All samples manifesting baterial or fungal growths on innoculation into nutrient broth culture were discarded.

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Estimation of cell population in cultures.

Viable cell population was estimated by simple haemocytometer methods, using a Fuchs-Rosenthal counting chamber, and scoring with a tally counter.

The criteria of cell viability adopted were phase contrast morphology and vital toluidine blue uptake. For counting, the culture was agitated with the sampling pipette. to ensure even cell suspension, and 0.04 ml of culture placed on a glass slide. An equal volume of 0.025% vital toluidine blue (Gurr, England) in TC 199 was added and the two mixed thoroughly with a teated pipette. The mixture was then charged into the counting chamber, and counting undertaken after 3 minutes, using a phase contrast microscope.

Viable Burkitt's lymphoma cells appear intense purple under a phase microscope within 3 minutes of exposure to vital toluidine blue; dead cells are not stained, but dying cells may take up some stain.

Culture containers.

Stock cultures were maintained in 120 ml medical flats. All test cultures were set up in 6" x ⅝" test tubes, tightly stoppered with white rubber bungs, and incubated stationary at 37°C with the tubes standing upright.

GROWTH EXPERIMENT 1:- Effect of serum concentration in TC 199 on growth of CB1 cells.

Procedure: A serial dilution of human serum was made in TC 199 in a total volume of 2 ml as shown in the following scheme:-

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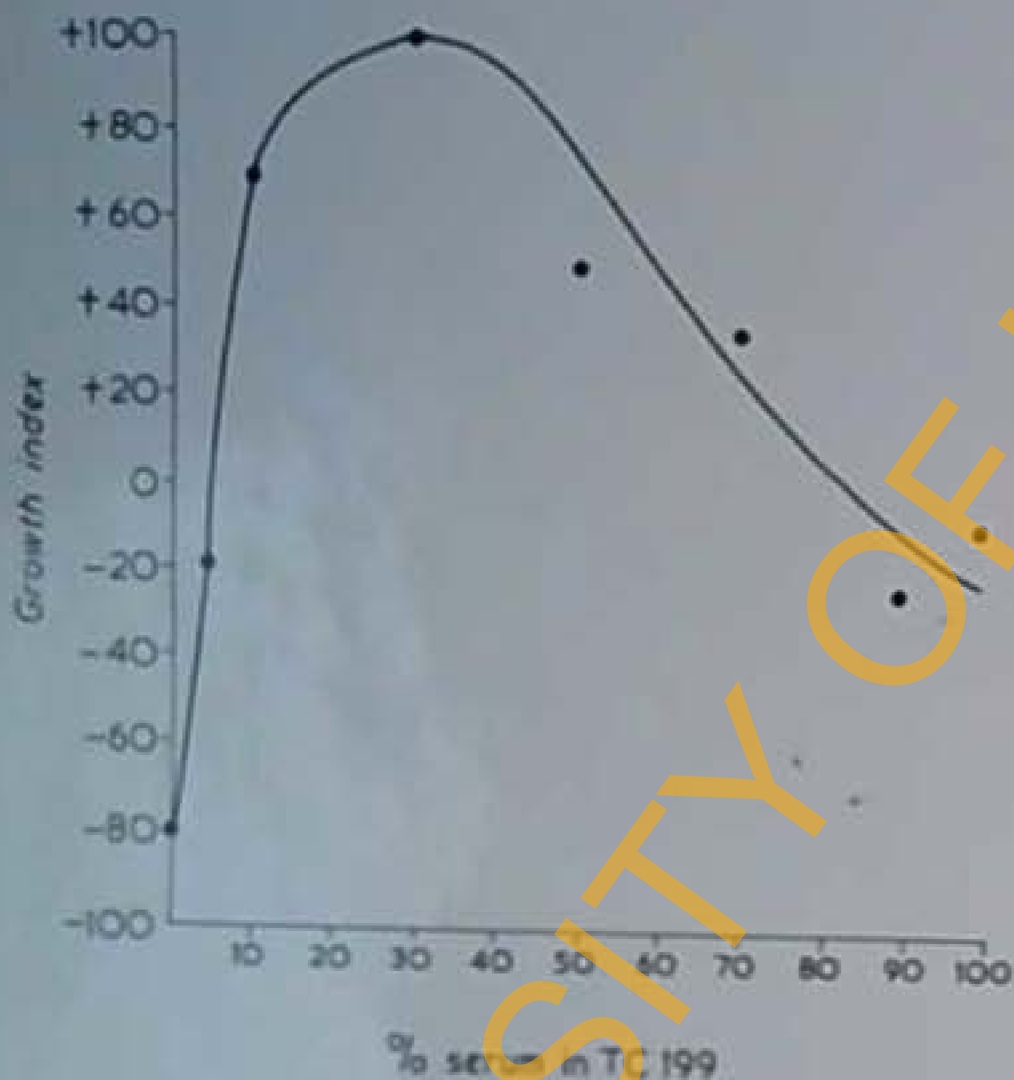

Fig. 37 Growth of OB1 cells in various concentration of human serum in TC 199. (Growth Expt. [illegible])

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Tube No. --	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Serum (ml) -	0	0.1	0.2	0.6	1.0	1.4	1.8	2.0
TC 199 (ml) -	2.0	1.9	1.8	1.4	1.0	0.6	0.2	0
% serum in TC199	0	5.0	10.0	30.0	50.0	70.0	90.0	100.0

OB1 cells were harvested, washed in 2 changes of TC 199 by centrifugation at 250g for 3 minutes and resuspended in 1.0 ml of TC 199. 0.1 ml of cell suspension was added to each tube and the cultures incubated at 37°C. Viable cell counts were carried out at 0 hour and again at 72 hours.

The growth of OB1 cells in each tube was expressed as a <u>growth index</u> which is the difference between viable cell population per unit volume at 72 hours and the viable cell population of 0 hour viz:-

Growth index = (viable cells per c. mm at 72 hours)

minus

(viable cells per c. mm at 0 hour)

<u>Results:</u> Fig. 97, shows the relative growth of OB1 cells in various concentration of the same sample in TC 199. Growth was not supported at serum concentration below 6% or above 80%. Maximum growth was achieved in the 30% serum culture.

<u>GROWTH EXPERIMENT 2.</u> Effect of size of cell innoculum on OB1 culture growth.

<u>Procedure:</u> 2 ml aliquots of a 30% serum in TC 199 were measured into a row of test tubes. OB1 cells harvested from stock culture were washed twice and resuspended in TC 199. A two-fold serial dilution of the cell suspension was made, and 0.1 ml of each diluted cell suspension

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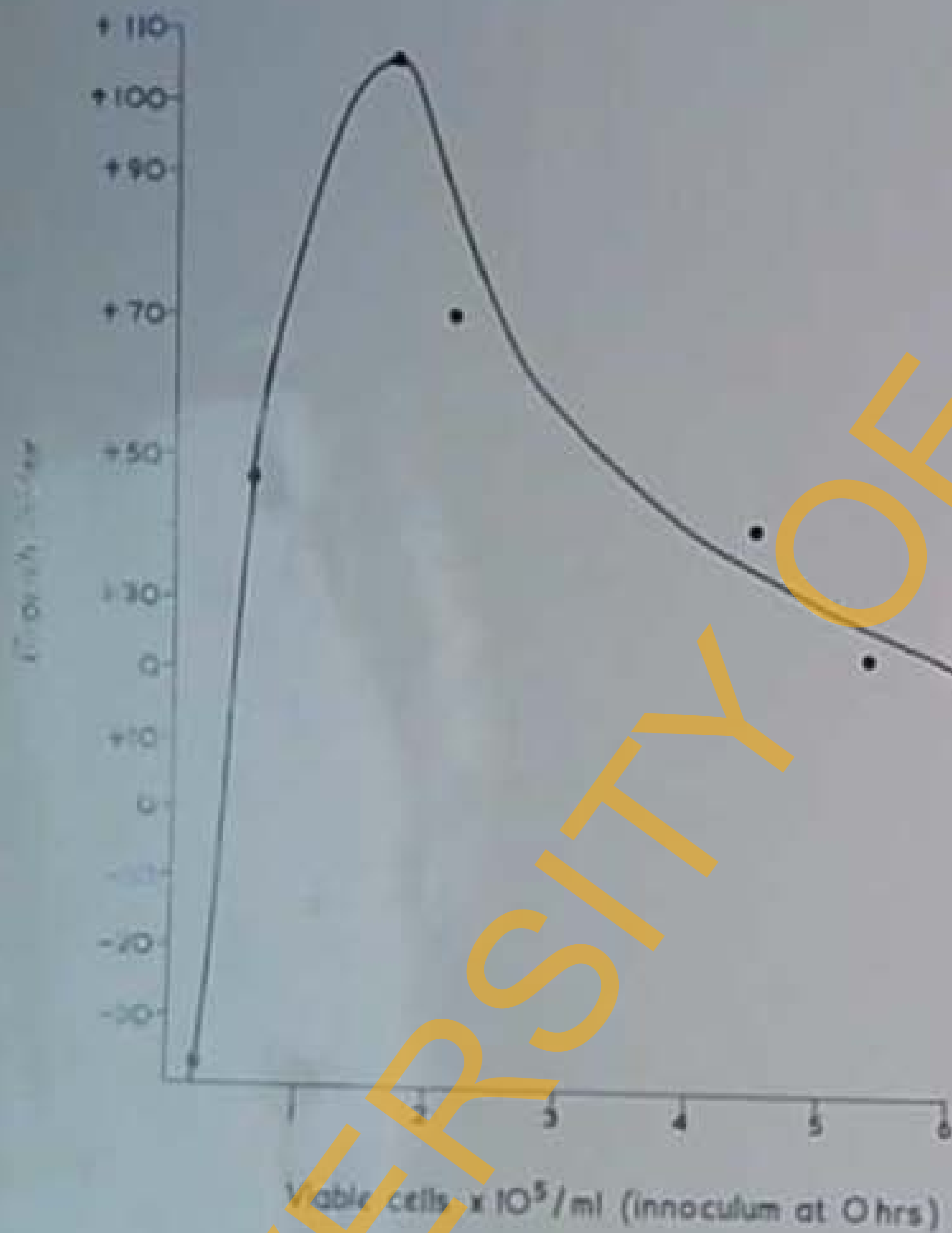

Fig. 98 Effect of size of cell innoculum on rate of growth of OB1 cells in tissue culture. (Growth Expt. 2)

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was added to each of the culture tubes. Cultures were incubated stationary at 37°C. Viable cell counts were carried out on each test culture at 0 hour and again at 72 hours. Growth in each test culture was expressed as a growth index, derived as in growth experiment 1.

Results: Fig. 98 depicts the dependence of growth on the concentration of subcultured cells. From the graph it may be concluded that continuous propagation of OB1 cells may not be possible if subcultured cells are less than 0.4×10^5 viable cells per ml. of culture. The optimal concentration of cells for growth was 1.5 to 2.0×10^5 viable cells per ml. of fresh medium.

GROWTH EXPERIMENT 3: - (i) Growth curve of OB1 cells

(ii) Effect of sera from various individuals on the growth curve of OB1 cells.

Procedure: 0.6 ml. serum from each of 4 Burkitt's lymphoma patients in remission, and 2 blood donors, were measured into test tubes. 1.4 ml. TC 199 was added to each tube to give 2 ml. of 30% test serum culture medium per tube.

An estimated volume of OB1 stock culture required to give an initial cell count of 1.5 to 2.0×10^5 per ml. in each test culture was sacrificed. The required volume was calculated from the formula:

$$V = \frac{x}{y} \times m$$

where v, is the volume in ml. of stock culture required.

x, is the number of viable cells per unit volume of stock culture.

y, is the required number of viable cells per unit volume of test culture.

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GROWTH CURVE OF OBI CELLS IN TISSUE CULTURE

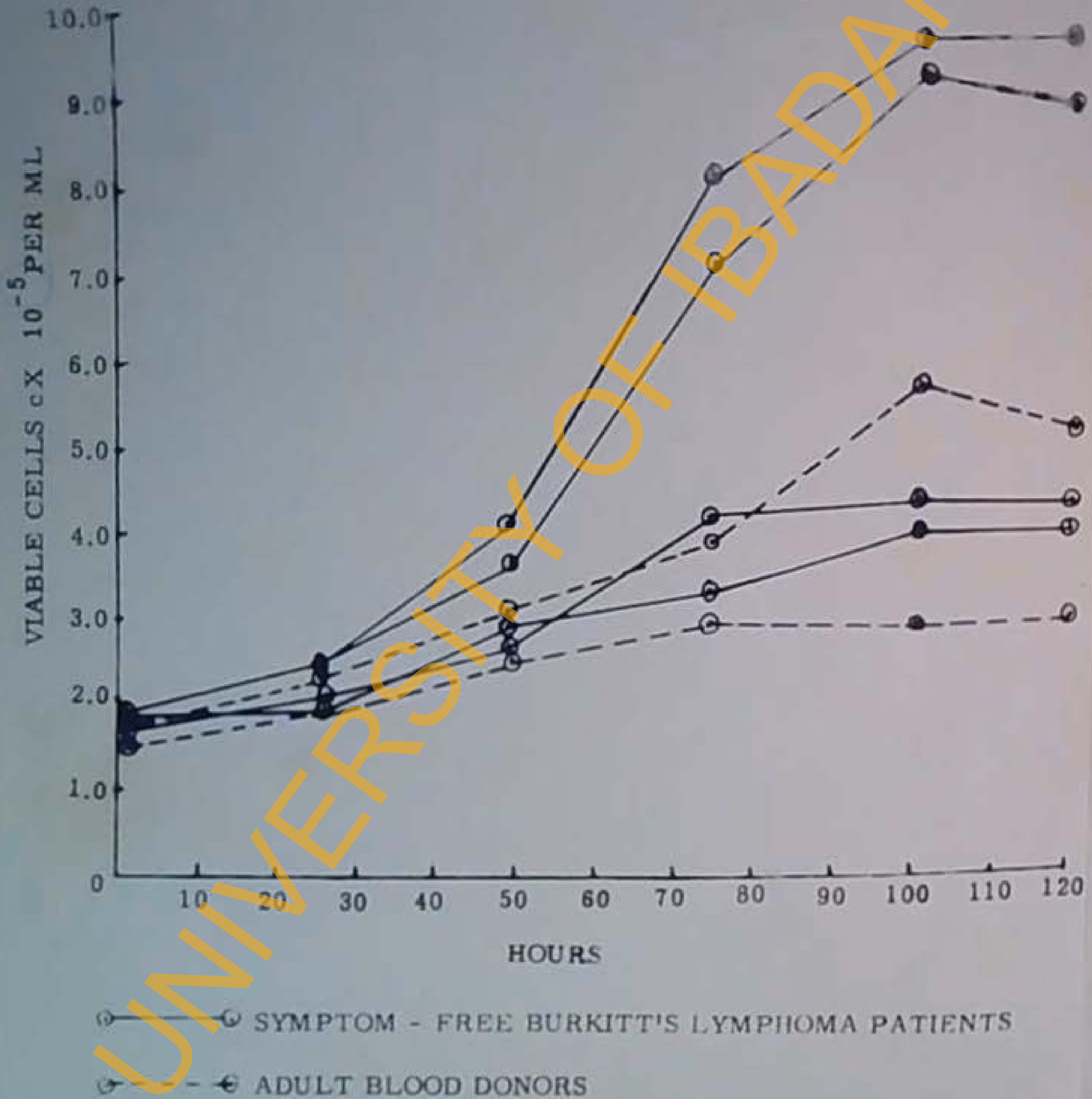

Fig. 99 Growth curves of OBI cells in different human sera. (Growth Expt. 3).

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TABLE 14

Effect of sampling on growth of OB1 cell cultures

		Viable Cells Per C.MM			
		0 hr.	18 hrs.	48 hrs.	75 hrs.
Serum A	Culture (1)	83	n.c.	n.c.	93
	Culture (2)	106	105	115	65
Serum B	Culture (1)	87	n.c.	n.c.	175
	Culture (2)	100	115	115	127

n.c. - not counted

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m, is the volume of each test culture

and n, is the number of test cultures in the experiment.

The tubes were incubated stationary at 37°C and viable cell counts carried out on each tube at roughly 24 hour intervals, up to 100 hours.

<u>Results:</u> Fig. 99 depicts the growth curve of OB1 cells in each test culture, and shows clearly a wide difference in the growth effect of human serum samples on an established strain of Burkitt's lymphoma cells.

Maximum differences in growth effect was manifested at about 100 hours, corresponding to the peak of the growth curves, and end of the exponential growth-phase.

<u>GROWTH EXPERIMENT 4.</u> - Assessment of growth-disturbance due to repeated sampling of culture for serial cell counts.

<u>Procedure:</u> Duplicate cultures were set up on each of two test sera from blood donors. Viable cell counts was carried out on all cultures at 0 hours. Serial counts at 18 hours and 42 hours was however carried out on one of each pair of test cultures. Viable cell counts was again carried out on all cultures at 75 hours.

<u>Results:</u> Table 14 shows details of cell counts. In both test cultures in which serial cell counts had been carried out, there were less viable cells at 75 hours than in the corresponding test culture in which less numbers of sampling was made.

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It was therefore concluded that there was better chance of optimal growth if counts on test cultures were reduced to the barest minimum, that is, zero-hour and final -hour counts only.

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DEFINITIVE GROWTH EXPERIMENTS.

From the foregoing and other preliminary experiments it was possible to evolve a standard system for the testing and comparison of the effect of serum samples from different sources on the growth of established Burkitt's lymphoma cell lines. Such a system it was envisaged, should permit

(1) the use of small quantities of serum and cells

(2) optimal growth of the target cells in test serum, and

(3) reproducibility.

The system adopted for the definitve growth experiments presented in this thesis may be defined as a 2 ml culture containing 0.6 ml (30%) test serum in TC 199 which 3 to 4 x 10^5 (150-200/c.mm) viable test cells are seeded; viable cell population is estimated by haemocytometer counting immediately after setting up of test culture (0 hour) and again at 96 hours after incubation at 37°C as a stationary suspension culture.

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GROWTH EXPERIMENT 5.

Effect of sera from various groups of individuals on the growth of Burkitt's lymphoma cells (CB1) in vitro.

PROCEDURE:

Burkitt's lymphoma cells - CB1 cells were used in all experiments.

Test Sera - 5 - 20 ml. blood was collected from

(a) 100 adult Nigerian blood donors at the Blood bank, U.C.H., Ibadan.

(b) 13 untreated Burkitt's lymphoma patients.

(c) 7 Burkitt's lymphoma patients symptom-free three months to three years following complete withdrawal of chemotherapy.

(d) 38 healthy children aged two months to six years attending an infant Welfare clinic at the Rural Health Centre, Araromi-Obu (Ondo Province, Western Nigeria) for routine measurements.

(e) 30 cord blood samples from normal deliveries at the Labour Ward, U.C.H., Ibadan.

Serum was separated from all samples within 36 hours of blood collection, preserved with neomycin (33 units/ml) and mycostatin (50 units/ml.) and stored at -10°C until use.

Standard Serum - This was 120 ml. serum separated from 300 ml. clotted blood collected from a healthy adult Nigerian at a single bleeding. The serum was preserved with neomycin and mycostatin and stored at -20°C in 2 ml. aliquots.

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Setting up of test cultures:

The system used was that developed in the preliminary growth experiments. Serum samples were tested in batches of 10 to 20. Duplicate cultures of the standard serum was included in each batch of test cultures.

0.6 ml. of each serum was measured into test tubes, and 1.4 ml. TC 199 added. An aliquot of OB1 stock culture calculated to contain enough viable cells to give an initial viable cell population of 1.5 to 2.0 x 10^5 cells per ml. in each tube was removed, and the contained cells spun down at 250g for 3 minutes. The cells were then washed by centrifugation in two changes of TC 199 and resuspended in an appropriate volume of TC 199. 0.1 ml of the cell suspension was then added to each tube. The tubes were tightly stoppered and incubated stationary at 37°C.

Viable cell counts were carried out on each tube at 0-2 hours and again at 96-98 hours.

Each serum sample was innoculated into nutient broth culture medium and incubated at 37°C. Cultures of sera yielding bacterial or fungal growth at or before 96 hours were withdrawn from the experiment; these included serum from 4 adults, one child and one cord blood.

Assessment of culture growth.

Culture growth in each cultures was measured by the difference in viable cell counts at 96 hours and at 0 hour. The growth effect of each test serum was expressed as a growth index, which is the ratio of growth in test culture to growth in the standard serum culture, the later having a growth index of 1.0. The growth effect of each test serum on OB1 cells was therefore calculated as follows:-

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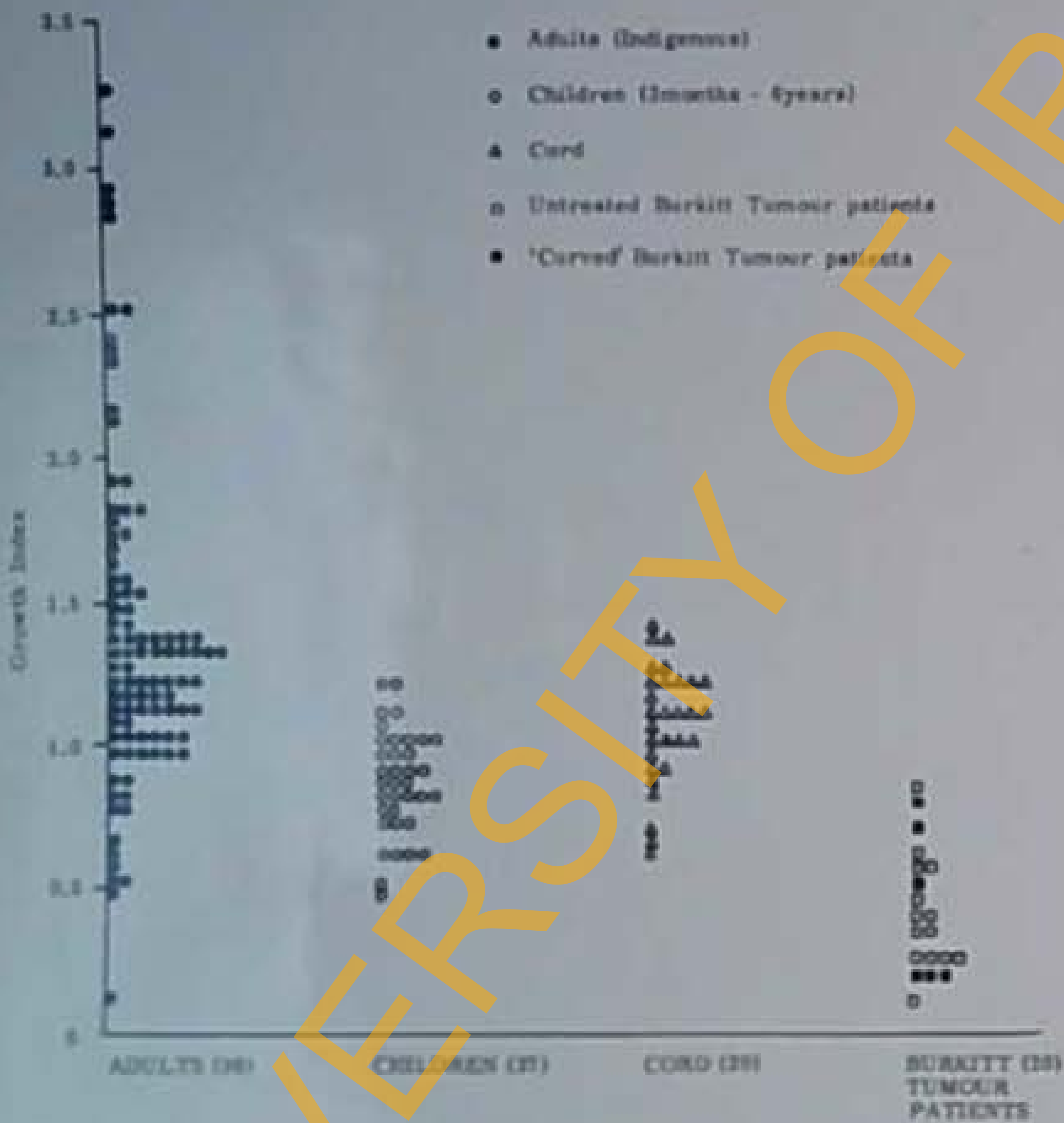

Fig.100 Scattergram show the relative growth effect of serum samples from various individuals on growth of Cb1 cells in vitro. (Growth Expt. 5).

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TABLE 15

Relative growth effects of serum samples from different groups of individuals on OB1 cells in vitro

Groups of Individuals	No. tested	Growth Index (G.I.)*			
		Range	Median	Mean + S.D.	% with G.I. Less than 0.9
Adults (Indigenous)	96	0.14 - 3.28	1.25	1.37 + 0.96	13
Children (2 months - 6 years	37	0.48 - 1.21	0.80	0.86 + 0.42	50
Cord Blood	29	0.65 - 1.40	1.10	1.08 + 0.43	16
Burkitt's	20	0.12 - 0.89	0.35	0.43 + 0.15	100

$$* \text{ G.I.} = \frac{\text{Culture growth in test serum}}{\text{Culture growth in standard serum}}$$

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$$\text{Growth index} = \frac{\text{culture growth in test serum}}{\text{culture growth in standard serum}}$$

$$= \frac{\text{increase in viable population in test culture}}{\text{increase in viable cell population in standard serum culture}}$$

RESULTS: 1. The relative growth effects of the various groups of human sera on CB1 cells are shown in the scattergram in Fig. 100, and summarised in Table 15. There was a wide variation in the growth effect of test sera on CB1 cells.

2. The Burkitt's lymphoma group showed the lowest growth promoting effect on CB1 cells, with a growth index range of 0.12 - 0.89 and a median of 0.35. Sera from normal children had a growth index range of 0.48 - 1.21 with a median of 0.80. The range of growth indices for cord serum samples was 0.65 - 1.80 (median 1.10)

3. The widest range of growth effect was encountered in the adult (blood donors) group. The growth index in this group ranged 0.14 - 3.30 (median [illegible]). A few individuals in this group produced sera with marked growth promoting effects on CB1 cells. Data on ABO and Rh blood groups and haemoglobin genotype was available on all individuals in this group; there was no correlation between the blood group and haemoglobin genotype of the individuals and the growth index of their respective serum samples.

4. CB1 cell growth is significantly lower in the Burkitt's lymphoma sera than in sera from any of the other groups. For example, the difference between the mean of the growth indices of the Burkitt's patients and healthy children groups. (0.43) is greater than thrice the

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standard error of the difference between the two means (0.41), a significance level of $p < 0.01$.

COMMENT. The differences shown in the growth on CB1 cells between sera from different individuals, and perhaps of more significance, between sera from different groups of individuals, cannot at this stage find full explanation. Although, there is some overlap in the effects of the different groups, it is interesting to note that growth index was less than 0.9 in 13% of adults, 16% of cord samples, 50% of young children and 100% of Burkitt's lymphoma patients. (Table 15).

The immediate question to be answered is whether the effects manifested by the different sera are a result of active growth stimulation by growth promoting factor(s), or varying degrees of growth suppression of a normally rapidly growing cell strain. Attempts were made to answer this question in the next few growth experiments.

GROWTH EXPERIMENTS 6: - Growth of CB1 cells in mixtures of adult human sera of known (opposite) growth effects.

Procedure: Two serum samples, one with a very high growth index (serum X) the other with a very low growth index (serum Y), were selected from the adult blood donors group of growth experiment 5. The two test sera were measured into a series of tubes in reciprocal proportions as shown:-

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Effect of good growth promoting serum (x) on OB1 cells in the presence of serum with low growth Index (Y)

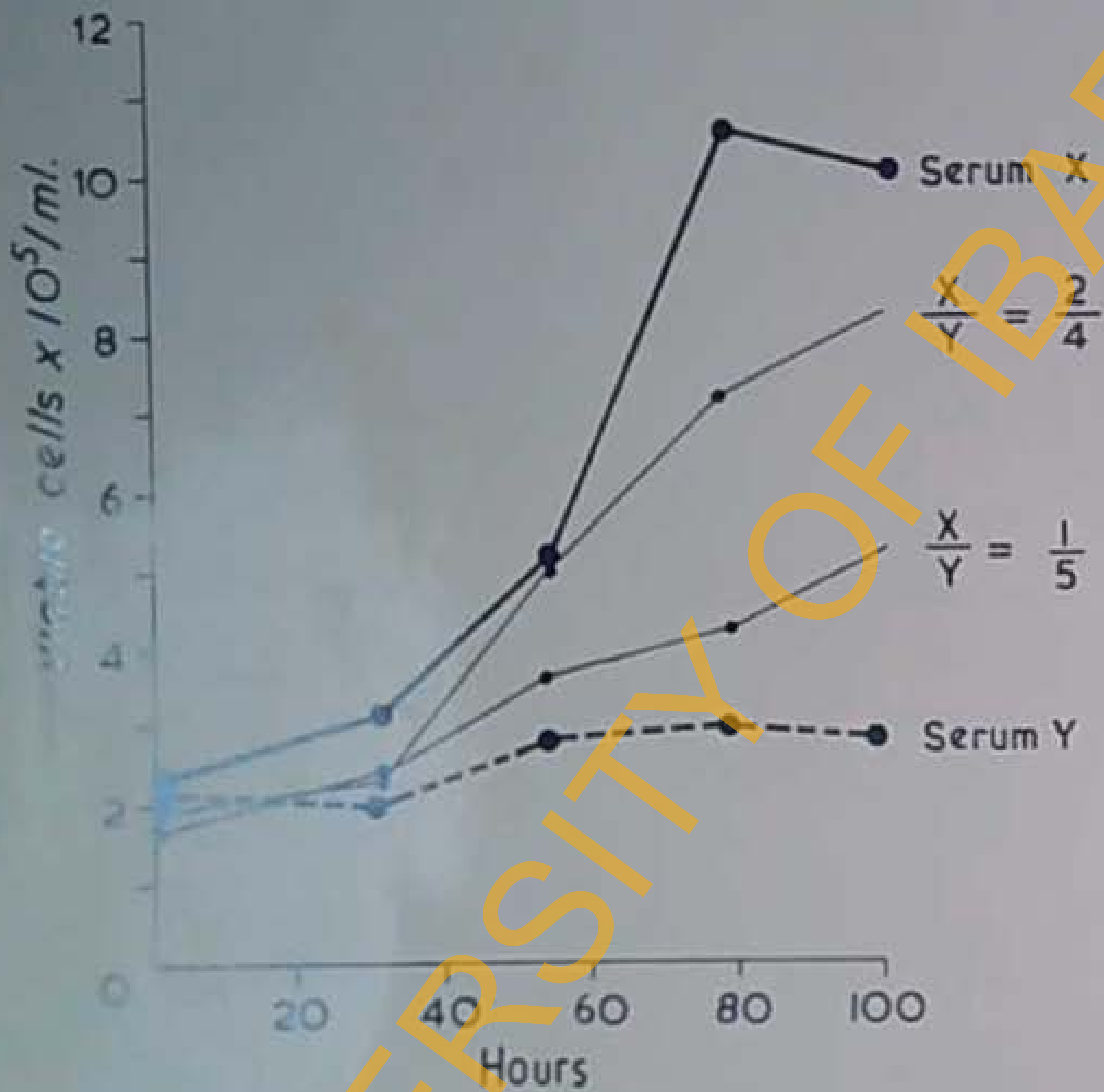

Fig.101 Effect of growth promoting serum on OB1 cells in the presence of serum of low growth index. (Growth Expt. 6).

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Tube	-	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Serum X (ml)	-	0.6	0.5	0.4	0.3	0.2	0.1	0
Serum Y (ml)	-	0	0.1	0.2	0.3	0.4	0.5	0.6
TC 199 (ml)	-	1.4	1.4	1.4	1.4	1.4	1.4	1.4

The culture volume in each tube was made up to 2 ml with 1.4 ml. TC 199, and washed CB1 cell suspension added. The tubes were stoppered and incubated at 37°C. Serial cell counts were carried out at 0.5, 31, 54, 78 and 100hours.

Tubes 2, 3 and 4 had to be withdrawn from the experiment because the cultures were found to have set into a gel at 0.5 hours. The cause was not immediately known but may be due to the high betamacrogloblin and total protein content of serum X (Total protein 10.8gm/100ml), features which may not be unrelated to the unusually high growth promoting effect of the serum on CB1 cells.

<u>RESULTS:</u> Growth curves of cultures in tubes 1 ,5, 6 and 7 are shown in Fig. 101. The growth promoting effect of serum X was dominant on the growth inhibitory property of serum Y. It required a relatively small amount of serum X (1/6 of total serum volume), to effect a substantial increase in growth in the presence of 83% serum Y concentration.

<u>COMMENT:</u> These results suggest that the probable difference between growth promoting and growth inhibitory adult human sera on CB1 cells is in the amount of a growth stimulating factor that they contain. Growth suppression may therefore be a passive effect resulting from lack of the necessary Burkitt's lymphoma cell growth-stimulating factor.

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Whatever the nature of the factor(s) is, it appears (from the scattergram in Fig. 100) to be relatively high in most adult Nigerians, relatively low in most infants and young children, and is very low in all Burkitt's lymphoma patients tested. Its fairly high concentration in cord serum would suggest among other possibilities that this factor can cross the placenta from maternal into foetal circulation. It is most tempting to regard the hypothetical growth stimulating factor as a "nutritional" factor necessary for the metabolism of CB1 cells, and present in varying concentration in human sera.

It should be remembered however that fresh Burkitt's lymphoma cells autolyse rapidly in the presence of sera from many indigenous adults. Since CB1 cells on which this experiment was carried out, as well as other cell lines established in this laboratory were isolated and propagated in media containing adult human serum, the cell clones that are now in culture are in fact selected cells. These selected cells are produced from a very small proportion of explanted cells in the primary culture (see Fig. 20). They have therefore survived in the presence of, and may probably be responding in a different manner to the very factor(s) which have caused the destruction of the majority of cells present in the primary explant; the response now being increased mitotic activity.

The evidence for presence in adult human serum of actively growth promoting factors for CB1 cells observed in this experiment, do not however completely exclude the concomitant presence of growth suppressive factors. Such growth suppressive factors might be more readily demonstrable in sera of Burkitt's lymphoma patients since it was

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this group that showed the most profound growth inhibition of OB1 cells as compared with other groups. It seems desirable therefore to compare the effect of a growth promoting serum, on a growth inhibiting adult serum and Burkitt's lymphoma serum of comparable growth index.

<u>GROWTH EXPERIMENT 7</u>: - Relative growth of OB1 cells in adult human serum and Burkitt's serum of comparable growth indices, in the presence of equall amounts of growth promoting serum.

<u>Procedure</u>: Equal aliquots of selected serum samples used in Experiment 5 were pooled in three groups (A1, A2, and B) viz:-

<u>Group A1</u>:- Adult human serum, growth promoting; five samples with an average growth index of 2.[illegible] were selected and pooled.

<u>Group A2</u> :- Adult human serum, growth inhibiting; five samples with average G. I. of 0.[illegible] selected and pooled.

<u>Group B</u>: Burkitt's serum. Five samples with average G. I. of 0.40 selected and pooled.

The pooled test sera were measured into tubes as shown below:-

Tube	-	(1)	(2)	(3)	(4)	(5)
Serum A1 (ml)	-	0.6	0	0	0.1	0.1
Serum A2 (ml)	-	0	0.6	0	0.5	0
Serum B (ml)	-	0	0	0.6	0	0.5
TC 199 (ml)	-	1.4	1.4	1.4	1.4	1.4

Washed OB1 cells were added to all tubes. Cultures were incubated at 37°C and cell counts carried out at 0, 30, 54 and 96 hours.

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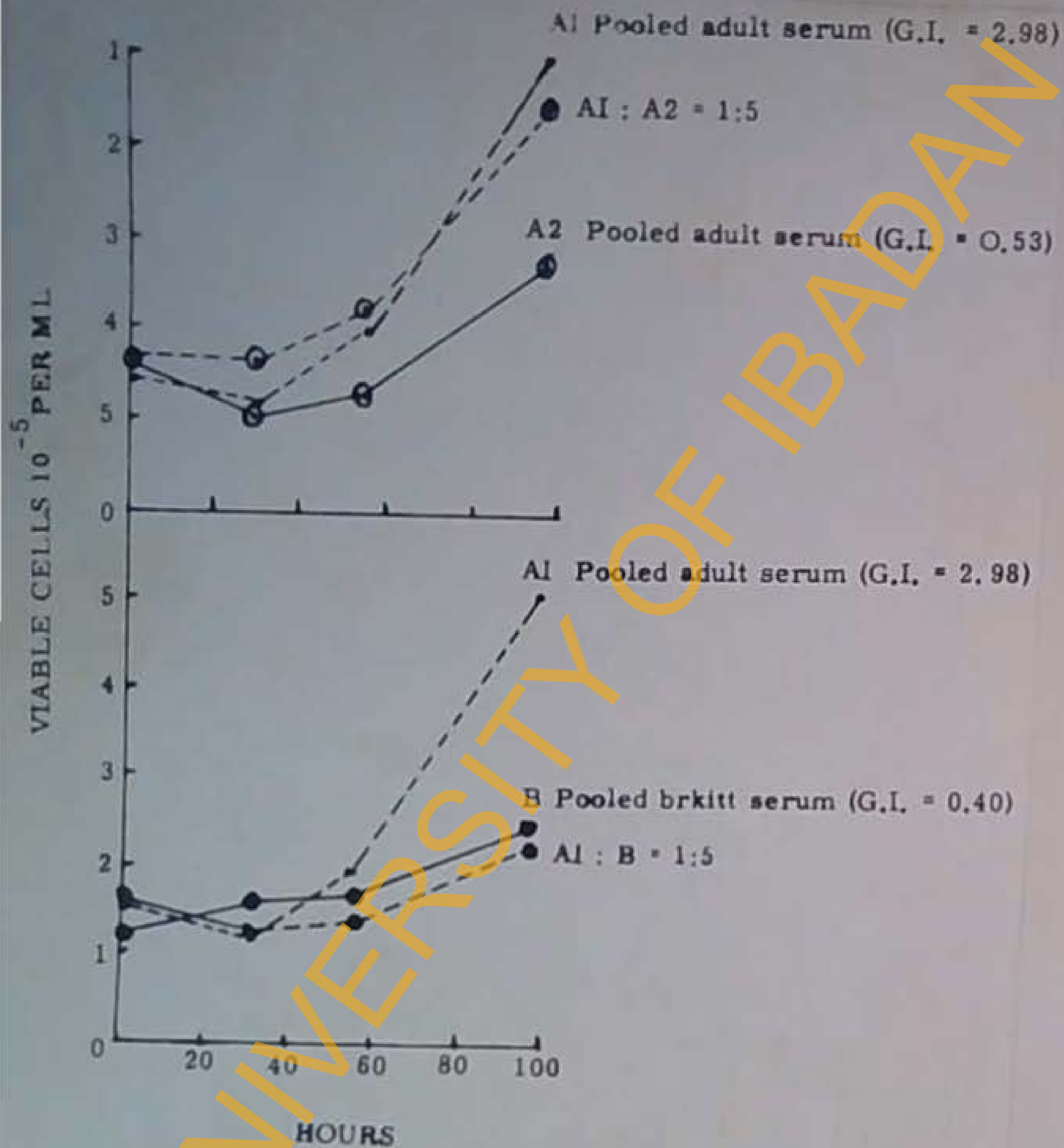

Fig.102 Growth of CB1 cells in adult and Burkitt's sera of comparable growth indices, in the presence of a growth promoting serum. (Growth Expt. 7)

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RESULTS: Fig. 102, depicts the growth of OB1 cells in the various test cultures. Serum A1 produced almost maximal improvement in growth when added to serum A2 in the proportion 1 : 5. Growth was not improved by addition of A1 serum to B serum in the same proportion 1 : 5.

COMMENT: The difference between the effect of growth promoting serum on adult serum and Burkitt's serum of comparable growth effects on OB1 cells, would suggest that in addition to lacking the growth promoting factors present in many indigenous adult sera, Burkitt's lymphoma sera also contain factors which actively suppress the growth of OB1 cells in vitro. This suggestion finds support in the outcome of the next experiment.

GROWTH EXPERIMENT 8. Prolonged growth suppression of OB1 cells by serum from a Burkitt's lymphoma patient in remission.

Procedure: The serum samples were :-

(1) **Serum B:** One of the three "cured Burkitt's" sera with the lowest growth index in growth experiment 5 (see Fig. 100); the serum was from a patient symptom-free three years after treatment for Burkitt's lymphoma of the jaw.

(2) **Serum A:** Adult serum with good growth effect on OB1 cells.

The lay-out of the experiment is as shown in Fig. 103. 0.6 ml. of each each serum sample were measured into each of two tubes, one of which was then heated at 56°C for 30 minutes to inactivate complement.

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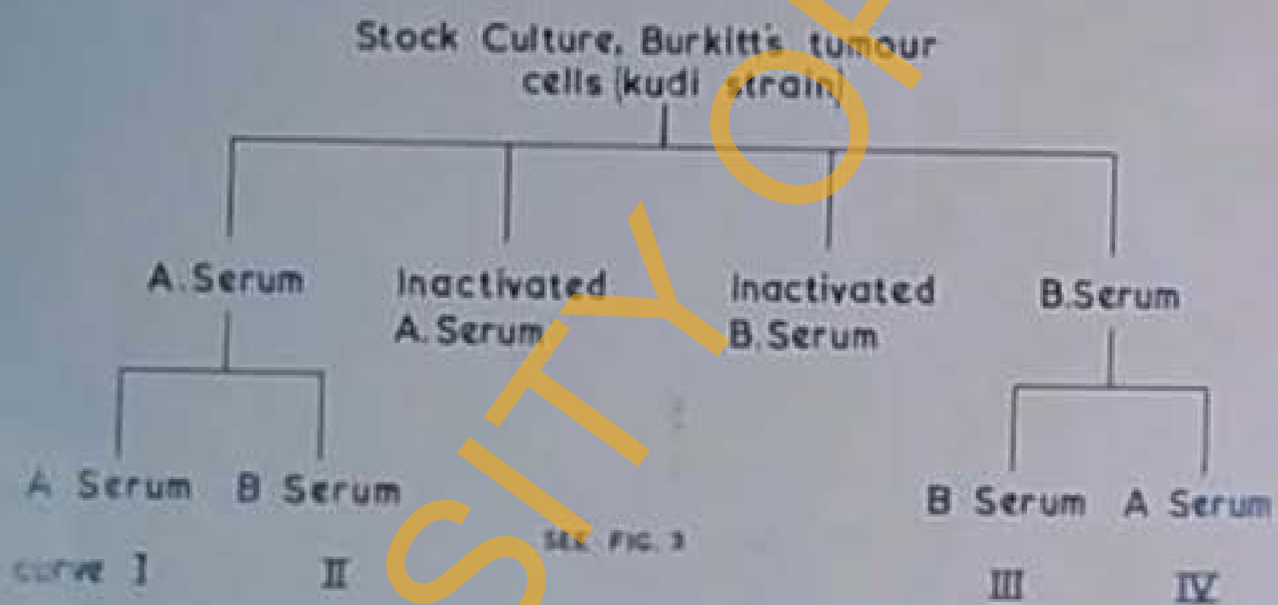

Fig. 103 Schema showing cell passages during Growth Experiment 8.

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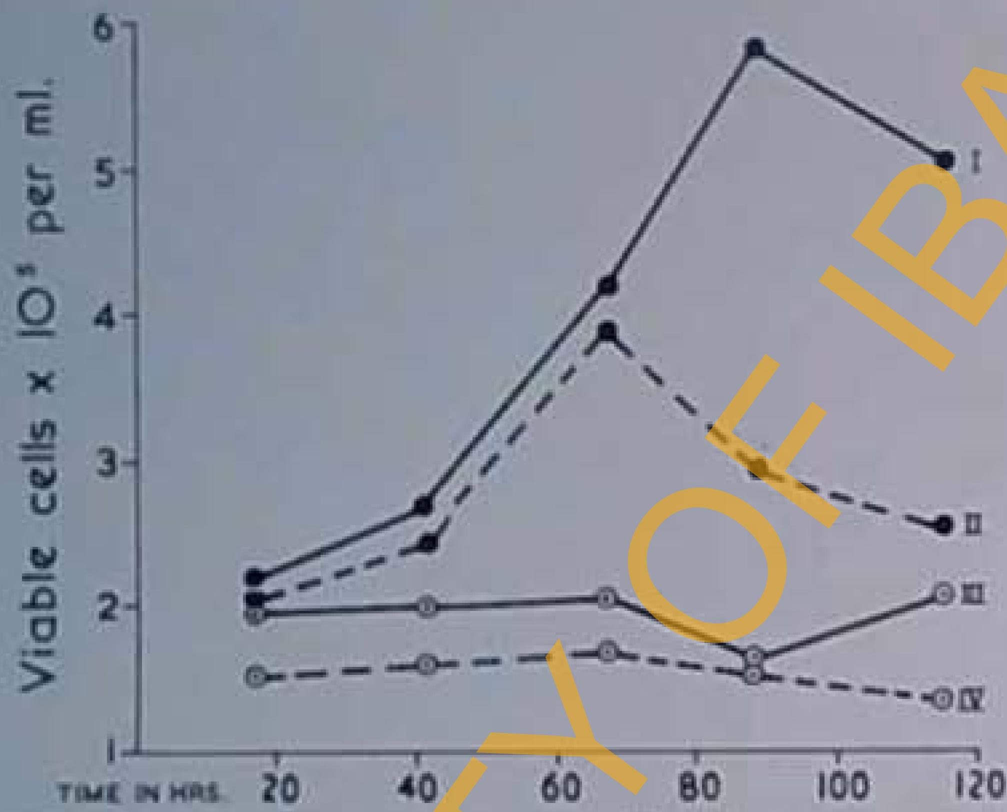

Fig. 2: Growth curves of Burkitt's tumour cells in Normal Adult (A) Serum & cured Burkitt's (B) Serum. Culture medium contained 30 % Serum in T.C. 199.

Curve I ●——● Normal Adult (A) Serum.
II ●- - -● Inactivated Normal Adult (A) Serum.
III ⊙——⊙ 'Cured' Burkitt's (B) Serum.
IV ⊙- - -⊙ Inactivated 'Cured' Burkitt's (B) Serum.

Fig. 104. Growth curves of CBM cells in Growth Experiment 6.

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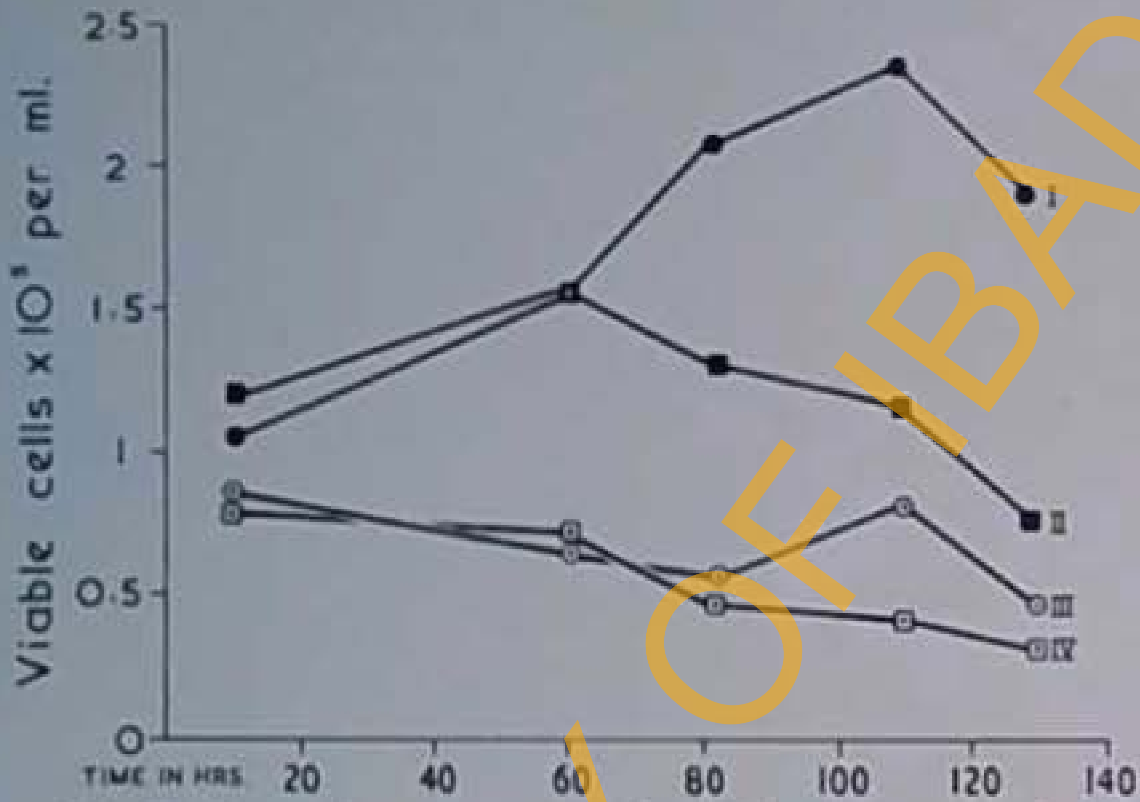

Fig. 3: Growth curves of Burkitt's tumour cells in cross-experiment.

Curve I ●——● Ex-A Serum cells, in A Serum
II ■——■ Ex-A Serum cells, in B Serum
III ⊙——⊙ Ex-B Serum cells, in B Serum
IV ⊡——⊡ Ex-B Serum cells, in A Serum

Fig. 105 Growth curves of OB1 cells after "crossing" cell cultures. (Growth Expt. 8)

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1.4 ml. TC 199, and 0.1 ml of washed CB1 cell suspension were added to each tube. The cultures were incubated at 37°C and serial counts carried out.

At 120 hours a cross-experiment was initiated. Cells in tubes containing uninactivated serum A were spun down and washed four times with TC 199 by centrifugation. Equal volumes of the washed cell suspension were then added to each of two tubes, one containing 2 ml. 30% serum A in TC 199, and the other 2 ml. 30% serum B in TC 199. Cells from uninactivated serum B cultures were similarly treated. Cultures were again incubated at 37°C and serial cell counts carried out.

RESULTS: Fig. 104 shows CB1 growth curve in the growth promoting serum A and growth suppressive serum B.

Is should be mentioned briefly that heating of sera to inactivate complement did not improve their growth; if anything, there were relative suppression of growth, as shown in the heated serum A culture.

Positive growth was maintained by washed cells transferred from serum A into fresh medium of same composition. (Fig. 105), curve I). Washed cells transferred from serum A into fresh serum B culture manifested positive growth during the first 60 hours with subsequent rapid decline of viable cell population (Fig. 105), curve II). Cells washed and transferred from serum B to fresh medium of same composition showed no change in growth behaviour (Fig. 105, curve III). When washed cells from serum B culture were however transferred to fresh serum A medium, there was a steady decline of viable cell population Fig. 105, curve IV); the cells have apparently been rendered refactory to growth promoting factors, by their previous exposure to serum B.

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COMMENT:- These results demonstrate conclusively that under the conditions of the experiment; serum from a symptom-free Burkitt's lymphoma patient not only suppressed the growth of CB1 cells, but also rendered the cells refractory to the influence of a culture medium of proved growth promoting potency for the cells. The observation finds striking analogy in animal experiments in which there was inhibition of tumour growth when malignant cells exposed in vitro to specific isoantisera were reinjected back into susceptible syngeneic hosts (Flax, 1956).

While purely nutritional factors cannot be ruled out, it is likely that immunological phenomena are involved in the suppression of CB1 proliferation by the Burkitt's serum tested. It is desirable therefore to utilise standard immunological techniques to investigate possible interaction between immunoglobulins in human serum and specific antigens in Burkitt's lymphoma cells.

Before proceeding to present the results of such experiments, observations made in some further experiments relevant to the effect of sera on growth of Burkitt's lymphoma cells will be briefly described.

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Table 16:- Effect of heat inactivation of serum on growth of CB1 cells in vitro (Growth Experiment 9).

Sample No.	Growth Index				Sample No.
	Untreated serum	Heated* serum	Untreated serum	Heated serum	
1	0.96	0.45	3.15	1.15	21
2	1.10	0.66	2.55	0.01	22
3	0.98	1.12	3.28	2.10	23
4	2.32	0.34	1.59	1.64	24
5	1.43	1.29	2.52	0.03	25
6.	1.75	1.00	1.28	0.86	26
7	1.69	1.21	0.99	0.17	28
8	1.84	0.74	1.78	0.22	29
9	2.12	0.89	1.58	0.74	30
10	1.91	0.94	1.31	0.01	31
11	1.45	1.34	1.50	0.22	32
12	1.13	1.31	0.86	0.37	33
13	1.34	1.12	1.20	0.29	34
14	1.15	1.48	1.83	0.84	35
15	0.95	0.96	1.33	0.29	36
16	1.19	1.44	1.38	0.29	37
17	1.52	1.18	1.18		
18	1.30	1.22			
19	1.12	1.83			
20	1.84	0.02			

* Serum heated at 56°C for 30 minutes before use.

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MISCELLANEOUS GROWTH EXPERIMENTS

In these experiments attempts were made to establish whether or not:-

(1) complement plays any role in the growth effect of human sera on Burkitt's lymphoma cells;

(2) growth effect of human sera on Burkitt's lymphoma cell lines is specific for cells derived from the Burkitt's lymphoma, and

(3) the technique and system adopted for comparative studies on the effect of serum samples on Burkitt's lymphoma cells lines permits reproducibility.

GROWTH EXPERIMENT 9: - Effect of heat inactivation of sera on growth of OB1 cells.

Procedure:- Thirty-seven serum samples from blood donors were tested. The layout of the experiment was as in growth experiment 5, except that duplicate cultures were set up on each serum sample. One of each pair of cultures contained test serum pre-heated at 56°C for 30 minutes.

RESULTS:- Table 16 lists the growth indices of the heat-inactivated sera and their respective unheated controls. In most cases growth was markedly less in the heated serum cultures as compared with the corresponding controls.

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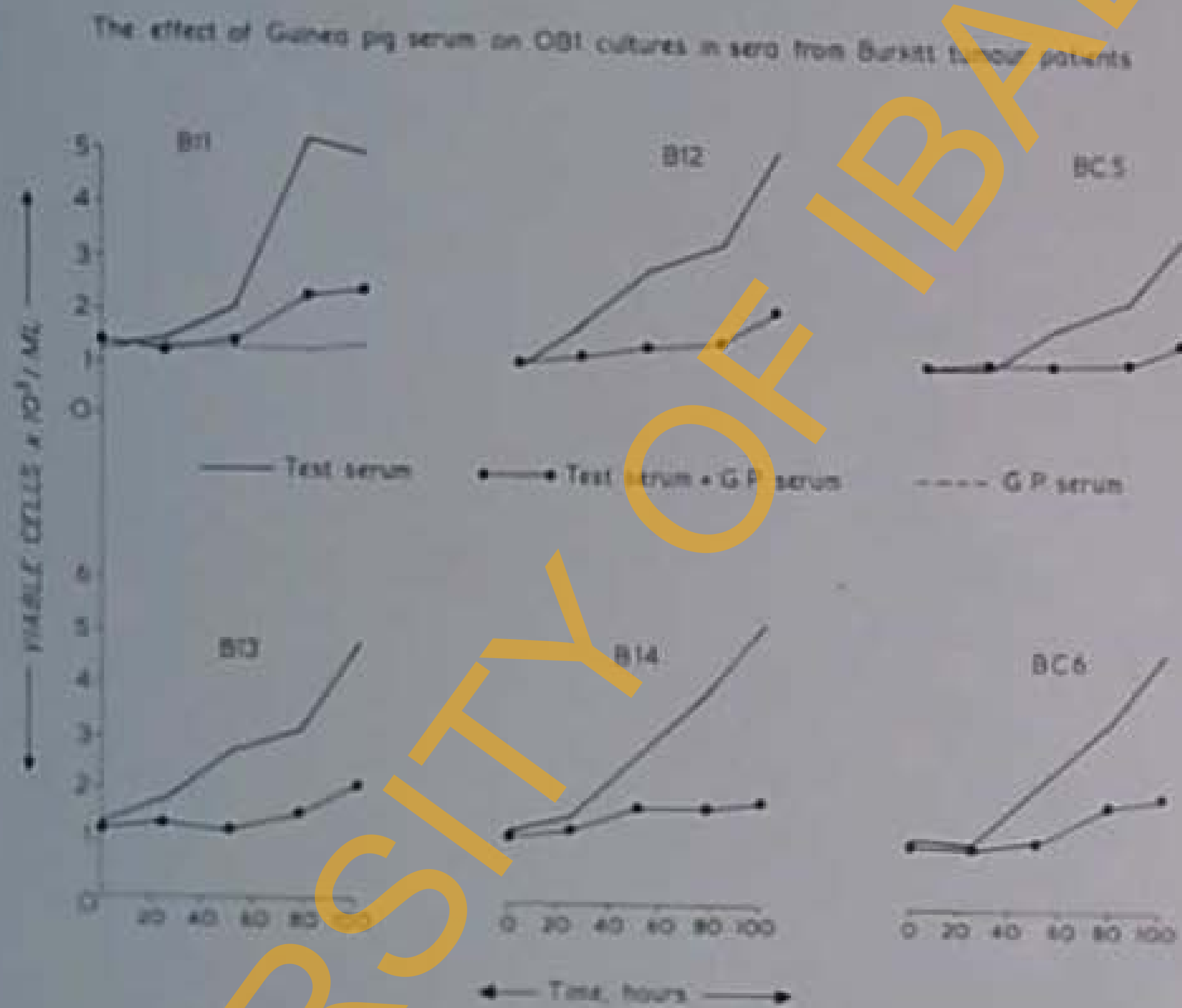

Fig. 106 Growth curves showing the effect of guinea pig serum on OB1 human cultures. (Growth Experiment 10)

COMMENTS:- Complement is presumed destroyed in the heated serum samples. Poorer growth in such samples suggests that the modus operandes of growth inhibition by certain sera on OB1 cells is different from that usually manifested by serum samples regarded as "toxic" in tissue culture work,- culture growth is improved by heating of "toxic" sera (Parker, 1964).

The possibility that complement inactivation by heating also destroys growth promoting factors for OB1 cells, cannot be excluded. The addition of complement to cultures, it was thought, might yield more conclusive results.

GROWTH EXPERIMENT 10:- Effect of guinea pig serum on growth of OB1 cells in human serum cultures.

Procedure:- Six human serum samples were tested. Test cultures were set up (see growth experiment 5) in duplicate, and to one of each pair was added 0.1 ml of pooled fresh guinea-pig serum obtained from 8 guinea pigs bought from a local market. An additional culture containing 30% guinea-pig serum only in TC 199 was also set up. Serial cell counts were made on all cultures and the respective growth curves plotted (Fig.106).

RESULTS:- Guinea-pig serum inhibited OB1 cell growth. The growth inhibition was so marked as to overshadow the growth effects of growth-promoting human sera present in the medium. Complete suppression of growth was apparent during the first 50 hours of culture, after which there was slight increase in viable cell population (Fig. 106), but to

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a degree far less than in the respective control serum culture. The viable cells in all cultures were normal in appearance.

COMMENT:- Many transplantable tumours in animals have been found to be susceptible in vivo to inhibitory effect of heterologous serum from unimmunised donors. Lymphomas have been the most susceptible and normal guinea-pig serum the most toxic (Kidd, 1953; Ainis et al, 1958; Jameson et al, 1958). The inhibitory effects were shown to involve natural antibodies as well as complement. Demonstrable cytotoxic effects included loss of glycolytic activity in the tumour cells. (Landy et al, 1960).

The inhibitory effect of fresh guinea-pig serum on OB1 cells is undoubted, and calls for deeper study of Burkitt's lymphoma/guinea-pig serum system for elucidation of the mechanisms involved in growth suppression. It is not unlikely that the mechanisms are similar to those which obtain in the animal experiments cited above.

GROWTH EXPERIMENT 11:- Effect of human serum on the growth of Burkitt's lymphoma and Hela cells. A comparative study.

Test cells and Serum:- The Burkitt's lymphoma cell-line used in this experiment was strain OB3 (Table 9). Hela (Bristol) cells were obtained from the Department of Virology, U.C.H, Ibadan through the kindness of Mr. G. O. Coker. The Hela cells had been maintained in 10% calf serum in Hand's medium. Two weeks before use in this experiment the cells were adapted to the culture medium in which Burkitt's

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lymphoma cell-lines were routinely maintained i.e 30% human serum in TC 199. Good growth was maintained, monolayers being available in four 120 ml medical flats for use in the experiment.

Serum samples were obtained from 6 blood donors and 5 Burkitt's lymphoma patients, and tested on the OB3 and Hela cells for growth effects.

Procedure: Hela cell-monolayers in two bottles were stripped by versenation (exposure to 1: 20,000 sodium ethylene-diaminetetra-acetate, EDTA, in PBS pH 7.2, at 37°C for 10 minutes), and the cell suspension washed twice by centrifugation in TC 199. 1.4 ml of a suspension of the washed cells in TC 199 were measured into test tubes containing 0.6 ml of test serum, and two tubes containing 0.6 ml of the standard serum. All tubes were well stoppered and incubated stationary on their side at 37°C.

Viable cell counts were carried out on the tubes at 0 hour and again at 96 hours; at 96 hours, the cultures were terminated by addition of 0.1 ml of 0.25% trypsin to each tube and left at 37°C for 30 minutes. The monolayer of cells were consequently brought into suspension, and vigorous agitation of the tubes facilitated complete dispersal of the cells into single cells. Criterion of cell viability was vital dye (0.1% trypan blue) exclusion.

OB3 test cultures were set up as usual using the same test and standard sera. Viable cell counts were carried out on all tubes at 0 hour and again at 96 hours.

Growth indices of each serum for the test cells was calculated by finding the difference between the increase in viable cell per c.mm in the test and standard serum.

Growth Index = growth in test serum
minus
growth in standard serum.

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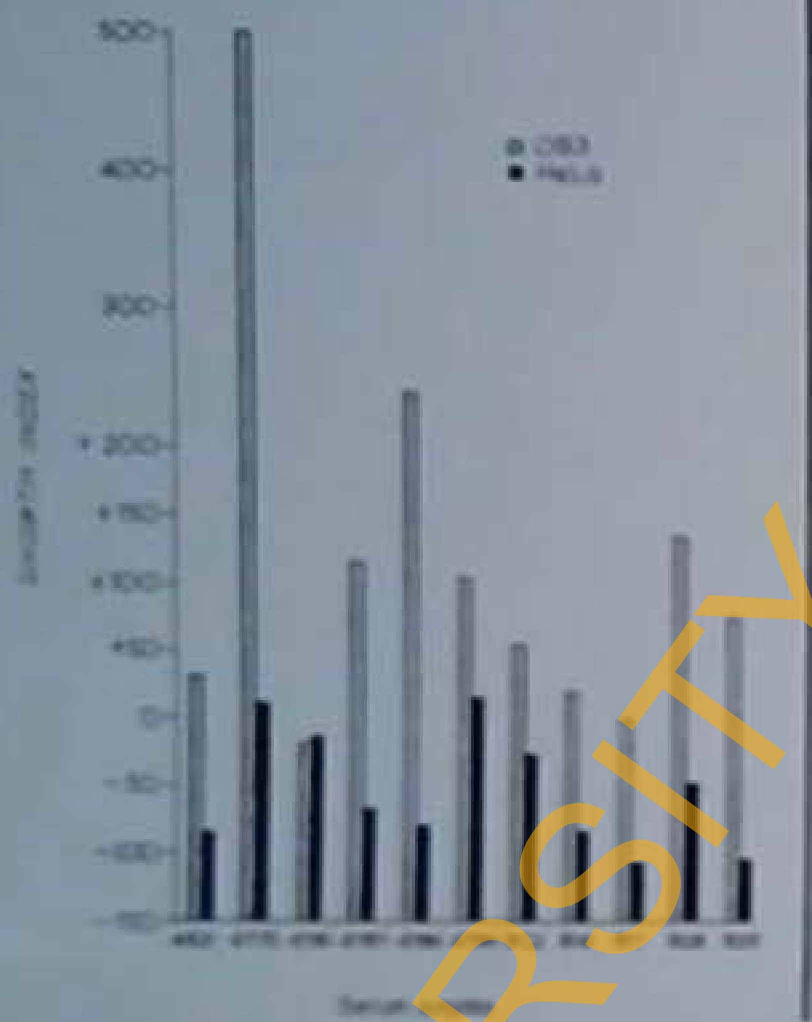

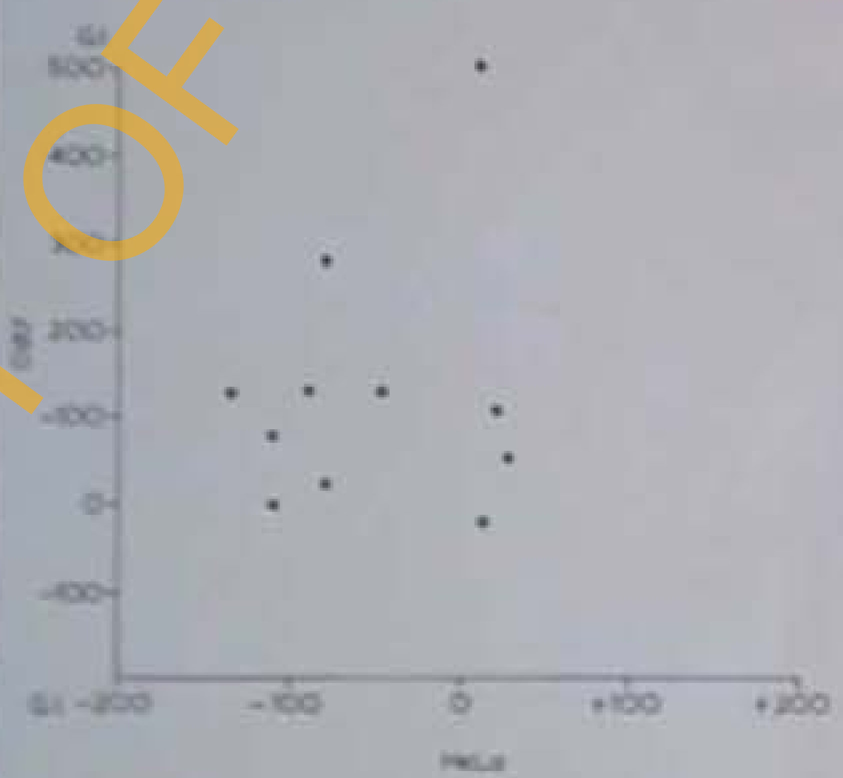

Fig. 107 Histogram and scattergram showing comparison of the growth effect of serum samples on OB3 and HeLa cells in tissue culture. (Growth Experiment 11).

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Table 17: Variability of observations on growth of CB1 cells in a single serum sample.

Tube	0 hr count cells/c.mm	100 hrs count cells/c.mm	Growth*	Growth Index.**
T1	140	700	557	1.36
T2	154	720	577	1.41
T3	178	770	627	1.53
T4	168	770	627	1.53
T5	142	696	533	1.36
T6	146	758	615	1.51
Standard (1)	144	540	407	
Standard (2)	146	560		

* Growth = (Cell population at 100 hours)

minus

(Average cell population at 0 hour)

** Growth Index = $\frac{\text{Growth in test culture}}{\text{Growth in standard culture.}}$

Mean Growth Index = 1.45

Standard deviation = ± 0.08

Coefficient of variation = 5.5%

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Test serum cultures in which the growth was greater than in standard serum culture have a positive growth index, those with less growth have a negative growth index.

Results: In all cases, HeLa cells did not thrive as well as OB3 cells in the test sera. On the whole there was no correlation between the growth effect of individual test serum samples on the two cell strains. This is well demonstrated in Fig. 107, in which the growth index of each serum for OB3 is plotted against that for HeLa cells.

Comment: It may be inferred from this experiment that the effect of serum from Burkitt's lymphoma cell lines is more involved than the mere capacity of a serum sample to support the growth of a stable long-established cell line of human neoplastic tissue origin.

GROWTH EXPERIMENT 12. Assessment of the degree of experimental error inherent in the system adopted for the comparative study of effect of different serum samples on growth of Burkitt's lymphoma cells.

Procedure: Six parallel OB1 cultures were set up as usual using the same serum specimen in all cultures. Duplicate cultures of the standard serum were also set up, and viable cell counts carried out on all tubes at 0 hour and again at 100 hours.

Results:- Table 17 shows the actual readings recorded in the experiment. The range of growth index calculated for the test serum was 1.36 - 1.53 with a mean of 1.45 ± 0.08 S.D., and a coefficient of variation of 5.5%

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COMMENT:- From the above figures it is reasonable to believe that the CB1 system is sensitive enough to distinguish between two test sera with a growth index difference of 0.24 (i.e thrice the standard error) or more. Possible sources of experimental error are differences in the size of cell innoculum, inaccuracy in cell-counting and/or variations pertaining to the culture tubes prior to or during the experiment; a loose stopper is a good example of the later

General summary and conclusions on growth experiments:

Certain conclusions are readily drawn from the series of experiments described on the growth of CB1 cells in vitro.

(1) It may not be possible to maintain long-established Burkitt's lymphoma cell lines previously propagated in 30% human serum in TC 199, in media containing less than 6% or more than 80% human serum in TC 199.

(2) Cultures may no longer render themselves amenable to continuous propagation if the population of viable cells subcultured routinely is less than 0.4 x 10^5 cells per ml. of culture.

(3) Optimal rate of increase in viable cell population is achieved by subculturing 2.0 x 10^5 viable cells per ml. of fresh medium consisting of 30% human serum in TC 199.

(4) It was possible to develop a system for measurement and comparison of the effect of small (0.6 ml) serum samples on the growth of Burkitt's lymphoma cells in vitro. With this system it was demonstrated that serum samples obtained from indigenous Nigerians vary widely in their growth effects on Burkitt's cells. The factors which are

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responsible for the variation would of necessity include non-specific substances required by Burkitt's lymphoma cells for metabolic activities and growth in vitro.

(5) Burkitt's lymphoma patients clearly constituted the group which produced serum samples with the highest degree of growth inhibition of the cells. This was considered due to active suppression of growth by humoral substances, such that the cells were in some way injured and prevented from replicating, (for ease of reference, this hypothetical substance(s) may be referred to as <u>factor B</u>).

(6) Factor B do not appear to be present in indigenous healthy adults, who however possess active growth-stimulating substances. While such growth stimulating factors (<u>Factor A</u>) are most likely "nutritional" in character, manifestation of a quasi-immunological reaction analogous to the phenomenon of tumour enhancement in vivo, remains a possiblity.

(7) Factor A is relatively high in most adult Nigerians, relatively low in most infants and young children, and is very low in Burkitt's tumour patients. Its fairly high concentration in cord serum would suggest among other possibilities that this factor can probably cross the placenta from maternal into foetal circulation. It is also possible that the growth-promoting effect of any factor A which may be present in Burkitt's lymphoma patients is masked by the concomitant presence of Factor B.

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(8) These factors do not have comparable effects on a long-established malignant cell-line of epithelial origin (Hela).

(9) The observations reported in this section may well be the expression of immunological interactions between humoral factors (in the sera of Burkitt's lymphoma patients and indigenous Nigerians) and homologous antigens in Burkitt's lymphoma cells.

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SECTION 2.

IMMUNOFLUORESCENCE TESTS ON BURKITT'S LYMPHOMA CELLS.

The findings in the last section relate largely to experience with only one Burkitt's lymphoma cell line. It is of interest to find out whether or not the same holds true for other cell lines derived from Burkitt's lymphoma. It is also desirable to search for more concrete evidences for the immunological nature of the humoral anti-Burkitt's lymphoma factors suggested by findings in the growth experiments.

In this section therefore, another cell line was used in the comparative study of growth effects of sera from an even more diverse groups of individuals, on Burkitt's lymphoma cells. Immunofluorescence studies were combined with the growth experiments, with the aim of detecting any immunoglobulin in test sera which may react with the cultured cells.

MATERIALS AND METHODS.

Burkitt's lymphoma cells:

The target cells used in these series of experiments were OB3 cells (Table 9). The cells have been maintained, in culture for about nine months at the time they were tested.

Viable Burkitt's lymphoma cells harvested from stock cultures maintained in this laboratory have been observed to regularly show a positive membrane immunofluorescence when simply washed thrice in phosphate buffered saline (PBS pH 7.2), and stained with fluorescein-labelled rabbit anti-human-globulin. The membrane immunofluorescence is totally prevented if the harvested cells are exposed to 0.25% trypsin

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in TC 199 for 30 minutes, at 37°C, before washing in three changes of PBS, pH 7.2.

Test Sera: Single blood samples were obtained from various groups of individuals;

These comprise:-

(a) 107 Nigerian blood donors aged 18 to 50 years presenting at the Blood bank, U.C.H. Ibadan.

(b) 39 American negro blood donors aged 25 to 51 years, in New York, through the kindness of Drs. Burchenal and Oettgen.

(c) 44 American caucasian blood donors aged 20 to 59 years, in New York, through the kindness of Drs. Burchenal and Oettgen.

(d) 15 members of the American Peace Corps, aged 22 to 27 years, within a week of their arrival in Nigeria, through the kindness of Dr. Friedland.

(e) 21 members of the American Peace Corps, aged 23 to 27 who have lived continuously in various parts of Nigeria for two or more years, through the kindness of Dr. Friedland.

(f) 20 untreated Burkitt's tumour patients aged 4 to 14 years and one aged 20 years.

(g) 16 Burkitt's tumour patients, symptom-free 3 months to 4 years after complete withdrawal of cytotoxic therapy.

(h) 21 sick Nigerian children aged 3 to 12 years presenting with miscellaneous acute diseases at the General Out Patient Department, U.C.H. Ibadan.

(i) 8 mothers and 6 fathers of Burkitt's lymphoma patients, total 14.

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(j) 14 untreated Nigerian patients with histological diagnosis of malignant lymphoma. These include 5 Hodgkin's-type lymphoma, 1 stem cell lymphoma, 1 myeloma and 1 case of chronic myeloid leukaemia presenting with generalised superficial lymphadenopathy.

Serum was separated from the blood samples within 36 hours of collection. Serum specimens were kept at 4°C until use in this study, after which they were deep frozen at -10°C for further studies. Specimens were generally tested at least one, but not more than four weeks after collection. Serum specimens from individuals in New York were shipped by air to Ibadan at cabin temperature. Such specimens arrived in this laboratory within 36 hours of dispatch.

Standard Serum:

This was a 90 ml serum sample, separated from 200 ml clotted blood collected from a healthy adult Nigerians at one bleeding. Samples were kept frozen at -20°C in 2 ml aliquots.

Tests: Serum samples were tested for their effects on Burkitt's tumour cells using two parameters viz:-

(i) the effect on survival and growth of CB3 cells in vitro and

(ii) detection of anti-Burkitt's (CB3) cell globulins in the test sera, by Moller's indirect membrane immunofluorescence ("ring") test (Moller, 1961) on viable CB3 cells recovered from cultures in (i).

(1) Growth Experiments:

The layout of the experiments was as described in growth experiment 5 in the last section. A major modification of the previous work was the rendering of cells harvested from stock cultures free of any

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adsorbed or immunologically reactant surface globulin, by trypsinisation and washing as described on page 118-119.

Serum samples were tested in batches of twenty. 0.6 ml of each serum was measured into test tubes. 1. 4 ml. of TC 199 was then added to each tube to give a 2 ml culture medium containing 30% test serum. 0.1 ml of the cell suspension of washed trypsinised cells calculated to give a viable cell population of 1-2 x 10^5 cells per ml. culture was then added to each tube. Duplicate cultures containing the standard serum were included in each batch of experiments. The culture tubes were tightly stoppered and incubated stationary at 37°C. Viable cell counts were carried out on all cultures at 0-2 hours and again 96-98 hours. Serum samples proved by nutrient broth culture to be contaminated were excluded from growth experiments.

The growth effect of each test serum was expressed as a <u>growth index</u>, which is the growth of CB3 cells in the test serum relative to growth in the standard serum. This was calculated simply as the difference between the increase in viable cell population in the test serum culture and the standard serum culture. Test sera in which there is less increase in numbers of viable cells per unti volume of culture than in the standard sera, have a negative growth index; those in which viable numbers are greater than in the standard serum have a positive growth index.

(ii) <u>Membrane immunofluorescence tests:</u>

After the final cell count (at 96-98 hours) in the growth experiments, each test culture was transferred into a small (Kahn) test tube, and centrifuged at 250g for 3 minutes. The cells were washed twice by centrifugation in PBS pH 7.2, and resuspended in 0.04 ml PBS.

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Fig. 103 Indirect Membrane immunofluorescence in CB3 cells. Two positive cells in centre of field showing complete ring of fluorescence. Several others (non-viable cells) with intense diffuse intracellular fluorescence.

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Fig. [illegible] Membrane immunofluorescence in OB3 cells. Negative test. Note fluorescent particles not forming a complete ring on surface of (viable) cell on left, and diffuse intracellular fluorescence in dead cell on right.

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0.04 ml of a 1 in 5 dilution of fluorescein - labelled anti-human globulin (Burroughs and Wellcome) was added and the tubes left standing at room temperature (25^{0}C) with intermittent shaking, for 20 minutes. The cells were then washed in three changes of PBS, pH 7.2 and resuspended in two drops of 50% glycerine. One drop of the cell suspension was then mounted on a glass slide, and the preparation examined immediately under a Leitz fluorescence microscope using an Osram HBO-200W mercury vapour lamp, and BG 12 primary and Blau 530 secondary filters.

Scoring of observations:- The degree of fluorescence of each preparation was recorded as one of three categories (0, +, or ++). Preparations in which no cells showed surface fluorescence were scored as 0. Those in which a distinct complete fluorescent ring outlined the cell surface in a good number of cells (usually 10-50%) were scored as + (Fig. 108). Those showing bright surface fluorescence in the majority of cells (usually over 60%) were scored as ++. The latter two categories were regarded as positive tests. In some preparations fluorescent particles were present on the cell surface of many cells without forming the characteristic ring (Fig. 109). These, as well as cells showing intracellular fluorescence (Fig. 108 & 109), a manifestation of non-viability, were ignored in the assessment of category of the preparation.

Cells from the standard serum culture served as controls in all batches of tests. The standard serum was a postive serum; out of the 16 batches of tests that constituted this experiment, the control cells showed a ++ positively 14 times, and a + positivety on two occasions.

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Fig. 110 Scattergram showing relative effects of serum from various individuals on the growth of CB3 cells in vitro.

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TABLE 18.

Table

Growth effect of sera from various individuals on CB 3 cells.

Groups of individuals	Number tested	Growth index *	
		Range	Median
Nigerian blood donors	105	-20 to +500	+120
Parents of Burkitt tumour patients	14	-50 to +250	+130
Burkitt tumour patients. Untreated	21	-10 to +140	+60
Burkitt tumour patients. In remission	16	-120 to +250	+40
Sick Nigerian children with miscellaneous complaints	20	-110 to +280	+90
Malignant lymphoma patients	13	+10 to +190	+120
New York blood donors. Negroes	37	-50 to +220	+70
New York blood donors. Caucasians	42	-40 to +190	+80
American Peace Corps volunteers. Newly arrived in Nigeria	15	-60 to +430	+220
American Peace Corps volunteers. 2 yrs resident in Nigeria	18	-80 to +440	+130

* Growth index = (increase in viable cell population in Test serum culture) minus (increase in viable cell population in Standard serum culture).

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RESULTS.

Growth Experiments:

The growth index of serum samples from the difference groups of individuals is as shown in the scattergram (Fig. 110). There was wide variation in the growth effect of the test sera on OB3 cells; the close similarity between these results and those obtained in OB1 growth experiment 5 is remarkable.

The widest variation was in the Nigerian blood donor group with growth indices ranging from -20 to +500, (Table 18) with a median value of +120. There was no significant difference between the effects of sera from negro and caucasian New York blood donors, median values being +70 and +80 respectively. Both groups however, lack individuals with unusually high growth promoting effect present in the adult Nigerian group. The effect, if any, of shipping on the growth effect of sera from New York is yet to be determined.

The Peace Corps groups show a distribution not unlike those of Nigerian blood donors, the long resident members of the corps tending to manifest lower growth indices. The numbers tested in the Peace Corps sub-groups were unfortunately too small to allow definite conclusions to be drawn from the apparent difference between the newly arrived (median, +220) and the long resident members (median, +130) of the American Peace Corps in Nigeria.

As a group, the sera from Burkitt's tumour patients showed the lowest growth promoting effects. The growth index range was -100 to +250. In this group, sera from patients in remission show lower growth promoting effects (median +40) than in the untreated tumour-bearing

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TABLE 19.

Table

Membrane immunofluorescence reaction on OB 3 cells cultured in sera from various individuals.

Groups of individuals	Age (yrs).	Number tested	Number of positive sera	% Positive sera
Nigerian blood donors	18 - 50	107	44	41
Parents of Burkitt tumour patients	Not known	14	9	64
Burkitt tumour patients	4 - 20	37	21	57
Sick Nigerian children with miscellaneous complaints	3 - 12	21	4	19
Malignant lymphoma patients. (Nigerians)	20 - 50	14	6	43
New York blood donors. Negroes	25 - 51	39	9	22
New York blood donors. Caucasians.	20 - 59	44	8	18
American Peace Corps volunteers. Newly arrived in Nigeria.	22 - 27	15	1	7
American Peace Corps volunteers. 2 years resident in Nigeria.	23 - 27	21	7	33

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patients (median 460). The three patients with the lowest growth indices had been symptom-free 2 or more years after treatment.

The distribution of growth indices of sera from parents of Burkitt's tumour patients, patients with malignant lymphoma other than Burkitt's tumour, and other sick children of the same age group as Burkitt's tumour patients were essentially within the range of those of most healthy Nigerian blood donors. The two sick children with sera with unusually low growth indices (Fig. 110) both had "fever and anaemia of unknown cause".

Membrane immunofluorescence tests on viable cultured cells.

OB3 cells are capable of showing a positive membrane immunofluorescence reaction when challenged with suitable human test sera. Using the criteria listed above for scoring of a positive test, sera from 41% of Nigerian blood donors showed a positive reaction. 22% and 18% of New York blood donors of negro and caucasian ethnic groups respectively, gave a positive reaction (Table 19).

Only one out of 15 (17%) newly arrived American Peace Corps volunteers produced a positive serum. Seven out of 21 (33%) of the volunteers that have been living in Nigeria for two or more years were positive.

Sera from parents of Burkitt's tumour patients showed the highest proportion of positive samples (64%), followed closely by the Burkitt's tumour patient group (57%).

Sera from adult patients with various types of malignant lymphomas showed a positive rate (43) closely similar to that of healthy Nigerian blood donors.

Lastly only 4 out of 21 (19%) . sick children with miscellaneous complaints produced positive sera.

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There was no correlation between the growth effect or outcome of the fluorescent antibody tests of sera from the Nigerian donors and their respective ABO and Rh blood groups. There was also no correlation between the growth effect of individual serum samples and their respective membrane immunofluorescence reaction on OB3 cells. Thus, a serum sample with a low growth index did not necessarily give a positive membrane immunofluorescence reaction.

COMMENT:- The knowledge gained in the growth experiments are closely similar to that reported in the earlier study in which another Burkitt's lymphoma cell line (Strain OB1) and different series of sera from adult Nigerians and Burkitt's lymphoma patients were used (see growth experiment 5).

Several conclusions are readily drawn from the set of figures shown in (Table 19). We can now say that:-

(i) cells that have been propagated in tissue culture for more than nine months after isolation from a patient suffering from Burkitt's tumour bear on their surface, moieties perhaps of an antigenic character, which react with human serum globins to such a degree as to be detectable by the indirect (sandwick) fluorescent antibody reaction.

(ii) Some Nigerians as well as North Americans do possess serum globulins which have affinity for the surface of these cells.

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(iii) Blood donors in New York have a lower incidence of individuals possessing such globulins as compared with Nigerian blood donors ($x^2 = 9.124 \quad p < 0.005$).

(iv) Burkitt's tumour patients show more tendency of having this type of globulin than sick Nigerian children of comparable age ($x^2 = 6.306 \quad p < 0.025$).

(v) Apparently a higher proportion of Americans 2 years resident in Nigeria produce this serum globulin, than a comparable newly arrived group ($0.05 < p < 0.10$).

(vi) The New York American caucasian is as likely to possess this globulin as the New York Negro.

The interpretation of these findings can, at this stage of our studies, be only a matter of conjecture. If the positive "ring" test (Moller, 1961) is a genuine index of an immunological reaction, then it would appear that antibodies to surface antigens on CB3 cells are present in detectable concentration in a little less than half of Nigerian blood donors, and about a fifth of New York blood donors. The incidence of positive individuals is quite low in Nigerian children unless they are suffering or have suffered from Burkitt's lymphoma.

The nature of the reactant antigens on the surface of Burkitt's tumour cells is not known. The probability that the antigens are Burkitt's tumour specific has much to commend itself, but several other possibilities have to be considered for exclusion, particularly if cell lines are used in the search for experimental proof of this surmise.

The non-correlation of positive sera with ABO and rehmus blood groups of the Nigerian donors has been mentioned already. The combined incidence of Anti-M, N and S haemagglutinins in the sera of Nigerian

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blood donors is less than 10%(LUZZATTO 1966), a figure too low to account for incidence of over 40% positive sera in the Nigerian blood donors studied. Klein and colleagues(1966) were also able to exclude the involvement of blood group antigens in the interpretation of results obtained on membrane immunofluorescent tests on fresh Burkitt's lymphoma cells.

The increasing reports of contamination of cell cultures by pleuropneumonia-like organisms (PPLO), with their characteristic tendency to aggregate on cell surfaces, brings this class of organisms under consideration in any immunological tests involving the cell surface of cultured cells (EDWARDS and FOGH, 1960; KRAEMER et al, 1963; FOGH and FOGH, 1964; HOLMGREN and PAYNE, 1966). Electron microscopic examination have so far failed to demonstrate mycoplasma (PPLO) in OB3 cultures (Dalton, 1966).

There is striking similarity between the results of the present investigations and reports on some serological investigations carried out in East Africa in which it was shown that 53 out of 72 (75%) Burkitt's lymphoma patients had demonstrable antibodies to reovirus type 3 as compared with 12 out of 65 (18%) healthy East Africa children, a highly significant difference (Bell, 1966).

While awaiting the characterisation of the cell surface antigens on OB3 and other Burkitt's lymphoma cells, the results of the present study would suggest that some of these antigens may be related to the pathogenesis of Burkitt's lymphoma, since it is children with this tumour as well as their relatives that produced the highest incidence of positive sera.

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This conclusion supports the concept of Burkitt's lymphoma being a rare aberrant response to an infective, probably viral, agent which occurs, commonly in tropical Africa, (Haddow and McCallum, 1962; Burkitt, 1963).

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Indirect fluorescent antibody tests on fixed Burkitt's lymphoma cell preparations.

One of the limitations of the membrane immunofluorescence reaction is its inability to yield information concerning intracellular antigens. As an extension of the last experiment, it was thought necessary to carry out immunofluorescence tests which would involve both surface and intracellular antigenic moieties.

It was of interest also to test sera against a battery of target cells derived from Burkitt's lymphoma. With these aims, serum samples from Burkitt's lymphoma patients were reacted against fixed cell-preparations from fresh as well as cultured Burkitt's lymphoma blast cells.

Materials and Methods.

<u>Target cells:-</u> The sources of Burkitt's lymphoma cells used were stock cultures of CB1, CB3, CB6 and MOBB, and fresh cells from a biopsy of Burkitt's lymphoma of the jaw (TC 222). The cells were trypsinised for 20 minutes at 37°C, to remove adsorbed or reactant globulins, washed thrice in TC 199, and resuspended in PBS pH 7.2. One drop of the cell suspension was placed on each of several acid-haemosol-cleaned, grease-free microscopic slides, and allow to dry at room temperature.

The dry cell preparations were fixed for 5 minutes in 0.3% hydrochloric acid, washed once in PBS pH 7.2 for 15 minutes, and stored at 4°C in a dry state until use.

<u>Test Sera:</u> These were samples from 20 untreated and 6 symptom-free Burkitt's lymphoma patients.

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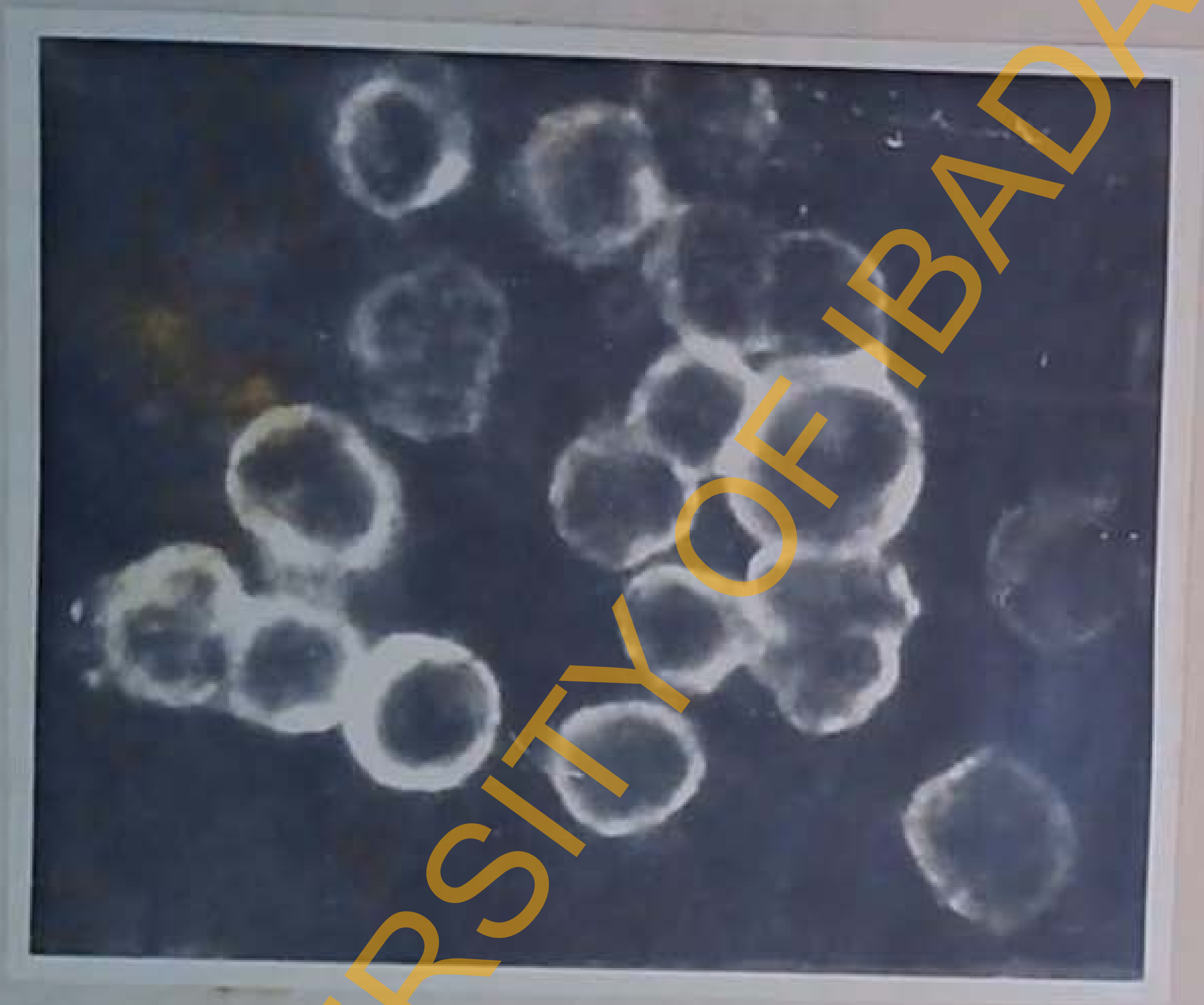

Fig. 111 Fluorescent antibody tests on fixed Burkitt's lymphoma cell preparation. Note marginal and cytoplasmic fluorescence.

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<u>Fluorescent antibody:</u> The fluorescein-conjugated anti-human globulin used was the same as in the last experiment. (Burroughs and Wellcome, 1 : 5 dilution; absorbed with guinea pig liver powder).

<u>Fluorescent antibody reaction:</u> Each slide was washed in PBS pH 7.2 for 15 minutes, and one drop of test serum placed on the fixed cell preparation. Reaction was allowed to take place for 20 minutes, after which slides were washed for 15 minutes in two changes of PBS pH 7.2, stained for 20 minutes with the fluorescein-labelled anti-human globulin and again washed twice for 15 minutes in PBS pH 7.2. All reactions were carried out at room temperature (22-25°C) in a perspex moist chamber. The cell preparations were mounted in 10% glycerine, sealed with nail-varnish, and examined under the fluorescence microscope.

<u>Controls:</u> Negative control slides were stained directly with the fluorescent anti-human globulin. Fixed cell preparations from a solitary plasmacytoma of the cavicle were used as the positive control.cells. Control cells for tumour specificity were the normal small lymphocytes and red blood cells present in fresh Burkitt's lymphoma cell preparation.

RESULTS.

<u>Scoring of observations:</u> Preparations which show no fluorescence (as in the negative control slides) were scored as 0. Those in which many cells show definite fluorescence were scored as + (Fig. 111). Preparations in which most cells show intense fluorescence were scored as ++.

It should be mentioned in passing that a test serum giving a + positivity with CB3 or CB6 cells, could be rendered negative by absorption with 12.0 x 10^6 cells per ml. for 1 hour at 37°C and 2 hours at 4°C.

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Table 20: Indirect fluorescent antibody reaction; Burkitt's sera against Burkitt's lymphoma cells.

Test sera	Cell preparations				
	TC 222	OB1	OB3	OB6	MOB8
B 13	N.T	+	+	+	+
14	N.T	0	0	0	+
15	+	+	+	+	+
17	0	++	0	+	++
18	0	+	++	0	0
19	0	+	+	0	+
20	+	0	+	+	+
21	+	++	+	0	+
22	+	•	0	+	+
23	+	+	0	0	+
24	0	+	+	0	+
25	0	++	++	++	0
26	0	+	+	+	0
27	+	+	+	0	0
28	0	++	++	+	+
29	+	+	++	+	+
30	+	0	++	+	+
31	+	+	+	+	+
32	N.T	++	++	•	+
33	N.T	++	++	+	+
BC 2	0	+	+	+	+
3	•	++	0	+	+
5	+	++	++	++	+
7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

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The pattern of fluorescence in most positive tests was more or less an outline, or marginal fluorescence, in which only the margins of cells fluoresced. An occasional cell may be seen showing intracellular (usually cytoplasmic and nucleolar) fluorescence.

The pattern of fluorescence in MOB8 cell preparations was different from the other cell preparations. There was usually no distinct marginal fluorescence, but rather, a diffuse fluorescence both in the cytoplasm and nucleus, with the nucleoli appearing as even more fluorescent dots.

Table 20 shows the detailed scoring of results. All the test sera were positive against one or more cell preparations. The same test serum may however show different degrees of positivity. Autologous serum (B28) for the fresh Burkitt's (TC 222) cells, did not react with the cells although it reacted strongly with other cell preparations.

COMMENT.

The different reactions of the same serum sample with different Burkitt's cells would suggest that there is some differences between Burkitt's lymphoma antigens.

This experiments also demonstrates that cells in long establish Burkitt's lymphoma cultures do retain structures against which human humoral factors can react.

From the degree of positivity obtained on each cell preparation, it would appear that some target cells(TC 222, and MOB8) are weak reactants, while CB1, and [illegible] cells, the oldest cell lines tested are relatively better reactants. The significance of such differences are at the moment not clear.

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SECTION 3

IN VITRO CYTOTOXICITY OF SERUM FROM ILL AND HEALTHY INDIVIDUALS ON BURKITT'S LYMPHOMA CELLS.

Derangement of cell-membrane integrity consequent upon addition of complement to a population of cells suspected of carrying antigen-antibody complexes at or near the cell surface, is a well recognised immunological phenomenon (Mayer, 1961). The derangement manifests as abnormal permeability and ultimate cell death and/or cytolysis (Goldberg and Green, 1959; Green et al, 1959). The vital dye exclusion method, which Gorer and O'Gorman (1956) adapted for use on non-erythroid cells remains a simple, sensitive though time consuming test. The method has been extensively used particularly in experiments on tumours of lymphoid and haemopoietic tissues in animals.

The knowledge gained in the previous sections of this chapter strongly suggests the presence of anti-Burkitt's lymphoma globulins in the patients as well as some healthy individuals. The demonstration by immunofluorescence of globulins on the surface of target Burkitt's lymphoma cells when reacted with some serum samples was almost conclusive evidence for the presence of antibodies to Burkitt's lymphoma cells. The simple adsorption to cell surfaces of a non-immunologically reactant globulin however, remains a possibility. Derangement of cell membrane integrity by addition of complement to such cells, would prove that the globulins demonstrated on the surface of the cells by other techniques were in fact part of antigen-antibody complexes.

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MATERIALS AND METHODS.

Burkitt's lymphoma cells: CB3 cells were used in all experiments. The cell line had been maintained in continuous culture for about a year at the time used.

Complement: Eight guinea pigs (Body weight 250 to 400 gms) were bought from a local market. The animals were bled by cardiac puncture, and the blood samples allowed to stand at room temperature for 4 hours after which serum was separated. The serum samples were pooled, and stored at -20°C in 1 ml. aliquots.

Test Sera: Serum samples were obtained from:

(1) 55 Nigerian blood donors all of whom were males.

(2) 14 parents of Burkitt's lymphoma patients (7 mothers and 7 fathers).

(3) 8 Burkitt's lymphoma patients with no evidence of tumour 3 months to 4 years after treatment.

(4) 16 untreated Burkitt's lymphoma patients and

(5) 27 children aged 3 to 14 years with miscellaneous non-malignant diseases at the G.O.P., U.C.H. Ibadan.

Most of the samples had been used in growth/membrane immunofluorescence experiment described in Section 2 of this chapter. The samples were stored at 4°C.

Reference Serum: This was the standard serum used in the immunofluorescent experiment (see section 2).

Procedure: Harvested CB3 cells were trypsinised for 30 minutes at 37°C, washed three times and resuspended in TC 199.

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Test sera were tested in batches of 10. For each batch of experiment, 1 tube of frozen guinea pig serum (complement) was thawed and divided into two, one of which was subsequently heated at 56°C for 30 minutes.

<u>Setting up of Tests:</u> Tests were carried out in small (Kahn) tubes. Two tubes were set up for each test serum, one being the test and the other the control containing fresh and heat-inactivated guinea pig serum respectively.

0.04 ml. of each test serum sample were placed in each of the two tubes, and an equal volume of CB3 cell suspension added. 0.04 ml of untreated guinea pig serum was added to the test, and the same volume of the heat-inactivated serum to the control tube.

The number of unstained cells per unit area was immediately counted on both tubes, using the trypan blue (dye exclusion) method. The tubes were then incubated at 37°C for exactly 1 hour when unstained cell counts were again carried out.

The cytotoxicity of each test serum for CB3 cells was calculated from the following formula:

$$\text{cytotoxicity} \quad c = \frac{b}{a} \times 100 \quad \ldots \quad \ldots (1)$$

where c, is the cytotoxicity of the contents of each tube for CB3 cells;

a, is the No. of unstained cells per unit area at 0 hour;

b, is the No. of unstained cells per unit area at 1 hour.

The cytotoxic index of each test serum was then expressed as the ratio of the difference between the cytotoxicity of control and

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CYTOTOXICITY OF SERUM FROM VARIOUS INDIVIDUALS ON BURKITT TUMOUR CELLS (OB3) INVITRO

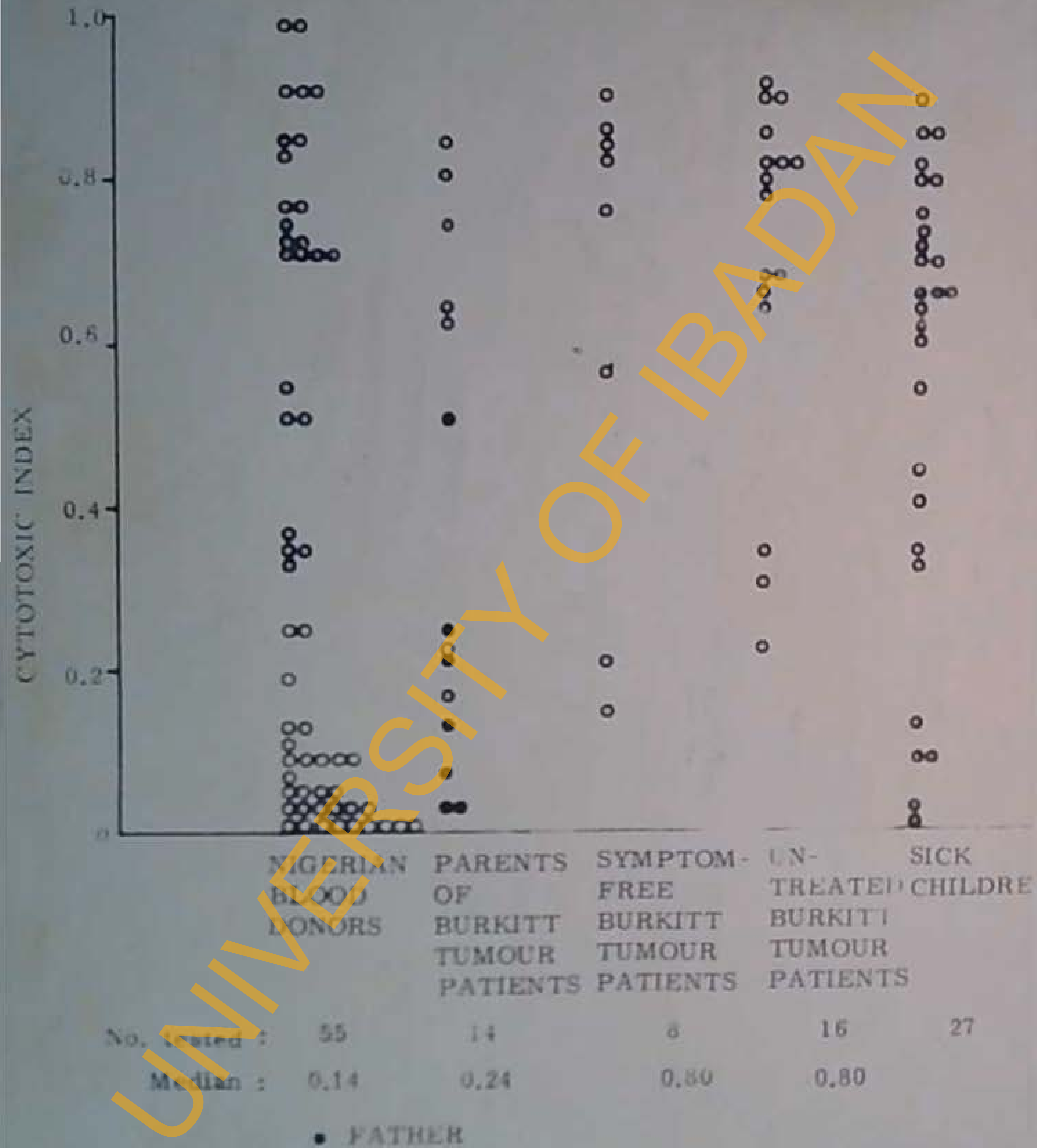

Fig. 112. Scattergram showing relative cytotoxicity of human sera on [illegible]

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Table 21. Cytotoxicity of sera from various individuals on Burkitt's lymphoma cells (CB3) in vitro.

Group of Individuals	No. tested	Cytotoxic Index		
		Range	Median	% over 0.2
Nigerian blood donors (males)	55	0-0.99	0.14	47.3
Parents of Burkitt's lymphoma patients - Fathers	7	0.04-0.52	0.14	30.0
Parents of Burkitt's lymphoma patients - Mothers	7	0.18-0.85	0.64	85.7
Symptom-free Burkitt's lymphoma patients	8	0.16-0.91	0.80	75.0
Untreated Burkitt's lymphoma patients	16	0.22-0.94	0.80	93.8
Sick children	27	0.01-0.92	0.66	81.5

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and the test to the cytotoxicity of the control viz:-

$$\text{Cytotoxic index, C.I.} = \frac{C^c - C^t}{C^c} \quad \ldots \quad \ldots(2)$$

where C^c, is the cytotoxicity of the control, and

C^t, is the cytotoxicity of the test.

RESULTS

The cytotoxic indices of the groups of serum samples tested are summarised in Table 21, and depicted as a scattergram in Fig. 112. All groups have a wide range of cytotoxic indices.

The medians for both the blood donors and fathers of Burkitt's lymphoma patients are 0.14; mothers of Burkitt's lymphoma patients have in contrast, a median of 0.64.

Sera from Burkitt's lymphoma patients both untreated and in remission have the highest median, of 0.80. Sera from many of the sick children with acute illnesses have quite high cytotoxic indices, with a median value of 0.66.

There is no correlation between the cytotoxic index and the ABO or Rh blood group of the individuals whose serum were tested.

The variability of observation in the system used is small, since the cytotoxic index calculated for the reference serum in 21 tests performed on different dates and with different batches of CB3 cells ranged from 0 to 0.16.

COMMENT

The cytotoxic tests confirms previous conclusions that the serum of Burkitt's lymphoma patients as well as some healthy individuals do possess antibodies to surface antigens on Burkitt's lymphoma cells.

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A few curious patterns are discernable from the scattergram, despite the small numbers tested in some groups. The significance of the apparent difference between the cytotoxic index levels of the fathers and mothers of Burkitt's lymphoma patients is not known. Similarly, the close similarity between the scatter of values of cytotoxic index for untreated Burkitt's lymphoma patients on one hand and acutely ill children on the other, cannot as yet find full explanation. This observation finds a close analogy in the report of Ngu et al (1966) that the correlation noted between IgM level of sera and their respective growth effect on a Burkitt lymphoma cell line (OB6) was shown by Burkitt's lymphoma patients and acutely ill children, but not by healthy school children. The humoral substances which both Burkitt's lymphoma patients and acutely ill individuals may have common are likely to be "acute phase proteins" of which C-reactive protein is one. The latter has been shown (Macfarlane 1966) to be quite high in untreated Burkitt's lymphoma patients.

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CHAPTER VI.

GENERAL DISCUSSION AND CONCLUSIONS

"....... there is intrinsic virtue in simplicity, if it aids understanding without doing violence to the facts of observation and experiment". Burnet, (1959).

Brief comments have already been made on each of the various observations presented in the preceeding chapters. In this concluding chapter, an attempt is made to discuss the inter-relationships between the diverse findings, and to stress the probable significance and implications of such findings. Some degree of speculative submissions cannot be avoided, the excuse for this being the existence of many wide and important gaps in our knowledge of almost all aspects of the pathology of lymphomas and other neoplastic diseases.

The facts which emerge from the work presented fall under four aspects, each of which has some bearing on the Burkitt's lymphoma problem.

1. Lymphoblastic neoplasias in childhood.

Before Denis Burkitt called attention in 1958 to the peculiar clinical features of the malignant tumour now named after him, the two known neoplastic diseases involving the primitive cell-type of the lymphocytic series were undifferentiated lymphosarcoma (lymphoblastic lymphoma) and acute lymphoblastic leukaemia. A leukaemic picture indistinguishable haematologically from acute lymphoblastic leukaemia is a frequent occurrence, particularly in the terminal stages, in classical undifferentiated lymphosarcoma (Jones and Klingberg, 1963). This frequent association of childhood lymphosarcoma and leukaemia have

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led many to regard this as strong circumstantial evidence suggesting that the two clinical entities are no more than different manifestations of the same disease.

The relationship of Burkitt's lymphoma to classical childhood lymphosarcoma and acute lymphoblastic leukaemia is not clear. Burkitt and Wright (1966), and Wright (1966b) made use of differences in clinical presentation and gross pathological features as evidences for their concluding belief that Burkitt's lymphoma is an entity, different and distinguishable from conventional childhood lymphosarcoma. Dorfman (1966) do not however share this view, and was of the opinion that "there is a distinct overlap between what is described as Burkitt's lymphoma in Africa and conventional childhood lymphosarcoma of the lymphoblastic type".

The overlap between the two types of solid lymphoblastic proliferations (in age incidence, clinical features and gross and histo-pathological features) is undoubted. The occurrence of both types of childhood lymphoblastic tumours in Nigeria and presumably most other tropical African countries has not hitherto been stressed, perhaps as a result of the overlap in clinico-pathological features. There is an unfortunate tendency to designate all childhood lymphoblastic tumours in this and other African countries as Burkitt's tumour. This is excusable because of the overwhelming preponderance of true Burkitt's tumours in these areas, the novelty of the tumour, and lack of absolute criteria of distinction between it and conventional childhood lymphosarcoma. It is of interest therefore that some light was shed on the problem during the morphological studies presented in this thesis. The detection by phase contrast cytology of a cell-type distinguishable from

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typical Burkitt's tumour lymphoblasts, in the 4 cases in this series who presented with features (marked generalised superficial lymphadenopathy, lymphoma, pleural effusion and gross splenomegaly) characteristic of conventional childhood lymphosarcoma but unusual for Burkitt's lymphoma, is strong evidence in favour of regarding Burkitt's lymphoma and conventional childhood lymphosarcoma as distinct entities.

It should therefore be emphasised that the three types of presently known lymphoblastic neoplasias in childhood do occur in Nigeria. Burkitt's lymphoma is by far the commonest type, and judging from figures presented in Table 11, at least ten times as common as conventional childhood lymphosarcoma. The remarkably low incidence of acute leukaemia in Nigerian children has already been noted by Watson-Williams and Allan (1963), and confirmed during the present review of malignant diseases recorded in the files of the Ibadan Cancer Registry. It is relatively more common in Western countries than in Tropical Africa (Davies, 1959). It may be inferred from the reports of Wright (1966) O'Conor et al (1965), Dorfman (1965), Beltran (1966), and the present study, that Burkitt's lymphoma and conventional childhood lymphosarcoma do occur in Europe, America and tropical Africa, and perhaps throughout the world. The uneven geographical distribution of Burkitt's lymphoma has been one of the highlights of the disease. Its overwhelming preponderance over other childhood tumours in tropical Africa indicates the operation of environmental influences. It is unlikely to be another expression of childhood lymphosarcoma, since both occur side by side everywhere, and as already been pointed out, both are due to proliferation of different types of lymphoblasts.

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The geographical distribution of childhood lymphosarcoma appears to be more even than that of Burkitt's lymphoma, because the former is a rare disease in all areas from where it is reported. Access of humans to its cause is presumed therefore to be fairly even. The reverse is true for Burkitt's lymphoma. Its relatively high incidence in Nigeria and other tropical African countries suggests the presence of environmental influences which make the cause or causes of the lymphoma readily accessible to man.

2. On the aetiology of Burkitt's lymphoma.

Several hypotheses have been put forward concerning the cause and pathogenesis of Burkitt's lymphoma. Prominent among such hypotheses is the postulated increased risk of neoplasia in lymphoreticular tissues as a result of frequent reactive proliferative activity, in response to overwhelming numbers of infective agents so common in tropical Africa. Attention has been focused on specific infections, particularly malaria, as probable candidates (Edington et al, 1967; Dalldorf et al, 1964; Williams, 1966).

Pulvertaft (1966) suggested that dietary beans might be responsible for the high incidence of the tumour in parts of Africa where he claimed high consumption of such foodstuffs by weanlings and children. Though an attractive hypothesis in view of the blastic transformation of lymphocytes by the water-soluble extract, phytohaemagglutinin (PHA) of seeds of the legume, Phaseolus vulgaris, it ignores the world wide consumption of beans and other leguminous plants, and the capacity for blastic transformation of lymphocytes by certain soluble bacterial products (tuberculin, staphylococcus toxin, etc.) drugs such as phenytoin, and other non-leguminous vegetable products such as that

obtained from poke-weed.

The attractive hypothesis of virus-induced, insect-vectored aetiology of Burkitt's lymphoma (Davies, quoted by Burkitt, 1962a; Burkitt, 1962b; Stanley, 1966) has given much impetus to the search for viral agents in Burkitt's lymphoma materials. Several types of viruses have in consequence been demonstrated in fresh Burkitt's lymphoma biopsy material and stable cell lines. Herpes-type virus particles have been demonstrated in several Burkitt's lymphoma cell lines (Epstein et al, 1965; Steward et al, 1965; Rabson et al, 1966; Zeve, 1966). The point in time at which the cells were infected by this viral agent, as well as its importance, if any, in the aetiogenesis of the tumour are still subjects of controversy. Similarly the role of other group of viruses isolated from the tumour material is not known. Herpes simplex virus has been isolated directly from tumour biopsy material in a few out of several attempts (Woodall et al, 1965; Simons and Ross, 1965; Bell et al, 1965). Dalldorf and Bergamini (1964) isolated undentified filterable antigenically-related agents from tumour, bone marrow and faeces of 6 out of 6 East African children suffering from Burkitt's tumour. Lastly, reovirus type 3, a virus whose exact role in the pathogenesis of murine lymphoma is as yet not clear (Stanley, 1966; Stanley et al, 1966) have also been isolated from Burkitt's lymphoma biopsy materials, including ascitic fluid. (Bell et al, 1966; Bell, 1966). It may well be that each of these viral agents is capable of producing the tumour.

3. Anti-Burkitt's lymphoma factors in patients and healthy individuals.

The immunological studies reported in Chapter V demonstrated the presence of antibodies, to surface and intracellular Burkitt's lymphoma

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antigens, in the serum of Burkitt's lymphoma patients and many individuals with no history of Burkitt's lymphoma. Of utmost interest and significance is the contrast in the proportions of positive individuals in the groups tested.

Irrespective of what the cause or causes of Burkitt's lymphoma are, and despite the unknown nature of the Burkitt's lymphoma antigens, the probable epidemiological importance of the relative proportions of positive individuals in the various groups cannot be over-emphasised. Antibodies against Burkitt's lymphoma cells were shown to be present in relatively high proportions of treated and untreated Burkitt's lymphoma patients, parents of Burkitt's lymphoma patients and healthy Nigerian adults as compared to other sick Nigerian children of comparable age with Burkitt's lymphoma patients, and healthy caucasian and negro blood donors from New York. Some evidences suggest that such antibodies are relatively high in cord blood, and tends to increase or appear in young adults who have migrated from America to Nigeria.

These findings implicate simultaneously the epidermiological and hereditary parameters of age, geographic location, race, and present or past history of Burkitt's lymphoma. The findings suggest that:

(i) antibodies to antigen(s) in Burkitt's lymphoma are produced by tumour-bearing patients and those in remission from the disease, as well as individuals in Nigeria and America with no history of the disease;

(ii) the number of individuals in Nigeria producing the antibodies increases with age;

(iii) relatively less proportion of Americans than Nigerians produce detectable antibodies;

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(iv) there is no racial difference in the capacity to produce the antibodies.

These conclusions are in support of an infective (antigenic) aetiology for Burkitt's lymphoma, but do not exclude other as yet unknown co-factors in the pathogenesis of the tumour.

4. <u>On the histogenesis and probable pathogenesis of Burkitt's tumour.</u>

The blast cells of Burkitt's tumour were characterised in the preliminary stages of this study, as primitive cells of the lymphocytic series, closely resembling, as was already noted by Pulvertaft (1964) and Wright (1966a), phytohaemagglutinin (PHA) transformed lymphocytes. The strikingly close similarity of the cells to blast cells of germinal centres of lymph nodes showing reactive follicular hyperplasia, was also mentioned.

More recently it was found in preliminary experiments that Burkitt's tumour cells maintained in continuous cultures do synthesize and secrete trichloroacetic acid-precipitable proteins (unpublished observations). These proteins have been demonstrated by Dr. H. Macfarlane (1966, unpublished to be immunoglobulins, thus confirming the experiences of an American team working with cell lysates of other Burkitt's lymphoma cell strains (Fahey et al., 1966). This is conclusive evidence that Burkitt's tumour cells belong to the lymphocytic-plasma cell series, and that the tumour is a lymphoma.

Burkitt's lymphoma cell-lines have been shown to be capable of endogenous interferon production (Henle and Henle, 1965), an evidence for a persistent viral infection in the cell lines. This evidence is further supported by the absence of endogenous interferon production

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in the oldest Burkitt's lymphoma cell line (Strain Raji; see Table 12) in which no virus particles could be detected (Epstein et al, 1966).

Untreated tumour-bearing patients suffering from Burkitt's lymphoma have unduly high serum C-reactive protein levels, while those in remission have normal (undetectable) levels (Macfarlane, 1966). This suggests that the presence of Burkitt's lymphoma is associated with C-reactive protein production, and implies the existence of an acute inflammatory process of some sort. Apart from its heterophile immunological reaction with pneumococcal polysaccharide antigen, and macroglobulin structure, next to nothing is known about the biological significance, site of production of, and cell type(s) capable of producing C-reactive protein. After a comprehensive review of the subject, Wilson and Miles (1955) concluded that it "presumably comes from tissues newly formed or newly responding to an external stimulus; whether it comes from cells that later will produce specific antibody remains to be determined." The cytotoxicity of the serum of children with miscellaneous acute febrile illnesses for OB3 cells in vitro, and the report by Ngu et al (1966) of similar growth-behaviour of OB6 cells in cultures containing sera of Burkitt's lymphoma patients and sick children, both suggest possible interaction between Burkitt's lymphoma cells and C-reactive or related proteins, since this protein is likely to be present in both groups of children. A corollary to this premise is that the presence of C-reactive proteins in sera of Burkitt's lymphoma patients reflects probable response to an external agent. Although superadded bacterial infection of the tumour mass cannot be excluded, an infective cause of the neoplastic process remains a possibility.

From the foregoing studies, conclusions and speculations it is

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postulated that Burkitt's lymphoma may well be a recently recognised type of pathological lesion which, though characterised by a farily constant histological picture and cell-type in different individuals and at different sites in the same individual , can be produced by a variety of related external agents. This concept finds analogy in the multitude of known or unknown agents capable of producing the common histological entity, - the tuberculoid granuloma, which is simply a tissue reaction to a variety of agents such as mycobacteria, fungus, helminth ova, inanimate foreign bodies etc., and characterised by by epitheloid cell proliferation with or without giant cell formation and lymphocytic infilteration. The concept further implicates an infective agent as the cause of the appearance of the tumour in a particular individual, and regards such tumours as the outcome of disordered immunoproliferation manifesting as a neoplastic process. Just as not all individuals who come in contact with a pathogenic mycobacterium eventually develop symptoms and signs of disease, so is it possible that in only a proportion of "exposed" individuals does the Burkitt's type of immuno-neoplastic disorder become manifest.

In terms of cellular dynamics, Burkitt's lymphoma may well be a "block" in the normal chain of cellular reactions which under normal conditions culminate in a secondary immune response, - a unique block in which the presence of the provoking alien antigen is obligatory and actively involved. The predilection of Burkitt's lymphoma for certain anatomical sites and its paradoxical rarity in some lymphoid organs may be explained by the presumption that the site of the tumour in an individual coincides with the anatomical sites of predilection of the provoking external agent on entry into the body. This implies that

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tumour production is a local reaction to the presence of the alien aetiological agent.

The type of aetiological factors which fulfil all the criteria required for the hypothetical pathogenesis of Burkitt's lymphoma, and explains most of the experimental findings without doing violence to established concepts in respect of experimental animal lymphoid tumours, are undoubtedly viral agents. A virus on entry into the body, is an alien, an antigen, capable of provoking an immune response capable of inducing specific antibody production, capable of a "block" with neoplastic consequences by incorporation into the genome of the host cell, and unusually show predilection for anatomical sites; it is the only type of external agent isolated and/or demonstrated in Burkitt's lymphoma tissue. The several common and widely distributed viruses associated with the lymphoma, though not necessarily causative agents, do suggest in support of the above concept that Burkitt's lymphoma is a "generic" lesion that can be produced by a variety of related agents. In Ibadan, mycobacterium tuberculosis is the commonest known cause of a tuberculoid follicle, so is it possible that in a given geographical locus, a specific agent may far outweigh other potential causes of the Burkitt-type of immunoneoplastic lesion.

Burkitt's lymphoma may therefore, in summary, be an acute persisting viral infection, perpetuating in a neoplastic fashion, a "generic" localised usually multifocal tissue reaction with a histological appearance of a malignant lymphoid germinal centre.

The demonstration of immunoglobulin synthesis by Burkitt's lymphoma cells raises the hope for search and discovery of causative specific antigens, and ultimate immuno-therapeutic manoeuvres for eradication of

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this childhood malady: a hope tempered however by the as yet unknown nature of "antigens" specific for myeloma proteins.

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APPENDIX 1.

Proforma for histological studies on "normal" lymph nodes.

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APPENDIX 1

Review of Lymphoreticular Tumours - PRELIMINARY STUDIES

"NORMAL" LYMPHNODES

Slide No: Year: Serial No:

Name: Hosp. No. Age: Sex

Biopsy Site

Indication for biopsy

Clinical diagnosis

HISTOLOGY

1. Node — Intact / Disrupted / Section well preserved / Nuclear smudging
2. Perinodal Tissues — Cellular infiltration
3. Capsule — Cellular infiltration / Fibrosis
4. Follicles — Peripheral — Medullary
   - (i) Number
   - (ii) Size
   - (iii) Germinal Centres
     - Phagocytosis
     - Mitosis (per h.p.f.)
     - Predominant Cell-type
     - Multinucleated cells
     - Others
   - (iv) Round cell corona
5. Sinus — Proliferation / Catarrh / Compression / Distension
6. Cellular infiltration — Polymorph / Eosinophil
7. Pigment — Melanin / Malarial / Iron / Formalin
8. Miscellaneous — Fibrosis / Reticulin / Necrosis / Haemorrhage / Blood vessels / Fat cells / OTHERS

COMMENTS:

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APPENDIX 2

Review of Lymphoreticular Tumours - PRELIMINARY STUDIES (2)
NON-SPECIFIC REACTIVE HYPERPLASIA IN LYMPH NODES

Slide No: | Year | Serial No.
Hospital No. | Age | Sex:
Biopsy Site
Indication for biopsy
Clinical findings and Diagnosis

HISTOLOGY

1. Node — Intact / Disrupted / Section well preserved / Nuclear smudging

2. Perinodal tissues — Cellular infiltration

3. Capsule — Cellular infiltration / Fibrosis

4. Follicles — Peripheral / Medullary
    - Number
    - Size
    - Germinal centres
        - Size
        - Phagocytosis
        - Mitosis
        - Predominant cell type
        - Others
        - Multinucleated cells
        - Round cell corona
        - Thickness
        - Predominant cell type
        - Others

5. Medullary cords — Predominant cell type / Other cell types / Multinucleated cells / Mitosis

6. Sinus — Peripheral / Medullary
    - Proliferation
    - Catarrh
    - Compression
    - Distension
    - Cell content
        - Lymphocytes
        - Plasma cells
        - Free macrophages
        - Others
    - Perisinus round cells

7. Cellular infiltration — Polymorph / Eosinophils / Others

8. Pigment — Melanin / Malarial / Iron / Carbon / Formalin

9. Miscellaneous — Fibrosis / Reticulin / Necrosis / Haemorrhage / Blood vessels / Fat cells / Others

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COMMENTS

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APPENDIX 3.

Proforma for histopathological studies on lymphomatous lymph nodes.

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APPENDIX 3

REVIEW OF LYMPHORETICULAR TUMOURS U C H., IBADAN

A. Biopsy material (i) LYMPH NODES

Slide No. Year: Serial No.
Hospital No: Age: Sex:
Biopsy site
Clinical findings

Histological diagnosis

HISTOLOGY

1. Quality of slide
2. Lymph node
   - Architecture
   - Subcapsular sinus
   - Perinodal tissue
3. Distribution of tumour cells
   - Diffuse
   - Multifocal
   - Follicular
4. Cell types
   - Uniform
   - Multiple
   - Pleomorphic
   - Tumour giant cells
   - Stem cell
   - Reticulum cell
   - Histiocyte
   - Lymphoblast
   - Lymphocyte
   - Others
5. Predominant cell type
   - Size
   - Outline
   - Shape
   - N/C ratio
   - Nucleus
     - Shape
     - Size
     - Chromatin
     - Nuclear membrane
     - Nucleolus
   - Cytoplasm
     - Amount
     - Colour
     - Vacuolation
     - Fenestration
     - Inclusion
6. Cellular infiltration
   - Lymphocytes
   - Plasma cells
   - Polymorphs
   - Eosinophils
7. Miscellaneous
   - Phagocytosis
   - Karyophagocytosis
   - "Starry sky" effect
   - Fibrosis
   - Necrosis

Comment

Classification

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APPENDIX 4.

Tiyoda phase contrast microscope, complete with photomicrographic attachment and warm stage incubator.

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APPENDIX 4.

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APPENDIX 5.

Culture chambers. (Agar roller slide and Ring chambers).

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APPENDIX 6.

Incubator showing agar-slide rack mounted on a motor. Note also culture bottles.

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APPENDIX 7.

Leitz fluorescent microscope, light source and camera attachment.

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